Effect of Long-Term Application of Chemical Fertilizers on Microbial Biomass and Functional Diversity of a Black Soil^{*1}

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ABSTRACT

An experiment with seven N, P, K-fertilizer treatments, *i.e.*, control (no fertilizer), NP, NK, PK, NPK, NP₂K, and NPK₂ where P₂ and K₂ indicate double amounts of P and K fertilizers respectively, was conducted to examine the effect of long-term continuous application of chemical fertilizers on microbial biomass and functional diversity of a black soil (Udoll in the USDA Soil Taxonomy) in Northeast China. The soil microbial biomass C ranged between 94 and 145 mg kg⁻¹, with the NK treatment showing a lower biomass; the functional diversity of soil microbial community ranged from 4.13 to 4.25, with an increasing tendency from control to double-fertilizer treatments, and to triple-fertilizer treatments. The soil microbial biomass, and the microbial functional diversity and evenness did not show any significant differences among the different fertilizer treatments including control, suggesting that the long-term application of chemical fertilization would not result in significant changes in the microbial characteristics of the black soil.

Key Words: Biolog, black soil, chemical fertilizer, microbial biomass, microbial functional diversity

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INTRODUCTION

The black soil zone in Northeast China extends to about seven degrees of latitude (43–49° N) and three degrees of longitude (125–127° E). The black soil, equivalent to Udoll in the USDA Soil Taxonomy (Soil Survey Staff, 1999), has high fertility and superior physical and chemical characteristics and is one of the most fertile soils in China. As one of the most important regions for cereal grain production in China, the sustainable management of the black soil resource has received considerable attention in recent years. The major problems of the black soil are soil fertility deterioration and soil erosion. These have hampered agricultural development and sustainability in the region (Fan *et al.*, 2004). A long-term experiment from the Hailun Agricultural and Ecological Station of the Chinese Academy of Sciences, located in Hailun, Heilongjiang Province of Northeast China, has shown that the nitrogen (N) and phosphorus (P) supplying capacity of the soil declined when N and P fertilizers were not used for the period of 13 years, whereas the grain yield increased significantly with the application of the fertilizers (Wang *et al.*, 2003; Han *et al.*, 2005). The application of chemical fertilizers is becoming a common practice in the black soil region to increase grain yield and maintain soil fertility.

Soil microorganisms play important roles in ecological functions of soils, such as nutrient cycling

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and degradation of organic pollutants. Microbial parameters (*e.g.*, microbial biomass and functional diversity) are considered to be potential indicators of the soil quality (Bending *et al.*, 2004). Soil microorganisms and the processes that they govern are thus considered to be essential for sustainable agricultural production (Wardle *et al.*, 1999). Soil microbial community and functions can be affected by an array of factors, such as organic matter quality, nutrient input, soil types, vegetation, management practices, and soil contamination (Bending *et al.*, 2002; Johnson *et al.*, 2003; Renella *et al.*, 2004). The effect of soil nutrient management on microbial functional diversity has been the subject of various studies (Bulluck III *et al.*, 2002; He *et al.*, 2006). In a long-term field experiment, Mäder *et al.* (2002) showed that soils under organic farming regime had higher microbial functional diversity than those under conventional farming system. Soil amendments of crop residues or other organic materials were shown to affect the functional diversity of soil microbial community (Bending *et al.*, 2002). However, few studies are available on how different chemical fertilizers will influence the soil microbial characteristics in the black soil.

In a long-term experiment site in the black soil region of Northeast China, the soil nutrient contents, the microbial biomass, and the functional diversity under different chemical fertilizing treatments were measured to examine the impact of different fertilizing treatments on the soil microbial characteristics.

MATERIALS AND METHODS

Experimental site and design

A long-term field experiment was established at Hailun Agricultural and Ecological Station of the Chinese Academy of Sciences in Northeast China (47° 26' N and 126° 38' E) in 1990. In the experimental station area, the average annual rainfall is 550 mm with more than 80% precipitating from mid-June to late September; the average temperature is the lowest (−18 °C) in January and the highest (23 °C) in July; the annual soil frost period is 160 days. The climate is suitable for one crop per year and the rotation sequence is wheat-maize-soybean in three years. The soil is a clay loam with pH 7.0 and an organic C content of 28 g kg^{−1}.

