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Influence of 34-years of fertilization on bacterial communities in an intensively cultivated black soil in northeast China

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ABSTRACT

Although the effects of chemical fertilization management on microbial communities in soils have been well studied, few studies have examined such impacts of long-term chemical fertilizations on the microbial community in black soils common to northeast China. We applied high-throughput pyrosequencing and quantitative PCR of the 16S rRNA gene to investigate bacterial communities in a long-term fertilizer experiment started in 1980. The following fertilizer treatments were compared with control plots (no fertilizer): N_1 (low nitrogen fertilizer), N_2 (high nitrogen fertilizer), N_1P_1 (low nitrogen plus low phosphorus fertilizers) and N_2P_2 (high nitrogen plus high phosphorus fertilizers). All fertilization treatments resulted in decreases in soil pH and increases in wheat yield and concentrations of total nitrogen, organic matter and KCl-extractable NO_3^- and NH_4^+. Fertilization also led to a significant decrease in total 16S rRNA gene abundance and bacterial diversity. The phyla Proteobacteria, Acidobacteria and Actinobacteria dominated in all fertilized treatments. There was an increase in relative abundance of Actinobacteria, Proteobacteria, TM7 and Verrucomicrobia across all fertilized treatments compared to unfertilized controls, whereas phyla Acidobacteria and Nitrospirae decreased. The bacterial communities in unfertilized controls and lower-mineral fertilizers (i.e. N_1 and N_1P_1) were predominantly composed of Acidobacteria, Actinobacteria and Proteobacteria, and separated from the communities where more concentrated fertilizer regimes were used (i.e. N_2 and N_2P_2) based on principal coordinates analysis. Soil pH and NO_3^- concentration appeared to be the most important factors in shaping bacterial communities. Our findings suggested that long-term inorganic fertilizer regimes reduced the biodiversity and abundance of bacteria. The influence of more concentrated fertilizer treatments was greater than that of lower concentrations.

1. Introduction

The interaction between plants, soil and microorganisms is considered to be the major driver of ecosystem functions (Suleiman et al., 2013) and microorganisms respond quickly to any modification of vegetation or soil properties (Hallin et al., 2009; Zhao et al., 2014) The diversity and abundance of bacteria community in agroecosystems is critical to maintaining soil quality, productivity and ecological balance in cropland areas (C.H. Li et al., 2014; Y. Li et al., 2014). Over the past 100 years, extensive agricultural intensification has been implemented by high inputs of chemical fertilizers which, in turn, are resulting in serious degradation of soil physicochemical properties (Singh et al., 2014). The nutrient-based alteration and associated microbiota were not surprisingly reflected in significant shifts in nitrogen-fixing bacteria (Berthrong et al., 2014), ammonia-oxidizing bacteria (Ai et al., 2013), methanotrophs (Dai et al., 2013) and denitrifiers (Tang et al., 2010). Understanding the shifts in microbial community structure and composition following long-term fertilization may have significant implications for the development of better fertilizer regimes for

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agroecosystems (Shen et al., 2010). High-throughput sequencing technologies can provide significant insights into species and functional diversity of microbial populations with different fertilizer regimes (Hallin et al., 2009; Ye et al., 2013; Zhao et al., 2014). Recent results suggest that the change of potential denitrification under long-term fertilization regimes was closely related to the shift in denitrifying bacteria communities resulting from the variation of properties in black soil (Yin et al., 2015). In the long term, such modifications may result in shifts in the quality and functionality of the soils (Beauregard et al., 2010).

As different groups of microorganisms can differ in their ability to process various nutrient forms found in soil, fertilization can affect their growth competitiveness, which could make a great difference in the diversity, biomass and activity of the soil microbial community. For example, long-term N application resulted in changes in both the overall bacterial community (Rousk et al., 2010) and the composition of individual bacterial groups, such as ammonia-oxidizing bacteria, Nitrobacter-like bacteria (Wertz et al., 2012), the cellulolytic community (Fan et al., 2012), Actinobacteria (Jenkins et al., 2009) and Acidobacteria (Zhao et al., 2014). Although elevated inputs of N revealed no significant effects on bacterial diversity, the relative abundance of copiotrophic taxa (including members of the Proteobacteria and Bacteroidetes phyla) increased and oligotrophic taxa (mainly Acidobacteria) decreased in the high N plots in two long-term N addition experiments in the USA (Fierer et al., 2012). Furthermore, nitrogen induces a shift in the predominant microbial life-history strategies and inhibits soil microbial respiration rates regardless of the form of nitrogen applied (Ramirez et al., 2010).

In this study, soil samples were collected from 34-year fertilization regimes using N and P fertilizers in an agricultural soil in northeast China. Earlier studies on this site showed that long-term application of chemical fertilizers resulted in a decline in the catabolic activity of fast-growing or eutrophic bacteria as indicated by PCR-Denaturing Gradient Gel Electrophoresis methods (Wei et al., 2008). However, this technique only revealed the dominant bacteria in the soil and there is limited information on the relationship between bacterial community shift and size changes under long-term fertilizer application, as well as the interactions between environmental factors and specific groups of soil bacteria. Here, we hypothesized that the composition of soil bacterial communities would be impacted by long-term inorganic fertilizers inputs in the rich black soils common across Northeastern China; such a shift may directly or indirectly derive from increased soil NO_3^- concentration and decreased pH. Five different fertilizer treatments were employed to evaluate the soil chemical properties, bacteria community structure and their abundance in soils.

2. Materials and methods

2.1. Site description and sampling

The sampling site was located in an experimental field in Harbin city, Heilongjiang Province, China (45°40′N, 126°35′E and altitude 151 m). This region has a temperate continental monsoon climate with an average annual temperature of 3.5 °C and mean annual precipitation of 533 mm. The long-term fertilization experiment started in 1980, comprising five treatments with different applications of inorganic fertilizer: CK (without fertilizer), N_1 (150 kg N ha^{-1} y^{-1}), N_2 (300 kg N ha^{-1} y^{-1}), N_1P_1 (150 kg N plus 75 kg P_2O_5 ha^{-1} y^{-1}) and N_2P_2 (300 kg N plus 150 kg P_2O_5 ha^{-1} y^{-1}). Fertilizer treatments were maintained in the same plot location each year. N fertilizer was applied as urea while P fertilizer was as calcium super phosphate and ammonium hydrogen phosphate. Each treatment had three replicates. The wheat growing period started in early April and ended in late September and we collected soil after wheat harvest in late September 2013. Ten cores (2.5 cm in diameter) were randomly collected from the plough layer of soil (5–25 cm) in each replicate plot with an area of 9 m × 4 m for each plot. The cores from each replicate plot were mixed together, pooled in a sterile plastic bag and transported to the laboratory on ice. The samples were sieved through a 2.0-mm sieve and stored at −80 °C for further molecular analysis.

