Soil microbial community responses to short-term nitrogen addition in China's Horqin Sandy Land

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Abstract

Anthropogenic nitrogen (N) addition has increased soil nutrient availability, thereby affecting ecosystem processes and functions in N-limited ecosystems. Long-term N addition decreases plant biodiversity, but the effects of short-term N addition on soil microbial community is poorly understood. The present study examined the impacts of short-term N addition (NH_4NO_3) on these factors in a sandy grassland and semi-fixed sandy land in the Horqin Sandy Land. We measured the responses of soil microbial biomass C and N; on soil β-1,4-glucosidase (BG) and β-1,4-N-acetylglucosaminidase (NAG) activity; and soil microflora characteristics to N additions gradient with 0 (control), 5 (N5), 10 (N10), and 15 (N15) g N m^{-2} yr^{-1}. The soil microbial biomass indices, NAG activity, and soil microflora characteristics did not differ significantly among the N levels, and there was no difference at the two sites. The competition for N between plants and soil microbes was not eliminated by short-term N addition due to the low soil nutrient and moisture contents, and the relationships among the original soil microbes did not change. However, N addition increased BG activity in the N5 and N10 additions in the sandy grassland, and in the N5, N10, and N15 additions in the semi-fixed sandy land. This may be due to increased accumulation and fixation of plant litter into soils in response to N addition, leading to increased microbial demand for a C source and increased soil BG activity. Future research should explore the relationships between soil microbial community and N addition at the two sites.

Key words: Nitrogen addition, Soil microbial biomass, Soil enzyme activity, Soil microflora characteristic, Horqin Sandy Land

Introduction

Nitrogen (N) is the major growth-limiting elements for plant growth in most terrestrial ecosystems, especially in arid and semi-arid ecosystems [1-2]. Changing N availability is therefore an important component of the functions of terrestrial ecosystems, particularly under global climate change scenarios [3-4]. During the 20th century, humans have more than doubled the amount of N added to the biosphere [5]. Anthropogenic N addition in N-limited ecosystems is a primary component of global change, as it can influence the biogeochemical coupling of the soil carbon (C) and N cycles by altering organic matter decomposition [6], and it can profoundly alter soil microbial communities and their enzyme activities [7-8].

Arid and semi-arid ecosystems cover one-third of the world's land surface and account for approximately 15% of the global soil organic carbon pool; they therefore play an important part in maintaining the world's ecosystem functions [9-10]. The Horqin Sandy Land is the largest sandy land in China, and comprises a severely desertified area in China's agro-pastoral ecotone, which has undergone tremendous changes in climate, land use, and anthropogenic N addition [11]. Sandy grassland and semi-fixed sandy land ecosystems are sensitive to increased atmospheric N deposition [12]. The availability of N is an important driver of soil enzyme activity, microbes, and soil microflora characteristics in this area [4, 13].

Soil microbes are a highly sensitive and active component of terrestrial ecosystems, as they respond quickly to environmental changes, such as short-term N enrichment of terrestrial ecosystems, by driving changes in biomass activity and nutrient cycling, as well as changes in the soil microbial biomass, quantity, community structure, and diversity, as well as in soil enzyme activity [14-16].

Soil microbial biomass refers to the volume of soil less than 5000 μm^3 of total living

organisms excluding plant bodies, and which is the most active component of soil organic matter and the most active factor in the soil [17]. Soil microbial biomass is the driving force for the transformation and cycling of soil organic matter and soil nutrients; it is also a reserve for soil nutrients and an important source of nutrients that are available for plant growth, and can therefore be used as an important indicator of soil fertility [18-20]. Previous studies have shown that long-term N addition reduces soil microbial biomass [21-23]. Liu et al. (2010) [15] and Li et al. (2010) [24] showed that long-term N addition decreases soil microbial biomass carbon (SMBC) and nitrogen (SMBN) in temperate steppe and sandy grassland ecosystems in semi-arid areas. However, studies of the effects of short-term N addition on soil microbial biomass in arid and semi-arid ecosystems show considerable disagreement, with researchers reporting increases [25], decreases [24, 26], and no influence [27]. Since soil microbial biomass has such an important effect on nutrient transformations and flows between the soil and plants, it is particularly important to learn their response to N addition to improve our understanding of the mechanisms that underlie nutrient cycling.

Soil enzymes, which are mainly released by soil microbes, play a key role in the decomposition of soil organic matter [28-29]. Soil β-1, 4-glucosidase (BG) hydrolyzes disaccharides and trisaccharides from cellulose to produce smaller molecules, such as glucose, and has been used to characterize C cycles in the soil [30]. Soil β-1, 4-N-acetylglucosaminidase (NAG) participates in the N cycle and is secreted by microbes to hydrolyze chitin and peptidoglycan to produce glucosamine [31]. Soil dehydrogenase (DHA) is mainly found in living cells, and can be used to characterize the overall activity of microbes [32]. Changes in the activities of these soil enzymes can directly reflect the intensity and speed of soil nutrient

79 release by decomposition of organic matter [33].

80 The effect of long-term N addition on soil microbial biomass and soil enzyme activity in
81 farmland and forest ecosystems is reasonably well understood, but the feedbacks among soil
82 microbial biomass, soil enzyme activity, and soil microflora characteristics during the response
83 to short-term N addition in sandy grassland and semi-fixed sandy land ecosystems requires
84 further exploration. In the present study, we obtained data to provide a clearer picture of these
85 feedbacks.

86 Materials and methods

87 Site description and experimental design

88 The two sampling sites were established in a sandy grassland and a semi-fixed sandy land
89 near the Naiman Desertification Research Station of the Chinese Academy of Sciences
90 (42°55′N, 120°42′E), in a semi-arid region of China's Horqin Sandy Land. The distance
91 between the two sampling sites was about 1.5 km. The terrain at the study site is flat and open,
92 with an elevation of 377 m asl. The region has a continental semi-arid monsoon temperate
93 climate, with an annual mean temperature of 6.8°C, with mean monthly temperatures ranging
94 from -9.63 °C in January to 24.58 °C in July, and with an annual mean precipitation of 360 mm,
95 70% of which occurs during the period from May to September. The soils of the two sampling
96 sites were chestnut soils (Chinese soil classification). Table 1 summarizes the physical and
97 chemical properties, initial values of soil microbial indices, and enzyme activity of the topsoil
98 (to a depth of 20 cm) at both sampling sites. The dominant native plant species of the sandy
99 grassland were *Messerschmidia sibirica*, *Setaria viridis*, and *Eragrostis pilosa*, and those of the
100 semi-fixed sandy land were *Caragana microphylla*, *Setaria viridis* and *Echinops gmelinii*. The

vegetation cover was 60 and 30 %, respectively.

Table 1 The physical and chemical properties, initial values of soil microbial indices, and enzyme activity of the topsoil (to a depth of 20 cm) at the sandy grassland and semi-fixed sandy land sites. Values represent means ± SD (*n* = 24).

Parameter	Sandy grassland	Semi-fixed sandy land
SOC (g kg^{-1})	1.67±0.001a	3.04±0.001b
TN (g kg^{-1})	0.12±0.009a	0.32±0.014b
TP (g kg^{-1})	0.20±0.013a	0.19±0.005a
pH	8.15±0.027a	8.50±0.150b
EC (μS cm^{-1})	16.76±0.517a	26.75±0.680b
SMBC (mg kg^{-1})	32.24±2.600a	26.05±2.706a
SMBN (mg kg^{-1})	4.80±0.766a	5.86±0.734a
BG (U g^{-1})	5.29±0.353a	16.32±1.165b
NAG (U g^{-1})	0.89±0.143a	2.30±0.323b
DHA (U g^{-1})	n.d.	n.d.

Note: Values of a parameter followed by different letters differ significantly between the two sampling sites (One-way ANOVA followed by LSD test, $P < 0.05$). SOC, soil organic C; TN, total nitrogen; TP, total phosphorus; EC, electric conductivity; SMBC, soil microbial biomass carbon; SMBN, soil microbial biomass nitrogen; BG, soil β-1,4-glucosidase activity; NAG, soil β-1,4-N-acetylglucosaminidase activity; DHA, soil dehydrogenase activity; n.d., not detected

We established 24 plots, each 1 m × 1 m, in May 2019. The treatments were a control and nitrogen addition at 5 g N m^{-2} yr^{-1} (N5), 10 g N m^{-2} yr^{-1} (N10), and 15 g N m^{-2} yr^{-1} (N15), are these values based on current atmospheric deposition levels (0.50 g N m^{-2} yr^{-1}) and the predicted levels in 10 (N5), 20 (N10) and 30 (N15) years [34]. The blocks were separated by a 2.0-m-wide buffer strip, and the plots within each block were separated by a 1.0-m-wide buffer strip to minimize disturbance from neighboring plots. Nitrogen (NH_4NO_3) addition was applied once, before it rained, in mid-May 2019.

We used a 2.5-cm-diameter auger to collect topsoil samples (to a depth of 20 cm) on 15 May 2019, early in the growing season, and on 15 August 2019, at the peak of the growing season from minimally disturbed natural soils. At each site, we collected topsoil at five random locations within each plot (1 m ×1 m) and homogenized them to provide a single composite soil sample, which we packed in sterilized polyethylene bags and transported to the lab in coolers portable car refrigerators as quickly as possible. All visible roots, residues, and stones were removed by sieving (with a 2-mm square-aperture mesh). Every sample was divided into two equal subsamples. One was stored at 4 ℃ to determine the soil properties, and the other was stored at -80 ℃ until to DNA extraction.

Measurement of microbial biomass

We used a fumigation-extraction method to measure SMBC and SMBN [35]. In summary, three fresh 50-g soil samples were placed in separate 100-mL beakers, and were then incubated in the dark for 7 days at 25 ℃ and a relative humidity of 70%. One soil sample was used as the control, and another was fumigated for 24 h with ethanol-free $CHCl_3$. The last soil sample was used to measure the soil moisture content. The control and the fumigated soil samples were transferred into 250-mL Erlenmeyer flasks, then 100 mL of 0.5 M K_2SO_4 was added, and the solution was shaken for 30 min at 25 ℃ to obtain soil extracts. Extracts were filtered through 0.45-μm cellulose ilters and stored at -20 ℃ until analysis. The SMBC and SMBN contents were measured using an Elementar Vario TOC (Elementar, Langenselbold, Germany). SMBC and SMBN were calculated from the difference between the extractable C and N contents in the fumigated and control samples using conversion factors: kEC for C and kEN for N were both equal to 0.45.

Enzyme activity

The enzyme activities of the soil BG and NAG were quantified using commercial enzyme kits following the manufacturer's protocol (BG Assay kit and NAG Assay kit; Solarbio, Beijing, China). Briefly, BG decomposes p-nitrobenzene-β-D-glucopyranoside to form p-nitrophenol, and NAG decomposes p-nitrobenzene β-N-acetylglucosamine to also form p-nitrophenol, which has a maximum absorption peak at 400 nm. We used a UV-VIS spectrophotometer (UV-1800, Mapada Instruments Co., Shanghai, China) to measure the absorbance. BG and NAG activities were calculated by measuring the rate of increase in absorbance. DHA activity was also measured using a commercial enzyme kit (the DHA Assay kit, Solarbio). 2, 3, 5-triphenyl tetrazolium chloride is reduced to triphenyl formazone after receiving hydrogen during cell respiration. Triphenyl formazone is red and has a maximum absorption peak at a wavelength of 485 nm, and its absorbance was also measured by UV-VIS spectrophotometry to obtain the DHA activity.

DNA Extraction

From each sample, total DNA was extracted from 0.5 g of soil using the PowerSoil kit (Omega Laboratories Inc., Mogadore, OH, USA) according to the manufacturer's instructions. The integrity of the DNA was determined by electrophoresis in 1.0% agarose gels, and the purity and concentration of the DNA were measured spectrophotometrically with a NanoDrop ND5000 (Thermo Fisher Scientific Inc., USA).

Quantitative Real-time Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed using a Line-Gene 9600 Plus Cycler (Thermo Fisher Scientific Inc.). The hyper-variable 444 bp V3 to V4 region of the bacterial

16S rRNA was amplified for each sample using two primers (338F, 5′-ACTCCTACGGGAGGCAGCAG-3′; 806R, 5′-GGACTACHVGGGTWTCTAAT-3′) [36]. Similarly, the 317-bp ITS1 region of the fungal ITS rRNA was amplified for each sample using two primers (ITS1F, 5'-CTTGGTCATTTAGAGGAAGTAA-3'; ITS2R, 5'-GCTGCGTTCTTCATCGATGC-3') [37].

