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Differential responses of soil bacterial taxa to long-term P, N, and organic manure application

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Abstract
Purpose Soil microorganisms and their interactions with environmental factors govern critical ecosystem processes. However, the changes of soil microbial communities (e.g., relative abundance changes of different phylotypes) and the links between specific environmental factors and microbial communities are not well understood.
Materials and methods We applied high-throughput sequencing of 16S rRNA gene amplicons to investigate the effects of mineral fertilizers P (superphosphate), N (urea), and NP and organic manure fertilizer (M) and its combined with mineral fertilizers (NM, PM, NPM) on bacterial and archaeal communities in rain-fed winter wheat soils in a 30-year experiment in the Loess Plateau of northwest China.
Results and discussion Dramatic changes of soil respiration and the concentrations of total organic C, total N, and microbial biomass C and N were found in manure application soils (M, NM, PM, NPM) and some of them in NP soil. Soil microbial community structure shifted after fertilization, and a significant difference of prokaryotic community structure was found between mineral fertilizer soils (P, N, and NP) and manure application soils (M, NM, PM, NPM) except the soils between PM and P. The prokaryotic community structure in M soil was different from that in NM and NPM soils and differed between N and P and NP soils. Acidobacteria, Actinobacteria, and Proteobacteria were the predominant phyla (55.5–76.5 % of abundance) and, together with some other phyla, were changed by fertilization at the phylum or lower taxon ranks. No fertilizer soil had the highest relative abundances of phyla WS3 and Gemmatimonadetes. P soil changed the relative abundances of phyla Acidobacteria, Gemmatimonadetes, and Verrucomicrobia, but only enriched the bacteria at the family level (Micrococcaceae) when combined with N or M application (NP, PM, and NPM). Some copiotrophic bacteria showed different responses to nitrogen and manure applications, e.g., Actinobacteria increased in abundance in nitrogen application soils (N, NP, NM, and NPM), whereas Bacteroidetes and Gammaproteobacteria increased in abundance in manure application soils (M, NM, PM, and NPM). The above patterns of the relative abundance vs nitrogen or manure application were correlated to soil C and N contents or C/N ratio.
Conclusions These results supported the hypothesis that different bacterial taxa would be favorable in P, N, and manure application soils and suggested that the changes of bacteria taxa in fertilized soils appeared to be more driven by nitrogen and manure applications than P application.

Keywords 16S rRNA · Community structure · Copiotrophic bacteria · MiSeq sequencing · Rain-fed farmland

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1 Introduction

Soil harbors a wide variety of microorganisms which play key roles in terrestrial ecosystem processes such as C and N cycles

(van der Heijden et al. 2008; Whitman et al. 1998). The rapidly advancing molecular methods, especially high-throughput sequencing technologies, allow a rapid and detailed study of prokaryotic communities (e.g., relative abundances of microbial phylotypes) and factors that lead to the community changes of the prokaryote (Prosser 2012; Zimmerman et al. 2014). Soil bacterial community composition is influenced by numerous biotic and abiotic factors ranging from their geographic location (Fulthorpe et al. 2008) to site-specific environmental conditions (Fierer and Jackson 2006). Ecosystem processes may be affected by the changes of soil bacterial communities through altering bacterial activities, compositions, or interactions (Prosser 2012). However, the responses of microbial communities and community structure to specific environmental factors induced by nutrient amendments are not clear.

Fertilizer inputs are historically used to increase crop yields and maintain soil fertility in agriculture. The effects of long-term (more than 10 years) N fertilization or N combined with other mineral fertilizers (e.g., NP, NK, NPK) are intensively studied, for example, for determining the fertilization strategies effects on crop yields and soil quality (Zhao et al. 2013) or for assessing fertilization effects on soil microbial biomass, activities, and community structure (Zhong and Cai 2007). It is well established that long-term application of N or N combined with other fertilizers affects the N-cycling processes and the associated microbial communities (Chu et al. 2007; He et al. 2007; Hallin et al. 2009; Ning et al. 2015). Several studies have found that long-term N fertilization alters the overall soil microbial community structure and selects for some bacterial taxa which are related to the changed ecosystem processes (Yao et al. 2014; Turlapati et al. 2013). Long-term manure application including plant residue, animal manure, and composted organic matter usually increases soil microbial biomass and activity and changes bacterial community structure (Jangid et al. 2008; Zhang et al. 2012; Li et al. 2014) and the abundances of N-cycling-related microorganisms (Chan et al. 2013; Wang et al. 2014). Compared with the studies on N or manure addition, the long-term effect of P on soil microbial communities was only found in a few studies. Long-term P effect on soil microbial communities was not consistent among studies with no changes of soil microbial communities (Shi et al. 2012; Pan et al. 2014) or shifts of soil bacterial and fungal communities (Beauregard et al. 2010; Tan et al. 2012; Wakelin et al. 2012). Generally, the long-term N application appears to select for copiotrophic taxa (Ramirez et al. 2012) which rely on more labile organic C pools and higher nutrient availabilities with fast growth rates. However, the impact of long-term P or manure fertilization on the overall belowground microbial community dynamics remains largely unknown.

This study focuses on a winter wheat farmland in the Loess Plateau of northwest China which is a traditional and typical rain-fed farming region, facing the threat of drought and nutrient deficiency with low inherent soil fertility and total N concentration 0.042 to 0.077 % (Zhu et al. 1983). Previous research has found that N or N combined with other fertilizers improves soil water content or water using efficiency (Huanga et al. 2003). soil total organic C and N concentrations, and crop yields in this region (Guo et al. 2011; Wei and Hao 2011; Zhengchao et al. 2013). The long-term effects related to fertilization have caused changes of soil microbial biomass and activity in addition to differentiated soil properties and crop yields (Fan and Hao 2003; Lai et al. 2004; Guo et al. 2011). however, the shifts of soil microbial communities associated with fertilization have obtained less attention. In the current study, the shifts of microbial communities among long-term P, N, NP, M, NM, PM, and NPM and no fertilizer soils were examined by the high-throughput sequencing of 16S ribosomal RNA (rRNA) gene amplicons in a long-term experiment which was established in 1984 in Shaanxi Province, China. The objective was to determine the responses of microbial communities to long-term P, N, and manure applications and the link between the microbial communities and soil properties. We hypothesized that the long-term fertilization and the changes induced by long-term fertilization had formed distinct ecological niches for bacteria and would provide favorable conditions for different bacterial taxa in P (P, NP, PM, and NPM), N (N, NP, NM, and NPM), and manure (M, NM, PM, and NPM) application soils.

