Distinct responses of active and total bacterial communities to inorganic fertilization in a 30-year experimental site

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Abstract

Soil microbial communities play a vital role in mediating nutrient turnover, thus enhancing growth and development of plants. Understanding the dynamics of microorganisms in soils can provide insight into the influence of fertilization practices on improving soil fertility and plant growth in agricultural ecosystems. In this study, we compared the abundances and compositions of total (DNA-based, 16S rRNA gene) and active (RNA-based, 16S rRNA) bacterial communities at a 30-year experimental site in different inorganic fertilization treatments with different key elements (nitrogen, phosphorus, and potassium). The inorganic fertilizer amendments did not affect the abundance of total bacteria but significantly affected the abundance of active bacteria due to changes in microbial biomass carbon and NH_4^+-N contents. *Cyanobacteria* and *Proteobacteria*, especially for some dominant orders (e.g. *Nostocales*, *Pseudanabaenales* and *Nitrosomonadales*) were the dominant phyla in the active microbial community and differed proportionally in nitrogen and phosphorus fertilized soil. Soil N speciation (e.g. total N, NH_4^+-N and NO_3^--N) were the main determinants controlling the *Cyanobacteria* and *Proteobacteria* communities. Our results indicated that the unbalanced fertilization could reduce the abundance of active bacteria and significantly changed the dominant phyla compared with balanced fertilization. These findings provided an insight of composition and ratio of nutrient elements including nitrogen, phosphorus and potassium for managing future fertilization regimes in agricultural ecosystem.

1 Introduction

The application of chemical fertilizers is an important agricultural practice that affect soil bacterial communities because soil bacteria are crucial to maintain the soil fertility and crop productions. Soil microbes mediate diverse soil processes in agricultural ecosystems, including decomposition, nutrient cycling and soil fertility. Shifts of soil microbial community is recognized as an indicator of quality and soil fertility, so understanding the responsive mechanisms of microbial groups in soils fertilized by different chemicals is important for developing an efficient system for agricultural application (Tang et al., 2017; Wang et al., 2018b; Ma et al., 2019).

Because of slow changes in soil quality and relatively steady state after changing field managements in long-term fertilization experiments, a recent series of studies have extensively explored the response of communities of soil microorganisms under long-term fertilization practices (Ahn et al., 2012;Su et al., 2015;Suleiman et al., 2016) and found that the communities were regulated by a wide range of biotic and abiotic parameters (Chen et al., 2016a; Li et al., 2016; Fierer, 2017; Zhang et al., 2017). Most studies of long-term fertilization, however, have focused on analyzing the DNA of microbial communities. Because a large amount of cells and most operational taxonomic units (OTUs) that represent bacteria in soils may be inactive (Lennon and Jones, 2011), analysis based on DNA only revealed the total bacterial community in the soil and will inevitably miss functional microbiota (Kim et al., 2013) and thus may not represent the relationship between a microbial community and environmental stress compared to the potentially active state (Baldrian et al., 2012).

RNA turns over quickly and can therefore be used as an indicator to gain insight into active microbial communities, which can directly use soil substrates and mediate essential functions of agricultural ecosystems (Moeseneder et al., 2005). The abundance (Sheng et al., 2016; Tang et al., 2017) and community composition (Freedman et al., 2015) of the active microbial community in several ecosystems differed significantly from the total microbial community (Zhang et al., 2014b;Li et al., 2019). For example, Tang et al. (2017) found more close relationship between active bacteria and activity of N fixation in long-term fertilized regions. Li et al. (2019) recently reported that the variation of bacterial communities was correlated with an oxygen gradient and root exudation in paddy soils and that a comparison between DNA and RNA sequencing data clearly indicated the dynamics of the rhizospheric microbial community. Therefore, rRNA-based molecular technologies can provide a feasible method for analyzing the dynamics of active microbiomes in agricultural ecosystem.

