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Effect of long-term mineral fertilizer application on soil enzyme activities and bacterial community composition

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ABSTRACT

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Soil bacteria are critical to maintain soil fertility. In this study, soil chemical properties, enzyme activities and soil bacterial community from a long-term fertilizer experiment (37 years) were analysed to elaborate the effects of long-term mineral fertilizer application on soil enzyme activities and bacterial community composition. Compared with control treatment, bacterial community richness was reduced in low nitrogen (N) fertilizer and high N fertilizer treatments and increased in high N fertilizer and phosphorus (P), high N fertilizer and potassium (K) (N2K), and high N fertilizer, P and K (N2PK) treatments. The distribution of each phylum and genera was obviously changed and the range of the dominant phyla was not affected in all fertilization treatments. Principal component analysis showed that soil bacterial community in the N2K treatment was clearly different than in the N2PK treatment. The N2PK treatment had much higher available P, total organic carbon, invertase, urease and phosphatase activities than the N2K treatment, which might change soil bacterial community composition. In conclusion, fertilization with combined application of P, K and N in appropriate proportions is an optimum approach for improving soil quality and soil bacterial community abundance in non-calcareous fluro-aquic soils in the North China Plain.

Keywords: soil bacterial diversity; soil ecosystem; taxonomic coverage; 16S rRNA sequencing; macronutrient

Soil bacteria are critical to maintain soil fertility and soil ecosystem function and are often sensitive to mineral fertilizer inputs (Ramirez et al. 2012). Many fertilization experiments showed that application of nitrogen (N) fertilizer had a significant influence on the soil bacterial community. However, the effect of N fertilizer inputs on soil bacterial community varied considerably. Nitrogen fertilizer application clearly increased soil bacterial biomass (Li et al. 2012) and the ratio of fungal bacterial biomass (Yevdokimov et al. 2012) reduced soil bacterial community abundance and changed the bacterial community composition (Leff et al. 2015). However, Börjesson et al. (2012) found that soil bacterial community in soils with N fertilizer inputs was not significantly different from that in the unfertilized soils. These different responses might be contributed by management and environmental factors, such as fertilizer combination, soil nutrition content and climate (Böhme et al. 2005).

Although N fertilizer is critical to crop yield, phosphorus (P) and potassium (K) fertilizers are also indispensable. When compared with N fertilizer inputs, investigations of the responses of soil bacterial communities to P and K fertilizer inputs remain limited. Leff et al. (2015) showed that P addition led to a small increase (0.5%) in

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bacterial diversity, but significantly affected bacterial community composition. It is unclear how soil bacterial community respond to long-term P, K and N fertilizer application in non-calcareous fluro-aquic soil. In this study, a high-throughput 16S rRNA sequencing technology was used to investigate soil bacterial community development of different mineral fertilizer treatments. The study aimed at understanding the effects of long-term N fertilizer application alone and the combined application of P, K and N fertilizers on soil bacterial community.

MATERIAL AND METHODS

Experimental site. The experimental field was set at the Laiyang long-term Ecological Research Station in Yantai, Shandong province, Northern China. This long-term fertilization experiment was initiated in 1978. According to the USDA Soil Taxonomy system, the site is a typical, non-calcareous fluro-acquic soil (Soil Survey Staff 1998). At the start of the experiment, the topsoil (0–20 cm) had a pH_{H_2O} of 6.8, 0.50 g/kg total N, 15.0 mg/kg of available P, 38.0 mg/kg of available K and 2.4 g/kg total organic carbon.

Experimental design. This experiment used summer maize and winter wheat rotations, and adopted a completely randomized design with three replicates. The total area of each replicate plot was 33.3 m^2. Twelve treatments were set at the start of the experiment and six treatments were selected for this study. Six treatments are: CK – soil without fertilizer; N1 – low N fertilizer; N2 – high N fertilizer; N2P – N2 fertilizer and P; N2K – N2 fertilizer and K; N2PK – N2 fertilizer, P and K. 138 kg N/ha and 276 kg N/ha was applied in N1 and N2 treatments, respectively. 39 kg P/ha and 112 kg K/ha was applied in the N2PK treatment.

