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Effect of molecular weight of dissolved organic matter on toxicity and bioavailability of copper to lettuce

Xudong Wang*, Xianni Chen, Shuai Liu, Xizu Ge

College of Resource and Environment, Northwest Agriculture and Forestry University, Shaanxi 712100, China. E-mail: wangxudong01@126.com

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Abstract

To clarify the effects of molecular weight of dissolved organic matter (DOM) on the toxicity and bioavailability of copper (Cu) to plants, DOM extracted from chicken manure was ultra-filtered into four fractions according to their molecular weights by means of sequential-stage ultrafiltration technique. Lettuce seeds were germinated by being exposed to the solutions containing Cu^{2+} with or without different fractions of DOM. The concentration of copper in roots, leaves, sprouts and the length of roots were investigated. The results showed that not all fractions of DOM could improve copper availability or toxicity. The fraction of DOM with larger molecular weight more than 1 kDa had higher complexation stability with Cu^{2+} and caused lower concentration of free Cu^{2+} ion in the solution of copper plus the fraction, resulting in lower availability and toxicity of copper to lettuce, but the fraction with molecular weight less than 1 kDa had the opposite function. Therefore, the molecular weight of 1 kDa may be the division point to determine DOM to increase or decrease copper availability and toxicity.

Key words: fraction of dissolved organic matter; complexation stability; copper availability

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Introduction

In the past few decades, heavy metals in the environment are increasingly conspicuous as a result of anthropogenic activities, which pose significant threats not only to soil ecosystem but also to human health. The environmental risk caused by these metals depends on their mobility and bioavailability to food chain (Zehl and Einax, 2005; Khan et al., 2006). Effects of organic matter, including humic substance and synthetic chelators on mobility or availability of copper and other heavy metals have been reported (Inaba and Takenaka, 2005; Miyazawa et al., 2002; Lu and Tu, 2009). However, the results or conclusions from authors are sometimes contradictory. On one hand, researchers used materials enriched in organic matter such as bio-solid or synthetic chelators to immobilize heavy metals and reduce their toxicity in environment (Basta et al., 2005; Kochian, 1991; Chen et al., 1989), on the other hand, compost and synthetic chelators sometimes are used to improve the availability of copper, zinc and other heavy metals for plant growth (Garcia-Mina et al., 2004; Schwab et al., 2007). This contradiction needs to be clarified to make more effective use of phytoremediation or improve availability of heavy metals.

Heavy metals, which exist in soil solution as soluble ions, are in the form of inorganic and organic complexes. For ecological considerations, not only the total concentration but also the kind of heavy metal species present in the soil solution is of primary importance, because metal mobility and availability are closely related to the composition of the solution (Brummer et al., 1986). Copper is a common environmental contaminant and usually presents high stability constants with natural dissolved organic materials (DOM). Hodgson et al. (1965, 1966) and Geering et al. (1969) found that most Cu in soil solution of limy or alkaline soils was present in the form of organic complexes. It has become clear that Cu can not be available unless soluble organic complexed Cu is able to withstand the unfavorable milieu of alkaline or calcareous soils. Nevertheless, chelate stability must not be so high that plants are unable to take up the chelated metal (Lucena et al., 1987). The interactions between humus and metals are different in their extents and ways to affect the mobility, bioavailability and phyto-toxicity of heavy metals (Lagier et al., 2000). The effect of humus on heavy metals' mobility and availability must be directly related to the properties of metal-humus complexes, and essentially to the molecular weight of humic substance or the metal-humus complexes. Humic substances are the main composition of dissolved organic matter in soil solution. Humic substances are a series of relatively high molecular weight substances which are formed by secondary synthesis reactions (Stevenson, 1994) and vary in molecular weight from hundreds Dalton (Da) to millions Da. Recent studies have investigated the stability and availability of

* Corresponding author. E-mail: xdwang365@yahoo.com

humic substance-copper complexes intensively (Badea et al., 2003). However, information about the influences of molecular weight of humic substance on copper availability or toxicity is insufficient. Although the fact that large molecular complexes are not easily absorbed by plant roots has been reported by some researchers (Kochian, 1991), little research has been done to clarify how the series molecular weight of DOM affects availability or mobility of heavy metals.