2.2. Chemical characteristics

For chemical characterization, soil samples were air dried at room temperature and sieved through a 2-mm screen. Soil pH was determined using a glass combination electrode with soil:water of 1:1 (Li et al., 2013). The OM and total nitrogen (TN) were determined according to Strickland and Sollins (1987). Soil KCl-extractable NO_3^- and NH_4^+ were determined by extraction with 2 M KCl, steam distillation and titration (Mulvaney, 1996). Available P was analyzed by resin extraction following a protocol modified from Hedley and Stewart (1982).

2.3. Total community DNA extraction

The total DNA was extracted from 0.25 g of soil using the Power Soil DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer. To minimize the DNA extraction bias, three successive extractions of microbial DNA were combined and purified using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The total DNA was checked on 1.0% agarose gel and DNA concentration and quality (A260/A280) of the extracts were estimated visually using a NanoDrop ND-1000 UV–vis spectrophotometer (ThermoScientific, Rockwood, TN, USA).

2.4. 16S rRNA gene amplification and sequencing

To amplify the bacterial fragments of the appropriate size for MiSeq, primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) which target V4 hypervariable regions of bacterial 16S rRNA genes were selected (Caporaso et al., 2012). Both forward and reverse primers were tagged with adapter, pad and linker sequences. Each barcode sequence (12 mer) was added to the reverse primer for pooling multiple samples in one run of MiSeq sequencing. The PCR conditions were 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min of extension, followed by 72 °C for 6 min. PCR products were purified and combined in equimolar ratios with the quantitative DNA binding method in order to create a DNA pool that was further used for sequencing from the adaptor. The 16S rRNA gene fragments were sequenced using the Illumina Miseq platform.

2.5. Quantitative PCR (qPCR) analysis

The abundances of bacterial 16S rRNA gene copies were quantified in duplicate using the ABI 7500 Real-Time PCR detection system (USA). The reaction mixture (20 μL) contained FastFire qPCR PreMix (SYBR Green) (Tiangen, China), 10 nM of each primer, ROX Reference Dye, and 1 μL of 1/10 diluted DNA. Bacterial assays used primers 515F and 806R and the following thermal program: 95 °C for 1 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 34 s and 72 °C for 15 s (Lauber et al., 2013). The standard for measuring the quantity of the 16S rRNA was developed from a clone with the correct insert. A plasmid DNA preparation was obtained from the clone using a Miniprep kit (Qiagen, Germantown, MD, USA). The R^2 of the standard curve was >0.99. The qPCR reactions were run in quadruplicate with the DNA extracted from each soil sample.

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2.6. Bioinformatic analysis

Sequence analysis was based on the software QIIME as described by Fadrosh et al. (2014). In brief, sequence reads belonging to the samples were extracted from the data obtained from the Illumina Miseq platform. The primers were removed, and the sequences were trimmed to remove low quality sequences. Operational taxonomic units (OTUs) were clustered at a dissimilarity of 0.03, and a representative sequence for each phylotype was aligned using the Ribosomal Database Project Reference file and a cutoff of 80% of the bootstrap value (Bodenhausen et al., 2013). The results were deposited into the NCBI short reads archive database (accession number: SRX671545). Shannon and Simpson diversity indices, as well as Chao and ACE richness estimators, were calculated using Mothur software to estimate bacterial diversity and richness. Rarefaction curves and diversity indices including microbial community diversity, phylogenetic diversity (Faith's PD) and coverage were calculated in Mothur (Yousuf et al., 2012).

2.7. Statistical analysis

The concentrations of OM, TN, NO_3^-, NH_4^+ and available P and pH in soil samples were tested for differences among samples using one-way analysis of variance (ANOVA), and paired comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$, using SPSS BASE ver. 19.1 statistical software (SPSS, Chicago, IL, USA) (Ahn et al., 2012).

Principal coordinates analysis (PCoA) based on the Fast UniFrac metric qualified sequences were imported into the ARB software package (version 5.2) and aligned using the SINA aligner (version 1.1) (Pruesse et al., 2012) and the SILVA 16S rRNA database (Pruesse et al., 2007). A phylogenetic tree was constructed using Fast-Tree (Price et al., 2009), and the resulting tree and the abundance data were imported into the Fast UniFrac environment (http://bmf.colorado.edu/FastUniFrac/) (Hamady et al., 2009). Weighted Fast UniFrac distances between the samples were calculated and PCoA was performed on the basis of the distance measured, and coordinates were used to draw 3D graphical outputs. In addition, multiple variations of correlation between environmental variables (OM, TN, available P, NO_3^-, NH_4^+ and pH) and community composition were analyzed using a redundancy analysis (RDA) by CANOCO 5.0. A manual forward-selection procedure was implemented in the RDA to determine significance of environmental variables ($P < 0.05$) using a Monte Carlo test with 499 permutations.

3. Results

3.1. Effects of long-term fertilization on soil chemical properties and wheat yield

The 34-y fertilization regimes significantly ($P < 0.05$) changed wheat yield and soil properties including soil pH, and soil concentrations of TN, NO_3^-, NH_4^+, OM and available P (Table 1). The ANOVA showed that soil pH in all fertilized treatments decreased significantly, with the highest decrease for N_2 (pH 4.64). However, all fertilized treatments significantly increased TN, available P, OM, NO_3^-, NH_4^+ and wheat yield, compared with the unfertilized controls. In addition, increases in TN and OM did not significantly differ between N_2, N_1P_1 and N_2P_2. The increases in wheat yield did not significantly differ between N_1 and N_2. Such changes in soil properties may affect the composition and abundance of bacterial communities, which were further examined by qPCR and Illumina MiSeq platform.