Soil sampling, soil property and enzyme activity measurements. Soil samples (0–20 cm) were randomly taken in each plot after the maize harvest in September 2014. Five soil cores from each treatment were combined which was homogeneously passed through a 2 mm screen. One part of soil samples were air-dried to determine soil total organic carbon, pH alkali-hydrolyzable N, available K and P as described by Chen et al. (2017). The other parts were used for enzyme activity and microbe analysis. Soil invertase, urease, catalase and phosphatase activities were measured as described by Li et al. (2008).

Isolation of DNA from soil. Fifteen soil cores from three replicates of each treatment were combined as a composite sample and used for analysis of microbial characteristics. The cetyl trimethyl ammonium bromide (CTAB) method was used to extract total metagenomic DNA from samples (Hess et al. 2011).

16S rDNA bacterial sequencing and data processing. The 16S rDNA bacterial sequencing and data processing were the same as described by Chen et al. (2017). A ribosomal database project classifier was assigned to taxonomic data for each representative sequence (12000) (Caporaso et al. 2011).

Statistical analysis. ANOVA was used to analyse the data of enzyme activities, nutrient concentrations and yield among different treatments and compared the differences by least-significant difference tests ($P < 0.05$) using the SPSS Statistics 18 (Armonk, New York, USA). The Uparse Software (San Francisco, USA) was used to perform unit-based operational taxonomic comparisons (Edgar 2013) and Microsoft Excel 2010 (Armonk, New York, USA) and Canoco 4.5 (Ithaca, New York, USA) were used to create histogram and PCA, respectively.

RESULTS AND DISCUSSION

Soil nutrient concentrations, enzyme activities and maize yield. Soil alkali-hydrolyzable N and total organic carbon were significantly increased while compared with CK treatment in the long-term mineral fertilizer application treatments (N1, N2, N2P, N2K and N2PK) (Table 1). Available P was significantly increased in N2P and N2PK treatments and there was no change in N2, N1 and N2K treatments when compared with CK treatment. Available K was significantly increased in N2K and N2PK treatments and significantly decreased in N2, N1 and N2P treatments when compared with CK treatment. These results illustrate that soil available P could be maintained after long-term repeated N and K fertilizer additions while soil available K declined after long-term repeated N and P fertilizer additions. Soil pH declined in the long-term mineral fertilizer application treatments (N1, N2, N2P, N2K and N2PK), especially for the N2PK treatment (Table 1).

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Table 1. Soil chemical properties of different mineral fertilizers treatments

Treatment	Alkali-hydrolyzable N	Available P	Available K	Total organic carbon (g/kg)	pH (1:2.5 g/v)
	(mg/kg)				
CK	49.8^{d}	3.9^{c}	41.2^{c}	4.5^{c}	6.7^{a}
N1	61.3^{c}	4.4^{c}	35.2^{d}	5.4^{b}	6.5^{a}
N2	64.5^{c}	3.9^{c}	33.3^{d}	5.5^{ab}	6.3^{a}
N2P	71.1^{b}	61.2^{a}	34.9^{d}	5.7^{ab}	6.2^{a}
N2K	85.2^{a}	4.2^{c}	66.7^{a}	5.2^{b}	6.5^{a}
N2PK	60.7^{c}	51.4^{b}	52.4^{b}	6.1^{a}	6.1^{a}

Three replicates of soil were used for each treatment. Numbers followed by different letters indicate significant differences among different treatments ($P < 0.05$). CK – soil without fertilizer; N1 – 138 kg N/ha; N2 – 276 kg N/ha; N2P – 276 kg N/ha and 39 kg P/ha; N2K – 276 kg N/ha and 112 kg K/ha; N2PK – 276 kg N/ha, 39 kg P/ha and 112 kg K/ha

