Nitrogen deposition experiment mimicked with NH₄NO₃ overestimates the effect on soil microbial community composition and functional potential in the Eurasian steppe

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Abstract

Background: The nitrogenous compound deposited from the atmosphere to the soil is complex, but most field experiments mimic nitrogen deposition with the acid NH₄NO₃ alone. Thus, whether the acid and non-acid nitrogenous compounds have similar effects on biodiversity and ecosystem functions remains understudied. We mimicked nitrogen deposition with acidic NH₄NO₃ and (NH₄)₂SO₄, and non-acidic urea, slow-released urea and NH₄HCO₃ in a temperate steppe, and quantified soil microbial taxonomic and functional gene composition with amplicon sequencing and shotgun metagenomics, respectively.

Results: While NH₄NO₃ and (NH₄)₂SO₄ significantly altered the soil microbial taxonomic and functional composition as well as their carbon decomposition potential, the other three compounds had smaller effects.

Conclusion: Our results suggested that previous nitrogen deposition experiments mimicked with NH₄NO₃ or (NH₄)₂SO₄ alone may have overestimated the effect on biodiversity and ecosystem functions in the Eurasian steppe and similar ecosystems affected by mainly nonacidic nitrogen deposition.

Keywords: Decomposition, Fertilization, Metagenomics, Microbial diversity, Nitrogen deposition

Background

Due to the agricultural fertilization and combustion of fossil fuels, the nitrogen (N) deposition rate has increased from the pre-industrial levels of approximately 0.1–0.3 to as high as 10 g N/m²/year in some developed countries [1–4], and it is predicted that N deposition rate will increase similarly over the next 50 years in many developing countries [3]. N deposition causes a series of ecological consequences, such as changing biological diversity and altering their ecosystem functions. The majority of previous studies focus on the effect of N deposition on plant communities [5–7]. For example, N deposition was found to significantly reduce plant diversity of a mature grassland ecosystem, but had a smaller impact on a nearby degraded ecosystem of the same type [8]. Although soil microbial communities harbor perhaps the highest biodiversity on the planet and they are responsible for many important ecosystem functions such as carbon (C) and nitrogen (N) cycling [9, 10], the effect of N deposition on soil microbial diversity and ecosystem functions remains relatively understudied when compared to the plant communities.

Following the rapid development of amplicon sequencing technologies in the last decade [11, 12], multiple...
studies have focused on the effects of N deposition on soil microbial taxonomic diversity in various ecosystems and the underlying mechanisms [13, 14]. N deposition was found to shift soil microbial diversity and community composition through elevating soil nutrient content, changing soil pH, as well as altering plant productivity and community structure [15–17]. In addition, N deposition was found to affect soil microbial diversity and community composition through mediating both the deterministic (e.g., environmental filtering and interspecific competition) and stochastic processes (e.g., ecological drift and species migration/colonization) [18–20]. In fact, the functional gene content of soil microbial communities is likely to be more closely associated with ecosystem functioning than the taxonomic diversity [21], and the molecular and computational methods allowing for the quantification of functional gene diversity have recently become widely accessible [10, 22]. However, the effect of N deposition on the functional gene diversity and composition of soil microbial communities still remains relatively underexplored.

Numerous field simulation experiments have been conducted to explore the ecological effects and to reveal the underlying mechanisms of N deposition on biodiversity and ecosystem functions [13, 14]. However, the majority of these experiments (>50%) mimicked N deposition by adding NH₄NO₃ [14, 23, 24]. The component of N deposited from the atmosphere to the soil is complex, and includes compounds with different acid levels. For example, (NH₄)₂SO₄, which is a strong acid and weak base salt, is often the main deposited component in areas with intensive animal husbandry [25], while organic N compounds, which are generally neutral, are the main component in remote ecosystems without anthropogenic influences [26]. Across China, neutral N compounds (e.g., organic N compounds) account for an average of 28% of the total N deposited from the atmosphere, and even up to 50% in the steppe ecosystem in Inner Mongolia [27]. Therefore, determining whether NH₄NO₃ and other N compounds have similar effects on soil microbial diversity and ecosystem functions is crucial.

Methods

Site description and experimental design

We conducted a three-year field experiment in a steppe ecosystem (50° 10' 46.1" N, 119° 22' 56.4" E) in Inner Mongolia, China, which is floristically and ecologically representative of much of the Eurasian steppe region. The mean annual precipitation of the site is approximately 363 mm, and the mean annual air temperature is −2.45 °C [28]. The vegetation is dominated by Stipa baicalensis, Leymus chinensis and Carex duriuscula, and the soil is classified as chernozem according to the Food and Agricultural Organization of the United Nations classification [29]. The experiment was established in May 2014, following a randomized complete block design that consisted of six treatments (control, NH₄NO₃, (NH₄)₂SO₄, urea, slow-released urea and NH₄HCO₃) and four replicates for each treatment. The ambient N deposition rate in this region is <1.5 g N m⁻² yr⁻¹ [30] and it is predicted to increase in the future [31]. The addition rate for each N compound is 10 g m⁻² yr⁻¹, mimicking the long-term accumulative effects of N deposition in this region. In each plot, N compound was blended with fine sand (500 g) and applied when the grassland turned green (often early June; once every year). Each plot was 10 × 10 m in area.