With these consideration, we used Illumina sequencing and quantitative PCR(qPCR) of the 16S rRNA gene(DNA) and 16S rRNA(RNA) extracted from soils with an approximately 30-year history of different fertilizations to determine the effects of applying different chemical fertilizers on active and total bacterial communities. We also estimated various soil characteristics including water content, soil pH, organic matter, total carbon, total nitrogen, total phosphorus, available phosphorus, NH_4^+-N, NO_3^--N, microbial biomass carbon, nitrogen and phosphorus to identify the relationship between changes in microbial abundance, community composition and environmental status. We hypothesized that: (1) the abundance of active bacteria would be more

influenced by long-term chemical fertilization and be more correlated with changes in soil properties than total bacteria and (2) the composition of the active bacterial community, especially of some dominant taxa, would respond more strongly to different fertilization treatments and be more sensitive to altered environmental factors than the total bacterial community.

2 Materials and methods

2.1 Research site and soil sampling

Soil samples were collected from the Fengqiu State Agro-ecological Experimental Station in Henan Province, China (35°00′N, 114°24′E). A field experiment was established in 1989 and has continued for almost 30 years with a consecutive integrated wheat-maize rotation system, testing four replicates of seven treatments: (1) CK, no fertilizer; (2) PK, chemical P and K fertilizer; (3) NK, chemical N and K fertilizer; (4) NP, chemical N and P fertilizer; and (5) NPK, N, P and K fertilizer. A detailed plot design and management practices have been reported in previous studies(Meng et al., 2005; Zheng et al., 2017).We collected five core samples of fresh surface soil (0–20 cm layer)from each plot of five of the seven treatments, CK, NK, NP, PK and NPK, on September 2015.Stones, soil animals and visible plant debris were manually removed. The samples were then sieved (<2 mm) and thoroughly mixed. Subsamples for chemical and biological analyses were pooled and stored at 4°C. Samples for DNA extraction were stored at -80°C, and samples for RNA extraction were storedat-20°C after adding a solution to protect RNA (GENEray Biotechnology, Shanghai, China), following the manufacturer's instructions, until molecular analysis.

2.2 Chemical and biological analyses

Gravimetric water content (WC) was determined by weighing before and after oven-drying at 105°C to a constant weight. Soil pH was measured in a 1:2.5 (w/v) soil: $CaCl_2$ solution using a pH meter (Accumet, Westford, USA). An elemental analyzer (Elementar, Hanau, Germany) was used to analyze total C (TC), N (TN) and sulfur contents. NH_4^+-N and NO_3^--N contents were determined by extraction with 2 M KCl (Abuqaoud et al., 1991) on a flow-injection analyzer (Lachat, Loveland, USA).Soil total P (TP) was digested with sulfuric acid (98% H_2SO_4) and measured using the moldable-blue method. Available P (Olsen-P) content was estimated by adding 0.5M $NaHCO_3$ (pH 8.5)(Olsen, 1954).Total K (TK) content was determined by flame emission after acid digestion (Inesa, Shanghai, China). Microbial biomass carbon (BC), N(BN) and P(BP) contents were measured using chloroform fumigation extraction, as described by Wu et al. (1990), Jenkinson (1988) and Wu et al. (2000), respectively.

2.3 DNA and RNA extraction and reverse transcription

Genomic DNA (gDNA) was extracted from the same amounts of moist soil (0.50 g) the day after sampling using a FastDNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, USA) following the manufacturer's instructions. A detailed protocol for RNA extraction has been reported previously(Li et al. 2019). The extracted RNA were purified using an RNeasy® Mini Kit (QIAGEN, Hilden, Germany). RNA fractions from the soil samples were reverse transcribed to complimentary DNA (cDNA) using a PrimeScipt™ RT-PCR Kit (Takara, Dalian, China), and the cDNA was stored at -80°Cuntil further use. The reaction mixtures and thermal-cycling conditions followed

the manufacturer's protocols.

