RESEARCH ARTICLE

Thirty-year amendment of horse manure and chemical fertilizer on the availability of micronutrients at the aggregate scale in black soil

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Abstract
Purpose This study evaluates manure and chemical fertilizer effects on micronutrient (Fe, Mn, Cu, and Zn) content and availability in crops.
Methods Seven treatments were selected, including three conventional fertilization treatments (NP, horse manure (M), and NP plus M (NPM)), three corresponding double rate fertilization (N2P2, M2, and N2P2M2), and a CK. Soil samples were collected and separated into four aggregates by wet-sieving in September 2009. Corn samples were collected and analyzed simultaneously.
Results Treatment N2P2 increased DTPA extractable Fe, Mn, and Cu in soil by 732%, 388%, and 42%, whereas M2 decreased the corresponding values by 26%, 22%, and 10%, respectively, compared to CK. DTPA extractable Zn in soil and Zn in corn grain were higher in the M and M2 treatments than in the other treatments, and DTPA Zn was significantly correlated with soil organic carbon (SOC) in large macroaggregate, microaggregate, and silt + clay fractions. The Mn concentrations in corn stalks and grain were significantly correlated with DTPA extractable Mn in bulk soil and microaggregates, and Zn in stalks were significantly correlated with DTPA Zn in bulk soil, microaggregates, and large macroaggregates.
Conclusions Long-term application of horse manure could increase soil Zn availability and uptake by corn, possibly due to its activation by SOC. In contrast, chemical fertilizer application increased DTPA extractable Fe, Mn, and Cu in soil by reducing soil pH. Our results also suggest that Mn uptake by corn originated mainly in microaggregates, whereas Zn in crops was primarily sourced from large macroaggregates and microaggregates.

Keywords Long-term fertilization · Micronutrients · Aggregates · Availability · Plant uptake · Black soil

1 Introduction

Micronutrients are essential for the growth of microorganisms, plants, and animals, but are required in much smaller amounts than primary nutrients (Senesi et al. 1999). Micronutrients in crop grain directly affect food quality and are closely related to human and livestock nutrition. Micronutrient deficiencies can cause anemia, decreased immunity, slow growth, and nyctalopia (Li et al. 2007). Similarly, an excess of micronutrients can have serious effects on plant growth and human health. Excessive agroecosystem inputs of micronutrients such as Fe, Mn, Cu, and Zn, which are heavy metals, can possibly lead to toxicity in plants and animals and consequently pose a threat to human health through the food chain (Westfall et al. 2005; Soriano-Disla et al. 2010).

The availability and behavior of micronutrients in soil are strongly affected by changes in soil characteristics, such as pH, soil organic matter (SOM), and by other nutrients (Li et al. 2007). The application of NH_4^+-forming fertilizer was found to decrease soil pH from 6.8 to 6.0, in turn increasing the

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available Zn concentration in soil solution by 5–15-fold over a 30-day period (Lorenz et al. 1994). Zhang et al. (2001) found that the content of SOM has a significant direct impact on the availability of Fe, Mn, and Zn but not Cu. The application of farmyard manure at a rate of 10 t ha^{-1} year^{-1} for 21 years increased soil organic carbon (SOC) and Zn contents by 28.9% and 46%, respectively, compared to CK (Parat et al. 2005). In addition, the interaction between macronutrients and micronutrients affects the amount of micronutrients taken up by crops (Aulakh and Malhi 2005). For example, phosphorus application can alter the surface properties of soil colloids and increase exchangeable Cu by 65% to 90% (Kaushik et al. 1993), but it was also found to reduce the availability of Zn in soil, possibly due to precipitation of $Zn_3(PO_4)_2$, resulting in a Zn deficiency in corn (Li et al. 2007).

The effect of fertilization and cultivation on the availability of micronutrients in soil has been linked not only to the concentrations of micronutrients in bulk soils but also to their spatial location in soil micro-sites, namely, their distribution in aggregates (Hardy and Cornu 2006; Acosta et al. 2011). Therefore, aggregate-size separation has been widely used to distinguish the active and inert pools of SOM and nutrients (Agbenin and Tiessen 1995; Christensen 2001). The status and behavior of nutrients in soil are strongly affected by the properties and composition of the soil matrix (Zhang et al. 2003). Eary et al. (1990) found that microelements are attached to the smaller particle sizes and exist mainly on soil particle surfaces. Macroaggregates (>250 μm) contain 30% to 45% of the Cu and Zn in soil, whereas the corresponding value in the silt + clay fraction is 13% to 19% (Zhang et al. 2003). Qian et al. (1996) found that 54.1% of DTPA extractable Cu in soil was sourced from the silt fraction (2–50 μm). These data suggest that the availability of microelements in aggregates is highly variable. Up to date, little information is available on the concentration and availability of microelements in aggregates and the relationship with the amount of microelements taken up by crops.

