

Article

Vertical Patterns and Responses of Soil Microbial Functional Groups to Chronic Nitrogen Addition and Their Linkages to Nutrient Dynamics in a Typical Steppe

Muqier Hasi ^{1,†}, Xinyi Jiang ^{2,†}, Shilin Wang ², Yasong Chen ², Canran Yang ², Jianhui Huang ³ 
and Guoxiang Niu ^{2,3,4,*} 

¹ College of Grassland Science, Inner Mongolia Agricultural University, Hohhot 010011, China; hasimuqier@imau.edu.cn

² Jiangxi Provincial Key Laboratory of Carbon Neutrality and Ecosystem Carbon Sink, Lushan Botanical Garden, Jiangxi Province and Chinese Academy of Sciences, Jiujiang 332900, China; jiangxy@lsbg.cn (X.J.); wangsl@lsbg.cn (S.W.); chenys@lsbg.cn (Y.C.); yangcr@lsbg.cn (C.Y.)

³ Key Laboratory of Vegetation and Environmental Change, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China; jhhuang@ibcas.ac.cn

⁴ Senckenberg Museum of Natural History Görlitz, 02826 Görlitz, Germany

* Correspondence: niuguoxiang15@mails.ucas.ac.cn

† These authors contributed equally to this work.

Abstract

Despite the pivotal importance of soil microbial communities in regulating the terrestrial carbon (C) cycle and ecosystem functioning, their vertical patterns and their responses to long-term nitrogen (N) deposition, in terms of microbial biomass and relative abundance, still remain unclear, particularly for fungi versus (vs.) bacteria and Gram-positive (GP) vs. -negative (GN) bacteria, where they traditionally have contrasting biological characteristics. In this study, we investigated the responses of GP and GN bacteria, total bacteria (TB), and fungi (TF) and their relationships with four nutrient ratios to 10 years of N addition with three N addition rates (control, N2, and N10 representing 0, 2, and 10 g m⁻²yr⁻¹, respectively) across a 100 cm soil profile (0–10–20–30–40–70–100 cm) in a semiarid grassland, both in terms of absolute biomass and relative abundance represented by the amount and percentage of phospholipid fatty acids (PLFAs) (e.g., GP bacterial PLFAs: total PLFAs) of specific microbial groups, respectively. Our results showed that N addition has significant negative effects on the biomass of these four microbial groups across the whole soil profile, while N addition significantly decreased the relative abundance of GN bacteria and increased those of TF and GP bacteria. Additionally, N addition significantly decreased the biomass of TF and GP bacteria in 0–10 cm soil, and of TB and GN bacteria in 0–10, 40–70, and 70–100 cm soils, with the significant increase or decrease mainly occurring at the N10 rate. Our results also showed that the biomass of these four microbial groups decreased with soil depth regardless of N addition, but that of TB and GN bacteria decreased faster than that of TF and GP bacteria did, and further caused the relative abundance of TF and GP bacteria to increase with soil depth. Significant Pearson correlations among the biomass and relative abundance of these microbial taxa with soil pH, total phosphorus, NH₄⁺-N, and NO₃⁻-N were mainly found in 0–10 cm soil. C:N and NH₄⁺-N: NO₃⁻-N ratios increased with increasing soil depth, while C: phosphorus (P) and N:P ratios decreased. N addition significantly increased C:P and NH₄⁺-N: NO₃⁻-N ratios and decreased N:P ratios, especially under the N10 rate. Among these four nutrient ratios, N:P significantly explained the highest variations in F:B (76%) and GP:GN (38%) across the whole soil profile. Together, these results underline the different responses of soil microbial relative abundance and biomass along the soil profile under long-term N addition. They also suggest a relationship



Academic Editor: Concepción Avila

Received: 4 June 2026

Revised: 15 July 2026

Accepted: 17 July 2026

Published: 30 July 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and

conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

between soil microbial diversity and biomass relationships, and how this links to nutrient ratios in the subsoil needs to be investigated in future.

Keywords: nitrogen deposition; soil microbiome; temperate grassland; subsoil; Mongolian Plateau

1. Introduction

Over the last century, reactive nitrogen (N) generated by human activities has far exceeded the total reactive N produced by natural processes in the Earth's surface system over the same period, leading to a high reactive N content ratio in the atmosphere, which further significantly alters N cycling processes between atmospheric and terrestrial systems [1–4]. China's grassland accounts for approximately 35% of the total national land area, underlying the sustainability of ecosystem products and services regionally [5–7]. Soil microorganisms, representing the unseen majority residing in soil, dominate terrestrial biogeochemical cycling and are essential for plant growth [8–11]. Increased mineral N fertilizers may cause adverse effects on soil microorganisms in grasslands via decreasing soil pH and aggravating nutrient competition [12–15]. However, the responses of microorganisms and their relationships with soil pH, NH_4^+ -N, and NO_3^- -N in grassland soils to N addition still remain unclear.

Fungi and bacteria play fundamental roles in key terrestrial ecosystem processes, including nutrient cycling (e.g., N; phosphorus, P), carbon (C) cycling and sequestration, and litter decomposition [16–19]. Moreover, Bar-On et al. (2018) [20] reported that there is 12 gigatons (Gt) of fungal biomass on earth, constituting about 3% of the global biomass, while there is 70 Gt of bacterial biomass, although bacteria mostly reside in aquifers and subseafloor sediment. Fungi, as the decomposers of relative recalcitrant soil organic matter (SOM), generally play dominant roles in ecosystems (e.g., forest ecosystems) with slow nutrient cycling [10,16]. As the most abundant of all bacteria taxa, Gram-positive (GP) and -negative (GN) bacteria have received considerable attention in recent decades [21–23]. GP bacteria can use more recalcitrant SOM, while GN bacteria use more labile SOM, derived from plants [24]. Although soil microbial community composition under N addition has received much research attention, we still lack enough field work to recognize the responses of these four microbial functional groups to long-term N addition.

Most previous studies indicated that N addition affects the growth of these four microbial groups differently by decreasing soil pH and increasing N availability [25–29]. The main reasons are that N addition can directly affect ecosystem N status (such as NH_4^+ -N and NO_3^- -N contents and their ratios) and soil pH, and further affect soil microbial activities. Generally, the decrease in soil pH could cause greater negative effects on GN bacteria than GP bacteria, because GP bacteria have thicker cell walls [28,30]. By contrast, the increment in N availability induced by N addition could favor bacteria than fungi, because bacteria generally have higher nutrient requirements [28]. Moreover, these aforementioned studies generally focus on the responses of microbial taxa to N addition in terms of only biomass or relative abundance, although their responses may differ [31,32]. For instance, Zhou et al. (2017) [27] analyzed the data according to 1408 paired observations and reported that N addition significantly reduced fungal biomass and increased the relative abundance of GP bacteria. However, another meta-analysis reported that the relative abundance of fungi increased with no significant change in GP biomass under N addition [27]. Divergent conclusions from existing meta-analyses could be attributed to not only enough data at the time but also inconsistent data analysis methods.

Most microbial studies are restricted to surface soil, although soil microorganisms play important roles throughout the soil profile [33–36]. Moreover, our knowledge of soil depth variations in fungi and bacteria, in terms of biomass and relative abundance, was very limited. Based on previous studies, microbial biomass and activity decreased with soil depth because available C and other nutrients decreased with soil depth [37–39]. However, the relative abundance of TF, TB, GP, and GN bacteria did not always decrease with soil depth [40], which reflects the different vertical patterns of these microbial taxa. Moreover, the relative abundance can reflect the environmental (e.g., pH, water, and nutrient availability) adaptability of a specific microbial functional group in the whole soil microbial community at a specific soil depth. Additionally, few studies have directly investigated the response of these microbial taxa to long-term N addition across the whole soil profile, particularly under field conditions. Due to the significant difference in soil environment among different soil layers, their response to N addition in top- and subsoil may be remarkably different. Therefore, it is of great interest to study the vertical pattern and responses of Gram-bacteria, total bacteria, and fungi to N addition across the soil profile.

Chinese grasslands cover 12.5% of the world's grassland area and 41.14% of the land area of China [7], playing an important role in ecosystem services and functioning at global and regional scales. Fungi and bacteria are pivotal to the control of the global terrestrial C cycle and climate change at least in the 100 cm soil profile, but they play different roles (TF vs. TB; GP vs. GN bacteria) in C cycling due to distinct biological characteristics [21,23,41]. In this study, based on a N addition field experiment in a semiarid steppe, we investigated their vertical patterns at the 100 cm soil depth and their responses and main nutrients after 10 years of N addition from the absolute biomass and relative abundance represented by phospholipid fatty acid (PLFA) amounts and percentages, respectively. Furthermore, we explored how these four functional soil microbial groups and two microbial ratios linked to soil properties and nutrient ratios across the 100 cm soil profile (Figure 1).