3.2. Effects of long-term fertilization on bacterial 16S rRNA gene copy numbers

The 34-y fertilization regimes influenced the size of the soil bacterial community as estimated by qPCR of the bacterial 16S rRNA genes (Fig. 1A). These effects were significant on gene copy numbers both per gram of soil and per ng of DNA extracted (data not shown). The numbers of bacterial 16S rRNA genes in 1 g of soil ranged from 2.2×10^9 to 4.3×10^9. There were significant decreases ($P < 0.05$) for all fertilized treatments compared with unfertilized controls. The N_2 and N_2P_2 plots had lower 16S rRNA gene copy numbers compared to those of N_1 and N_1P_1. The 16S rRNA gene copy numbers were positively correlated (Spearman's rank correlation) with soil pH in all fertilized soils ($R^2 = 0.724$, $P = 0.005$; Fig. 1B), whereas there were no significant correlations with other soil properties (i.e. soil TN, OM, available P, NH_4^+ and NO_3^-).

3.3. Bacterial α-diversity

In bacterial community analysis of the 15 soil samples, a total of 157,421 high quality sequences (69% of the total 227,196) were obtained. They could be classified for a mean of 10,495 classifiable sequences per sample used in the subsequent analysis (range 7905–13,502) with a mean read length of 270 bp. The Good's coverage values were in the range of 0.81–0.88 at 97% similarity cutoff, indicating that the current numbers of sequence reads were sufficient to capture the bacterial diversity in these soils. Based on Mothur clustering, the numbers of OTUs in soil samples were in the range of 2065–2635 in the five fertilizer treatments, with N_2 treatment having the lowest number of total OTUs and N_1 the highest (Table S1).

There were significant differences in microbial richness and diversity ($P < 0.05$) for Shannon, coverage, ACE, Chao1 and Simpson measures but not for OTUs (Table S1). ACE and Chao1 were higher in N_1 and N_1P_1 and lower in the N_2 and N_2P_2 treatments (Table S1). Furthermore, Shannon's index was lower in all fertilized than in unfertilized treatments (Fig. S1C). Rarefaction analysis showed that bacterial diversity was higher in N_1P_1 and N_1 and lower in N_2 and N_2P_2 plots (Fig. S1A).

The relationship of α-diversity with soil properties is shown in Table 2. The values of Chao1 and ACE were negatively correlated

Table 1
Properties of soil samples under different fertilizer treatments.

Fertilizer regimes	pH (1:1H_2O)	Total N (g kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	Available P (mg kg^{-1})	Organic matter (g kg^{-1})	Wheat yield (kg ha^{-1})
CK	6.36 (0.03)d	1.20 (0.01)a	7.68 (0.05)a	33.82 (1.09)a	9.29 (0.31)a	27.26 (0.43)a	1548.1 (280.1)a
N_1	5.64 (0.03)c	1.28 (0.01)b	8.47 (0.06)b	36.91 (2.51)ab	10.89 (0.64)b	28.26 (0.92)ab	2140.0 (230.5)b
N_2	4.64 (0.03)a	1.42 (0.03)c	24.54 (0.08)e	40.75 (1.15)c	15.30 (0.31)c	29.47 (0.99)b	2155.2 (248.1)b
N_1P_1	5.59 (0.08)c	1.38 (0.04)c	10.50 (0.06)c	34.77 (0.54)ab	70.88 (0.83)d	29.86 (0.10)b	3352.0 (244.7)d
N_2P_2	4.79 (0.03)b	1.38 (0.03)c	22.29 (0.23)d	39.07 (1.74)bc	84.97 (0.72)e	29.89 (0.31)b	2617.0 (329.5)c
ANOVA *P*-values	<0.001	<0.001	<0.001	0.001	<0.001	0.002	<0.001

Values are mean ± standard deviation ($N = 3$). Values within the same column followed by the different letters indicate significant difference ($P < 0.05$).
Fertilizer regimes: CK (without fertilizer), N_1 (150 kg N ha^{-1}), N_2 (300 kg N ha^{-1}), N_1P_1 (150 kg N ha^{-1} plus 75 kg P ha^{-1}), N_2P_2 (300 kg N ha^{-1}plus 150 kg P ha^{-1}).

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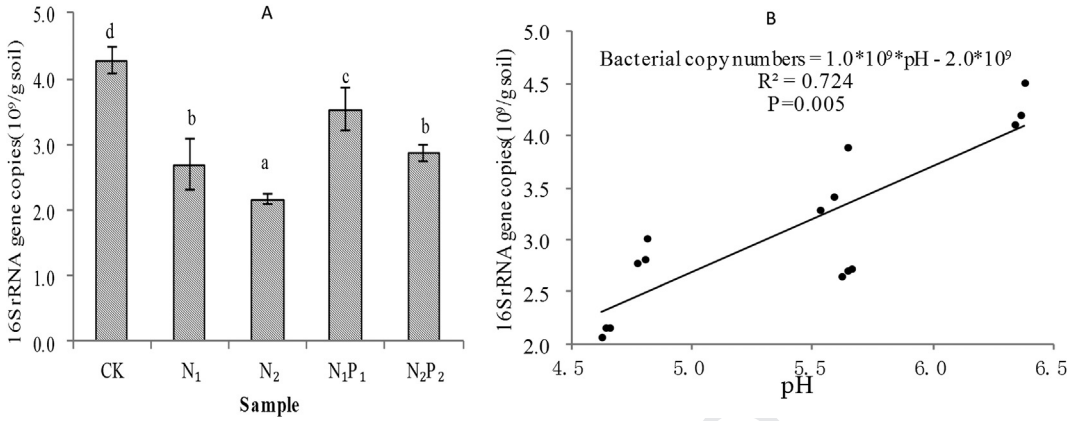

Fig. 1. The abundance of bacteria as indicated by the numbers of 16S rDNA copies measured using quantitative PCR. (A) Bacterial abundance (16S rRNA gene copies g^{-1} soil). Same letters above columns denote no significant difference ($P < 0.05$, Tukey's test). (B) Linear functions were used to describe the relationship between bacterial copy numbers and pH.