Black soil (Udic Mollisols) is the most fertile and productive soil in China and is mainly distributed in Northeast China, which is one of three major black soil zones in the world. The SOM concentration in intensively cultivated black soils has generally decreased over the last 20 years, mainly due to the removal of crop straw (Xie et al. 2007). As a result, chemical fertilizers have been applied at higher rates in an effort to improve soil productivity. Therefore, we hypothesized that long-term application of different fertilizers may lead to different distribution pattern of micronutrients in soil aggregates, which may influence their uptake by crops. In 1979, a long-term field experiment was established to monitor the influence of chemical fertilizers and horse manure on soil fertility and production. The objectives of this study were (1) to determine the distribution of extractable micronutrients (Fe, Mn, Cu, and Zn) in aggregate fractions and (2) to gain a better understanding of the relationship between crop uptake and extractable concentrations of micronutrients in soil and aggregates.

2 Materials and methods

2.1 Study site and experimental design

The long-term experiment was established at the Harbin Black Soil Agro-Ecological Experimental Station, Harbin, Heilongjiang province, China (45°41′N, 126°37′E). The altitude is 151 m. According to the Thornthwaite classification, the territory has a mesothermal mainland monsoon climate. The 30-year mean annual temperature is 3.5°C, and the mean annual precipitation is 533 mm, 88% of which falls between May and September. There is a frost-free period of about 135 days, and the cumulative temperature (≥10°C) range from 2,400°C to 2,500°C. The experimental field was located on a flat plain that was native prairie before it was cleared for cropping more than 100 years ago. The soil, derived from loamy loess and classified as Udic Mollisol, had a clayey loam texture and a pH of 7.22, and contained 15.5 g organic C kg^{-1}, 1.47 g total N kg^{-1}, 1.07 g total P kg^{-1}, and 25.5 g total K kg^{-1} prior to the start of the experiment.

The field experiment commenced in 1979 with a three-year rotation of spring wheat (*Triticum aestivum* L.), soybean (*Glycine max* L.), and corn (*Zea mays* L.). To evaluate and compare the effect of N plus P fertilizers and horse manure on the availability of micronutrients, seven treatments with three replicates were selected in this study, including three conventional fertilization treatments: fertilizer NP (NP), horse manure (M), and fertilizer NP plus horse manure (NPM); three excessive fertilization treatments: double fertilizer NP (N2P2), double horse manure (M2), and double fertilizer NP plus horse manure (N2P2M2); and a control without fertilization (CK). Each plot had an area of 56 m^2. Horse manure was applied every three years before wheat was sown, whereas fertilizer urea and calcium superphosphate were added every year. The application rate of urea was 75 kg N ha^{-1} for soybean and 150 kg N ha^{-1} for wheat and corn in the NP and NPM treatments; and 150 kg N ha^{-1} for soybean and 300 kg N ha^{-1} for wheat and corn in the N2P2 and N2P2M2 treatments, respectively. The application rate of calcium superphosphate was 65.5 kg P ha^{-1} for soybean and 32.7 kg P ha^{-1} for wheat and corn in the NP and NPM treatments; and 131 kg P ha^{-1} for soybean and 65.5 kg P ha^{-1} for wheat and corn in the N2P2 and N2P2M2 treatments, respectively. The amount of horse manure applied was 18,600 kg ha^{-1} (75 kg N ha^{-1}) in the M and NPM treatments and 37,200 kg ha^{-1} (150 kg N ha^{-1}) in the M2 and N2P2M2 treatments. All fertilizers applied as basal fertilizers were broadcast onto the soil surface and immediately incorporated into the plowed soil (0–20 cm) by hand-hoeing. Crops were

sown in the rows of each plot by hand in April and harvested in September.

2.2 Soil sampling and fractionation

Soil samples were collected in September 2009 after the corn harvest. Soil samples were taken from the 0–20 cm soil layer at 10 different locations in each plot for each treatment, and were carefully mixed to form a composite. The fresh soils were stored at 4°C and rapidly transported to the laboratory for sieving. Aggregate size separation was conducted by a wet-sieving method (Six et al. 1998). One hundred grams of fresh soil samples (on an oven-dried basis) was submerged in deionized water on top of a 2-mm plastic sieve. After 5 min of slaking, the sieve was manually moved 50 times up and down within a distance of 3 cm over a 2-min period. The fraction remaining on the 2-mm sieve was collected, and sieving was repeated for the other fractions. Soils <0.053 mm were centrifuged and collected. All aggregate fractions were oven dried at 60°C, weighed, ground, and homogenized. Thus, four fractions were obtained: large macroaggregates (>2 mm), small macroaggregates (0.25–2 mm), microaggregates (0.053–0.25 mm), and silt + clay fraction (<0.053 mm). The mass recovery ratio of aggregates was 99.0±0.9%.

2.3 Soil analysis

Soil pH was determined in a soil/water ratio of 1:2.5 (v/v). Organic matter concentrations in soils and aggregates were determined by the wet oxidation-redox titration method (Skjemstad and Badlock 2006). Total soil P was determined by the molybdate–ascorbic acid method after concentrated $H_2SO_4/HClO_4$ acid digestion (O'halloran and Cade-Menun 2006). Total micronutrients (Fe, Mn, Cu, and Zn) in soils were determined according to the method of Amacher (1996) after a tri-acid (HF, HNO_3, and $HClO_4$) digestion. The available Fe, Mn, Cu, and Zn in soils and aggregates were extracted with diethylenetriamine pentaacetic acid (DTPA, 0.005 M DTPA, 0.1 M triethanolamine, and 0.01 M $CaCl_2$ at pH 7.3) in a soil/solution ratio of 1:2 (v/v; Lindsay and Norvell 1978). The concentrations of Fe, Mn, Cu, and Zn in the digestion solution and extracts were determined using an IRIS Advantage inductively coupled plasma atomic emission spectrometry (ICP-AES) system (Thermo Scientific Inc., USA). The recovery rate of DTPA extractable microelements in aggregates was 92% to 98% relative to DTPA microelements in bulk soil, and unrecovered microelements were mainly water dissolved microelements.