The objective of this work was to assess the effects of molecular weight of DOM on the toxicity and bioavailability of inorganic copper in solution. DOM extracted from chicken manure (compost) was ultra-filtered into four fractions according to their molecular weight. Copper (Cu) ion was added to the fractions of DOM to form complexes. Germination tests using lettuce seeds were conducted in copper-DOM's fraction solution. The effects of molecular weight of DOM's fraction on toxicity and bioavailability of copper were investigated.

1 Materials and methods

1.1 Composting of chicken manure

Fresh chicken manure was collected from the agroexperimental station of Northwest Agriculture and Forestry University. The chicken manure sample was characterized by a C/N ratio of 21.5, organic carbon of 440.5 g/kg and pH 7.3. Water content of chicken manure was adjusted to 60% (*W/W*). Manure was placed into a plastic container of 42 L for composting with forced aeration (air flux rate is 30 L/(m^3·min)) during the first 15 days, 15 L/(m^3·min) from day 15 to day 30 without aeration). The compost was turned, mixed, and sampled on day 60. The samples were placed in polyethylene bags, and stored at 4°C.

1.2 Extraction and fractionation of DOM

DOM was extracted from 1000 g of the air dried compost sample by adding 5000 mL distilled water (the weight ratio of water to compost was 5:1) and shaking for 24 hr. Subsequently, the extraction was centrifuged and the supernatant was filtered by using 0.45 μm membrane. Metal ions in the supernatant were removed by treating with necessary amount of cation exchange resin (Amberlite IRA-118 H^+ Sigma-aldrich Inc., USA) until pH values were in the range of 3.5 to 4. The resin was separated by filtration (Whatman no. 42 filter paper), and the supernatant containing DOM was obtained.

DOM in the filtered supernatant was fractionated into four fractions according to molecular weight of humus by means of sequential-stage ultrafiltration technique (Burba et al., 1998), which were expressed as F1 (> 10 kDa), F2 (10–5 kDa), F3 (5–1 kDa) and F4 (< 1 kDa), respectively. The organic carbon content of the fraction was determined by oxidation with 0.01 mol/L $KMnO_4$.

1.3 Germination of lettuce seeds

Ten seeds of lettuce (*Lactuca sativa* L.) were sown on filter paper in a dish for each treatment with triplication, and 5 mL of treatment solution was supplied to each dish. We prepared 72 treatment solutions containing 10 μg Cu^{2+}/mL of $CuSO_4$ along with one of four fractions including F1, F2, F3 to F4 at three level concentrations. Distilled water added with or without Cu^{2+}, or distilled water added with one of four fractions, respectively, was conducted as control. Other nutrients such as N, P, K except Cu were added into every dish based on the formula of Hogland solution. The germination experiment lasted for three weeks. Table 1 shows the pH and total organic carbon (TOC) content of the treatment solutions.

1.4 Determination of stability constant and Cu speciation in solution

The stability constant of Cu with DOM fractions was determined by cation exchange equilibrium method (Schnitzer and Hansen, 1970). The dissolved Cu speciation in the solution was determined by Nafion-coated thin mercury film electrodes (Hurst and Bruland, 2005).

