Technical Notes

A Modified Multisite Stern−Volmer Equation for the Determination of Conditional Stability Constants and Ligand Concentrations of Soil Fulvic Acid with Metal Ions

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In this work, we modify the multisite Stern−Volmer (MSV) equation for fitting fluorescence titration curves. Under the condition of a static quenching mechanism, the MSV postulates an underlying 1:1 fulvic acid (FA)/copper coordination ratio at multisites. Approximates of six fitting parameters characterize the stability constants (K_1 and K_2) of FA ligands with Cu^{2+}, micromolar ligand site concentrations (C_{L1} and C_{L2}), the unquenched, steady-state fractional fluorescence contributions (f_x), and the residual fluorescence intensity (I_{RES}). Prior to its application to actual FA titration data, the MSV function is simulated, and its predictive ability is confirmed by titrating a mixture of model fluorophores, glycyl-L-tryptophan and L-tryptophan with Cu^{2+} at pH 6. Molecular fluorescence measurements of FA are acquired at a fixed spectral position (λ_{ex} = 335 nm; λ_{em} = 450 nm), and FA is titrated with copper in triplicate at three pH values—5, 6, and 7. An objective analysis of log K_1 and K_2 values supports several site organization schemes, including (i) subtle, cooperative interaction, (ii) interfering molecular conformations, and (iii) aggregate forms. Site densities (C_{L1} and C_{L2}) are consistent across varied pH. The f_1 is indicative of a pH-induced spectral shift of a fluorophore and convincingly associates with a transect in the $\Delta\lambda$ = 25 synchronous fluorescence spectrum and with the preexponential terms describing the time-dependent fluorescence decay. The MSV and its parent one-site version are equivalent for data fitting but are only simple approximations of a FA ligand system with more complex molecular fundamentals.

The coordination of metal ions with acidic functional groups of organic molecules in dissolved fulvic acids (FA) is well documented.[1] By complexing to FA ligands, these metal ions become less bioavailable and thus less toxic to a variety of aquatic

species (for example, see Knezovich et al.[2]). These complexes partition between liquid−solid and solid−solid phases, thereby affecting the environmental fate and transport of metals. In accord, interest in developing innovative data treatments to investigate metal binding by these macromolecules has surfaced. The functionality and chemical reactivity of FA can be effectively examined with total luminescence spectra, constant wavelength offset ($\Delta\lambda$) synchronous fluorescence spectra (SyF), and emission spectra.[3−5] These scanning methods are relatively fast and inexpensive and provide advantages over competing electrochemical and equilibrium dialysis methods, which are not as sensitive as fluorescence to the complex molecular environment of FA.

Paramagnetic cations (Mn^{2+}, Co^{2+}, Cu^{2+}) that bind to FA can evoke a proportional quenching response in its fluorescence spectra.[6] This response can be used to describe the solution equilibria in quantitative terms.[3,7] The titrimetric approach of Ryan and Weber[7] is widely practiced, assumes a simple 1:1 metal−ligand coordination ratio, and treats quenching or enhancement; trivalent Be^{3+} and Al^{3+} can demonstrate an enhancement response commensurate with complexation.[8−10] However, the linear relationship between formed complex and fluorescence quenching it assumes is hotly contested.[11,12] Fluorescence measurements of Cu^{2+}-titrated solutions of a Laurentian FA propose that this

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relationship may be slightly nonlinear.[13] This nonlinearity is dependent on sample type, pretreatment, and age and may, among other possibilities (or combinations of possibilities), be due to (a) overlap of fluorophores that typify chemically different complexation sites, (b) the wavelengths (or scanning method) at which the fluorescence measurements are acquired, (c) the varied physicochemical microenvironments of the FA structure as characterized by either a single fluorophore or fluorophore set, (d) cooperational and molecular configuration changes due to metal loading or ionization state, or (e) shielding of binding sites or fluorescence (i.e., controlling photophysical effects).

With the high probability of the occurrence of these effects, a data treatment to account for this nonlinear fluorescence response for the metal−FA system is needed. Thus, we turn to the Stern−Volmer equation, which, by means of quenching constants (K_{SV}) and (unquenched, steady-state) fractional fluorescence contributions (f), is frequently extended to describe the nonlinear relationship between titrant (quencher) concentration and fluorescence at a given excitation−emission wavelength pair. Fluorescence titrations of residual proteinaceous matter[14] and of transition-metal complex oxygen sensors in polymer support materials[15] offer examples of its successful application.