2.4 Crop sampling and analysis

Crop samples were collected in September 2009 when the corn was harvested. Corn plants were separated into stalks and grain. All plants were rinsed with deionized water and dried at 70°C for 24 h. Dried plants were acid-digested with 5 ml concentrated HNO_3 and 1 ml mixed acid ($HNO_3/HClO_4=$ 87:13, v/v; Zhao et al. 1994). A reagent blank and a standard reference material (Shrub, GBW 07602, National Certified Reference Materials, Beijing) were used to verify the accuracy and precision of the digestion procedure and subsequent analysis. The concentrations of Fe, Mn, Cu, and Zn in digestion solutions were determined using ICP-AES. The standard reference material contained a certified Fe, Mn, Cu, and Zn concentrations of 1,020±67, 58±6, 20.6±2.2, and 5.2± 0.5 mg kg^{-1}, respectively. We obtained 979±116, 54±2, 20.0±2.7, and 5.4±0.7 mg kg^{-1}, respectively.

2.5 Data analysis

Statistically significant differences between treatments were identified using one-way ANOVA and least significant difference calculations at $P<0.05$. Correlation analyses were used to test relationships between micronutrient in crop plants and in soil or aggregates. All statistical analyses were performed with SPSS 13.0 software for Windows (SPSS Inc., Chicago, IL).

3 Results

3.1 Soil characteristics and total and DTPA extractable Fe, Mn, Cu, and Zn

Soil pH, SOC, and total P were significantly different among treatments (Table 1). Soil amended with chemical fertilizer (NP, NPM, N2P2, and N2P2M2) showed significantly lower pH but higher total P concentrations than the unfertilized control (CK) and soil amended with manure alone (M and M2). Compared to CK, the content of SOC increased significantly in all treatments except N2P2.

Manure and chemical fertilizer applications significantly increased soil total Fe and Zn concentrations (Table 1). All fertilizer-added treatments showed higher total Mn and Cu concentrations compared with treatment CK, although the difference was not significant in treatments NPM and M2 for Mn or in treatments NP and M2 for Cu.

Treatments N2P2, N2P2M2, NP, and NPM showed significantly higher DTPA extractable Fe and Mn concentrations than the other treatments. The highest DTPA extractable Cu concentration was found in the N2P2 and N2P2M2 treatments, and the highest DTPA Zn extractable concentration was found in the M, M2, and N2P2M2 treatments. Correlation analysis showed that DTPA extractable Fe in soil was significantly correlated with DTPA Mn and DTPA Cu, while DTPA Mn was significantly correlated with DTPA Cu (Table 2). However, DTPA extractable Zn was not significantly correlated with DTPA extractable Fe, Mn, or Cu. In soil, DTPA extractable

Table 1 Effects of 30 years of chemical fertilizer and horse manure applications on soil pH, organic carbon (SOC), cation exchange capacity (CEC), total N, P, K, and total and DTPA extractable micronutrient concentrations ($n=3$)

Treatment	pH	SOC (g C kg⁻¹)	CEC (cmol kg⁻¹)	Total N (g kg⁻¹)	Total P (g kg⁻¹)	Total K (g kg⁻¹)	Total Fe (g kg⁻¹)	Total Mn (mg kg⁻¹)	Total Cu (mg kg⁻¹)	Total Zn (mg kg⁻¹)	DTPA extractable micronutrients (mg kg⁻¹)			
											Fe	Mn	Cu	Zn
CK	6.47b	14.1d	24.45ab	1.37d	0.38c	22.2ab	15.8b	264.9b	10.5c	19.7d	13.7f	11.4f	1.64c	1.15cd
NP	5.73d	16.1c	24.44ab	1.55b	0.62b	22.1ab	22.4a	453.5a	19.5bc	79.4a	60.1c	37.0c	1.76b	1.23c
M	6.48b	18.6ab	25.53a	1.49c	0.44c	23.2a	21.7a	428.7a	25.2ab	53.8c	20.9e	15.7e	1.71bc	1.49ab
NPM	6.07c	18.2b	25.41a	1.52b	0.61b	22.5ab	21.2a	378.3ab	23.3ab	69.1abc	31.4d	20.0d	1.74bc	0.96d
N2P2	4.73f	13.4e	23.99b	1.40d	0.86a	21.0ab	23.8a	446.8a	23.3ab	63.4bc	114.0a	55.4a	2.33a	1.19cd
M2	6.57a	15.6c	24.64ab	1.48c	0.43c	20.1b	22.9a	370.2ab	19.0bc	71.3ab	10.1f	8.9f	1.49d	1.48b
N2P2M2	5.08e	19.0a	25.65a	1.68a	0.92a	23.1a	23.8a	431.1a	32.8a	64.8abc	93.0b	46.9b	2.31a	1.90a

Means followed by different letters within the same column are different at $P<0.05$

Fe, Mn, and Cu concentrations showed a significant negative correlation with pH and positive correlation with total P content (Table 2). However, no significant relationship was observed between DTPA extractable Fe, Mn, and Cu and their total contents in soil.