2 Materials and methods

2.1 Experimental site and sampling

The experiments were conducted in the Changwu Agro-ecological Experimental Station on the Loess Plateau 107° 40′ E, 35° 12′ N, altitude 1220 m, Shaanxi Province, China. A long-term fertilizer experiment was established in 1984 with a winter wheat (*Triticum aestivum* L.) continuous grown system, including eight treatments with triplicates in a incompletely random plot design: control without fertilizer (CK), mineral fertilizer nitrogen (N), mineral fertilizer phosphate (P), organic manure (M), N and P (NP), N and M (NM), P and M (NM), and NP plus organic manure (NPM) (Guo et al. 2011). The fertilizers N and P applied in the form of urea (120 kg N ha^{-1} per year) and superphosphate (40 kg P_2O_5 ha^{-1} per year) and M were mainly from cow excretion (equivalent to 87 kg N ha^{-1} per year). This site has a semiarid climate with an annual rainfall of 584 mm (1957–2001) and annual average temperature of 9.1 °C and represents a typical rain-fed agricultural area in the warm temperate zone of China. The soil is loam developed from loess deposits. The soil samples were collected in May 2014 at depths 0–20 cm. Five cores were taken from each plot and mixed to form one composite

sample. Each sample was placed in a sterile plastic bag, sealed, and placed on ice during transportation to the laboratory. All samples were passed through a 2.0-mm sieve and stored at −80 °C for DNA extraction and at 4 °C for other analyses.

2.2 Soil properties

Soil pH was determined with a soil to water ratio of 1:5. Ammonium (NH_4^+-N) was extracted from the soil by horizontal shaking with 2 M KCl (1:10) for 1 h and determined with sodium nitroprusside/dichloro-S-triazine (Searle 1984). total organic carbon (TOC) with dichromate oxidation method, and total nitrogen (TN) with the Kjeldahl method. Soil microbial biomass C and N (MBC and MBN) were measured by the chloroform fumigation-extraction method (Joergensen and Brookes 1990). and the organic C extracted in unfumigated soil was considered as soil dissolved organic C (DOC). Soil basal respiration (R) was measured according to the method described by Enwall et al. (2007). The respiratory quotient Qco_2, was calculated based on Meyer et al. (1996).

2.3 Soil DNA extraction and MiSeq sequencing of 16S rRNA gene amplicons

Soil DNA was extracted from 0.5 g soil using the FastDNA® Spin Kit for Soil (MP Biomedicals, Cleveland, OH, USA) according to the manufacturer's instructions. The purified DNA was diluted with 50 μl sterilized water and checked for quality and quantity using a NanoDrop Spectrophotometer.

DNA was amplified using the primers 515F (50-GTGCCAGCMGCCGCGGTAA-30) and 806R (50-GGACTACHVGGGTWTCTAAT-30) designed to be universal for bacteria and archaea (Caporaso et al. 2011). Primers were tagged with unique barcodes for each replicate DNA sample. PCR reactions were carried out in a 30-μl mixture with 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of each primer, and about 10 ng template DNA. The thermal cycling was as follows: 98 °C for 1 min; 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min. Negative controls using sterilized water instead of soil DNA were included to avoid primer or sample DNA contamination. Each DNA sample was amplified in three technical replicates and then quantified with electrophoresis and mixed in one tube. All samples were pooled together with equal molar amounts from each sample and purified with the GeneJET gel extraction kit (Thermo Scientific). The purified library was generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) and mixed with the index codes. The library quality was assessed in the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Then, the library was sequenced on an Illumina MiSeq platform by which 250 bp/300 bp paired-end reads were generated.

All sequence reads were merged using FLASH (Magoc and Salzberg 2011) and assigned to each sample according to their barcodes. Sequence analysis was performed by UPARSE software package using the UPARSE-OTU and UPARSE-OTUref algorithms (Edgar 2013). Sequences with ≥97 % similarity were clustered into operational taxonomic units (OTUs). The aligned 16S rRNA gene sequences were used for a chimera check using the Uchime algorithm (Edgar et al. 2011). Taxonomy was assigned using the Ribosomal Database Project classifier (Wang et al. 2007). Each sample was rarefied to the same number of reads (28,318 sequences) for both alpha-diversity (Chao1 estimator of richness, observed species and Shannon's diversity index) and beta-diversity (NMDS and UniFrac) analyses. The original sequence data are available at the European Nucleotide Archive (ENA) with accession number PRJEB11700 (http://www.ebi.ac.uk/ena/data/view/PRJEB11700).

2.4 Statistical analysis

Differences in relative abundances of microbial taxa and soil properties between samples were tested by one-way analysis of variance (ANOVA). The linear correlations between microbial diversity, species abundance, and soil properties were analyzed using SPSS 17.0 software. Significant difference was $p<0.05$.

With the untransformed microbial relative abundance at the OTU level as input data, the overall structural changes of soil microbial communities were evaluated by nonmetric multidimensional scaling (NMDS) with *PC-ORD* 5.0 (MjM software, www.pcord.com/). The 3D stress indicates how well the plot represents the variability in the data. A 3D stress <10 is considered to represent a good reflection of the resemblance matrix (Peck 2010). Significant differences in microbial community structure between treatments were determined by MRPP (*PC-ORD* 5.0, MjM software, www.pcord.com/). The Mantel test was applied to evaluate the correlations between microbial communities and environmental variables using PASSaGE (http://www.passagesoftware.net/). Environmental variables with higher Pearson's correlation coefficients with microbial communities were selected using the BioEnv procedure, and variance partitioning analysis (VPA) based on redundancy analysis procedure was performed to quantify the relative contributions of environmental variables using the *varpart* procedure in the R package Vegan (http://cran.r-project.org/web/packages/vegan/index.html).

3 Results

3.1 Soil chemical and microbial properties

All the measured soil chemical and microbial properties did not show differences among the CK, P, N, and NP soils with a few exceptions (Table 1). Soil respiration (R) and DNA concentration in NP soil were higher than those in CK, P, and N soils. The concentrations of ammonium and dissolved organic carbon were highest in N soil. Soil respiratory quotient Qco_2 was significantly increased in NP application soils (NP and NPM).

Soil pH was lower in NM soil than in CK soil by 0.2 units (Table 1). Soil respiration and the concentrations of total organic C, total N, microbial C (MBC), microbial N (MBN), and DNA were generally higher in manure application soils (M, NM, PM, and NPM) than in CK and mineral fertilizer soils (P, N, and NP). Compared to CK, manure application combined with N and NP increased the concentrations of total organic C and total N by more than 59 and 61 %, respectively.

