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Full Length Research Paper

The community structure of microbial in arable soil under different long-term fertilization regimes in the Loess Plateau of China

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To explore the long-term impact of different fertilization regimes on microbial communities, we targeted total bacteria and the genus *Pseudomonas* at a field site established in 1979, in the Loess Plateau of China. Soils were sampled thrice during the growing period of winter wheat in 2008, with treatments: N (inorganic N), NP (inorganic N and P), MNP (farmyard manure, inorganic N and P), M (farmyard manure), SNP (straw, inorganic N and P), and CK (no fertilized control). Soil samples were analyzed by PCR-DGGE (16S rRNA gene fragments), cloning and sequencing techniques. The recovered bacteria sequences were grouped into five major taxa: *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Gemmatimonadetes*, and *Actinobacteria*. Most of *Pseudomonas* phylotypes were similar to species *Pseudomonas* sp., *Pseudomonas fluorescens*, and *Pseudomonas putida*. The community structures microbes we detected were influenced diversely by the different fertilization regimes and plots amended with NP (NP, MNP and SNP) fostered more bacterial taxa as compared to N, M, or CK treatments, but application of organic fertilizers (MNP, M and SNP) enhanced the richness of *Pseudomonas* compared to mineral fertilizers treated alone (N and NP), or unfertilized control. Furthermore, application of fertilizers, was beneficial to the accumulation of soil organic carbon and microbial taxon with respect to the unfertilized control. Meanwhile, a reduction in bacterial taxon richness throughout the growing season was also manifested. Factors of sampling time, edaphic characteristics, such as soil moisture, organic carbon, and pH contributed much to the shifts of community structures. Balanced-fertilization should be encouraged for the maintaining of soil quality and agricultural sustainability in such a typical rain-fed agroecosystem.

Key words: Bacteria, polymerase chain reaction - denaturing gradient gel electrophoresis, *Pseudomonas*, soil chemical characteristics, canonical correspondence analysis, Loess Plateau.

INTRODUCTION

Soil micro-organisms play a key role in the processes of significant bio-geochemical cycling, soil aggregate formation, and plant health (Madsen, 2005). In agroecosystems, fertilization represents an important agricultural practice for increasing nutrient availability and crop yields, but it accompanies a disturbance to the soil, which may affect the composition and function of soil micro-organisms (Hallin et al., 2009). Recently, increased attention has been paid to responsible use and maintenance of microbial diversity in arable soil for the sustainability of agroecosystems (Kennedy, 1999;

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Abbreviations: Treat, Treatments; **SM,** soil moisture; **TC,** total carbon; **OC,** organic carbon; **TN,** total nitrogen; **TP,** total phosphorus; **AP,** available phosphorus.

Brussaard et al., 2007). As a result, the impacts of cropping systems and fertilization regimes on micro-organisms attracted researchers' attention (Esperschütz et al., 2007; Cruz et al., 2009; Jangid et al., 2011). Monitoring changes in microbial communities induced by long-term fertilization regimes is suggested to be an important step towards sustainable agriculture.

In recent years, extensive studies have focused on soil microbial biomass, enzyme activities, and community structure at long-term field sites in different continents (Böhme et al., 2005; Chu et al., 2007; Hamel et al., 2006). Most of experimental stations with different soil types, such as Brown Chernozem, Upland Red soil, Calcareous Purple soil, Black soil were investigated simultaneously (Cruz et al., 2009; He et al., 2008; Wei et al., 2008). Actually, fertilization regimes generally consist of different combinations of organic or inorganic fertilizers applied in different doses and times ranging from few decades to centuries (Ge et al., 2008a; Zhong et al., 2009; Börjesson et al. 2012). The impact of fertilization regimes on microbial community shifts were significant (Ge et al., 2008a; Sun et al., 2004; Zhang et al., 2012), or insignificant (Sessitsch et al., 2001; Crecchio et al., 2007); whereas organic fertilizers play an important role in determining soil microbial biomass and activities versus mineral fertilizers (Parham et al., 2002; Peacock et al., 2001; Plaza et al., 2004).

While screening of entire bacterial communities has proven to be a successful approach for assessing the composition of dominant community members, the resolution may not be sufficient to obtaining information on the structure of some specific microbial subgroups. For example, the genus *Pseudomonas* (sensu stricto), which represents a subgroup of micro-organisms directly involved in functions concerning plants, animals, and human health; some of them exhibit plant pathogen-suppressing properties useful in biological control, or express metabolic versatilities helpful in biotechnology and bioremediation (Stephen et al., 1998). Recently, the occurrence and distribution of *Pseudomonas* in agricultural management systems such as the long-term field trials has intrigued scientists greatly (Pesaro and Widmer, 2006).

Little is known about the composition of microbial community under long-term fertilization regimes in the Loess Plateau of China, which is a vast, semi-arid region with an average annual precipitation ranging from 300 to 600 mm. More than 90% of the cropland in this area receives no irrigation. The main crops are wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.), which are periodically rotated. There are about 1.3 million hectares of wheat and corn rotations that produce about 40% of local food needs. Consequently, knowledge about microbial composition and community shifts after long-term application of different fertilizers could improve our understanding of soil processes, and thus help us to develop proper field managing strategies in this area.

One of such long-term fertilization experiment site at Gaoping County of Pingliang city, Gansu Province, China, established in 1979, was selected for this study. The previous studies of this site showed that manure is more effective in building soil carbon than straw; yields and water-use efficiency declined significantly with lapse of time, but long-term additions of organic fertilizers to soil could increase the size of water-stable aggregates, the soil water-holding capacity, and the soil enzymatic activity (Fan et al., 2005, 2008; Liu et al., 2010).

The target of this study was to examine shifts of community structures of the total bacterial and *Pseudomonas* (sensu stricto) after a long-term difference in fertilization. Moreover, as the seasonality of microbes is a general pattern in agroecosystem (Toljander et al., 2008; Ge et al., 2009), we tested if there are obvious temporal changes in these communities during the wheat growing period, and further explored the main factors that can induce these changes. On the basis of the results of studies described earlier, we hypothesized that the community structures of the total bacteria and *Pseudomonas* would change after long-term application of mineral or organic fertilizers. To test this hypothesis, we performed microbial community analyses using PCR-DGGE (denaturing-gradient gel electrophoresis), cloning and sequencing techniques.

MATERIALS AND METHODS

Field sites and experimental design

A long-term fixed fertilization experiment has been conducted since April 1979 at the Gaoping Agronomy farm station of Pingliang City, Gansu Province, China. The site is in the central part of the Shizi highland plateau (107°30' E, 35°16' N, 1, 254 m above sea level) in Pingliang. Its dark loess soils were classified as calcarid regosols, with a soil bulk density of 1.30 g cm^{-3}. This area has an annual precipitation of 500 to 600 mm, about 60% of which occurs from July through September. May through June is the driest period for crop growth.