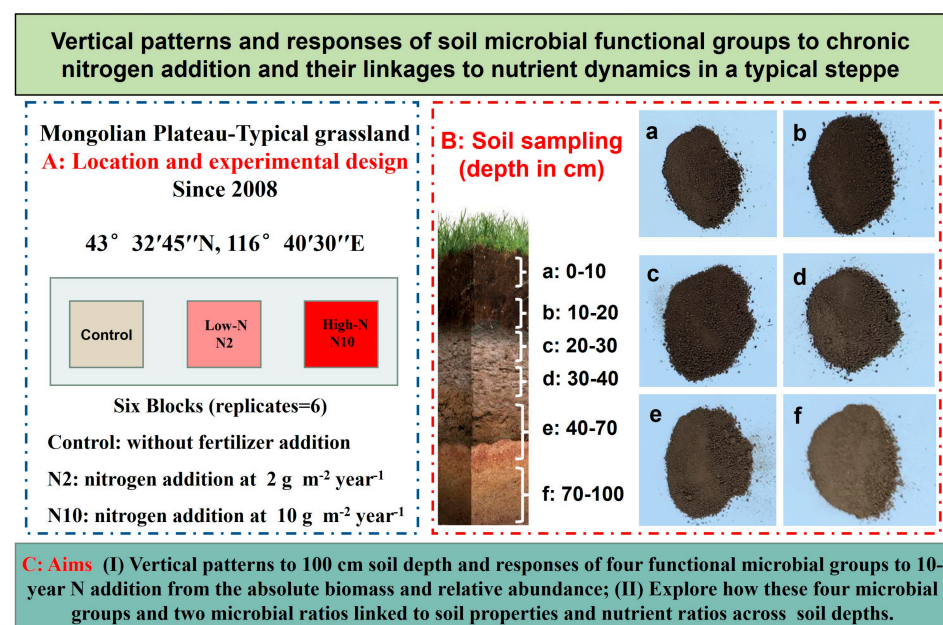


Figure 1. Overall research framework of the present study.

2. Materials and Methods

Our study site is located in a semiarid grassland in Xilingol, Inner Mongolia, north of China (43°32' N, 116°40' E, elevation ~1200 m a.s.l.). The study area, an important part of the Eurasia grasslands, has a semiarid continental climate with a mean annual temperature

of 0.9 °C and mean annual precipitation of 321.8 mm for the period of 1985–2017. Moreover, the mean monthly air temperature of the growing season (from May to August) is about 16 °C, with approximately 60–80% precipitation falling during the growing season in this area. The soil is classified as a Haplic Calcisol (the FAO soil classification system) or a chestnut (Chinese classification system). The content of soil clay, silt, and sand in the 0–10 cm soil is 5.2%, 44.2%, and 50.6%, respectively. The background annual N deposition at this site is less than 2 g m⁻² yr⁻¹ [42]. No fertilizer was applied at this site before our long-term experiment.

The study area has been fenced off since 1999 to exclude large animal grazing, and a field N addition experiment was established in 2008. The experimental design was described previously in detail by Niu et al. (2021) [43]. Briefly, we used a complete randomized block design and set up ten 45 m × 70 m blocks in total, with each block having thirty-eight 8 m × 8 m experimental plots, with 2 m walkways among blocks and a 1 m walkway buffer zone to separate plots. Here, three N addition rates (0, 2, and 10 g m⁻² yr⁻¹ are described as the control, N2, and N10, respectively) were selected to investigate the effects of N addition on total bacteria and fungi, and GP and GN bacteria, both in terms of absolute abundance or biomass and relative abundance along the 100 cm soil profile. The N deposition rate of 2 g m⁻² yr⁻¹ may become a reality in the near-future, whereas 10 g N m⁻² yr⁻¹ is similar to the N saturation threshold in this area [44]. N, as the form of ammonium nitrate (NH₄NO₃), was added to the plots on the first days of June and November equally for each year. Meanwhile, to simulate seasonal wet and dry N deposition, and ensure an even distribution in plots, N was added in the wet form of NH₄NO₃ solution (NH₄NO₃ particles mixed with 9.0 L of purified water) on June 1st and the dry form of NH₄NO₃ particles (mixed with 0.5 kg of sand) on November 1st. Hence, the experimental plot received less than 1 mm of additional water annually.

We collected 108 soil samples in total (3 treatments × 6 soil depths × 6 replicates) in September 2018, when the plots (not including control plots) received 10 consecutive years of N addition treatment. For each plot, a composite soil sample was taken from five scattered points, using a 5 cm diameter corer from six depth increments (0–10, 10–20, 20–30, 30–40, 40–70, and 70–100 cm). To remove visible plant materials, all soil samples were passed through a 2 mm sieve and mixed. Then, each collected soil sample was divided into three parts: one was used to analyze soil physicochemical properties after being air-dried; one was stored at 4 °C to measure soil ammonium N (NH₄⁺-N) and nitrate N (NO₃⁻-N); and the other was frozen at −20 °C for later use to measure soil microbial PLFAs.

The pH of air-dried soil samples was measured using a pH meter (sartorius PB-10; Goettingen, Germany) with a 1:2.5 (wt/vol, 10 g/25 mL) soil/distilled water suspension. Prior to analyses of soil total carbon (TC), N (TN), and P (TP), 20 grams (g) of each air-dried soil sample was ground using a ball mill. Soil TC and TN contents were determined by a C/N analyzer (Analytik-jena multi N/C 3100; Jena, Germany), while TP concentration was measured using the mixed strong acid extraction method. Fresh soil NO₃⁻-N and NH₄⁺-N were extracted with 0.5 mol L⁻¹ of K₂SO₄ (1:5, soil/solution) and the concentrations of the extracts were measured with a flow injection analyzer (Skalar; Breda, The Netherlands). We further calculated four nutrient ratios: C:N, C:P, N:P, and NH₄⁺-N: NO₃⁻-N.

Phospholipid fatty acids (PLFAs) of each frozen soil sample were extracted [45–47] and then analyzed via gas chromatography with a flame-ionization detector (Agilent 6850, MIDI V.6.2; Agilent Technologies, Palo Alto, CA, USA). On the basis of 19:0 internal standard concentrations, we calculated each PLFA concentration and we express it as nmol per g of dry soil (nmol g⁻¹). As one of the most commonly used methods for quantifying soil microbial biomass and analyzing microbial community structure (relative abundance), the PLFA method extracts PLFAs from the cell membranes of living soil microorganisms. Based

on the resulting PLFA profiles, specific microbial groups (e.g., TB, TF, and GP bacteria) can then be identified using their corresponding PLFA biomarkers [36,48,49]. Therein, total fungal biomarkers included 16:1 w5c, 18:2 w6c, and 18:2 w9c, while total bacteria biomarkers included all the indicators of GP and GN bacteria, and the PLFAs 14:00, 15:00, 16:00, 17:00, 18:00, and 20:00. The indicators of GP bacteria included i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0, while those of GN bacteria included 16:1 w7c, 16:1 w9c, 17:1 w8c, cy17:0 w7c, 18:1 w7c, 18:1 w9c, and cy19:0 w7c. Total PLFAs were calculated as the sum of total fungi, total bacteria, and other identifiable PLFAs, including 10me16:0, 10me17:0, and 10me18:0. The ratios of fungi to bacteria (F:B) and GP:GN were calculated. The relative abundances of these four microbial taxa were also calculated by using the corresponding PLFA of those divided by total PLFAs and are expressed as a percentage.

We ran all statistical analyses in R 3.6.2 (<https://www.r-project.org>, accessed on 12 December 2019) and the level of significance was accepted at $p < 0.05$, while we found that all data met the homogeneity and normality requirements for data analyses after a test in the “Car” package. Two-way analysis of variance (ANOVA) was used to assess the effects of N addition and soil depth on soil properties, the PLFAs and relative abundance of these four microbial groups, and F:B and GP:GN ratios. Then, one-way ANOVA with Tukey’s HSD was used to test the effects of N treatments or soil depth on those aforementioned indices. After one-way ANOVA, we used the Pearson correlation analyses to show the relationships among microbial PLFAs, relative abundance with soil properties, and nutrient contents. Linear regression analysis was used to assess the relationships between two microbial ratios and four nutrient ratios (also including $\text{NH}_4^+\text{-N}$: $\text{NO}_3^-\text{-N}$ with pH) across the whole soil profile. For some cases (e.g., GP:GN ratio with $\text{NH}_4^+\text{-N}$: $\text{NO}_3^-\text{-N}$; $\text{NH}_4^+\text{-N}$: $\text{NO}_3^-\text{-N}$ with pH), which may not fit with general linear regression analysis, we also found the break point using the bent stick approach in the “segmented” package.

2.1. Soil Properties

Based on two-way ANOVA, N addition and soil depth significantly affected all our measured soil factors (Figures 1 and 2). Additionally, for soil pH and the concentrations of $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and TP, the interactions of N addition $\times$ depth were also significant (Figure 1). As compared with the control plots, N addition significantly decreased soil pH and increased $\text{NO}_3^-\text{-N}$ content in the N10 treatment in all soil layers (Figure 2a,c). N addition increased soil $\text{NH}_4^+\text{-N}$ and TN contents, and the increase in $\text{NH}_4^+\text{-N}$ was significant in the N10 treatment in the upper three soil layers and 70–100 cm layer, while the increase in TN was significant in the N2 and N10 treatments in the 10–20 cm soil layer and in the N10 treatment in the 30–40 and 40–70 cm soil layers (Figure 2b,e). The TC content in the N10 treatment was significantly higher than in the control and N2 treatments (Figure 2d). N addition decreased soil TP content, with this decrease being significant in the N2 and N10 treatments in the 0–10 cm soil layer, in the N2 treatment in the 20–30 cm soil layer, and in the N10 treatment in the 70–100 cm soil layer (Figure 2f). Soil pH significantly increased with increasing soil depth, while the contents of soil $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, TC, TN, and TP decreased significantly with soil depth (Figure 2). Significant linear relationships between $\text{NH}_4^+\text{-N}$: $\text{NO}_3^-\text{-N}$ ratio and pH were also observed within the 100 cm soil profile (Figure 3). Regardless of N addition, C:N and $\text{NH}_4^+\text{-N}$: $\text{NO}_3^-\text{-N}$ ratios significantly increased with increasing soil depths, and C:P and N:P decreased (Figure 4). As compared with the control, high N addition (N10) significantly decreased in C:N ratio at the 30–40 cm soil depth (Figure 4a). N addition generally increased soil C:P and N:P ratios at all soil depths, and the effects of this addition became more significant under N10 treatments (Figure 4b,c). N addition decreased the $\text{NH}_4^+\text{-N}$: $\text{NO}_3^-\text{-N}$ ratio, with this decreasing effect generally being significant at 10–100 cm soil depths (Figure 4d).