Table 2
Pearson's correlation coefficients between soil properties and α-diversity.

	Chao1	ACE	Simpson	Shannon	OTUs[a]
pH (1:1H_2O)	0.341	0.405	−0.678**	0.924**	0.353
Total N	−0.163	−0.193	0.660**	−0.734**	−0.207
	−0.539*	−0.594*	0.458	−0.881**	−0.498
	−0.314	−0.333	0.417	−0.699**	−0.295
Available P	0.046	−0.036	0.703**	−0.490	0.082
Organic matter	0.056	−0.038	0.741**	−0.609*	0.101

**$P < 0.01$, *$P < 0.05$.
[a] OTUs: operational taxonomic units (97% similarity).

with NO_3^- concentration (R = −0.539 and −0.594, respectively, both $P < 0.05$). Shannon's index was positively correlated with pH ($R = 0.924$, $P < 0.05$), but negatively correlated with NO_3^- concentration (R = −0.734, P < 0.05). However, Simpson's index was negatively correlated with pH (R = −0.678, P < 0.05) (Table 2).

3.4. Bacterial community composition

The relative abundances of different phyla in the 15 samples are shown in Fig. 2. The phyla Acidobacteria, Actinobacteria and Proteobacteria occupied 55–60% of the bacterial sequences obtained from the fertilized soils and were followed by Planctomycetes (7.2–8.1%), Bacteroidetes (7.0–9.5%), Verrucomicrobia (6.1–9.4%), Gemmatimonadetes (6.0–7.3%) and Crenarchaeota (1.1–2.4%). The sequence affiliated with the phylum Proteobacteria occupied the highest proportions (27.8–36.0%) of the bacterial sequences in all soil samples (Fig. 2).

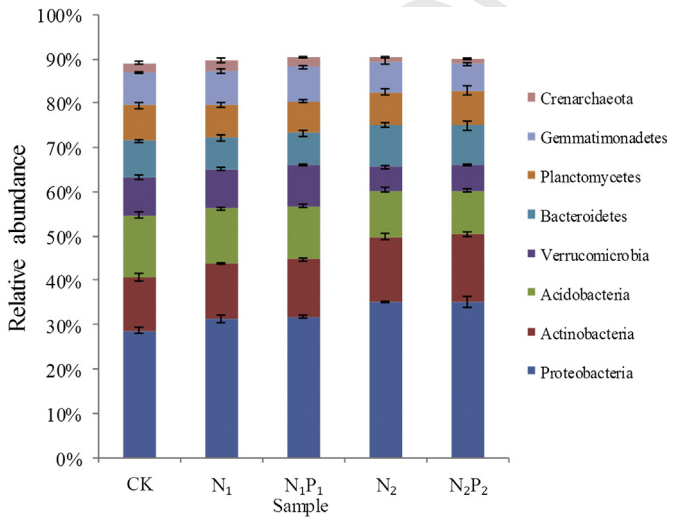

Fig. 2. Relative average abundances of the eight most abundant phyla across soils from different fertilizer regimes (values represent % of total redundant sequences). Error bars indicate the standard deviation of relative abundance between three replicate samples.

Different fertilization regimes significantly changed the relative abundance of the main phyla (>1%) (Table S2). The relative abundance of phyla Actinobacteria, Proteobacteria, TM7 and Verrucomicrobia increased in all fertilizer treatments, and phyla Acidobacteria and Nitrospirae decreased, compared to unfertilized controls (Fig. 2).

There were no significant ($P > 0.05$) differences between treatments for abundant classes Acidobacteria, Cytophagia, Gemmatimonadetes, Phycisphaerae, Planctomycetia and Betaproteobacteria, but there were significant ($P < 0.05$) differences between treatments for the nine most abundant classes shown in Fig. 3. Notably, the relative abundance of the different proteobacterial classes responded differently to the different fertilizer regimes. Classes Alphaproteobacteria and Gammaproteobacteria were higher in all fertilized soils, and class Deltaproteobacteria was lower, compared to those in controls. For the phylum Acidobacteria, classes Solibacteres and Acidobacteria were higher in all fertilized soils while Chloracidobacteria was lower compared to unfertilized controls (Fig. 3). Furthermore, the relative abundance of Alphaproteobacteria, Gammaproteobacteria, Solibacteres and Sphingobacteriia increased significantly in all fertilized soils; and for N_2 and N_2P_2, the relative abundance of these four abundant classes increased to a greater extent compared to N_1 and N_1P_1 treatments (Fig. 3A–D). However, the relative abundance of Thaumarchaeota, Chloracidobacteria, Nitrospira, Pedosphaerae and Thermoleophilia decreased significantly in all fertilized soils; and for N_2 and N_2P_2, the relative abundance of these five abundant classes decreased to a greater extent compared to N_1 and N_1P_1 treatments (Fig. 3E–I).