The mean monthly maximum and minimum temperatures of the winter wheat growing period (October 2008 to July 2009) ranged from 21.1 to -12.9°C. Physical and chemical characteristics of the initial field soil (0 to 15 cm) in 1979 were as follows: pH (H_2O) 8.2; Soil organic C (SOC) 6.15 g kg^{-1}; total N (TN) 0.95 g kg^{-1}; total P (TP) 5.7 g kg^{-1}; available P (AP) 0.0072 g kg^{-1} and available K (AK) 0.165 g kg^{-1}. The experiment site was established in 1979 with a maize crop. The crop rotation was two years maize followed by four years wheat, except for 1 year of soybean (in 1999) and 1 year of sorghum (in 2000) in the course of planting. The experimental treatments were arranged in a randomized complete block design (RCBD) with three replications.

Each plot was about 220 m^2 (16.7 m ×13.3 m) with a buffer zone of 1.0 m in every margin. The six fertilization treatments were: N (inorganic N fertilizer annually), NP (inorganic N and P fertilizer annually), MNP (farmyard manure plus inorganic N and P added annually), M (farmyard manure added annually), SNP (straw plus inorganic N annually but P fertilizer added every second year), and CK (no fertilization). Urea (inorganic N sources) and superphosphate (P) were used at rates of 90 kg N ha^{-1} and 30 kg Pha^{-1} respectively. Manure was applied at a rate of 75 t ha^{-1} (wet weight), roughly equal to 40 kg N ha^{-1}, 200 kg P ha^{-1}, and 840 kg Kha^{-1} in manure to crops. For SNP treatment, 3.75 t ha^{-1} of wheat

straw cut into pieces (approximately, 10 cm in length), was returned to the soil before plowing. For the other treatments, there was very little wheat straw or corn residues left, because crops were harvested close to the ground and residues were removed as thoroughly as possible from the plots before threshing the grain. Winter wheat was planted in rows 14.7 cm apart at a rate of 165 kg ha^{-1} on 20th September each year when planting wheat.

Sample collection

Each treatment contained three plots and samples were collected from each plot at three physiological stages of the wheat crop, namely, jointing, dough and maturity (ready for harvest), in 26th April, 28th May and 22nd June 2008, respectively. The second year of wheat planting in the two years cycles of maize followed four years of wheat rotation. In each plot, the rhizosphere soils were randomly sampled from each of the 10 wheat individuals by digging up a 15 cm soil profile near the wheat stem, and all 30 samples of each treatment were then pooled together, mixed thoroughly, and divided into two parts. Sub-samples for DNA extraction were stored in sterilized aluminium specimen boxes at -20°C. Sub-samples for soil properties analysis were stored in plastic bags, air-dried, sieved (2 mm) and stored at 4°C.

Soil properties analysis

Soil pH was analyzed in a 1: 5 soil: KCl solution (1 M), soil available P (AP) by the Olsen method (Olsen et al., 1954) and total P following the $HClO_4$-H_2SO_4 Colorimetry method. Soil organic C and total N were measured using the CHNS-analyzer system (Elementar Vario EL, Elementar Analysensysteme GmbH, Hanau, Germany) with the burning method at 450 and 1250°C, respectively (Analysis and Test Center of Lanzhou University). Soil moisture (SM) was determined after drying at 105°C for 24 h. All chemical results are means of triplicate analysis and expressed on an oven-dry based weight.

DNA extraction

Total DNA of soil was extracted from 0.5 g sub-samples, using Fast DNA SPIN Kit PowerSoil^{TM} (MOBIO Laboratories, Soanabeach, CA, USA), as described by the manufacturer. Isolated DNA was visualized under UV light after electrophoresis on agarose gels followed by staining in ethidium bromide and extracted DNA was stored at -20°C until further use.

PCR and DGGE analysis

The extracted DNA from soils was subjected to PCR subsequently. For the bacterial, amplification of the 16S rRNA gene was performed with the universal bacterial primer pair 341F-GC and 803R (Sheffield et al., 1989; Labbé et al., 2007); and followed by a touchdown amplification procedure. The starting annealing temperature was 65°C, which was decreased by 1°C every cycle until it reached 55°C, and then 20 more cycles were performed at 55°C; Denaturation was carried out at 94°C for 1 min; the annealing time was 1 min, and primer extension was carried out at 72°C for 3 min and the final extension at 72°C for 10 min. The *Pseudomonas* group was amplified using *Pseudomonas*-selective primers Ps-f and Ps-r firstly (Widmer et al., 1998).

The parameters of the program used were as follows: initial denaturation was carried out at 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, annealing was performed at 66°C for 1 min, extension of annealing was done at 72°C for 1 min, and a final extension was done at 72°C for 10 min. Subsequently, the products were diluted 100 times with the specific primer pair UNI-b-f/520-r-GC for the second round of PCR. The parameters of the program used were as follows: denaturation was carried out at 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, annealing was performed at 59°C for 1 min, extension of annealing was done at 72°C for 1 min, and a final 10 min elongation step was done at 72°C (Widmer et al., 1998). All PCR were carried out in a 25 µL reaction volume with 2 µL template (10 ng μL^{-1}), 1 Unit Taq polymerase (NEB, New England Biolabs), 0.5 µM of each primer, 0.2 mM of each dNTP, and 2 mM $MgCl_2$. PCR products were examined on a 1.5% (w/v) agarose gel with ethidium bromide staining to confirm product integrity and correct size (by comparison to marker DNA of known molecular weight).

All DGGE analyses were performed with Dcode^{TM} Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) using the method described by Kowalchuk and Stephen (2001) and modified as follows: 8% (w/v) polyacrylamide gel (acrylamide: bis-acrylamide [37.5: 1], 1 × TAE buffer, 16 cm × 16 cm, 1.5 mm thick gel) containing a linear denaturing gradient of 30 to 55% for the total bacteria, 45 to 65% for *Pseudomonas*, with 100% of denaturant corresponding to 7 M L^{-1} urea and 40% (v/v) deionized formamide. Electrophoresis was run in 1.0 × Tris-acetate-EDTA buffer at 80 V 60°C 14 h, 70 V 60°C 14 h respectively. Gels were silver stained (Sanguinetti et al., 1994) and images were captured digitally using a scanner (Epson, Japan).