Measurement of plant and soil physicochemical indices

In mid-August of 2016 (the time with the highest plant biomass), all aboveground plants were harvested in a 1 × 1 m quadrats from each plot. The plants were sorted by species, and then oven-dried at 65 °C for 48 h and weighted. The total weight of all these plants was calculated as the aboveground plant biomass, and the total species number was counted to represent plant richness. To quantify belowground plant biomass, one soil core with a diameter of 8 cm was collected from each quadrant at 10 cm depth. Roots were collected carefully by a 2 mm sieve, and then oven-dried at 65 °C for 48 h to obtain belowground biomass.

In mid-August of 2016, nine soil cores (10 cm deep, 3.5 cm diameter) were also collected randomly from each plot and mixed to yield one composite sample. Soil samples were stored in a cool box at 4 °C to transport to the laboratory, where the roots and stones were removed using a 2 mm sieve. Part of the composited soil samples was frozen (−20 °C) for DNA extraction, whereas the remaining portion was used to measure soil pH, soil total organic carbon (TOC) content, total N (TN) content, total phosphorus content, dissolved organic carbon content, NH₄⁺-N and NO₃⁻-N content, available phosphorus content and soil moisture. Soil pH was measured in 1:2.5 (W/V) suspensions of soil in deionized water. TOC and TN content were determined by the potassium dichromate-vitriol oxidation method and the Kjeldahl acid-digestion method, respectively [32]. NH₄⁺-N and NO₃⁻-N concentrations were determined on a FIAstar 5000 analyzer (Foss Tecator, Denmark) following 2 M KCl (1:50 w/v) extraction for 30 min [32]. Total phosphorus and available phosphorus concentrations were measured by the ammonium molybdate method after persulfate oxidation. The dissolved organic carbon concentration was determined in soil extracts (1:5 soil water ratio) filtered through a 0.45 mm membrane filter using a TOC analyzer (multi NC 2100S, Analytik Jena AG, Jena,
Germany). Soil moisture was determined as the weight loss after drying for 24 h at 105 °C.

**Amplicon sequencing and sequence processing**

Soil DNA was extracted from 0.25 g fresh soil using the MoBio Power Lyzer Power Soil DNA isolation kit according to the manufacturer’s instructions. To obtain sufficient DNA for subsequent analysis and to overcome the experimental constraints of soil habitat heterogeneity, 4 or 5 extraction replications were mixed to form a composite genetic pool representing the total DNA composition of each sample. The DNA concentration and purity were determined with a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA). The quality was checked on 1% agarose gel. Isolated total DNA was stored at −80 °C for further analysis.

Illumina Miseq sequencing was adopted to quantify bacterial OTU (operational taxonomic unit) diversity. We amplified the fragments of bacterial 16S rRNA V4-V5 using the primers 515F/907R. The primers contained a unique paired barcode sequence for each sample to distinguish samples sequenced in a run. All amplifications were performed in 20 μl reactions containing 4 μl Fast-Pfu Buffer (5×; Transgen), 2 μl of 2.5 mM dNTPs, 10 ng of DNA template, 0.4 μl of each primer (5 μM), 0.2 μl of BSA and 0.4 μl of FastPfu Polymerase (Transgen). The PCR conditions are described as follows: 95 °C for 3 min (denature), 27 cycles of 94 °C at 30 s, 55 °C for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR reactions were performed in triplicate. The PCR products were purified by a AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). Following the quantification by Quantus™ Fluorometer (Promega, USA), the PCR products were pooled at equimolar concentrations for pair-end sequencing with Illumina MiSeq PE300 (Illumina, San Diego, USA) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into NCBI under the project accession number SRP338702.

Raw reads of the 16S rRNA gene were assigned into different sample libraries based on the barcodes and the primers of each read were trimmed. Low quality (Q score < 20) and short reads (length < 300) were discarded. Paired-end reads were merged by FLASH (version 1.2.11) [33] according to at least a 20 bp overlap and < 5% mismatches. Any joined sequences with an ambiguous base or a length of < 300 bp were removed. Thereafter, the OTUs were clustered by UPARSE [34] at a 97% identity [34, 35]. Chimeric sequences were identified and removed using UCHIME [36]. The OTU taxonomic classification of the 16S rRNA gene sequences was assigned using the SILVA database and Ribosomal Database Project Classifier (version 2.11) with 70% confidence estimates [37]. To minimize the effect of unequal sampling on the following calculated indices, 21,093 reads were randomly selected from each sample.

**Shotgun metagenomic sequencing and sequence annotation**

Shotgun sequencing was performed on the Illumina Hiseq 2000 platform at Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). A sequencing library was prepared by NEXTFLEX Rapid DNA-Seq (Bio Scientific, Austin, TX, USA) following the standard procedure of the manufacturer. Briefly, DNA extract was randomly fragmented to roughly 210 bp using a Covaris M220 instrument (Gene Company Limited, China). Adapters containing the full complement of sequencing primer hybridization sites were ligated to the blunt-end of the fragments. The prepared libraries were then sequenced. The sequence data have been deposited in the NCBI Short Read Archive database (Accession Number: SRP338302). Shotgun sequencing resulted in 89.8 ± 2.6 million sequences (mean ± one standard error) or 13.5 ± 0.4 Giga base pairs (Gbp) of sequences per sample.

