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Nitrogen fertilizer is a key factor effecting the soil chemical and microbial communities in a Mollisol

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Abstract: Microbial communities drive geochemical cycles in soils. Relatively few
studies have assessed the long-term impacts of different types of soil amendments
under field conditions in long-term experiments. The response of soil microbial

organisms in a Mollisol cultivated with maize for 35 years was examined. Treatments involved the use of N, P and K fertilizers and two doses of straw residue incorporation, in isolation and combined Real-time PCR and Illumina Miseq sequencing methods were used to characterize the microbial community. The results showed that addition of nitrogen fertilizers decreased soil pH, but this was mitigated when a high dose of straw was also incorporated. Long-term application of inorganic fertilizers was able to alter the abundance of functional soil microbial population. Application of inorganic N fertilizer resulted in distinctive changes on N-cycle microorganisms. Phosphate-solubilizing functional genes abundance was lower in plots with no phosphate fertilizer. Sequencing analysis showed that the presence or absence of N in the fertilizer mix is a key factor affecting bacterial community diversity of agricultural soil, pH TOC and TN showed a high correlation with bacterial community composition. Nitrogen addition increased the N concentration in the soil which could cause changes in the soil pH and changed the soil bacterial community. Our findings proved that interaction of N fertilizer with other fertilizers can affect microbial communities.

Keyword: Nitrogen fertilizer; microbial communities; real-time PCR; Illumina Miseq sequencing

Introduction

Soils are complex biological systems inhabited by a large number of microorganisms, which play a vital role in the decomposition of soil organic matter

1 and in the biogeochemical cycling of several key nutrients, including N and P.
2 Agricultural systems have intensified during the last 50 years. Soil microbial
3 communities can be significantly disturbed by agricultural modern agricultural
4 practices, including long-term fertilization(Marschner et al.2003; Ding et al.2017;
5 Cinnadurai and Gopalaswamy.2013; Segal et al.2017). Some studies have reported
6 that the use of inorganic fertilizers can lead to adverse impacts on soil quality,
7 including physico-chemical properties and soil microbial communities. It is well
8 established that inorganic nitrogen fertilization leads to decreases in soil pH (Belay et
9 al.2002). More uncertainty surrounds the long-term fate of soil organic carbon pools.
10 Belay et al. (2002) reported that inorganic fertilization could result in a decrease of
11 soil organic carbon. This will have associated impacts in soil microbial communities
12 (Zheng et al. 2017). Application of N fertilizer can reduce the decomposition of
13 organic matter in rhizosphere soil (Zang et al. 2016). The application of N fertilizer
14 influenced soil microbial composition, particularly fungal community (Guo et al.
15 2018). Liu et al. (2019) found that applying N fertilizer not only increase rhizo-C flow
16 into microorganisms but it may also increase the effect of rhizosphere on bulk-soil
17 microorganisms and subsequent processes related to soil C-cycling. Similarly,
18 phosphorus fertilization provides an important nutrient to crops. However, previous
19 studies have found that increased soil phosphorus availability could accelerate organic
20 matter decomposition (Mao et al.2015).

21 In general, combining inorganic fertilizer application with organic amendments

1 promotes biological interactions to maintain cropping systems sustainability (Li et
2 al.2017; Chen et al.2016; Lazcano et al.2013)

3 Crop straw is an important source of soil organic matter. Straw contains nutrients
4 including nitrogen, phosphorus, potassium, magnesium, calcium, and sulfur. China
5 produces about 700 million tons of straw, being one of the highest producers in the
6 world. As a consequence of a relatively low level of mechanization in the field and a
7 shift in population from urban to rural areas, most of this straw is managed
8 inadequately. Thus, less than 50% of the straw is returned to the soil, with most of the
9 remainder being burnt (Hu et al.2012).

10 Mollisols are predominant in North-East China, in the Russian steppe and East of
11 the Rocky Mountains in America. Mollisols are highly fertile and, as a consequence,
12 can lead to high crop productivity. However, long-term cultivation and fertilization
13 has occasionally led to fertility deterioration. This has been attributed to soil erosion
14 and acidification.