Cloning, sequencing and phylogenetic analysis

Dominant bands were excised from the DGGE gel and washed twice with sterilized deionized water, mashed and incubated in 50 µL ddH_2O at 4°C overnight to recover the DNA fragments for PCR. After centrifuging at 6000 g for 20 s, a volume of 1 µL supernatant was used for a further PCR amplification using the primers 341F/803R, and UNI-b-f/520-r respectively, and PCR conditions were the same as described earlier. The fragments recovered from the PCR were subjected to DGGE again to confirm the equal mobilities compared with that of the soil samples. If a single band appeared in the DGGE gel for one sample, the retrieved DNA fragments were purified with PCR Clean-Up System (Promega, Madison, WI) according to manufacturer's instructions. The purified PCR products from each mobility group was ligated into pGEM-T (Promega) and cloned into *Escherichia coli* DH5α according to manufacturer's recommended protocol. The transformed cells were plated onto LB (Luria-Bertani) medium (1.0% Bacto-Tryptone, 0.5% Bacto-yeast extract, 1.0% NaCl, 1.5% Bacto-agar, pH 7.0) containing ampicillin (50 µg ml^{-1}) and X-Gal (0.1 mM) to identify white-colored recombinant colonies. The presence of inserts of the expected size was confirmed by PCR using the primers 341F/803R, and UNI-b-f/520-r respectively (PCR conditions as described earlier). Reconfirmed clones were sequenced by Majorbio Tech Co., LtdM (Shanghai, China).

All sequences were edited and assembled using the CONTIGEXPRESS program of Vector NTI Suite 6.0 (Informax, MD). Sequences of possible chimeric origin were detected using the online CHIMERA DETECTION program (http://rdp8.cme.msu.edu/html/analyses.html). Sequences were quality trimmed at their 3' and 5' ends to a common size fragment; blasted in GenBank (http://www.ncbi.nlm.nih.gov/), and subsequently registered in GenBank database under accession numbers GU183722-GU183752. For phylogenetic analysis, sequences with gaps were treated as missing data, and a neighbor-joining tree (1000 replicates) including the obtained sequences and their closest relatives was constructed using MEGA version 4.0 with the Maximum Composite Likelihood model.

Statistical analysis

Before analysis, the percentage variables (including total carbon, organic carbon, total nitrogen, soil moisture) were arcsine square root transformed, and other variables (including available phosphate and total phosphate) were natural log transformed. The effect of treatments and growing period on soil characteristics was subjected to one-way ANOVA.

The frequency of occurrence of each bacterial taxon was recorded in terms of presence (1) or absence (0) in samples before richness estimating and ordination analysis. The relative abundance of the major bacterial phyla was estimated by counting the percentage of phylum member in all retrieved sequences. Richness of the bacterial taxon was analyzed according to phyla detected by PCR-DGGE and sequencing in samples of different treatments. Total richness of bacterial and *Pseudomonas* taxon were analyzed by adding phyla that appeared in samples of the same treatment together respectively.

To analyze the potential influence of environmental variables and sampling time on the total bacterial and *Pseudomonas* community composition, a canonical correspondence analysis (CCA) with Monte-Carlo permutation tests (n=999) were performed using Canoco for Windows version 4.52.

RESULTS

Soil properties

The impacts of six fertilization treatments and three different plant developmental stages on soil characteristics were subjected to one-way ANOVA (Table 1). The soil properties varied greatly after long-term application of different mineral or organic fertilizers. Long-term manure supply (M, MNP) greatly changed the soil characteristics, especially, for the levels of soil organic carbon and total nitrogen ($P < 0.05$).

The soil organic carbon (SOC) at the beginning of the study in 1979 was 6.1 g kg^{-1} in the 0 to 15 depth, whereas, the concentration increased for all treatment, even the lowest values of CK treatment was 6.41 g kg^{-1} at Maturity stage. But we detected that the soil pH decreased dramatically after 30 years localized fertilization; the properties of soils changed gradually from alkalescent with pH 8.2 towards nearly neutral with pH 7.3 for most soils. Meanwhile, the soil properties varied with the growth of aboveground wheat plants, especially, for soil moisture, pH, and available phosphorus.

Phylogenetic analysis and community composition

All soil samples (18) were successfully analyzed by PCR-DGGE for the community of the total bacteria and genus *Pseudomonas*. The DNA fragments with distinct mobility for the total bacteria (6 to 18 sequences), and *Pseudomonas* (3 to 9 sequences), were retrieved from each DGGE profile respectively. Prominent bands (32 in total) were excised and reconfirmed as earlier described in this study's materials and methods section.

Bacterial community composition

Bacterial community DGGE profiles generally showed more bands after long-term fertilization with respect to the unfertilized control, especially, for treatments with manure or straw (Figure 1a). The signal intensity of bands with the same mobility varied in different samples, as band b11 and b12 are dominant bands and band b13 turning to faint in June samples.

A total of 18 unique types of bacterial sequence (taxon) were retrieved from DGGE bands (Figure 2a). Phylogenetic sequence analysis revealed a high diversity of putative bacterial community. Members of these bacteria were affiliated to phylum *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Gemmatimonadetes*. Most of these taxa had a uniform distribution among samples; only a few extra taxa were detected in specific samples, for example, taxa retrieved from band b1, b3, b10 and b18. The fertilization regimes had considerable effects on the community structure of total bacteria; a group of taxa just appeared in plots applied with mineral fertilizer; sequences retrieved from band b1 and b18 belonged to phylum *Bacteroidetes* and *Firmicutes* respectively, appeared in N treatment merely and sequence of band b10 affiliated to phylum *Firmicutes* appeared in treatments N and NP. However, the community structures of the total bacteria shifted at different wheat growing stages. The bands b3 and b17 belonging to phylum *Gemmatimonadetes* and δ-*Proteobacteria*, appeared in April samples only while band 11, which was affiliated to γ-*Proteobacteria* appeared in May and June samples. Additionally, members of *Actinobacteria* just appeared in soils collected in April, but members of *Gemmatimonadetes* disappeared in most of the soils sampled in June.

The percentage of taxa affiliated to different bacterial phylum varied in different samples (Figure 3a); the dominant bacterial groups responded discrepantly in soils under different fertilization regimes, and fertilizer treatments influenced the incidence of some individual taxa while members of *Firmicutes* in plots M and CK were less than plots with mineral fertilizers (Figure 3a).

After all, richness of the total bacterial taxon declined at three different sampling occasions and plots amended with NP (NP, MNP, SNP) enhanced bacterial richness as compared with treatments N, M, or unfertilized CK (Figure 3b).

Pseudomonas community structure

Bands (p1 to p13) in the DGGE gel were excised for sequencing (Figure 1b). Bands p3, p10, and p14 were the dominant bands in all samples and the signal intensity of band p1 and p12 increased after long-term application organic fertilizers, whether manure or straw. Cluster analysis revealed that 9 of 13 sequences matched the target range of the *Pseudomonas*-selective

Table 1. Variation of soil chemical characteristics in different fertilization treatment during wheat growing stages (Jointing in 26 April, Dough in 28 May, Maturity in 22 June, 2008) at soil depths of 0-15 cm.