1.5 Determination of copper concentration in lettuce

The dishes with lettuce were placed in a climate-controlled chamber (light/dark, 16 hr/8 hr, 20°C/14°C) for three weeks, then lettuce sprouts were collected from each

Table 1 Content of DOM with different molecular weight and copper distribution

Treatment*	Molecular weight of DOM (kDa)	Concentration of the fractions (μg C/mL)	$CuSO_4$ (μg Cu/mL)	pH	TOC (μg C/mL)	Organically complexed Cu (%)	Inorganic Cu (%)
CK	–	0	10	5.20	–	0	100 h
F11	> 10	10	10	7.65	6.05	83	17 cd
F12	> 10	20	10	7.42	12.03	90	10 b
F13	> 10	40	10	7.15	23.96	95	5 a
F21	10–5	10	10	7.36	5.89	79	21d
F22	10–5	20	10	7.18	11.57	84	16 c
F23	10–5	40	10	7.05	23.30	91	9 b
F31	5–1	10	10	7.26	5.54	72	28 e
F32	5–1	20	10	7.08	11.02	77	23 d
F33	5–1	40	10	6.96	23.94	81	19 cd
F41	< 1	10	10	7.24	5.23	55	45 g
F42	< 1	20	10	7.11	10.26	58	42 g
F43	< 1	40	10	7.03	20.46	63	37 f

The results of inorganic Cu ion concentration in each treatment of DOM with Cu^{2+} or in the Cu^{2+} control indicated by different letters are significantly different at $p < 0.05$.
* The treatments from F11 to F43 mean Cu^{2+} with fractions of F1, F2, F3, F4 at three different levels.

dish and washed with distilled water prior to use. The sprouts were cut into leaf/root parts and the root length was measured. These plant materials were oven dried at 60°C for 48 hr before determining their dry weight, and then wet-digested with HNO_3 in a microwave sample processor. Copper in the digested solution was assayed by AAS (Atomic Absorption Spectroscopy, AA320, Shanghai, China).

All data sets which were presented as mean value were statistically analyzed using the software of SAS (version 8.1).

2 Results

2.1 Effects of DOM on Cu toxicity to root lengths

Figure 1 shows the root lengths of lettuce sprouts germinated in the solution of DOM's fraction with or without 10 μg/mL Cu^{2+}. In the no-copper control, the roots were about 2.2 cm long, but in the Cu^{2+} control, the roots were only 0.75 cm long, suggesting the copper toxicity to lettuce roots. The roots of the lettuce in the treatments of Cu^{2+} with F1 at three different levels of concentration expressed as F11, F12 and F13, respectively, were longer than in the Cu^{2+} control. Similarly, the roots of the lettuce were longer in the treatments of Cu^{2+} with F2 at three levels expressed as F21, F22 and F23. The results suggest that fraction 1 (F1) and fraction 2 (F2) of DOM can reduce copper toxicity to lettuce roots. However, in the treatment of Cu^{2+} with F4 at three levels expressed as F41, F42 and F43, the roots were shorter than the Cu^{2+} control, indicating that fraction 4 (F4) of DOM improves copper toxicity. Compared with the Cu^{2+} control, there was no significant influence on copper toxicity in the treatment of F31, but copper toxicity decreased significantly in the treatment of F33, suggesting that the ratio of humus to copper is also a factor to affect the copper toxicity. The higher the ratio of humus to copper, the lower copper toxicity is. This is consistent with those reported in the previous work (Inaba and Takenaka, 2005).

The stimulation function of DOM's fractions can be also seen in Fig. 1. Compared to the no copper control, the

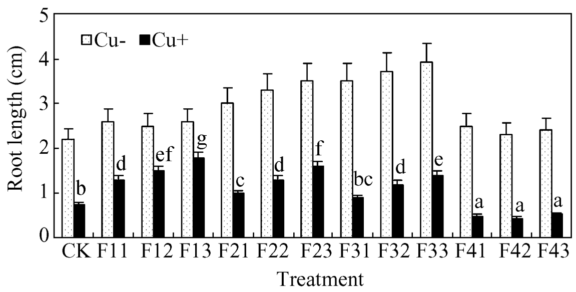

Fig. 1 Root length of lettuce sprouts germinated in each treatment. Light bars (Cu-) mean no copper treatment; dark bars (Cu+) mean treatment containing 10 μg/mL Cu^{2+}. CK means without DOM, with or without Cu^{2+}; treatments from F11 to F43 mean Cu^{2+} with each DOM fraction from F1 to F4 at three concentrations of 10, 20, and 40 μg C/mL. The results from the Cu^{2+} only control and treatments with each DOM in the presence of Cu^{2+} indicated by different letters are significantly different at $p < 0.05$.

roots length in the treatments of no copper with DOM fraction from F1 to F3 were longer, but the root length in the treatments of no copper with F4 has not changed at a significant level, suggesting that fraction 1, fraction 2 and fraction 3 of DOM had obvious stimulating function, but fraction 4 had little stimulating function.