In this effort, the Stern−Volmer equation is modified (MSV), simulated, validated for predictive capability with a suitable model compound set, and applied to fluorescence titration data of a well-characterized FA. Copper is utilized because it capably quenches fluorescence and binds to FA at pH values characteristic of natural waters. The soil FA sample isolated by Weber and Wilson[16] is utilized here. Comprehension of its fluorescence properties may now imply a subtle nonlinearity between the complex and fluorescence quenching. A fit of the time-dependent decay equation to time-resolved fluorescence data resolves two principal fluorescent constituents near the excitation−emission maximums.[17] A minor third fluorescence component is rendered, but the two principal constituents account for 89% of the preexponential terms. Total luminescence and SyF show these constituents and suggest they respond uniquely to added copper, are distinguishable, and partially eclipsed at the main excitation−emission maximum.[17,18] The set of MSV model-simulated curves profiles these fluorescent species as independent to better comprehend their potential to vary the overall fluorescence response. Copper hydrolysis is accounted for at pH 7. Molar concentrations of the mono- and dihydroxy species formed are determined by MINTEQA2/PRODEFA2 (MINPRO), a computer modeling program developed by the U.S. EPA for geochemical assessment of environmental systems.

THEORY

The quenching of FA's fluorescence by Cu^{2+} is static, meaning that a ground-state complex between Cu and FA is observed.[19]

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The MSV approach assumes a simple 1:1 coordination ratio between Cu^{2+} and the fluorescent ligand components, giving the following reversible solution equilibria (charges are omitted for simplicity): $M + L1 \leftrightarrows ML1$ and $M + L2 \leftrightarrows ML2$. L1 and L2 are the free ligand species (all forms of metal-free ligand) at sites 1 and 2, respectively, M is free metal, and ML1 and ML2 are the metal-bound species at these sites. Other reaction stoichiometries are possible and reliant on metal loading. For example, at low metal loadings, chelation (2:1 = ligand:metal) may be induced, contorting the FA shape and affecting fluorescence. Evidence for the effects these molecular formation changes have on fluorescence are scant, inconclusive, and not easily quantified.

The MSV approach requires measuring the intensity at the excitation−emission maximum, I, which is equal to the sum of the intensities of fluorescence species present in solution at any point in the titration and given by

$$I = I_{L1} + I_{ML1} + I_{L2} + I_{ML2} \qquad (1)$$

For (1), I_{L1} and I_{L2} are the intensities of metal-free fluorescence ligand components 1 and 2, respectively, and I_{ML1} and I_{ML2} are the metal-bound fluorescence intensities of components 1 and 2. Where $I_{L1} = Q_{L1}[L1]$, $I_{L2} = Q_{L2}[L2]$, $I_{ML1} = Q_{ML1}[ML1]$, and $I_{ML2} = Q_{ML2}[ML2]$. Q_{ML1}, Q_{ML2}, Q_{L1}, and Q_{L2} are the complex and free ligand component quantum efficiencies, assumed to be constant at fixed wavelength and equal to the slope of a linear plot (forced through 0,0) of I versus [L].

Before metal is added, [ML1] = 0, [ML2] = 0, [L1] = C_{L1}, and [L2] = C_{L2}. Additionally, because I_{O1} and I_{O2} are the initial fluorescence intensities of components 1 and 2, $I_{L1} = I_{O1}$ and $I_{L2} = I_{O2}$, respectively. Equations 2 and 3 describe the start of the titration

$$I_{O1} = Q_{L1}C_{L1} \qquad (2)$$

$$I_{O2} = Q_{L2}C_{L2} \qquad (3)$$

Near the titration end, a fluorescence signal remains. Termed the residual intensity (I_{RES}), it is due to material that (a) fluoresces but does not complex, (b) fluoresces, forms complex, and quenches only partially, or (c) fluoresces, forms complex, and does not quench. To simplify, it is assumed that I_{RES} is bound, fluorescing material and equal to the sum of I_{RES1} and I_{RES2}, which are the residual intensities of components 1 and 2, respectively. As metal titrant is added, [L1] → 0, [L2] → 0, [ML1] → C_{L1}, [ML2] → C_{L2}, I_{ML1} → I_{RES1}, and I_{ML2} → I_{RES2}. The residual intensities for each component are

$$I_{RES1} = Q_{ML1}C_{L1} \qquad (4)$$

$$I_{RES2} = Q_{ML2}C_{L2} \qquad (5)$$

By rearranging eqs 1−5, expressions used to systematically treat equilibria (i.e., K_1 and K_2 that describe 1:1 conditional stability constants characteristic of components 1 and 2), and chemical mass balance equations, it is possible to derive (6). K parameters

are considered average constants, which characterize the bulk ligand properties of an array of molecular entities.

$$I = (I_{RES} - I_O)[\{f_1 K_1[M]/(K_1[M] + 1)\} + \{f_2 K_2[M]/(K_2[M] + 1)\}] + I_O \quad (6)$$

The f_1 and f_2 values are the unquenched, steady-state fractional contributions of individual fluorescence components at the monitoring wavelength, $f_1 = C_{L1}/[C_{L1} + (\gamma)C_{L2}]$, where $\gamma = (Q_{ML2} - Q_{L2})/(Q_{ML1} - Q_{L1})$. Further analysis shows that $f_1 + f_2 = 1$, so $f_2 = 1 - f_1$.