Growing stages	Treat	Soil chemical characteristics (g kg^{-1} soil)						
		SM	pH	TC	OC	TN	TP	AP
Jointing	CK	120±0.05^{ab}	7.22±0.03^{c}	14.19±0.03^{a}	7.66±0.01^{d}	0.53±0.02^{d}	0.47± 0.06^{d}	0.006±0.0002^{e}
	N	123.3±5.78^{a}	7.47±0.02^{a}	14.61±0.05^{a}	7.88±0.03^{c}	0.48±0.01^{e}	0.61± 0.07^{b}	0.003±0.0001^{f}
	NP	110±0.05^{c}	7.33±0.06^{b}	13.97±0.02^{a}	7.54±0.05^{f}	0.45±0.01^{f}	0.64± 0.04^{a}	0.013±0.0002^{d}
	MNP	110±0.05^{c}	7.35±0.04^{b}	17.23±0.18^{a}	9.30±0.21^{a}	0.64±0.01^{b}	0.59± 0.12^{b}	0.051±0.0001^{a}
	M	120±0.06^{ab}	7.32±0.03^{b}	15.95±0.06^{a}	8.45±0.05^{b}	0.75±0.02^{a}	0.62± 0.01^{ab}	0.016±0.0001^{c}
	SNP	111.6±5.8^{b}	7.35±0.05^{b}	14.13±0.05^{a}	7.62±0.02^{e}	0.57±0.01^{c}	0.53± 0.04^{c}	0.020±0.0001^{b}
Dough	CK	66.7±0.1^{a}	7.30±0.03^{c}	14.03±0.05^{a}	7.42±0.02^{e}	0.51±0.02^{e}	0.57± 0.002^{c}	0.003±0.0001^{e}
	N	60.3±0.05^{b}	7.42±0.02^{b}	12.88±0.05^{a}	6.83±0.01^{f}	0.48±0.02^{f}	0.53± 0.005^{d}	0.001±0.0001^{f}
	NP	50.7±0.05^{c}	7.44±0.01^{b}	14.23±0.03^{a}	7.54±0.03^{d}	0.53±0.03^{d}	0.73± 0.002^{a}	0.032±0.0001^{b}
	MNP	50.2±0.15^{c}	7.52±0.03^{a}	17.58±0.07^{a}	9.32±0.05^{a}	0.83±0.01^{a}	0.72± 0.008^{a}	0.033±0.0001^{a}
	M	60.8±0.06^{b}	7.47±0.03^{ab}	16.02±0.03^{a}	8.47±0.01^{b}	0.65 ±0.01^{b}	0.46± 0.006^{e}	0.023±0.0001^{c}
	SNP	56.7±0.1^{b}	7.52±0.02^{a}	14.87±0.01^{a}	7.89±0.12^{c}	0.58±0.03^{c}	0.62± 0.007^{b}	0.011±0.0001^{d}
Maturity	CK	120±0.31^{a}	7.23±0.02^{d}	12.13±0.01^{a}	6.41±0.02^{f}	0.46±0.03^{d}	0.59± 0.009^{c}	0.002±0.0001^{f}
	N	120.3±0.60^{a}	7.32±0.02^{c}	13.14±0.05^{a}	6.95±0.01^{e}	0.49±0.01^{c}	0.57± 0.002^{d}	0.003±0.0001^{e}
	NP	90±1.20^{a}	7.24±0.01^{cd}	13.78±0.02^{a}	7.32±0.02^{d}	0.45±0.01^{d}	0.72± 0.003^{a}	0.022±0.0001^{b}
	MNP	80±0.10^{a}	7.62±0.01^{a}	15.38±0.16^{a}	8.21±0.01^{b}	0.59±0.02^{b}	0.64± 0.004^{b}	0.017±0.0001^{a}
	M	130±0.02^{a}	7.51±0.03^{b}	15.84±0.10^{a}	8.41±0.01^{a}	0.69±0.01^{a}	0.58± 0.007^{cd}	0.009±0.0001^{d}
	SNP	120±0.45^{a}	7.30±0.03^{c}	13.82±0.05^{a}	7.46±0.01^{c}	0.47±0.02^{d}	0.63± 0.005^{b}	0.014±0.0001^{c}

Data are expressed as means ± standard error (n=3). In a column of the same growing stage (Jointing, Dough, or Maturity), values followed by the different letters are statistically significantly different ($P < 0.05$) by the LSD Test. Soil properties of the initial field (0 to 15 cm) in 1979 were as follows: pH, 8.2; OC, 6.15 g kg^{-1}; TN, 0.95 g kg^{-1}; TP, 5.7 g kg^{-1}; AP, 0.0072 g kg^{-1}.

primers and were associated within *Pseudomonadaceae* (Figure 2b). Sequences retrieved from band p1 and p7 were much similar to *Pseudomonas fluorescens* and *fluorescens putida*; both of them represent the common species in natural systems. Band p3 retrieved sequence was similar to uncultured *Pseudomonas* sp, but p10 was associated with the other uncultured bacterium which is affiliated to β-*proteobacteria*. Band p6 appeared in June samples only and was associated with the genus *Methylobacterium* affiliated to α-*proteobacteria*. Plots amended with organic fertilizers (MNP, M and SNP) have higher *Pseudomonas* richness compared to mineral fertilizers treated alone or unfertilized CK (Figure 3c), indicating organic fertilizers were more efficient to improve the richness of Pseudomonas in this study site and community shifts occurred after long-term application of organic fertilizers. Additionally, the sources of most referred sequences were retrieved from soils, especially, farmland soil, polluted soil, or chemical factory soil and some of them originated from water habitats.

CCA analysis

The resulting ordination biplots showed the occurrence of the total bacteria and *Pseudomonas* species with respect to each of the environmental variables (Figure 4a and b). The length of these arrows indicated the relative importance of the environmental factor in explaining variation of community structures, while the angle between the arrows indicated the degree to which they were correlated. In the CCA-biplot, the total bacterial communities were separated into three different groups, generally corresponding to the sampling times respectively (Figure 4a). The ordination showed that community structures in treatments applied with NP were more closely related to each other than other treatments. Samples from the MNP plots were markedly separated from the other treatments. Among all determined soil parameters, the significance of the factors were ranged as SM, pH, OC, and AP in determining the clustering of the total bacterial communities. The community of *Pseudomonas* in the plots where M was applied (MNP and M) were much similar, and different from the other treatments (Figure4b). The community structure of *Pseudomonas* was strongly influenced by our determined parameters as OC, TN, AP, and pH.