3.2 Soil prokaryotic diversity and structure

In total, 1,539,393 high quality and chimera-free reads were obtained by MiSeq sequencing of 16S rRNA gene amplicons with 28,313 to 123,463 reads per sample. Both sequencing and quantitative real-time PCR data showed that the relative abundance of bacteria was more than 97.5 % while archaea was less than 2.5 % in fertilized soils (P, N, NP, M, NM, PM, and NPM). The observed species and Chao1 richness were highest in P soil and lowest in CK soil, whereas the Shannon's diversity index and OTU numbers were highest in M soil and lowest in CK soil (Table 2). The abundances of bacteria and archaea quantified by quantitative real-time PCR were generally higher in manure application soils (M, NM, PM, and NPM) than in mineral fertilizer soils (P, N, and NP) by 9.5 to 127 % and 5 to 77 %, respectively (data not shown).

The proportion of OTUs shared between CK and fertilization soils was occupied 71.6–84.0 % in CK soil, 75.2 % in P soil, 76.6 % in N soil, 76.4 in NP soil, and 72.1 to 74.9 % in manure application soils, respectively (data not shown). This was further supported by the changes of prokaryotic community structure affected by fertilization. The overall prokaryotic community structure generally differed between CK and fertilization soils (Fig. 1 and Table S1, Electronic supplementary material). There was a significant difference of prokaryotic community structure between mineral fertilizer soils (P, N, and NP) and manure application soils (M, NM, PM, NPM) except that between PM and P. The prokaryotic community structure in M soil was different from that in NM and NPM soils and differed between N and P and NP soils. The prokaryotic community structure affected by fertilization was also

Table 1 Soil chemical and microbial properties

Soil properties	CK	P	N	NP	NM	NPM	M	PM
pH	8.68±0.07a	8.64±0ab	8.66±0.02ab	8.56±0.02ab	8.49±0.02b	8.58±0.03ab	8.71±0.06a	8.64±0.01ab
TN (mg kg^{-1})	0.8±0.03b	0.78±0.01b	0.82±0.05b	0.95±0.04b	1.29±0.01a	1.33±0.04a	1.22±0.05a	1.23±0.06a
TOC (mg kg^{-1})	6.59±0.18c	6.44±0.25c	6.63±0.25c	7.9±0.3b	10.48±0.2a	10.73±0.2a	10.01±0.38a	10.29±0.22a
C/N	16.35±0.4a	16.55±0.21a	16.13±0.37a	16.56±0.23a	16.16±0.23a	16.17±0.72a	16.38±0.04a	16.81±0.59a
NH_4^+-N (mg kg^{-1})	3.95±1.84b	4.25±0.9b	7.81±0.52a	3.95±1.24b	3.39±0.45b	7.18±1.27a	3.57±0.58b	3.33±0.11b
DOC (mg kg^{-1})	116.62±3.15 cd	145.71±12.25bcd	245.3±31.12a	81.9±7.76d	213.22±28.24ab	142.35±5.12bcd	169.56±17.63bcd	126.28±6.44 cd
MBC (mg kg^{-1})	50.44±3.74b	48.12±9.47b	60.55±7.71b	51.55±4.3b	113.42±11.33a	62.01±5.39b	104.54±0.3a	106.33±11.82a
MBN (mg kg^{-1})	12.17±0.48 cd	11.61±0.65 cd	9.38±0.34d	17.33±0.1bc	23.04±3.4a	18.53±0.54ab	20.55±0.17a	19.94±1.01a
R (mg CO_2-C kg^{-1})	0.2±0.01d	0.22±0.02d	0.21±0.01d	0.29±0.11c	0.41±0.01a	0.36±0ab	0.34±0ab	0.37±0.01ab
Qco_2 (mg CO_2-C g^{-1} MBC day^{-1})	4.11±0.47abc	4.92±0.71abc	3.47±0.4c	5.71±0.36ab	3.65±0.27bc	5.95±0.53a	3.29±0.02c	3.53±0.32c
DNA (ng μl^{-1})	4.8±0.65d	5.7±0.45d	5.2±0.1d	7.1±0.22c	9.5±0.12b	10.5±0.01ab	10.7±0.31a	9.8±0.22ab
Wheat yield (kg hm^{-2})	989±20b	756±25a	1382±50c	2728±102de	2844±105e	3148±70f	3104±75f	2608±95d

Values with different letters in a row mean significant difference at $p<0.05$. Values are means of three replicates±SE

TN total nitrogen, *TOC* total organic C, *DOC* dissolved organic C, *MBC* microbial biomass C, *MBN* microbial biomass N, *R* respiration, Qco_2 respiration quotient

Table 2 Prokaryotic diversity indices at 97 % sequence similarity of 16S rRNA gene sequence calculated based on 28,313 sequences for each sample

Treatments	Chao1 estimator of richness	Observed species	Shannon's diversity index	OTU number
CK	3341±103c	2951±99c	9.82±0.08d	3398±103d
P	4973±102a	3482±34a	10.05±0.02ab	4789±32a
N	3370±134c	3098±63bc	9.83±0.05d	4213±53bc
NP	4592±68ab	3279±32ab	9.88±0.04bcd	4578±128ab
NM	4349±173b	3348±78ab	10.06±0.02ab	4277±26bc
NPM	4507±13ab	3395±33ab	10±0.04abc	4388±130bc
M	4453±97b	3359±89ab	10.12±0.02a	4880±44a
PM	4530±23ab	3409±68ab	10.04±0.03ab	4084±20c

Values with different letters in a column mean significant difference at $p<0.05$. Values are means of three replicates±SE

confirmed by hierarchical clustering based on the UniFrac distance matrix which showed distinct clusters among CK, mineral fertilizer soils, and manure application soils (Fig. 1b).

3.3 Phylogenetic composition of soil prokaryotic community

Acidobacteria, Actinobacteria, and Proteobacteria were the predominant phyla in CK and fertilization soils, with relative abundances of 12.5–19.2, 19.7–27.9, and 23.3–29.4 %, respectively. The relative abundances of phyla Acidobacteria and Verrucomicrobia were lowest in CK soil and highest in P soil, whereas the phyla WS3 and Gemmatimonadetes were highest in CK soil and lowest in PM soil. P application soils (P, NP, PM, and NPM) increased the relative abundance of the family Micrococcaceae (Table 3). The relative abundance of Chloroflexi increased in nitrogen application soils (N, NP, NM, and NPM) and manure application soils (M, NM, PM, and NPM) (Table 3). Actinobacteria increased in abundance in nitrogen application soils, whereas Bacteroidetes increased in abundance in manure application soils at the phylum level. The above patterns of the relative abundance vs nitrogen or manure application were observed at the lower taxon ranks in addition to the phylum level. The relative abundance of Gammaproteobacteria was increased in manure application soils at the class level and lower taxon ranks. The relative abundance of Actinobacteria was negatively correlated to soil C/N ratio, whereas the relative abundances of Bacteroidetes and Gammaproteobacteria were positively correlated to soil total organic C, total N, and R (Fig. 2 and Table S2, Electronic supplementary material).