To estimate bacterial and fungal small-subunit rRNA gene abundances, we generated standard curves using a 10-fold serial dilution with a plasmid containing a full-length copy of either the *Escherichia coli* 16S rRNA gene or the ITS rRNA gene. Quantitative PCR (*q*PCR) was performed with 25 mg of the sample mixed with 12.5 mL of ChamQ SYBR Color qPCR Master Mix (2X) (Vazyme Biotech Co., Ltd, Nanjing, China), 0.5 mL solutions (10 mM) of each forward and reverse primer, and 9.5 mL of sterile, double-distilled H_2O. Standard and environmental DNA samples were added at 2.0 mL per reaction. The reaction was carried out on a Line-Gene 9600 Plus Cycler (Thermo Fisher Scientific Inc.). The cycling program was an initial denaturation at 95 ℃ for 3 min, followed by 40 cycles of 94 ℃ for 30 s, 53 ℃ for 30 s, and 72℃ for 45 s, with a final extension at 72 °C for 5 min. Melting curve and gel electrophoresis analyses were performed to confirm that the amplified products were of the appropriate size. Bacterial and fungal gene copy numbers were generated using a regression equation for each assay that related the cycle threshold (*Ct*) value to the known number of copies in the standards. All of the qPCR reactions were run in triplicate for each soil sample. The average bacterial PCR efficiency was 92.22% with an R^2 of the standard curves of 0.9991, and the fungal PCR efficiency was 91.99% with an R^2 of the standard curves of 0.9995.

PCR Amplification and Illumina MiSeq Sequencing

PCR was carried out in triplicate in a 20-μL reaction volume that contained 4 μL of 5-fold reaction buffer, 4 μL of dNTPs (2.5 mM), 0.8 μL of each primer (5 μM), 1 μL of template DNA (ca. 10 ng), and 0.4 μL of Pfu DNA Polymerase (TransStart-FastPfu DNA Polymerase, TransGen Biotech, Beijing, China), with double-distilled H_2O to bring the solution to the final volume. The PCR program included an initial denaturation at 95°C for 3 min; 35 cycles at 94°C for 30 s, annealing at 55°C for 30 s, and an extension at 70°C for 45 s; and a final extension at 72 °C for 10 min. PCR was performed using an ABI GeneAmp 9700 Cycler (Thermo Fisher Scientific Inc.).

Different barcode sequences were added at the 5′ end of the forward primer to separate corresponding reads from the data pool that was generated in a single sequencing run. The amplicons were extracted by electrophoresis in 2.0% agarose gels, purified by using a Gel Extraction Kit (Axygen Co., Hangzhou, China) according to the manufacturer's instructions, and quantified using a QuantiFluor-ST Fluorimeter (Promega, Fitchburg, WI, USA). The purified amplicons were pooled in an equimolar and paired-end sequence (2×300) on an Illumina MiSeq PE300 Sequencer (Majorbio Co. Ltd., Shanghai, China) according to the manufacturer's standard protocols.

Statistical analysis, processing, and analysis of the sequencing data

We tested for differences in the soil properties, soil microbial biomass indices, and soil enzyme activity between the sandy grassland and semi-fixed sandy land with different N addition levels using one-way analysis of variance (one-way ANOVA). Site type and N addition were used as treatment factors to conduct two-factor ANOVA for soil microbial indicators and enzyme activity. The data were tested to confirm normality and homogeneity of variance

(Levene's test) prior to ANOVA. When the ANOVA results were significant, we used the least-significant-difference test to identify significant differences between pairs of values, with significance at $P < 0.05$. The analyses were performed using version 19.0 of the SPSS software (https://www.ibm.com/analytics/spss-statistics-software).

Raw FASTQ files were de-multiplexed and quality-filtered using version 0.35 of the Trimmomatic software (http://www.usadellab.org/cms/?page=trimmomatic) with the following criteria: (i) The 300-bp reads were truncated at any site that obtained an average quality score less than 20 over a 50-bp sliding window, and truncated reads shorter than 50 bp were discarded. (ii) We removed the extracted matching barcodes, and any two-nucleotide mismatches in the primer matching and reads that contained ambiguous characters. (iii) Only overlapping sequences longer than 10 bp were assembled according to their overlapping sequence. Reads that could not be assembled were discarded.

Quality sequences were aligned in accordance with the SILVA alignment database (https://www.arb-silva.de/) [38] and clustered into operational taxonomic units (OTUs) using version 7.1 of the USEARCH software (https://www.drive5.com/usearch/). OTUs with a 97% or better similarity level were used for the rarefaction curve, and we calculated the α-diversity indices, including the ACE, Chao, Shannon, and Simpson diversity indices, and performed coverage analysis using version 1.30.2 of the mothur software (https://www.mothur.org/) [39]. Taxonomic assignments of the OTUs with at least 97% similarity were performed using mothur in accordance with the SILVA (132) or Unite (8.0) databases with a 70% confidence interval. For taxonomic analysis, we used the SILVA database and the Unite database (http://unite.ut.ee/index.php) for bacteria and fungi, respectively. For β-diversity analysis, we

performed principal-components analysis (PCA) and generated a hierarchical heatmap using version 2.5-6 the vegan package (https://cran.r-project.org/web/packages/vegan/index.html) for version 3.2.0 of the R statistical software (https://www.r-project.org/).

Data deposition

All sequencing data associated with this study have been deposited at the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under project accession number PRJNA615072.

Results

Changes in microbial biomass indices

In the sandy grassland, the soil biomass microbial indices (SMBC, SMBN, and SMBC/SMBN) did not differ significantly among N additions ($P > 0.05$, Table 2). All N addition levels were significantly decreased SMBC, and control level was significantly decreased SMBN compared with the background ($P < 0.05$, Table 2). In the semi-fixed sandy land, N addition significantly decreased SMBC and SMBN compared with the control ($P < 0.05$, Table 2), but with no significant differences among N addition levels ($P > 0.05$). SMBC/SMBN did not differ significantly from the control at any N addition level. In compared with background, control level was significantly increased SMBC, and all N addition levels were significantly increased SMBC/SMBN ($P < 0.05$, Table 2). We also compared the soil microbial biomass indices in a given treatment between the sampling sites (Table 2). In the control, SMBC and SMBN were significantly higher in the semi-fixed sandy land ($P < 0.05$), but their ratio did not differ significantly. However, the SMBC, SMBN, and SMBC/SMBN did not differ significantly between the two sampling sites in any N treatment ($P > 0.05$). In the

249 background, the SMBC/SMBN of sandy grassland were significantly higher in the semi-fixed

250 sandy land ($P < 0.05$).

Table 2 The soil microbial biomass indices (soil microbial biomass carbon (SMBC), soil microbial biomass nitrogen (SMBN), and SMBC/SMBN ratio) and soil enzyme activities (β-1,4-glucosidase (BG), soil N-acetyl-β-D-glucosidase (NAG), and soil dehydrogenase activity (DHA)) in the topsoil (to a depth of 20 cm) in the sandy grassland and semi-fixed sandy land. Nitrogen addition treatments are no N addition (Control), 5 g N m^{-2} yr^{-1} (N5), 10 g N m^{-2} yr^{-1} (N10), and 15 g N m^{-2} yr^{-1} (N15).

Parameter	Sandy grassland					Semi-fixed sandy land				
	Background	Control	N5	N10	N15	Background	Control	N5	N10	N15
SMBC (mg kg^{-1})	32.24±2.60Ab	17.16±2.59Aa	16.32±4.37Aa	16.81±4.45Aa	13.48±1.97Aa	26.05±2.71Aa	62.99±14.02Bb	25.50±1.98Aa	20.36±6.87Aa	23.57±3.93Aa
SMBN (mg kg^{-1})	4.80±0.77Ab	2.06±0.36Aa	4.07±0.57Aab	3.28±0.80Aab	2.34±0.38Aab	5.86±0.73Aab	8.31±1.65Bb	4.16±0.89Aa	3.10±0.99Aa	4.36±1.38Aa
SMBC/SMBN	7.84±0.90Ba	7.79±2.72Aa	4.79±1.49Aa	6.44±1.84Aa	5.24±0.62Aa	4.77±0.41Aa	7.49±0.61Ab	6.75±1.57Aab	5.31±1.44Aab	6.03±0.68Aab
BG(U g^{-1})	5.29±0.35Aa	11.98±0.36Bc	14.26±0.60Ad	19.49±0.60Be	6.99±0.43Ab	16.32±1.17Bb	6.44±0.70Aa	11.67±1.00Aab	11.09±0.71Aab	14.06±0.29Bb
NAG (U g^{-1})	0.89±0.14Aa	1.48±0.14Aa	1.21±0.22Aa	2.64±0.16Ab	2.79±0.27Ab	2.30±0.32Ba	3.50±0.83Ba	2.73±0.32Ba	2.63±0.18Aa	2.85±0.84Aa
DHA(U g^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Note: Values in a column with different lowercase letters represent significant difference between different N addition levels under same site (One-way ANOVA followed by LSD test, $P < 0.05$); those with different capital letters represent significant difference between different site under same N addition level ($P < 0.05$). Background, Initial value measured in mid-May; n.d., not detected.

The results of two-way ANOVA showed that site type, N addition and their interactions had a significant effect on SMBC, and SMBN was significantly affected by site type and the interactions between site type and N addition ($P < 0.01$, Table 3).

Table 3 Two-way ANOVA results of site type, N addition and their interactions on soil microbial biomass indices (soil microbial biomass carbon (SMBC), soil microbial biomass nitrogen (SMBN), and SMBC/SMBN ratio) and soil enzyme activities (β-1,4-glucosidase (BG), soil N-acetyl-β-D-glucosidase (NAG), and soil dehydrogenase activity (DHA)) in the topsoil (to a depth of 20 cm).

Parameter	Site type	N addition	Site type×N addition
SMBC	20.12**	4.83**	5.15**
SMBN	10.81**	1.25	4.64**
SMBC/SMBN	0.02	1.29	0.43
BG	29.91**	41.86**	53.64**
NAG	8.36**	2.31	3.00
DHA	——	——	——

Note: Significant levels (ANOVA followed by LSD test): **, P < 0.01; *, P < 0.05. SMBC, soil microbial biomass carbon; SMBN, soil microbial biomass nitrogen; BG, soil β-1,4-glucosidase activity; NAG, soil β-1,4-N-acetylglucosaminidase activity; DHA, soil dehydrogenase activity.

Changes in soil enzyme activities

N addition changed soil enzyme activities, but the effect depended on the enzyme and the sampling site (Table 2). DHA activity at both sampling sites was below the detection limit, so in the rest of this paper, we focus on changes of the BG and NAG activities. In the sandy grassland, N addition significantly increased BG activity compared with the control in N5 and N10, but significantly decreased BG activity in N15 ($P < 0.05$). All N addition significantly

increased BG activity compared with the background ($P < 0.05$). NAG activity had no significantly difference in control and N5, but increased significantly in N10 and N15 ($P < 0.05$). In the semi-fixed sandy land, N addition significantly increased BG activity compared with the control in all three treatments ($P < 0.05$), but there was no significant difference between N5, N10, and N15 ($P > 0.05$). NAG activity did not differ significantly among the treatments and background ($P > 0.05$). The results of two-way ANOVA showed that site type, N addition and their interactions had a significant effect on BG, and NAG was significantly affected by site type and the interactions between site type and N addition ($P < 0.01$, Table 3).

Microbial abundance

We used *q*PCR to determine the gene copy numbers for the total bacteria and fungi species at the two sampling sites (Fig 1). For bacteria, the 16S RNA gene copy numbers in the sandy grassland and semi-fixed sandy land ranged from $7.88\times10^7 \pm 1.41\times10^7$ to $9.91\times10^7 \pm 1.09\times10^7$ copies/g and from $7.11\times10^7 \pm 0.80\times10^7$ to $20.7\times10^7 \pm 6.09\times10^7$ copies/g, respectively. There were no significant differences in soil bacterial abundance between the sandy grassland and semi-fixed sandy land ($P > 0.05$). For fungi, the ITS RNA gene copy numbers in the sandy grassland and semi-fixed sandy land ranged from $2.44\times10^6 \pm 0.47\times10^6$ to $5.19\times10^6 \pm 2.41\times10^6$ copies/g and from $1.15\times10^6 \pm 0.16\times10^6$ to $11.3\times10^6 \pm 5.52\times10^6$ copies/g, respectively. There were also no significant differences in soil fungal abundance between the sandy grassland and semi-fixed sandy land ($P > 0.05$). In addition, the bacterial and fungal abundance did not differ significantly between sandy grassland and semi-fixed sandy land at any N addition level ($P > 0.05$).