Paired-end shotgun metagenomic sequences were trimmed and quality controlled using Sickle (https://github.com/najoshi/sickle) and SeqPrep (https://github.com/jstjohn/SeqPrep) with default parameters [22]. Clean reads were then merged using FLASH [33]. Following this, reads > 50 bp were retained and aligned to eggNOG 5.0 and Swiss-Prot [38] using Blastx (E-value < 10−5, coverage > 80%, identity > 30%). A count matrix of the summarized COG (cluster of orthologous groups of proteins) function terms was obtained to evaluate the functional richness and composition of the soil microbial community. Another count matrix was generated to summarize the occurrence of each Swiss-Prot entry in each sample. The corresponding Gene Ontology Annotations of functions and processes for each Swiss-Prot entry were obtained from the uniprot_sprot.dat file provided on http://www.uniprot.org/downloads (downloaded on July 2017). To estimate the effects of N compound on soil carbon degradation potential of soil microbial communities, we focused on the genes involved in the soil carbon decomposition.

**Statistical analysis**

The differences in soil physicochemical indices, microbial diversity indices and the relative abundance of carbon decomposition genes were determined by a general linear model, followed by the least significant difference (P < 0.05) test using SPSS (version 21.0). Bray–Curtis distances calculated from the relative abundances of OTUs and COG terms were used to assess the pairwise distance between taxonomic and functional community
composition, respectively. Bray–Curtis distances calculated from the biomass of plant species were used to represent the dissimilarity in plant community composition. Principle co-ordinates analysis (PCoA) was used to visualize changes in microbial taxonomic and functional community composition. PERMANOVA (permutational multivariate analysis of variance) was performed to test significant changes in microbial taxonomic and functional community composition. The Mantel test was used to reveal the relationship between the soil microbial community structure and environmental factors (soil physicochemical and plant community indices). The spearman correlation was used to analyze the effects of environmental variables on compositional PCoA axis 1 and the relative abundance of dominant bacterial taxa. The statistical significance was determined at $P<0.05$ for all analyses. Partial least squares path modeling (PLS-PM) was performed to further evaluate the direct and indirect effects of environmental factors (soil properties and the plant biomass) on soil microbial taxonomic and functional communities (R plspm package). The path coefficients and explained variability ($R^2$) reflected the cause-and-effect relationships among observed and latent variables. A goodness of fit (GOF) test was used to evaluate the predictive power of the model [39]. Various statistical analyses were carried out using R v4.1.0 software unless otherwise indicated.

**Results and discussion**

General linear model revealed that among these measured indices, the treatments only significantly affected above- and below-ground plant biomass, soil pH, NH$_4^{+}$-N and NO$_3^{-}$-N content (Table 1). Multiple comparisons further revealed that the five N compounds had significantly different effects on these indices (Table 1). In particular, when compared to the control, only NH$_4$NO$_3$, (NH$_4$)$_2$SO$_4$ and NH$_4$HCO$_3$ significantly decreased belowground biomass, while only NH$_4$NO$_3$, (NH$_4$)$_2$SO$_4$ and urea significantly decreased soil pH. NH$_4$NO$_3$ and (NH$_4$)$_2$SO$_4$ are strong acid and weak base salts, NH$_4$HCO$_3$ is a weak acid and weak base salt, and urea and slow-released urea are organic N compounds. Thus, NH$_4$NO$_3$ and (NH$_4$)$_2$SO$_4$ had the largest acidification effect on the soil, inhibiting ammonia volatilization and plant root growth, resulting in higher NH$_4^{+}$-N content and lower belowground plant biomass.

Permutational multivariate analysis of variance (PERMANOVA) revealed that the treatments had a non-significant effect on plant community composition based on species relative biomass ($P>0.05$). In contrast, they had a significant effect on soil bacterial taxonomic composition ($P<0.05$; Fig. 1a). In particular, multiple comparisons revealed that NH$_4$NO$_3$ and (NH$_4$)$_2$SO$_4$ were statistically different from the others (Additional file 1: Table S1), which was also reflected in the axis 1 of the principal coordinate analysis (PCoA; Fig. 1a). More specifically, NH$_4$NO$_3$ and (NH$_4$)$_2$SO$_4$ significantly increased the relative abundances of phylum Actinobacteria and Alphaproteobacteria, while decreased those of Acidobacteria and Chloroflexi (Fig. 2), agreeing with other studies [10, 16]. The Mantel test and correlation analysis revealed that soil NH$_4^{+}$-N content was the main factor controlling soil bacterial community composition and the relative abundances of the main bacterial taxa (Table 2, Additional file 2: Table S2).

