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Cu(II) complexation of high molecular weight (HMW) fluorescent substances in root exudates from a wetland halophyte (*Salicornia europaea* L.)

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High molecular weight (HMW) fractions are important components in root exudates. However, there is little available information concerning complexation of Cu(II) to the HMW fractions in root exudates. In the present study, complexation of root exudates from *Salicornia europaea* L. with Cu(II) was investigated using excitation emission matrix (EEM) fluorescence spectroscopy. Two protein-like fluorescence peaks were identified in the EEM spectra of root exudates. Fluorescence of both peaks was clearly quenched by Cu(II). The increase of conditional stability constant with increasing temperature indicates that the fluorescence quenching of the protein-like fluorescence by Cu(II) may be controlled by a dynamic process. The values of conditional stability constants ($\log K_a$) were in the range of 4.32–4.69, which were close to those of complexation of fulvic acid with Cu(II). This shows that the HMW fluorescent substances in root exudates from *S. europaea* L. were strong organic ligands for Cu(II). Our study suggests that the HMW fluorescent substances may affect chemical forms, mobility, and thus the fate of copper in wetland.

[**Key words:** Binding; Copper; Fluorescence quenching; Halophyte; Root exudates]

Salt marshes are important sink for heavy metals (1). Copper is one of the commonly found heavy metals that accumulate in salt marshes at high levels (2). Concentrations of Cu in salt marshes can be from several hundreds to several thousands of mg kg^{-1} soil (2,3). Copper is an essential trace element for plant growth, but it is highly toxic to plants or animals (4) or microorganisms (5,6) at high levels.

Plants can release soluble organic substances from their roots, including low molecular weight (LMW) organic acids and high molecular weight (HMW) polysaccharides and other organic substances (7). The root exudates may provide nutrients for plant growth and carbon source for rhizospheric microorganisms. They have also been demonstrated to affect solubility, mobilization, and phytoavailability of heavy metals (8)]. A lot of studies show that root exudates can improve the solubility of heavy metal ions, such as Cu(II), Pb(II), and Cd(II), in the soil (9,10), and thus enhance metal accumulation in plants (11–13). The metal mobilization of root exudates are frequently attributed to the strong metal complexing ability of the LMW fraction of root exudates, especially the LMW organic acids (11). On the contrary, few studies reported that root exudates did not enhance the mobilization and phytoavailability of metals (14,15). Besides, Hill and Lion (15) showed that phytosiderophore 2′-deoxymugineic acid (DMA) reduced Cd accumulation in *Zea mays* due to the formation of DMA–Cd complex, which might decrease the availability of Cd to *Z. mays*.

The HMW fraction is mainly composed of polysaccharides and proteins (16,17). The HMW fraction can act as cation exchangers. Limited studies showed that they had high binding capacity for metals (18,19). Nevertheless, very limited information was available on quantitative analysis of complexation of HMW fraction with metal ions.

The fluorescence excitation emission matrix (EEM) spectrometry is a rapid and sensitive modern technique that provides qualitative and quantitative information on interaction of fluorescent organic matter and metal ions (20–22). EEM fluorescence spectroscopy has been extensively used to investigate interaction of dissolved organic matter (DOM) and metals (20,21). Since proteins are also key components of root exudates and they show fluorescent properties, EEM fluorescence spectroscopy should also be a powerful technology for characterization of the fluorescent components in root exudates and their interaction with metals.

The aim of the present study was to investigate complexation of the HMW fraction of root exudates from *Salicornia europaea* with Cu (II) using EEM fluorescence spectroscopy.

MATERIALS AND METHODS

Collection of root exudates *S. europaea* is selected as the test plant species because it is a wetland halophyte species (Chenopodiaceae) that is commonly found in coastal and inland salt marshes (23). Three-month-old *S. europaea* L. seedlings were collected from one salt marsh near Urumqi, Xinjiang, China. The soil attached to roots of the seedlings was gently removed with tap water. The seedlings were then hydroponically grown in Hoagland solution (24) containing 400 mM NaCl at day/

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night temperatures of 28/18°C with an average PAR at midday of 1000 μmol m^{-2} s^{-1}. After 3 weeks, root exudates were collected according to the method of Luo et al. (12). The collected root exudate solution was filtered through 0.22-μm membrane into an autoclaved glass tube. Once the root exudates were collected, they were immediately used for fluorescence titration test.