The experimental design was a randomized block with four replicates per treatment, including seven different N, P, K-fertilizer treatments, *i.e.*, control (no fertilizer), NP, NK, PK, NPK, NP₂K, and NPK₂, where P₂ and K₂ represent double amounts of P and K fertilizers, respectively. The application rates of fertilizers were: N 112.5 kg ha^{−1} for maize and wheat, and 13.5 kg ha^{−1} for soybean; P (P₂O₅) 45.0 kg ha^{−1} for maize and wheat, and 34.5 kg ha^{−1} for soybean, and K (K₂O) 60.0 kg ha^{−1} for all the three crops. Each plot was 15 m × 4.2 m and the plots were separated from each other by concrete sunk to a depth of 0.2 m.

Seven soil cores (2.0 cm in diameter and 20 cm in depth), which were subsequently bulked, were randomly taken from each plot in early August, 2003. All soil samples were stored in an icebox and transferred to the laboratory for further analyses. One half of each sample was air-dried and passed through a 2-mm sieve for chemical analyses. Another half of each fresh sample was kept at 4 °C for the analyses of the microbial characteristics.

Soil chemical parameters

Soil available P was extracted with 0.5 mol L^{−1} NaHCO₃ (pH 8.5) at a solution to soil ratio of 20:1 for 30 min, and determined by the ascorbic acid method at 880 nm absorbance (Lu, 1999). The available K was extracted by NH₄OAc (pH 7.0) and determined by atomic absorption spectrometry. The total organic carbon was measured using the acid dichromate method (Lu, 1999). Available N was measured according to the method of Lu (1999). Briefly, the air-dried soil samples with 1 mol L^{−1} NaOH were incubated in a sealed glass container at 40 °C for 24 h, NH₃ volatilized from the soil was trapped by H₃BO₃ (pH 4.5), and the NH₄⁺ in the H₃BO₃ was then determined by back-titration with standard H₂SO₄ solution.

Soil microbial biomass C

Microbial biomass C was measured by the fumigation-extraction method (Wu *et al.*, 1990). Briefly, the fresh soil samples were carefully dried to 60% of water holding capacity in the dark, passed through a 2-mm sieve, and incubated for two weeks at 25 °C. The moist sub-samples (15 g dry weight equivalent) were fumigated for 24 h with ethanol-free CHCl_3 (Jenkinson and Powlson, 1976). The soluble organic C was then extracted from the fumigated and the unfumigated samples with 60 mL 0.5 mol L⁻¹ K_2SO_4 for 30 min on an end-over-end shaker. The extracts were filtered through 0.45- μm filters and frozen at -20 °C prior to the analysis for organic C with a total organic carbon analyzer (Phoenix-8000, Tekmar-Dohrmann, NY). The microbial biomass C was calculated from the difference between dissolved organic C in the fumigated and the unfumigated samples using a conversion factor of 0.45. All results were expressed on an oven-dried soil basis and were the mean of four replicates.

Microbial functional diversity

The whole microbial community was extracted from the fresh soil samples and inoculated into Biolog GN plates (Schutter and Dick, 2001). Triplicate 10 g fresh samples were suspended in 100 mL sterile saline solution (9 g kg⁻¹ NaCl) with 5 g of 3 mm glass beads on a rotary shaker at 300 r min⁻¹ for 10 min at 25 °C. The suspensions were allowed to settle for 10 min before 10-fold diluted samples were prepared. 150 μL aliquots of dilutions were added to each plate well. The absorbance of the wells at 590 nm was read using an automated Biolog MicroplateTM Reader (Biolog, Hayward, CA) and data were collected using the Microlog 4.01 software. The plates were then sealed inside a plastic bag and incubated at 25 °C in darkness, and read every 24 h over 7 days.

Data analyses

The Biolog plate well absorbance values were adjusted by subtracting the absorbance of the control well, which did not contain a substrate. The average well color development (AWCD), calculated as the average optical density across all wells per plate, was used as an indicator of general microbial activity (Garland and Mills, 1991; Garland, 1997). The microbial functional diversity (H') was calculated as the Shannon index, $H' = -\sum P_i \ln P_i$, where P_i was calculated by subtracting the blank from each substrate absorbance and then dividing this value by the total color change recorded for all 95 substrates. The microbial evenness (E) was calculated as $E = H' / \ln R$, where R was richness, which referred to the number of substrates used (Zak *et al.*, 1994; Garland, 1997). The substrates were divided into six categories according to their chemical nature, *i.e.*, carbohydrates, carboxylic acids, amines and amides, amino acids, polymers, and miscellaneous (Preston-Matham *et al.*, 2002). Shannon diversity and evenness were determined by ANOVA with the SPSS software (SPSS Inc., Chicago, IL). Biolog data were interpreted by principal component analysis (PCA).