3.2 Organic carbon and DTPA extractable Fe, Mn, Cu, and Zn in aggregates

The SOC concentration in microaggregates, ranged from 21.8 to 39.6 g C kg⁻¹, was highest among four fractions (Fig. 1). Compared to CK, chemical fertilizer alone application (NP and N2P2) increased the SOC concentration by 9.9% in large macroaggregates, by 20.4% in small macroaggregates, by 6.1% in microaggregates, and by 78.4% in the silt + clay fraction. Manure alone application (M and M2) caused a more marked increase in SOC concentration in large macroaggregates (17.0%), microaggregates (47.1%), and the silt + clay fraction (87.3%) as compared with chemical fertilizer addition.

DTPA extractable Fe, Mn, Cu, and Zn concentrations in the aggregates among treatments differed significantly (Fig. 2). In large macroaggregates, treatments NPM and M2 had notably higher DTPA extractable Fe than the other treatments. Extractable Mn and Cu concentrations in large macroaggregates with the application of manure alone (M and M2) were significantly higher than the corresponding concentrations in the other treatments, except CK. Treatments with double application rates of chemical fertilizer and manure had notably higher DTPA extractable Zn in large macroaggregates (1.7–2.1 mg kg⁻¹) than the other treatments (1.1–1.4 mg kg⁻¹). In the small macroaggregates of treatment CK, DTPA extractable Fe and Zn concentrations were significantly higher than in the NP treatment but lower than in the other treatments, whereas DTPA extractable Mn was remarkably lower than in the N2P2 treatment and higher than in the other treatments. DTPA extractable Cu in the CK treatment was not significantly different from that in the M treatment but it was significantly higher than in the other treatments.

Compared to CK, manure and chemical fertilizer addition significantly increased DTPA extractable Fe, Mn, Cu, and Zn in microaggregates by 32% to 144%, 9% to 182%, 14% to 54%, and 20% to 298%, respectively (Fig. 2) Manure alone application (M and M2) significantly increased DTPA extractable Fe, Mn, Cu, and Zn in the silt + clay fraction.

3.3 Fe, Mn, Cu, and Zn concentrations and total amounts in corn stalks and grain

The Fe concentrations in corn stalks ranged from 225 to 237 mg kg⁻¹ in treatments NP, NPM, and N2P2M2, exceeding the levels in treatments CK, N2P2, and M2 (Table 3). The lowest Fe concentration in stalks was found in the M treatment. In corn grain, no significant difference in Fe concentration was

Table 2 Relationship between DTPA extractable micronutrients and soil properties in bulk soil and aggregates

		DTPA-Fe	DTPA-Mn	DTPA-Cu	DTPA-Zn
Bulk soil	pH	−0.99**	−0.99**	−0.96**	−0.19
	SOC	−0.12	−0.10	−0.01	0.46
	Total N	0.29	0.32	0.31	0.58
	Total P	0.95**	0.94**	0.94**	0.32
	Total K	0.05	0.08	0.21	0.31
	Total Fe	0.59	–	–	–
	Total Mn	–	0.68	–	–
	Total Cu	–	–	0.65	–
	Total Zn	–	–	–	0.14
	DTPA-Fe	1	–	–	–
	DTPA-Mn	0.99**	1	–	–
	DTPA-Cu	0.95**	0.93**	1	–
	DTPA-Zn	0.15	0.13	0.27	1
Large macroaggregates	SOC	−0.32	0.27	0.18	0.95**
Small macroaggregates	SOC	0.08	−0.08	−0.28	0.09
Microaggregates	SOC	0.23	0.65 (0.10)	0.74 (0.06)	0.93**
Silt+clay fraction	SOC	−0.32	0.73 (0.06)	0.63	0.86*

*P<0.05; **P<0.01; P value given in parentheses when 0.10≤P≤0.05

observed between CK and fertilizer-added treatments, although treatment N2P2 had significantly higher grain Fe concentrations than treatments NP and M2. The highest Mn concentration in corn stalks was found in treatment N2P2M2 and the lowest concentration in treatment M. The Mn concentration in corn grain was significantly different among treatments showing an increasing order: CK, M2<NP, M, NPM<N2P2<N2P2M2. These results indicate that chemical fertilizer exhibited a greater impact than manure on Mn uptake by corn.

There was no significant difference in the Cu concentrations of corn stalks between CK and fertilizer-added treatments. However, Cu concentrations in corn grain were significantly higher in the CK, M, NPM, and N2P2M2 treatments than in the NP, M2, and N2P2 treatments. The Zn concentration in corn

stalks was 10.39 and 12.30 mg kg^{-1} in the M2 and N2P2M2 treatments, respectively, which were significantly higher than in the CK, M, and NPM treatments. In corn grain, Zn concentrations were higher in the CK, M, and M2 treatments than in the other treatments, especially N2P2, indicating that the application of horse manure increased Zn uptake by corn.