Fig 1. Comparison of the bacterial 16S rRNA gene and fungal ITS rRNA gene copy

numbers from the soils at the two sampling sites as determined by qPCR. Values are means ± SD. Nitrogen addition treatments: control, no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15, 15 g N m^{-2} yr^{-1}.

MiSeq sequencing and α-diversity indices

We obtained 1,131,376 valid reads and 2397 OTUs from the 24 samples through Illumina MiSeq sequencing analysis and classified the bacteria in these samples. Each library contained from 40,037 to 51,789 reads, with 1811 to 2086 different phylogenetic OTUs. The average length of high-quality sequences ranged from 412.779 to 417.805 bp. Similarly, we obtained 1,604,773 valid reads and 473 OTUs for fungi, and each library contained from 56,652 to 73,089 reads, with 161 to 373 different phylogenetic OTUs. The average length of high-quality sequences ranged from 230.261 to 250.127 bp.

Rarefaction curves approached saturation in all samples, indicating that the data volume in the sequenced reads was reasonable, and the discovery of a high number of reads contributed relatively little to the total number of OTUs. The curves show that only a very small fraction of the new phylotypes of the bacteria was retrieved after 50,000 sequencing reads, while the fungi was retrieved after 10,000 sequencing reads. This rarefaction curve indicated the presence of low variation in the total number of OTUs among the different samples (S1 Fig).

We estimated the α-diversity based on the observed species using the ACE, Chao, Shannon, and Simpson diversity indices. The results for bacterial and fungal diversity are summarized in S1 and S2 Tables, respectively. The observed species score (number of OTUs) for the bacterial communities ranged from 1811 to 2086, and the ACE and Chao scores ranged from 4242.927 to 5532.818 and from 4119.895 to 5489.310, respectively. The Shannon and Simpson scores

ranged from 6.159 to 6.840 and 0.0030 to 0.0153, respectively. The species score (number of OTUs) for the fungal communities ranged from 161 to 373, and the ACE and Chao scores ranged from 173.659 to 395.885 and from 172.143 to 397.459, respectively. The Shannon and Simpson scores ranged from 1.386 to 4.081 and 0.039 to 0.581, respectively.

Taxonomic composition

The samples yielded different numbers and abundance of OTUs. Sequences that could not be classified into any known group or that had an undetermined taxonomic position were assigned as unclassified or no rank group, respectively.

The bacterial OTUs were assigned into 26 phyla, 236 families, and 475 genera. Of the prokaryotic phylotypes, 10 of the 26 phyla were common to the 24 libraries: Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Nitrospirae, Patescibacteria, and Proteobacteria (Fig 2A), and comprised more than 98% of the total reads in every library. Proteobacteria and Actinobacteria were the two most abundant groups, comprising approximately 31.6% (357,483 reads) and 30.7% (347,423 reads) of the total reads across all samples, respectively. However, the proportions of Firmicutes and Actinobacteria varied widely among the samples, with values ranging from 19.8 to 48.1% and from 13.5 to 38.3%, respectively. The proportion of Proteobacteria reached their lowest value in sample G3N15, which was significantly different from that in samples of G1N15 and G5N15. (Sample names are defined as the site type [G, sandy grassland; S, semi-fixed sandy land] followed by the N addition treatment.) Acidobacteria and Chloroflexi were the third- and fourth-most abundant groups, comprising 14.7% (166,330) and 10.17% (115,083) of the reads, respectively, across all samples. Members of the Bacteroidetes, Gemmatimonadetes, Firmicutes,

Patescibacteria, Cyanobacteria, and Nitrospirae accounted for 3.4% (38,273 reads), 3.2% (36,720 reads), 2.4% (27,384 reads), 1.3% (14,819 reads), 0.6% (6,537 reads), and 0.5% (6,046 reads) of the reads in all libraries combined. The other groups represented a small fraction (ca. 1.4%) of the total bacterial community.

Fig 2A. Relative abundance of phylotypes in the bacterial community. Sample names are the sampling location (G, sandy grassland; S, semi-fixed sandy land) followed by the nitrogen addition treatment: Control, no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15, 15 g N m^{-2} yr^{-1}.

The fungal communities were assigned to 8 phyla, 105 families, and 167 genera. Among them, Ascomycota was the dominant group, comprising 69.0% (1,107,374 reads) of the total reads (Fig 2B). Mortierellomycota was the second-largest group, accounting for 26.6% (427,349 reads) of the total reads. However, the proportions of Ascomycota and Mortierellomycota varied widely among the samples, accounting for 19.2 to 93.0% of the reads and 14.9 to 80.4% of the reads, respectively. The proportion of Ascomycota reached its lowest value in S1CK, and was significantly different from the proportions in samples of S3CK and S5CK The other fungal phyla accounted for only 4.4% of the total (70,050 reads): Basidiomycota (2.1%, 34,190 reads), Fungi_unclassified (1.9%, 30,215 reads), Chytridiomycota (0.2%, 3201 reads), Calcarisporiellomycota (0.1%, 1,383 reads), Olpidiomycota (<0.1%, 826 reads), and Glomeromycota (<0.1%, 235 reads).

Fig 2B. Relative abundance of phylotypes in the fungal community. Sample names are the sampling location (G, sandy grassland; S, semi-fixed sandy land) followed by the nitrogen addition treatment: Control, no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15,

15 g N m^{-2} yr^{-1}.

Microbial community structure

To analyze the similarity of the bacterial communities in the different samples, we constructed a heatmap using hierarchical cluster analysis. For bacteria, the heatmap was based on the 50 most abundant bacterial genera, and this divided the bacteria into two main groups (Fig 3A). One was mainly composed of genera from the SN10 group, including S3N10 and S5N10, and grouped them with S5N15, S1N15, S1N10, and S3N15; the other grouped the members from the other samples together. The PCA results also revealed that bacterial communities from samples of SN10 group and SN15 group were grouped together at the right side of the graph along PC1, whereas the other samples were grouped at the left along PC1, with PC1 accounting for 39.4% of the total variations (Fig 4A). PC2 only accounted for 19.8% of the variation, but again separated the samples of SN10 and SN15 group from the other samples.

Fig 3A. Heat map representations and cluster analysis for the microbial community based on 24 samples from the two sampling sites. Bacterial distributions for the 50 most-abundant genera and families. The double hierarchical dendrogram shows the bacterial and fungal distribution. Bacterial and fungal phylogenetic trees were calculated using the neighbor-joining method. Sample names are composed of the sampling site (G, sandy grassland; S, semi-fixed sandy land) and nitrogen addition: Control (CK), no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15, 15 g N m^{-2} yr^{-1}.

Fig 4A. Results of the principal-components analysis (PCA) on bacterial communities. Plots are based on the UniFrac distance. Sample names are composed of the sampling site

(G, sandy grassland; S, semi-fixed sandy land) and nitrogen addition: Control (CK), no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15, 15 g N m^{-2} yr^{-1}.

For fungi, the heatmap was based on the top 50 families (Fig 3B). The samples could be divided into two clusters at the family level: one was mainly composed of the samples G1N10, S5C, S5N15, S3N5, and grouped with S1N10, S1N5, S5N5, G3N15, G5N5, S1C, S5N10, S1N15, and S3N15, and the other cluster grouped the rest of the samples together. The PCA plot grouped the fungal communities from the samples G5N5, S1N5, S3N15, G3N15, S5N10, and S1C together to the right along PC1, which accounted for 38.8% of the variation, and PC2 (which accounted for 12.5% of the variation) produced the same separation of the two sample groups (Fig 4B).

Fig 3B. Heat map representations and cluster analysis for the microbial community based on 24 samples from the two sampling sites. Fungal distributions for the 50 most-abundant genera and families. The double hierarchical dendrogram shows the bacterial and fungal distribution. Bacterial and fungal phylogenetic trees were calculated using the neighbor-joining method. Sample names are composed of the sampling site (G, sandy grassland; S, semi-fixed sandy land) and nitrogen addition: Control (CK), no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15, 15 g N m^{-2} yr^{-1}.

Fig 4B. Results of the principal-components analysis (PCA) on fungal communities. Plots are based on the UniFrac distance. Sample names are composed of the sampling site (G, sandy grassland; S, semi-fixed sandy land) and nitrogen addition: Control (CK), no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15, 15 g N m^{-2} yr^{-1}.

Discussion

The effects of N addition on soil microbial biomass indices

Our result showed the peaking growing season of SMBC and SMBN were significant higher those of background (Table 2), which in consistent with previous studies [40-41]. The SMBC and SMBN were lower in the peak growing season and higher in the dormancy season. This may be due to the large demand for soil nutrients by plants at the peak of the growing season limited the availability of nutrients by soil microbes, so SMBC and SMBN were decreased in the peaking growing [42].

Our goal was to determine the effects of changes in the soil microbial biomass indices (SMBC, SMBN, SMBC/SMBN) resulting from N addition at different levels. We found no significant effects of short-term N addition on these indices in the sandy grassland, which agrees with previous reports [43-45]. In the short term, the activity of soil microbes is regulated more strongly by plants than by the direct effects of N addition [45]. The plants competed for nutrient elements strongly than the microbes for the available N, and the original relationships among soil microbes may not change immediately in response to N addition. This may explain why we observed no difference in soil microbial biomass among the short-term N addition treatments in the sandy grassland. This could be attributed to the small changes in the plant community during the first growing season [45]. However, the effect of N addition on grassland soil microbial biomass depended on the amount, type, time, and initial level of nitrogen addition in grasslands in previous research. It is widely accepted that long-term N addition decreases soil microbial biomass [46-48], mainly because under natural conditions, the promoting effect of N addition on plant growth will decrease over time, resulting in a reduction of the amount of plant residues and litter that is input into the soil, and the increase of soil microbial biomass

will then be inhibited by a lack of sufficient carbon [47]. We plan to continue the N addition experiments at the same sites to explore long-term changes in the microbial biomass indices.

In the semi-fixed sandy land, short-term N significantly decreased SMBC and SMBN, but their ratio was unaffected by the N addition. *Caragana microphylla* is the dominant species in the ecosystem, and the species has solid nitrogen capacity, so N addition could reduce the quantity and quality of its root exudates, thereby decreasing SMBC and SMBN [49]. Some studies have shown that the addition of N alleviated the N limitation of the soil, but that the plants decreased their allocation of resources to belowground biomass by decreasing root growth and releasing of easily decomposed materials, thereby inhibiting the growth of microorganisms [50-51]. In addition, the lack of C input to the soils represents a substrate limitation that can decrease SMBC. Dijkstra et al. (2005) [52] showed that short-term N application reduced SMBC and SMBN in tall grasses in Minnesota (United States), and explained that this resulted from a reduction of plant root secretion, resulting in an insufficient carbon source available to soil microbes, which is similar to the present results.

SMBC and SMBN in the semi-fixed sandy land were significantly higher than those in sandy grassland in the control. At the same N addition level, there was no significant difference in the microbial indices between the two sampling sites. The differences of SMBC and SMBN between the two sampling sites may have resulted from differences in the dominant plants. The nitrogen-fixing effect of the root system of *Caragana microphylla* and the existence of a large amount of root exudates may increase the soil microbial biomass compared with an annual or perennial herb-based ecosystem such as that in the sandy grassland [53].

The effects of N addition on soil enzymes

451 The N5 and N10 additions significantly promoted BG activity in the sandy grassland,
452 whereas N15 significantly decreased BG activity. N5, N10, and N15 additions significantly
453 increased the activity of BG in the semi-fixed sandy land. Thus, N addition increased the overall
454 activity of BG at both sampling sites. The change of BG activity reflects the variety of organic
455 matter in the soil. With increasing N content, the N limitation for microbe decreases, and N
456 addition promoted the accumulation and fixation of plant litter in the soil, leading to an
457 increased carbon source for soil microorganisms to meet their demand and increasing BG
458 activity in the soil [54]. Many studies have shown that N addition can promote BG activity [13,
459 55-56], and our results agree with that previous research. There were no differences in BG
460 activity between the two sampling sites for other N addition treatment, but BG activity in the
461 control and N10 were significantly higher in the sandy grassland than in the semi-fixed sandy
462 land. This may be because SOC and SMBC were lower in the sandy grassland than in the semi-
463 fixed sandy land. Soil enzyme activities increase to maintain efficient utilization of soil carbon
464 in areas with a low soil organic carbon content [57]. This may explain why the BG enzyme
465 activity was relatively high in the sandy grassland.