15 The application of high-throughput sequencing technology to study the effects of
16 long-term organic and inorganic fertilization on soil microbial community has been
17 increasingly used (Chen et al.2016; Davide et al.2015; Zhou et al.2015). However,
18 relatively few studies have compared the effects of different fertilizers on soil
19 microbial communities (Zhu et al. 2018), and how long-term fertilization affects the
20 microbial community of soil rhizosphere. To address these knowledge gaps, a
21 long-term field experiment receiving several types of inorganic and

1 organics-fertilizers, for 35 years was selected. Quantitative real-time PCR (q-PCR)
2 and Illumina Miseq sequencing technique were used, to analyze the soil bacterial
3 community structure in fourteen different treatments. This study is expected to
4 provide insights into the effects of several soil amendments on the composition of soil
5 microbial communities. We examined: (1) whether soil amendment can significantly
6 affect the microbial community of soil rhizosphere. (2) the type of N fertilization
7 more conducive of shifts in soil bacterial community.

8 **Materials and Methods**

9 The long-term experimental site is located at Jilin Agricultural University,
10 Changchun city, Jilin Province, China (43°47'N, 123°20'E). The area belongs to the
11 monsoon-influenced hot-summer humid continental climate (Dwa according to
12 Koppen) with an annual mean temperature of 4.8°C and an average annual
13 precipitation of 570mm. The soil type in the area is a Mollisol where maize (*Zea mays*
14 *L.*) with continuous cropping is cultivated in summer by conventional tillage.
15 Fourteen treatments with three replicates (42 plots) in completely randomized blocks
16 were established in 1984, The soil had originally a pH value of 7.12, soil organic
17 matter of 22.8g kg⁻¹ and 1.39g kg⁻¹ total nitrogen. Total phosphorus was 0.53g kg⁻¹,
18 total potassium was 23.1g kg⁻¹, alkali-hydrolyzable nitrogen was 125mg kg⁻¹,
19 available phosphorus as 13.6mg kg⁻¹ and available potassium was 218 mg kg⁻¹. Each
20 plot had an area of 2m². The treatments were: (1) control without fertilizer (CK), (2)
21 mineral N fertilizer (ON), (3) mineral P fertilizer (OP), (4) mineral K fertilizer (OK),

(5) mineral N and potassium (K) fertilizer (NK), (6) mineral PK fertilizer (PK), (7) mineral NP fertilizer (NP), (8) mineral NPK fertilizer (NPK), (9) Low levels of maize straw ($S_{0.25}$) (10) High levels of maize straw ($S_{0.5}$), (11) Low levels of maize straw plus NPK fertilizers ($NPK+S_{0.25}$), (12) High levels of maize straw plus NPK ($NPK+S_{0.5}$), (13) Low levels of maize straw plus PK fertilizers ($PK+S_{0.25}$), (14) High levels of maize straw plus PK ($PK+S_{0.5}$). The amounts of fertilizer applied to the crop were $150.0 \text{ kg N ha}^{-1} \text{ years}^{-1}$, $75.0 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1} \text{ years}^{-1}$, and $75.0 \text{ kg K}_2\text{O ha}^{-1} \text{ year}^{-1}$. P and K fertilizer were applied all at once during the spring, 1/3 of the N fertilizer was applied together with the phosphate and potassium fertilizers, while the remaining two-thirds were applied as a top dressing. Maize straw was buried in the soil to a depth of 20cm and applied at two levels, namely $2500 \text{ kg ha}^{-1} (S_{0.25})$ and $5000 \text{ kg ha}^{-1} (S_{0.50})$.

In October 2016, six soil cores (5 cm diameter, 0-15 cm depth) were randomly collected from each replicate plot and pooled into one composite sample per plot. Thus, a total of 42 samples were collected and brought to the laboratory. After visible stones and plant residues were removed, the soil was homogenized and sieved to 2mm for biological and chemical analyses. All samples were divided into two parts, one portion was air-dried to determine the general soil properties, and another part was preserved at -80°C for DNA extraction and molecular biology analysis. The detailed experiment design and fertilizer application are listed in Table S1.

Soil Biochemical Characterization

1 Soil pH was measured on a 1:2.5 (w/v) ratio in distilled water using a pH meter;
2 soil organic matter(SOM) was determined after dichromate oxidation and titration
3 with ferrous ammonium sulfate (Mebius. 1960). Soil total nitrogen (TN) was
4 determined by semi micro-Kjeldahl (KDY-9820) digestion (Bremner. 2010). Soil
5 total phosphorus (TP) and soil available phosphorus (AP) were determined
6 colorimetrically using the molybdate method (Murphy and Riley. 1962) and the Olsen
7 method (Olsen et al.1953) respectively. Soil total potassium (TK) was extracted by
8 incubation with sodium hydroxide, and soil available potassium (AK) was extracted
9 by incubation with 1.0 mol L⁻¹ ammonium acetate for 0.5 h, followed by filtration ,
10 Soil total potassium and available potassium content was determined using a flame
11 photometer (Lu. 1999). The concentrations of soil NH₄⁺ and NO₃⁻ were extracted for
12 30 minutes using a 0.01 M CaCl₂ solution (1:10 of soil: solution w:v) and then
13 measured using a continuous-flow stream autoanalyser (Autoanalyzer 3, Bran
14 Luebbe, Norderstedt, Germany).

