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Molecular and Quantitative Analysis of Metal Ion Binding to Humic Acids from Sewage Sludge and Sludge-Amended Soils by Fluorescence Spectroscopy

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Humic acids (HAs) isolated from sewage sludge (SS) and control and SS-amended soils were characterized by fluorescence spectroscopy. The main feature of fluorescence spectra was a broad band with the maximum centered at excitation/emission wavelengths that were much shorter for SS-HA (340/438 nm) than for any soil HA (440/510 nm). Titration with Cu(II), Zn(II), Cd(II), and Pb(II) ions decreased fluorescence intensities of HAs. Titration data were fitted to a single-site fluorescence quenching model, and metal ion complexing capacities of each HA sample and stability constants of metal ion–HA complexes were calculated. The binding capacities of HAs and strengths of metal ion–HA complexes followed the order Pb(II) > Cu(II) > Cd(II) > Zn(II). With respect to the control soil HA, SS-HA, which showed the smallest contents of acidic functional groups and the lowest humification degree, was characterized by much smaller binding capacities and stability constants. The binding capacities and affinities of SS-amended soil HA were intermediate between those of native soil HA and SS-HA but closer to the former, thus suggesting a partial incorporation of HA fractions of SS into native soil HAs. These effects are expected to have a great impact on the behavior of metals in SS-amended soils.

Introduction

Land application is considered as the most environmentally safe, economically advantageous option for sewage sludge (SS) disposal, based on the agronomic benefit of adding nutrients and organic matter to soil (*1–5*). However, relatively high levels of potentially toxic trace metals, including Cu, Zn, Pb, and Cd, which are typically present in SS from heavily urbanized and industrialized areas, may pose a serious threat to the environment (*6–8*). In particular, land-applied trace metals derived from SS may accumulate to phytotoxic levels in SS-amended soils (*9*), may be taken up by crops in amounts potentially dangerous to food chain (*10*), and/or may be transported through runoff and leaching processes affecting surface and groundwater quality (*11*).

The total contents of trace metals in SS-amended soil are governed by the total content of metals in SS and by the rate of SS application (*12*). However, the mobility and bioavailability of trace metals in SS-amended soils are closely related to the chemical transformations that these metals undergo in the soil environment and to their distribution in soil solid and liquid phases (*12*, *13*). According to Sposito et al. (*12*), the application of SS tended to increase the organic fractions of Cu, Zn, Pb, and Cd in SS-amended soils.

Humic substances (HS) are the most abundant and reactive components of soil organic matter. Humic acids (HAs) and fulvic acids (FAs) represent the major fraction of HS which feature a colloidal, polydispersed, and polyelectrolytic character, and a mixed aliphatic and aromatic nature. The capacity to bind metal ions is one of the most important physicochemical properties of HS. This property is mainly attributed to the large content of oxygenated reactive functional groups of HS, which include carboxylic, phenolic, alcoholic, and enolic hydroxyl groups, and carbonyl functionalities of various types. Nitrogen-, S- and P-containing functional groups may also be involved in metal ion binding by HAs (*14*, *15*). Most processes in which metal ions are involved in soils, including mobility and transport, fixation and accumulation, chemical reactivity, and bioavailability, are affected by their interaction with HS (*16*). For these reasons, the effects of SS application on metal ion binding properties of native soil HS is of great environmental and agronomic importance.

Depending on the pH value, the presence of salts (ionic strength effect), and the degree of saturation of binding sites, HS can form either soluble or insoluble complexes with metal ions and, therefore, play a double role in soil. Because of their smaller molecular weight and larger contents of acidic functional groups, FAs can form metal complexes that are more soluble, bioavailable, and mobile than those formed by HAs. Thus, FAs can act as carriers of metal ions in soil solution, whereas HAs tend to immobilize and accumulate metal ions in soil solid phases (*14*).