The increase of Acidobacteria relative abundance in mineral fertilizer soils and manure application soils was driven by increased abundances of dominant OTUs, such as OTU23 (order iii1-15), OTU4503 (order iii1-15), OTU53 (order iii1-15), and OTU7358 (Acidobacteria subdivision 6). The relative abundance of Chloroflexi increased in nitrogen and manure application soils, which was mainly driven by the changes of class Thermomicrobia. The increase in Actinobacteria relative abundance in nitrogen application soils was mainly driven by the changes of families Geodermatophilaceae, Nocardioidaceae, and Solirubrobacteraceae. However, the family Rubrobacteraceae was decreased in nitrogen application soils. The phylum Bacteroidetes was dominated by order Sphingobacteriales and its increase in manure soils was mainly driven by the family Flammeovirgaceae. The Micrococcaceae which increased abundance in P application soils was dominated by the genus *Arthrobacter*.

Based on sequence data, archaea were mainly found in the phylum Crenarchaeota (92–99 % of total archaeal reads), and its abundance decreased in mineral fertilizer soils and manure application soils compared to CK soil (Fig. 3). The abundance

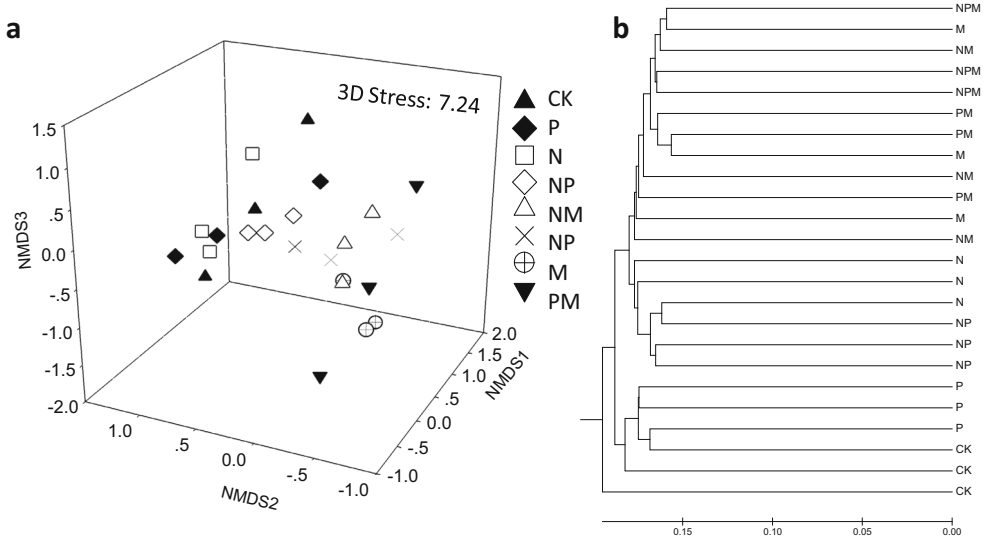

Fig. 1 NMDS plot of the overall prokaryotic communities in CK and fertilization soils based on all OTUs' relative abundance (**a**) and clustering of soil prokaryote based on the UniFrac distances (**b**)

Table 3 Relative abundances of soil bacterial communities changed by fertilization at phylum (a), class (b), order (c), family (d), and genus (e) levels