Total amount of Fe and Mn in corn stalks were significantly higher in the NP, N2P2, and N2P2M2 treatments than in the other treatments (Table 4). And treatment N2P2 showed notably higher grain Fe and Mn amounts than the other treatments, which were mainly due to high grain yield and stalk biomass of N2P2 treatment. The amount of Cu in corn stalks was 36.3 g ha^{-1}, which was significantly higher than CK, M, and N2P2M2 treatments. Compared to CK, N2P2 and NPM treatments markedly increased Cu amount in corn grain by 37% and 29%. Total amount of Zn in corn stalks was significantly different among treatments showing an increasing order: CK, M, NPM<NP, M2<N2P2, N2P2M2. However, no significant difference was observed in the grain Zn amount between CK and fertilizer-added treatments.
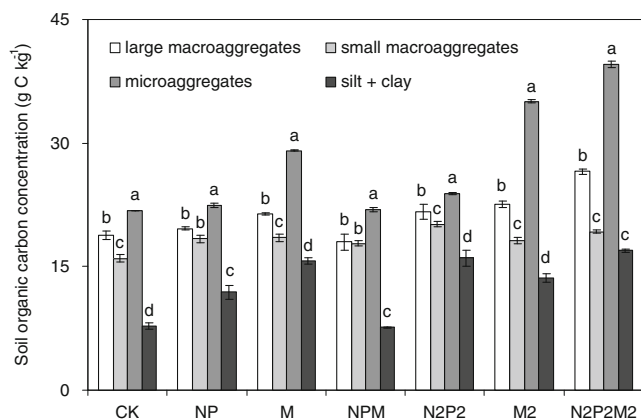

Fig. 1 Soil organic carbon concentration in aggregate fractions as affected by the 30-year applications of chemical fertilizer and horse manure. *Different letters* indicate significant differences between aggregate fractions at *P*<0.05

4 Discussions

4.1 Manure and chemical fertilizer effects on the availability of micronutrients

The DTPA extractable Fe, Mn, Cu, and Zn concentrations in all treatments were much higher than the critical deficiency levels of 4.5 mg kg^{-1} for Fe, 1.0–5.0 mg kg^{-1} for Mn, 0.2 mg kg^{-1} for Cu, and 0.5–0.8 mg kg^{-1} for Zn (Lindsay and Norvell 1978;

Fig. 2 DTPA extractable Fe, Mn, Cu, and Zn concentrations in aggregate fractions as affected by the 30-year application of chemical fertilizer and horse manure. *Different letters* indicate significant differences between treatments at *P*<0.05
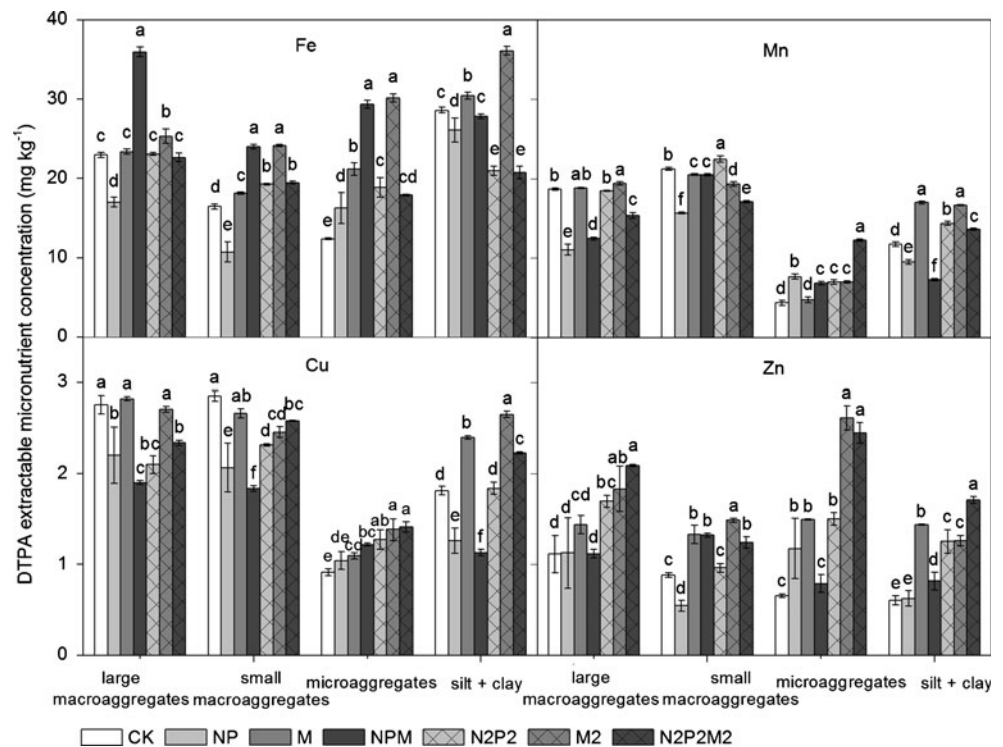
Lin and Li 1997), but much lower than the maximun limit values for crop toxicity of 140–200 mg kg^{-1} for Mn, 17–25 mg kg^{-1} for Cu, and 10–20 mg kg^{-1} for Zn (Sillanpää 1982). Our results indicate that black soil even without fertilization for 30 years did not appear to show a deficiency of available Fe, Mn, Cu, and Zn, and that horse manure and chemical fertilizer application may not result in a surplus of Fe, Mn, Cu, and Zn for crop growth.