RESULTS

Soil nutrients under different fertilizing treatments

The soil organic matter C contents ranged between 23.8 and 29.9 g kg⁻¹ (Table I). The NPK₂ treatment showed the highest soil organic matter C, and the PK treatment showed the lowest. Compared to the initial result in 1990, the soil organic C of the PK treatment decreased by 15% and that of control by 10%. N-fertilizer application showed positive effects on maintaining and even increasing the soil organic matter content. Fertilizing also affected the soil available-N contents. The PK and NPK₂ treatments had the highest available-N contents (65.7 mg kg⁻¹), followed by the NPK, NK, and NP₂K treatments, and then by the NP and control treatments (57.8 mg kg⁻¹). All treatments with P-fertilizer showed a higher soil available P content than those without P. Compared to the initial available P in

1990, the P application was able to maintain or even increase the soil available P, but the control and NK treatments significantly decreased the available P in soil. On the other hand, soil available K did not show any significant differences among the treatments. These results implicated that N and P application for the black soil was essential to maintain soil available nutrients and organic matter contents and thus soil fertility and productivity.

TABLE I

Soil nutrient contents under different fertilizing treatments after a long-term field experiment

Year	Treatment ^{a)}	Organic C	Available N	Available P	Available K
		g kg ⁻¹		mg kg ⁻¹	
1990		28.0	0.0	17.9	190.8
2003	Control	25.2bc ^{b)}	57.8c	13.8bc	220.6a
	NK	26.4bc	62.8bc	11.2c	196.3a
	NP	27.0bc	57.8c	17.6b	208.7a
	PK	23.8c	65.8ab	23.2a	219.8a
	NPK	28.2ab	63.0bc	16.3b	216.1a
	NP ₂ K	27.4ab	60.9bc	23.3a	214.9a
	NPK ₂	29.9a	65.6ab	16.1b	215.3a

^{a)} N, P, and K represent N, P, and K fertilizers, respectively. P₂ and K₂ indicate double application amounts of P and K fertilizers.

^{b)} Means followed by the same letter(s) in a column are not significantly different at $P < 0.05$ ($n = 4$).

Microbial biomass C under different fertilizing treatments

As seen in Fig. 1, the soil microbial biomass C ranged between 94 and 145 mg kg⁻¹. The NK treatment showed a lower biomass than the other treatments, probably due to its lowest available P content. However, the variation among its four replicates was high (107.5 ± 14.2 mg kg⁻¹). Statistically, there was no significant difference in the microbial biomass C among the different fertilizing treatments.

Fig. 1 Soil microbial biomass carbon under different N, P, K-fertilizer treatments. See Table I for the descriptions of NK, PK, NP, NPK, NP₂K, and NPK₂. Vertical bars show the standard error of the mean ($n = 4$).

Microbial functional diversity under different fertilizing treatments

Shannon diversity and evenness indices are shown in Fig. 2. The diversity ranged from 4.13 to 4.25. The Shannon diversity tended to increase from control to NK, NP, and PK double-fertilizer treatments, and to NPK, NP₂K, and NPK₂ triple-fertilizer treatments. The triple-fertilizer application tended to promote the microbial diversity. For the soil microbial evenness, there was a similar tendency with the diversity, with the control lowest of 0.915 and the NPK treatment highest of 0.945.

As a general indicator of the microbial activity, AWCD changes with incubation time are shown in Fig. 3. Obviously, the microbial activity tended to increase with incubation time during the 7-day

Fig. 2 Soil microbial functional diversity and evenness. Vertical bars show the standard error of the mean ($n = 4$). See Table I for the descriptions of NK, PK, NP, NPK, NP₂K, and NPK₂.