466 NAG is the terminal enzyme in the mineralization of soil organic nitrogen, and its
467 degradation products can be directly used by plants and microbes. Its activity can therefore
468 characterize soil nitrogen turnover [58]. We found that the N10 and N15 addition levels
469 significantly increased NAG enzyme activity in the sandy grassland. The reason may be that
470 the addition of N increased the input of plant biomass to the soil, which increased the soil
471 organic nitrogen content and induced NAG secretion [59-60]. NAG activity in the semi-fixed
472 sandy land did not differ among the N addition levels and at a given N addition, only the result

473 at background, control, and N5 differed significantly between the sites, with a higher value in
474 the semi-fixed sandy land, this may be due to the original differences between two sites. Short-
475 term N addition did not alter overall NAG activity in the soil, and this may be because the
476 microbial community structure did not change [61].

477 DHA can catalyze the redox reaction in soil, and always was used to characterize the
478 overall activity of soil microbes [32]. Previous study had shown that long-term nitrogen
479 addition significantly inhibits DHA activity by reducing soil pH [62]. Our result showed that
480 the DHA was no detected, this could be attributed to low soil nutrient and low soil microbial
481 activity in the study area, DHA activity was below the detection limit. Further research will be
482 needed to examine DHA activity in future N addition treatment.

483 The effects of N addition on the soil microflora characteristics

484 To compare the soil microflora characteristics of the sandy grassland and semi-fixed sandy
485 land, we used *q*PCR and an Illumina MiSeq high-throughput sequencer to reveal differences in
486 the microbial abundance, diversity, and community structure between the two sampling sites.

487 For bacteria, the number of 16S rRNA gene copies decreased compared with the control,
488 from 20.7×10^7 to 7.8×10^7, in response to increasing N addition in the semi-fixed sandy land (P
489 >0.05), but the number of bacterial gene copies changed little after the treatment in the sandy
490 grassland. For fungi, the dynamics of the ITS rRNA gene copies were similar to those for
491 bacteria at both sampling sites. For example, the ITS rRNA gene copies reached the highest
492 score, at 1.13×10^7, without N addition in the semi-fixed sandy land, and the number of copies
493 decreased with increasing N addition. Thus, there were no significant differences in the soil
494 bacterial and fungal abundance at either site between samples without and with N addition.

The sequencing results showed a high bacterial diversity and abundance, but a relatively low fungal diversity and abundance, at both sampling sites in the Horqin Sandy Land. For the bacteria, the Proteobacteria, Actinobacteria, and Acidobacteria were the dominant bacterial taxa. Proteobacteria and Actinobacteria are considered to be the dominant bacterial groups in many terrestrial environments, whereas Firmicutes have high resistance to high temperatures and soil moisture, and are frequently associated with arid terrestrial environments. For the fungi, the Ascomycota and Mortierellomycota were dominant. There was no obvious change of the bacterial and fungal community structures at both sites in response to the N addition treatments.

In the present study, soil microbial abundance, phylogenetic α-diversity, and the community's taxonomic structure were insensitive to the short-term N addition in the Horqin Sandy Land, which can be partly attributed to the poor soil nutrient conditions and limited moisture supply, leading to rapid uptake of the added N by the vegetation before it could become part of SMBN. This would remain true if the competition for N between plants and soil microbes was not alleviated by the N addition [62-63]. The stability of the microbial community may result from more than the community diversity and structure; it is also likely to be linked to a range of other vegetation and soil properties, including the plant species, the abundance and size of soil aggregates and the substrate quality. The resistance and resilience (stability) of soil microbial communities are governed by soil physical and chemical structures through their effect on the microbial community composition and physiology [64-65]. In the present study, the N addition probably failed to change the soil's physical and chemical properties and microbial community structure.

Previously, many studies concentrated on the effects of long-term nitrogen addition on

soil microorganisms, and researchers found that the nitrogen application had a dual effect on soil physical-chemical properties, and these changes then altered the diversity of soil microbes and their community structure. On the one hand, application of sufficient nitrogen could improve soil nutrient conditions and facilitate microbial growth and reproduction [66-67]. On the other hand, excessive nitrogen application can lead to eutrophication and acidification of soils and can inhibit fungal growth and reproduction, especially for arbuscular mycorrhizal fungi, and this can lead to changes in the overall soil microbial community. In addition, long-term nitrogen application can significantly decrease microbial diversity in grassland soils [68-71]. It will be necessary to conduct long-term nitrogen application experiments in the Horqin Sandy Land to determine how the present results will change over time.

Conclusion

Short-term N addition had no significant effects on the three soil microbial indices (SMBC, SMBN, and SMBC/SMBN), on the activity of NAG, and on soil microflora characteristics (soil microbial abundance, phylogenetic α-diversity and taxonomic structure) at the three N addition levels in the sandy grassland and semi-fixed sandy land. This appears to be because the short-term N addition did not alleviate the competition for N between plants and soil microbes, and because the relationships among the original soil microbes may not have changed sufficiently to affect the community structure. However, the soil BG activity increased with increasing N addition at both sites. The N addition in the first growing season had no significant impact on the measured soil microbes. Long-term studies will be needed to examine the responses of the soil microbial indices, the activity of soil enzymes, and the soil microflora characteristics at the two sites.

References

1. Macko S, Wang L, D'Odorico P. Nitrogen uptake preferences by plants in arid and semiarid ecosystems. In: Proc. AGU Fall Meeting Abstracts. 2005.

2. Iversen CM, Bridgham SD. Kellogg LE, Scaling plant nitrogen use and uptake efficiencies in response to nutrient addition in peatlands. Ecology 2010; 91(3):693-707. https://doi.org/10.1890/09-0064.1.

3. Lebauer DS, Treseder KK. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecology 2008; 89(2):371-379.

4. Shi Y, Sheng L, Wang Z, Zhang X, He N, Yu Q. Responses of soil enzyme activity and microbial community compositions to nitrogen addition in bulk and microaggregate soil in the temperate steppe of Inner Mongolia. Eurasian Soil Sci 2016; 49(10):1149-1160. https://doi.org/10.1134/s1064229316100124.

5. Holtgrieve GW, Schindler DE, Hobbs WO, Leavitt PR, Ward EJ, Bunting L, Lisac MJ. A coherent signature of anthropogenic nitrogen deposition to remote watersheds of the northern hemisphere. Science 2011; 334(6062):1545-1548. https://doi.org/10.1126/science.1212267.

6. Neff JC, Townsend AR, Gleixner G, Lehman SJ, Turnbull J, Bowman WD. Variable effects of nitrogen additions on the stability and turnover of soil carbon. Nature 2002; 419(6910):915-917. https://doi.org/10.1038/nature01136.

7. Sala OE, Chapin FS, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, et al. Global biodiversity scenarios for the year 2100. Science 2000: 287(5459):1770–1774. https://doi.org/10.1126/science.287.5459.1770.

8. Wei C, Zheng H, Li Q, Lü X, Yu Q, Zhang H et al. Nitrogen addition regulates soil nematode

561 community composition through ammonium suppression. PLOS One 2012; 7(8):e43384.
562 https://doi.org/10.1371/journal.pone.0043384.
563 9. Lal R. Carbon sequestration in dryland ecosystems. Environ Manage 2004, 33(4):528–544.
564 https://doi.org/10.1007/s00267-003-9110-9.
565 10. Nielsen UN, Ball BA. Impacts of altered precipitation regimes on soil communities and
566 biogeochemistry in arid and semi-arid ecosystems. Global Change Biol 2015; 21(4):1407-1421.
567 https://doi.org/10.1111/gcb.12789.
568 11. Li YQ, Zhao XY, Wang SK, Zhang FX, Lian J, Huang WD. Carbon accumulation in the bulk soil
569 and different soil fractions during the rehabilitation of desertified grassland in Horqin Sandy Land
570 (Northern China). Polish J Ecol 2015; 63(1):88-101.
571 https://doi.org/10.3161/15052249PJE2015.63.1.008.
572 12. Phoenix GK, Leake JR, Read DJ, Grime JP, Lee JA. Accumulation of pollutant nitrogen in
573 calcareous and acidic grasslands: evidence from N flux and ^{15}N tracer studies. Water Air Soil Poll
574 2004; 4(6):159-167. https://doi.org/10.1007/s11267-004-3026-0.
575 13. Stursova M, Crenshaw CL, Sinsabaugh RL. Microbial responses to long-term N deposition in a
576 semiarid grassland. Microbial Ecol 2006; 51(1):90-98. https://doi.org/10.1007/s00248-005-5156-y.
577 14. Anderson TH. Soil microbial biomass and activity estimations in forest soils. In: Teller A, Mathy P,
578 Jeffers JNR (eds.) Responses of Forest Ecosystems to Environmental Changes Springer, Dordrecht
579 1992; 738-739.
580 15. Liu W, Xu W, Hong J, Wan S. Interannual variability of soil microbial biomass and respiration in
581 responses to topography, annual burning and N addition in a semiarid temperate steppe. Geoderma
582 2010; 158(3-4):259-267. https://doi.org/10.1016/j.geoderma.2010.05.004.

583 16. Wang NN, Han DX, Sun X. Effects of precipitation change on soil microbial functional diversity in
584 the primitive Korean pine and broadleaved forests (in Chinese). Acta Ecologica Sinica 2017;
585 37(3):1-9. https://doi.org/10.5846/stxb201509101873.
586 17. Jenkinson DS. Microbial biomass in soil: measurement and turnover. Soil Biol Biochem 1981;
587 5:415-471.
588 18. Sparling GP. Soil microbial biomass, activity and nutrient cycling as indicators of soil health. In:
589 Pankhurst CE, Doube BM, Gupta VVSR (Eds.) Biological Indicators of Soil Health, CABI,
590 Wallington. 1997; pp: 97-119.
591 19. Powlson DS, Hirsch PR, Brookes PC. The role of soil microorganisms in soil organic matter
592 conservation in the tropics. Nutr Cycl Agroecosys 2001; 61(1-2):41-51.
593 https://doi.org/10.1023/A:1013338028454.
594 20. Pan F, Zhang W, LiangY, Liu S, Wang K. Increased associated effects of topography and litter and
595 soil nutrients on soil enzyme activities and microbial biomass along vegetation successions in karst
596 ecosystem, southwestern China. Environmental Science and Pollution Research 2018;
597 25(17):16979-16990. https://doi.org/10.1007/s11356-018-1673-3.
598 21. Keeler BL, Hobbie SE, Kellogg LE. Effects of long-term nitrogen addition on microbial enzyme
599 activity in eight forested and grassland sites: implications for litter and soil organic matter
600 decomposition. Ecosystems 2009; m12(1):1-15. https://doi.org/10.1007/s10021-008-9199-z.
601 22. Spohn M, Pötsch EM, Eichorst SA, Woebken D, Wanek W, Richter A. Soil microbial carbon use
602 efficiency and biomass turnover in a long-term fertilization experiment in a temperate grassland.
603 Soil Biol Biochem 2016; 97:168-175. https://doi.org/10.1016/j.soilbio.2016.03.008.
604 23. Liu HM, Zhang HF, Huangfu CH, Jie LI, Zhou GF, Yang DL, et al. Effects of different long-term

nitrogen addition on soil microbial diversity of *Stipa baicalensis* steppe in Inner Mongolia, China (in Chinese). J Agric Environ Sci 2017; 36:709-717.

24. Li LJ, Zeng DH, Yu ZY, Fan ZP, Mao R. Soil microbial properties under N and P additions in a semi-arid, sandy grassland. Biol Fert Soils 2010; 46(6):653-658. https://doi.org/10.1007/s00374-010-0463-y.

25. Zhou XB, Zhang YB, Downing A. Non-linear response of microbial activity across a gradient of nitrogen addition to a soil from the Gurbantunggut Desert, northwestern China. Soil Biol Biochem 2012; 47:67-77. https://doi.org/10.1016/j.soilbio.2011.05.012.

26. Treseder KK. Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. Ecol Lett 2008; 11(10):1111-1120. https://doi.org/10.1111/j.1461-0248.2008.01230.x.

27. McCrackin ML, Harms TK, Grimm NB, Hall SJ, Kaye JP. Responses of soil microorganisms to resource availability in urban, desert soils. Biogeochemistry 2008; 87:43-155. https://doi.org/10.1007/s10533-007-9173-4.

28. Triebwasser DJ, Tharayil N, Preston CM, Gerard PD. The susceptibility of soil enzymes to inhibition by leaf litter tannins is dependent on the tannin chemistry, enzyme class and vegetation history. New Phytologist 2012; 196(4):1122-1132. https://doi.org/10.1111/j.1469-8137.2012.04333.x.