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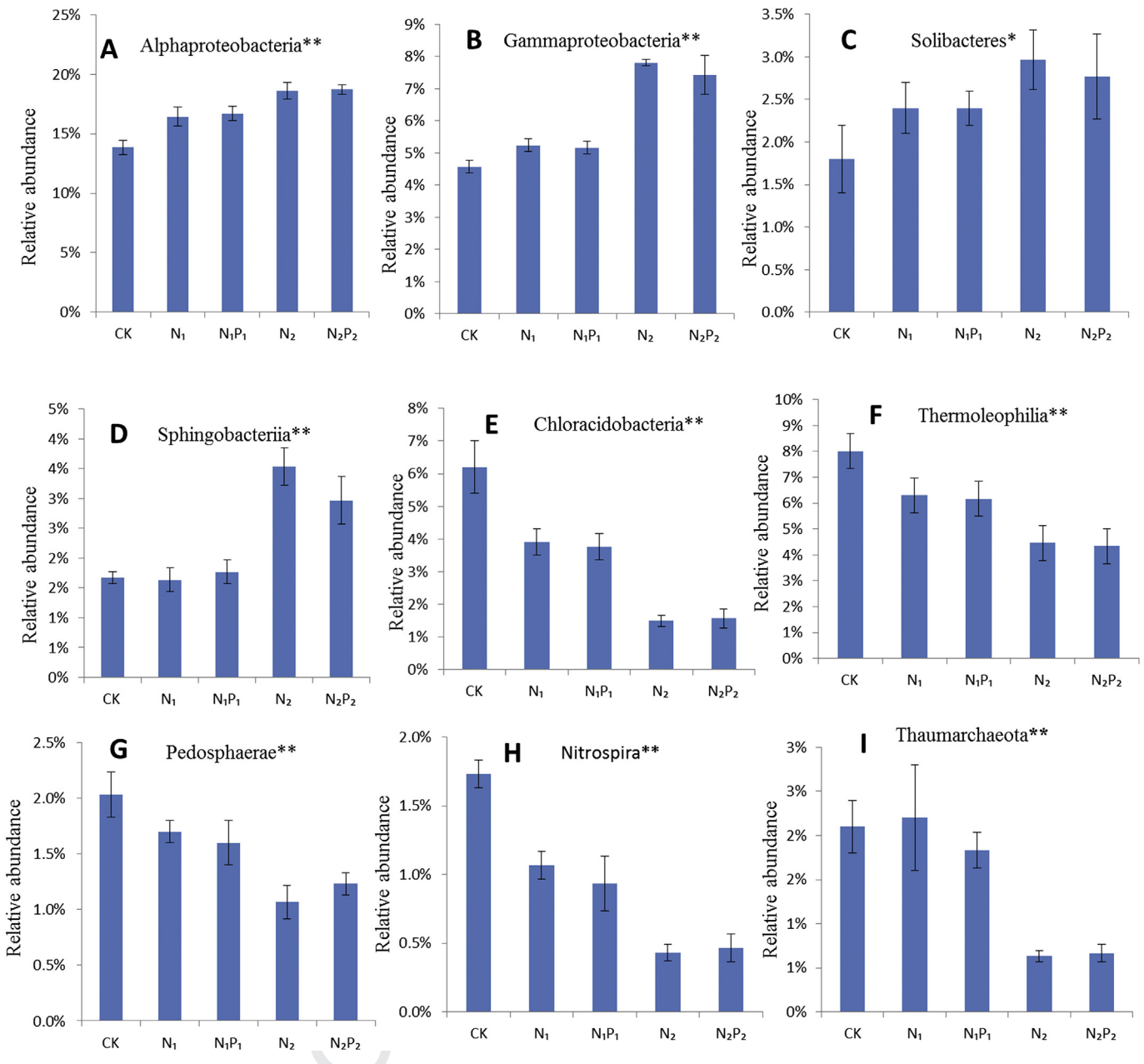

Fig. 3. Relative average abundances of the nine most abundant classes under different long-term fertilizer treatments. Error bars indicate the standard deviation of relative abundance between three replicate samples.

3.5. OTU-level bacterial β-diversity analysis

The variations in bacterial communities caused by long-term fertilization were investigated using PCoA based on weighted Fast UniFrac metrics. The first three principal coordinates represented 59% (PC1), 12% (PC2) and 4.4% (PC3) of the variation in bacterial communities, respectively (Fig. 4A). PC1 generally distributed the bacterial communities along with soil pH: the bacterial communities from plots with relatively acidic pH (4.64–4.79, N_2 and N_2P_2) were generally located in the upper left part; whereas those from plots with a slightly higher pH (5.59–5.64, N_1 and N_1P_1) were in the middle-lower; and the unfertilized control, with the highest pH, was in the bottom left (Table 1, Fig. 4A). These results show that the bacterial communities were affected by pH alteration due to long-term fertilization.

3.6. Relationships between bacterial community and environmental variables

According to forward-selection option in CANOCO, soil pH ($F = 2.8$, $P = 0.002$) and NO_3^- concentration ($F = 1.7$, $P = 0.002$) were the two most important contributors to the variation in bacterial communities, and individually accounted for 17.5 and 10.5% of the variation, respectively. All the environmental variables together explained 60.86% of the variation in microbial communities between samples. The order of influence was: pH > NO_3^- concentration > available P > wheat yield > OM > NH_4^+ concentration. Based on this model, a total of 29.11% of the total variance was explained by the first two constrained axes of the RDA: the first axis explaining 18.34% and the second explaining 10.77% (Fig. 4B). The first canonical axis ($F = 1.1$, $P = 0.002$) and the sum of all

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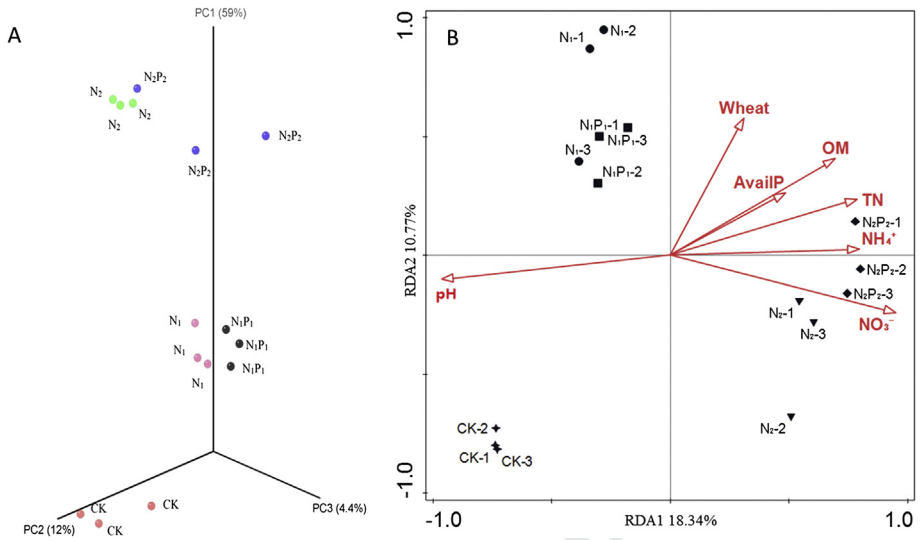

Fig. 4. Principal coordinates analysis (PCoA) and redundancy analysis (RDA). (A) PCoA of the pyrosequencing reads obtained from soils subjected to different fertilization regimes based on the weighted Fast UniFrac metric. The first three axes are drawn and the percent of variance explained by each axis is given. (B) RDA of soil bacterial communities and soil characteristics for individual samples. Soil factors indicated in red text include AvailP (available phosphorus), pH, NH_4^+ (soil concentration of NH_4^+), NO_3^- (soil concentration of NO_3^-), TN (total nitrogen), OM (organic matter) and Wheat (wheat yield).

canonical axes ($F = 1.4$, $P = 0.002$) were significantly different according to the Monte Carlo test. The bacterial communities in unfertilized controls and the low-concentration fertilizer treatments (i.e. N_1 and N_1P_1) were grouped respectively and separately from the high-concentration fertilizer treatments (N_2 and N_2P_2) along the first axis.