15 **Real-time PCR and 16S rDNA**

16 Freeze-dried soil samples (0.5g) were used for DNA extraction using the Power
17 Soil DNA Isolation Kit (Mo Bio), according to the manufacturer's protocol. Extracted
18 DNA was then purified using the GV-High-Efficiency Agarose Gel DNA Purification
19 Kit, DNA concentrations were determined using the Qubit quantification platform
20 with Quant-iT™ ESDNA BR Assay Kit (Invitrogen). DNA was diluted to 10 mg
21 mL⁻¹ and stored at -80°C prior to molecular analysis.

The abundances of genes (*nifH*, archaeal *amoA*, bacterial *amoA*, *nosZ*) encoding the key enzymes for biological N cycling were quantified by real-time PCR (IBA7500) for all soil samples. These methods were modified from their original descriptions (references following primers) as (detailed in Table S2). Gel-purified PCR products from a common DNA mixture (equal amounts of DNA from all samples) were used to prepare sample-derived quantification standards (Chen et al. 2007). The copy number of genes in each standard was calculated by DNA concentration ($\text{mg}\cdot\text{mL}^{-1}$, measured by Qubit) divided by average molecular weight (PCR product length and GC content were obtained from barcoded-pyro sequencing results) of the amplified gene fragment (Mao et al. 2012).

The 16S rDNA high-throughput sequencing was performed by the Genewiz Genomics Institute (Suzhou, China) using the Illumina MiSeq platform. The sequences have been deposited in NCBI and the accession numbers are listed in Table 1. The 16S V3-V4 region was amplified using the primers U341F (ACT CCT ACG GGA GGC AGC AG) and U806R (GGA CTA CHV GGG TWT CTA AT). The clustering of the operational taxonomic unit (OTU), at 97% similarity, was conducted using the UPARSE pipeline (Edgar. 2013). Paired end reads with sufficient overlap (minimum 20 base overlap between forward and reverse reads) were merged into full length sequences by FLASH (Zhang et al. 2015). OTU clustering analysis was performed using QIIME software at 97% similarity to obtain OTUs for species classification. Each OTU was considered to represent a species, and a sequence was

1 selected from each OTU as a representative sequence of the OTU. The RDP method
2 was used to analyze the species levels at 97% similarity. A profiling prism and
3 performing heat map analysis was done. QIIME software was used to calculate the α
4 diversity index (chao1 index, Shannon, Simpson), and a dilution curve was made to
5 analyze the content of each group in the sample (Segata et al. 2011).

6 **Statistical analysis**

7 To determine main and interactive treatment effects on microbial functioning, R
8 statistical software was utilized. Wilcox.test was used to determine differences among
9 soil microbial communities. The number of phylo types (the number of OTUs) was
10 used to estimate the community richness; Treatment differences among these metrics
11 were assessed using Wilcox. Test as described above. The abundance differences of
12 these selected OTUs under each pair of native and invaded soil samples were further
13 tested by Wilcox. Test. The OTUs with a P -value < 0.05 were selected and identified
14 as the phylo types that were significant. Multivariate permutation tests per MANOVA
15 were used to determine if beta diversity differed significantly among treatments (999
16 permutations). Weighted Unifrac scores between all pairs of samples were used as the
17 basis for PER MANOVA and for non-metric multidimensional scaling to visualize
18 the patterns of soil communities. The correlations between the abundance of C, P and
19 N-cycling functional genes and soil general properties were characteristics using
20 Pearson correlations. Statistical analyses were performed in the corrplot package in R.