DISCUSSION

Change of soil properties

The soil properties changed significantly after long-term

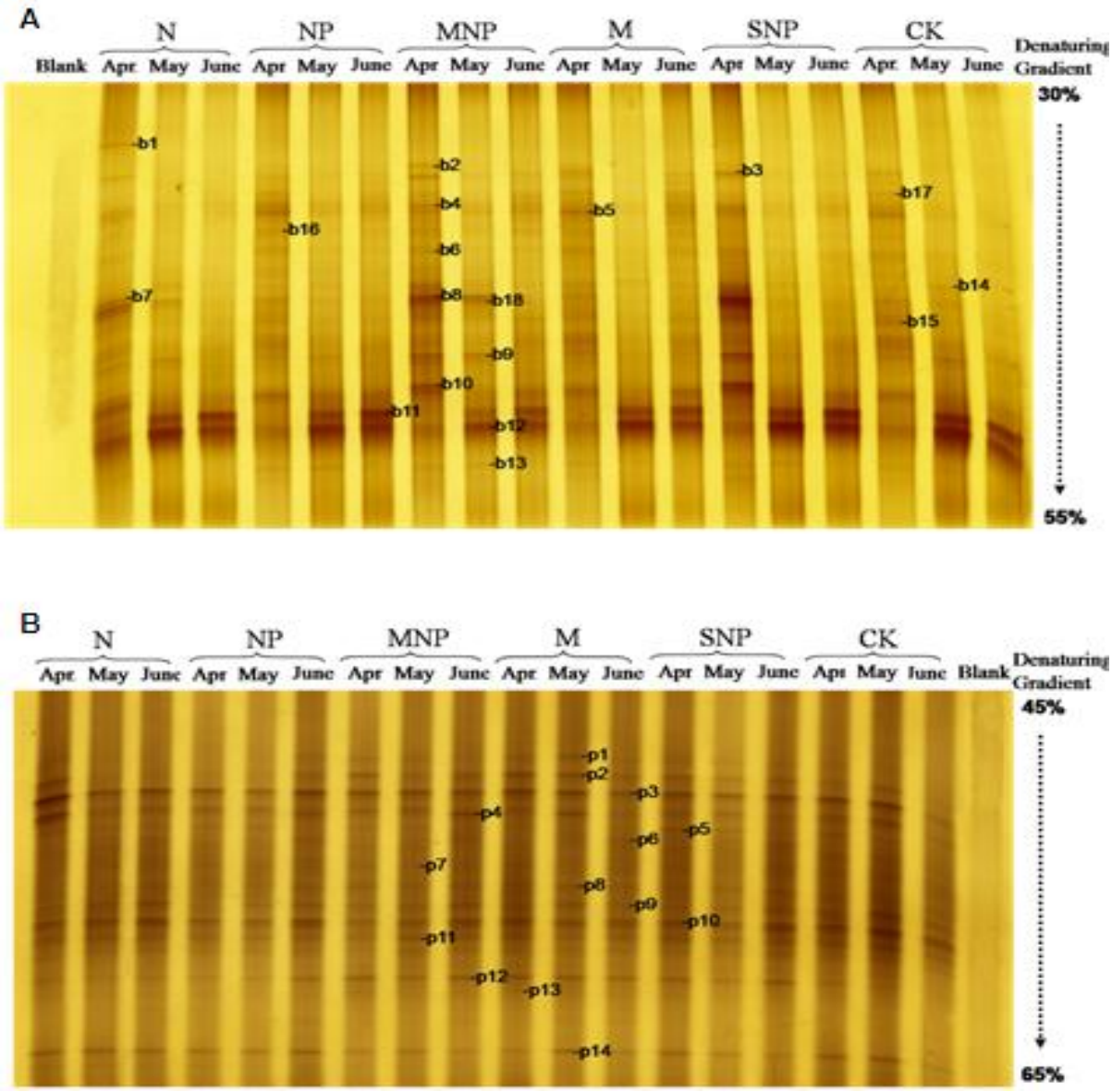

Figure 1. DGGE profile of partial 16S rRNA gene sequences of bacteria (1a); *Pseudomonas*-specific fragments retrieved from the soil (1b). b1-b18, p1-p14 represent bands retrieved from soils collected in April, May, and June (at wheat growing stages of Jointing, Dough, and Maturity), bands marked were excised and sequenced. Treatments including: *CK* (no fertilization), *N* (inorganic N added annually), *NP* (inorganic N and P fertilizer annually), *MNP* (farmyard manure plus inorganic N and P annually), *M* (farmyard manure annually), and *SNP* (straw plus inorganic N annually but P every second year). The denaturing gradient ranged from 30% to 55%, 40% - 60%, and 45% - 65% for the total bacteria and *Pseudomonas* respectively.

application of mineral and organic fertilizers when compared with the unfertilized control. Farmyard manure input and return of straw residues were effective in helping to maintain the organic carbon of cultivated land in this kind of soil. The results were consistent with the studies of this site that stated the benefits of manure and straw to soil for organic carbon accumulation (Fan et al., 2008; Liu et al., 2010). Obviously, improvement of SOC is mainly due to the input of manure and straw as anorganic carbon source. Enhancement of SOC with the application of chemical fertilizers was attributed to greater input of root biomass due to better crop productivity (Izaurralde et al., 2000; Zhao et al., 2009). As a result, we highlight that long-term fertilization, especially manure application is helpful in improving soil fertility. By comparison with the initial value, soil pH values decreased in all soils and similar results was also reported lately by Liu et al. (2010). This is mainly due to the fact that most