Preparation of copper solution Stock copper solution (0.5 M) was prepared by dissolving $CuSO_4$ of analytical grade in Milli-Q water.

Fluorescence quenching titration The fluorescence spectra of root exudates were recorded with a fluorescence spectrophotometer (F-7000, HITACHI, Japan). A 450-W Xenon lamp was used as the excitation source. EEM were collected every 5 nm over an excitation range of 200–400 nm, with an emission range of 200–550 nm by 2 nm. The excitation and emission slits were set to 5 and 5 nm of band pass, respectively. Scan speed was 1200 nm/min. The fluorometer's response to a Milli-Q water blank solution was subtracted from the fluorescence spectra recorded for samples containing root exudates and Cu(II) under the same conditions. The EEM data were processed using the software SigmaPlot 10.0 (Systat, US). All the experiments were triplicated, and the mean values were used.

Root exudate solution was titrated with incremental μl additions of 0.1 M Cu(II) at 278, 298, and 308 K, respectively. After each addition of Cu(II), the solution was fully mixed using a magnetic stirrer for 15 min, and the fluorescence spectra were recorded.

Data analysis All the fluorescence titration data were fitted with the Stern–Volmer equation (25):

$$\frac{F_0}{F} = 1 + K_{sv}[\mathrm{Cu(II)}] = 1 + k_q\tau_0[\mathrm{Cu(II)}] \quad (1)$$

where F_0 and F are the steady-state fluorescence intensities in absence and in presence of quencher, respectively. K_{sv} is the Stern–Volmer quenching constant, and [Cu(II)] is the concentration of quencher. k_q is the quenching rate constant of the biological macromolecule. τ_0 is the average lifetime of the molecule without any quencher, and the fluorescence lifetime of the biopolymer is 10^{-8} s (25). A linear Stern–Volmer plot generally indicates the presence of one class of fluorophores that are equally accessible to quencher. For dynamic quenching, the maximum dynamic quenching rate constant (k_q) of various quenchers is 2.0×10^{10} l/(mol s) (25).

The quenching data were further analyzed using the modified Stern–Volmer equation (25):

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} = \frac{1}{f_a K_a}\frac{1}{[Cu(II)]} + \frac{1}{f_a}. \quad (2)$$

where K_a is the conditional stability constant (effective quenching constant) for the accessible fluorophores, and f_a is the fraction of accessible fluorescence.

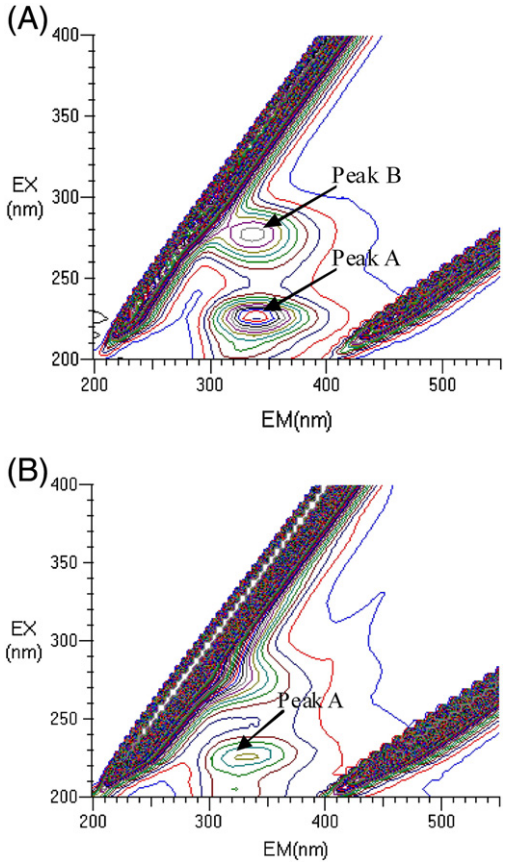

FIG. 1. EEM spectra of root exudates in absence (A) and in presence (B) of 0.1 M Cu(II) at 308 K.