Similar to the taxonomic composition, PERMANOVA revealed that the treatments had a significant effect on soil microbial functional gene composition ($P<0.05$; Fig. 1b), and multiple comparisons revealed that NH$_4$NO$_3$ and (NH$_4$)$_2$SO$_4$ were statistically different from the other compounds (Additional file 1: Table S1), which was also reflected in the PCoA axis 1 (Fig. 1b). Moreover, NH$_4$NO$_3$ and (NH$_4$)$_2$SO$_4$ significantly increased the relative abundances of genes for the degradation of relatively recalcitrant organic matters, including aromatics, phenolics, cellulose, lignin, lipids, and polysaccharides (Fig. 3). The Mantel test revealed that functional gene composition and C-decomposition genes were both correlated with soil NH$_4^{+}$-N content, soil pH and belowground biomass (Table 2).

Our results demonstrated that acid N compounds (NH$_4$NO$_3$ and (NH$_4$)$_2$SO$_4$) had stronger effects on soil microbial community and functional potential than the non-acid N compounds (urea, slow-released urea and NH$_4$HCO$_3$). Consistent with our study, Wang et al. [40] in 2018 revealed that NH$_4$NO$_3$ reduced the soil microbial bacterial/fungi ratio, while urea had no significant effect on the ratio in a temperate forest in China. Previous meta-analyses also demonstrated the NH$_4$NO$_3$ exerted a negative impact on microbial biomass and bacterial diversity at the global scale, while urea had no significant impact [24, 41]. Similarly, Wang et al. [42] in 2019 reported that the abundances of the majority of N cycling and C decomposition genes under (NH$_4$)$_2$SO$_4$ addition were significantly higher than those under neutral N compound (KNO$_3$) addition, which was due to the lower soil pH and higher soil ammonium content caused by (NH$_4$)$_2$SO$_4$ addition.

As previously mentioned, NH$_4$NO$_3$ and (NH$_4$)$_2$SO$_4$ are strong acid and weak base salts, thus leading to a stronger soil acidification effect after application [42, 43]. The acidification can impact soil bacterial communities directly through influencing bacterial growth. The maximum values of bacterial growth were observed to be in neutral soils [44, 45]. Therefore, bacterial growth would be inhibited in acidic soils, resulting in the exclusion of
some intolerant bacterial groups [46–48] and a shift in the bacterial function. Alternatively, the higher acidification effects of acid N compounds may impact the bacterial community indirectly through inhibiting ammonia volatilization, nitrification, and the growth of plant roots [49–51] (Additional file 3: Fig. S1). Ammonium is preferentially used by soil organisms due to its low energy cost [52], and has thus been identified as the determinant factor for soil bacterial community composition [53]. In addition, the carbon originating from rhizodeposition is pivotal for soil microbial growth [54–56], while its reduction would definitely impact bacterial growth and their carbon metabolism. Interestingly, we indeed detected that NH4NO3 and (NH4)2SO4 had stronger effects on the bacterial potential function relating to the carbon metabolism. Taken together, NH4NO3 and (NH4)2SO4 had the largest soil acidification effect and higher soil NH4+ content and also decreased plant root biomass, which together led to the changes in soil microbial taxonomic composition, functional gene composition, and also the degradation potential of soil organic matter. Overall, our results demonstrated that acid N compounds (NH4NO3 and (NH4)2SO4) had stronger effects on soil microbial community and functional potential than the non-acid N compounds (urea, slow-released urea and NH4HCO3). Thus, it would be unsuitable to mimic the effects of N deposition by adding NH4NO3 or (NH4)2SO4 alone in areas where non-acidic N compounds (e.g., organic N compounds) contribute the most of N deposition, such as the Eurasian steppe [27].

Table 1  Effects of different forms of N addition on soil, plant and microbial properties