Prokaryotic communities	CK	P	N	NP	NM	NPM	M	PM
(a) Phylum								
Acidobacteria	12.5±1.1b	19.15±0.78a	18.17±0.4a	18.25±0.81a	15.27±1.59ab	17.6±0.25a	18.06±0.85a	16.93±0.3a
Actinobacteria	19.67±0.43c	22.24±1.11bc	27.87±0.33a	26.14±0.46ab	24.36±0.84ab	24.5±1.8ab	22.45±0.69bc	22.05±1.14bc
Bacteroidetes	2.14±0.48c	2.77±0.13c	2.19±0.16c	2.73±0.01c	4.59±0.13ab	4.41±0.39ab	4.14±0.08b	5.4±0.23a
Chloroflexi	4.82±0.56c	5.37±0.06bc	7.18±0.31ab	7.43±0.15a	6.79±0.37ab	6.69±0.06ab	7.5±0.18a	7.33±0.32 a
Gemmatimonadetes	7.76±0.06a	6.32±0.26bc	7.35±0.22ab	6.39±0.1abc	7.74±0.49a	7.06±0.36ab	5.98±0.16bc	5.62±0.3c
Planctomycetes	3.01±0.09b	4.14±0.19ab	2.86±0.11b	3.74±0.2ab	3.77±0.83ab	3.58±0.18b	6.04±0.53a	4.06±0.83ab
Verrucomicrobia	1.38±0.11b	2.49±0.08a	1.41±0.01b	1.71±0.04b	1.52±0.19b	1.71±0.08b	2.33±0.15a	2.3±0.03a
WS3	0.55±0.01a	0.53±0.13a	0.31±0.04bc	0.39±0.02abc	0.26±0.03c	0.27±0.01bc	0.31±0.02bc	0.21±0c
(b) Class								
Acidobacteria subdivision 6	4.58±0.65b	8.71±0.19a	8.77±0.36a	8.55±0.56a	7.37±0.27ab	8.97±0.34a	10.19±0.69a	8.91±0.51a
Actinobacteria	7.59±0.08b	8.81±0.85ab	11.83±0.9a	11.15±0.33a	10.81±0.21ab	11.09±1.04a	10.37±0.22ab	9.81±0.9ab
Rubrobacteria	1.35±0.11a	1.19±0.16ab	0.82±0.02ab	1.21±0.29ab	0.63±0.08b	0.77±0.04ab	0.68±0.05b	0.74±0.06ab
Thermoleophilia	5.46±0.83c	7.32±0.53abc	9.69±0.15a	8.4±0.18ab	7.95±0.56abc	7.73±0.66abc	7.07±0.43bc	6.66±0.37bc
Chloracidobacteria	2.85±0.14d	5.83±0.24a	5.01±0ab	5.03±0.01ab	3.23±0.46 cd	4.02±0.11bc	3.77±0.13 cd	3.29±0.33 cd
Thermomicrobia	0.53±0.1c	1.34±0.08b	1.85±0.03a	1.37±0b	1.32±0.03b	1.44±0.07b	1.33±0.03b	1.31±0.04b
Anaerolineae	0.94±0b	1.22±0.17b	0.95±1.18b	1.18±0.12b	1.34±0.12ab	1.17±0.05b	1.9±0.12a	1.16±0.2b
Gemmatimonadetes	4.31±0.08a	3.04±0 cd	3.67±0.08abc	3.07±0.01bcd	3.76±0.31ab	3.28±0.09bcd	3.23±0.08bcd	2.85±0.2d
Gemm-3	0.51±0e	0.81±0.06 cd	1.12±0.04a	0.86±0.02bc	1.02±0.04ab	0.91±0.04bc	0.67±0.03de	0.78±0.03 cd
Gemm-5	0.71±0.12b	0.82±0.12ab	1.05±0.15ab	0.81±0.02ab	1.25±0.06a	1.08±0.11ab	0.73±0.04b	0.97±0.11ab
Gammaproteobacteria	4.06±0.31d	4.66±0.54bcd	4.85±0.06bc	4.18±0.11 cd	5.86±0.16a	5.36±0.14ab	5.11±0.13b	4.99±0.11b
Sphingobacteria	2.4±0.46d	2.69±0.2 cd	2.67±0.15 cd	2.68±0 cd	4.21±0.27ab	3.04±0.71bcd	3.78±0.14abc	4.97±0.44a
(c) Order								
iii1-15	3.09±0.31b	6.32±0.14a	6.4±0.24a	5.71±0.05a	5.18±0.9a	6.26±0.32a	7.18±0.49a	6.24±0.32a
Actinomycetales	7.38±0.08c	7.69±0bc	11.57±0.92a	10.86±0.3a	10.56±0.19a	10.54±1.03a	10.14±0.21ab	7.44±0.93c
Acidimicrobiales	3.08±0.01ab	2.72±0.04b	2.92±0.08ab	2.86±0.07ab	3.27±0.21a	3.03±0ab	2.62±0.12b	3.0±0.07ab
Solirubrobacterales	5.66±0.57ab	4.86±0.45b	6.95±0.13a	5.86±0.16ab	5.67±0.36ab	5.17±0.22b	4.97±0.27b	4.63±0.33b
Sphingobacteriales	3.24±0.51ab	3.1±0.32ab	2.38±0.32b	2.68±0b	4.21±0.27ab	3.69±0.71ab	3.78±0.14ab	4.97±0.44a
Xanthomonadales	3.51±0.03bcde	2.61±0.02e	3.19±0.26de	3.4±0.11 cde	4.41±0.42ab	4.51±0.03a	4.21±0.14abc	3.88±0.04abcd
(d) Family								
Geodermatophilaceae	2.15±0.2b	2.4±0.46ab	3.89±0.42a	2.72±0.21ab	2.73±0.16ab	2.5±0.36ab	2.34±0.14b	1.97±0.34b
Gaiellaceae	1.65±0.29b	2.37±0.08ab	2.42±0.08a	2.43±0.02a	2.16±0.2ab	2.05±0.19ab	1.99±0.15ab	1.94±0.02ab
Micrococcaceae	1.33±0.08d	1.95±0.12ab	1.36±.0.11 cd	2.22±0.11a	1.63±0.15bcd	2.04±0.09ab	1.53±0.01 cd	1.9±0.03ab
Nocardioidaceae	0.75±0.06e	0.92±0.02de	1.38±0.06abc	1.25±0.04bcd	1.77±0.13a	1.64±0.08ab	1.22±0.04 cd	1.37±0.13abc
Rubrobacteraceae	1.35±0.11a	1.04±0.05ab	0.82±0.02bc	0.93±0.05bc	0.63±0.08c	0.74±0bc	0.68±0.05c	0.74±0.05bc

Table 3 (continued)

Prokaryotic communities	CK	P	N	NP	NM	NPM	M	PM
Solirubrobacteraceae	0.67±0.07b	0.81±0.02b	1.45±0.1a	1.16±0.06a	1.25±0.04a	1.13±0.08a	1.14±0.05a	0.74±0.01b
Micromonosporaceae	0.56±0.01b	0.57±0.07b	0.79±0.03b	0.67±0.08b	0.72±0.04b	0.73±0.07b	1.13±0.05a	0.64±0.08b
Pirellulaceae	0.55±0.12b	0.85±0.23ab	0.52±0.06b	0.81±0.07ab	0.75±0.16ab	0.65±0.05ab	1.25±0.11a	0.71±0.14ab
Chitinophagaceae	2.22±0.22a	1.2±0.04bc	0.84±0.05c	1.29±0.03bc	1.76±0.17ab	1.22±0.08bc	1.53±0.06abc	2.26±0.29a
Flammeovirgaceae	0.34±0.14b	0.42±0.11b	0.37±0.03b	0.36±0.03b	0.91±0.04a	0.67±0.17ab	0.69±0.12ab	0.84±0.13ab
Sphingomonadaceae	2.91±0.5ab	3.01±0.2ab	3.63±0.4a	2.94±0.15ab	2.9±0.39ab	3.04±0.08ab	1.96±0.07b	2.95±0.27ab
Hyphomicrobiaceae	1.07±0.02b	1.03±0.05b	0.91±0.07b	1.08±0.09b	1.32±0.03ab	1.36±0.02ab	1.38±0.05ab	1.56±0.16a
Rhodospirillaceae	0.96±0.01b	1.21±0.05ab	1.23±0.06ab	1.21±0.05ab	1.38±0.08a	1.34±0.03a	1.23±0.03ab	1.43±0.07a
Xanthomonadaceae	1.26±0.14c	1.59±0.14bc	1.84±0.16abc	1.96±0.09abc	2.49±0.27a	2.44±0.16a	2.24±0.1ab	2.05±0.18abc
Sinobacteraceae	1.69±0.05a	1.03±0.04c	1.09±0.03c	1.34±0bc	1.55±0.23ab	1.33±0.02bc	1.56±0.04ab	1.47±0.21ab
(e) Genus								
Actinomadura	0.07±0.01d	0.06±0.01d	0.09±0.01d	0.08±0.01d	0.26±0.01ab	0.19±0.01bc	0.31±0a	0.18±0.02c
Arthrobacter	1.31±0.08 cd	1.95±0.09ab	1.31±0.1 cd	2.2±0.1a	1.06±0.12d	1.75±0.09bc	1.35±0.01 cd	1.86±0.05ab
Modestobacter	0.21±0b	0.25±0.03b	0.44±0.05a	0.31±0.02ab	0.36±0.01ab	0.31±0.04ab	0.31±0ab	0.25±0.05b
Nocardioides	0.15±0.02d	0.21±0.01 cd	0.33±0.03bc	0.24±0bcd	0.51±0.04a	0.42±0.02ab	0.32±0.03bc	0.28±0.06bcd
Streptomyces	0.45±0.02b	0.44±0.03b	0.64±0.03a	0.64±0.02a	0.61±0.02a	0.54±0.04ab	0.43±0.03b	0.42±0.07b
Modestobacter	0.21±0b	0.25±0.03b	0.44±0.05a	0.31±0.02ab	0.36±0.01ab	0.31±0.04ab	0.31±0ab	0.25±0.05b
Rubrobacter	1.35±0.11a	1.19±0.16ab	0.82±0.02ab	1.22±0.02ab	0.63±0.08b	0.77±0.03ab	0.68±0.05b	0.74±0.06b
A4	0.34±0.14c	0.42±0.11bc	0.37±0.03c	0.36±0.03c	0.91±0.04a	0.67±0.17b	0.69±0.12b	0.75±0.16ab
Rhodoplanes	0.59±0.11ab	0.62±0.11ab	0.52±0.04b	0.64±0.06ab	0.73±0.02ab	0.81±0.06ab	0.83±0.03ab	0.96±0.13a
Hyphomicrobium	0.14±0.03ab	0.09±0.01b	0.12±0ab	0.11±0.01ab	0.18±0a	0.15±0.01ab	0.17±0.01a	0.13±0.02ab
Sphingomonas	0.51±0.05ab	0.55±0.03ab	0.72±0.05a	0.58±0.07ab	0.58±0.06ab	0.59±0.04ab	0.34±0.01b	0.44±0.09b
Lysobacter	0.28±0.33b	0.36±0.05b	0.44±0.05ab	0.42±0.01ab	0.47±0.03ab	0.57±0.05a	0.45±0ab	0.46±0.01ab
DA101	0.38±0.03ab	0.45±0.05a	0.32±0.04abc	0.32±0.04abc	0.18±0.04c	0.24±0.06bc	0.34±0.02abc	0.3±0abc