2.4 Real-time qPCR

Copies of the 16S rRNA gene from both the gDNA and cDNA pools were quantified in triplicate using the Light-cycler Roche 480 System (Roche, Basel, Switzerland) with the universal primer pair 515f (5'-GTGCCAGCMGCCGCGG-3') (Gomez-Alvarez et al., 2016) and 907r (5'-CCGTCAATTCMTTTRAGTTT-3') (Long et al., 2018; Chen et al., 2016b), which amplify the hyper variable V4-V5 regions(~375bp). The 20-μl reaction master mix contained10μl of SYBR Green Premix Ex Taq (1×, Takara, Dalian, China), 0.8μl of each primer (10μM), 2μl of DNA template (~60-100 ng), 0.5μl of bovine serum albumin (0.1mg ml^{-1}) and 5.9μl of nuclease-free water. The PCR conditions began with an initial denaturation at 95°C for 5min, followed by 45 cycles of 95°C for 10s, 53°C for 45s, 72°C for 45s and 84°Cfor 15s (fluorescence-intensity detection) and a final extension at72°C for 60s, followed by a melting-curve analysis. A standard curve was produced by quantifying 10-fold serial dilutions of plasmids containing the fragments of the16S rRNA gene. All qPCR efficiencies were >90%. The abundances of 16S rRNA gDNA and cDNA were calculated as copies g^{-1} dry soil.

2.5 MiSeq sequencing of gDNA and cDNA

We used the 515f/907r primer pair described above for the PCR amplification of both16S rRNA gDNA and cDNA. Unique barcode sequences(6bp) were added to each reverse primer to distinguish between the samples of gDNA and cDNA. The reaction master mixes and PCR conditions have been described by Chen et al. (2016). PCR

products were purified, and their concentrations were measured using a Qubit 2.0 Fluorimeter (Thermo Scientific, Waltham, USA). The purified products from all samples were mixed in equimolar amounts and sent to Novogene (Novogene, Beijing, China) for MiSeq sequencing.

2.6 Processing of sequencing data

The data for the bacterial 16S rRNA gene were processed using QIIME 1.9.1 (Quantitative Insights Into Microbial Ecology, http://www.qiime.org) (Caporaso et al., 2010) with default parameters unless otherwise noted. In brief, high-quality reads were binned into operational taxonomic units (OTUs) at a similarity of 97%, and the most abundant read from each OTU was selected as a representative sequence. Each out was taxonomically classified using RDP tools (version 11, http://rdp.cme. msu.edu/), and a phylogenetic tree was created using FastTree (Price et al., 2009).Singletons from the OTUs were filtered, followed by removing representative sequences identified as Archaea, mitochondrial DNA or chloroplast DNA.A table of filtered OTUs was generated for further statistical analysis using R version 3.2.3 (https://www.r-project.org).All raw sequencing data were deposited in the NCBI Sequence Read Archive with the accession number SRP072392.

2.7 Statistical analysis

We used a one-way ANOVA combined with Fisher's least significant difference (LSD) test as a *post hoc* test to analyze the effects of the fertilization treatments on soil properties and bacterial abundance and community composition. A path analysis based on stepwise regression was performed to identify the relationships between

environmental factors and the absolute or relative (the ratio of cDNA to gDNA abundance) abundance of the 16S rRNA gene.

We selected the most active bacterial community at the phylum level by comparing the difference of the relative abundances between active and total bacteria. Standardized OTUs, using the percentage of total OTUs, were used for further statistical analysis: permutational multivariate analysis of variance (PERMANOVA), Monte Carlo permutation (MCP) test, canonical analysis (CCA), variation partition analysis (VPA) and multiple regression tree (MRT)processed with the VEGAN (Oksanen et al., 2013) and MVPART (Therneau et al., 2013) packages in R 3.2.3. A SIMPER analysis was used to account for the differences of OTUs between the treatments based on PAST (Hammer et al., 2001), and a one-way ANOVA was used to determine statistical significance.