29. Zhou X, Wang SS, Chen C. Modeling the effects of tree species and incubation temperature on soil's extracellular enzyme activity in 78-year-old tree plantations. Biogeoscience 2017; 14(23):5393-5402. https://doi.org/10.5194/bg-14-5393-2017.

30. Allison SD, Jastrow JD. Activities of extracellular enzymes in physically isolated fractions of restored grassland soils. Soil Biol Biochem, 2006; 38(11): 3245-3256.

627 https://doi.org/10.1016/j.soilbio.2006.04.011.

628 31. Brunner K, Peterbauer CK, Mach RL, Lorito M, Zeilinger S, Kubicek CK, et al. The Nag1 N-
629 acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin and
630 of major relevance to biocontrol. Curr Genet2003; 43(4):289-295. https://doi.org/10.1007/s00294-
631 003-0399-y

632 32. Zhou LK. Soil Enzymology (in Chinese). Science Press. Beijing. 1987; pp: 116-206.

633 33. Niu XY, Sun XM, Chen DS, Zhang SG. Soil enzyme activities of the litter in *Larix kaempferi*
634 plantation (in Chinese). Scientia Silvae Sinicae 2015; 51(4):16-25. https://doi.org/10．11707
635 /j．1001-7488．20150403.

636 34. Jia YL, Yu GR, He NP, Zhan XY, Fang HJ, Sheng WP, et al. Spatial and decadal variations in
637 inorganic nitrogen wet deposition in China induced by human activity. Scientific reports 2014;
638 4(3763):1-7. https://doi.org/10.1038/srep03763.

639 35. Joergensen RG. The fumigation-extraction method to estimate soil microbial biomass: calibration
640 of the kEC value. Soil Biol Biochem 1996; 28(1):25-31. https://doi.org/10.1016/0038-
641 0717(95)00102-6.

642 *36.* Xu N, Tan G, Wang H, Gai X. Effect of biochar additions to soil on nitrogen leaching, microbial
643 biomass and bacterial community structure. Eur. J. Soil Biol. 2016; 74, 1-8.
644 https://doi.org/10.1016/j.ejsobi.2016.02.004.

645 37. Adams RI, Miletto M, Taylor JW, Bruns TD. Dispersal in microbes: fungi in indoor air are
646 dominated by outdoor air and show dispersal limitation at short distances. ISME Journal 2013;
647 7(7):1262-1273. https://doi.org/10.1038/ismej.2013.28.

648 38. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA

649 gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;
650 41(D1):590-596. https://doi.org/10.1093/nar/gks1219.
651 39. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur:
652 open-source, platform-independent, community-supported software for describing and comparing
653 microbial communities. Appl Environ Microbiol 2009; 75(23):7537-7541.
654 https://doi.org/10.1128/AEM.01541-09.
655 40. Barbhuiya AR, Arunachalam A, Pandey HN, Arunachalam K, Khan ML, Nath PC. Dynamics of
656 soil microbial biomass C, N and P in disturbed and undisturbed stands of a tropical wet-evergreen
657 forest. Eur J Soil Biol 2004; 40(3-4):113-121. https://doi.org/10.1016/j.ejsobi.2005.02.003.
658 41. Jefferies RL, Walker NA, Edwards KA, Dainty J. Is the decline of soil microbial biomass in late
659 winter coupled to changes in the physical state of cold soils? Soil Biol Biochem 2010; 42(2):129-
660 135. https://doi.org/10.1016/j.soilbio.2009.10.008.
661 42. Jaeger IIICH, Monson RK, Fisk MC, Schmidt SK. Seasonal partitioning of nitrogen by plants and
662 soil microorganisms in an alpine ecosystem. Ecology 1999; 80(6):1883-1891.
663 https://doi.org/10.2307/176666.
664 43. Johnson D, Leake JR, Read DJ. Liming and nitrogen fertilization affects phosphatase activities,
665 microbial biomass and mycorrhizal colonisation in upland grassland. Plant Soil 2005; 271(1-2):157-
666 164. https://doi.org/10.1007/s11104-004-2267-z.
667 44. Stark S, Kytöviita MM. Simulated grazer effects on microbial respiration in a subarctic meadow:
668 implications for nutrient competition between plants and soil microorganisms. Applied Soil Ecology
669 2006; 31:20-31. https://doi.org/10.1016/j.apsoil.2005.04.002.
670 45. Liu W, Xu W, Han Y, Wang C, Wan S. Responses of microbial biomass and respiration of soil to

topography, burning, and nitrogen fertilization in a temperate steppe. Biol Fert Soils 2007; 44(2):259-268. https://doi.org/10.1007/s00374-007-0198-6.

46. Lovell RD, Jarvis SC, Bardgett RD. Soil microbial biomass and activity in long-term grassland: effects of management changes. Soil Biol Biochem 1995; 27(7):969-975. https://doi.org/10.1016/0038-0717(94)00241-R.

47. Compton JE, Watrud LS, Porteous LA, DeGrood S. Response of soil microbial biomass and community composition to chronic nitrogen additions at Harvard forest. Forest Ecol Manag 2004; 196(1):143-158. https://doi.org/10.1016/S0378-1127(04)00197-5.

48. Wallenstein MD, McNulty S, Fernandez IJ, Boggs J, Schlesinger WH. Nitrogen fertilization decreases forest soil fungal and bacterial biomass in three long-term experiments. For Ecol Manage 2006; 222(1-3):459-468. https://doi.org/v10.1016/j.foreco.2005.11.002.

49. Bakken LR. Microbial growth and immobilization/mineralization of N in the rhizosphere. Symbiosis 1990; 9:37-41.

50. Liu W, Jiang L, Hu S, Li L, Liu L, Wan S. Decoupling of soil microbes and plants with increasing anthropogenic nitrogen inputs in a temperate steppe. Soil Biol Biochem 2014; 72:116–122. https://doi.org/10.1016/j.soilbio.2014.01.022.

51. Zhang J, Ai Z, Liang C, Wang G, Xue S. Response of soil microbial communities and nitrogen thresholds of *Bothriochloa ischaemum*, to short-term nitrogen addition on the Loess Plateau. Geoderma2017; 308:112-119. https://doi.org/10.1016/j.geoderma.2017.08.034.

52. Dijkstra FA, Hobbie SE, Reich PB, Knops JM. Divergent effects of elevated CO_2, N fertilization, and plant diversity on soil C and N dynamics in a grassland field experiment. Plant Soil 2005; 272:41-52. https://doi.org/10.1007/s11104-004-3848-6.

693 53. Liu YL, Liu XL, Xu F, Wu YN, Li Z J. Numerical simulation of plant roots protection effects on the
694 silty clays slope with low liquid limit. Sixth International Conference on Intelligent Systems Design
695 and Engineering Applications (ISDEA). IEEE2016; pp: 533-537
696 54. Zeglin LH, Stursova M, Collins SSL. Microbial responses to nitrogen addition in three contrasting
697 grassland ecosystems. Oecologia 2007; 154(2):349-359. https://doi.org/10.1007/s00442-007-0836-
698 6.
699 55. Saiya-Cork KR, Sinsabaugh RL, Zak DR. The effects of long term nitrogen deposition on
700 extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biol Biochem 2002; 34(9):1309-
701 1315. https://doi.org/10.1016/S0038-0717(02)00074-3.
702 56. Henry HAL, Juarez JD, Field CB, Vitousek PM. Interactive effects of elevated CO_2, N deposition
703 and climate change on extracellular enzyme activity and soil density fractionation in a California
704 annual grassland. Soil Biol Biochem 2005; 11(10):1808-1815. https://doi.org/10.1111/j.1365-
705 2486.2005.001007.x.
706 57. Wang B, Xue S, Liu GB, Zhang GH, Li G, Ren ZP. Changes in soil nutrient and enzyme activities
707 under different vegetation in the Loess Plateau area, northwest China. Catena 2012; 92:186-195.
708 https://doi.org/10.1016/j.catena.2011.12.004.
709 58. Pan FJ, Liang YM, Zhang W, Zhao J, Wang KL. Enhanced nitrogen availability in karst ecosystems
710 by oxalic acid release in the rhizosphere. Front Plant Sci 2016; 7(11636):687.
711 https://doi.org/10.3389/fpls.2016.00687.
712 59. Creamer CA, Filley TR, Olk DC, Stott DE, Dooling V, Boutton TW. Changes to soil organic N
713 dynamics with leguminous woody plant encroachment into grasslands. Biogeochemistry 2013;
714 113(1-3):307-321. https://doi.org/10.1007/s10533-012-9757-5.

715 60. Yang S. Effects of water and fertilizer addition on soil microbial characteristics in a typical grassland
716 of Inner Mongolia (in Chinese). Dissertation. Shenyang University 2014.
717 61. Stark CH, Condron LM, O'Callaghan M, Stewart A, Di HJ. Differences in soil enzyme activities,
718 microbial community structure and short-term nitrogen mineralisation resulting from farm
719 management history and organic matter amendments. Soil Biol Biochem 2008; 40(6):352-1363.
720 https://doi.org/10.1016/j.soilbio.2007.09.025.
721 62. Wang R, Filley TR, Xu Z, Wang X, Li MH, Zhang Y et al. Coupled response of soil carbon and
722 nitrogen pools and enzyme activities to nitrogen and water addition in a semi-arid grassland of Inner
723 Mongolia. Plant Soil 2014; 381:323-336. https://doi.org/10.5194/bg-14-2155-2017.
724 63. Zhang TA, Chen-Han, YH, Ruan HH. Global negative effects of nitrogen deposition on soil
725 microbes. ISME Journal 2018; 12(7):1817-1825. https://doi.org/10.1038/s41396-018-0096-y.
726 **64.** Griffiths BS, Philippot L. Insights into the resistance and resilience of the soil microbial community.
727 FEMS Microbiology Reviews 2013; 37(2):112-129. https://doi.org/10.1111/j.1574-
728 6976.2012.00343.x.
729 65. He JZ, Li J, Zheng YM. Thoughts on the microbial diversity-stability relationship in soil ecosystems
730 (in Chinese). Biodiversity Science 2013; 21(4):411-420.
731 https://doi.org/10.3724/SP.J.1003.2013.10033.
732 66. Bardgett RD, Denton CS, Cook R. Below-ground herbivory promotes soil nutrient transfer and root
733 growth in grassland. Ecology Letters 1999a; 2(6):357-360. https://doi.org/10.1046/j.1461-
734 0248.1999.00001.x.
735 67. Frey SD, Knorr M, Parrent JL, Simpson RT. Chronic nitrogen enrichment affects the structure and
736 function of the soil microbial community in temperate hardwood pine forests. For Ecol Manage

2004; 196(1):159-171. https://doi.org/10.1016/j.foreco.2004.03.018.

68. Smolander A, Kurka A, Kitunen V, Mälkönen E. Microbial biomass C and N, and respiratory activity in soil of repeatedly limed and N and P-fertilized Norway spruce stands. Soil Biol Biochem 1994; 26(8):957-962. https://doi.org/10.1016/0038-0717(94)90109-0.

69. Bardgett RD, Lovell RD, Hobbs PJ, Jarvis SC. Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biol Biochem1999b; 31(7):1021-1030. https://doi.org/10.1016/S0038-0717(99)00016-4.

70. Yoshida LC, Allen EB. Response to ammonium and nitrate by a mycorrhizal annual invasive grass and native shrub in southern California. Am J Bot 2001; 88(8):1430-1436. https://doi.org/10.2307/3558450.