Values with different letters in a row mean significant difference at $p<0.05$. Values are means of three replicates±SE

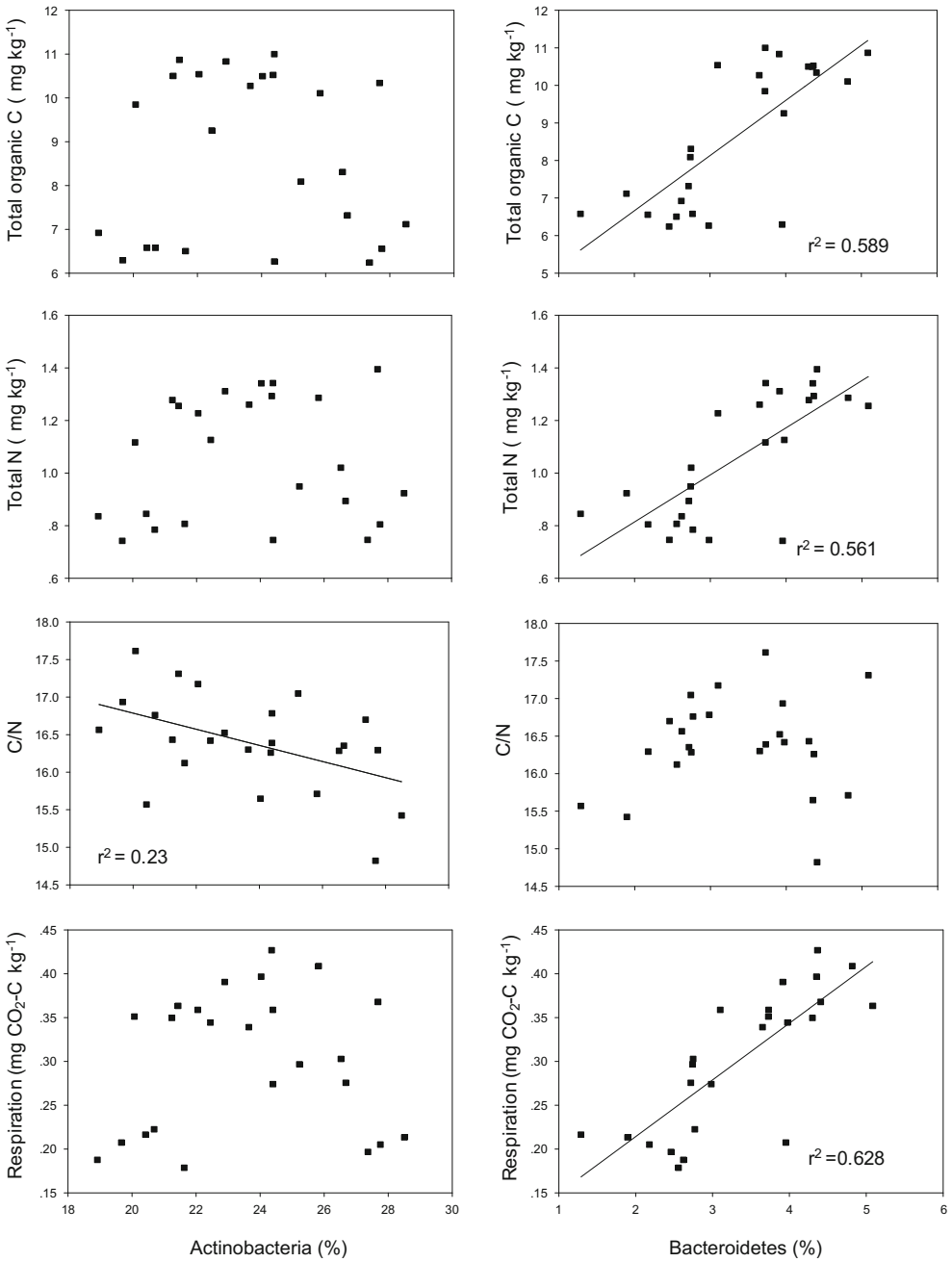

Fig. 2 Relationships between the relative abundances of Actinobacteria and Bacteroidetes and soil total organic C, total N, C/N ratio, and respiration

of Euryarchaeota and other phyla was 1.8–8 % in fertilized soils. The phylum Crenarchaeota was dominated by class Thaumarchaeota, in which the family Nitrososphaeraceae and genus *Candidatus* Nitrososphaera were higher in CK soil than in mineral fertilizer soils and manure application soils by 201 to 576 %.

3.4 Relationships between prokaryotic community structure and soil properties

The Mantel test was employed to understand the relationships between the prokaryotic community structure, diversity, biomass, and soil properties. Soil total organic C, total N, MBC, MBN, and R showed the highest Pearson's correlation ($p<0.05$) with prokaryotic communities (OTUs), diversity, and biomass. VPA was performed to quantify the relative contributions of different environmental variables to the changes of prokaryotic community structure (at the OTU level) by the *varpart* procedure. DOC alone explained 6.5 %, and total organic C, total N, MBC, MBN, and R explained 11.6 %, leaving 71.3 % of the variation unexplained (Fig. S1, Electronic supplementary material).