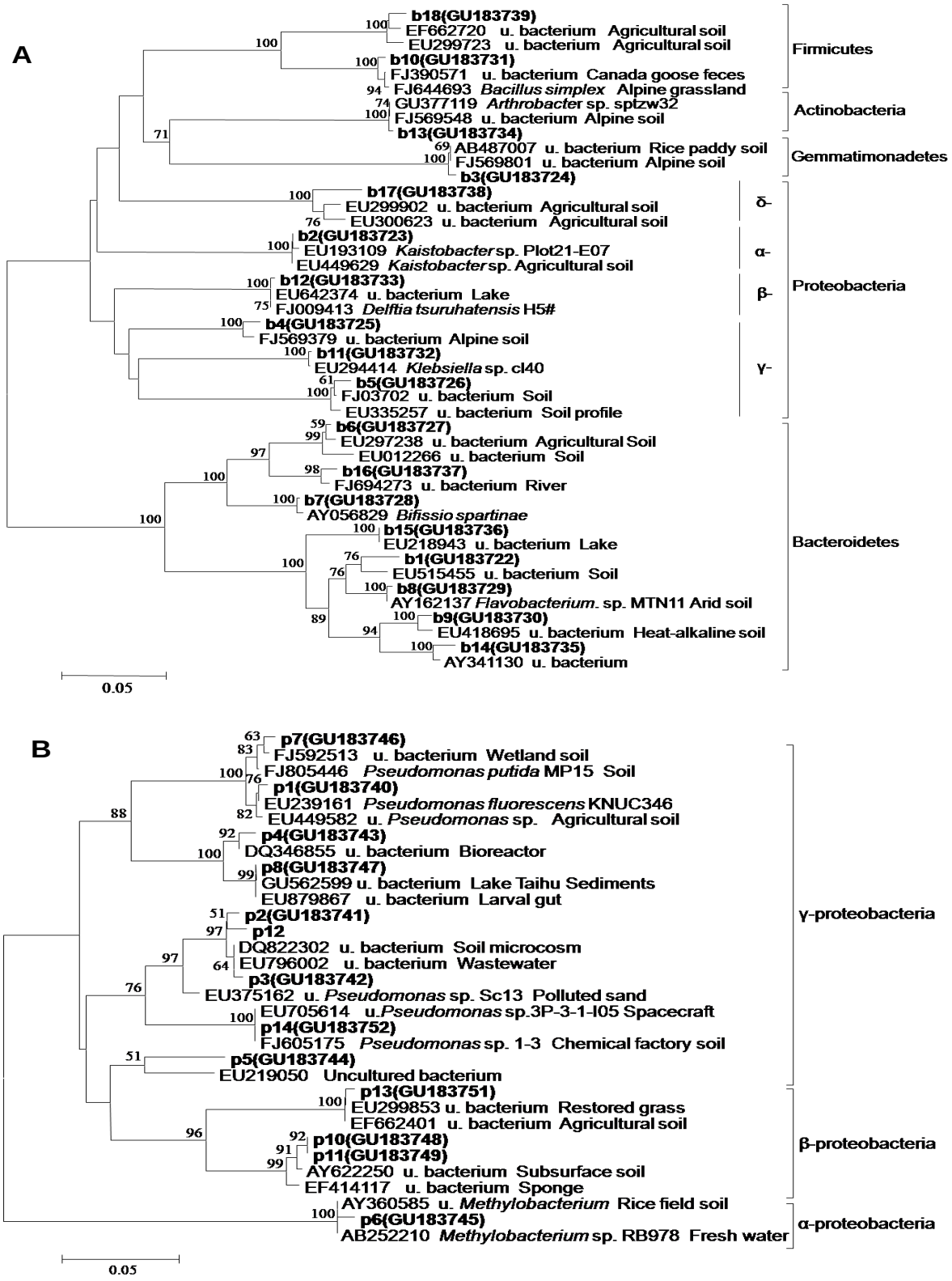

Figure 2. Neighbor-joining phylogenetic tree of the total bacterial (2a); and *pseudomonadaceae* (2b) based on 16S rRNA gene sequences; with referenced sequences in soil samples; Numbers above branches denote bootstrap values from 1000 replicates. Sequences obtained in the present study were shown in boldface. They were labeled with the GenBank database accession number (e.g. GU183722-GU183752). The internal identification number (e.g. b1, p1) represents sequences retrieved from DGGE profile band b1 and p1. The sources of referenced sequences were listed after the basic data. The right boxes show the phylum or clusters delimitations of the phylotypes.

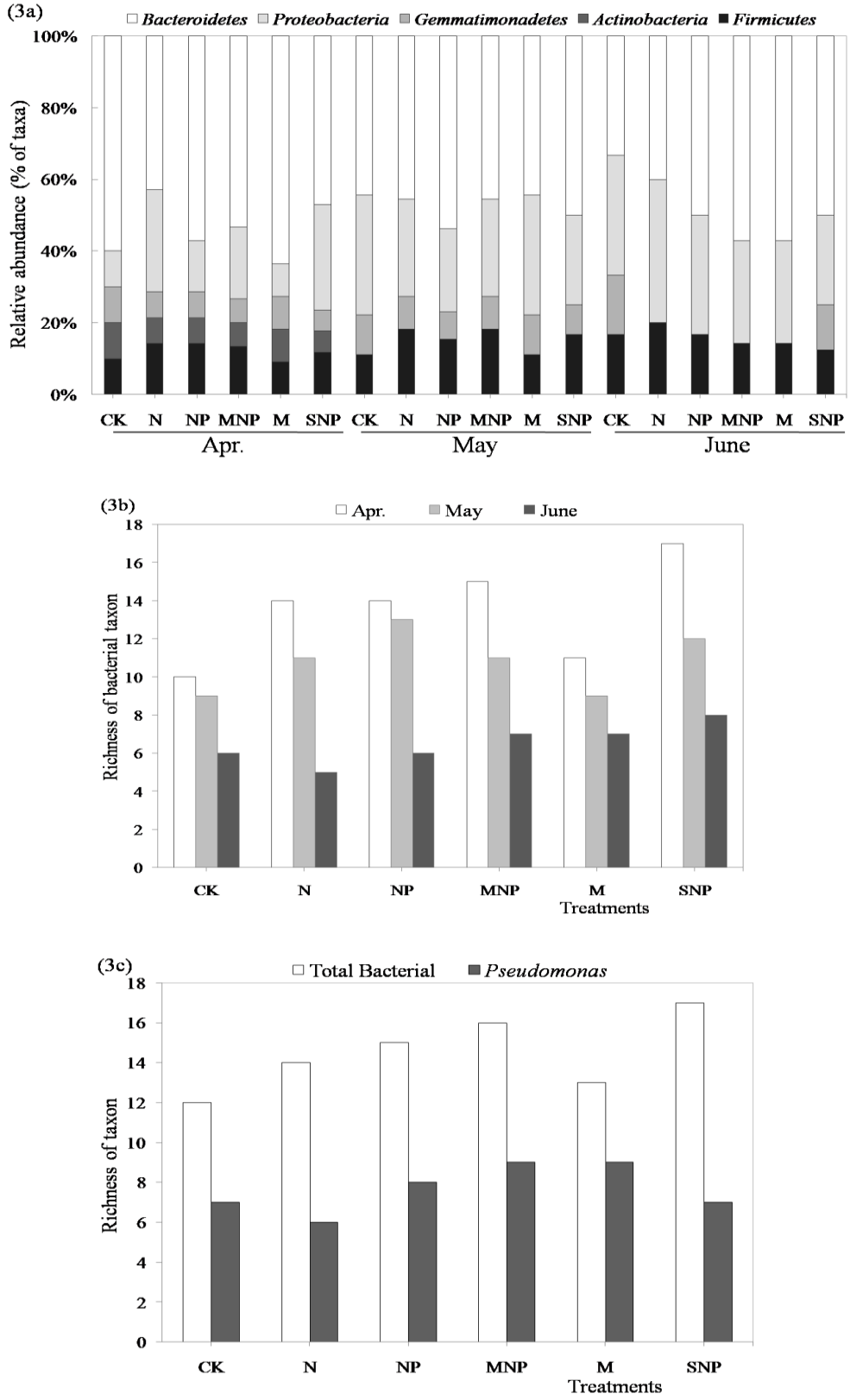

Figure 3. Relative abundance of the major bacterial phyla (a); numbers of bacterial taxa (b); and richness of the total bacterial and *Pseudomonas* taxon (c); presence in samples of different treatments collected in April, May, and June. *CK*, *N*, *NP*, *MNP, M*, and *SNP* indicate fertilizer treatments (See Figure 1).