Pearson's correlation coefficient was used to evaluate relationships between abundant phyla (i.e. abundance >1%) and environmental factors (Table 3). Most of the abundant phyla were significantly correlated with some of the environmental factors, while only phyla Armatimonadetes, Bacteroidetes and Planctomycetes were not significantly correlated with any of the environmental factors. The abundances of Crenarchaeota, Acidobacteria, Chloroflexi, Nitrospirae and Verrucomicrobia were negatively correlated with soil TN, NO_3^- and NH_4^+, whereas Actinobacteria, Proteobacteria and TM7 were positively correlated. This was opposite to the results for pH: the relative abundances of phyla Crenarchaeota, Acidobacteria, Chloroflexi, Nitrospirae and Verrucomicrobia were positively correlated with soil pH; whereas Actinobacteria, Proteobacteria and TM7 were negatively correlated. Only phylum Acidobacteria was negatively correlated with soil available P. Relative abundances of phyla Proteobacteria, Actinobacteria and TM7 were positively correlated with soil OM; however, those of Crenarchaeota, Acidobacteria and Nitrospirae were negatively correlated with soil OM (Table 3).

Although phylum or class was not correlated with wheat yield, some orders, including Acidimicrobiales and MND1 were negatively correlated with wheat yield, while Sphingomonadales and Legionellales were positively correlated (Table 4).

4. Discussion

4.1. Long-term fertilizer application increased nutrient contents and decreased pH

All fertilizer treatments increased the wheat yield, with the highest increase being observed with N_1P_1; whereas further improvement in yield was not achieved with higher fertilization levels. All fertilizer treatments induced an increase in soil nutrients (i.e. TN, available P, NO_3^-, NH_4^+ and OM), while a decrease in pH was observed, which could acidify the soils (Guo et al., 2010). Specifically, consistent with previous results (Ahn et al., 2012; Körschens et al., 2013; Zhang et al., 2014), there was an

Table 3
Pearson's correlation coefficients between soil characteristics and abundant phyla (relative abundance >1%).

Phylum	pH (1:1H_2O)	Total N (g kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	Available P (mg kg^{-1})	Organic matter (g kg^{-1})	Wheat Yield (kg ha^{-1})
Crenarchaeota	0.806**	−0.697**	−0.891**	−0.595*	−0.417	−0.607*	−0.197
Acidobacteria	0.918**	−0.837**	−0.864**	−0.723**	−0.535*	−0.731**	−0.426
Actinobacteria	−0.897**	0.751**	0.904**	0.725**	0.488	0.669**	0.306
Armatimonadetes	−0.168	−0.107	0.140	0.095	0.229	−0.105	−0.132
Bacteroidetes	−0.479	0.276	0.695**	0.514	−0.062	0.042	−0.301
Chloroflexi	0.841**	−0.635*	−0.921**	−0.789**	−0.164	−0.410	0.016
Gemmatimonadetes	0.572*	−0.373	−0.711**	−0.479	−0.359	−0.299	0.052
Nitrospirae	0.972**	−0.898**	−0.859**	−0.786**	−0.471	−0.786**	−0.500
Planctomycetes	0.178	−0.239	−0.019	−0.022	−0.005	−0.250	−0.311
Proteobacteria	−0.967**	0.875**	0.915**	0.775**	0.437	0.748**	0.377
TM7	−0.921**	0.746**	0.966*	0.841**	0.166	0.562*	0.064
Verrucomicrobia	0.815**	−0.573*	−0.945**	−0.816**	−0.099	−0.377	0.130

**$P < 0.01$, *$P < 0.05$.

accumulation of OM in soils, which could be attributed to elevation of wheat yield that may be passed to soil via wheat residues.

4.2. Long-term fertilizer application decreased bacterial richness and diversity

All fertilizer treatments decreased the biodiversity of agricultural soils and thus concurring with the recent results of Geisseler and Scow (2014) . In particular, Shannon's index was higher in N_1P_1 and N_1 and lower in N_2 and N_2P_2 plots, indicating a stronger influence on microbial diversity of N_2 and N_2P_2 than of N_1 and N_1P_1 treatments. Furthermore, Shannon's index was significantly positively correlated with soil pH and significantly negatively correlated with NO_3^- concentration, indicating that soil pH and NO_3^- concentration may be important factors in decreasing the diversity of bacteria.

4.3. Long-term fertilizer application decreased 16S rRNA gene copy numbers

Shifts in microbial community structure may be explained by the low pH from N- and P-fertilized soils. These results are consistent with previous observations (He et al., 2007; Shen et al., 2010; Jorquera et al., 2014). In this study, soil pH decreased from 6.36 (unfertilized control) to 4.64 after 34 y of N and combined N and P fertilizer application (Table 1). The significant correlation between bacterial abundance and soil pH also indicated that soil pH could have a major effect on the population sizes of bacteria. Recently, it was reported that the abundance of bacteria showed a consistent relationship across a gradient of soil pH (Ahn et al., 2012). These narrow pH optima for bacterial strains would explain the strong relationship between the population sizes of bacteria and soil pH (Rousk et al., 2010).