The structural chemistry of HAs and FAs in SS and in SS-amended soils has been extensively studied previously (*2*, *3*, *17–21*). Generally, HAs and FAs in organic amendments feature smaller acidic functional group contents, larger aliphatic character, and smaller degrees of aromatic polycondensation and humification than native soil HAs and FAs. Application of SS is known to affect the status of soil HA and FA and influence their properties on dependence on SS nature, origin, and application rate. Further, extensive investigations on the trace metal reactions of HAs and FAs isolated from SS and SS-amended soils have been carried in the past (*22–28*). However, to the best of our knowledge metal ion binding to HAs from SS and SS-amended soils has not been studied yet by use of fluorescence spectroscopy.

Fluorescence spectroscopy is a simple, sensitive, and nondestructive analytical technique that has the potential to provide invaluable information on the molecular structure, functionalities, and conformation, as well as intramolecular and intermolecular interactions of HS (*29–31*). In particular, fluorescence spectroscopy has been revealed as a very promising technique for the study of metal ion binding to HS (*16*, *32–39*). In general, metal ions, and especially paramagnetic metals, are able to quench the intrinsic fluorescence of HS ligands (*16*). Monitoring the changes of fluorescence spectra of HAs upon titration with metal ions can thus provide unique qualitative and quantitative information on metal solution equilibria. In particular, metal ion complexing capacities of HA and stability constants of metal

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ion−HA complexes, which are important parameters for predicting the behavior and performance of metal ions in soils, can be determined using the fluorescence titration technique and the mathematical approach proposed by Ryan and Weber (*40*). This method is based on the existence of a linear relationship between the fraction of the total ligand bound and fluorescence intensity and on the assumption of a simple 1:1 stoichiometry for metal ion−ligand binding.

The objectives of this work were (a) to determine and discuss comparatively the Cu(II), Zn(II), Cd(II), and Pb(II) complexing capacities of SS-HA and stability constants of complexes of SS-HA with these ions, with respect to those of unamended soil HA; (b) to investigate the effect of SS application on metal ion binding behavior of soil HAs. To reach these objectives, fluorescence titration and the single-site model proposed by Ryan and Weber (*40*) were used.

Experimental Section

Sewage Sludge, Soils, and Humic Acids. The SS sample used in this work was collected in a SS treatment plant located in Bari, Italy, where SS were subjected to a conventional biological treatment followed by aerobic stabilization. The composition of the SS sample was the following: dry matter content, 16.4 g kg^{-1}; pH, 6.9; electrical conductivity, 3.2 dS m^{-1}; total organic C, 384.7 g kg^{-1}; total N content, 31.6 g kg^{-1}; and total contents of Cu, Zn, Cd, and Pb, 254.3, 682.0, 19.3, and 40.3 mg kg^{-1}, respectively.

The soil amended with 40 tons ha^{-1} of SS (SS40) and the corresponding nonamended control soil (SS0) were sampled from the top layer (Ap horizon, 0−15 cm depth) of a Typic Xerorthent in an agricultural field in the Bari province, having a silty clay texture (sand, 183 g kg^{-1}; silt, 388 g kg^{-1}; clay, 429 g kg^{-1}); pH (H_2O), 8.1; pH (HCl), 7.0; electrical conductivity (soil/water = 1:10), 0.6 dS m^{-1}; cation exchange capacity, 28.2 cmol kg^{-1}; total organic C, 11.2 g kg^{-1}; total N content, 1.07 g kg^{-1}; and total contents of Cu, Zn, Cd, and Pb, 52.3, 118.7, 1.3, and 44.4 mg kg^{-1}, respectively. Soil samples were collected approximately 9 months after SS application, which is generally considered to be enough time for allowing an adequate incorporation of organic amendment into native soil organic matter.

Plant residues and stones were removed from the soil, and soil and sludge samples were then air-dried and passed through a 2-mm sieve. Carbonates were removed from soil samples by mechanical stirring with H_3PO_4 2 M for 30 min. The treatment was repeated three times. Then soil samples were washed with distilled water until the suspension reached a pH of 7. Humic acids were isolated from the SS sample and the free-carbonate soils according to conventional procedures (*41*).