Trace element load in agricultural soils is the sum of metal input from atmospheric deposition and the addition of fertilizers, biosolids, metal-containing pesticides, and sometimes wastewater (Adriano 2001). Previous studies have shown that Fe and Mn are primarily sourced from parent material, whereas Cu and Zn are associated with anthropogenic activities (Luo et al. 2007; Franco-Uría et al. 2009). Averaged Fe, Mn,

Cu, and Zn contents in P fertilizer used in north China were 2,108, 82.38, 7.36, and 60.96 mg kg^{-1}, respectively (Wang and Ma 2004). In this study, we used 65.5 kg P ha^{-1} in double rate treatments. Assuming a soil density of 1.15 g cm^{-3}, a plough depth of 20 cm and taking no account of crop uptake and removal, 30-year application of P fertilizer at double rate would increase total Fe, Mn, Cu, and Zn by 25.78, 1.01, 0.09, and 0.75 mg kg^{-1}, respectively. This increase is only about 0.1–1.2% of total elements content in soil (Table 1). Luo et al. (2009) also reported that the inputs of trace elements to agricultural soils in China mainly attributed to atmospheric deposition and livestock manures and that chemical fertilizers only accounted to 1.3% and 1.5% of total Cu and Zn input. Therefore, micronutrients input from chemical fertilizers may contribute to their availability in soil but this impact is limited.

Table 3 Effects of 30 years of applications of chemical fertilizer and horse manure on Fe, Mn, Cu, and Zn concentrations (mg kg^{-1}) in corn stalks and grain (means ± SD, *n*=3)

Treatment	Stalks				Grain			
	Fe	Mn	Cu	Zn	Fe	Mn	Cu	Zn
CK	199±14bc	23.7±0.7cd	5.30±0.47abc	5.95±1.73cd	22.8±22.8abc	3.43±0.19e	1.32±0.01a	18.4±2.1ab
NP	225±19ab	29.4±2.5b	4.32±0.40c	8.64±1.04b	19.7±2.3c	4.33±0.03c	1.01±0.03b	14.4±2.0bc
M	142±14d	18.4±0.5e	4.14±0.01c	5.16±1.72d	31.8±3.4ab	4.04±0.11cd	1.24±0.05a	19.3±2.2a
NPM	231±32ab	24.9±2.0cd	6.00±1.91ab	3.68±0.67d	27.7±4.6abc	3.91±0.06d	1.24±0.02a	15.0±1.6bc
N2P2	191±17c	27.0±5.6bc	4.50±0.51bc	8.21±0.90bc	38.9±17.2a	5.46±0.26b	1.05±0.01b	11.5±3.1c
M2	217±19abc	21.4±1.8de	6.52±1.96a	10.39±1.38ab	24.0±3.0bc	3.24±0.35e	1.07±0.15b	16.8±3.8ab
N2P2M2	237±16a	32.7±2.5a	4.10±0.02c	12.30±2.05a	25.8±4.9abc	6.22±0.28a	1.21±0.06a	14.3±2.5bc

Means followed by different letters within the same column are different at *P*<0.05

Table 4 Effects of 30 years of applications of chemical fertilizer and horse manure on total amount of Fe, Mn, Cu, and Zn (g ha^{-1}) in corn stalks and grain (means ± SD, n=3)

Treatment	Stalks				Grain			
	Fe	Mn	Cu	Zn	Fe	Mn	Cu	Zn
CK	944±69c	112±3bc	25.2±2.3b	28.2±8.2c	152±152b	22.9±1.3e	8.80±0.10bc	123±14ab
NP	1485±128ab	194±17a	28.5±2.7ab	57.1±6.8b	173±20b	38.1±0.2c	8.87±0.29bc	126±18ab
M	914±90c	118±3bc	26.6±0.1b	33.1±11.0c	245±26b	31.1±0.9d	9.53±0.31b	149±17a
NPM	1310±183b	141±11b	34.1±10.9ab	20.9±3.8c	255±42b	35.8±0.6c	11.37±0.15a	138±15a
N2P2	1541±136a	217±45a	36.3±4.1a	66.2±7.2b	445±198a	62.6±3.0a	12.03±0.12a	132±36ab
M2	1093±94c	108±9c	32.8±9.8ab	52.2±6.9b	206±26b	27.9±3.0 d	9.17±1.23bc	144±33a
N2P2M2	1604±108a	221±17a	27.7±0.2b	83.1±13.9a	183±35b	44.2±2.0b	8.60±0.46c	101±18b

Means followed by different letters within the same column are different at $P<0.05$

Furthermore, horses feeding on alfalfa and/or grass hay with almost no additives produced metal-poor manure, in which Fe, Mn, Cu, and Zn concentrations were 284–1,173, 108–111, 20–29, and 129–205 mg kg^{-1}, respectively, which were much lower than that in pig, chicken and cattle manures (Moreno-Caselles et al. 2002). Previous study verified that horse manure did not contribute extra metal contaminants to soils (Shtangeeva et al. 2004). In the present study, no significant difference was found between manure treatments (M and M2) and chemical fertilizer treatments (NP and N2P2) for total micronutrients content (Table 1), suggesting that horse manure we used contained low contents of Fe, Mn, Cu, and Zn. Furthermore, our previous results suggested that atmospheric deposition was a main source of heavy metals in the black soil (Fan et al. 2012).