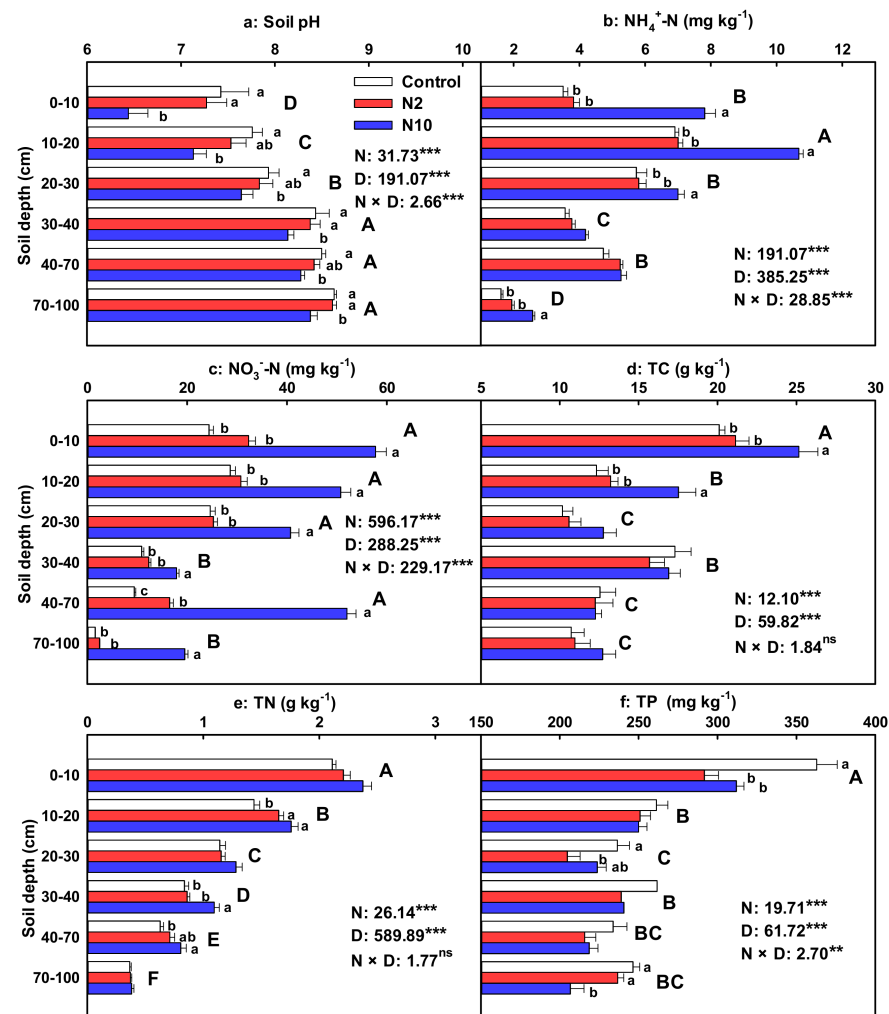


Figure 2. Effects of chronic N addition on soil properties within the 100 cm soil profile in a typical grassland. Notes: Data are shown as means with standard errors, and some raw data come from a previous study [43]. TC, TN, and TP correspond to soil total carbon, nitrogen, and phosphorus. The different lowercase letters show significant differences ($p < 0.05$) among N treatments for each soil depth, while the different capital letters indicate the same among different soil depths. The results of two-way ANOVA are shown in the lower or upper right corner of the figures. Asterisks ** and *** indicate significant levels at $p < 0.01$ and < 0.001 , respectively. The ns means no significant effect.

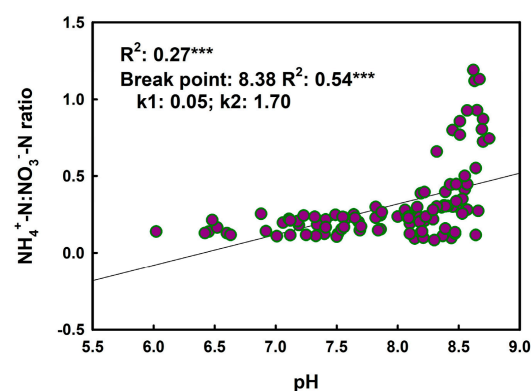


Figure 3. Relationships of $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ ratio with pH within the 100 cm soil profile in a typical grassland. Notes: The R^2 value and its significance of linear regression are shown in the corner for all significant cases. Results (break point, R^2 value, slopes (k_1 and k_2) for the line before and after the break point, and the significance) are shown using the bent stick approach. Asterisks *** indicate significance levels at $p < 0.001$.

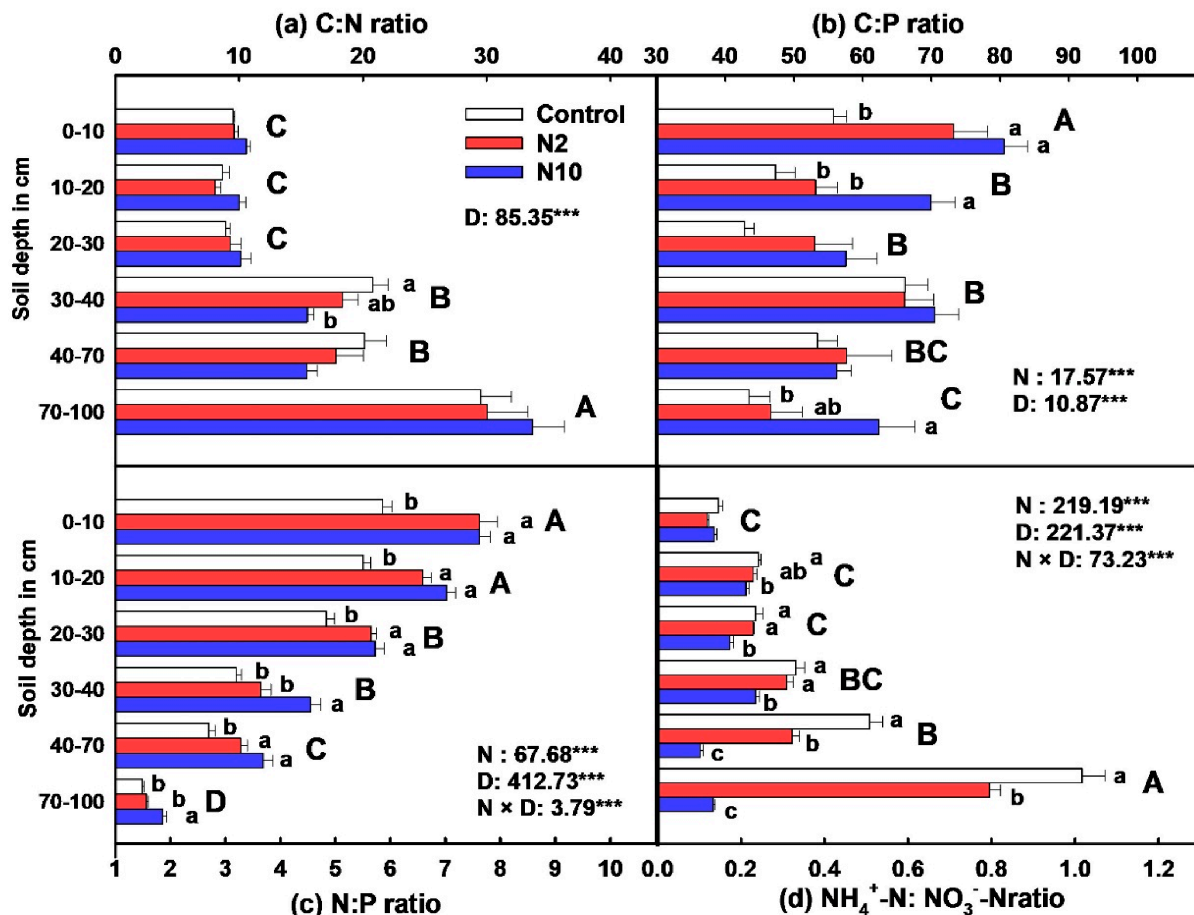


Figure 4. Effects of chronic N addition on four soil nutrient ratios within the 100 cm soil profile in a typical grassland. The different lowercase letters show significant differences ($p < 0.05$) among N treatments for each soil depth, while the different capital letters indicate the same among different soil depths. The results of two-way ANOVA are shown in the lower or upper right corner of the figures. Asterisks *** indicate significant level at <0.001 , respectively.