An expression that takes the form of a third-order polynomial (7) can also be derived from the deliberate ordering of these equations.

$$K_1 K_2[M]^3 + \{K_1 K_2(C_{L1} + C_{L2} - C_M) + K_1 + K_2\}[M]^2 + \{C_{L1}K_1 + C_{L2}K_2 - C_M(K_1 + K_2) + 1\}[M] - C_M = 0 \quad (7)$$

Hays et al.[18] provided an extensive description of the function of a similar model. Briefly, equations 6 and 7 predict the fluorescence intensity value I corresponding to a given value of total added metal concentration (C_M). We first solve (7) numerically to find [M], and then we use (6) to calculate I. Of the seven parameters in this model, one (I) is measured experimentally and six (K_1, K_2, C_{L1}, C_{L2}, I_{RES}, f_1) are estimated numerically using the standard least-squares procedure. We measure the value of I that corresponds to several different values of C_M, and we then choose the values of K_1, K_2, C_{L1}, C_{L2}, I_{RES}, and f_1 to minimize the sum of squares of the differences between the experimentally measured intensity values and the intensity values predicted by the model. Because of measurement error, there is no way to find the exact parameter values. The method of least squares is a standard procedure for finding a set of parameter values which best fit the experimental data.

MATERIALS AND METHODS

Reagents. Glycyl-L-tryptophan (GlyTrp; Sigma Chemical Co., St. Louis, MO) and L-tryptophan (Aldrich Chemical Co., Milwaukee, WI) were used as model compounds that simulate copper binding and fluorescence response behavior of FA. In dissolved organic matter, certain fractions of intermediate polarity are enriched in amino acids.[20] These compounds also contain aromatic structures and carbonyl and carboxylic groups that typify our FA sample. The FA material (University of New Hampshire, Durham, NH) used in our experiments was isolated from the B$_2$ horizon of a Podzol soil (Conway, NH). Characterization of it was provided by Weber and Wilson.[16] (Found: C, 53.1; H, 3.24; N, 0.90. Total acidity, 13.4 mequiv/g; oxygen-containing functional groups as carboxyl, 8.2 mequiv/g; phenol, 5.2 mequiv/g; carbonyl, 3.5 mequiv/g; number average molecular weight, 644). Values are given as percentages unless otherwise noted.

Stock solutions of GlyTrp (800 μM; Sigma Chemical Co.), L-tryptophan (Trp) (1000 μM; Aldrich Chemical Co.), and FA (100 mg/L) were prepared in deionized water (17 MΩ, Barnstead-Nanopure). Before use, the FA solution was filtered (0.4-μm

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polycarbonate, Costar Corp., Cambridge, MA). For all experiments, NaOH and HClO$_4$ (0.001−2 M) were used to adjust pH, and the ionic strength was adjusted to 0.1 M with an inert electrolyte (NaClO$_4$) prior to titrating. The copper ion standard was prepared from its reagent grade perchlorate salt and standardized by direct current plasma atomic emission spectrometry.

Copper titrations of 15 mg/L dissolved FA solution and of the GlyTrp (50 μM) and Trp (20 μM) mixture (50 mL) were performed at pH 5, 6, and 7 and at pH 6 ($\pm$0.02 pH unit), respectively. All titrations were performed in triplicate.

Apparatus. A complete description of the titration procedure and apparatus utilized here is given elsewhere.[7,18] Briefly, a spectrofluorometer (Farrand Optical Co. Inc., Valhalla, NY) with refurbished electronics (Optical Technology Devices Inc, Elmsford, NY) was utilized to obtain the fluorescence data. It was equipped with two grating monochromators, an emission bandpass of 5 nm, an excitation band-pass of 10 nm, a 150-W dc xenon arc source, a 10-mm cell holder, and a photomultiplier tube. Intensity and wavelength data could be read directly from the instrument's displays or fed to an analog-to-digital converter (Metrabyte, Kiethly, Taunton, MA) on board a personal computer (Dell Dimension, Dell Computer Corp., Austin, TX).

Titrations were performed in a capped, temperature-jacketed titration cell (Princeton Applied Research, Princeton, NJ), in which constant stirring was maintained. Inserted through the cap was a combination pH electrode (8104 Ross; Orion, Boston, MA) attached to an autochemistry system (960 pH meter, Orion) with readout. A 10-mm quartz cuvette (NSG Precision Cells, Farmingdale, NY) was used for all experiments. A constant-temperature water bath (VWR Scientific, Boston, MA) outfitted with pump maintained solution temperature at 25.0 $\pm$ 2.0 °C. Microliter reagent additions and solution transfers from the titration cell to the cuvette were made with autoclavable pipets (Eppendorf; Brinkmann Instruments Inc., Westbury, NY). All glassware and pipet components were soaked in 19% v/v HNO$_3$ for a minimum of 12 h.