2.2 Effects of molecular weight of DOM on Cu^{2+} bioavailability

Copper concentration in lettuce sprouts in different treatments is shown in Fig. 2. The Cu concentration in lettuce sprouts amounted to 460 mg/kg in the Cu^{2+} treatment (control). In the treatments of Cu^{2+} with DOM from F1 to F3 at three levels (expressed as F11, F12, F13, and F21, F22, F23, and F31, F32, F33), with molecular weight being larger than 1 kDa, Cu concentration in the lettuce sprouts decreased significantly compared to the control. The larger the molecular weight of the fractions, the lower the copper concentration is in the sprouts. Furthermore, in the treatments with the same fraction, copper concentration in the sprouts decreased with elevating concentration of the fraction. However, in the treatments of Cu^{2+} added with fraction 4, including the treatment of F41, F42 and F43 whose molecular weights were less than 1 kDa, copper concentration in sprouts increased significantly compared to the Cu^{2+} control treatment, and was enhanced as the concentration of the fraction increased. Therefore, molecular weight of DOM and the ratio of DOM to Cu^{2+} are important parameters affecting Cu bioavailability. Our results reveal that when the molecular weight of DOM's fractions are larger than 1 kDa, the fraction of DOM complexed with Cu^{2+} could decrease the copper uptake. However, the fraction with molecular weight less than 1 kDa could increase copper availability and uptake. For the same fraction of DOM, the positive or negative effect of DOM on copper uptake could be intensified by the elevated ratio of the fraction to Cu^{2+}.

Copper concentration in lettuce leaves is shown in Fig. 3a. Copper concentration in leaves was 32 mg/kg in the control of Cu^{2+} treatment. In the treatments of Cu^{2+} added with DOM with molecular weight larger than 1 kDa, from F1 to F3, copper concentration in leaves ranged from 6 to 28 mg/kg. However, copper concentration in leaves was enhanced by the treatments of Cu^{2+} with DOM with molecular weight less than 1 kDa. Copper concentration in

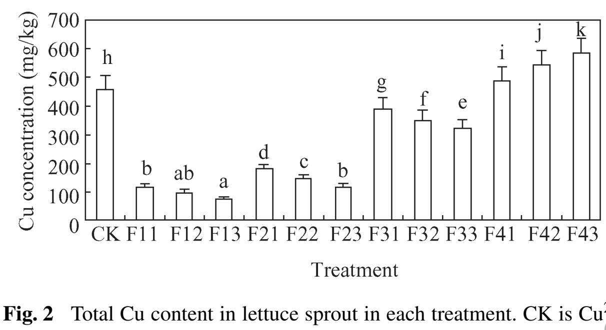

Fig. 2 Total Cu content in lettuce sprout in each treatment. CK is Cu^{2+} without DOM. The results from the treatments with each DOM in the presence of Cu^{2+} indicated by different letters are significantly different at $p < 0.05$.