The application of horse manure and chemical fertilizer over a 30-year period caused a significant increase in Fe, Mn, Zn, and Cu concentrations in black soil (Table 1), mainly due to the effect of fertilization on soil characterisitcs (Mortvedt 1996; Shtangeeva et al. 2004; Wang and Ma 2004; Luo et al. 2009). Compared to CK, chemical fertilizer applications significantly increased DTPA Fe, Mn, and Cu concentrations in bulk soil. Manure alone application (M and M2) and treatment N2P2M2 significantly increased DTPA Zn in bulk soil (Table 1). This finding is in agreement with Singh et al. (2010) showing that fertilizer NPK application significantly increased Cu and Mn phytoavailability whereas farmyard manure application markedly reduced it. We found that the significant increase of DTPA Fe, Mn, and Cu in chemical fertilizer-added soil occurred in microaggregates rather than in large macroaggregates, small macroaggregates or the silt + clay fraction compared with CK (Fig. 2). However, manure application significantly increased DTPA Zn in all aggregates, particularly microaggregates.

In the present study, there was a significant relationship among DTPA extractable Fe, Mn and Cu concentrations in soils (Table 2), indicating that Fe, Mn, and Cu in soil were interdependent and that their distribution and availability in

soil may be affected by similar soil factors (Sharma et al. 2002; Sidhu and Sharma 2010). The availability of microelements in soil is affected by their chemical characteristics and by soil properties like soil pH (Smith 2009). We found that DTPA extractable Fe, Mn, and Cu in soil was negatively correlated with soil pH, and positively correlated with soil P content (Table 2). A significant negative relationship was also observed between soil pH and soil P content (r=−0.961, $P<0.01$). Guo et al. (2010) demonstrated that inputs of NH$_4^+$-forming fertilizer decreased soil pH through nitrification. Furthermore, after superphosphate application, dissolved monocalcium phosphate (MCP) is transformed into slightly soluble dicalcium phosphate (DCP) and phosphoric acid, which in turn dissociates into phosphate and H$^+$, resulting in the reduction of soil pH (Bolan et al. 1999). Therefore, the increase in DTPA Fe, Mn, and Cu was mainly due to the decrease in soil pH driven by both urea and superphosphate application. However, DTPA extractable Zn in soil was not significantly correlated with soil pH or P content, indicating that Zn availability in soil may not be controlled by soil pH and P content. It is suggested that although the decrease in soil pH could also increase the amount of dissolved Zn, the formation of Zn$_3$(PO$_4$)$_2$·4H$_2$O may lower Zn availability in soil (Verma and Minhas 1987). In the present study, we found that DTPA extractable Zn in aggregates was significantly correlated with SOC in large macroaggregates, microaggregates, and the silt + clay fraction (Table 2), as reported by Behera et al. (2011) and Sidhu and Sharma (2010). Lair et al. (2006) pointed out that Zn is more weakly bound to soil surfaces than other metals such as Cu, but it could be combined with organic matter, especially labile organic matter, which would promote its extractability (Hodgson 1963; Chahal et al. 2005; Behera et al. 2011). While the SOC concentration was highest in microaggregates compared with the other fractions, and that in large macroaggregates was also higher than in small macroaggregates and in the silt + clay fraction (Fig. 2). Moral et al. (2005) demonstrated that the concentration of dissolved organic C was significantly higher

Table 5 Relationship between micronutrient concentrations in corn stalks or grain and DTPA extractable micronutrient in bulk soil or aggregates as affected by 30 years of applications of chemical fertilizer and horse manure

	Fe		Mn		Cu		Zn	
	Stalks	Grain	Stalks	Grain	Stalks	Grain	Stalks	Grain
Soil	0.22	0.48	0.77*	0.91**	−0.62	−0.16	0.76*	0.08
Large macroaggregates	0.18	0.22	−0.33	−0.18	0.02	0.33	0.80*	−0.29
Small macroaggregates	0.12	0.33	−0.53	−0.21	−0.22	0.45	0.01	0.31
Microaggregates	0.16	0.11	0.82*	0.77*	0.18	−0.33	0.82*	−0.12
Silt + clay fraction	−0.22	−0.36	−0.12	0.05	0.03	0.04	0.52	−0.09

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

in horse and chicken manure than in pig and cow manure and accounted for 5.2% to 6.6% of total organic C. Therefore, we argue that SOC may enhance the availability of Zn by binding labile organic C with Zn in microaggregates and large macroaggregates (Marschner 1993). However, DTPA extractable Zn in small macroaggregates was not significantly corelated with SOC (Table 2), which was mainly due to the low SOC content and small range (16.0 to 20.1 g C kg^{-1}) in this fraction (Fig. 1). Correlation analysis also showed that DTPA extractable Mn in aggregates was marginally correlated with SOC in microaggregates and in the silt + clay fraction ($0.10<P<0.05$; Table 2). DTPA extractable Mn was significantly correlated with SOC (Benbi and Brar 1992) and cation exchange capacity (CEC) increased with increasing of SOC ($r=0.964$, $P<0.01$). Therefore, we argue that the increase in CEC caused by SOC may promote an increase in exchangeable Mn in microaggregates and Mn uptake by the crop.