Soil fertility is largely affected by soil enzymes. Urease plays an important role in soil quality (Sun et al. 2014). In the present study, long-term mineral fertilizer application significantly increased urease activity, invertase activity and phosphatase activity (Table 2). No significant differences were obtained for catalase activity between CK treatment and fertilization treatments (Table 2). Compared with CK treatment, maize yield was significantly increased by 232, 235, 297, 241, and 378% in the N1, N2, N2P, N2K and N2PK treatments, respectively (Table 2). This result might be partly contributed by the increase of soil total organic carbon, urease activity, invertase activity and phosphatase activity (Table 3). The increase of yield conversely facilitated the abundance of plant residues returned to the soil, resulting in increasing soil total organic carbon (Geisseler and Scow 2014) and improving soil enzyme activity to reduce the soil C/N ratio (Kieft et al. 1994), through decomposing abundant plant residues contributed by the higher matter accumulated in the long-term mineral fertilizer application treatments (Table 2).

Richness. For each treatment, more than 12000 valid reads were got using a sequence optimization process (Figure 1). Taking CK treatment as a benchmark, more than 70 and 160 estimated operational taxonomic unit richness (OTUs) were reduced in the N1 and N2 treatment, which was consistent with Lu et al. (2011) who suggested that increasing N inputs suppressed soil community richness. However, more than 40 additional OTUs were increased in the N2P, N2K, N2PK treatments (Table 4). This indicated that P and K additions might change the soil bacterial community composition in the opposite direction to

Table 2. Soil enzyme activity and maize yield of different treatments

Treatment	Invertase (glucose, mg/g, 24 h, 37°C)	Urease (NH_3-N, mg/g, 24 h, 37°C)	Catalase (hydrogen peroxide, mg/g, 20 min, 37°C)	Phosphatase (phenol, mg/g, 24 h, 37°C)	Yield (kg/ha)
CK	0.82^{d}	4.56^{d}	0.65^{a}	36.81^{c}	2110^{d}
N1	0.98^{c}	8.87^{a}	0.68^{a}	38.33^{b}	7014^{c}
N2	1.12^{b}	5.86^{c}	0.63^{a}	38.70^{b}	7064^{c}
N2P	1.28^{a}	7.45^{b}	0.64^{a}	39.02^{b}	8377^{b}
N2K	1.16^{b}	8.03^{a}	0.68^{a}	39.15^{b}	7195^{c}
N2PK	1.30^{a}	8.33^{a}	0.70^{a}	41.61^{a}	10 086^{a}

Three replicates of soil were used for each treatment. Numbers followed by different letters indicate significant differences among different treatments ($P < 0.05$). CK – soil without fertilizer; N1 – 138 kg N/ha; N2 – 276 kg N/ha; N2P – 276 kg N/ha and 39 kg P/ha; N2K – 276 kg N/ha and 112 kg K/ha; N2PK – 276 kg N/ha, 39 kg P/ha and 112 kg K/ha

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Table 3. Correlations between soil nutrient concentrations, enzyme activities and maize yield

	Linear equation	Correlation coefficients (R^2-value)
Alkali-hydrolyzable nitrogen	$y = 103.7x + 188.67$	0.2151
Available phosphorus	$y = 62.006x + 5641.2$	0.4002
Available potassium	$y = 35.345x + 5420.9$	0.0307
Total organic carbon	$y = 4820.6x - 19\,057$	0.9461**
pH	$y = -10696x + 75\,253$	0.8033**
Invertase	$y = 13\,328x - 7819.7$	0.8467**
Urease	$y = 1239.8x - 1931.8$	0.5902*
Catalase	$y = 39\,529x - 19\,246$	0.1649
Phosphatase	$y = 1546.4 - 53\,236$	0.8211**

*$P < 0.05$; **$P < 0.01$

N addition. Chao1 was higher in CK treatment than in N2 and N1 treatments, but lower than in N2P, N2K and N2PK treatments, indicating that the bacterial community richness was reduced in the N1 and N2 treatments and increased in the N2P, N2K and N2PK treatments. Taking CK treatment as a benchmark, the Shannon indices showed no change in different fertilization treatments, illustrating that long-term different mineral fertilizer application did not influence the bacterial community diversity. For the rank abundance curve, no difference was found between CK and fertilized treatments (Figure 2), which indicated that OTU abundance was approximately equally distributed among community members.