Fluorescence Measurements. For both the FA and model compound titrations, fluorescence measurements were made at fixed λ_{ex} and λ_{em}. The GlyTrp ($\lambda_{ex} = 285$ nm and $\lambda_{em} = 362$ nm) and Trp ($\lambda_{ex} = 283$ nm and $\lambda_{em} = 355$ nm) maximums were slightly offset, but their fluorescence bands were predominately overlapped. This condition made the detection of their simultaneous quenching possible; $\lambda_{ex} = 285$ nm and $\lambda_{em} = 360$ nm were selected as optimal. Fluorescence data for the overlapping fluorophores of this FA were collected at its main excitation−emission maximum, $\lambda_{ex} = 335$ nm and $\lambda_{em} = 450$ nm.

Data Treatment. The curve-fitting routine of the MSV model was optimized using a Nelder−Meade simplex search method in MATLAB (Version 4.0, Math Works Inc., Natick, MA). A computer program was designed using the method of least squares, in which the parameter values (K_1, K_2, C_{L1}, C_{L2}, f_1, I_{RES}) were chosen to minimize the sum of the squares of the differences between measured intensity values (data points $\geq$25) and the values predicted by the model. During the fitting operation, the maximum number of iterations performed was 600 (100 $\times$ the number of parameters) and the termination tolerances for the parameter vector and scalar valued function were arbitrarily set to 1%. Equation 7 was solved numerically for [M] by the ROOTS
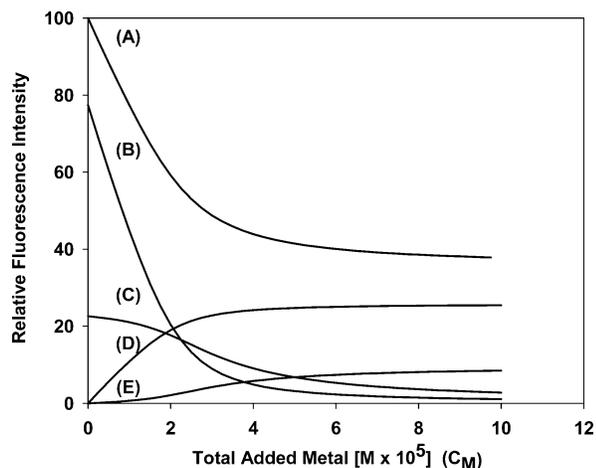
Figure 1. MSV-simulated fluorescence quenching response and fluorescent component contributions for the titration of two ligands with metal ion: log $K_1 = 6$; log $K_2 = 5$; $C_{L1} = 20$ μM $C_{L2} = 10$ μM; $I_{RES} = 10$. (A) I; (B) I_{L1}; (C) I_{L2}; (D) I_{ML1}; (E) I_{ML2}.

Figure 2. MSV-fitted fluorescence quenching data of GlyTrp (50 μM) and Trp (20 μM) titrated with Cu^{2+} at 0.1 M ionic strength, 25 °C, and pH 6: (circles) experimental data; (solid line) MSV-predicted intensity values.

command in MATLAB, which found all three roots of the equation by creating a 3×3 matrix and then finding the eigenvalues of this matrix. To find eigenvalues, MATLAB uses algorithms from the public domain collection of algorithms called EISPACK. We were able to show that there is always exactly one positive real root of eq 7 and chose the positive real root.

Geochemical Model. A computer model for geochemical systems, MINPRO [MINTEQA2/PRODEFA2] Version 3.0, U.S. EPA),[21] was applied to determine the concentrations of solid and dissolved copper hydroxy species in dilute, aqueous solutions of FA at each pH. MINPRO characterized copper complexation strengths of dissolved FA ligand sites (30 μM) using a log-normal distribution function: geometric mean log $K = 4.90$ and σ (standard deviation) = 1.7. A series of iterations at each C_M value was performed by MINPRO, and by means of its extensive thermodynamic database, the percentage of all the species formed relevant to this specific problem was calculated.

RESULTS AND DISCUSSION

Hypothetical MSV Model Curves. Figure 1 provides a series of MSV model-simulated curves that hypothetically profile the fluorescence intensities of individual species. The curves (A–E in Figure 1) are nonlinear and a function of the solution equilibrium. For a fluorescence titration experiment, only curve A is observed; it is formed from eq 1, which scopes the entire fluorescence response versus C_M. For this simulation, fluorescence quantum efficiency values are arbitrarily selected, fixed, and lower for complex than for free ligand. This order simulates quenching at curve A. Selected parameter values, $K_1 = 10^6$, $K_2 = 10^5$, $C_{L1} = 20$ μM, $C_{L2} = 10$ μM, $f_1 = 0.8$, $f_2 = 0.2$, and $I_{RES} = 10$, closely approximate those of our FA sample.