| Indices                                      | Mean (± se) under each treatment | General lineal models |
|----------------------------------------------|----------------------------------|-----------------------|
|                                              | Control | NH4NO3 | Slow-released urea | Urea | NH4HCO3 | (NH4)2SO4 | Block N addition |
| Soil total organic carbon content (g kg⁻¹)   | 42.509 (4.332) | 35.292 (1.609) | 40.679 (2.912) | 40.329 (2.287) | 37.47 (1.409) | 37.462 (0.622) | 0.304 | 0.358 |
| Soil total N content (g kg⁻¹)                | 2.555 (0.070) | 2.593 (0.137) | 2.940 (0.169) | 2.753 (0.072) | 2.705 (0.038) | 2.788 (0.104) | 0.325 | 0.183 |
| C/N                                          | 16.608 (1.578) | 13.72 (0.895) | 13.905 (0.923) | 14.643 (0.697) | 13.88 (0.702) | 13.512 (0.677) | 0.645 | 0.302 |
| Soil total phosphorus content (g kg⁻¹)       | 0.118 (0.008) | 0.108 (0.001) | 0.111 (0.003) | 0.108 (0.003) | 0.105 (0.002) | 0.109 (0.002) | 0.421 | 0.383 |
| Soil NO3⁻-N content (mg kg⁻¹)                | 11.295 (1.556) | 29.540 (5.842) | 49.817 (2.790) | 45.975 (5.173) | 29.924 (1.236) | 27.888 (0.104) | 0.325 | 0.183 |
| Soil NH4⁺-N content (mg kg⁻¹)                | 1.673 (0.316) | 28.950 (6.953) | 22.750 (5.562) | 6.513 (1.379) | 2.213 (0.224) | 115.240 (12.247) | 0.237 | <0.001 |
| Soil dissolved organic carbon content (mg g⁻¹) | 351.345 (61.620) | 258.636 (50.380) | 260.702 (39.512) | 235.843 (34.609) | 216.965 (28.755) | 222.135 (16.549) | 0.414 | 0.268 |
| Soil available phosphorus content (mg g⁻¹)   | 6.450 (1.393) | 6.775 (0.642) | 7.050 (0.972) | 6.650 (0.096) | 6.900 (0.670) | 6.275 (0.256) | 0.486 | 0.984 |
| Soil pH                                       | 6.888 (0.081) | 6.590 (0.079) | 6.735 (0.093) | 6.548 (0.094) | 6.668 (0.029) | 6.030 (0.094) | 0.111 | <0.001 |
| Soil moisture (%)                             | 15.392 (0.271) | 15.414 (0.264) | 15.705 (0.229) | 14.791 (0.747) | 15.401 (0.179) | 15.799 (0.401) | 0.791 | 0.633 |
| Aboveground plant biomass (g m⁻²)            | 130.283 (11.184) | 240.610 (40.884) | 209.565 (25.066) | 214.028 (27.775) | 169.780 (19.101) | 146.265 (23.029) | 0.178 | 0.040 |
| Belowground plant biomass (g m⁻²)            | 110.650 (6.916) | 57.340 (5.901) | 115.420 (27.360) | 93.663 (6.534) | 63.250 (6.680) | 48.168 (10.128) | 0.598 | 0.009 |
| Plant species richness                        | 14.750 (2.462) | 16.500 (1.500) | 13.000 (1.291) | 11.250 (1.031) | 13.750 (1.652) | 15.750 (3.227) | 0.638 | 0.408 |
| Microbial biomass carbon (mg g⁻¹)            | 0.927 (0.081) | 0.779 (0.067) | 0.957 (0.091) | 0.873 (0.069) | 0.993 (0.037) | 0.810 (0.044) | 0.988 | 0.313 |

Different letters within a row indicate significant differences between treatments (P < 0.05)
**Fig. 1** Principal coordinate analysis (PCoA) of soil bacterial composition **a** and soil microbial functional gene composition **b** under different N forms. PERMANOVA: permutational multivariate analysis of variance

**Fig. 2** Relative abundances of dominant bacterial phyla (> 1%) under different forms of N addition. Results are reported as mean±se (n = 4). Different letters indicate significant (P < 0.05) differences among treatments.
In other words, the N deposition experiments simulated with NH₄NO₃ alone are likely to have overestimated the effect on soil microbial diversity and functions in the Eurasian steppe. For example, when the addition of NH₄HCO₃ increased the relative abundances of phylum Actinobacteria by only 9.68% (Table 3), adding NH₄NO₃ resulted in an increase of 24.97%. Hence if the NH₄NO₃ addition is used to mimic N deposition in areas where the actual deposited N is NH₄HCO₃, the effect on the Actinobacteria relative abundance will be overestimated by 157% ((24.97–9.68)/9.68%). Similarly, when the NH₄HCO₃ addition increased the relative abundances of cellulose-degradation genes by only 1.70% (Table 3), adding NH₄NO₃ resulted in an increase of 5.63%, and consequently, a 231% ((5.63–1.70)/1.70%) overestimation.

**Table 2** Relationship between soil microbial community composition (or C-decomposition genes composition) and environmental attributes revealed by Mantel test

|                                | Bacterial community composition | Microbial functional composition | C-decomposition genes composition |
|--------------------------------|---------------------------------|----------------------------------|----------------------------------|
|                                | R  | P  | r   | P  | r   | P  | r   | P  | r   | P  | r   | P  |
| Soil total N content           | 0.118 | 0.837 | 0.048 | 0.663 | 0.079 | 0.218 |
| Soil total organic carbon content | 0.146 | 0.123 | 0.159 | 0.102 | 0.168 | 0.086 |
| Soil NH₄⁺-N content            | 0.201 | 0.052 | 0.073 | 0.201 | 0.156 | 0.064 |
| Soil NO₃⁻-N content            | 0.075 | 0.274 | 0.253 | 0.028 | 0.292 | 0.010 |
| Soil pH                        | 0.138 | 0.048 | 0.201 | 0.003 | 0.222 | 0.005 |
| Aboveground plant biomass      | 0.075 | 0.274 | 0.253 | 0.028 | 0.292 | 0.010 |
| Belowground plant biomass      | 0.131 | 0.122 | 0.240 | 0.010 | 0.255 | 0.008 |

Bold r values indicate significant correlation.