3 Results

3.1. 16S rRNA gene and 16S rRNA abundances and its relationships with environmental factors

The 16S rRNA gene was quantified in the fertilizer-amended soils, and its abundance ranged from 2.08×10^{11} to 8.79×10^{11} copies g^{-1} dry soil (Fig. S1). There were no significant differences among each treatment, except for NP treatments (Fig S1, $P>0.05$). For active bacteria, its abundances varied from 2.65×10^{9} to 7.61×10^{10} copies g^{-1} dry soil, and reached the highest in NPK treatments ($F=28.41$, $P<0.001$) (Fig.1a). The proportion of active bacteria in total bacteria was highest in NPK (15.07%), followed by NP (10.37%), NK (5.00%) and PK (3.92%), and was lowest in CK (0.54%)

198 (Fig. 1b). Fertilization thus had a significant effect on the relative abundance of active
199 bacteria (F=65.19, P<0.001) compared to CK.

200 The soil chemical characteristics of the treatments are presented in Table S1. The
201 path analysis indicated that pH, BC and NH_4^+-N contents explained 82.8% of the total
202 variation in the abundance of active bacteria (Fig. 2a). NH_4^+-N and BC contents in this
203 model had positive effects on the number of active bacteria, and pH had a negative
204 effect. BC content particularly affected the number of active bacteria. BC, BN and TN
205 contents in the regression model of relative abundance of active bacteria accounted for
206 91.3% of the total variation, and BC content had the strongest positive direct effect.

207 *3.2 Community composition of active bacteria and relationships with environmental*
208 *factors*

209 Taxonomic compositions at phylum level based on the RNA- and DNA-derived
210 microbial communities are shown in Fig. 3 and S2. The relative abundances of
211 *Cyanobacteria* and *Proteobacteria* were consistently 2.60-13.83% and 20.53-32.85%
212 higher, respectively, in the RNA-derived than the DNA-derived microbial community,
213 indicating the most abundant and active taxa in the long-term fertilized soils. The
214 variation in relative abundance of *Cyanobacteria* differed significantly among the
215 treatments (F=26.39, P<0.001), especially in CK and PK where the increases in relative
216 abundances were significantly higher than in the treatments with N fertilization (NK,
217 NP and NPK). The average abundances of *Proteobacteria* were >30% higher in NP and
218 NPK after both N and P fertilizers were added, and relative abundances were about 20%
219 higher in the CK, PK and NK treatments.

Totals of 3786 OTUs derived from 375801 *Cyanobacteria* sequences and 21258 OTUs derived from 1226650 *Proteobacteria* sequences were detected from the active microbial community. Some orders affiliated within active *Cyanobacteria* and *Proteobacteria* varied among the treatments (Fig. S3). N but not P or K fertilization had a significant effect on the compositions of the *Cyanobacteria* community (Table 1). For example, the abundances of *Nostocales* and *Stramenopiles*, the dominant orders, were significantly lower in the treatments with N than in those without N fertilization. The order *Pseudanabaenales* had the opposite trend, with a higher relative abundance in the treatments with N (F=23.51, P<0.001) (Fig. S3a). The *Myxococcales*, *Entotheonellales* and *MND1* orders of *Proteobacteria*, which were a large proportion of the active community, were highly abundant in NPK (Fig. S3b), indicating that the importance of balanced fertilization increased the relative abundances of active *Proteobacteria*. The relative abundances of the order *Nitrosomonadales* were significantly higher in NK (13.31%), NP (5.48%) and NPK(8.63%) than that in CK(1.24%) and PK(1.02%) (F=42.33, P<0.001), whereas the orders *Rhizobiales* and *Rhodospirillales* did not follow the similar trend.

The MRT analysis indicated that the compositions of the *Cyanobacteria* communities in the treatments segregated with N speciations (TN, BN andNO_3^--N) and TK contents (Fig. 4a), indicating that N played important roles controlling the composition of the *Cyanobacteria* community. The tree of the relative abundances of *Proteobacteria* is shown in Fig. 4b. The first node in the tree was defined byNO_3^--N content, and the following nodes were subsequently segregated with BC and SOM

contents and finally with TN content, demonstrating different environmental variables as predictors compared with those determining *Cyanobacteria* community composition.