Results

Soil General Properties

Soil pH, organic matter content and nutrient concentrations are shown in Table 2. Addition of nitrogen fertilizer (NPK, NP, NK, ON, and NPK+S_{0.25}) resulted in a lower pH (6.18~6.82) than for the control soil (pH=7.20). The treatment S_{0.25} increased soil organic carbon. However, several treatments (S_{0.50}, NPK+S_{0.25}, NPK+S_{0.50}, N, NK and, PK+S_{0.50}) resulted in lower organic matter. Total nitrogen contents were not altered by the different soil treatments. With respect to inorganic nitrogen, few differences were found among the control and the other treatments. Thus, NH₄⁺-N levels were similar in all treatments, while nitrate was lower in the S_{0.50} treatment. TP was significantly higher under treatments with phosphorus NPK, NPK+S_{0.25}, NPK+S_{0.50} NP, PK, K, PK+S_{0.25} and PK+S_{0.50}. Total potassium and available phosphorus did not differ within treatments. Available potassium, was enhanced by PK+S_{0.5}, S_{0.5} and K.

Relative Abundance of Functional Microbial

For nitrogen cycling function microbial, AOA abundance in some of the treatments receiving long-term addition of straw (S_{0.25}, S_{0.5}, , NPK+S_{0.5}) was lower than in the control soil ($P<0.05$, Table3). AOB abundance in most treatments containing nitrogen fertilizer (NPK, NK, ON, NPK+S_{0.25}, and NPK+S_{0.5}) was higher than in the control ($P<0.05$, Table3). The abundance of nifH was lower in the

1 treatments NPK and N ($P>0.05$). The abundance of *nosZ* were considerably less in
 2 treatments receiving high doses of straw ($S_{0.5}$, NPK+ $S_{0.5}$, PK+ $S_{0.5}$) and in N. For
 3 C-fixing functional gene, the plots assigned to treatments N and PK had a lower value
 4 than the control. Phosphate-solubilizing functional genes abundance was similar
 5 across all treatments (Table3).

6 AOA abundance was significant negatively correlated with TOC ($r=-0.845$,
 7 $P<0.001$) and TN ($r=-0.801$, $P=0.001$, Table4). The value of soil pH was negatively
 8 correlated with AOB ($r=-0.694$, $P=0.006$) and positive correlated with *nifH* ($r=0.564$,
 9 $P=0.036$) and *cbbl* ($r=0.541$, $P=0.046$) abundance. AOB abundance was negatively
 10 correlated with AK ($r=-0.622$, $P=0.018$, Table4). TOC and *cbbl* abundance were
 11 positively correlated ($r=0.618$, $P=0.019$). The abundance of *nosZ* was negatively
 12 correlated with TOC ($r=-0.735$, $P=0.003$) and TN ($r=-0.722$, $P=0.004$).
 13 Phosphate-solubilizing functional genes abundance was positively correlated with soil
 14 AP ($r=0.801$, $P=0.001$) and TP ($r=0.772$, $P=0.001$, Table4).

15 **Compositions of bacterial communities**

16 After basal quality control, a total of 2,556,777 bacterial sequences were
 17 obtained from the 14 samples. The sequences were grouped into 8261 OTUs of
 18 bacteria at the 97% similarity cut-off level. The most abundant phylum of bacteria
 19 was *Acidobacteria*, which accounted for an average 33.2% of the total sequences,
 20 followed by *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, which accounted for
 21 18.4%, 17.6% and 10.6% of the total sequences, respectively (Fig. 1).

By grouping comparison, it was found that the addition of different fertilizers had little effect on OTU richness ($P>0.05$, Table S3). However, correlation analysis showed that OTU richness was significantly positively ($r^2=0.31$, $P=0.021$) correlated with soil pH (Fig.2). For a relative abundance of major phyla, the difference analysis showed that the *Gemmatimonadetes* abundance in plots with nitrogen fertilizer was significantly higher than in those without nitrogen fertilizer ($P<0.05$, Fig.3), However, the former exhibited lower abundance in *Latescibacteria* and *Thaumarchaeota* than the later ($P<0.05$, Fig.3). Other fertilizer treatments had little influence on the major phyla, and there was no significant difference among them.

The heatmap shows pairwise comparisons of the bacterial community structures between all samples (Fig. 4). Two major clusters, A and B, were identified (Fig. 4). Cluster A was mainly composed of nitrogen fertilizer with other fertilizer samples and cluster B mostly samples without nitrogen, with the exception of the treatment NPK+S_{0.5}. Non-metric multidimensional scaling (NMDS) was used to further verify the effect of N-fertilizer addition on microbial community structure. The taxa that responded to microbial significantly clustered by treatment in NMDS ordination space (ANOSIM: $R = 0.20$, $P = 0.045$, Fig. 5). The addition of other amendments, such as phosphate fertilizer, potassium fertilizer, and straw, could not significantly alter the soil microbial community structure.