**Fig. 3** Relative abundances of functional genes involved in C degradation. The complexity of carbon is presented in order from labile to recalcitrant. Results are reported as the mean ± se (n = 4). Different letters indicate significant (P < 0.05) differences among treatments.
Conclusion
In this study, we investigated whether NH₄NO₃ and other N compounds had similar effects on microbial communities and the corresponding functions. Acidic NH₄NO₃ and (NH₄)₂SO₄ significantly altered soil microbial taxonomic and functional composition as well as their carbon decomposition potential, while non-acidic urea, slow-released urea and NH₄HCO₃ had smaller effects. This indicates that previous N deposition experiments mimicked with acidic NH₄NO₃ alone in the Eurasian steppe and similar ecosystems may have overestimated the effect on biodiversity and ecosystem functions, and that the actual deposited N compound or even the mixtures of different N compounds should be used to simulate atmospheric N deposition in future studies. Our study was conducted in a meadow steppe with simulated N deposition for just three years. Therefore, the results in this study may differ from those of long-term experiments and other ecosystems. In particular, the effect of these non-acidic N compounds on soil microbial diversity and ecosystem functions may turn to be more significant as the treatment time lasts much longer. Further work is required to test the generality of these results in long-term experiments and other ecosystems.

Table 3  Percent changes in the relative abundances of bacterial phyla and C-decomposition gene categories under different N compound addition treatments when compared with the control

| Bacterial phyla/gene categories | NH₄NO₃ (%) | Slow-released urea (%) | Urea (%) | NH₄HCO₃ (%) | (NH₄)₂SO₄ (%) |
|---------------------------------|-----------|------------------------|---------|------------|--------------|
| Actinobacteria                  | 24.97     | 0.83                   | −2.63   | 9.68       | 19.79        |
| Acidobacteria                   | −40.48    | 2.54                   | 7.83    | −2.88      | −29.95       |
| Alphaproteobacteria             | 34.66     | 0.52                   | −0.02   | 5.87       | 22.81        |
| Chloroflexi                     | −30.27    | −8.54                  | −11.01  | −10.09     | −23.36       |
| Verrucomicrobia                 | −43.85    | 17.99                  | 9.26    | −31.72     | −14.36       |
| Gemmatimonadetes                | −0.44     | −11.23                 | −0.64   | −0.81      | −9.68        |
| Bacteroidetes                   | 2.54      | −11.12                 | −7.62   | −4.54      | −14.37       |
| Betaproteobacteria              | 17.38     | −1.01                  | 10.11   | −4.70      | −14.60       |
| Deltaproteobacteria             | −11.89    | −14.54                 | −12.68  | −4.16      | −19.84       |
| Planctomycetes                  | −27.42    | 19.52                  | 15.53   | −18.46     | −2.13        |
| Nitrospirae                     | −30.23    | −5.47                  | −5.18   | −3.36      | −29.17       |
| Other                           | 7.81      | −6.35                  | 3.07    | 1.39       | 19.58        |
| Monosaccharides                 | −0.22     | −1.25                  | −0.82   | 0.01       | 1.66         |
| Polysaccharides and disaccharides | 6.87   | 3.65                   | 1.83    | 5.27       | 8.36         |
| Sugar acids and alcohols        | 0.71      | 0.36                   | 0.99    | 0.21       | 2.24         |
| Carboxylic acids                | 10.83     | 8.02                   | 4.63    | 6.76       | 12.73        |
| Cellulose, lignin and lipids    | 5.63      | 0.20                   | 3.45    | 1.70       | 7.76         |
| Phenolics                       | 9.24      | 5.87                   | 7.00    | 4.66       | 10.16        |
| Other aromatics                 | 6.33      | 2.70                   | 3.28    | 2.57       | 8.21         |

Abbreviations
PERMANOVA: Permutational multivariate analysis of variance; PCoA: Principal coordinate analysis; C-decomposition: Carbon decomposition; TOC: Total organic carbon; TN: Total nitrogen.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s40793-022-00441-1.

Additional file 1: Table S1. Pairwise PERMANOVA of microbial taxonomic (upper right) and functional (lower left) community composition under different treatments. PERMANOVA: permutational multivariate analysis of variance. Data are the p values.

Additional file 2: Table S2. Correlation between relative abundance of dominant bacterial taxa and environmental variables.

Additional file 3: Fig. S1. Partial least squares path models (PLS-PM) for bacterial communities and C-decomposition potential.

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Author contributions
X-GH and X-MZ designed the research. Y-PM, Y-QS, Z-JZ and G-JY performed the research. T-TL analyzed the data and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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**Availability of data and materials**

The amplicon sequencing and shotgun metagenomic sequencing data in this study was deposited in NCBI under the project accession number SRP338702 and SRP338302, respectively.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

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**References**

1. Vitousek PM, Hooper DU, Levy DE, Ewel CJ, Pickett STA, Reiners W. Human domination of Earth’s ecosystems. Science. 1997;277(5325):494–9.

2. Bakker JP, Berendse F. Constraints in the restoration of ecological diversity in grassland and heathland communities. Trends Ecol Evol. 1999;14(2):63–8.

3. Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA, Karl DM, Michaels AF, Porter JH, Townsend AR, Viessman JW. Nitrogen cycles: past, present, and future. Biogeochemistry. 2004;70:153–226.

4. Stevens CJ, O’Neill RB, Muir AJ, Gowing DJ. Impact of nitrogen deposition on the species richness of grasslands. Science. 2004;303(5665):1876–9.