4.4. Long-term fertilizer application changed bacterial community composition

The relative abundances of phyla Proteobacteria, Acidobacteria and Actinobacteria were greater in all fertilized treatments. As the addition of N and P fertilizers increased, the relative abundance of Acidobacteria declined, while that of Proteobacteria and Actinobacteria increased (Fig. 2). The relative abundance of Acidobacteria was significantly lower in N_2 and N_2P_2 treatments than in unfertilized controls. This was in accordance with results of Stroobants et al. (2014) in which some Acidobacteria subdivisions were negatively or positively correlated with soil pH and from this study; additionally, Chloracidobacteria was positively correlated with this parameter in the present study ($F = 0.975$, $P < 0.01$, Table 4).

Proteobacteria became more dominant in the high-concentration fertilizer treatments (N_2P_2 and N_2). For example, in the N_2 treatment, the relative abundance of Proteobacteria was 35.1%, among them the relative abundance of class Gammaproteobacteria increased by 62.7% compared to the unfertilized controls (Fig. 3B). Previous work also suggested that Gammaproteobacteria were more abundant in long-term fertilized soils compared with controls (Jangid et al., 2008; Campbell et al., 2010).

Our results showed that N and P fertilizer application significantly decreased the relative abundance of the nitrifying community (i.e. Nitrospirae), although its relative abundance was very low (0.47–1.73%) (Table S2 and Fig. 3H). The relative abundance of Nitrospirae was higher in the unfertilized controls (Table 3) and was similar to observations in some previous studies (Ramirez et al., 2010; Wang et al., 2012). The N_2 and N_2P_2 treatments also decreased the relative abundance of Crenarchaeota, which was inconsistent with the recent result that inorganic fertilizer

Table 4
Pearson's correlation coefficients between soil characteristics and abundant classes and orders (relative abundance >1%).

Class	Order	pH (1:1H_2O)	Total N (g kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	Available P (mg kg^{-1})	Organic matter (g kg^{-1})	Wheat yield (kg ha^{-1})
Thaumarchaeota		0.860**	−0.721**	−0.941**	−0.730**	−0.372	−0.495	−0.175
Solibacteres		−0.788**	0.761**	0.706**	0.531*	0.272	0.701**	0.327
Acidobacteria		−0.337	0.331	0.271	0.372	−0.275	0.159	0.006
Chloracidobacteria		0.975**	−0.852**	−0.887**	−0.806**	−0.429	−0.747**	−0.412
Acidimicrobiia		−0.820**	0.793**	0.724**	0.597*	0.161	0.686**	0.299
	Acidimicrobiales	0.885**	−0.873**	−0.770**	−0.636*	−0.542*	−0.751**	−0.581*
Thermoleophilia		0.951**	−0.806**	−0.870**	−0.829**	−0.445	−0.695**	−0.405
Actinobacteria		−0.808**	0.794**	0.898**	0.687**	0.310	0.640*	0.265
Cytophagia		0.458	−0.275	−0.447	−0.486	−0.133	−0.007	−0.011
Sphingobacteriia		−0.859**	0.707**	0.951**	0.773**	0.165	0.569*	0.037
	Sphingomonadales	−0.879**	0.831**	0.739**	0.629*	0.549*	0.718**	0.565*
Ellin6529		0.642**	−0.332	−0.827**	−0.711**	0.033	−0.177	0.333
Gemmatimonadetes		−0.073	0.235	−0.121	0.074	−0.079	0.183	0.356
Nitrospira		0.967**	−0.919**	−0.861**	−0.759**	−0.472	−0.789**	−0.499
Phycisphaerae		−0.255	0.105	0.286	0.097	0.241	0.161	0.072
Planctomycetia		0.688**	−0.743**	−0.574*	−0.607*	−0.26	−0.592*	−0.393
Alphaproteobacteria		−0.948**	0.868**	0.841**	0.810**	0.462	0.638*	0.465
Betaproteobacteria		0.301	−0.126	−0.318	−0.372	−0.314	−0.092	−0.030
	MND1	0.953**	−0.914**	−0.817**	−0.754**	−0.474	−0.771**	−0.565*
Deltaproteobacteria		0.778**	−0.757**	−0.752**	−0.698**	0.098	−0.461	−0.118
Gammaproteobacteria		−0.945**	0.765**	0.976**	0.886**	0.279	0.607*	0.146
	Legionellales	−0.241	0.296	−0.051	0.140	0.530*	0.477	0.614*
Pedosphaerae		0.929**	−0.827**	−0.881**	−0.761**	−0.333	−0.593*	−0.340
Spartobacteria		0.697**	−0.429	−0.882**	−0.726**	−0.001	−0.271	0.288

**$P < 0.01$, *$P < 0.05$.

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increased the relative abundance of Nitrospirae and Crenarchaeota (C.H. Li et al., 2014; Y. Li et al., 2014). Crenarchaeota may be the dominant group of microorganisms governing NH_4^+ oxidation (Leininger et al., 2006) and a massive decline at pH 4.7–5.2 was previously observed (Bengtson et al., 2012). We observed that the N_1 treatment had the highest relative abundance of Crenarchaeota, which indicated that the higher soil NH_4^+ concentration and lower pH resulting from other more concentrated fertilizer treatments (N_1P_1, N_2 and N_2P_2) may inhibit their growth. In summary, 34 y of inorganic fertilizer application significantly decreased the relative abundance of nitrifying archaea and bacteria in this black soil.

In the high-concentration fertilizer treatments (N_2 and N_2P_2), compared to the N_1 and N_1P_1 treatments, the relative abundances of Alphaproteobacteria, Gammaproteobacteria, Solibacteres and Sphingobacteria increased, whereas those of Thaumarchaeota, Chloracidobacteria, Nitrospirae, Pedosphaerae and Thermoleophilia decreased (Fig. 3). This indicated a greater influence of high-concentration fertilizer treatments (N_2 and N_2P_2) on these groups compared to N_1 and N_1P_1. This was consistent with the RDA axis that separated the bacterial communities of lower fertilizer treatments and the unfertilized control from those of higher fertilizer concentrations.

4.5. Relationships between bacterial community and soil properties

The structure of bacterial communities was closely correlated to soil pH ($P < 0.01$), as reported in a number of studies (Shen et al., 2010; Zhao et al., 2014). A recent study of a number of ecosystems showed that pH exerted a strong influence on the composition of soil microbial communities, with soil pH being a good predictor of bacterial community composition (Geisseler and Scow, 2014). Rousk et al. (2010) hypothesized that bacterial communities were directly influenced by soil pH as most bacterial taxa exhibit relatively narrow growth tolerances.