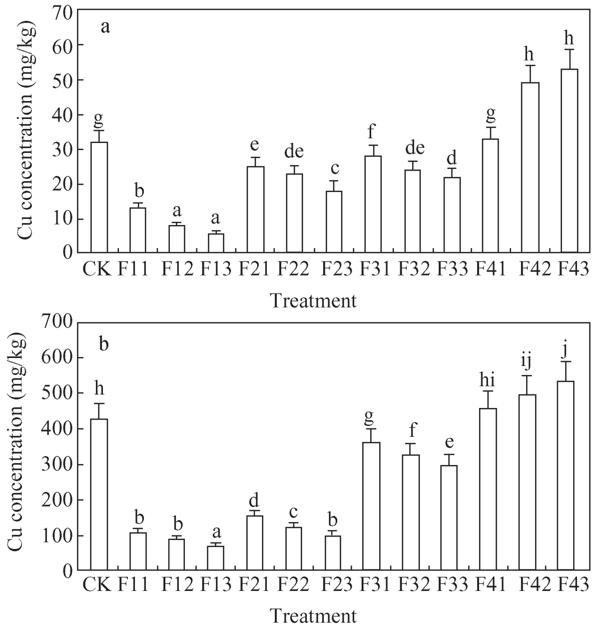

Fig. 3 Copper concentration in leaves (a) and roots (b) of lettuce in each treatment. CK is Cu^{2+} without DOM. The results from the Cu^{2+} only control treatment and those of the treatments with each DOM in the presence of Cu^{2+} indicated by different letters are significantly different at $p < 0.05$.

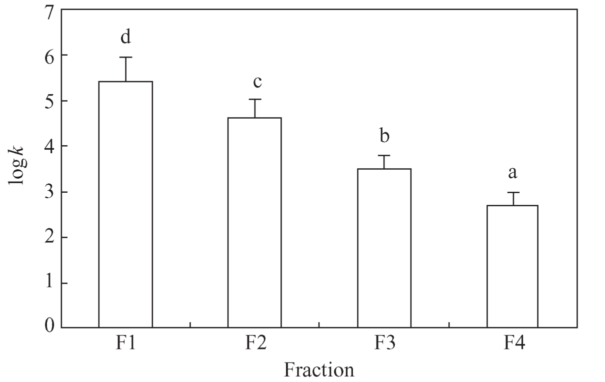

Fig. 4 Stable constant (log$k$) of copper complexes of F1 (> 10 kDa), F2 (10–5 kDa), F3 (5–1 kDa), F4 (<1 kDa). The results of each DOM-Cu^{2+} complexes indicated by different letters of a, b, c, d are significantly different at $p < 0.05$.

roots was 428 mg/kg in the Cu^{2+} treatment (Fig. 3b) being higher than in leaves. Influences of molecular weight of DOM on copper concentration in roots were similar as that in leaves. Compared with the control, copper concentrations in roots were decreased by the treatments of Cu^{2+} added with DOM fraction from F1 to F3. However, when the molecular weight of DOM was less than 1 kDa, in the treatments such as F41, F42 and F43, copper concentration in roots was higher than that in the Cu^{2+} treatment. This means that DOM in large molecular weight could make copper lower available and result in lower uptake in roots, but DOM in smaller molecular weight was on the contrary.

2.3 Stability of DOM-copper complexes

The stability of DOM's fractions complexed with copper was studied in this article. Figure 4 shows that as molecular weight of the fractions including F1, F2, F3 to F4 decreased, the stability constant (log$k$) changed from 5.4, 4.6, 3.5, to 2.7, respectively, indicating that the fraction of DOM with larger molecular weight has higher complexes ability. Copper speciation distribution showed that in the solution of Cu^{2+} added with DOM fraction, more than 70% of total copper was in organically complexed form when the molecular weight of the DOM fraction was larger than 1 kDa (Table 1). However, only about 50% of total copper was in organically complexed form when the molecular weight of DOM fraction was less than 1 kDa. Therefore, DOM's fraction with higher molecular weight is benefit to form the organically complexed copper due to high complexes ability. The ratio of DOM to copper also influences the formation of organically complexed copper. At the same copper concentration, higher concentration of the DOM could form more organically complexes of copper and resulted in less inorganic copper ions in the solution.