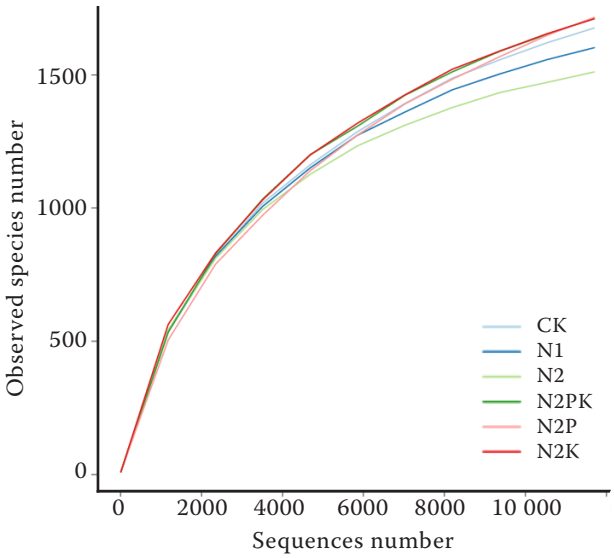

Figure 1. Rarefaction for species-abundance data. CK – soil without fertilizer; N1 – 138 kg N/ha; N2 – 276 kg N/ha; N2P – 276 kg N/ha and 39 kg P/ha; N2K – 276 kg N/ha and 112 kg K/ha; N2PK – 276 kg N/ha, 39 kg P/ha and 112 kg K/ha

Taxonomic coverage. The sequences were classified into 10 phyla using a mothur program. The 10 phyla represented in the sequences did not change (Figure 3), the two dominant phyla were *Proteobacteria* and *Acidobacteria*, which accounted for more than 50% of the species. In each treatment, it was indicated that N, P and K fertilizers have similar effects at the phylum level on soil bacterial community composition (Mazzola and Strauss 2013). However, the distribution of each phylum varied in the different fertilization treatments (Figure 3). When compared with CK treatment (4% *Actinobacteria*, 4% *Chloroflexi*, 2% *Verrucomicrobia*, 2% *Planctomycetes*, 11% *Crenarchaeota* and 5% *Nitrospirae*), the long-term fertilized treatments increased the percentage of *Actinobacteria* (N1 by 1%, N2 by 3%, N2K by 4% and N2PK by 2%), *Chloroflexi* (N2 by 1%, N2P by 4%, N2K by 2% and N2PK by 2%), *Verrucomicrobia*

Table 4. Comparison of the estimated operational taxonomic unit (OTU) richness and diversity indexes of the 16S rRNA gene libraries for clustering at 97% identity as obtained from the Illumina MiSeq (250) analysis

Treatment	Observed OTUs	Shannon	Chao1
CK	1676	8.91	2067.5
N1	1602	9.05	1825.3
N2	1511	9.01	1685.0
N2P	1716	9.14	2397.8
N2K	1710	9.27	2098.0
N2PK	1713	9.01	2118.8

CK – soil without fertilizer; N1 – 138 kg N/ha; N2 – 276 kg N/ha; N2P – 276 kg N/ha and 39 kg P/ha; N2K – 276 kg N/ha and 112 kg K/ha; N2PK – 276 kg N/ha, 39 kg P/ha and 112 kg K/ha