Fluorescence Measurements. A stock solution was prepared for each HA by dissolving 50 mg of freeze-dried HA in 100 mL of 1 M KOH. The mixture was stirred for 30 min under N_2 gas, and then 100 mL of 1 M HNO_3 was added. Subsequently, the solution was brought to a volume of 950 mL with deionized distilled water, the pH was adjusted to a value of 6 by addition of 0.1 M KOH, and the volume was finally brought to 1 L with deionized distilled water. Aliquots of 100 mL of the stock solution of HA were titrated in 150-mL thermostatic vessels with 0.01 M $Cu(NO_3)_2$, $Zn(NO_3)_2$, $Cd(NO_3)_2$, and $Pb(NO_3)_2$ by using an automatic syringe. To maintain a constant pH, 0.1 M KOH was dispensed using another automatic syringe. Samples were maintained at a constant stirring speed, at a temperature of 298 K, and under N_2 atmosphere throughout the titrations. After each addition of titrant, the solution was circulated via a peristaltic pump through a quartz flow-through cell for fluorescence spectral recording and back to the titration vessel for 15 min.

Three-dimensional fluorescence spectra in the form of excitation−emission matrix (EEM) plots were recorded using a Perkin-Elmer (Norwalk, CT) LS 55 luminescence spectrometer equipped with the WinLab 4.00.02 software (Perkin-Elmer, Inc., 2001, Norwalk, CT) for data processing. Emission and excitation slits were set at a 5-nm bandwidth, and a scan speed of 500 nm min^{-1} was selected for the emission monochromator. The wavelength emission range was from 350 to 550 nm, while the excitation wavelength was increased sequentially from 300 to 500 nm by 5-nm steps. Excitation−emission matrix plots were generated from fluorescence spectral data using the Surfer 8.01 software (Golden Software, Inc., 2002, Golden, CO). Since the HA concentration used in the present study was relatively low, fluorescence spectra were not corrected for inner filter and absorption effects. Similarly, these effects were not accounted in several previous studies of metal ion binding to HS by fluorescence spectroscopy (*16*, *32−39*).

Determination of Complexing Capacities and Stability Constants. The Cu(II), Zn(II), Cd(II), and Pb(II) complexing capacities of HAs and the stability constants of Cu(II), Zn(II), Cd(II), and Pb(II) complexes with HAs were determined by the single-site fluorescence quenching model of Ryan and Weber (*40*). This model is based on the assumption that metal ion binding occurs at identical and independent binding sites or ligands and only 1:1 metal/ligand complexes are formed. Although the assumption of 1:1 stoichiometry is not accurate for metal ion binding to HAs, and may result in small complexing capacity values as compared with the corresponding acidic functional group contents, this assumption simplifies the theoretical analysis and is used and justified in most studies on metal ion binding to HS (*16*, *32−40*).

The complexation of a metal ion (M) by an organic ligand (L) (i.e., the formation of an ML complex) at constant pH and ionic strength may be described by the conditional stability constant

$$K_M = \frac{[ML]}{[M][L]} \tag{1}$$

where [M] denotes the free metal molar concentration, [L] is the molar concentration of all forms of ligand which are not bound to M, and [ML] is the molar concentration of the ML complex.

The mass balance equations for the metal ion and the ligand are

$$C_M = [M] + [ML] \tag{2}$$

$$C_L = [L] + [ML] \tag{3}$$

where C_M and C_L are the stoichiometric concentrations of the metal ion and the ligand, respectively. The complexation capacity (CC_M), i.e., the amount of active binding sites per unit mass of HA, is related to C_L by

$$CC_M = \frac{C_L}{(HA)_{total}} \tag{4}$$

where $(HA)_{total}$ is the total concentration of HA.