The relative abundances of prokaryotic microorganisms (at the family level) were significantly correlated to soil total

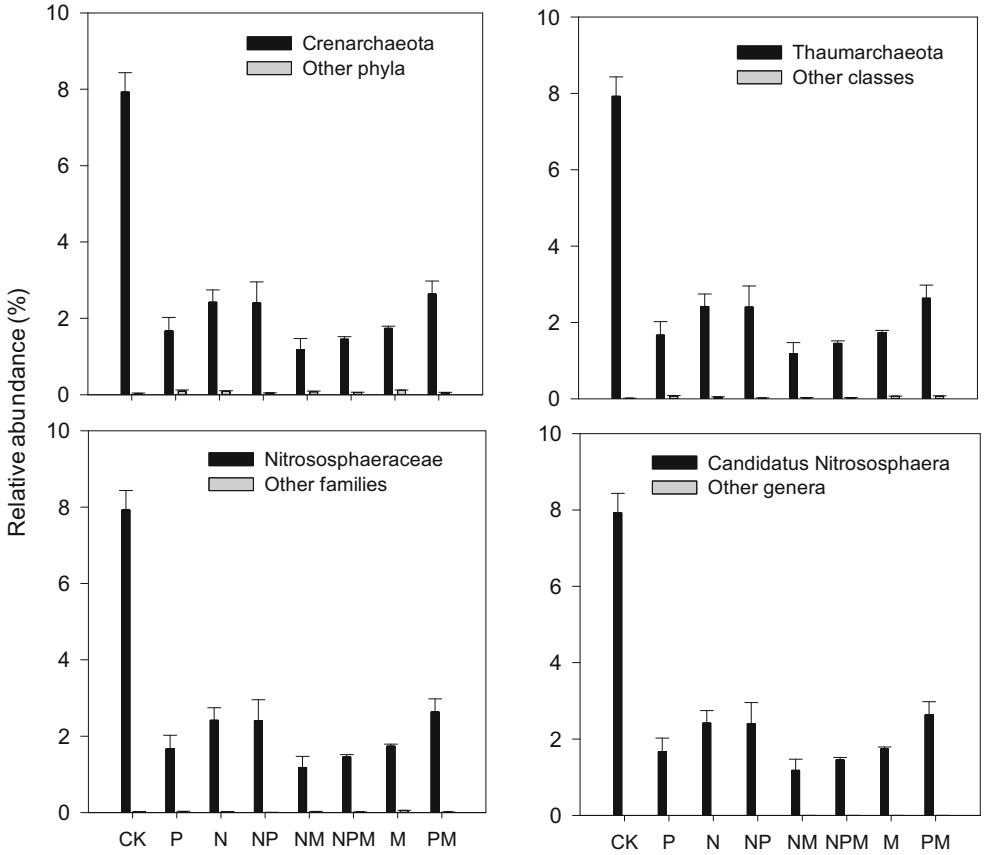

Fig. 3 Relative abundance (% of total reads) of archaeal 16S rRNA gene

organic C (22 families), total N (22), MBC (20), MBN (20), and respiration (25) ($p<0.05$, Table S2, Electronic supplementary material). Among these families, 16 of them were significantly correlated to all above variables, such as Rubrobacteraceae, Microbacteriaceae, Flammeovirgaceae, and Saprospiraceae. They were sensitive to the environmental condition changes induced by mineral fertilizer and/or manure application. In addition, there was a significant correlation between environmental variables (pH, TOC, TN, and R) and microbial biomass ($p<0.05$, Table S3, Electronic supplementary material).

4 Discussion

This study showed that prokaryotic community structure generally differed between no fertilizer soil, mineral fertilizer soils, and manure application soils, and the soil bacterial communities exhibited specific responses to nitrogen and manure applications at the phylum and lower taxon levels and at the family level in P application soils.

4.1 Soil prokaryotic community structure changed by fertilizer application

Prokaryotic community structure differed between no fertilizer and fertilizer application soils, and similar results are found in other long-term fertilization studies (Beauregard et al. 2010; Pan et al. 2014; Yao et al. 2014). P soil showed different prokaryotic community structures from those in N, NM, NPM, and M soils (Fig. 1 and Table S1, Electronic supplementary material). This is in agreement with a tropical rain forest study in which soil microbial community structure in P soil differed from that in N or cellulose addition soils (Fanin et al. 2015; Su et al. 2015). However, the changes of microbial community structure between P soil and N soil and manure application soils were not found in other long-term fertilization studies in grassland or agriculture ecosystems (Ge et al. 2008; Pan et al. 2014). These results indicate that P effect on soil prokaryotic community structure is variable and likely ecosystem- or site-dependent. The prokaryotic community structure was significantly different between N soil, NP soil, and manure application soils and differed between M and NM and NPM soils (Fig. 1 and Table S1, Electronic supplementary material). Organic fertilizer application has a significant influence on soil microbial community structure which differs from that in mineral fertilizer soils revealed by several studies (Ge et al. 2008; Jangid et al. 2008; Wu et al. 2011; Li et al. 2014). However, soil bacterial community structure was relatively nonresponsive to mineral fertilization but application rates (e.g., N rates) (Fierer et al. 2012; Yao et al. 2014) and showed similar patterns among mineral fertilizer soils (He et al. 2008; Wu et al. 2011; Pan et al. 2014). The differences

in prokaryotic community structure among mineral fertilizer soils in the present study may be due to the rain-fed agriculture without irrigation. In irrigated agriculture, fertilizers are always applied after or at the same time as irrigation. This will cause the leaching of fertilizer and other soil nutrients to subsoil and reduce the fertilization effect on soil microorganisms in top soil. Li et al. (2014) found that soil prokaryotic community structure showed significant difference in subsoil but similar community structure in top soil after long-term fertilization with irrigation.