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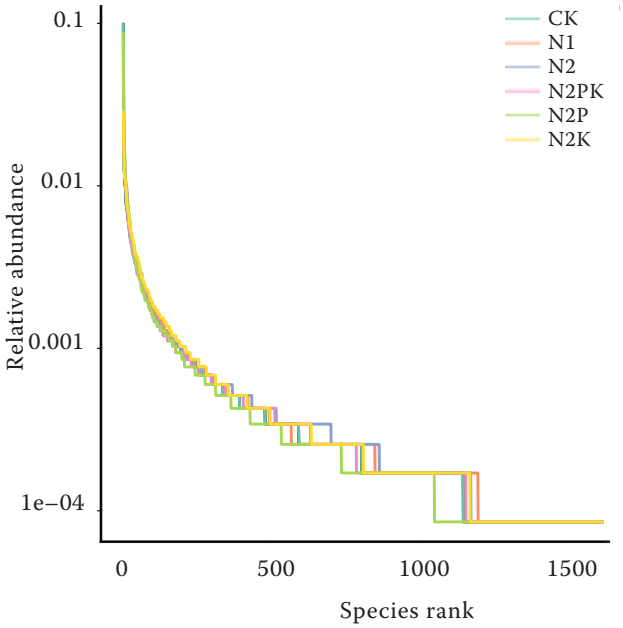

Figure 2. Rank abundance curve of different treatments. CK – soil without fertilizer; N1 – 138 kg N/ha; N2 – 276 kg N/ha; N2P – 276 kg N/ha and 39 kg P/ha; N2K – 276 kg N/ha and 112 kg K/ha; N2PK – 276 kg N/ha, 39 kg P/ha and 112 kg K/ha

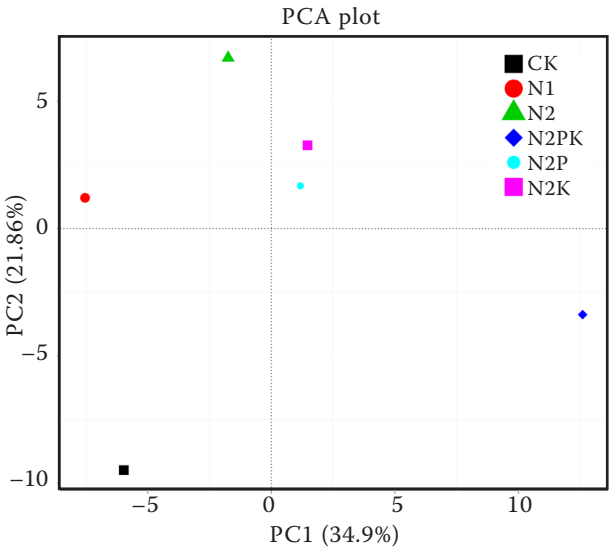

Figure 4. Principal component analysis (PCA) on the relative abundance of bacterial genera. CK – soil without fertilizer; N1 – 138 kg N/ha; N2 – 276 kg N/ha; N2P – 276 kg N/ha and 39 kg P/ha; N2K – 276 kg N/ha and 112 kg K/ha; N2PK – 276 kg N/ha, 39 kg P/ha and 112 kg K/ha

(N2 by 1%, N2P by 3%, N2K by 5% and N2PK by 3%), and *Planctomycetes* (N2P by 2%, N2K by 4% and N2PK by 1%), but reduced the percentage of *Crenarchaeota* (N1 by 7%, N2 by 9%, N2P by 10%, N2K by 9% and N2PK by 9%), and *Nitrospirae* (N2 by 2%, N2P by 3%, N2K by 4% and N2PK by 2%). There was no obvious change in the percentage of *Actinobacteria* in N2P treatment, and in the percentage of *Chloroflexi* and *Verrucomicrobia* in N1 treatment. More unclassified species were detected in N2K treatment than in other fertilized treatments (Figure 3). These taxonomic shifts