We conducted the interdependence between 16S rDNA transcript abundance of

individual phylo types and single environmental factors using redundancy analysis (RDA, Fig. 6). Of all the environmental variables tested, soil pH showed the highest correlation with community composition ($r^2=0.71$, $P=0.001$, Table S4). Other factors, such as TOC ($r^2=0.42$, $P=0.048$) and TN ($r^2=0.46$, $P=0.038$) also showed a high ($P<0.05$) correlation with bacterial community composition (Table S4).

Discussion

Effect of fertilization on soil characters

The results of the present study demonstrated that after the 35-year application of organic amendments and/or inorganic fertilization, some soil physicochemical properties significantly changed, which concurs with previous studies (Wang et al.2014; Wang et al.2017). Long-term nitrogen fertilization results in soil acidification due to the release of H^+ by nitrification(Vries and Breeuwsma. 1987). In our study, the addition of nitrogen fertilizer (NPK, NP, NK, ON, NPK+S_{0.25}, and NPK+S_{0.5}) resulted in a lower pH (6.18~6.82) than for CK (pH=7.20), with pH declining significantly with adding nitrogen fertilizer. Williams et al. (2013) suggested that soil pH declines with increasing N which is in accordance with our results. Long-term application of phosphorus fertilizer can promote soil phosphorus accumulation; the results showed that long-term application of phosphate fertilizer, could significantly increase soil total phosphorus content. Especially with Single phosphate fertilizer treatment (OP), the accumulation of phosphorus in soil was the most. When combined with other fertilizers, the total phosphorus content of the soil was less than that of

1 single phosphate fertilizer. This may be due to the effect of formula fertilization on
2 the soil. It provides soil with a variety of nutrients, promotes soil nutrient cycling,
3 microbial activity, and makes soil phosphorus absorbed and utilized by plants.
4 Similarly to the application of phosphorus fertilizer, potassium fertilization can also
5 effectively increase the accumulation of potassium in the soil.

6 **Effects of fertilization on Relative Abundance of Functional Microbial** 7 **Communities**

8 Nutrient cycling microorganisms are important for soil nutrient cycling and plant
9 nutrient utilization(Davide et al. 2016; Banerjee 2016). Long-term fertilization can
10 directly change the abundance of certain bacterial phyla (Chen et al.2016; Helgason et
11 al. 2016). Our long-term observation data shows adding nitrogen fertilizer influence
12 the relative abundance of N-cycling function microorganism in the soil's rhizosphere.
13 Among them, AOB abundance in the plots without fertilizer was less than in the plots
14 with nitrogen fertilizer, while AOA abundance was significant negatively correlated
15 with TOC ($r=-0.845$, $P<0.001$) and TN ($r=-0.801$, $P=0.001$). This supports the general
16 view that soil AOB is favored by high soil N availability, whereas AOA is less
17 responsive to N availability (Simonin et al. 2015; Wertz et al. 2012; Chen et al.2014).
18 It was suggested that AOB have a lower affinity for N substrate than AOA and are
19 generally favored by higher N levels (Martens-Habbena et al. 2009). Plots without N
20 fertilizer (i.e., PK, OP, OK, PK+S_{0.25} and PK+S_{0.5} treatments) had little impact on
21 nifH abundance. This means that long-term application of nitrogen fertilizer has

different effects on different functional microorganisms(Ma et al. 2016).

Tang et al. (2011) found that P fertilization resulted in a higher abundance of targeted bacterial phyla and of the whole bacterial and fungal communities. Zheng et al. (2017) suggested that N rather than P or K fertilization significantly affected the PSB community structure and abundance and suggested that P-fertilization may scarcely affect the phosphorus solubilizing bacteria community. These results are similar to the ones found in our study. Soil TOC and cbbl abundance exhibited a significant positive correlation, in line with the view that soil organic carbon and pH are the most significant factors influencing the community composition, abundance, and activity of the cbbl bacteria(Yuan et al. 2012; Long et al. 2015). The value of soil pH was also significantly correlated with the abundance of AOB and nifH. Addition of nitrogen fertilizer caused soil pH reduction and different functional microbial were sensitive to N. Hence soil pH can affect the abundance of functional microbes. The above results show that fertilization can change the soil nutrient content and change in soil nutrients content could affect the abundance of nutrient cycling microbial and different functional microbes.

Effect of Soil Properties and Nitrogen Addition on Functional Microbial Communities.