At high C_M, [ML1] and [ML2] and their corresponding intensity values (curves D and E) approach a maximum as [L1] and [L2] (curves B and C) near a minimum. At this section of curve A, the overall fluorescence response is low. At low C_M, a
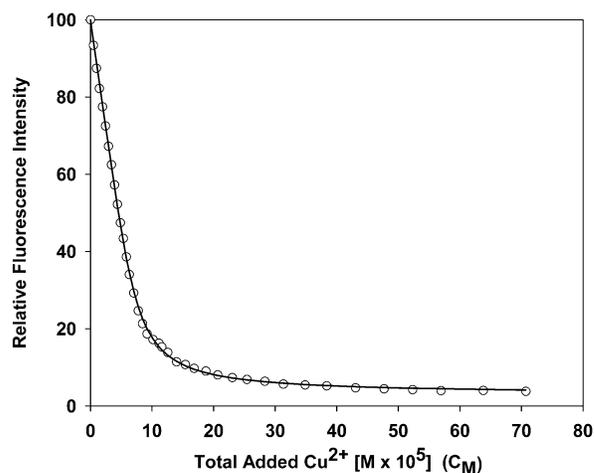
high fraction of fluorescent L1 and L2 strongly binds metal to form weakly or nonfluorescing ML1 and ML2. This stage of the complexation scheme largely causes the observed quenching. Evidence of fluorescing complex can be inferred from results of electron spin resonance and molecular fluorescence experiments with a leaf litter extract.[3] The MSV can also be broadened to simulate fluorescence enhancement, which is evidenced by Al and Be being bound to FA.[8–10] Forming the basis of the enhancement case, ML1 and ML2 possess quantum efficiency values greater than those of L1 and L2.

Model Validation. Although the chemical composition of FA varies by source, phenol, carbonyl, amino, and carboxylic functional groups are consistently detected in them and considered responsible for binding copper.[3,16,20] For this reason, two model compounds (the dipeptide GlyTrp and the amino acid Trp) that comprise these functional groups, complex Cu^{2+} at varying strength, exhibit fluorescence maximums at similar excitation–emission wavelengths, and quench proportionally when bound to Cu^{2+} are chosen to test and validate the predictive capability of the MSV approach. Particularly interesting is recent evidence that shows significant binding at low [Cu^{2+}] by a small concentration of nitrogen-containing amino groups in dissolved organic matter.[20] The GlyTrp simulates a terdentate binding involving the carboxyl and amino groups and the dipeptide linkage.[22] Because of the difficulty in mimicking the complex macromolecular structure of FA and its binding properties as they relate to conformational influences, formation of aggregates, and fluorescence, only the predictive competency of the MSV model is verified by this mixed-ligand model system. In addition to these constraints, the fluorescence spectroscopy technique itself is not fully sensitive to molecular specific features. Regardless, past work shows that this approach to model validation is effective.[7]

Experimental data (C_M vs fluorescence) and the model-predicted fluorescence for a titration of 50 μM GlyTrp and 20 μM Trp with Cu^{2+} at pH 6 are given in Figure 2. For this system, linear fluorescence quenching is shown when $0 \leq C_M$ (Cu^{2+}) $\leq$ 100 μM. This result indicates that the model compounds strongly

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Table 1. Average MSV Model-Predicted Equilibrium Parameters for Cu^{2+}-Titrated Solutions of GlyTrp and Trp[a]

parameter estimated	literature or fixed	MSV model (SD)
$\log K_1$ (GlyTrp)	5.85 or 6.02	5.81 (± 0.17)
$\log K_2$ (Trp)	4.88	4.90 (± 0.15)
C_{L1} (GlyTrp)	50.0 μM	50.0 μM (± 0.2)
C_{L2} (Trp)	20.0 μM	17.7 μM (± 4.0)
f_1	0.48[a]	0.52 (± 0.02)
f_2	0.52[a]	0.48 (± 0.02)
I_{RES}	0.0	1.8 (± 0.8)

[a] Values determined experimentally.

Figure 3. MSV-fitted fluorescence quenching data of soil fulvic acid (15 mg/L) titrated with Cu^{2+} at 0.1 M ionic strength: (circles) pH 5 data; (squares) pH 6 data; (triangles) pH 7 data; (solid lines) MSV-predicted intensity values.

complex Cu^{2+}. Fluorescence is mostly quenched near the end of the titration, associating limited or no fluorescence with Cu−Trp and Cu−GlyTrp. Experimental fluorescence titration data (circles) corroborate model predictions (solid lines). Average MSV curve-fitting parameters determined from the replicate ($N = 3$) copper titrations of the GlyTrp−Trp mixture are summarized in Table 1. Conditional log K values for GlyTrp and Trp are both within 1% of their theoretical values, which are calculated using thermodynamic stability constants, pK_a's (two pK_a values (8.00 and 8.20) for GlyTrp are reported, and therefore, two conditional log K values appear in the table), and experimental pH.[23−25] Analytical precisions within ± 0.25 are reported (Table 1) for these MSV-computed log K values.