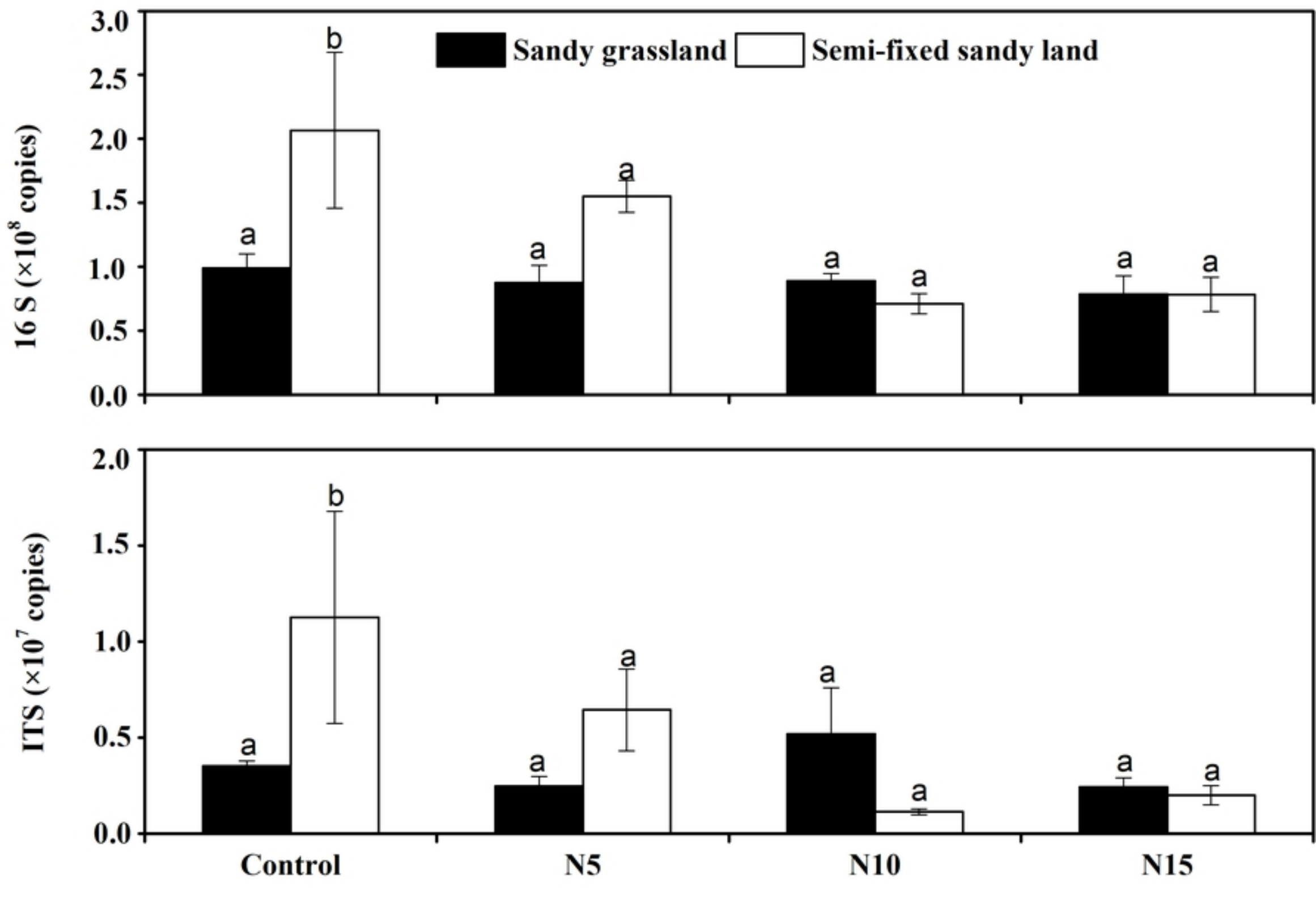
71. Chung H, Zak DR, Reich PB, Ellsworth DS. Plant species richness, elevated CO_2, and atmospheric nitrogen deposition alter soil microbial community composition and function. Global Change Biol 2007; 13:980-989. https://doi.org/10.1111/j.1365-2486.2007.01313.x.

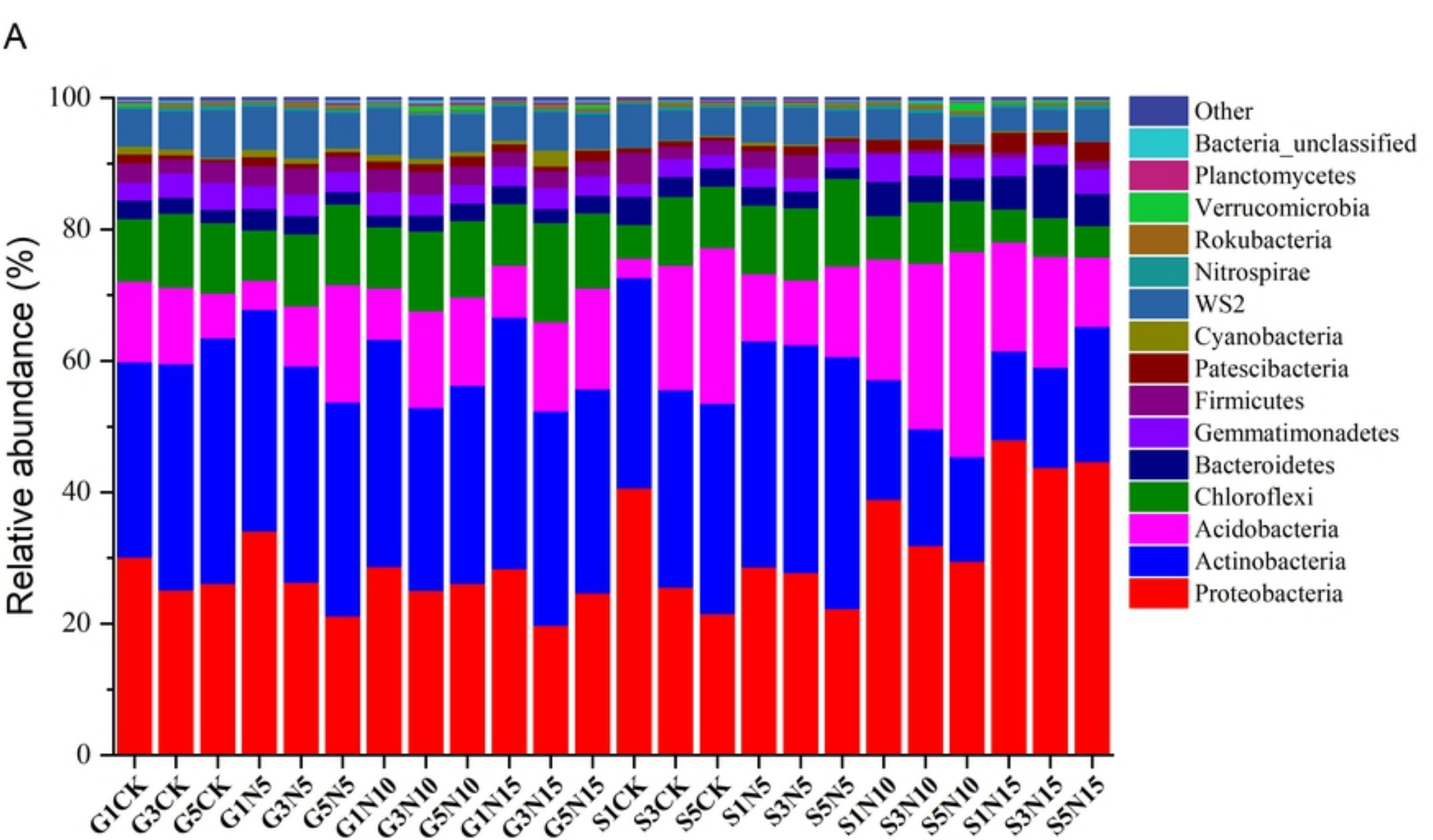
Supporting information

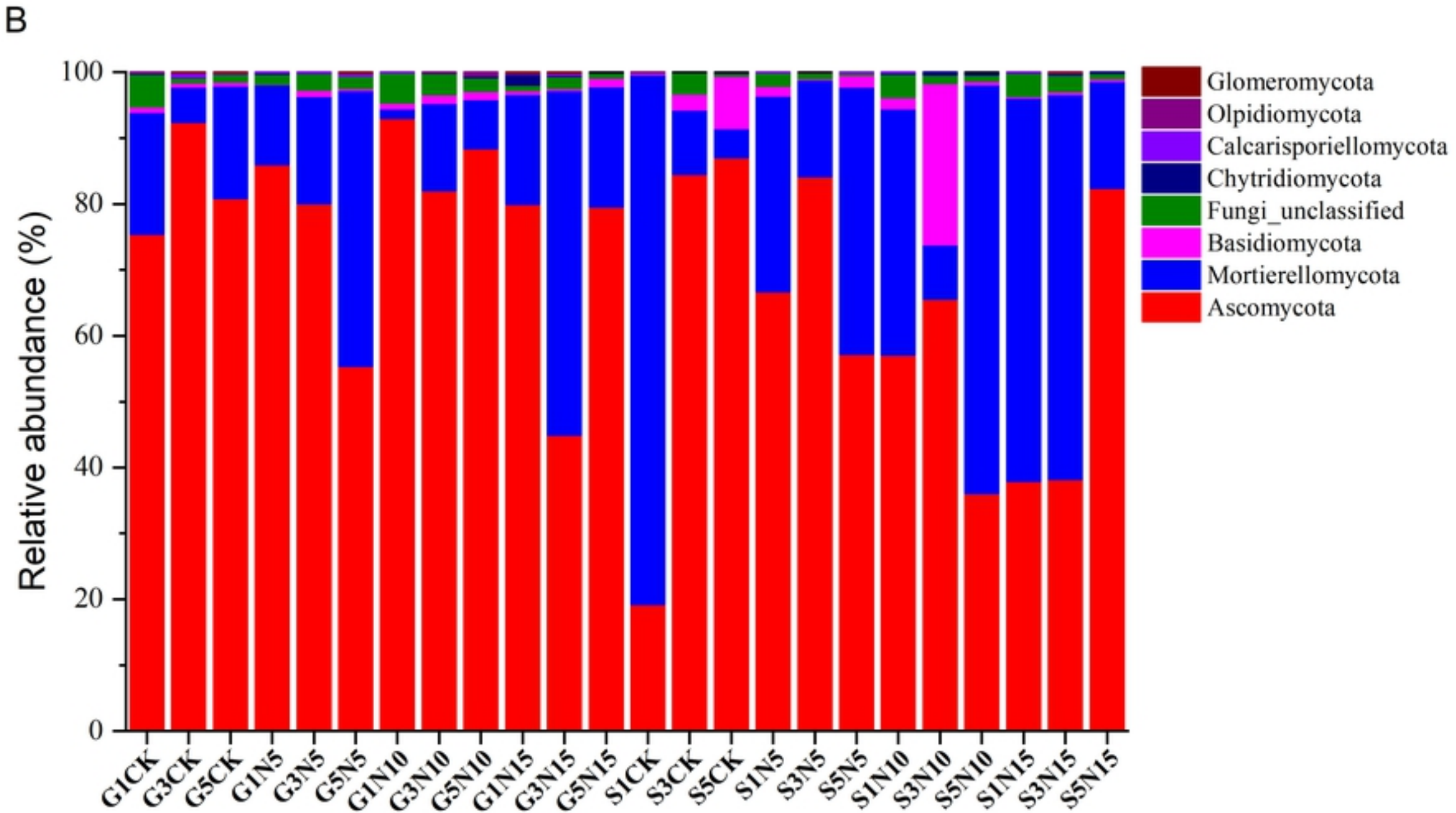
S1 Table. Phylotype coverage and diversity estimation based on the bacterial 16S rRNA gene libraries for the samples from the MiSeq sequencing analysis. Sample names include the site type (G, sandy grassland; S, semi-fixed sandy land) and nitrogen addition treatment: control (CK), no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15, 15 g N m^{-2} yr^{-1}.

S2 Table Phylotype coverage and diversity estimation based on the fungal ITS rRNA gene libraries for the samples from the MiSeq sequencing analysis. Sample names include the site (G, sandy grassland; S, semi-fixed sandy land) and nitrogen addition treatment: control (CK), no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15, 15 g N m^{-2} yr^{-1}.