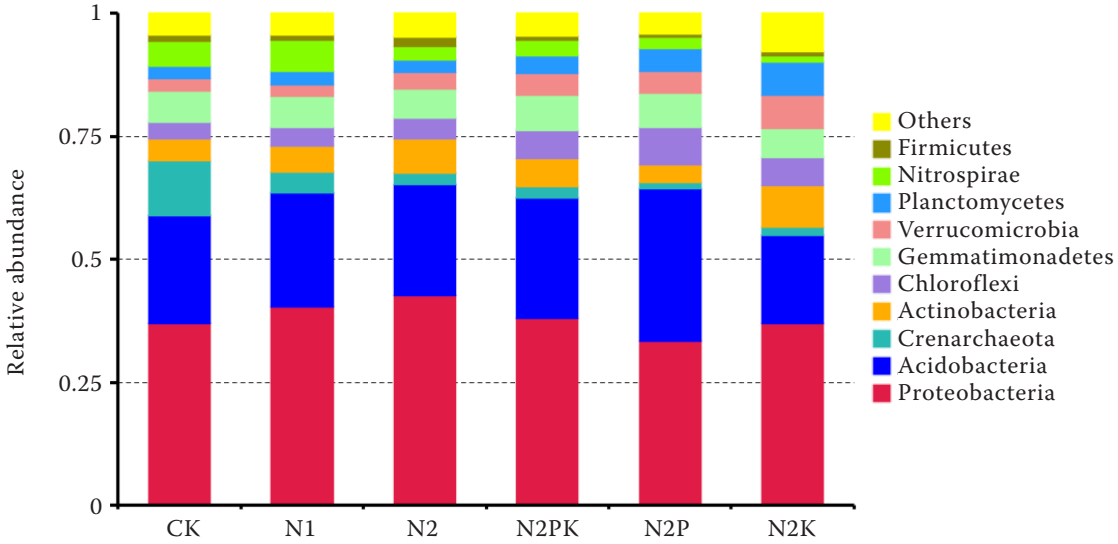

Figure 3. Comparison of the bacterial communities at the phylum level. Relative read abundance of different bacterial phyla within different communities. Sequences that could not be classified into any known group were labelled 'Others'. CK – soil without fertilizer; N1 – 138 kg N/ha; N2 – 276 kg N/ha; N2P – 276 kg N/ha and 39 kg P/ha; N2K – 276 kg N/ha and 112 kg K/ha; N2PK – 276 kg N/ha, 39 kg P/ha and 112 kg K/ha

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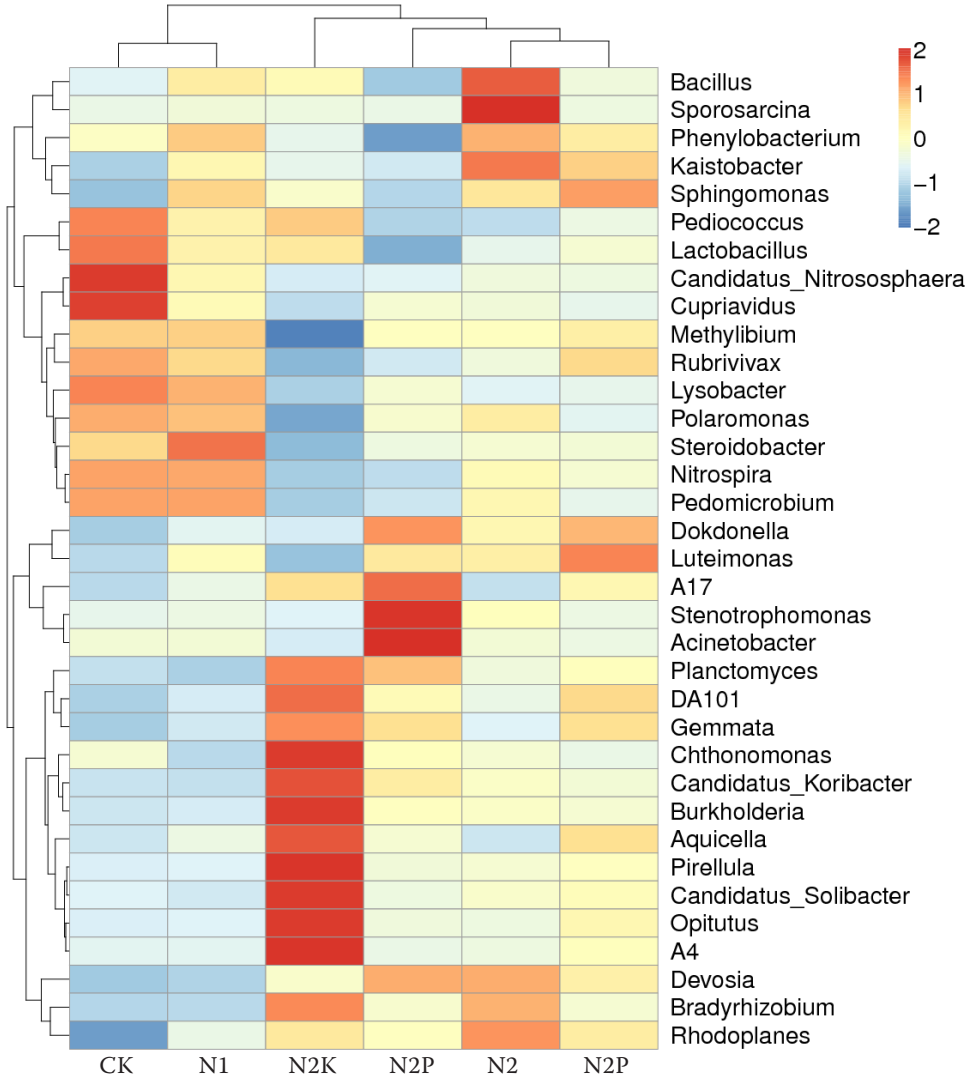