Equations 1 and 3 may be combined to obtain the fraction of ligand bound (ν) expressed in terms of the stability constant and free metal ion concentration:

$$\nu = \frac{[ML]}{C_L} = \frac{K_M[M]}{1 + K[M]} \tag{5}$$

Combining with eq 2, eq 5 assumes the form

$$\nu = \frac{K_M(C_M - \nu C_L)}{1 + K_M(C_M - \nu C_L)} \quad (6)$$

Equation 6 can be solved for ν giving

$$\nu = \left(\frac{1}{2K_MC_L}\right)(1 + K_MC_L + K_MC_M - \sqrt{(1 + K_MC_L + K_MC_M)^2 - 4K_M{}^2C_LC_M}) \quad (7)$$

According to Ryan and Weber (*40*), the measured fluorescence intensity (I) varies linearly with the fraction of the total ligand bound yielding the following relationship

$$\nu = \frac{(I_0 - I)}{(I_0 - I_{ML})} \quad (8)$$

where I_0 is the fluorescence intensity at the beginning of titration (i.e., the fluorescence intensity of the metal-free HA) and I_{ML} is the limiting value below which the fluorescence intensity does not decrease due to the addition of metal ion (i.e., the fluorescence intensity of the metal-saturated complex).

Combination of eqs 7 and 8 and rearrangement yield

$$I = I_0 + (I_{ML} - I_0)\left(\frac{1}{2K_MC_L}\right)(1 + K_MC_L + K_MC_M - \sqrt{(1 + K_MC_L + K_MC_M)^2 - 4K_M{}^2C_LC_M}) \quad (9)$$

By introducing the fluorescence intensity values determined experimentally at the various total metal ion concentrations used, eq 9 can be solved by nonlinear regression analysis for K_M, C_L, and I_{ML}. The optimum set of fitting parameters for each HA sample was thus obtained by iteratively varying the adjustable parameter values until the sum of the squares of the differences between observed and fitted values of I was minimized. Full, unconstrained optimization was achieved using the Quasi-Newton algorithm. The computer program Statistica 4.0 for Windows (Statsoft, Inc., 1993, Tulsa, OK) was used for calculations.

Results and Discussion

Acidic Functional Group Contents of Humic Acids. The acid–base properties of HAs examined in this paper have been previously described (*42*). Briefly, results obtained indicated that the total acidity and carboxyl and phenolic OH group contents of SS-HA (3.38, 2.17, and 1.21 mmol g^{-1}, respectively) are smaller than those of nonamended soil HA (6.62, 4.42, and 2.20 mmol g^{-1}, respectively), whereas these contents (5.54, 3.65, and 1.89 mmol g^{-1}, respectively) are intermediate for SS-amended soil HA. In general, the larger acidic functional group contents measured for soil HAs, with respect to that of SS-HA, may be related to the aerobic conditions in which the former samples are formed. Well-aerated conditions existing in soils, in contrast to the prevalent anaerobic conditions in which SS are produced, may favor oxidation reactions leading to the formation of acidic oxygenated functional groups.

Fluorescence Spectra of Humic Acids. The fluorescence spectra of the HAs measured in the absence and presence of Cu(II), Zn(II), Cd(II), or Pb(II) at a total concentration of 40 μM are shown in Figure 1 as EEM plots. The fluorescence EEM spectrum of SS-HA is characterized by a unique main fluorophore centered at an excitation/emission wavelength pair of 340/438 nm whose relative intensity is 176 arbitrary units (a.u.). The fluorescence EEM spectrum of SS0-HA is very different from that of SS-HA and features a prominent peak of greater relative intensity (320 a.u.) at long excitation/emission wavelengths (440/510 nm) accompanied by a broad shoulder that extends to shorter excitation wavelengths and a less intense peak (247 a.u.) at 325/508 nm. The fluorescence EEM spectrum of SS40-HA resembles that of SS0-HA but is characterized by less intense fluorescence peaks at 440/510 nm (215 a.u.) and 325/509 nm (198 a.u.).

In general, the fluorescence features of HA samples here examined are typical of HAs of similar origin and nature (*30*, *38*, *43*). The short wavelength measured for the main fluorescence peak of SS-HA suggests the presence of simple structural components of wide molecular heterogeneity and small molecular weight, low degree of aromatic polycondensation, low level of conjugated chromophores, and low humification degree (*29*). On the contrary, the long wavelength of the major peak of both soil HAs may be ascribed to the presence of an extended, linearly-condensed aromatic ring network and other unsaturated bond systems capable of a great degree of conjugation in large molecular weight units of great humification degree (*29*).