Fig. 3 Average well color development (AWCD) of soil microbial communities with incubation time. See Table I for the descriptions of NK, PK, NP, NPK, NP₂K, and NPK₂.

period. However, the triple-fertilizer treatments usually had higher AWCD values than other treatments, indicating that these three treatments had a higher microbial activity. The AWCD value at 72-h was often used to describe the difference of soil microbial activities among the different treatments. In this experiment, the AWCD value at 72-h increased in the following order for the different treatments: control < NK < PK < NP < NPK < NPK₂ < NP₂K.

The principal component (PC) analysis results (Fig. 4) indicated that PC1 explained 32.5% of the total variance in the 95 carbon source data, mainly due to substrates of amino acids, carboxylic acid, and amine (Table II). PC2 explained 9.1% of the variance by mainly representing carbohydrate, polymer, and miscellaneous substrates. The distribution of the different treatments in the PCA pattern was almost random with considerable variation among their replicates. The tendency of different treatments between PC1 and PC2 was not identified.

DISCUSSION

Microbial biomass and functional diversity in different fertilizer treatments did not show significant differences. The lack of significant differences in the microbial properties of the black soil was not surprising owing to the following considerations: (i) the natural fertility of the black soil was relatively high, and the application of chemical fertilizers did not result in any significant change in microbial substrates (*e.g.*, soil organic carbon), which thus did not result in significant changes in microbial biomass

Fig. 4 Ordination of principal component (PC) analysis on 95 substrate utilizations by the soil microbial communities. See Table I for the descriptions of NK, PK, NP, NPK, NP₂K, and NPK₂.

TABLE II

Correlation coefficients between the principal components (PCs) and carbon sources of the Biolog GN microplate

Category of substrates	PC1	PC2
Amino acid	0.833**	0.454*
Carbohydrate	0.333	0.893**
Carboxylic acid	0.763**	0.448*
Polymer	0.434*	0.794**
Amine	0.858**	0.330
Miscellaneous	0.527**	0.738**

*, **Significant at $P < 0.05$ and $P < 0.01$ ($n = 4$), respectively.

and functional diversity; (ii) the experimental period of 13 years was not long enough to show the significant differences in the microbial characteristics although some tendencies were observed, *e.g.*, triple-fertilizer treatments tended to have higher Shannon diversity and evenness than other treatments; (iii) the soil microbial characteristics will be subject to the large spatial and temporal variations, and perhaps more replications and sample frequency will be required to deal with the spatial and temporal variability.

The mechanisms governing soil microbial communities and their ecosystem functions are not well understood (Torsvik *et al.*, 2002). Microbial community composition and diversity in soils are driven by factors such as crop types, soil physicochemical properties, and other environmental disturbances (*e.g.*, heavy metal contamination, fertilization, and tillage) (McCaig *et al.*, 1999, 2001; Nusslein and Tiedje, 1999; Smit *et al.*, 2001; Brodie *et al.*, 2002; Ellis *et al.*, 2003; Johnson *et al.*, 2003; He *et al.*, 2005, 2006). However, there has been no conclusive results regarding the importance of individual factors. McCaig *et al.* (2001) did not detect the differences in bacterial communities in improved and unimproved grassland; Chow *et al.* (2000) reported no significant differences in bacterial communities among several treatments, including whole-tree harvesting with and without heavy soil compaction and whole-tree harvesting with complete surface organic matter removal and heavy soil compaction. Ellis *et al.* (2003) found that the denaturing gradient gel electrophoresis profiles of whole-community and clone libraries of soil samples with patchy metal contamination were very similar to pristine soil.

Black soil has high natural fertility with organic C of 28 g kg⁻¹ in the control treatment. After 13 years crop production, the organic C decreased by 10% as compared to the initial value (Table I). This is a relatively short time span. Yu *et al.* (2004) studied the organic C changes of the cultivated black soils for the period of the past two hundred years and found that the organic C tended to decrease with increasing tillage years. However, after 130 years of tillage, the soil organic C remained at a relatively

stable value. Our experimental site has a long cultivation history and the organic C content is in a relatively stable stage. The soil with the different fertilizing treatments was yielding the same crops including soybean in a three-year rotation. Although fertilization had resulted in crop yield increase (Wang *et al.*, 2003), it was not sufficient to trigger a significant change in the soil microbial properties. However, from a longer time view, how different fertilizing treatments affect soil microbial properties and how to use soil microbial indicators to assess soil quality change need to be further studied. In conclusion, 13 years of continuous application of chemical fertilizers did not result in a significant change in the soil microbial biomass and the functional diversity of the black soil.

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