3.3Structure of the active bacterial community and relationships with environmental factors

The PERMANOVAs analysis showed that the *Cyanobacteria* (F=3.388, P=0.011) and *Proteobacteria* community structures in N fertilization amendments treatments were different from the those without N fertilization amendment (CK) (F=3.247, P<0.001, Table1). The community structures of *Proteobacteria* also differed significantly between treatments with P and those without P fertilization (F=2.447, P=0.003). The SIMPER analysis was performed to identify the OTUs contributing most to the differences in active *Cyanobacteria* and *Proteobacteria* communities between fertilization treatments (Table 2). The ten dominant OTUs classified as *Cyanobacteria* and *Proteobacteria* together explained 35.12 and 22.35% of the variation, respectively. The most dominant *Cyanobacteria* OTU (OTU57909) was classified in the family *Nostocaceae* and explained 6.54% of the total variation, and its relative abundance was significantly higher in treatments with than without N fertilization (F=8.17, P=0.001). In contrast, OTU117089 (family *Nostocaceae*) and OTU603876 (family *Pseudanabaenaceae*) explained 2.9% and 2.07% of the difference but their relative abundances were significantly increased with N fertilization. OTUs 499318 and 439795, which were affiliated with the family *Nitrosomonadaceae*, notably contributed about11% of the total difference in the *Proteobacteria* community, indicating that the long-term fertilized soils contained the most dominant and active species.

The environmental variables that correlated most with the structure of the *Cyanobacteria* or *Proteobacteria* community based on the MCP analysis were selected for CCA and VPA after testing for variance inflation factors (Table S2). The CCA separated the treatments with and without N fertilization for the *Cyanobacteria* community (Fig. 5a), and data points for the five treatments indicated different distributions along the first two axes for the *Proteobacteria* community, with NK, PK and NPK clearly separated from CK (Fig. 5b). The VPA was conducted to identify the contributions of the environmental variables to the *Cyanobacteria* and *Proteobacteria* communities. The environmental parameters together explained 45.5 and 38.0% of the community structures of the *Cyanobacteria* and *Proteobacteria*, respectively (Fig. 6a and b). TN, NH_4^+-N and NO_3^--N contents explained 13.83% of the total variation in the *Cyanobacteria* community, and TN, NH_4^+-N and BN contents explained 11.8% of the total variation in the *Proteobacteria* community, indicating the important influence of N in both communities. pH and WC and SOM contents contributed 5.14, 3.95 and 5.52% of the variation in the *Cyanobacteria* community and 3.86, 4.93 and 4.58% of the variation in the *Proteobacteria* community, respectively, and were the most important environmental parameters affecting both active communities.

4 Discussions

4.1. Changes in abundances of the 16SrRNA gene and 16S rRNA in the long-term fertilized soils

The N fertilization treatments did not affect the abundance of the total microbial community (Fig. S1), consistent with previous studies in most agricultural ecosystems

(Shen et al., 2008;Wu et al., 2011;Ding et al., 2016). In contrast, Zhou et al. (2015) reported that total 16S rRNA gene abundance was lower in N- and P-amended soils than in the control treatment, most likely due to the large variation of soil pH that in turn negatively affects bacterial population sizes. Interestingly, the abundance of active bacteria in our study differed significantly among the treatments, unlike total bacterial abundance. The response of transcripts of functional genes associated with N fixation (Tang et al., 2017), ammonia oxidation (Nicol et al., 2008), methane production and oxidation (Sheng et al., 2016) differed significantly among fertilization practices and was correlated with nutrient status. Our path analysis indicated that the abundance of active bacteria was positively correlated with BC and NH_4^+-N contents (Fig. 2), suggesting that increasing nutrient availability by long-term fertilization increased the transcriptional activity and bacterial growth.