2.2. Soil Microbial Taxa Biomass (PLFA Amount)

The effects of N addition, soil depth, and its interactive effects on the biomass (PLFAs) of TF, TB, GP, and GN bacteria were all significant (Figure 5). When compared with control plots at a total soil depth of 0–100 cm, N addition significantly decreased TF PLFAs by 6.83%, TB PLFAs by 10.62%, GP bacteria PLFAs by 8%, and GN bacteria PLFAs by 14.27% (Figure S1). From 0–10 to 70–100 cm soil, the PLFAs of TF, TB, GP, and GN biomass significantly decreased by 51.9%, 74.3%, 67.0%, and 77.8%, respectively (Table S1). Hence, in comparison to TF and GP bacteria PLFAs, TB and GN bacteria PLFAs decreased faster with increasing soil depth and N inputs. N addition significantly decreased the biomass of GP bacteria and TF at the N2 and N10 rates in the 0–10 cm soil layer, except for TF at the N2 rate, while N addition had no significant effects below 10 cm soils as compared with control plots (Figure 5a,c). Additionally, N addition significantly decreased GN bacteria and TB PLFAs at the N2 and N10 rates in the 0–10, 40–70, and 70–100 cm soil layers, except for TB biomass at the N10 level at the 40–70 cm depth and GN bacteria biomass at the N10 level at the 70–100 cm depth (Figure 5b,d). Despite PLFAs of four microbial taxa significantly decreasing with soil depth, those in the upper three soil layers had significant differences, while relatively small differences occurred in 30–40, 40–70, and 70–100 cm soils (Figure 5, Table S1).

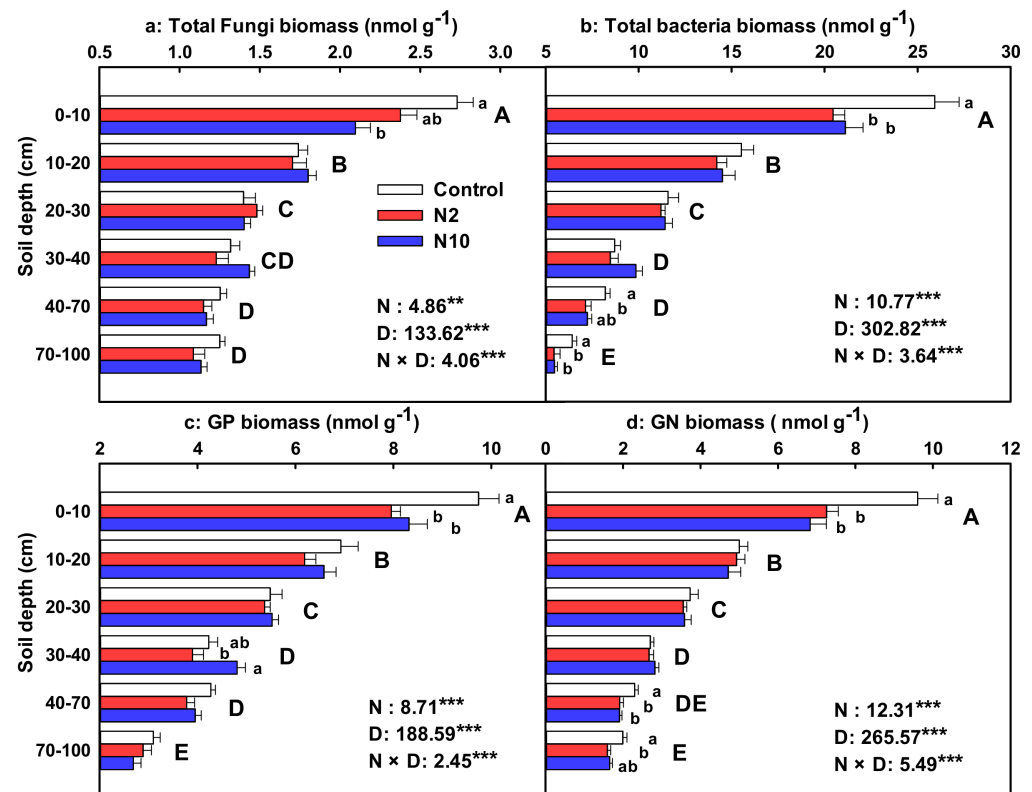


Figure 5. Effects of chronic N addition on the PLFAs of soil microbial groups within the 100 cm soil profile in a typical grassland. Notes: TF, total fungi; TB, total bacteria; GP, Gram-positive bacteria; GN, Gram-negative bacteria. The different lowercase letters show significant differences ($p < 0.05$) among N treatments for each soil depth, while the different capital letters indicate the same among different soil depths. The results of two-way ANOVA are shown in the lower or upper right corner of the figures. Asterisks ** and *** indicate significant levels at $p < 0.01$ and < 0.001 , respectively.

2.3. Relative Abundances of Soil Microbial Taxa (PLFA Percentages)

Soil depth and N addition significantly influenced the relative abundance of TF, TB, GP, and GN bacteria and the F: B and GP: GN ratios, while the interactive effects of N addition and soil depth were also significant (Figure 6). At a soil depth of 0–100 cm, N addition significantly increased the relative abundance of TF and GP bacteria by 3.1% and 1.8%, respectively, while significantly decreasing that of GN bacteria by 3.4% in comparison to the control plots (Figure S1). N addition also increased F: B and GP: GN ratios from 1.33 and 0.12 in the CK plots to 1.43 and 0.13 in the N addition plots ($p < 0.05$), respectively. The relative abundances of TF and GP bacteria in the 0–10 cm soil increased by 71.3% and 18.1% as compared with that in 70–100 cm soil, whereas those of TB and GN bacteria decreased by 8.9% and 20.1% (Figure 6, Table S2), respectively. When compared with control plots, N addition significantly increased the relative abundance of TF in 0–10, 10–20 and 40–70 cm soils and increased that of GP in 0–10 and 40–70 cm soils (Figure 6a,c). By contrast, that of TB significantly increased in 0–10 cm soil, but decreased in 20–30 cm soil under N-addition treatments (Figure 4b). That of GN also decreased in 0–10, 30–40 and 40–70 cm soils but increased in 10–20 cm soil (Figure 6d, $p < 0.05$). Nitrogen addition significantly increased the F: B ratio in all soil layers, except for 30–40 cm soil, while the GP: GN ratio also significantly increased in 0–10, 30–40, and 40–70 cm soils (Figure 6e,f). The relative abundance of TF and GP bacteria and the F: B and GP: GN ratios increased with increasing soil depth, while that of TB and GN bacteria decreased with soil depth (Figure 6, $p < 0.05$).

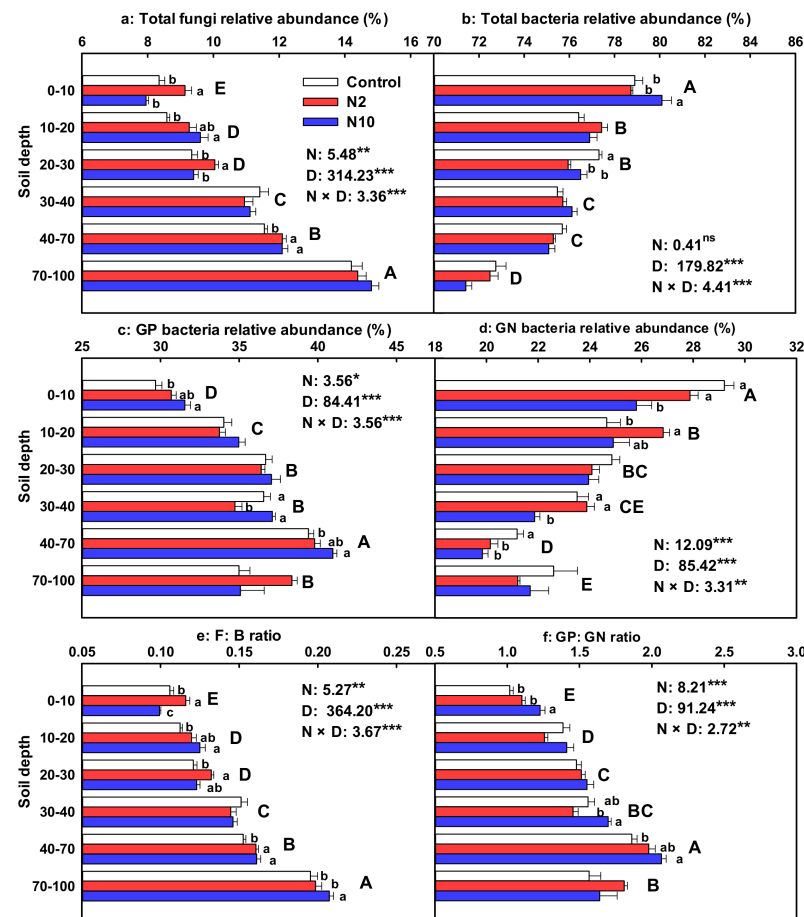


Figure 6. Effects of chronic N addition on the relative abundance of soil microbial groups within the 100 cm soil profile in a typical grassland. Notes: TF, total fungi; TB, total bacteria; GP, Gram-positive bacteria; GN, Gram-negative bacteria. The different lowercase letters show significant differences ($p < 0.05$) among N treatments for each soil depth, while the different capital letters indicate the same among different soil depths. The results of two-way ANOVA are shown in the lower or upper right corner of the figures. Asterisks *, **, and *** indicate significance levels at $p < 0.05$, < 0.01 , and < 0.001 , respectively. The ns means no significant effect.