Generally, SS-HAs are characterized by small free radical concentrations and relatively large contents of carbohydrates and electron-withdrawing carboxylic groups (*2*, *3*). These structural features may be responsible of the small fluorescence intensity observed for SS-HAs (*29*, *31*, *44*). On the contrary, the large fluorescence intensity of the main peak of SS0-HA would reflect a relatively large presence of electron-donating substituents, such as hydroxyl and methoxyl groups, which are reported to enhance fluorescence by increasing the transition probability between the singlet and ground state (*29*, *31*, *44*). The smaller fluorescence intensity of the main peak of SS40-HA, with respect to that of SS0-HA, suggests a partial incorporation of simple and low humified components of SS-HA into native soil HA.

Addition of Cu(II), Zn(II), Cd(II), or Pb(II) ions to HA solutions causes relevant changes of fluorescence EEM spectra on dependence of HA origin and the metal species, which suggest the occurrence of marked modifications of electronic structure of HAs upon interaction with metal ions. In particular, in agreement with previous findings on similar systems (*36*), HA fluorescence is quenched by addition of Zn(II) and Cd(II), and especially Cu(II) and Pb(II) (Figure 1). Further, Cu(II), Cd(II), and Pb(II) produce a slight increase (i.e., a red shift) of the excitation maximum wavelength of SS-HA, which may be related to aggregation of HA macromolecules upon binding to metal ions (*45*). On the contrary, the wavelengths of excitation/emission peaks of SS0-HA remain almost constant upon any metal addition. In the case of SS40-HA, Cu(II) and Pb(II) cause an apparent shift of the emission maximum wavelength to shorter values (i.e., a blue shift), most probably because of the large quenching effect of these metal ions on HA fluorescence at long excitation and emission wavelengths.

Metal Ion Binding Parameters of Humic Acids. The experimental values (dots) of fluorescence intensity of the main HA fluorophore as a function of metal ion concentration and the non linear regressions generated by best-fitting these data in the model of Ryan and Weber (*40*) are shown in Figure 2. The corresponding best-fit parameters obtained for each HA, i.e., the fluorescence intensities of metal ion-saturated–HA complexes (I_{ML}), the correlation coefficients of predicted versus measured fluorescence intensity (r_M), the stability constants (log K_M) of metal ion–HA complexes, and the metal ion complexing capacities (CC_M) of HAs are listed in Table 1. The large values found for the correlation coefficients (r_M) indicate that the model fits very well to the experimental data sets for the HAs examined, as was previously demonstrated in other works on similar systems (*33*, *36*, *38*, *46*). The stability constants of metal ion–HA complexes and the complexing capacities of HAs are described and discussed comparatively in the following text.