4.2. Changes in compositions of the active Cyanobacteria and Proteobacteria communities in the long-term fertilized soils

Cyanobacteria accounted for 1.4-19.0% of the active bacterial community, more than the total bacterial community (0.3-1.9%) (Figs. 3 and S2), and was one of the most dominant and active phyla in the long-term fertilized soils. These findings differed from studies that did not analyze *Cyanobacteria* because of their low average proportions based on DNA analyses (Carson and Zeglin, 2018; Kumar et al., 2018). However, sequence analyses based on gene transcripts could provide more useful information than other DNA analyses for understanding the potential activity of specific microbial communities. The presence of extracellular DNA (relic DNA) in the soil can be up to

40% of the total DNA yield, which baffle our understanding of the full extent of living microbial cells (Carini et al., 2017; Pathan et al., 2021). Furthermore, the presence of intracellular DNA from dormant and potentially inactive cells in soil could over-estimate the actual microbial communities' variability under environmental changes (Carini et al., 2020). Thus, gene transcripts are more indicative of active groups than the total microbial community. Some orders affiliated with N-fixing *Cyanobacteria* varied significantly among the fertilization treatments. For example, we used SIMPER analysis to identify *Nostocales*, a dominant diazotrophic taxon, as an indicator of the orders and as a dominant group (Fig. S3 and Table 2). This result was supported by several studies reporting the dominance of *Nostocales* in various environments and their significant contributions to N fixation (Dodds et al., 1995;Potts, 1994; Yeager et al., 2007; Che et al., 2018).

The relative abundance of *Nostocales* was significantly lower in NK, NP and NPK than other treatments, similar to the 8-23% of relative abundance lower after N fertilization reported by Kuppusamy et al., 2018. The relationship between TN content and *Nostocales* abundance was supported by the MRT analysis (Fig. 4). The higher relative abundance of the active *Nostocales* community was therefore more evident in treatments without than with N fertilization. In contrast, the order *Pseudanabaenales* had the opposite trend, where the average proportion was higher when N fertilizers were added (Fig. S1). Bergman et al. (1997) suggested that *Cyanobacteria* communities contained both non-heterocystous and heterocystous bacteria. *Pseudanabaenales*, most of whose OTUs belonged to the genus *Leptolyngbya*(Table 2), are non-heterocystous

bacteria (Gallon, 2004). Non-heterocystous bacteria such as *Leptolyngbya* fix only a small amount of N, unlike heterocystous *Cyanobacteria* (e.g. *Nostoc*)(Belnap, 2002; Garcia-Pichel et al., 2003). The shift between non-heterocystous and heterocystous communities under different amounts of available N may partly account for the different responses of the community compositions of *Nostocales* and *Pseudanabaenales* to N fertilization. Further study is clearly needed to determine the ecological relationships among these *Cyanobacteria* groups.

Proteobacteria, the largest and most active phylum in the long-term fertilized soils, mainly consisted of dominant orders that responded differently to the treatments. For example, the relative abundances of the orders *Myxococcales*, *Entotheonellales* and *MND1* were the highest in NPK (Fig. S3), indicating that these active heterotrophic bacterial communities could flourish with large amounts of available nutrients, in accordance with other studies targeting the16S rRNA gene (Zhou et al., 2014; Zhou et al., 2015). In addition to the changes in the proportions of the orders in the *Proteobacteria* community, changes at the genus and even OTU levels were significantly affected by the treatments. Specifically, some OTUs identified by the SIMPER analysis as dominant species in the order *Nitrosomonadales* had higher average proportions in NK and NPK (Table 2). This finding was in accordance with a previous study, high $NH4^+$-N-induced the increase in *Nitrosomonadales* abundance (Li et al., 2014). Such results demonstrated that long-term fertilization could increase the amount of available N, and subsequently promote the growth of nitrifying bacteria. The relative abundance of the order *Rhodospirillales* differed significantly between

treatments with and without N fertilization, but the key genus *Skermanella* was more abundant without N fertilization, suggesting that the community was still sensitive to variation in N content, even though it was not involved in N fixation(Luo et al., 2012;Zhu et al., 2014). Recent studies have reported that *Skermanella* was dominant in neutral agricultural soil(Hu et al., 2018) and negatively correlated with available N content (Sun et al., 2014), supporting our results.