RESULTS AND DISCUSSION

Root exudates Two fluorescent peaks were identified in the EEM spectra of root exudates from *S. europaea* L. seedlings (Fig. 1A and B). Peak A was at Ex/Em = 225/336~340 nm, and peak B was at Ex/Em = 275/ 326-336 nm. Fluorescence of peaks A and B can be attributed to protein-like fluorescence (20,26–28). Fluorescence intensities of both peaks were significantly reduced after addition of 0.1 M Cu(II) (Fig. 1A and B). Peak B even disappeared in the presence of 0.1 M Cu(II). This means that 0.1 M Cu(II) can quench most of the peak B fluorescence because most of the binding sites of the fluorophores represented by peak B were occupied by 0.1 M Cu(II). For peak A, 0.1 M Cu(II) was not enough to occupy most of the binding sites of fluorophores represented by peak A.

Fluorescence quenching titration The fluorescence intensities of peaks A and B decreased markedly with increasing Cu(II) concentration at all experimental temperatures (Fig. 2A and B), indicating that fluorescence of peaks A and B was quenched by Cu(II). It was also shown that initial fluorescence intensities of both peak A and peak B decreased with increasing temperature.

Fluorescence quenching mechanisms Two types of quenching mechanisms are usually involved in fluorescence quenching: the dynamic quenching and the static quenching. The dynamic quenching originates from collision between the fluorophore and quencher, while the static quenching is attributed to the formation of a ground-state complexation of the fluorophore with the quencher (25).

Fig. 3A showed that all the fluorescence quenching titration data for peak A were well represented by the Stern–Volmer equation (R^2 = 0.969–0.972). This implies that the fluorescence quenching is governed by either a dynamic process or a static process singly. The values of K_{sv} and k_q were summarized in Table 1. k_q was greater than 2.0×10^{10} l/(mol s), suggesting that the fluorescence quenching may be a dynamic process. For peak B, the fluorescence titration data were poorly fitted by the Stern–Volmer equation (Fig. 3B, R^2 = 0.831–0.909). This nonlinearity of Stern–Volmer plot may be interpreted that the dynamic quenching and static fluorescence quenching occur simultaneously or that nonlinear binding isotherm involving a significant occupation of binding sites is present and the free quencher concentration decreases (29).

Good linear relationship between $F_0/(F_0-F)$ and $[\mathrm{Cu(II)}]^{-1}$ for peaks A and B was observed (Fig. 4, R^2 = 0.964–0.997). The values of conditional stability constant, logK_a, for peaks A and B were 4.32–4.51 and 4.32–4.69, respectively (Table 2). The close values of logK_a for peaks A and B imply that the fluorophores represented by peak A has similar binding capacity for Cu(II) to peak B. The value of logK_a for peaks A and B generally showed an increasing trend with increasing temperature, indicating that fluorescence quenching of the HMW root exudates by Cu(II) was governed by a dynamic process. The conditional stability constants for peaks A and B were close to those for complexation of fulvic acid with Cu(II) (4.34–5.06) (30) but smaller than those for Cu(II) complexation of organic ligands in river (7.21–7.31) and in lake (7.84–9.23) (20). The fluorescence quenching of fulvic acid by Cu(II) was attributed to changes of the electronic polarization of both the metal ion and the binding site in humic substance molecules and effects of paramagnetic metal ions (31). The difference of conditional stability constants for Cu(II) complexation of earthworm mucus and river organic ligands may be due to their different structures and binding site concentrations (30).

The values of logK_a for complexation of HMW fraction of root exudates with Cu(II) in the present study were close to overall