The mean values of model-calculated [GlyTrp] and [Trp] correspond exactly and to within 12% of their experimentally fixed concentrations. The inexact MSV-predicted value for [Trp] is likely due to its relatively low concentration in solution and weak affinity for Cu^{2+}. The MSV-determined values of f_1 and f_2 are within 8% of those estimated using $f_1 = C_{L1}/C_{L1} + (\gamma)C_{L2}$, where C_{L1} and C_{L2} are known, Q_{L1} and Q_{L2} are determined by measuring I_{L1} and I_{L2} in separate experiments at $\lambda_{ex} = 285$ nm and $\lambda_{em} = 360$ nm and setting $Q_{ML1} = 0$ and $Q_{ML2} = 0$. In summary, the MSV accurately and reproducibly approximates the solution equilibria of a mixed-ligand system with overlapping fluorescence components.

MSV Model Applied to Fluorescence Titrations of FA with Copper. Representative fluorescence titrations of FA with Cu^{2+} at pH values of 5, 6, and 7 are shown in Figure 3. MSV model predictions (solid lines) correspond well to the nonlinear titration data. Fluorescence quenching increases with pH, as does the accessibility of the FA complexation sites to Cu^{2+}. The average ($N = 3$) MSV-computed conditional log K values (Table 2) indicate a rise in Cu^{2+}−ligand binding strength with pH − except for component 1 at pH 7. This exception is due in part to the formation of mono- ($CuOH^+$) and dihydroxy $Cu(OH)_2$ species in solution. The pH 7 log K for this component increases by implementing the correction procedure, where MINPRO[21] accounts for the micromolar concentrations of copper hydroxides and C_M is revised to reflect only the $[Cu^{2+}]$ (Table 2).

At pH values of 5 and 6, log $K_1 >$ log K_2 by nearly 1 log unit. This difference in site complexation strengths may indicate
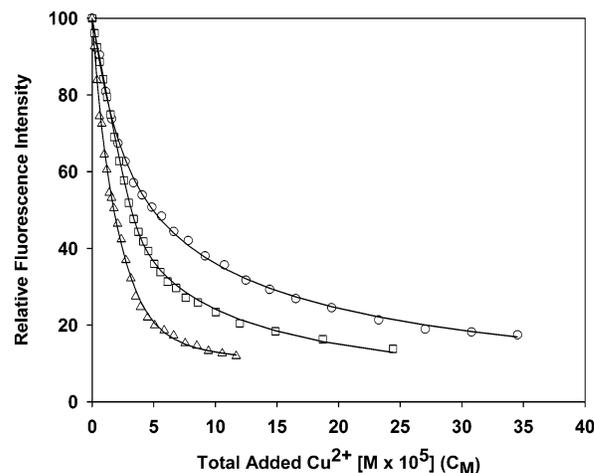
"pseudochelation". This effect is synergistic, demonstrating a subtle electrostatic association between a vacant ligand (site 2 in this case) and the molecular orbitals of organically bound Cu (at site 1).[26,27] With increasing $[Cu^{2+}]$, these vacant ligand atoms may eventually chelate the engaged Cu^{2+}. Presumably, the fluorescence is sensitive to each phase of this cooperative interaction. Quenching of these fluorescent constituents could also be mutually dependent on Cu without chelation. For this condition, a Cu atom would have to interact with a single ligand site joined to uniquely fluorescing and disorderly featured (polymer-like) molecular constituents and synchronously quench them both. The local molecular environment about the organically bound Cu may effectively control the quenching efficiency of each constituent. Luster et al.[3] show the probability for this sort of binding environment is quite high by stressing that up to four donor atoms (N, O, and combinations thereof) will associate with a central Cu atom. With this advance, binding in the context of molecular aggregates must not be overlooked. In either of the latter cases, the MSV assumption of a 1:1 coordination ratio would conflict with the underlying Cu:FA binding mechanism. On the basis of its design, the original MSV equation indexes the quenching of multiple fluorescence regions by a single atom. And only under the condition of dynamic quenching is this interpretation unconditionally valid. Though, a mutidentate site that is linked to and quenches a single fluorescent unit potentially conserves the 1:1 assumption.