Environmental changes could alter soil microbial community structure and diversity to some extent (Burns et al. 2013). Deep 16S rDNA Illumina Miseq sequencing methods were used to investigate soil bacterial community in the Mollisol

1 treated with various types of amendments. Overall, there were no large differences
2 among different amendments with and without straw. Bacterial diversity was
3 significantly correlated with soil pH, which concurs with other studies (Geisseler and
4 Scow, 2014; Li et al. 2015). Consistently with previous studies on agricultural soils,
5 the dominated phyla of bacterial communities across all treatments were
6 *Acidobacteria*, *Proteobacteria* and *Bacteroidetes* (Ding et al. 2016; Zhong et al.
7 2015). Significant differences of other major phyla abundance (*Gemmatimonadetes*,
8 *Latescibacteria*, and *Thaumarchaeota*) were also observed with soil samples treated
9 with and without nitrogen fertilizer, but other fertilizer treatments had little influence
10 on the major phyla. This could mean that nitrogen fertilizer addition has more
11 influence on soil microorganisms than other fertilizers, as suggested in previous
12 studies (Freitag et al. 2005; Lagomarsino et al. 2007).

13 The heatmap analysis shows pairwise comparisons of the bacterial community
14 structures between all samples which reveal that repeated application N-fertilizer for
15 35 years facilitated the development of a soil bacterial community structure distinct
16 from no N-fertilization. Non-metric multidimensional scaling (NMDS) was useful to
17 further verify the effect of N-fertilizer addition on microbial community structure.
18 The taxa that responded to microbial significantly clustered by treatment in NMDS
19 ordination space (Fig. 5). Based on this, N fertilizer may tentatively be considered as
20 a key factor which interacts with other fertilizers and impacts soil microbial
21 communities (Lagomarsino et al. 2007; Yao et al. 2014). The presence or absence of

1 other amendments, including P and K fertilizers and straw return have little effect on
2 soil microbial community structure. The RDA ordination plot (Fig.6) shows the
3 relationships between soil properties and bacterial community structures. Soil pH
4 ($r^2=0.71$, $P= 0.001$), TOC($r^2=0.42$, $P= 0.048$) and TN ($r^2=0.46$, $P= 0.038$) showed a
5 high ($P< 0.05$) correlation with bacterial community composition. While pH declined
6 significantly with increasing N application. All these results indicated that the N
7 addition increased the N concentration in the soil (Li et al. 2014), which could cause
8 changes in the soil pH and changed the soil bacterial community.

9 **Conclusions**

10 Our results clearly showed that the addition of nitrogen fertilizer decreased soil
11 pH. Long-term amendments were, in general, not able to change the abundance of
12 functional microbial community, with an exception being N fertilizers. The alteration
13 in soil pH as a consequence of fertilizer addition seems to play a key role in the
14 changes in soil microbial community. The results obtained in this study offer a key
15 insight into soil microbial community and its changes after long-term amendment.
16 The study assisted the identification of the main variables shaping soil microbial
17 communities. The results indicated that straw return to the soil can help mitigate the
18 bacterial impacts brought about by N addition.

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5 **Author Contributions** Yan Du, Tianye Wang, Chengyu Wang, Paul-Simon Anane,
6 Shuxia Liu and Jorge Paz-Ferreiro conceived the study. Yan Du, Tianye Wang and
7 Chengyu Wang performed the experiments. Yan Du, Tianye Wang and Paul-Simon
8 wrote the manuscript. All authors reviewed the manuscript and agree with its
9 contents.

10 **Corresponding author** Shuxia Liu and Jorge Paz-Ferreiro

11 **Conflict of interest:** The authors declare that they have no conflict of interest.

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14 under consideration by another journal, and has not been published previously. All
15 authors read and approved the final manuscript.

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12 **Figure legends**

13 **Fig. 1** The relative abundances of microorganisms at the phyla level under the
14 long-term fertilization.

15 **Fig. 2** Correlation analysis between OTU abundance and soil pH according to the
16 Duncan test.

17 **Fig. 3** Differences in the major phyla levels abundances of soil microbial between
18 with and without nitrogenous fertilizers.

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20 Note: Horizontal clustering means that the abundance of each species in the sample is
21 similar. The closer the distance, the shorter the branch length, indicating that the more

1 similar the two species are among the samples. Longitudinal clustering shows
2 similarities of all species in different samples. Similar to horizontal clustering, the
3 shorter the distance, the shorter the branch length, indicating more similar species
4 composition and abundance. Sample grouping information, the first line in the figure
5 is sample grouping information, and the color corresponds to the map column.