5. Xia JY, Wang SQ. Global response patterns of terrestrial plant species to nitrogen addition. New Phytol. 2008;179(2):286–309.

6. Deng Q, Hui D, Dennis S, Reddy KC. Responses of terrestrial ecosystem phosphorus cycling to nitrogen addition: a meta-analysis. Global Ecol Biogeogr. 2017;26(6):713–28.

7. Bai TS, Wang P, Ye CL, Hu SJ. Form of nitrogen input dominates N effects on root growth and soil aggregation: a meta-analysis. Soil Biol Biochem. 2021;151:108251.

8. Bai YF, Wu JG, Jia C, Pan QM, Huang JH, Zhang LX, Han XG. Tradeoffs and thresholds in the effects of nitrogen addition on biodiversity and ecosystem functioning: evidence from Inner Mongolia grasslands. Glob Change Biol. 2009;16(1):358–72.

9. Van Der Heijden MG, Bardgett RD, Van Straalen NM. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett. 2008;11(3):296–310.

10. Fierer N, Lauber CL, Ramirez KS, Zaneveld J, Bradford MA, Knight R. Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J. 2012;6:1007–17.

11. Besser J, Carleton HA, Gerner-Smith P, Lindsey R, Trees E. Next-generation sequencing technologies and their application to the study and control of bacterial infections. Clin Microbiol Infect. 2018;24(4):335–41.

12. Ji Z, Li W, Li J. Mblmpmute: an accurate and robust imputation method for microbiome data. Genome Biol. 2021;22(1):192.

13. Zhou Z, Wang CK, Luo YQ. Meta-analysis of the impacts of global change factors on soil microbial diversity and functionality. Nat Commun. 2020;11:3072.

14. Wang C, Liu DW, Bai E. Decreasing soil microbial diversity is associated with decreasing microbial biomass under nitrogen addition. Soil Biol Biochem. 2018;120:126–33.

15. Zhang XM, Han XG. Nitrogen deposition alters soil chemical properties and bacterial communities in the inner Mongolia grassland. J Environ Sci. 2012;24(8):1483–91.

16. Li H, Xu ZW, Yang S, Li XB, Top EE, Wang RZ, Zhang YG, Cai JP, Yao F, Han XG, Jiang Y. Responses of soil bacterial communities to nitrogen deposition and precipitation increment are closely linked with aboveground community variation. Microb Ecol. 2016;71:974–9.

17. Ning QS, Hattenschwiler S, Lu TY, Kardol P, Zhang YH, Wei CZ, Xu CY, Huang JH, Li A, Yang J, Wang J, Peng Y, Penuelas J, Sardans J, He JZ, Xu ZH, Gao YZ, Han XG. Carbon limitation overrides acidification in mediating soil microbial activity to nitrogen enrichment in a temperate grassland. Glob Change Biol. 2021;27(22):5976–88.

18. Zhang XM, Liu W, Bai YF, Zhang GM, Han XG. Nitrogen deposition mediates the effects and importance of change in biodiversity. Mol Ecol. 2011;20(2):429–38.

19. Yang J, Xu MJ, Yang S, Gao LL, Zhang JZ, Wang ZP, Zhang YH, Han XG, Zhang XM. Disturbance-level-dependent post-disturbance succession in a Eurasian steppe. Sci China Life Sci. 2022;65(1):142–50.

20. Li TT, Zhang XM. Research progress of the maintaining mechanisms of soil microbial diversity in Inner Mongolia grasslands under global change. Biodiv Sci. 2020;28(6):749–56.

21. Burke C, Steinberg P, Rusch D, Kjelleberg S, Thomas T. Bacterial community assembly based on functional genes rather than species. Proc Natl Acad Sci U S A. 2011;108(34):14288–93.

22. Zhang XM, Johnston ER, Li LH, Konstantinidis KT, Han XG. Experimental warming reveals positive feedbacks to climate change in the Eurasian Steppe. ISME J. 2017;11:885–95.

23. Treseder K. Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. Ecol Lett. 2008;11:1111–20.

24. Liu LL, Greaver TL. A global perspective on belowground carbon dynamics under nitrogen enrichment. Ecol Lett. 2010;13(7):819–29.

25. Evgenios A, Yu WL, Georgina N, Kota G, Costas JS, Mitsutoshi K, Takayoshi K. Effects of ozone and ammonium sulfate on cauliflower: emphasis on the interaction between plants and insect herbivores. Sci Total Environ. 2019;659:995–1007.

26. van Breemen N. Natural organic tendency. Nature. 2002;415:381–2.

27. Zhang Y, Song L, Liu X, Li WQ, Li SH, Zhong LY, Bai ZC, Cai GY, Zhang FS. Atmospheric organic nitrogen deposition in China. Atmos Environ. 2012;46:195–204.

28. Feng X, Wang RZ, Yu Q, Cao YZ, Zhang YG, Li DJ, Jia YJ, Zhang YH. Decoupling of plant and soil metal nutrients as affected by nitrogen addition in a meadow steppe. Plant Soil. 2019;443:337–51.