The fact that fluorescence component 2 binds copper more strongly than component 1 is only evident at pH 7. MSV predictions for conditional log K values of FA seem to vary from the systematic aquatic equilibria typically exhibited by simpler molecules. Possible explanations for this occurrence are that (a) due to their nonuniform distribution about the main structure, carboxyl and phenol groups in the chemically heterogeneous FA have nonideal, competitive affinity for copper and protons[28] or (b) the binding sites associated with the FA fluorophore at this fixed

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Table 2. Average MSV Model-Predicted Equilibrium Parameters for Cu^{2+}-Titrated FA Solutions

pH	$\log K_1$	$\log K_2$	C_{L1} (μM)	C_{L2} (μM)	I_{RES}	f_l
			Solutions			
5	4.78 (0.09)	3.68 (0.18)	23.5 (7.4)	4.4 (1.5)	4.2 (0.4)	0.57 (0.02)
6	5.45 (0.36)	4.56 (0.27)	22.2 (7.8)	6.2 (2.6)	7.5 (3.6)	0.52 (0.06)
7	5.35 (0.18)	6.06 (0.27)	34.8 (4.4)	5.7 (2.7)	11.0 (2.0)	0.46 (0.03)
7^a	5.70 (0.03)	6.54 (0.20)	19.0 (1.7)	3.2 (2.3)	13.0 (3.0)	0.49 (0.25)
			Results from the Model of Ryan and Weber[7]			
5	4.68		22.1		40.2	
6	5.03 (0.37)		19.7 (6.1)		22.4 (1.4)	
7	5.45 (1.55)		19.6 (3.6)		20.7 (3.5)	

[a] Values obtained using MINPRO-adjusted Cu^{2+} concentrations. Standard deviation in parentheses.

set of wavelengths changes with pH. Evidence for fluorescence change as a function of the ionization state of the molecule is convincing and will be covered. An increase in pH likely corresponds to an increase in the repulsive forces for the FA polyelectrolyte. In turn, molecular conformation changes reorganize copper binding (or adsorption) properties, control FA and site solvation, and influence fluorescence. Still, the MSV parameters measured by fluorescence change should somehow reflect the molecular properties of the corresponding binding sites.[3] A prior discussion of coordinating conformations and their ability to affect fluorescence, molecular rigidity, and subsequent complex and ligand geometries is relevant here as well.[6]

Constant offset SyF and fluorescence lifetime measurements may also be drawn on to resolve the relationship between fluorescence and molecular binding assemblies of FA. For example, Cu-quenched peaks in the $\Delta\lambda = 20$ nm SyF of a Laurentian FA are putatively linked to fluorescence lifetime components.[13] For the soil FA sample used here, fluorescence lifetime measurements define two principal components among a third minor constituent.[17] Estimates of the solution equilibria ($\log K$ and C_{Lx}) modeled over multiple wavelengths using a $\Delta\lambda = 25$ nm SyF of this FA are somewhat similar to those modeled by the MSV. More precisely, components 1 and 2 correspond to the transect of the excitation–emission maximums (315/440 nm) and the 471/496 nm maximums in the $\Delta\lambda = 25$ SyF spectra. In the $\Delta\lambda = 25$ SyF, as the pH is raised, the transect peak shifts to longer wavelengths (toward the SyF maximums at 471/496 nm), narrows markedly, and generally increases in emission intensity. This spectral peak shift may be due to intensity changes of independent, collocated fluorophores, is not viewed at $\lambda_{ex} = 335$ nm, $\lambda_{em} = 450$ nm, but compellingly supports that the complexation sites fluorophore 1 measures vary with pH.

It is conceivable that a partially ionized FA experiences a molecular conformation change, such as a folding, a stretching, or a bending motion, which creates a steric hindrance, obstructing Cu^{2+} from the fluorescent ligands of component 2 at pH values of 5 and 6. After all, FAs are considered an inhomogeneous mixture of structurally flexible biopolymers. Data fitting of the SyF titrations show no aspect of this interference; intensity measurements for this component were consistently acquired at the peak's ($\sim$315/440 nm) shifting apex.[18] Scanning methods and the wavelengths at which the measurements were taken can also effect the spectrum (or fluorophore) appearance and which ligand sites are excited. The fixed wavelength quenching measurements used to obtain the MSV results may have physically missed a section of the constituent 2 fluorophore at pH values of 5 and 6, poorly characterizing the binding progression of this molecular array.

Average MSV model predictions of C_{L1} and C_{L2} for FA at individual pH values are given in Table 2. By $\sim$5-fold, C_{L1} is greater than C_{L2}. C_{Lx} values should not change with pH because $C_{Lx} = [L_x] + [ML_x]$. In accordance with this framework, C_{L2} values are similar at each pH studied. This assumption is also valid for C_{L1} at pH values of 5 and 6. And though C_{L1} seems overestimated at pH 7, accounting for the copper hydroxide yield in solution at this pH produces a distinctly lower C_{L1} more consistent with pH 5 and 6 values. C_{L2} is less affected by copper–hydroxy species formed in solution; C_{L2} values at all pHs are similar before and after revising. In the SyF, evidence of variations in peak shape, location, and emission intensity with pH is sparse at the 471/496 nm maximums (which likely is described by C_{L2}). Apparently, the ligand forms (protonated and deprotonated) of C_{L2} are shielded from the repulsive affects of dissociated groups, hydrogen-bonding forces, and other solute–solvent interactions that can effect fluorescence. From this evidence, it seems more plausible that the binding sites of C_{L2} are obstructed (or different) at the lower pH values rather than misread due to shifts in fluorescence. It is possible that this obstructive hindrance or interference complements the "pseodochelation" effect described earlier but to what extent or precisely how is unclear.