6 **Fig. 5** NMDS Analysis of Microbial Community Structure with Nitrogen Fertilizer.

7 Note: The No.1 and No. 2 indicate the treatment with and without nitrogen fertilizers.

8 **Fig. 6** Redundancy analysis between fertilization and environmental factors.

9 Note: Environmental factors are generally indicated by arrows. The length of arrow
10 connection represents the degree of correlation between an environmental factor and
11 community distribution and species distribution. The longer the connection, the
12 greater the correlation and the smaller the other.

13

1 **List of Tables**

2 Table 1. NCBI ratio on the results of bacterial

Sample	Accession	Query cover	Ident
CK	SRR7168815	100%	97%
S0.25	SRR7168814	100%	97%
S0.5	SRR7168809	100%	97%
NPK	SRR7168807	100%	97%
NPK+S0.25	SRR7168811	100%	97%
NPK+S0.5	SRR7168810	100%	97%
N	SRR7168813	100%	97%
NP	SRR7168812	100%	97%
NK	SRR7168819	100%	97%
PK	SRR7168808	100%	97%
P	SRR7168817	100%	97%
K	SRR7168816	100%	97%
PK+S0.25	SRR7168806	100%	97%
PK+S0.5	SRR7168818	100%	97%

3

Table 2. Effect of fertilizer treatments (full set of treatments) and the interaction between fertilizer input type and level on soil chemical characteristics in a long-term fertilizer input trial. The lower case letters ‘a’, ‘b’, ‘c’ and ‘d’ indicate values that are significantly different ($P < 0.05$) among treatment.

Sample	pH	SOM (g kg ⁻¹)	T-N (g kg ⁻¹)	T-P (g kg ⁻¹)	T-K (g kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	A-P (mg kg ⁻¹)	A-K (mg kg ⁻¹)
CK	7.20±0.25ab	29.21±6.64ab	1.40±0.09ab	6.20±0.10cd	5.08±0.15ab	1.78±0.27ab	0.74±0.12abc	3.07±0.38d	211.36±4.50cdef
S0.25	7.28±0.19ab	27.47±7.23b	1.59±0.37ab	6.84±1.83cd	4.42±0.16b	2.02±1.23ab	0.92±0.28abc	2.88±0.58d	202.36±4.37defg
S0.5	7.44±0.21a	32.08±2.04ab	1.50±0.09ab	5.77±0.28d	5.05±0.88ab	2.15±0.06ab	0.57±0.09d	4.20±1.22d	303.33±9.06ab
NPK	6.55±0.13def	28.68±6.61ab	1.55±0.07ab	9.92±0.33ab	5.00±0.64ab	1.77±1.45ab	1.56±0.55a	25.40±4.34c	187.70±4.24efg
NPK+S0.25	6.30±0.55ef	36.44±2.23ab	1.64±0.16ab	10.08±1.44ab	5.18±0.03ab	2.33±0.52ab	0.80±0.35abc	23.80±5.25c	220.36±3.17cdef
NPK+S0.5	6.82±0.31bcde	38.06±2.59a	1.66±0.30ab	9.86±2.55ab	5.15±0.09ab	2.05±0.46ab	0.60±0.25cd	18.60±4.8c	249.68±3.07bcde
N	6.18±0.27f	28.59±9.77ab	1.35±0.09b	6.00±0.08cd	4.93±0.46ab	2.46±0.23ab	1.48±1.36abc	2.42±0.41d	156.37±2.50fg
NP	6.61±0.28cdef	32.14±2.61ab	1.30±0.31b	10.64±0.68ab	4.85±0.30ab	1.82±0.05ab	0.78±0.06abc	26.1±6.32c	141.71±1.53g
NK	6.56±0.50def	31.55±3.07ab	1.41±0.02ab	5.78±0.16d	4.93±0.31ab	2.10±0.21ab	0.64±0.11bcd	2.62±0.41d	243.35±4.24bcde
PK	7.11±0.12abc	30.93±6.60ab	1.64±0.40ab	10.86±0.33ab	5.22±0.33ab	1.48±0.58b	1.14±0.33abcd	38.37±4.62ab	273.67±1.52abc
P	7.14±0.25ab	26.44±5.15b	1.46±0.03ab	11.58±0.61a	4.85±0.30ab	2.59±0.67ab	1.46±0.41abc	44.15±4.26a	207.36±2.53def
K	7.04±0.02abcd	32.20±2.74ab	1.49±0.05ab	5.71±0.15d	5.43±0.98a	1.84±0.77ab	1.00±0.21abc	3.80±1.04d	289.34±2.10ab
PK+S0.25	7.18±0.19ab	33.24±7.04ab	1.87±0.37a	9.77±3.03ab	5.20±0.01ab	3.09±0.55a	1.52±0.25ab	34.65±5.25b	257.27±2.19bcd
PK+S0.5	7.27±0.36ab	30.35±2.29ab	1.41±0.36ab	11.29±1.50a	5.20±0.05ab	1.92±0.35ab	0.66±0.08bcd	45.82±9.82a	325.70±3.81a