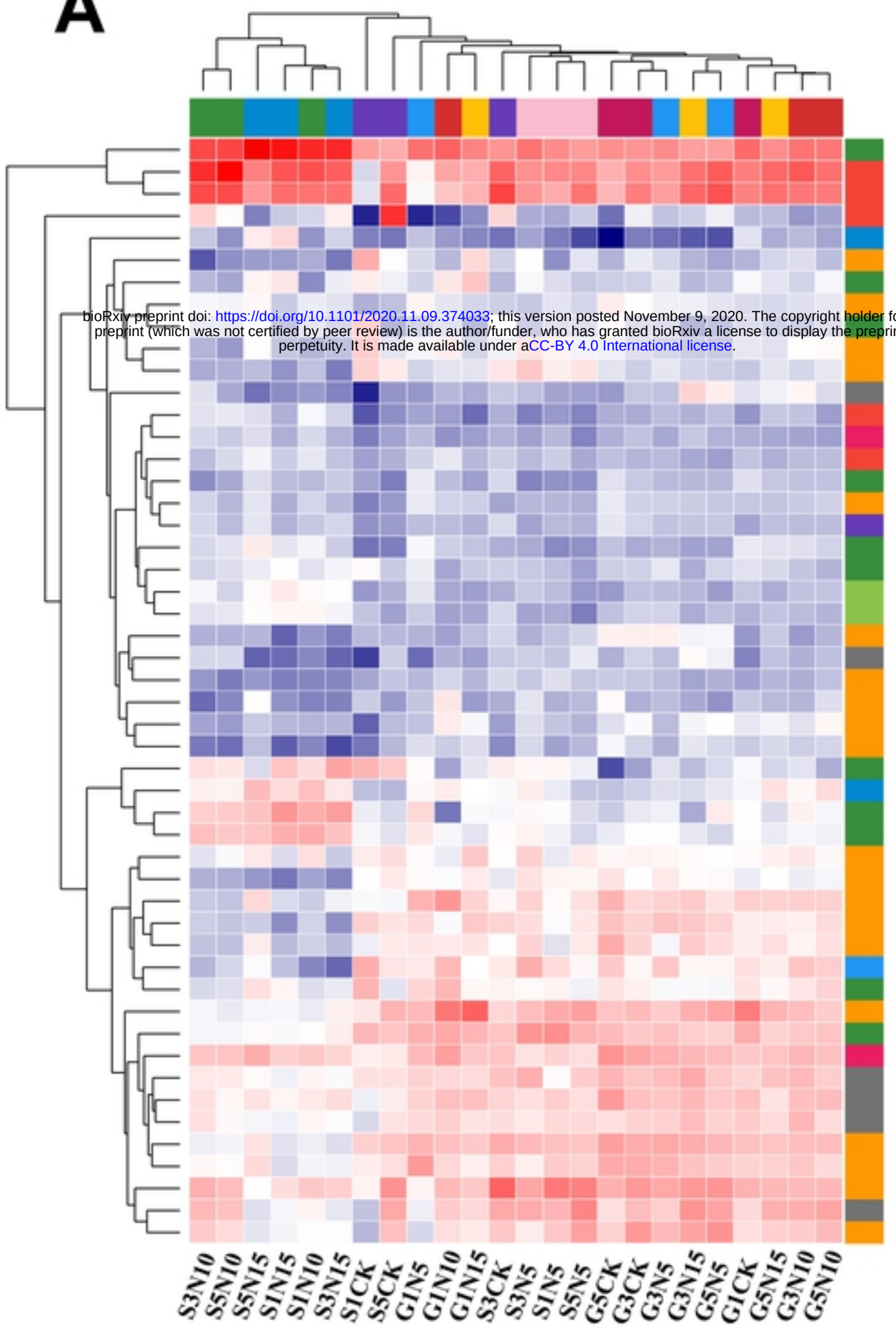
S1 Fig. Rarefaction curves. Rarefaction curves show the number of reads 97% sequence similarity level for the different samples. (A) Bacteria, (B) Fungi. Sample names include the site type (G, sandy grassland; S, semi-fixed sandy land) and nitrogen addition treatment: control (CK), no N addition; N5, 5 g N m^{-2} yr^{-1}; N10, 10 g N m^{-2} yr^{-1}; N15, 15 g N m^{-2} yr^{-1}.


Sandy grassland
Semi-fixed sandy land
16 S (×10^8 copies)
ITS (×10^7 copies)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2.0
1.5
1.0
0.5
0.0
a
b
a
a
a
a
a
a
a
b
a
a
a
a
a
a
Control
N5
N10
N15


A
Relative abundance (%)
100
80
60
40
20
0
G1CK
G3CK
G5CK
G1N5
G3N5
G5N5
G1N10
G3N10
G5N10
G1N15
G3N15
G5N15
S1CK
S3CK
S5CK
S1N5
S3N5
S5N5
S1N10
S3N10
S5N10
S1N15
S3N15
S5N15
Other
Bacteria_unclassified
Planctomycetes
Verrucomicrobia
Rokubacteria
Nitrospirae
WS2
Cyanobacteria
Patescibacteria
Firmicutes
Gemmatimonadetes
Bacteroidetes
Chloroflexi
Acidobacteria
Actinobacteria
Proteobacteria


B
Relative abundance (%)
100
80
60
40
20
0
Glomeromycota
Olpidiomycota
Calcarisporiellomycota
Chytridiomycota
Fungi_unclassified
Basidiomycota
Mortierellomycota
Ascomycota
G1CK
G3CK
G5CK
G1N5
G3N5
G5N5
G1N10
G3N10
G5N10
G1N15
G3N15
G5N15
S1CK
S3CK
S5CK
S1N5
S3N5
S5N5
S1N10
S3N10
S5N10
S1N15
S3N15
S5N15

A



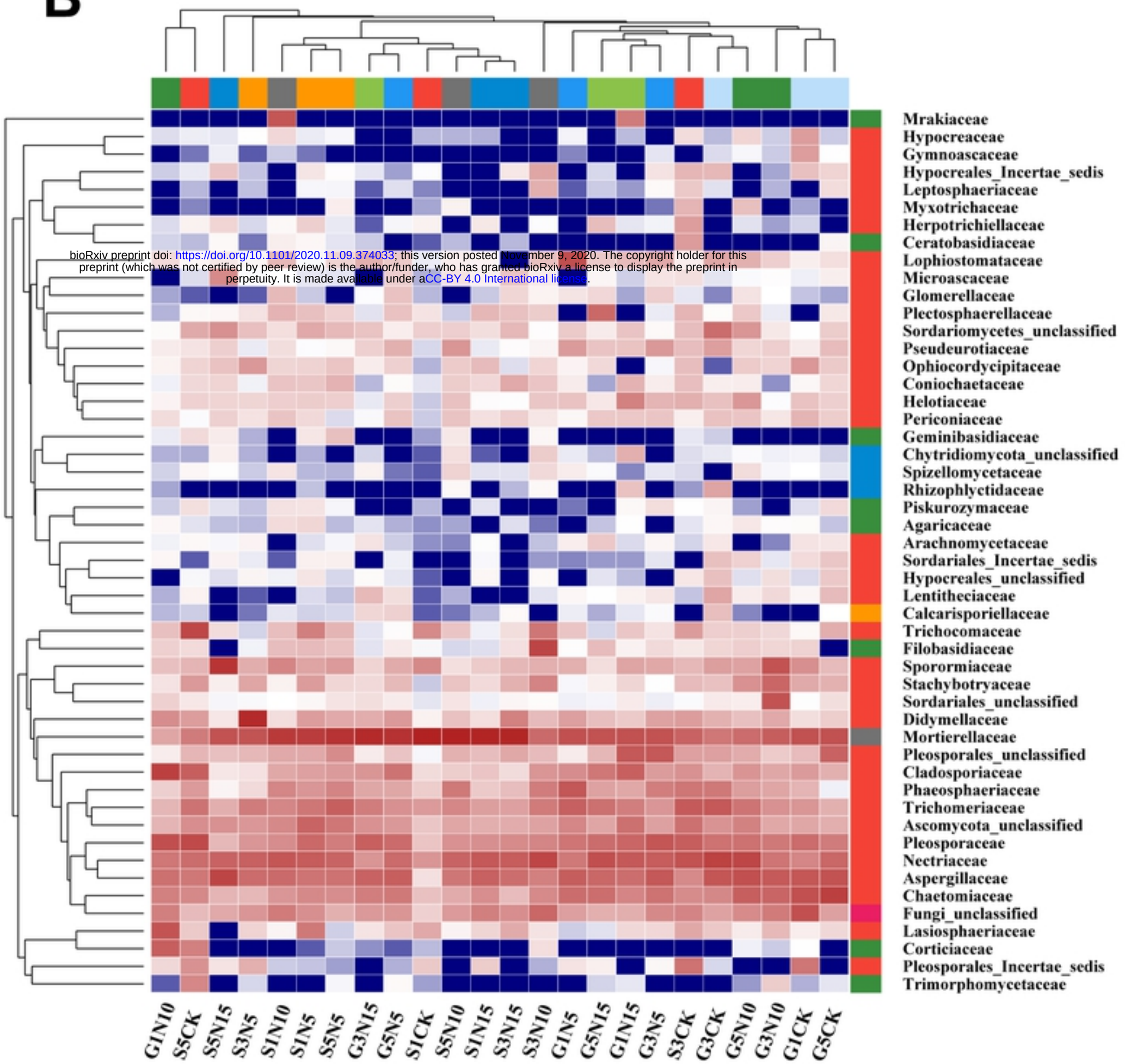
Sample group

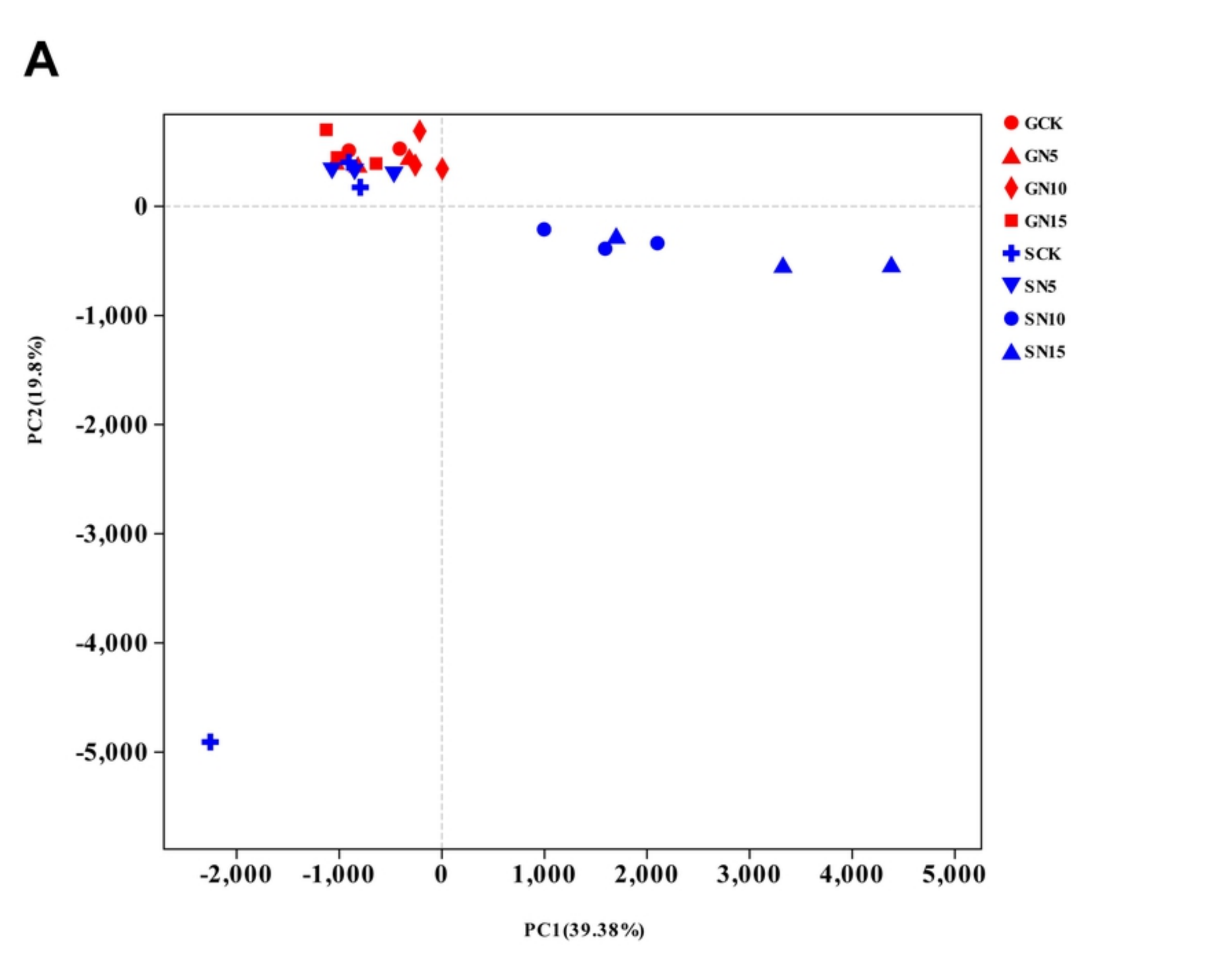
S-N10 S-N15 S-CK G-N5 G-N10 G-N15 S-N5 G-CK