4.2 Potential mechanisms controlling the shifts of prokaryotic communities in fertilization soils

Soil prokaryotic communities revealed by sequencing showed different responses to P, N, and manure applications. The changes in prokaryotic communities may be reflected in the trait differences of soil microbial taxa. Our results support the copiotrophic hypothesis, which predicts that bacteria with fast-growing rates (copiotrophs) prefer the environment with high organic C and nutrient contents, while bacteria with slow-growing rates (oligotrophs) likely thrive in low-nutrient conditions (Fierer et al. 2007). and nitrogen or manure application soils increased abundance in the predicted copiotrophic groups (e.g., Actinobacteria, Bacteroidetes, and Gammaproteobacteria) (Fierer et al. 2007, 2012; Eilers et al. 2010; Goldfarb et al. 2011). In previous studies, Proteobacteria (Gamma- or Beta-), Bacteroidetes, and Actinobacteria increased in abundance in high N soils (Fierer et al. 2012) or in response to labile substrates supply or C availability (Fierer et al. 2007; Eilers et al. 2010; Goldfarb et al. 2011). However, Actinobacteria, Bacteroidetes, and Gammaproteobacteria showed different responses to nitrogen and manure applications in this study with Actinobacteria increased in abundance in nitrogen application soils, whereas Bacteroidetes and Gammaproteobacteria increased in abundance in manure application soils (Table 3). The relative abundance of Actinobacteria was correlated to soil C/N ratio but not to soil carbon or nitrogen content, which is in line with several studies that show that Actinobacteria has no response to nitrogen or organic substrate addition (Cederlund et al. 2014; Li et al. 2014; Yao et al. 2014). Bacteroidetes and Gammaproteobacteria were positively correlated with soil C and N contents in the present study (Fig. 2 and Table S2, Electronic supplementary material), and increases in abundance have been found in soils with high C and N contents or carbon catabolic potential (Fierer et al. 2007, 2012; Ge et al. 2008; Wessén et al. 2010). Phylum WS3 occupied only 0.21–0.55 % in abundance and decreased abundance in nitrogen application soils and manure application soils. Although four single-cell-amplified genomes of WS3 retrieved from anoxic monimolimnion and sediments have been suggested to have an anaerobic fermentative mode of metabolism (Youssef et al. 2015). little is known about WS3 ecology and metabolism in soil. Their presence in environments across a wide range of habitats including soils, marine sediments, wastewater treatment bioreactors, deep sea hypersaline anoxic lakes, and contaminated environments (Berg et al. 2012; Youssef et al. 2015) suggests versatile metabolisms. The relative abundances of Acidobacteria and Chloroflexi were lowest in CK soil. However, Acidobacteria and Chloroflexi have been suggested to be oligotrophic groups (Fierer et al. 2007) and decreased abundances with nitrogen addition (Yao et al. 2014). These results suggest that some members of Acidobacteria and Chloroflexi may not be oligotrophic, e.g., Acidobacteria subdivision 6 which drove the changes of phylum Acidobacteria accounting for 39–58 % in abundance has shown higher abundance in rhizosphere than bulk soil (Kielak et al. 2009; Nunes da Rocha et al. 2013) which was suggested to be a result of the copiotrophic lifestyle. P application effect on soil microbial communities was minor compared to N or manure application. P soil and P combined with N or manure application soils only changed the relative abundance of the family Micrococcaceae which was dominated by the genus *Arthrobacter*. Some species in the genus *Arthrobacter* isolated from the environment have high ability to accumulate or solubilize phosphate (Banerjee et al. 2010; Chen et al. 2011). The accumulation of *Arthrobacter* in P application soils is in agreement with previous studies which found significant correlations between Actinobacteria and soil P status in pasture soils (Wakelin et al. 2012) and might increase soil P availability which is supported by a higher amount of available P and microbial P in P application soils (Lai et al. 2003, 2004). Further work is needed to determine the relationship of P mobilization potential and the accumulation of *Arthrobacter* in P application soils.

Fertilizer application had no significant impacts on nitrifying communities (e.g., phylum Nitrospirae, order Nitrosomonadales, and genus *Nitrosococcus*) except Crenarchaeota. The relative abundance of Crenarchaeota revealed by sequencing decreased after fertilization by 67–85 % (Fig. 3). This is not consistent with previous studies which found that the relative abundance of Crenarchaeota was increased with long-term fertilizer application (Li et al. 2014) or decreased only with high N application rates revealed by high-throughput sequencing (Ning et al. 2015). The Crenarchaeota in this study was dominated by *Candidatus* Nitrososphaera which has one culture from soil (*Candidatus* Nitrososphaera evergladensis) in public databases (Zhalnina et al. 2014) and potentially increases abundance with agricultural management (Zhalnina et al. 2013). The inconsistent response of Crenarchaeota to fertilizer suggests that more work is needed to understand the ecological preference of this archaeal phylum.

Preceding studies focused on fertilization effect mainly analyzed the changes of soil biochemical properties and soil

microbial community structure (by PLFA, DGGE, or T-RFLP) (Enwall et al. 2007; He et al. 2008; Dong et al. 2014). Some studies also used high-throughput sequencing technologies (Li et al. 2014). however, these are distinct from the current study due to differences in management practice (e.g., fertilization regimes and water management), soil type, and land use. Acidobacteria and Chloroflexi decreased with nitrogen or manure application in some investigations (Cederlund et al. 2014; Li et al. 2014; Yao et al. 2014). This contrasts to our study in rain-fed semiarid soil which showed distinct results. In a grassland study in Inner Mongolia, Actinobacteria was a dominant phylum and did not show any difference between different N rates and unfertilized soil (Yao et al. 2014). whereas the abundance of Actinobacteria was significantly increased in nitrogen application soils in the present study. Moreover, our work suggested that P, nitrogen, and manure applications showed differential effects on soil bacterial communities. In line with many previous findings (Fierer and Jackson 2006; Jangid et al. 2008; Campbell et al. 2010). the changes of soil prokaryotic communities and structure were correlated with soil C and N contents and soil respiration. However, the changes of soil prokaryotic structure after long-term P, nitrogen, and manure fertilizations remain largely unexplained (more than 70 % by VPA) and need more work to determine the inherent mechanisms.

5 Conclusions

This study showed that prokaryotic communities and community structure were mainly affected by long-term fertilization, and part of these changes could be explained by soil C and N contents. In general, the relative abundances of bacteria at the phylum level and also lower taxon ranks changed in response to changes induced by nitrogen and manure applications and also in the P soil, while a response that was general for all the soils receiving P application (P, NP, PM, and NPM) was only seen at a lower taxonomic level. Supporting our hypothesis that soils with P, N, and manure applications would be favorable for different bacterial taxa, we observed increases in the relative abundance of Actinobacteria in nitrogen application soils (N, NP, NM, and NPM) and Bacteroidetes and Gammaproteobacteria in manure application soils (M, NM, PM, and NPM), while the family Micrococcaceae increased abundance in P application soils (P, NP, PM, and NPM). The relative abundance of Actinobacteria, Bacteroidetes, and Gammaproteobacteria showed distinct relationships with soil properties, with Actinobacteria negatively correlated to soil C/N ratio, whereas Bacteroidetes and Gammaproteobacteria positively correlated to soil total organic C, total N, and R. More work is needed to elucidate the effect of altered soil prokaryotic communities on the function of rain-fed farmland ecosystems.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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