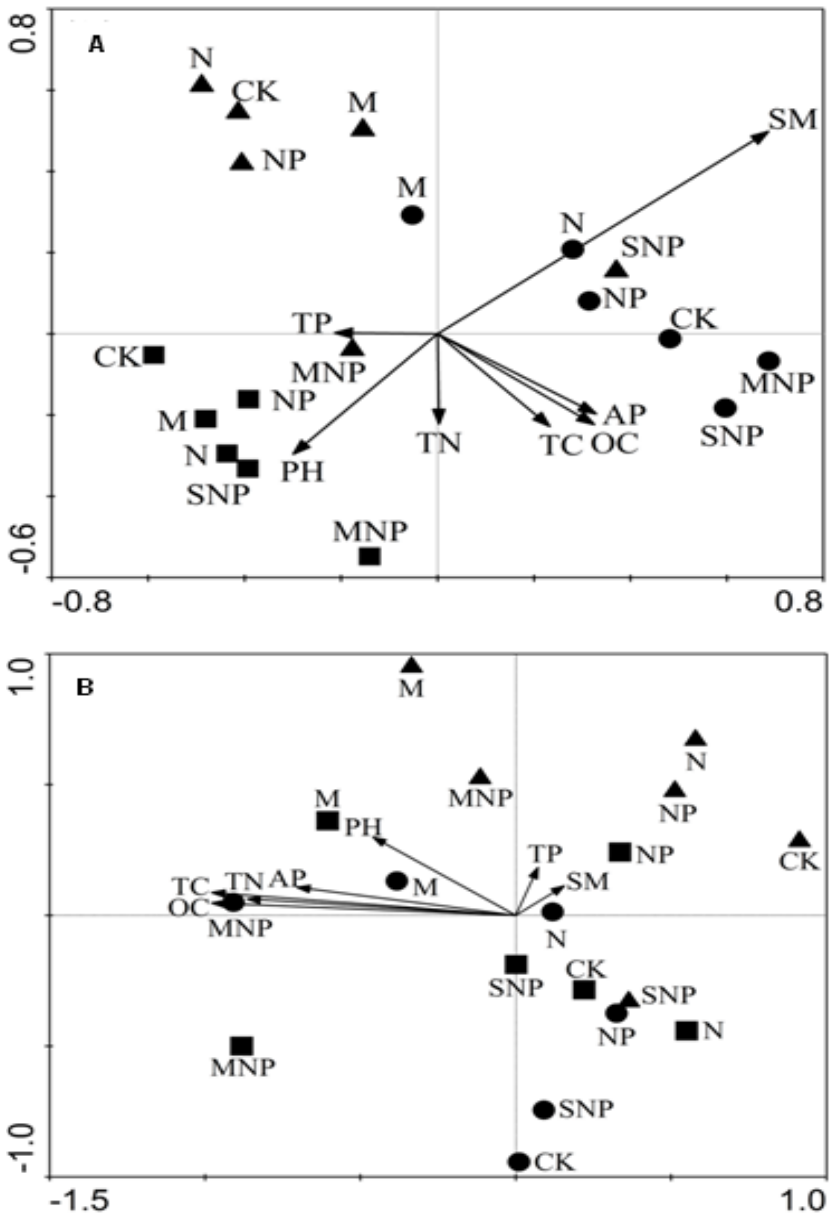

Figure 4. CCA-biplot depicting the relationship between soil properties and phylotypes of the total bacteria (4a); and *Pseudomonas* (4b); with regard to sampling time in treatments (*CK*, *N*, *NP*, *MNP, M*, and *SNP*, See Fig. 1). Phylotypes appeared in soils sampled in April, May, and June were noted as solid circles (●), square (■), and up-triangles (▲) respectively; Environmental factors denoted as arrows (soil parameters represented by SM, pH, TC, OC, AP, TP, TN).

nitrogenous fertilizers supply N as NH_4^+, which upon oxidation release H^+ ions and decrease soil pH (Magdof et al., 1997).

Furthermore, the soil chemical properties changed significantly with the growing periods of crop, especially for the soil moisture, pH, and available phosphorus in our soils. The soil parameters were reported as being changed with development of wheat physiological stages (Mandal et al., 2007). May is the driest season in this area, while the soil moisture in treatments NP and MNP had lower water contents than unfertilized CK in this period. This was probably due to the vigorous crop growth, with higher evapotranspiration in these plots.

Changes of soil bacteria community

The long-term fertilization regimes did affect DGGE

banding pattern and more specific bands appeared in manure or straw fertilized soils; the beneficial effects of organic fertilizer on DGGE fingerprints was stated previously by Chu et al., (2007). When compared with mineral fertilization or unfertilized control, organic amendment showed more superiority for enriching the richness and diversity of soil bacteria (Esperschütz et al., 2007; He et al., 2008; Widmer et al., 2006); and the effects on bacterial community shifts were positive (Zhong et al., 2009), or insignificant (Wei et al., 2008; Widmer et al., 2006). This may probably be due to the enhanced soil microbial biomass and activities in organic treated soils (Plaza et al., 2004; Islam et al., 2011).

Phylogenetic analysis suggested that most taxon affiliate to phylum *Bacteroidetes* in our soils, while *Proteobacteria* dominated the total bacteria taxa at other long-term fertilized study sites (Ge et al., 2008a; Sun et al., 2004) which generally was caused by the different soil type in these study fields. The soil type in our site was classified as calcarid regosols, definitely different from types of sandy loam (Ge et al., 2008a), or silt loam (Sun et al., 2004).

The fertilization regimes had clear impacts on bacterial community structure in our study. Some of the taxa just appeared in N and NP plots. Once the application of mineral fertilizers can modify the soil available N and P fertility, it can be the selective force causing structural shifts in the soil microbial community (Cruz et al., 2009). Another study suggested that soil bacteria were more sensitive to N fertilizers during the plant growth cycle (Crecchio et al., 2007). But P fertilizer was considered as a major factor to control the microbial community in Chinese upland red soil (He et al., 2008). Whether it was caused by one, both or even more of these factors still need further study.

The plots exhibited different bacterial composition, and long-term fertilization influenced the occurrence frequency of some bacterial taxa strongly. These results are in line with previous studies concerning community analysis (Ge et al., 2008a; Enwall et al., 2007). Meanwhile, there was a manifested reduction in bacterial taxon richness at three different sampling times; this declining trend of richness in fertilized soils has been reported previously by Toljander et al., (2008). Plots amended with NP (NP, MNP and SNP) fostered more bacterial taxa as compared to N, M, or CK treatments in our study. The recent study pointed out that organic manures enhanced the bacterial communities; whereas, impact of chemical fertilizers was vice versa indicating deficiency of organic carbon and nutrients in the soil (Zhang et al., 2012). As the studies suggested that application of fertilizers did not directly influence microbial parameters in soil, but did so indirectly by increasing the contents of these critical nutrients (Zhong et al., 2009), thus, promoting plant biomass and increasing soil organic accumulation (Zhong and Cai, 2007), and enhancing soil microbial biomass and activities (Plaza et al., 2004; Islam et al., 2011). Therefore, we speculated that different fertilization regimes affected plant growth differently, which may result in different soil organic carbon accumulation through aboveground turnover and root residues, thereby, affecting community composition and shifts in the present study.