4.2 The availability of micronutrients in aggregates for crop uptake

The application of chemical fertilizer increased Mn uptake by corn, but did not affect Zn uptake, whereas the application of manure enhanced Zn uptake (Tables 3 and 4). This finding is consistent with Singh et al (2010), who observed that Mn and Cu concentrations in *Beta vulgaris* L. shoot increased by 12.3% and 9.9% in fertilizer NPK-amended soil, but decreased by 4.4% and 16.7% in manured soil, respectively, compared to CK. Mn in corn stalks or grain and the Zn concentration in corn

stalks was significantly associated with DTPA extractable Mn and Zn in bulk soil (Tables 5 and 6), indicating that the increase in Mn and Zn uptake by corn was mainly attributable to the increase in DTPA extractable Mn in chemical fertilizer-added soil and DTPA Zn in manured soil.

Correlation analysis also showed that the Mn concentration in corn stalks and grain was significantly correlated with DTPA Mn in microaggregates, whereas Zn in corn stalks was significantly associated with DTPA Zn in microaggregates and large macroaggregates (Table 5). Furthermore, the total amount of Mn in corn stalks was also notably correlated with DTPA Mn in microaggregates, while Zn amount in stalks were significantly correlated with DTPA Zn in large macroaggregates and roughly correlated with Zn in microaggregates (Table 6). It is possible that Mn uptake by corn was mainly originated from microaggregates and Zn uptake by corn was primarily sourced from microaggregates and large macroaggregates. Therefore, we argued that the chemical fertilizer and manure applications caused more noticeable increases in DTPA Mn and Zn concentrations in microaggregates, as well as in large macroaggregates in the case of Zn, than in the other fractions, which in turn increased Mn and Zn concentrations in corn. This finding demonstrates that the amendment of chemical fertilizer and manure affected Mn and Zn availability in soil, particularly microaggregates and then uptake by corn.

The application of chemical fertilizer also accelerated Fe and Cu accumulation in corn stalks and grain, although the effect was not statistically significant (Table 3). The lack of a significant relationship between Fe or Cu concentrations

Table 6 Relationship between the amount of micronutrient in corn stalks or grain and DTPA extractable micronutrient in bulk soil or aggregates as affected by 30 years of applications of chemical fertilizer and horse manure

	Fe		Mn		Cu		Zn	
	Stalks	Grain	Stalks	Grain	Stalks	Grain	Stalks	Grain
Soil	0.64	0.49	0.82*	0.88**	0.27	0.18	0.52	0.04
Large macroaggregates	−0.11	0.15	−0.30	−0.04	−0.60	−0.26	0.76*	−0.22
Small macroaggregates	−0.11	0.23	−0.44	0.17	−0.64	−0.28	−0.16	0.37
Microaggregates	−0.09	0.16	0.92**	0.36	0.54	0.04	0.68 (0.08)	−0.09
Silt + clay fraction	−0.74	−0.32	−0.06	0.13	−0.27	−0.09	0.53	0.11

$*P<0.05$; $**P<0.01$; P value given in parentheses when $0.10\leq P\leq0.05$

and amounts in corn stalks or grain and DTPA extractable Fe or Cu in bulk soil and aggregates (Tables 5 and 6) suggests that DTPA Fe and Cu in soil were not strongly associated with the uptake of Fe and Cu by corn. O'Connor (1988) reported that DTPA extractable Fe in soil could be used to predict plant Fe response only when the concentration is close to the critical deficiency level, and otherwise it only reflects soil Fe loading. In the present study, the DTPA extractable Fe concentration was 2.2- to 25.3-fold higher than the critical value. Thus, an excessive amount of DTPA Fe in bulk soil or aggregates may mask the relationship between DPTA extractable Fe in soil or aggregates and the Fe concentration in corn. Lindsay and Norvell (1978) found that the significant correlation between DTPA extractable Cu and Cu concentrations in corn or oats appeared only when DTPA Cu concentrations in soil showed a wide range. In the present study, DTPA extractable Cu in different soils varied within a narrow range, possibly explaining the poor correlation between Cu concentrations in corn and DTPA Cu concentrations in bulk soil or aggregates (Table 5).

5 Conclusions

Chemical fertilizer over a 30-year period application significantly increased DTPA extractable Fe, Mn, and Cu concentrations in soil and subsequently promoted their contents in corn. In contrast, manure applications caused a more noticeable increase in the DTPA Zn in soil and the Zn content in corn. The Mn concentrations and amounts in corn stalks and grain were significantly correlated with DTPA extractable Mn in bulk soil and microaggregates, and Zn in corn stalks were correlated with DTPA extractable Zn in bulk soil, microaggregates and large macroaggregates. These results suggest that Mn uptake by corn was mainly originated from microaggregates, whereas Zn was possibly sourced from large macroaggregates and microaggregates. We found that SOC was the main factor controlling Mn and Zn uptake by corn. The increase in CEC caused by SOC may promote an increase in exchangeable Mn in soil and result in an increase in DTPA Mn in microaggregates and Mn uptake by the crop. And SOC, especially labile organic C, may enhance the availability of Zn by binding labile organic C with Zn in microaggregates and large macroaggregates.

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