2.4. Relationships Between Microbial Properties and Relative Abundances of Soil Microbial Taxa

Across the whole 100 cm soil profile, GP:GN and F:B ratios increased with increasing soil C:N and $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ ratios, and decreased with C:P and N:P ratios (Figure 5; Table S3). C:N, C:P, N:P, and $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ can significantly explain 3%, 3%, 76%, and 32% of the total variation in F:B, while C:N, N:P, and $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ can significantly explain 20%, 38%, and 6% of the total variation in GP:GN, respectively (Figure 7). Significant relationships of microbial properties (including PLFAs and the relative abundance of TF, TB, GP, and GN bacteria) with soil properties were mainly found in 0–10 cm soils. In 0–10 cm soil, soil pH was positively correlated with the PLFAs of TF and GN bacteria, while soil TC and TN were negatively correlated with TF PLFAs ($p < 0.05$, Table 1). Soil TP was also positively correlated with the PLFAs of these four microbial groups ($p < 0.05$, Table 1). The PLFAs of TF and GN bacteria correlated significantly positively with soil $\text{NH}_4^+\text{-N}$, and negatively with soil $\text{NO}_3^-\text{-N}$ (Table 1). Soil TP was also significantly positively correlated with TB PLFAs in 10–20 cm soil (Table 1). In 30–40 cm soil, soil pH was negatively correlated with PLFAs of TF, TB, and GP bacteria, while soil TN positively correlated with PLFAs of TB and GP bacteria (Table 1). Soil TN was negatively correlated with TF PLFAs in 70–100 cm soils (Table 1).

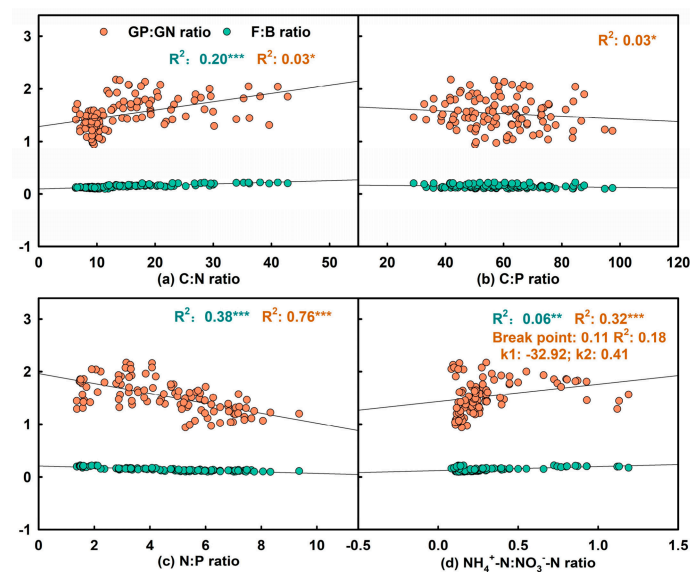


Figure 7. Relationships of GP:GN and F:B with C:N, C:P, N:P, and NH₄⁺-N:NO₃⁻-N ratios within the 100 cm soil profile in a typical grassland. Notes: The R² values and their significance of linear regression are shown in the corner for all significant cases. Results (break point, R² value, slopes (k1 and k2) for the line before and after the break point, and the significance) are shown using the bent stick approach. Asterisks *, **, and *** indicate significance levels at $p < 0.05$, < 0.01 , and < 0.001 , respectively.

Table 1. Pearson's correlation coefficients (r value) between the PLFAs of soil microbial groups and soil factors at different depth in a typical grassland.

Depth (cm)	PLFAs	pH	TC	TN	TP	NH ₄ ⁺ -N	NO ₃ ⁻ -N
0–10	GP	0.300	−0.119	−0.107	0.751 ***	−0.291	−0.320
	GN	0.527 *	−0.348	−0.311	0.789 ***	−0.531 *	−0.554 *
	TF	0.634 ***	−0.468 *	−0.421	0.620 **	−0.665 **	−0.657 **
	TB	0.372	−0.220	−0.205	0.798 ***	−0.351	−0.391
10–20	GP	−0.074	−0.169	0.058	0.436 *	0.037	0.033
	GN	−0.023	−0.165	0.140	0.407	−0.157	−0.128
	TF	0.013	−0.081	0.278	0.281	0.187	0.158
	TB	0.007	−0.224	0.065	0.494 *	−0.083	−0.078
20–30	GP	0.008	0.187	0.042	0.148	0.067	0.068
	GN	0.202	−0.099	−0.027	0.096	−0.201	−0.071
	TF	0.181	0.258	−0.065	−0.132	−0.052	−0.093
	TB	0.105	0.042	−0.022	0.095	−0.075	0.002
30–40	GP	−0.564 *	0.216	0.577 *	−0.214	0.293	0.446
	GN	−0.372	0.245	0.387	−0.121	0.201	0.121
	TF	−0.563 *	0.027	0.432	−0.225	0.311	0.350
	TB	−0.580 *	0.196	0.577 *	−0.240	0.334	0.419
40–70	GP	−0.313	−0.039	−0.217	0.156	−0.172	−0.141
	GN	−0.014	−0.076	−0.340	0.129	−0.332	−0.389
	TF	−0.331	−0.020	−0.169	0.107	−0.215	−0.146
	TB	−0.159	−0.040	−0.332	0.112	−0.261	−0.300
70–100	GP	−0.009	−0.117	−0.351	0.003	−0.195	−0.325
	GN	0.199	−0.278	−0.263	0.363	−0.303	−0.242
	TF	−0.119	−0.135	−0.468 *	−0.071	−0.134	−0.128
	TB	0.050	−0.225	−0.304	0.240	−0.304	−0.300

Notes: TF, total fungi; TB, total bacteria; GP, Gram-positive bacteria; GN, Gram-negative bacteria. Dots and asterisks *, **, and *** indicate significance levels at $p < 0.1$, < 0.05 , < 0.01 , and < 0.001 , respectively. All significant correlations are shown in bold.

For the relative abundances of these microbial taxa, the relationships with soil properties were different than for PLFAs. In 0–10 cm soil, soil pH significantly positively correlated with the relative abundance of GN bacteria, and negatively correlated with that of TB and GP bacteria (Table 2). Similarly, soil TC, NH_4^+ -N, and NO_3^- -N also positively correlated with the relative abundance of TB and GP bacteria, and negatively correlated with that of TF and GN bacteria ($p < 0.05$, Table 2). Soil TN was also significantly positively correlated with that of TB and GP bacteria. Soil TP was significantly positively correlated with that of GP bacteria, and negatively correlated with that of GN bacteria in 0–10 cm soil (Table 2). In 10–20 cm soil, the relative abundance of GP bacteria significantly negatively correlated with soil pH, and positively correlated with NH_4^+ -N and NO_3^- -N (Table 2). Soil TN was also positively correlated with the relative abundance of TB. Soil TC and NH_4^+ -N negatively correlated with the relative abundance of GN bacteria in 20–30 cm soil ($p < 0.05$, Table 2). In 30–40 cm soil, the relative abundance of GN bacteria negatively correlated with soil TN and NH_4^+ -N, while that of TB positively correlated with soil TN ($p < 0.05$, Table 2). Soil NO_3^- -N was significantly positively correlated with the relative abundance of GP bacteria and negatively with that of GN bacteria ($p < 0.05$, Table 2). The relative abundance of TF was also significantly negatively correlated with TP, while that of TB was positively correlated with soil NO_3^- -N in 70–100 cm soil (Table 2).

Table 2. Pearson’s correlation coefficients between the relative abundance of soil microbial groups and soil factors at different depth in a typical grassland.

Depth (cm)	Relative Abundance	pH	TC	TN	TP	NH_4^+ -N	NO_3^- -N
0–10	GP	−0.592 **	0.584 *	0.594 **	−0.433 *	0.557 *	0.603 **
	GN	0.676 **	−0.480 *	−0.365	0.536 *	−0.753 ***	−0.724 ***
	TF	0.447	−0.442 *	−0.343	−0.372	−0.570 *	−0.472 *
	TB	−0.623 **	0.479 *	0.553 *	0.116	0.632 **	0.607 **
10–20	GP	−0.467 *	0.224	0.155	−0.183	0.462 *	0.444 *
	GN	−0.102	0.005	0.236	0.020	−0.222	−0.164
	TF	−0.086	0.285	0.378	−0.361	0.449	0.398
	TB	−0.467 *	0.025	0.444 *	0.033	0.057	0.091
20–30	GP	−0.350	0.219	0.200	0.234	0.429	0.124
	GN	0.368	−0.488 *	−0.062	0.162	−0.423	−0.28
	TF	0.116	0.322	−0.123	−0.379	0.024	−0.201
	TB	−0.051	−0.375	−0.029	0.431	0.045	−0.197
30–40	GP	−0.363	0.265	0.433	−0.108	0.116	0.374
	GN	0.610 **	0.059	−0.524 *	0.304	−0.351	−0.676 **
	TF	−0.094	−0.349	−0.173	−0.024	0.032	−0.047
	TB	−0.35	0.387	0.513 *	−0.314	0.350	0.258
40–70	GP	−0.398	0.023	0.360	0.099	0.300	0.611 **
	GN	0.38	−0.128	−0.367	0.100	−0.427	−0.536 *
	TF	−0.403	0.038	0.362	−0.089	0.102	0.413
	TB	0.281	0.011	−0.670 **	−0.126	−0.345	−0.349
70–100	GP	−0.037	0.170	−0.130	−0.290	0.060	−0.221
	GN	0.338	−0.219	−0.023	0.383	−0.172	−0.069
	TF	−0.378	0.308	−0.284	−0.649 **	0.367	0.334
	TB	0.139	−0.099	−0.021	0.391	−0.401	−0.537 *

Notes: TF, total fungi; TB, total bacteria; GP, Gram-positive bacteria; GN, Gram-negative bacteria. Dots and asterisks *, **, and *** indicate significance levels at $p < 0.1$, < 0.05 , < 0.01 , and < 0.001 , respectively. All significant correlations are shown in bold.