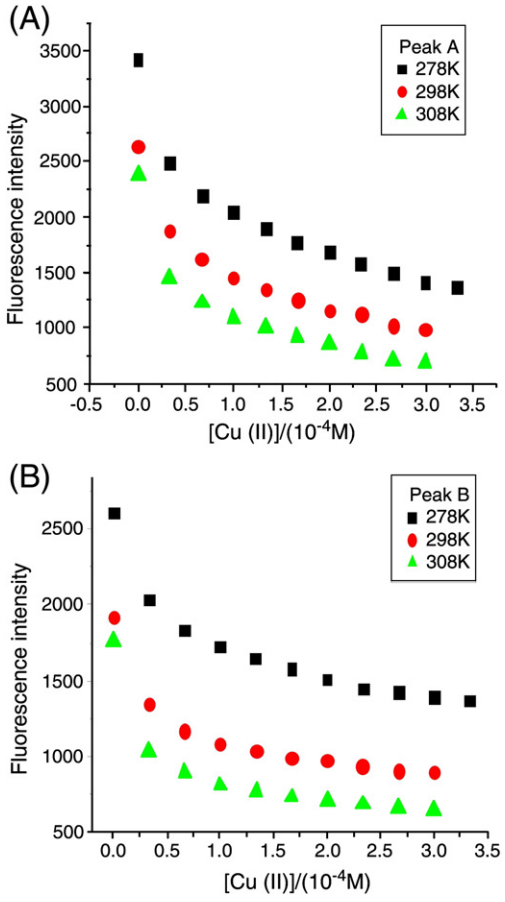

FIG. 2. Fluorescence intensities of peak A (A) and peak B (B) varies with increasing Cu(II) concentration.

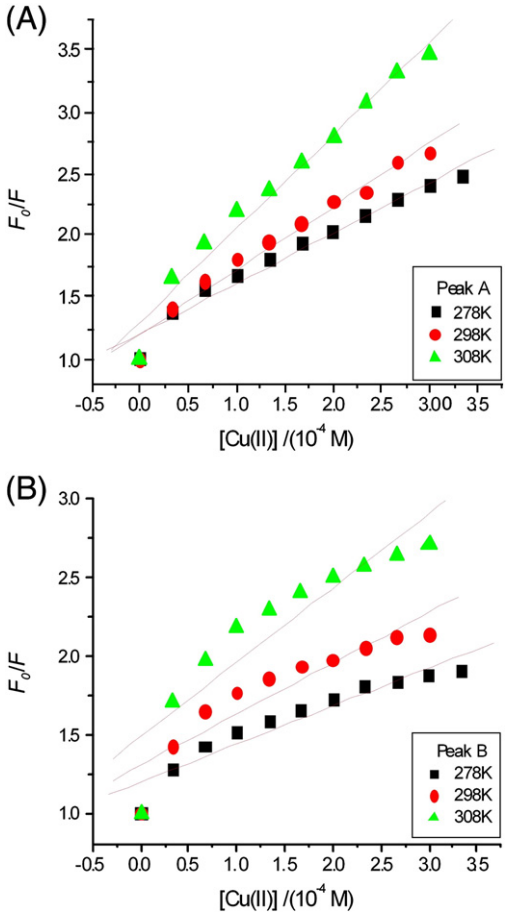

FIG. 3. Plots of F_0/F versus [Cu(II)] at various temperatures.

stability constants (log*K*) for complexation of the whole root exudates with Cu(II) and other heavy metals, determined by other methods (18,19). Morel et al. (18), reported that the overall stability constant (log*K*) values for complexation of root mucilage from maize (*Z. mays* L.) with Cu(II), Pb(II), and Cd(II) were 4.14–5.4, 4.17–5.6, and 4.17–5.3, respectively. In their study, the root mucilage was mainly composed of HMW polysaccharides, proteins, and uronic acids. The overall stability constants were determined from the data of equilibrium dialysis using the linear Langmuir equation. Mench et al. (19) , using dialysis and ion-selective electrode titrations, estimated the values of overall stability constants (log*K*) of complexation of soluble HMW exudates from maize (*Z. mays* L.) with metals (Pb, Cu, Cd, and Zn) to be 3.65–3.15. The overall stability constant of complexation of soluble HMW exudates with Cu(II) was 3.4. Although the values of stability constants for complexation of root exudates with heavy metals were close in these studies and the present study, caution should be taken when they are compared with each other because of the different methods used. In our study, the conditional stability constants were determined through EEM fluorescence titration. Despite of its advantages including rapidness, high sensitivity, and simplicity, EEM fluorescence titration was only applicable to the fluorescent components in root exudates. This means that binding capacity of other important HMW substances that do not emit fluorescence cannot be determined by this method and the conditional stability constant estimated by this method may be underestimated.