Table 3. Real-time PCR quantification of the N-transforming function genes (*nifH*, archaeal *amoA*, bacterial *amoA*, and *nosZ*) between fertilizer input type. The copy number of genes in each gram of dry soil was estimated based on the results of real-time PCR (copies per ng DNA). The standard curve of all these genes was > 0.99 . The lower case letters 'a', 'b', 'c' and 'd' indicate values that are significantly different ($P < 0.05$) among treatment. Each sample was measured in triplicate.

	AOA	AOB	<i>nifH</i>	<i>cbbL</i>	<i>nosZ</i>	<i>Gdh3</i>
CK	7.19±0.06a	6.68±0.43cdef	6.74±0.09abc	7.19±0.19ab	6.95±0.15ab	6.79±0.11ab
S0.25	6.81±0.16cd	6.73±0.10bcdef	6.73±0.01abc	7.17±0.21ab	6.83±0.24abc	7.71±0.27ab
S0.5	6.76±0.05d	6.86±0.51abcde	6.57±0.07cde	7.18±0.13ab	6.64±0.13cd	6.76±0.21b
NPK	7.11±0.09ab	7.17±0.06a	6.53±0.09de	7.10±0.16abc	6.82±0.13abc	7.08±0.63ab
NPK+S0.25	6.93±0.04abcd	7.14±0.11a	6.69±0.21	7.13±0.17abc	6.69±0.19bcd	7.13±0.11ab
NPK+S0.5	6.89±0.44bcd	7.10±0.11ab	6.64±0.12bcd	7.17±0.05ab	6.43±0.07d	7.03±0.07ab
N	7.20±0.06a	7.18±0.06a	6.41±0.16e	6.84±0.15d	6.62±0.15cd	6.97±0.10ab
NP	7.08±0.06abc	7.06±0.09abc	6.73±0.10abc	6.90±0.10bcd	7.04±0.09a	7.08±0.11ab
NK	7.10±0.14ab	7.11±0.23ab	6.70±0.12abcd	6.93±0.14abcd	6.94±0.13ab	7.01±0.18ab
PK	7.13±0.01ab	6.94±0.14abcd	6.88±0.07a	6.87±0.20cd	7.02±0.13a	7.13±0.09ab
P	7.11±0.03ab	6.99±0.10abcd	6.85±0.05a	6.99±0.13abcd	7.02±0.21a	7.11±0.11ab
K	7.19±0.05a	6.35±0.10f	6.83±0.06ab	7.18±0.15ab	7.01±0.10a	6.96±0.10ab
PK+S0.25	6.96±0.15abcd	6.61±0.15def	6.80±0.05ab	7.14±0.15abc	6.97±0.05ab	7.20±0.16a
PK+S0.5	6.97±0.02abcd	6.52±0.01ef	6.82±0.11ab	7.23±0.18a	6.58±0.20cd	7.19±0.08ab

1 Table 4. Pearson correlations between functional gene abundance involved in C, N, P-
 2 cycling and soil properties.

	AOA	AOB	<i>nifH</i>	<i>CbbL</i>	<i>nosZ</i>	<i>Gdh3</i>
TOC	-0.845**	—	—	0.618*	-0.735**	—
TN	-0.801**	—	—	—	-0.722**	—
TP	—	—	—	—	—	0.772**
AP	—	—	—	—	—	0.801**
AK	—	-0.622*	—	—	—	—
pH	—	-0.694*	0.564*	0.541*	—	—
Moisture	—	—	—	—	0.711**	—

3

Fig. 1 The relative abundances of microorganisms at the phyla level under the long-term fertilization.

Fig. 2 Correlation analysis between OTU abundance and soil pH according to the Duncan test.

Fig. 3 Differences in the major phyla levels abundances of soil microbial between with and without nitrogenous fertilizers.

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