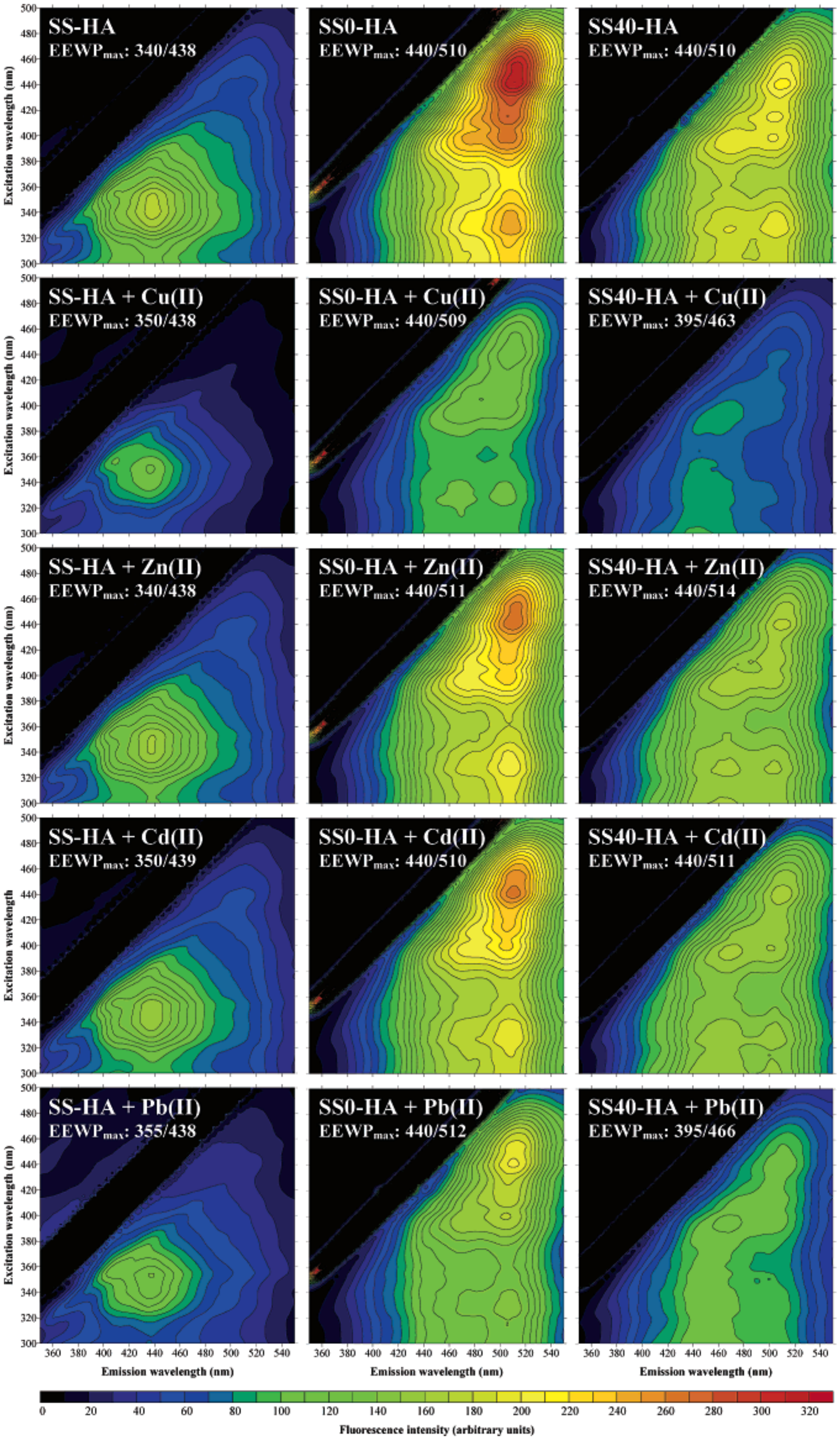

FIGURE 1. Fluorescence excitation−emission matrix spectra of humic acids (HAs) isolated from sewage sludge (SS), soil amended with SS at a rate of 40 tons ha^{-1} (SS40), and the corresponding nonamended control soil (SS0), in the absence or presence of Cu(II), Zn(II), Cd(II), and Pb(II) at a total concentration of 40 μM. EEWP$_{max}$: excitation/emission wavelength pairs at the maximum fluorescence intensity.

Metal Ion Binding Parameters of Humic Acids: Stability Constants. For any HA examined, the decreasing stability of metal ion−HA complexes, as indicated by the decreasing log K_M values, was in the order Pb(II) > Cu(II) > Cd(II) > Zn(II). This order of metal ion complex stabilities is in agreement with that reported by Irving and Williams (*47*), regardless of the nature of the complexing agent involved, and also agrees with findings of other authors for divalent metal ion complexes with HS of various origin and nature (*36*, *48*, *49*).