2.4 Relationship between complexes stability and copper availability

Correlation analysis (Table 2) showed that the stability constant (log$k$) was negatively correlated with total copper content in the sprouts, leaves and roots, but was positively correlated with the roots length. Inorganic copper concentration in solution was significantly correlated with total copper content in sprouts, roots and leaves, but organically complexed copper in the solution was on the contrary. The content of organically complexed copper in the solution was significantly correlated with the stability constant (log$k$). Therefore, DOMs with large molecular weight have high ability to form complexes with copper and decrease inorganic copper ions in solution resulting in lower copper toxicity to lettuces.

Table 2 Pearson correlation coefficient among different factors

Item	Total Cu in sprouts	Cu in leaves	Cu in roots	Root length	log$k$	Inorganic Cu	Organically complexed Cu
Total Cu	1						
Cu in leaves	0.936	1					
Cu in roots	0.999	0.926	1				
Root length	–0.952	–0.988	–0.944	1			
log$k$	–0.980	–0.942	–0.977	0.927	1		
Inorganic Cu	0.980	0.974	0.975	–0.992	–0.950	1	
Organically complexed Cu	–0.981	–0.974	–0.976	0.992	0.950	–1	1

$r_{0.05} = 0.878$; $r_{0.01} = 0.959$.

3 Discussion

Dissolved organic matter in environment is often considered as accelerant to improve heavy metals availability and toxicity because it can increase solubility of heavy metals and prevent them from precipitation by soil oxides, carbonates and clay minerals. However, in fact, the impact of DOM on heavy metals is dependent on the composition and properties. Inaba and Takenaka (2005) have found that DOM extracted from soil could decrease copper content in plant. DOM, like humic substance, is comprised of series substances such as organic acids, fulvic acids and humic acids. Although the chemical composition is complicated, sometimes it can be fractionated just by molecular weight size (Peuravuori and Pihlaja, 1997; Zhang et al., 2004). One reason for this is that higher molecular weight DOMs have higher ability to form Cu complex and the macro molecule of the complexes is not easy to translocate or to be uptaken by plant roots (Pandey et al., 2000). Another reason is related to the amount of inorganic copper ion in the solution of Cu added with DOM's fraction. Inorganic copper ion is always more toxic than humus complexed copper ion (Hurst and Bruland, 2005). The amount of inorganic copper in the solution is also influenced by the ratio of DOM to copper besides the complexation ability. At a certain concentration of copper, elevating DOM concentration is benefit to form more copper complexes and results in low concentration of inorganic copper ion in the solution. Therefore, the availability and toxicity of copper decreased with elevated DOM concentration in the treatments of F11 to F31, in which the molecular weight was more than 1 kDa. But this phenomenon could not be observed in the treatments of F41, F42 and F43, in which the molecular weight was less than 1 kDa, because low molecular DOMs such as organic acids could increase the toxicity of copper complexes (Inaba and Takenaka, 2005). Sauve et al. (2000) has indicated that the value of pH is another factor to affect toxicity and availability of copper or copper complex. In this work, the values of pH were almost at the similar level among the treatments except the control of copper only. Therefore, the variations in availability and toxicity of Cu in the treatments are caused by the nature of DOM or DOM-Cu complexes rather than by the value of pH.

4 Conclusions

The influences of DOM fractions on copper toxicity or availability were related to the molecular weight. When the molecular weight of DOM's fraction was less than 1 kDa, it could increase copper content in lettuce sprouts, roots and leaves, resulting in short roots and causing high toxicity to lettuce. However, when the molecular weight of the DOM's fraction was larger than 1 kDa, copper content in lettuce sprouts, roots and leaves decreased, and lettuce roots were longer. Therefore, the division index for the molecular weight of DOM fractions to accelerate or inhibit copper toxicity was 1 kDa. Copper in complexes with molecular weight more than 1 kDa had lower toxicity.

Effect of molecular weight of DOM on copper toxicity was determined by stability of the copper complex. The fractions of DOM with larger molecular weight had higher ability to form copper complex and resulted in higher stability constant (log$k$) and less free Cu^{2+} ion in solution, which caused the total copper content in the sprouts, or in leaves and roots to be lower, and resulted in lower toxicity.

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