4.3. The relationships of the active Cyanobacteria and Proteobacteria communities with environmental factors in the long-term fertilized soils

Nearly 30 years of chemical fertilization have strongly affected the structure of the active *Cyanobacteria* community. N fertilization greatly influenced this group (Table 1), as expected, in accordance with the CCA axis that separated the *Cyanobacteria* communities with and without N fertilization (Fig. 5a). *Cyanobacteria* abundance and community composition were both significantly correlated with TN, NO_3^--N and NH_4^+-N contents, with TN content being the second largest contributor to the variation of the *Cyanobacteria* group (Figs. 4 and 5, Table S2). This result highlights the important role that N plays in determining the composition of active *Cyanobacteria* communities in long-term fertilized soils.

N is considered as the main determinants controlling the structure of microbial communities (Chen et al., 2016a;Wang et al., 2016;Wang et al., 2018a). TN, NO_3^--N and NH_4^+-N contents differed significantly among the treatments, indicating that N concentration may directly affect the *Cyanobacteria* community. The input of N fertilizers may also induce variations in soil characteristics such as pH and SOM content

(Table S1), which has been widely observed in agricultural ecosystems (Zhou et al., 2015; Cui et al., 2017; Zhang et al., 2017).Variations in pH may shift the distribution of microbial communities (Jones et al., 2009; Lauber et al., 2009; Rousk et al., 2010; Wessén et al., 2010), indicating an indirect effect of N compounds on *Cyanobacteria* communities. Interestingly, N addition usually leads to the accumulation of SOM (Keeler et al., 2008) and in turn can affect bacterial communities because it supports C sources and improves microbial environmental conditions(Helgason et al., 2010; Zhang et al., 2014a).In contrast, N fertilization in our study decreased SOM content. This discrepancy maybe partly due to the transformation of the main *Cyanobacteria* group from light-dependent diazotrophs(e.g. *Nostocales*) to heterotrophic bacteria(e.g. *Pseudanabaenales*) that depend on SOM decomposition. The distribution of the active *Cyanobacteria* community could be influenced by the availability of N both directly and indirectly.

The contents of other nutrients such as TP and Olsen-P also had a strong effect influencing *Cyanobacteria* community. Vitousek et al. (2002) and Reed et al. (2011) suggested that P plays a vital role in limiting N fixation as an important source for the synthesis of adenosine triphosphate (ATP), which in turn directly affects the distribution of the N-fixing *Cyanobacteria* community. This proposal could account for the significantly higher relative abundance of *Nostocales* in PK than in CK (Fig. S3).We did not analyze the effect of plants, but recent studies have reported that P input could have a direct impact on plant growth, which would affect the quality and quantity of root exudates (Pandey et al., 2014; Šantrůčková et al., 2016; Wang et al., 2017),

396 subsequently leading to variation in the microbial communities (Li et al., 2016 and
397 2019). This source of variation provides new insight into the priming effect of root
398 exudation on specific microbial functional groups.

399 N in our study had the strongest impacts on the active *Proteobacteria* community
400 (Table 1). TN, BN, NH_4^+-N, SOM, TC, TK and Olsen-P contents contributed 11.8%
401 of the variation of microbial communities, indicating the importance of N in also
402 controlling the active *Proteobacteria* community. Gupta (2000) recognized
403 *Proteobacteria* as a functionally diverse group of bacteria and divided it into
404 chemorganotrophs and chemolithotrophs, which are involved in nutrient transformation
405 depending on the substrates they use. Dominant and active orders that are more
406 abundant than the DNA-derived orders in our study could positively participate in
407 biogeochemical cycling, such as N fixation (members of *Rhizobiales* and
408 *Rhodospirillales*), nitrification (members of *Nitrosomonadales*) and C sequestration
409 (members of *Myxococcales*), and the direct and indirect effects of N on these processes
410 mediated by microbes have also been demonstrated previously (Li et al., 2014;Zhou et
411 al., 2014;Che et al., 2018).