Figure 5. Relative abundance of top 35 genera in different treatments. CK – soil without fertilizer; N1 – 138 kg N/ha; N2 – 276 kg N/ha; N2P – 276 kg N/ha and 39 kg P/ha; N2K – 276 kg N/ha and 112 kg K/ha; N2PK – 276 kg N/ha, 39 kg P/ha and 112 kg K/ha

showed different trends in different fertilization treatments, which suggested that various long-term combinations of N, P and K fertilizers can stimulate different bacterial species and make them the main species (Teng et al. 2009, Leff et al. 2015).

Principal component analysis (PCA) was performed using the Canoco 4.5 on the relative abundance of bacterial genera among different fertilization treatments (Figure 4). A 2D plot was used to present the data, which showed that principal components 1 and 2 accounted for 35% and 22% of the total variation, respectively. Obvious differences were found in PC1 and PC2 among the 6 treatments. Higher similarity was found in the relative abundance of bacterial genera in N2P and N2PK treatments than in any of the other treatments. There was a distinct difference between the N2K and the two P fertilizer-containing treatments. *Chthonomonas*, *Burkholderia*, *Pirellula*, *Candidatus_Solibacter*, *Opitutus* and *A4* became the main genera in N2K treatment, while *Luteimonas* became the main genera in N2PK treatment (Figure 5). *Chthonomonas*, *Burkholderia*, *Pirellula*, *Candidatus_Solibacter*, *Opitutus* and *A4* belong to gram-negative bacteria (Palleroni and Bradbury 1993), and sensitive to nutrient deficiency conditions (Buyer et al. 2010), which might be stimulated by P deficiency in N2K treatment and made themselves the main species (Table 1, Figure 5). *Luteimonas* also belong to gram-negative group and is an important contributor to

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plant rhizosphere activities, since it is particularly abundant within fertilized soils (Eichorst et al. 2007), such as in N2PK treatment in this study. This suggested that P fertilizer had a substantial effect on the relative abundance of bacterial genera.

In conclusion, soil fertility and enzyme activity were all improved by long-term mineral fertilizer. The distribution of each phylum was changed, but the dominant range of phyla was not affected by long-term mineral fertilizer application. The bacterial community richness was reduced by N fertilizer application alone and increased by combined N, P and K fertilizer application. Soil bacteria communities were clearly affected by P fertilizer application in this long-term experimental soil. The response of soil bacterial communities in N2K and N2PK treatments were different, leading to distinct differences in soil bacterial community structures between N2K and N2PK treatments.

Compared with other treatment, fertilization with combined application of P, K and N in appropriate proportions is the optimum approach for improving soil quality, soil bacterial community abundance and maize yield in non-calcareous fluro-acquic soil.

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