3. Discussion

Generally, long-term N addition decreased the biomass of TF, TB, GP, and GN bacteria across the soil profiles, but increased the relative abundance of TF and GP bacteria and decreased that of TB and GN bacteria (Figures 3 and 4). This result reflected that long-term

N addition influenced the biomass and relative abundance of these four microbial groups differently. Additionally, our result indicated that low-N addition ($2 \text{ g m}^{-2} \text{ yr}^{-1}$) generally has no clear effects on the aforementioned microbial properties. These results are consistent with those of previous studies in grassland ecosystems, although those studies only reported changes in either biomass or relative abundance under N addition [27,28,50,51]. The decreasing availability of C and nutrients (e.g., N and P) with increasing soil depth also reflected the resource limitations for soil microbial communities.

The negative effects of N addition on soil microbes indicated that excessive N inputs can inhibit microbial growth. First, long-term N addition could increase the N availability in this N-limited grassland and may further intensify competition for other nutrients (e.g., P, K) among plants vs. microbes, and microbes vs. microbes [52–54]. The negative correlations among NH_4^+ -N and NO_3^- -N with the PLFAs of TF, TB, GN, and GP bacteria may indicate that soil microbial growth may not be limited by N after 10 years of addition at this site. Furthermore, the NH_4^+ -N: NO_3^- -N ratio generally increased with increasing soil pH (Figure 2), which may indicate that the relative influence of pH, NH_4^+ -N, and NO_3^- -N on soil functional microbial groups is coupled to a certain extent. Importantly, the relationship of our results to those of soil pH, GP: GN, and NH_4^+ -N: NO_3^- -N using the bent stick approach indicated that there are important breakpoints (e.g., soil pH: 8.37 and NH_4^+ -N: NO_3^- -N ratio: 0.11) among these ratios, suggesting that the more complex relationships should be reconsidered in future studies. We also found significant positive correlations among TP with the biomass of these four microbial groups in 0–100 cm soil (Table S3), indicating soil microbial growth may also face P deficiency. In addition, the decrease in soil microbe biomass may be related to organic material decomposition and root turnover [55,56]. For example, chronic N enrichment can inhibit the growth of white rot fungi or mycorrhizal fungi by decreasing lignin's activity and fine root growth [55,57]. Finally, the decrease in soil pH may be an important reason for the reduction in soil microbe biomass. Our results indicate that long-term N addition decreased the pH across the whole soil profile, while low-N addition decreased about 0.3 pH units even in the 70–100 cm soil layer. The N-induced lower pH and higher solubility of metal cations (e.g., Fe, Mn) can result in greater osmotic potential between soil microbe cells and soil solution, leading to the accumulation of metal cations that are toxic to soil microbes [13,52,58].

Changes in the relative abundance of soil microbial groups reflect the different physiologies of those groups and adaptive ability to the disturbance of N addition. Generally, bacteria and GN bacteria have higher N demands than fungi and GP bacteria, while fungi and GP bacteria can use relatively stable carbon fractions [59,60]. Hence, high N availability induced by chronic N addition inhibits the growth of TF and GP, but favors more active, copiotroph soil microbial groups, such as TB and GN bacteria. We found that N addition significantly decreased the biomass of TF and GP in 0–10 cm soil, but it significantly reduced those of TB and GN bacteria in 0–10, 40–70, and 70–100 cm soils (Figures 5 and 6). These results indicated that the responses of TB and GN bacteria were more sensitive to long-term N addition than TF and GN bacteria were, and therefore N addition increased the relative abundances of TF and GN bacteria. Our results are inconsistent with those of Zhou et al. (2017) [27], who reported that N addition had positive effects on microbial growth when the N addition dose was lower than $10 \text{ g m}^{-2} \text{ yr}^{-1}$, and with a decreased F: B ratio. The inconsistent results may be because our study has a longer duration of N addition (10 years).

As expected, we found an inconsistent vertical pattern of relative abundance of TB vs. TF, and GP vs. GN bacteria, although the biomass of those all decreased with increasing soil depth regardless of N addition. The relative abundance of TF and GP bacteria increased, whereas those of TB and GN bacteria decreased with soil depth. Meanwhile, we found

that the biomass of TB and GN bacteria decreased faster than that of TF and GP bacteria. Additionally, we found that F:B and GP: GN ratios increased with soil depth. These results are partly consistent with those of Fierer et al. (2003) [40]. Moreover, we found significant relationships between these two microbial ratios and four nutrient ratios across the whole soil profile, and C:N and N:P can explain more than 20% of the total variation in GP:GN, while those of N:P and $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ can explain more than 30% of total variation in F:B (Figure 7). There may be three reasons for these phenomena related to ratio shifts. First, the biological characteristics of different microbial groups seemed to prioritize the use of SOM or a variety of different qualities, and the SOM (C:N ratio) quality generally decreased with increasing soil depth due to less fresh plant inputs regardless of N addition [24,38,61,62]. Second, long-term N addition can accelerate the N cycle, altering the uptake of N and other nutrient elements by plants and microorganisms, further leading to limitations in the P ecosystem in our grassland ecosystems [21,22,53]. Moreover, the $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ ratio changes significantly under N addition even in the 70–100 cm soil layer, suggesting further studies need to focus on different N forms (e.g., $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and dissolved organic nitrogen). Third, as compared with GP:GN, the degree of dispersion of F:B ratio data was smaller across the whole soil profile (Figure 7). Although the scientific community has now developed various emerging microbial sequencing methods, we suggest that the PLFA method, which can indicate the absolute biomass of different microbes, can still can provide irreplaceable insights into the dynamics of soil microbial communities under global changes and human activities [23].

It is widely believed that microbial growth in subsoil is slower than that in topsoil, and the vertical pattern is connected with the diminishing C and nutrient resources across the soil profile [40,61,62]. We also showed that the biomass or PLFAs of TF, TB, GP, and GN bacteria all decreased from 0–10 to 70–100 cm, with significant relationships between these microbial groups and most soil factors along the whole soil profile (Figure 3; Table S3). Moreover, the contents of soil TC and other nutrients decreased with soil depth (Figure 1). It should be noted that the vertical pattern of TC is not completely consistent with that of TN and TP, due to the higher TC values at the 30–40 cm soil depth. Although direct evidence is lacking, we speculate that this pattern may be attributable to long-term plant inputs, especially root growth and its vertical distribution. In our study area, most root biomass was distributed in the surface soil [29], and we found almost no roots below a soil depth of 40 cm during soil sampling. Hence, low C and other nutrient resources may not favor microbial growth. Additionally, the faster decrease in TB and GN bacteria compared to TF and GP bacteria with soil depth resulted in an inconsistent vertical pattern of relative abundance of those microbes, and this result may correlate with their physiologies. For example, the TF biomass decreased 52% on average in the 70–100 cm soil when compared with that in 0–10 cm soil, but TB decreased 74% correspondingly (Table S1). The relative abundance of TF increased by 71% on average and that of TB decreased by 9% correspondingly (Table S2). Generally, fungi and GP bacteria have higher adaptive capacities under low C and other nutrient availability because they can use relatively recalcitrant C components compared to bacteria and GN bacteria [27,38].

4. Conclusions

In summary, our study investigated the vertical pattern to soil depths of 0–100 cm and the responses of Gram bacteria, total bacteria, and fungi to 10 years of N addition in a typical steppe. The biomass (represented by PLFAs) of all microbial groups and the relative abundance of TB and GN bacteria decreased with increasing soil depth, but the relative abundance of TF and GP bacteria and the F:B and GP: GN ratios increased. N addition decreased the biomass of all microbial groups and the relative abundance of

GN bacteria across the soil profile but increased the relative abundances of TF and GP bacteria and GP: GN and F:B ratios, although the effects were not significant in all soil layers. N:P significantly explains the highest variations in F:B and GP:GN across the whole soil profile. Our results distinguish the responses of biomass and the relative abundance of common microbial groups to N addition along the profiles and link microbial changes to nutrient ratio shifts in the 100 cm soil profile. Further studies are required to examine the frame responses of soil microbes and the interactions with C and nutrient cycling under N increment scenarios in grassland ecosystems.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/nitrogen7030077/s1>, Figure S1: Effects of chronic N addition on the biomass of soil microbial groups in the 100 cm soil profile in a typical steppe; Table S1: Variations in biomass of soil total fungi, bacteria, and Gram bacteria in different soil layers within the 100 cm soil profile under different N addition levels; Table S2: Variations in the relative abundance of soil total fungi, bacteria, and Gram bacteria in different soil layers within the 1 m soil profile under different N addition levels; Table S3: Pearson's correlation coefficients of the biomass and relative abundance of soil microbial groups with soil factors at different depths in a typical steppe.

Author Contributions: G.N. and J.H. developed this idea. Field data and functional analyses were collected and performed by M.H., X.J., S.W., Y.C. and C.Y.; and M.H., X.J. and G.N. wrote the first draft, which was further revised by other authors. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially supported by High-level Talent Research Start-up Project of Inner Mongolia Agricultural University (NDYB2021-4), Inner Mongolia Autonomous Region Natural Science Foundation (2023QN03011), National Natural Science Foundation of China (32301366), Jiu-jiang Outstanding Talent Program (XCYC2025074), Guangdong Basic and Applied Basic Research Foundation (2022A1515110439), and Special Research Project for the First-Class Discipline of Grassland Science at Inner Mongolia Agricultural University (YLXKZX-NND-035).

Data Availability Statement: The dataset supporting the conclusions of this article is included within the article and can be found in supporting material files.