CCA-biplot showed that the total bacterial communities were separated mainly due to the number of times sampling was carried out. The significance of determined soil factors ranged as SM, pH, OC and AP. The occurrence of rainfall on a dry soil must be the most important determinant for seasonal variation of bacterial communities at our study site, as the study explained that these sudden events was prone to seasonal variation of soil microbial biomass (Hamel et al., 2006). Whereas, growth of crops in different fertilized plots was always accompanied with various evapotranspiration and the soil moisture and microbial biomass changed afterwards. Studies showed that the soil pH can affect the composition of total bacterial communities, and changes in pH were mainly induced by the fertilization regimes (Toljander et al., 2008; Enwall et al., 2007). Furthermore, factors including phosphate and soil carbon also contributed significantly to the microbial community changes (Toljander et al., 2008). However, soil type was generally considered as the dominant factor determining microbial community characteristics in arable soils (Girvan et al., 2003; Larkin et al., 2006), even more important than fertilizer type (Suzuki et al., 2009).

As a result, the effects of fertilization regimes on community structures may be obscured due to the similar soil type among different treatments in our study site. Actually, the relative importance of these factors on soil bacterial diversity variation was ranked as sampling locations, soil profiles, sampling time, OM, and P after multivariate regression tree (MRT) and aggregated boosted tree (ABT) analysis (Ge et al., 2008b). Besides that, history of land-use was a stronger determinant of the composition of microbial communities than vegetation and soil properties (Jangid et al., 2011). Whereas, crop and soil management practices such as crop rotation, soil tillage and fertilization regimes etc interacted in our agroecosystem, thus, the main discriminator between different treatments cannot be resolved undoubtedly. We should attempt to verify the patterns and mechanisms of these phenomena, and find out the correlated factors in future studies.

Changes of *Pseudomonas* community

A PCR-DGGE strategy was used to generate specific *Pseudomonas* spp. fingerprints of 16S rRNA genes amplified from the total soil DNA. Cluster analysis revealed that most of sequences matched the target range of the *Pseudomonas*-selective primers and is associated with *Pseudomonadaceae.* The *Pseudomonas*-

selective PCR primers were highly specific and may represent a powerful tool for *Pseudomonas* population structure analysis and taxonomic confirmations (Widmer et al., 1998). Sequences retrieved from bands p1 and p7 were much similar to *Pseudomonas fluorescens* and *Pseudomonas putida*, both of which are common culturable *Pseudomonas* known for displaying antifungal properties (Costa et al., 2006a; Behn, 2008). Sequences retrieved from the prominent bands p3 and p14 in our DGGE profiles were similar to uncultured *Pseudomonas* sp. A group of sequences isolated from soils clustered apart from *Pseudomonas* (sensu stricto), sequence from band p10 was associated with other uncultured bacterium affiliated to β-*proteobacteria*, and band p6 was associated with the genus *Methylobacterium* affiliated to α-*proteobacteria*. Our results suggested that the primer pair UNI-b-f/520-r has some limitations for the discrimination of *proteobacteria* members completely.

The *Pseudomonas* population structure shift occurred after long-term application of organic fertilizers in our study site and plots amended with organic fertilizers (MNP, M and SNP), enhanced the richness of *Pseudomonas* compared to mineral fertilizers treated alone (N and NP), or unfertilized control.

Similarly, studies suggested that agricultural regimes, especially, long-term manure application, influenced the abundance and structure of *Pseudomonas* populations strongly in farmland soils (Garbeva et al., 2004; Van Bruggen and Semenov, 2000). But plant type had more influence than managing systems in the DOK field experiment (Pesaro and Widmer, 2006). The community structure of *Pseudomonas* was greatly influenced by OC, TN, AP, and pH according to CCA analysis; the sampling time has minor effects reversely, which was supported by the other findings (Costa et al., 2006b). Factors such as sampling site, plant species, and year-to-year variation were shown to significantly influence the community structure of *Pseudomonas* in their study (Costa et al., 2006a).

Finally, we are aware of the fact that application of organic manure may improve soil structure and function, enhance water and nutrient supplying capacity, and thus, promote plant growth, maintain high crop yield, increase soil microbial biomass and enzymatic activity in our study site (Fan et al., 2005; Fan et al., 2008; Liu et al., 2010). Consequently, greater microbial population and richness may be sustained indirectly through increased root turnover, and rhizosphere deposition of soil organic substrates (Izaurralde et al., 2000; Börjesson et al., 2012) which may stimulate the microbial activity and enhance decomposition conversely (Liu et al., 2010).

Additionally, most of the reference sequences were derived from soils, especially, from agricultural soil or even farmland that has fertilization history. These similarities of microbial composition may be promoted by such agricultural managing systems to a certain degree. The total bacterial community responded significantly to the long-term fertilization regimes and showed temporal changes in our study, but some different patterns were detected for the *Pseudomonas* communities. The difference may be explained by the fact that the total bacterial community represents a composite of various bacterial subgroups, which may display different responses, and relatively small groups may not be detected in bacterial community profiles (Muyzer et al., 1993).

Conclusions

In summary, the present study is the first investigation aimed to clarify the community structure of the total bacteria *Pseudomonas* status in arable soil after long-term different fertilization in the rain-fed Loess Plateau of China. Results of this study highlight that long-term fertilization, especially manure application is helpful to improve soil fertility. Long-term application of fertilization, whether in mineral or organic forms, is beneficial to the accumulation of soil organic carbon and microbial diversity than unfertilized control. Plots amended with manure are better for increasing the richness of *Pseudomonas*.

Consequently, higher microbial population and richness probably be sustained indirectly through increased root turnover and rhizosphere deposition of soil organic substrate in our study site. Meanwhile, a reduction in bacterial taxon richness throughout the growing season was also manifest. The effects of sampling time on microbial composition and community structure were more evident than fertilization regimes in our soils. Factors of sampling time, edaphic characteristics, and habitats interacted to affect the structures of microbial community.

Furthermore, the determined soil parameters such as soil moisture (SM), organic carbon, pH, and available phosphorus (AP) contributed much to the variation of the targeted microbial community structure in such a typical rain-fed semi-arid agroecosystem. As a results, our study emphasizing the necessity of functional subgroup analyses in further microbial ecology investigations. Balanced-fertilization should be encouraged for the maintaining of soil quality and agricultural sustainability.

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