412 It has been suggested that (Li et al. 2019) that the active bacterial community was
413 more sensitive than total bacteria to environmental change, because relic and dormant
414 DNA was not disturbed. The responses of the active *Proteobacteria* community to
415 various environmental factors are therefore reasonable. In addition to the effect of
416 nutrients, WC content and pH were significantly correlated with the *Proteobacteria*
417 group (Table S2), although these two soil parameters varied weakly among the

418 treatments. These findings indicated that the strong relationship between the changes
419 in the bacterial communities and the soil characteristics were due to long-term
420 fertilization.

421 5 Conclusions

422 The abundance of active bacteria responded more sensitive than total bacterial
423 abundance among the fertilization treatments, due to differences in BC and NH_4^+-N
424 contents. *Cyanobacteria* and *Proteobacteria* were the dominant phyla in the active
425 microbial community and differed proportionally in fertilized soil, indicating that the
426 two communities were more sensitive to changes in nutrient status than the
427 communities of other phyla. The correlation analysis indicated that N availability was
428 the main driver structuring the *Cyanobacteria* and *Proteobacteria* communities. This
429 study contributes substantially to our understanding of the dynamics of microbial
430 communities in agricultural ecosystems.

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Figure captions

Fig.1 Absolute (a) and relative (b) abundances (mean±SE) of active bacteria in the fertilization treatments. Relative abundance is the ratio of active to total bacterial abundance. CK, no fertilizer; PK, chemical phosphorus and potassium fertilizer; NK, chemical nitrogen and K fertilizer; NP, chemical N and P fertilizer; NPK, chemical N, P and K fertilizer. Different letters indicate significant differences.

Fig.2 Path analysis based on stepwise regression identifying the relationships among the environmental variables and the absolute (a) and relative (b) abundances of active bacteria. TN, total nitrogen content. The arrows between the environmental factors and the active bacteria denote direct effects, and the arrows among the environmental factors denote indirect effects. The number above the arrows represents path coefficient.

Fig.3 Changes in the relative abundances (mean±SE) of eight dominant phyla that represents the differences between the active and total bacteria in the treatments. The values (%) were the differences between relative abundances from RNA-derived and DNA-derived community. CK, no fertilizer; PK, chemical phosphorus and potassium fertilizer; NK, chemical nitrogen and K fertilizer; NP, chemical N and P fertilizer; NPK, chemical N, P and K fertilizer. Different letters indicate significant differences.

Fig. 4 Multiple regression tree analysis indicating the relationships between the environmental variables and the compositions of the active communities of *Cyanobacteria* (a) and *Proteobacteria* (b). SOM, soil organic-matter content(g/kg); TC, total carbon content(g/kg); TN, total nitrogen content(g/kg); TK, total potassium content(g/kg); BC, biomass carbon content(mg/kg); BN, biomass N content(mg/kg).

Fig. 5 Canonical correspondence analysis of the *Cyanobacteria* (a) and *Proteobacteria* (b) communities based on environmental data. The environmental

686 factors were selected after testing for variation inflation factors (VIF). WC, water
687 content; SOM, soil organic-matter content; TC, total carbon content; TN, total nitrogen
688 content; TP, total phosphorus content; TK, total potassium content; BC, biomass carbon
689 content; BN, biomass N content.

690 **Fig.6** Variance partitioning analysis based on a partial canonical correspondence
691 analysis indicating the effect of the environmental factors and their interactions on the
692 active communities of *Cyanobacteria* (a) and *Proteobacteria* (b). The columns on the
693 right represent the contributions of single factors on the variance of the microbial
694 communities. WC, water content; SOM, soil organic-matter content; TC, total carbon
695 content; TN, total nitrogen content; TP, total phosphorus content; TK, total potassium
696 content; BC, biomass C content; BN, biomass N content.