The interaction of root exudates and Cu(II) from *S. europaea* L. was investigated by EEM fluorescence spectroscopy. Two protein-like fluorescence peaks were identified in the EEM spectra of root exudates and fluorescence of both peaks could be quenched by Cu (II). The values of conditional stability constant (4.32–4.69) were close to those of complexation of fulvic acid with Cu(II), indicating that the HMW fluorescent components in root exudates from *S. europaea* L. were strong organic ligands for copper. The conditional stability constant generally increased with increasing temperature, suggesting that the fluorescence quenching of the protein-like fluorescence by Cu(II) may be controlled by a dynamic process.

The strong Cu(II) complexing ability of the HMW fluorescent substances in root exudates implies that they may affect transport and fate of copper in environments. Furthermore, the HMW fluorescent substances are essential components in root exudates and constantly released into environment in huge quantity; therefore, enough attention should be paid to their influence on chemical forms, mobility, bioavailability, and other biogeochemical processes of copper. Effects of the HMW fractions in root exudates on phytoavailability of heavy metals and adsorption/desorption of heavy metals in soil need further study.

In addition, although this study shows that fluorescence EEM spectroscopy is a powerful tool for examination of interaction of root

TABLE 1. The Stern–Volmer quenching constants for peak A.

T (K)	$K_{sv}(\times 10^3\ M^{-1})$	$k_q(\times 10^{11}\ M^{-1}\ s^{-1})$	R^2	SD
278	4.087	4.087	0.969	0.085
298	5.169	5.169	0.970	0.097
308	7.557	7.557	0.972	0.137

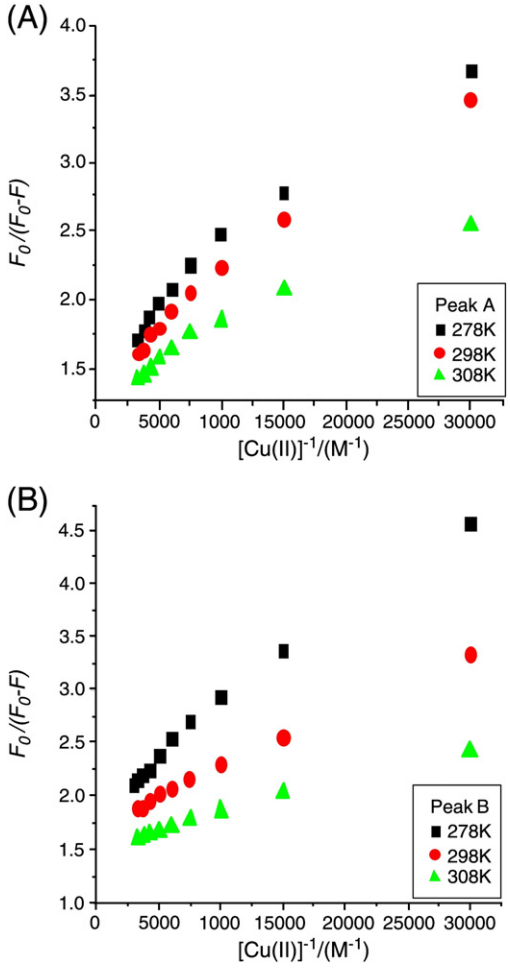

FIG. 4. Modified Stern–Volmer plots for the quenching of root exudates by Cu(II) at three different temperatures.

exudates and metals, this technology can only detect the fluorescent components but cannot detect other components in root exudates that do not emit fluorescence. Other methods such as infrared spectroscopy (IR), electrochemical analysis, and nuclear magnetic resonance (NMR) should be combinedly used in order to comprehensively understand the effects of root exudates on transport and fate of heavy metals in soil.

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TABLE 2. The conditional stability constant, logK_a, for peaks A and B derived from modified Stern–Volmer equation.

	Peak A		Peak B	
T (K)	logK_a	R^2	logK_a	R^2
278	4.35	0.973	4.32	0.987
298	4.32	0.985	4.50	0.997
308	4.51	0.964	4.69	0.990

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