The stability constants of Cu(II), Zn(II), Cd(II), and Pb(II) complexes with SS-HA are smaller than those with SS0-HA, whereas the log K_M values of SS-amended soil HAs are, as may be expected, intermediate between the former and the latter values. The relatively small stability constants of SS-HA may be ascribed to its ascertained typical aliphatic character and small degree of aromatic polycondensation and humification (*2*, *3*, *21*). On the other hand, the large stability constants measured for SS0-HA may be related to

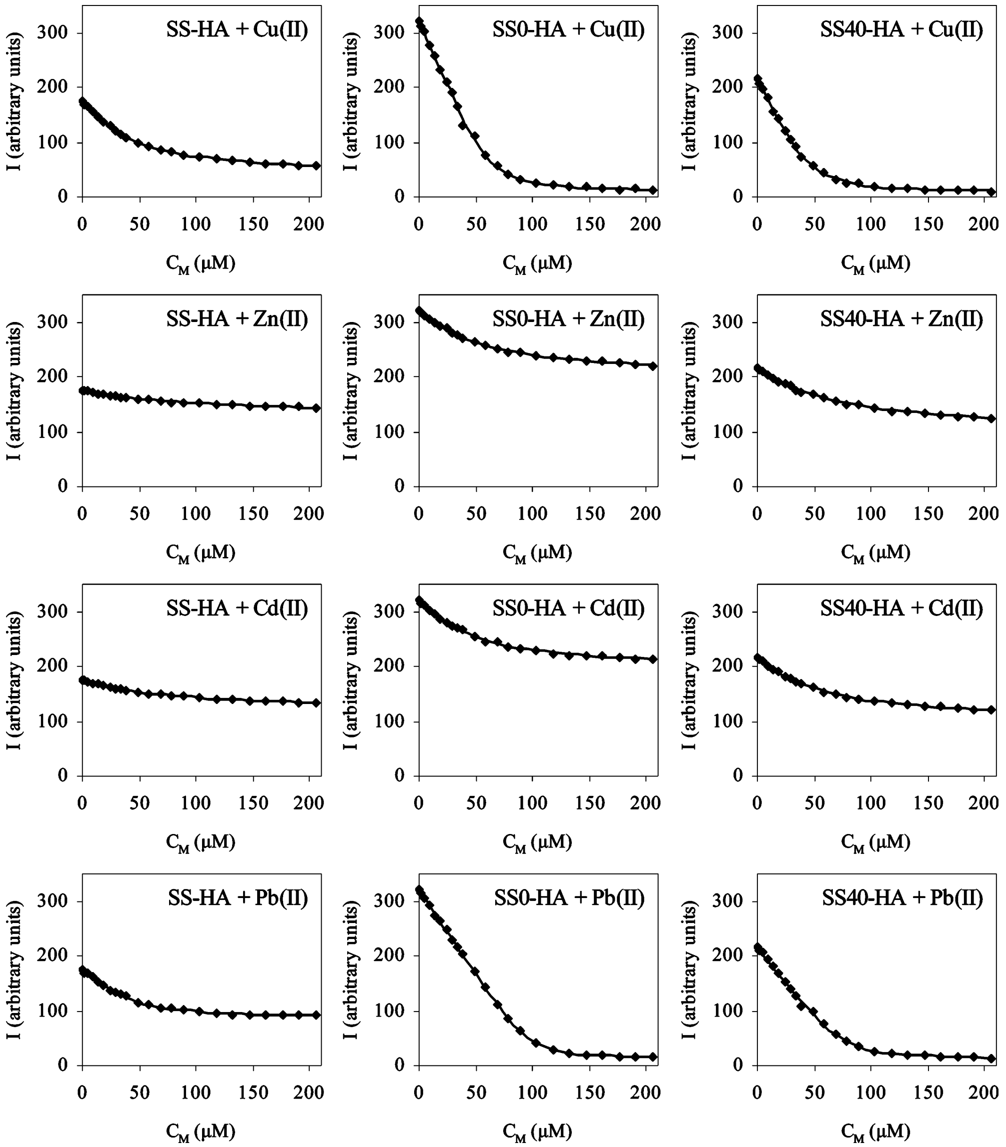

FIGURE 2. Experimentally determined values (dots) and model-derived nonlinear regressions of fluorescence intensity (I) of the main peak in fluorescence spectra of humic acids (HAs) isolated from sewage sludge (SS), soil amended with SS at a rate of 40 tons ha^{-1} (SS40), and the corresponding nonamended control soil (SS0) as a function of increasing total concentration (C_M) of Cu(II), Zn(II), Cd(II), and Pb(II).