Acknowledgments: The authors acknowledge Xingguo Han and Junjie Yang for coordinating the field operations since 2008. Guoxiang Niu acknowledges the supports from Chinese fundings for as a visiting researcher at the Senckenberg Museum of Natural History Görlitz Germany.

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

References

1. Galloway, J.N.; Townsend, A.R.; Erisman, J.W.; Bekunda, M.; Cai, Z.C.; Freney, J.R.; Martinelli, L.A.; Seitzinger, S.P.; Sutton, M.A. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. *Science* **2008**, *320*, 889–892. [[CrossRef](#)] [[PubMed](#)]
2. Canfield, D.E.; Glazer, A.N.; Falkowski, P.G. The Evolution and Future of Earth's Nitrogen Cycle. *Science* **2010**, *330*, 192–196. [[CrossRef](#)] [[PubMed](#)]
3. Dentener, F.; Drevet, J.; Lamarque, J.F.; Bey, I.; Eickhout, B.; Fiore, A.M.; Hauglustaine, D.; Horowitz, L.W.; Krol, M.; Kulshrestha, U.C.; et al. Nitrogen and sulfur deposition on regional and global scales: A multimodel evaluation. *Glob. Biogeochem. Cycles* **2006**, *20*, GB4003.6. [[CrossRef](#)]
4. Liu, X.J.; Zhang, Y.; Han, W.X.; Tang, A.; Shen, J.L.; Cui, Z.L.; Vitousek, P.; Erisman, J.W.; Goulding, K.; Christie, P.; et al. Enhanced nitrogen deposition over China. *Nature* **2013**, *494*, 459–462. [[CrossRef](#)] [[PubMed](#)]
5. Yu, G.R.; Jia, Y.L.; He, N.P.; Zhu, J.X.; Chen, Z.; Wang, Q.F.; Piao, S.L.; Liu, X.J.; He, H.L.; Guo, X.B.; et al. Stabilization of atmospheric nitrogen deposition in China over the past decade. *Nat. Geosci.* **2019**, *12*, 424–429. [[CrossRef](#)]
6. Wen, Z.; Xu, W.; Li, Q.; Han, M.J.; Tang, A.H.; Zhang, Y.; Luo, X.S.; Shen, J.L.; Wang, W.; Li, K.H.; et al. Changes of nitrogen deposition in China from 1980 to 2018. *Environ. Int.* **2020**, *144*, 106022. [[CrossRef](#)] [[PubMed](#)]
7. Li, L.H.; Chen, J.Q.; Han, X.G.; Zhang, W.H.; Shao, C.L. *Grassland Ecosystems of China*, 1st ed.; Springer Singapore: Singapore, 2020. [[CrossRef](#)]

8. van der Heijden, M.G.; Bardgett, R.D.; van Straalen, N.M. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol. Lett.* **2008**, *11*, 296–310. [[CrossRef](#)] [[PubMed](#)]
9. Fierer, N.; Strickland, M.S.; Liptzin, D.; Bradford, M.A.; Cleveland, C.C. Global patterns in belowground communities. *Ecol. Lett.* **2009**, *12*, 1238–1249. [[CrossRef](#)] [[PubMed](#)]
10. Bahram, M.; Hildebrand, F.; Forslund, S.K.; Anderson, J.L.; Soudzilovskaia, N.A.; Bodegom, P.M.; Bengtsson-Palme, J.; Anslan, S.; Coelho, L.P.; Harend, H.; et al. Structure and function of the global topsoil microbiome. *Nature* **2018**, *560*, 233–237. [[CrossRef](#)] [[PubMed](#)]
11. Fierer, N.; Wood, S.A.; de Mesquita, C.P.B. How microbes can, and cannot, be used to assess soil health. *Soil Biol. Biochem.* **2021**, *153*, 108111. [[CrossRef](#)]
12. Lu, X.K.; Mao, Q.G.; Gilliam, F.S.; Luo, Y.Q.; Mo, J.M. Nitrogen deposition contributes to soil acidification in tropical ecosystems. *Glob. Change Biol.* **2014**, *20*, 3790–3801. [[CrossRef](#)] [[PubMed](#)]
13. Tian, D.S.; Niu, S.L. A global analysis of soil acidification caused by nitrogen addition. *Environ. Res. Lett.* **2015**, *10*, 024019. [[CrossRef](#)]
14. Vitousek, P.M. Grassland ecology: Complexity of nutrient constraints. *Nat. Plants* **2015**, *1*, 15098. [[CrossRef](#)] [[PubMed](#)]
15. Niu, S.L.; Classen, A.T.; Dukes, J.S.; Kardol, P.; Liu, L.L.; Luo, Y.Q.; Rustad, L.; Sun, J.; Tang, J.W.; Templer, P.H.; et al. Global patterns and substrate-based mechanisms of the terrestrial nitrogen cycle. *Ecol. Lett.* **2016**, *19*, 697–709. [[CrossRef](#)] [[PubMed](#)]
16. Crowther, T.W.; Maynard, D.S.; Crowther, T.R.; Peccia, J.; Smith, J.R.; Bradford, M.A. Untangling the fungal niche: The trait-based approach. *Front. Microbiol.* **2014**, *5*, 579. [[CrossRef](#)] [[PubMed](#)]
17. Wardle, D.A.; Lindahl, B.D. Ecology. Disentangling global soil fungal diversity. *Science* **2014**, *346*, 1052–1053. [[CrossRef](#)] [[PubMed](#)]
18. Ke, P.J.; Miki, T.; Ding, T.S. The soil microbial community predicts the importance of plant traits in plant-soil feedback. *New Phytol.* **2015**, *206*, 3298–3341. [[CrossRef](#)] [[PubMed](#)]
19. Semchenko, M.; Leff, J.W.; Lozano, Y.M.; Saar, S.; Davison, J.; Wilkinson, A.; Jackson, B.G.; Pritchard, W.J.; De Long, J.R.; Oakle, S.; et al. Fungal diversity regulates plant-soil feedbacks in temperate grassland. *Sci. Adv.* **2018**, *4*, eaau4578. [[CrossRef](#)] [[PubMed](#)]
20. Bar-On, Y.M.; Phillips, R.; Milo, R. The biomass distribution on Earth. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 6506–6511. [[CrossRef](#)] [[PubMed](#)]
21. Crowther, T.W.; van den Hoogen, J.; Wan, J.; Mayes, M.A.; Keiser, A.D.; Mo, L.; Averill, C.; Maynard, D.S. The global soil community and its influence on biogeochemistry. *Science* **2019**, *365*, eaav0550. [[CrossRef](#)] [[PubMed](#)]
22. Fanin, N.; Kardol, P.; Farrell, M.; Nilsson, M.C.; Gundale, M.J.; Wardle, D.A. The ratio of Gram-positive to Gram-negative bacterial PLFA markers as an indicator of carbon availability in organic soils. *Soil Biol. Biochem.* **2019**, *128*, 111–114. [[CrossRef](#)]
23. Görka, S.; Darcy, S.; Horak, J.; Imai, B.; Mohrlok, M.; Salas, E.; Canarini, A. Beyond PLFA: Concurrent extraction of neutral and glycolipid fatty acids provides new insights into soil microbial communities. *Soil Biol. Biochem.* **2023**, *187*, 109205. [[CrossRef](#)]
24. Kramer, C.; Gleixner, G. Soil organic matter in soil depth profiles: Distinct carbon preferences of microbial groups during carbon transformation. *Soil Biol. Biochem.* **2008**, *40*, 425–433. [[CrossRef](#)]
25. Lauber, C.L.; Hamady, M.; Knight, R.; Fierer, N. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl. Environ. Microbiol.* **2009**, *75*, 5111–5120. [[CrossRef](#)] [[PubMed](#)]
26. Rousk, J.; Brookes, P.C.; Bååth, E. The microbial PLFA composition as affected by pH in an arable soil. *Soil Biol. Biochem.* **2010**, *42*, 516–520. [[CrossRef](#)]
27. Zhou, Z.H.; Wang, C.K.; Zheng, M.H.; Jiang, L.F.; Luo, Y.Q. Patterns and mechanisms of responses by soil microbial communities to nitrogen addition. *Soil Biol. Biochem.* **2017**, *115*, 433–441. [[CrossRef](#)]
28. Wang, C.; Liu, D.; Bai, E. Decreasing soil microbial diversity is associated with decreasing microbial biomass under nitrogen addition. *Soil Biol. Biochem.* **2018**, *120*, 126–133. [[CrossRef](#)]
29. Zhang, B.W.; Cadotte, M.W.; Chen, S.P.; Tan, X.R.; Han, X.G. Plants alter their vertical root distribution rather than biomass allocation in response to changing precipitation. *Ecology* **2019**, *100*, ecy2828. [[CrossRef](#)] [[PubMed](#)]
30. Joergensen, R.G. Phospholipid fatty acids in soil—Drawbacks and future prospects. *Biol. Fertil. Soils* **2022**, *58*, 1–6. [[CrossRef](#)]
31. Bastida, F.; Eldridge, D.J.; García, C.; Png, G.K.; Bardgett, R.D.; Delgado-Baquerizo, M. Soil microbial diversity–biomass relationships are driven by soil carbon content across global biomes. *ISME J.* **2021**, *15*, 2081–2091. [[CrossRef](#)] [[PubMed](#)]
32. Laine, M.B.; Taipale, S.J.; Tirola, M. Comparison of methods for assessing fungi-to-bacteria ratio of soil. *Biol. Fertil. Soils* **2025**, *61*, 941–954. [[CrossRef](#)]
33. Li, C.H.; Yan, K.; Tang, L.S.; Jia, Z.J.; Li, Y. Change in deep soil microbial communities due to long-term fertilization. *Soil Biol. Biochem.* **2014**, *75*, 264–272. [[CrossRef](#)]
34. Gu, Y.F.; Wang, Y.Y.; Lu, S.E.; Xiang, Q.J.; Yu, X.M.; Zhao, K.; Zhou, L.K.; Chen, Q.; Tu, S.H.; Zhang, X.P. Long-term Fertilization Structures Bacterial and Archaeal Communities along Soil Depth Gradient in a Paddy Soil. *Front. Microbiol.* **2017**, *8*, 1516. [[CrossRef](#)] [[PubMed](#)]
35. Liu, G.Y.; Chen, L.L.; Deng, Q.; Shi, X.R.; Lock, T.R.; Kallenbach, R.L.; Yuan, Z.Y. Vertical changes in bacterial community composition down to a depth of 20 m on the degraded Loess Plateau in China. *Land Degrad. Dev.* **2020**, *31*, 1300–1313. [[CrossRef](#)]