29. IUISS Working Group WRB. World reference base for soil resources 2014. International soil classification system for naming soils and creating legends for soil maps. World soil resources reports no. 106. Rome: FAO; 2014.

30. Li XL, Shi HQ, Wu JF, Liu X, Hou DY, Feng F, Yuan WP, Li LH, Xu SY. Seasonal and spatial variations of bulk nitrogen deposition and the impacts on the carbon cycle in the arid/semi-arid grassland of Inner Mongolia, China. PLoS ONE. 2015;10(12):e0144689.

31. Zhu JX, He NP, Wang QF, Yuan GF, Wen D, Yu GR, Jia YL. The composition, spatial patterns, and influencing factors of atmospheric wet nitrogen deposition in Chinese terrestrial ecosystems. Sci Total Environ. 2015;511:777–85.

32. Bao SD. Soil and agricultural chemistry analysis. 3rd ed. Beijing: China Agriculture Press; 2000.

33. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011;27(21):2957–63.

34. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10(10):996–9.

35. Stalkebrandt E, Goebel BM. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol. 1994;44(4):846–9.
36. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27(16):2194–200.

37. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73(16):5261–7.

38. The UniProt Consortium. UniProt: the universal protein knowledgebase. Nucleic Acids Res. 2017;45(1):158–69.

39. Sanchez G, Trinchera L. Pslpm: Partial least squares data analysis methods. R package version 0.2–2. 2012. URL. http://CRAN.R-project.org/package=plspm.

40. Wang Q, Liu SG, Wang YP, Tian P, Sun T. Influences of N deposition on soil microbial respiration and its temperature sensitivity depend on N type in a temperate forest. Agric For Meteorol. 2018;260–261:240–6.

41. Yang Y, Cheng H, Gao H, An SS. Response and driving factors of soil microbial diversity related to global nitrogen addition. Land Degrad Dev. 2019;31(2):190–204.

42. Wang ZJ, Lu GX, Yuan MT, Yu H, Wang S, Li X, Deng Y. Elevated temperature overrides the effects of N amendment in Tibetan grass island on soil microbiome. Soil Biol Biochem. 2019;136:107532.

43. Du YH, Guo P, Liu JQ, Wang CY, Yang N, Jiao ZX. Different types of nitrogen deposition show variable effects on the soil carbon cycle process of temperate forests. Glob Change Biol. 2014;20:3222–8.

44. Gupta UC, Wu K, Liang S. Micronutrients in soils, crops, and livestock. Earth Sci Front. 2008;15:110–25.

45. Rousk J, Baath E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R, Fierer N. Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J. 2010;4:1340–51.

46. Ratcliffe R, Gore J. Modifying and reacting to the environmental pH can drive bacterial interactions. PLoS Biol. 2018;16(3): e2004248.

47. Ling Z, Chen DM, Guo H, Wei JX, Bai YF, Shen QR, Hu SJ. Differential responses of soil bacterial communities to long-term N and P inputs in a semi-arid steppe. Geoderma. 2017;292:25–33.

48. Liu WX, Jiang L, Yang S, Wang Z, Tian R, Peng ZY, Chen YL, Zhang XX, Huang JX, Ling N, Wang SP, Liu LL. Critical transition of soil bacterial diversity and composition triggered by nitrogen enrichment. Ecology. 2020;101(8): e03053.

49. Gerendas J, Zhu ZJ, Bendixen R, Ratcliffe RG, Sattelmacher B. Physiological and biochemical processes related to ammonium toxicity in higher plants. J Plant Nutr Soil Sci. 1997;160(2):239–51.

50. He ZL, Alva AK, Calvert DV, Banks DJ. Ammonia volatilization from different fertilizer sources and effects of temperature and soil pH. Soil Sci. 1999;164(164):750–8.

51. Peng Y, Guo D, Yang Y. Global patterns of root dynamics under nitrogen enrichment. Glob Ecol Biogeogr. 2017;26:102–14.

52. Puri G, Ashman M. Microbial immobilization of 15N-labelled ammonium and nitrate in a temperate woodland soil. Soil Biol Biochem. 1999;31(6):929–31.

53. Nie YX, Wang MC, Zhang W, Ni Z, Hashidoko Y, Shen WJ. Ammonium nitrogen content is a dominant predictor of bacterial community composition in an acidic forest soil with exogenous nitrogen enrichment. Sci Total Environ. 2017;624:407–15.

54. Clemmensen KE, Bahr A, Ovaskainen O, Dahlberg A, Ekblad A, Wallander H, Stenlid J, Finlay RD, Wardle DA, Lindahl BD. Roots and associated fungi drive long-term carbon sequestration in boreal forest. Science. 2013;339(6127):1615–8.

55. Mazzilli SR, Kermanian AR, Ernst OR, Jackson RB, Piñeiro G. Greater humification of belowground than aboveground biomass carbon into particulate soil organic matter in no-till corn and soybean crops. Soil Biol Biochem. 2015;85:22–30.

56. Sokol NW, Bradford MA. Microbial formation of stable soil carbon is more efficient from belowground than aboveground input. Nat Geosci. 2019;12(1):46–53.

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