TABLE 1. Fitting Parameters of the Ryan−Weber Model, i.e., Fluorescence Intensity of Metal Ion-Saturated Complex (I_{ML}, Arbitrary Units), Correlation Coefficient of Predicted vs Measured Fluorescence Intensity (r_M), Stability Constant (log K_M), and Complexing Capacity (CC_M, mmol g^{-1}), for Cu(II), Zn(II), Cd(II), and Pb(II) Binding to Humic Acids (HAs) Isolated from Sewage Sludge (SS), Soil Amended with SS at a rate of 40 tons ha^{-1} (SS40), and the Corresponding Nonamended Control Soil (SS0)

	origin of HA sample				origin of HA sample		
parameter	SS	SS0	SS40	parameter	SS	SS0	SS40
I_{CuL}	42.1	7.0	5.8	I_{CdL}	121.1	199.1	102.7
r_{Cu}	0.9998[a]	0.9996[a]	0.9998[a]	r_{Cd}	0.9996[a]	0.9993[a]	0.9997[a]
log K_{Cu}	4.65	5.55	5.36	log K_{Cd}	4.24	4.63	4.47
CC_{Cu}	0.68	1.25	0.93	CC_{Cd}	0.50	0.77	0.68
I_{ZnL}	129.8	201.3	101.0	I_{PbL}	85.1	11.4	9.7
r_{Zn}	0.9988[a]	0.9994[a]	0.9995[a]	r_{Pb}	0.9992[a]	0.9999[a]	0.9996[a]
log K_{Zn}	4.08	4.43	4.31	log K_{Pb}	4.95	5.81	5.53
CC_{Zn}	0.39	0.67	0.60	CC_{Pb}	0.83	1.95	1.51

[a] $P < 0.001$.

the high content of acidic functional groups and other O-containing ligands groups, and the marked aromatic character and high humification degree typical of native soil HAs. Two adjacent aromatic carboxyl groups (i.e., a phthalic acid-like binding site) and an aromatic carboxyl group and adjacent phenolic OH group (i.e., a salicylic acid-like binding site), in the HA structure are known to form highly stable bidentate complexes with metal ions (*14−16*, *36*). The intermediate stability constant values of metal ion−SS40−HA complexes confirm the partial modification occurred in amended soil HA by partial incorporation of SS-HA structures into native soil HA.

Metal Ion Binding Parameters of Humic Acids: Complexing Capacities. The metal ion complexing capacity of any HA increases in the order Zn(II) < Cd(II) < Cu(II) < Pb(II). In agreement with the stability constant trends, the metal binding capacities of HAs examined follow the order SS-HA < SS40-HA < SS0-HA.

Although the binding capacities of the HAs examined appear to be related to their acidic functional group contents, the CC_M values are in any case much smaller than the corresponding acidic functional group contents (*42*). This effect may be due to (a) the involvement of bidentate, salicylic- and phthalic-type binding sites, (b) the formation of 2:1 complexes with the metal ion bridging two HA macromolecules, and/or (c) the presence of a number of carboxyl and phenolic hydroxyl groups that are unavailable for metal ion binding due to either sterical hindrance or proton competition or electrostatic effects (e.g., metal ion complexation at one site decreases the capacity of neighboring functional groups to complex other metal ions).

Environmental Significance. The binding of metal ions to HAs generally reduces their biovailability, mobilization, and transport in soil. The results obtained show that binding capacities and affinities of SS-amended soil HA are smaller than those of native soil HA, which suggest the occurrence of an averaging effect possibly due to a partial incorporation of the HA fractions of SS into native soil HAs. These effect might be expected to decrease the capacity of HA in SS-amended soils to retain metal ions, with important impacts on the bioavailability, mobilization, and transport of metals in SS-amended soils. Application of SS may thus represent an environmental risk for soil and groundwater pollution by metals. However, SS addition to soil has the potential of greatly increasing organic matter content (*1–3*) so that, on a total soil mass basis, the metal ion binding capacity of HA in SS-amended soil may be increased even though the metal binding capacity of the HA fraction decreases.

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