36. Yuan, X.B.; Yao, S.Z.; Zhou, G.Y.; Frew, A.; Dietrich, P.; Li, Y.; Wang, Y.; Ma, T.; Chen, N.; Zhang, Y.D.; et al. Depth-dependent mechanisms regulate accumulation of plant- and microbial-derived residues under long-term nitrogen addition in a semiarid grassland. *Funct. Ecol.* **2026**, *40*, 1790–1808. [\[CrossRef\]](#)
37. Jobbagy, E.G.; Jackson, R.B. The vertical distribution of soil organic carbon and its relation to climate and vegetation. *Ecol. Appl.* **2000**, *10*, 423–436. [\[CrossRef\]](#)
38. Soares, M.; Rousk, J. Microbial growth and carbon use efficiency in soil: Links to fungal-bacterial dominance, SOC-quality and stoichiometry. *Soil Biol. Biochem.* **2019**, *131*, 195–205. [\[CrossRef\]](#)
39. Dubeux, J.C.; Junior, M.d.A.L.; Simili, F.F.; Bretas, I.L.; Trumpp, K.R.; Bizzuti, B.E.; Garcia, L.; Oduor, K.T.; Queiroz, L.M.; Acuña, J.P.; et al. Deep soil organic carbon: A review. *CABI Rev.* **2024**, *19*, 1. [\[CrossRef\]](#)
40. Fierer, N.; Schimel, J.P.; Holden, P.A. Variations in microbial community composition through two soil depth profiles. *Soil Biol. Biochem.* **2003**, *35*, 167–176. [\[CrossRef\]](#)
41. Wang, C.L.; Liu, Y.T.; Xue, W.J.; Shi, B.K.; Gao, J.Q.; Zhang, W.J.; Gong, X.D.; Wang, D.Q.; Sun, W. Contrasting spring-fall microclimate and summer nutrient controls drive seasonal soil microbial biomass responses to nitrogen addition and grazing in a temperate meadow steppe. *Front. Environ. Sci.* **2026**, *14*, 1712702. [\[CrossRef\]](#)
42. Zhao, Y.H.; Zhang, L.; Chen, Y.F.; Liu, X.J.; Xu, W.; Pan, Y.P.; Duan, L. Atmospheric nitrogen deposition to China: A model analysis on nitrogen budget and critical load exceedance. *Atmos. Environ.* **2017**, *153*, 32–40. [\[CrossRef\]](#)
43. Niu, G.X.; Hasi, M.; Wang, R.; Wang, Y.; Geng, Q.; Hu, S.; Xu, X.; Yang, J.; Wang, C.; Han, X.; et al. Soil microbial community responses to long-term nitrogen addition at different soil depths in a typical steppe. *Appl. Soil Ecol.* **2021**, *167*, 104054. [\[CrossRef\]](#)
44. Bai, Y.F.; Wu, J.G.; Clark, C.M.; Naeem, S.; Pan, Q.M.; Huang, J.H.; Zhang, L.X.; Han, X.G. Tradeoffs and thresholds in the effects of nitrogen addition on biodiversity and ecosystem functioning: Evidence from inner Mongolia Grasslands. *Glob. Change Biol.* **2010**, *16*, 358–372. [\[CrossRef\]](#)
45. Zelles, L. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: A review. *Biol. Fertil. Soils* **1999**, *29*, 111–129. [\[CrossRef\]](#)
46. Grayston, S.J.; Campbell, C.D.; Bardgett, R.D.; Mawdsley, J.L.; Clegg, C.D.; Ritz, K.; Griffiths, B.S.; Rodwell, J.S.; Edwards, S.J.; Davies, W.J.; et al. Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using clpp, plfa and community dna techniques. *Appl. Soil Ecol.* **2004**, *25*, 63–84. [\[CrossRef\]](#)
47. Zhang, S.; Jiao, X.; Kang, H.; Yu, W. Efficiency evaluation of PLFA method based on lipid standards. *Front. Microbiol.* **2025**, *16*, 1587425. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Wang, R.Z.; Dorodnikov, M.; Dijkstra, F.A.; Yang, S.; Xu, Z.W.; Li, H.; Jiang, Y. Sensitivities to nitrogen and water addition vary among microbial groups within soil aggregates in a semiarid grassland. *Biol. Fertil. Soils* **2016**, *53*, 129–140. [\[CrossRef\]](#)
49. Chen, D.M.; Saleem, M.; Cheng, J.H.; Mi, J.; Chu, P.F.; Tuvshintogtokh, I.; Hu, S.J.; Bai, Y.F. Effects of aridity on soil microbial communities and functions across soil depths on the Mongolian Plateau. *Funct. Ecol.* **2019**, *33*, 1561–1571. [\[CrossRef\]](#)
50. Zhang, T.A.; Chen, H.Y.H.; Ruan, H.H. Global negative effects of nitrogen deposition on soil microbes. *ISME J.* **2018**, *12*, 1817–1825. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Yang, Y.; Cheng, H.; Gao, H.; An, S.S. Response and driving factors of soil microbial diversity related to global nitrogen addition. *Land Degrad. Dev.* **2020**, *31*, 190–204. [\[CrossRef\]](#)
52. Niu, G.X.; Wang, R.Z.; Hasi, M.Q.E.; Wang, Y.L.; Huang, J.H. Availability of soil base cations and micronutrients along soil profile after 13-year nitrogen and water addition in a semi-arid grassland. *Biogeochemistry* **2021**, *152*, 223–236. [\[CrossRef\]](#)
53. Reay, M.K.; Charteris, A.F.; Jones, D.L.; Evershed, R.P. ¹⁵N-amino sugar stable isotope probing (¹⁵N-SIP) to trace the assimilation of fertiliser-N by soil bacterial and fungal communities. *Soil Biol. Biochem.* **2019**, *138*, 107599. [\[CrossRef\]](#)
54. Yuan, X.B.; Niu, D.C.; Gherardi, L.A.; Liu, Y.B.; Wang, Y.; Elser, J.J.; Hua, F. Linkages of stoichiometric imbalances to soil microbial respiration with increasing nitrogen addition: Evidence from a long-term grassland experiment. *Soil Biol. Biochem.* **2019**, *138*, 107580. [\[CrossRef\]](#)
55. Liu, J.; Wu, N.N.; Wang, H.; Sun, J.F.; Peng, B.; Jiang, P.; Bai, E. Nitrogen addition affects chemical compositions of plant tissues, litter and soil organic matter. *Ecology* **2016**, *97*, 1796–1806. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Zheng, Z.; Bai, W.M.; Zhang, W.H. Root trait-mediated belowground competition and community composition of a temperate steppe under nitrogen enrichment. *Plant Soil* **2019**, *437*, 341–354. [\[CrossRef\]](#)
57. Treseder, K.K. Nitrogen additions and microbial biomass: A meta-analysis of ecosystem studies. *Ecol. Lett.* **2008**, *11*, 1111–1120. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Vitousek, P.M.; Aber, J.D.; Howarth, R.W.; Likens, G.E.; Matson, P.A.; Schindler, D.W.; Schlesinger, W.H.; Tilman, D. Human alteration of the global nitrogen cycle: Sources and consequences. *Ecol. Appl.* **1997**, *7*, 737–750. [\[CrossRef\]](#)
59. Zechmeister-Boltenstern, S.; Keiblinger, K.M.; Mooshammer, M.; Peñuelas, J.; Richter, A.; Sardans, J.; Wanek, W. The application of ecological stoichiometry to plant–microbial–soil organic matter transformations. *Ecol. Monogr.* **2016**, *85*, 133–155. [\[CrossRef\]](#)

60. Li, Y.; Liu, Y.H.; Wu, S.M.; Nie, C.; Lorenz, N.; Lee, N.R.; Dick, R.P. Composition and carbon utilization of soil microbial communities subjected to long-term nitrogen fertilization in a temperate grassland in northern China. *Appl. Soil Ecol.* **2018**, *124*, 252–261. [[CrossRef](#)]
61. Kuzyakov, Y.; Xu, X.L. Competition between roots and microorganisms for nitrogen: Mechanisms and ecological relevance. *New Phytol.* **2013**, *198*, 656–669. [[CrossRef](#)] [[PubMed](#)]
62. Loeppmann, S.; Semenov, M.; Blagodatskaya, E.; Kuzyakov, Y. Substrate quality affects microbial- and enzyme activities in rooted soil. *J. Plant Nutr. Soil Sci.* **2015**, *179*, 39–47. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.