The shifts in the relative abundance of specific taxonomic groups across different pH values are similar to the pH responses observed in other studies. For instance, the relative abundance of Actinobacteria had strong positive (Lauber et al., 2009; Rousk et al., 2010) or negative (Harald et al., 2014) correlations with higher pH. However, Actinobacteria and Proteobacteria demonstrated a negative trend with increasing pH in the present study as also found previously. The relative abundance of Proteobacteria was higher in N_2 and N_2P_2 than other treatments, and this was significantly correlated with the higher concentrations of TN, NH_4^+, NO_3^- and OM ($P < 0.01$) and lower pH ($P < 0.01$) (Tables 3 and S2). In particular, a higher relative abundance of Alphaproteobacteria and Gammaproteobacteria, but a lower Deltaproteobacteria, was observed in N_2 and N_2P_2 treatments (Fig. S1). Alphaproteobacteria and Gammaproteobacteria are considered to be organotrophic and can flourish in soils with large amounts of nutrients, and they utilize reduced inorganic compounds such as ammonia, nitrite or nitrate as energy sources (Ahn et al., 2012). Also, the available nutrients in the N_2 and N_2P_2 treatments were higher than in other fertilizer treatments and in controls. This is consistent with results from a recent study by Zhao et al. (2014), and other reports that the relative abundances of Alphaproteobacteria and Gammaproteobacteria increase with higher soil pH (Chu et al., 2010; Rousk et al., 2010). Thus, we hypothesize that there are other important factors, other than pH, shaping soil bacterial communities. In this study, RDA indicated that the structure of bacterial communities was closely correlated with NO_3^- concentration ($P = 0.006$) and soil pH ($P = 0.0036$). Another relatively abundant phylum was Actinobacteria in N_2 and N_2P_2 treatments (Table 3). The predominant Actinobacteria subgroups were Acidimicrobiia, Thermoleophilia and Actinobacteria (Table 4), which are known to be aerophilic or microaerophilic and are involved in the degradation of OM in soils. The relative abundance of the phylum Verrucomicrobia was high in N_2 and N_2P_2 treatments – this was not in agreement with recent findings indicating a correlation between Verrucomicrobia abundance patterns and conditions of limited nutrient availability in prairie soils (Fierer et al., 2013), because members of the Verrucomicrobia are generally thought to be oligotrophic (Nemergut et al., 2008; Ramirez et al., 2012; Turlapati et al., 2012). The relative abundance of Chloroflexi and Verrucomicrobia was negatively correlated with TN, and the same result was observed at Rothamsted Research in the UK (Zhalnina et al., 2015). The relative abundance of phylum Gemmatimonadetes increased in N-fertilized soils (N_1 and N_2) as previously described for N-fertilized forest soils (Nemergut et al., 2008). However, when combined with P fertilizer (i.e. N_1P_1 and N_2P_2), the relative abundance of Gemmatimonadetes decreased. This change in the relative abundance of Gemmatimonadetes in the N- and P-fertilized soils contradicts the results of Nemergut et al. (2008), indicating that they may be less ecologically consistent in their responses to N-fertilization (DeBruyn et al., 2011).

The relative abundance of orders Acidimicrobiales, Sphingomonadales, MND1 and Legionellales were significantly correlated with wheat yield. This study showed higher wheat yields in fertilizer-treated soils than in controls, and may be due to an increase in the relative abundance of Sphingomonadales and Legionellales accompanied by a decrease in Acidimicrobiales and MND1. Sphingomonadales have macromolecule-degrading and phytopathogen-suppressing abilities (Feng et al., 2011), and the presence of glycosphingolipids in their cell envelope is a distinguishing characteristic of many species of Sphingomonadales. This group includes phototrophic organisms (e.g. *Erythrobacter litoralis*) and can derive a significant fraction of their metabolic energy via anaerobic photosynthesis (Gupta and Mok, 2007). The higher wheat yield may be due to an increase in the metabolic energy in the rhizosphere. Based on these results, the order Legionellales may be associated with higher yields, and be beneficial due to ammonium assimilation (Vishnivetskaya et al., 2013). Acidimicrobiales members are considered responsive to changes in soil pH (Lauber et al., 2009), and their relative abundance increased with pH ($R^2 = 0.885$, $P < 0.05$; Table 4). Recently, Nelson et al. (2014) established associations of the relative abundance of Acidimicrobiales with elevated phytoplankton biomass and productivity in oceanic surveys. However, this was not consistent with results of the present study – the negative correlation of Acidimicrobiales with wheat yield was probably due to the differences between the ocean and soil environments. Originally MND1 was first detected in ferromanganous-coated sediment (Stein et al., 2001; Joseph et al., 2003) but showed no preferential distribution towards either aerobic or anaerobic environments (Spain et al., 2010). However, there is no report available concerning any association between MND1 and crop yield. The results provide further evidence on species-specific associations between wheat yield and heterotrophic bacteria which will be useful for understanding the interactions between wheat and their associated bacteria. The relative abundances of orders Acidimicrobiales, Sphingomonadales and MND1 were significantly correlated with soil properties (pH, TN, NO_3^- and NH_4^+; Table 4). These results showed significant correlations between the changes in soil properties, bacteria types and crop yield as a result of fertilization. Limited data are available on correlations between crop yield and specific microbes.

5. Conclusion

In the present study, soil properties, bacterial community abundance and composition were significantly affected by long-

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term fertilizer application; and differences in soil microbial community structure between fertilizer regimes were clearly distinguished. The change in soil pH due to continuous application of N alone and N plus P fertilizer appeared to be an important factor for bacterial community size and structure. In addition to pH, the NO_3^- concentration also appeared to alter community composition. Our findings suggested that long-term inorganic fertilizer regimes reduced the biodiversity and abundance of bacteria. The influence of high-concentration fertilizer treatments was stronger than that of lower concentrations. These results provide important insight into the microbial community structure in this distinct ecosystem and identified the major factors shaping the microbial community.

Uncited reference

Q6 Lin et al., 2012, Liu et al., 2012.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.07.005.

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