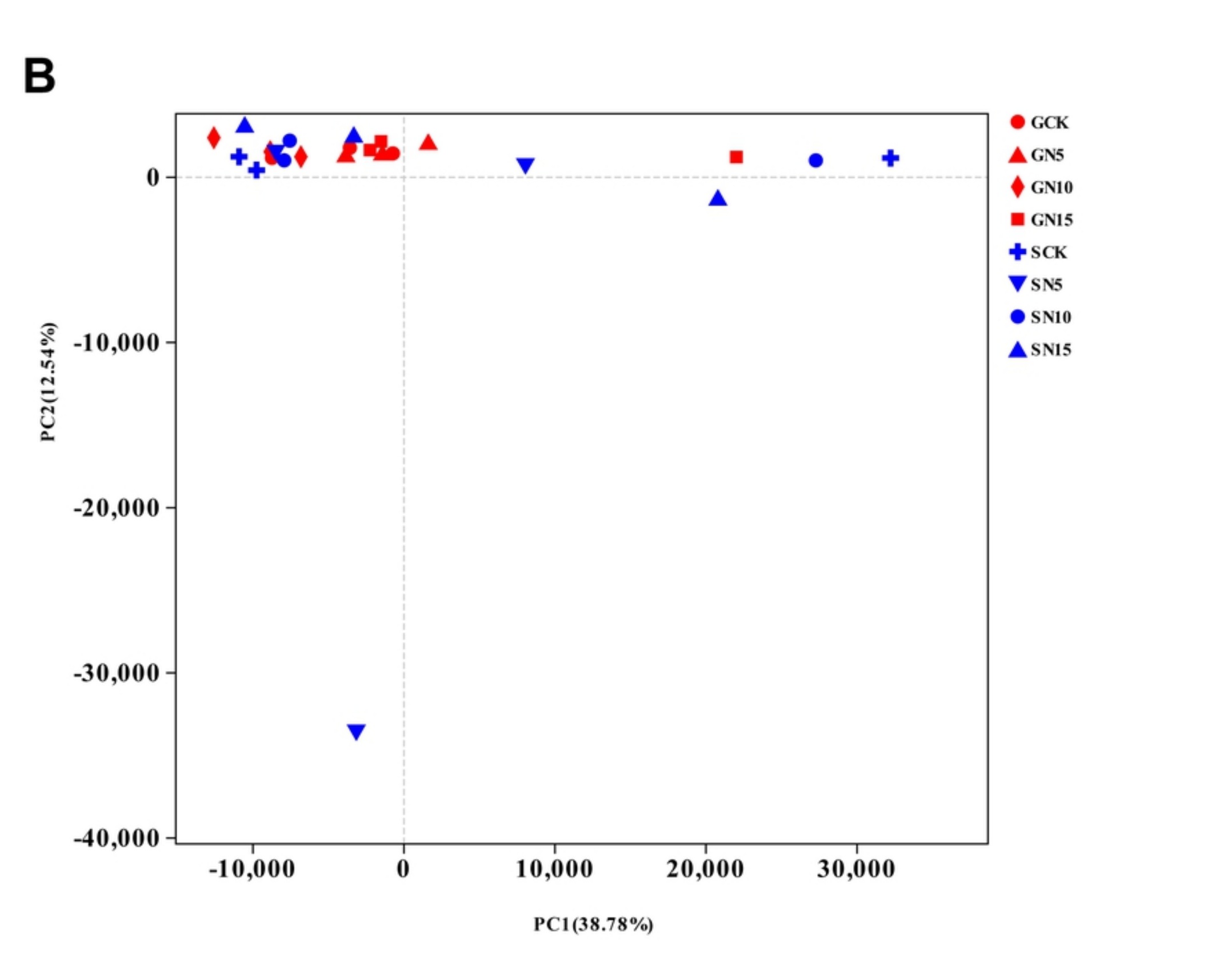
Taxon group

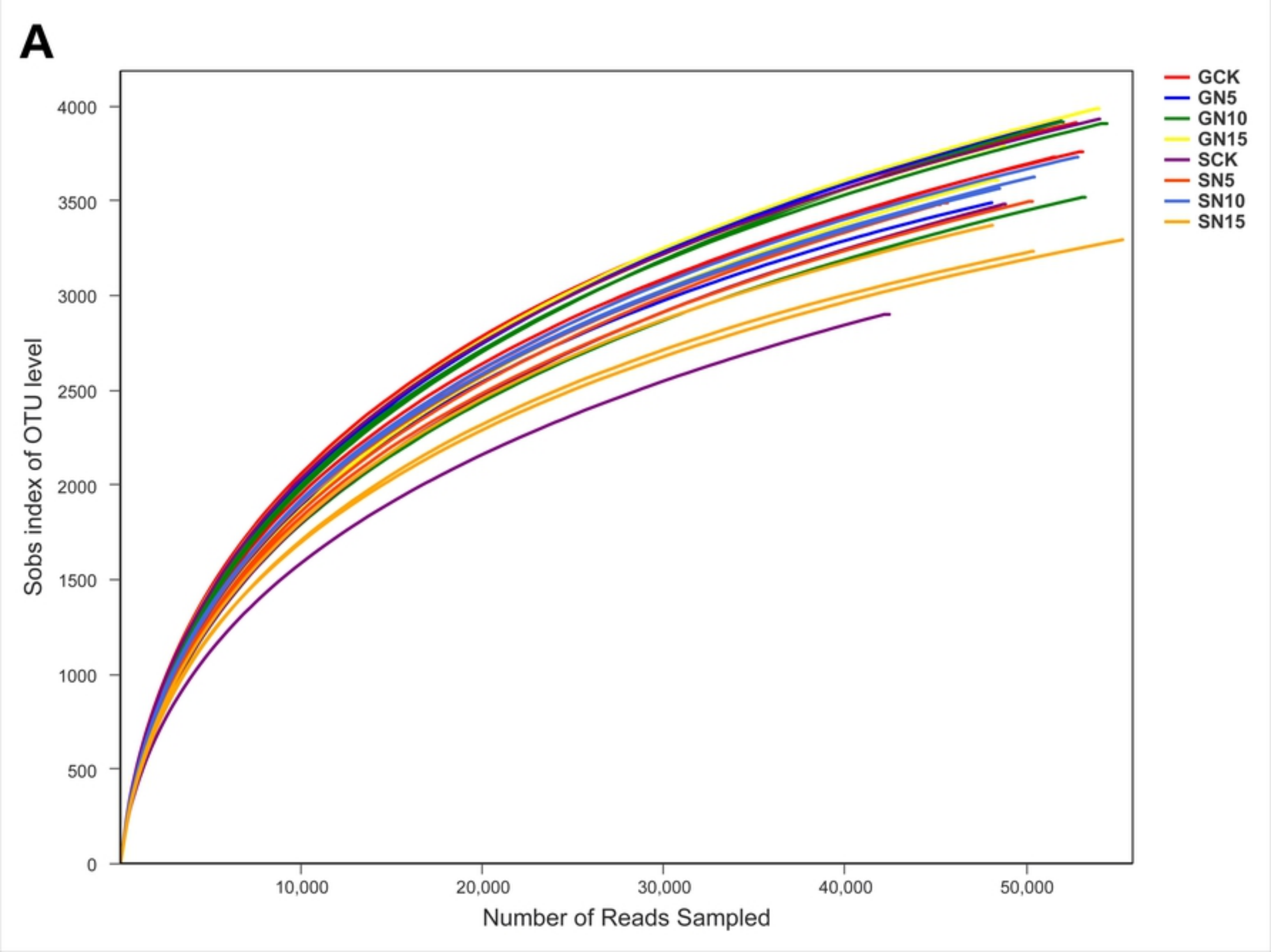
Proteobacteria Acidobacteria Patescibacteria
Actinobacteria Chloroflexi Gemmatimonadetes
Nitrospirae Bacteroidetes Firmicutes

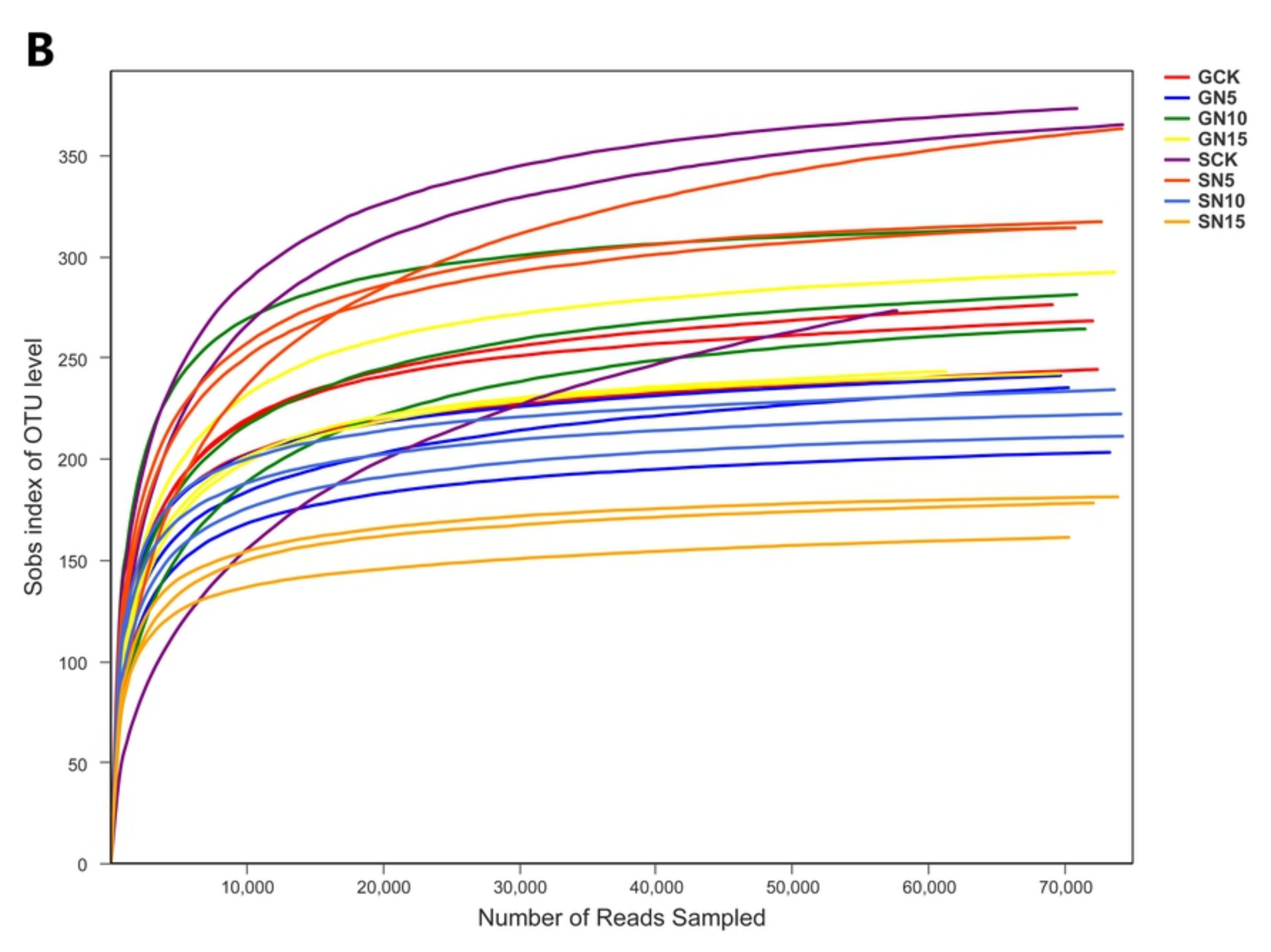
B




A
GCK
GN5
GN10
GN15
SCK
SN5
SN10
SN15
0
-1,000
-2,000
-3,000
-4,000
-5,000
PC2(19.8%)
-2,000
-1,000
0
1,000
2,000
3,000
4,000
5,000
PC1(39.38%)


B
GCK
GN5
GN10
GN15
SCK
SN5
SN10
SN15
0
-10,000
-20,000
-30,000
-40,000
PC2(12.54%)
-10,000
0
10,000
20,000
30,000
PC1(38.78%)


A
GCK
GN5
GN10
GN15
SCK
SN5
SN10
SN15
Sobs index of OTU level
0
500
1000
1500
2000
2500
3000
3500
4000
10,000
20,000
30,000
40,000
50,000
Number of Reads Sampled


B
GCK
GN5
GN10
GN15
SCK
SN5
SN10
SN15
Sobs index of OTU level
0
50
100
150
200
250
300
350
10,000
20,000
30,000
40,000
50,000
60,000
70,000
Number of Reads Sampled