Weber and Wilson[16] showed that 50 mL of our soil FA comprises $\sim$12 μM of phenolic, carboxylic, and carbonyl functional groups, whereas the average total ligand concentration calculated by the MSV is $\sim$2-fold greater (26 μM). Some fraction of this difference could be attributed to nitrogenous Cu^{2+}-complexing sites in the FA, but the nitrogen concentration (0.90 w/w %) in this terrestrial sample equates to an upper limit of just 0.48 μM unidentate sites. However, these sites are apt to complex Cu^{2+} early in the titration, possibly causing a disproportionate degree of quenching initially. An exchange to a Cu_2L from a CuL binding order with increasing C_M is also possible. Or, perhaps, the total C_L is overestimated by MSV because Cu^{2+} binds more strongly to nonfluorescing FA material. For this case, an increase in the upper limit of C_M may impart a bias that effectively inflates C_L. This explanation is by no means conclusive but reasonable because reportedly just 1% of FA is fluorescent.[29]

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In Table 2, the average model-calculated fluorescence fraction of component 1 (f_1) decreases as pH increases, indicating that $Q_{ML2} - Q_{L2}$ is increasing or $Q_{ML1} - Q_{L1}$ is decreasing as C_{L1} and C_{L2} remain almost unchanged and $f_1 = C_{L1}/C_{L1} + (\gamma) C_{L2}$, where $\gamma = (Q_{ML2} - Q_{L2})/(Q_{ML1} - Q_{L1})$. The steady-state fluorescence ascribed to these components is consistent with a pH-induced spectral shift of a fluorophore from shorter to longer wavelengths, giving additional evidence for the association between the $\Delta\lambda = 25$ SyF peaks (315/440 and 471/496 nm) and the fluorescence measured at $\lambda_{ex} = 335$ nm, $\lambda_{em} = 450$ nm (components 1 and 2). Even more profound is that the preexponential terms describing the time-dependent fluorescence decay (Found: A_1, [0.59]; A_2[0.30] + A_3[0.11]) match almost identically to the steady-state fluorescence fractions attributed to each component by the MSV.[17] However, we point out that there are known constraints to the exponential fits of complex decay curves from single-photon counting instruments, such as the one used to acquire these terms.[30] In brief, fluorescence decay data are analytically biased toward fitting a two-site equation.

I_{RES} inversely correlates to pH. Relative to pH 5, FA fluorescence at pH 7 is more often efficiently quenched near the titration end. For this FA, there is a fair degree of evidence indicating an inter-reliance between the molecular fluorescence constituents. For example, (i) at pH 5, calculated I_{RES} values of the multiwavelength data set (excitation emission matrix maximums) are nearly identical, (ii) the fluorophores synchronously quench over a similar C_M range, and (iii) the fluorescence bands of the excitation emission matrix and of the SyF overlap.[18]

The Ryan and Weber[7] approach is a single-component nested version of the MSV equation. In comparing the data-fitting results of the interrelated models, the following is observed for the identical FA (data are unadjusted for copper hydroxides): (1) single-site log K values (Table 2) describe a statistical median and approach the MSV log K_1 and K_2 mean at each pH, (2) after summing and normalizing them to a 10 mg/L FA concentration, C_{L1} and C_{L2} are within 84% (range of 73−96%) of their C_L on average, and (3) MSV estimates of I_{RES} are nearly 2-fold less than those of Ryan and Weber.[7] By 1 order of magnitude, the C_M values used for this work are relatively higher. This increase is enough to elicit more quenching and to precipitate the FA as evidenced by light-scattering experiments.[7] A precipitate containing clusters or fragments of hydrophobic fluorescent complex may proceed to artificially accelerate the quenching. These problems at high [Cu^{2+}] are compounded by Cu−hydroxide formation and may serve to explain the noticeable gap between the studies for C_L values at pH 7.

An advantage of the MSV equation is that it can be consistently solved for pH 5 titration data for our FA, eliminating the parent one-site version's past inability to converge. On a mathematical basis, the models are equivalent for data fitting, accentuating that the fitting of the model equation to the data does not necessarily validate the underlying binding mechanism of FA. It is likely that both models are only a simple approximation of a FA ligand system with more complex molecular fundamentals. What makes the application of a multisite model rational is the knowledge of the presence of an additional fluorophore that binds Cu^{2+} or the potential for a nonlinear interaction between Cu and the ligand fluorescence. From a computational standpoint, the one-site model is simpler to apply. The MSV is expandable. In the event a third K is added to the model, best-fit K values likely would distribute differently. In any case, the K values gather the complex molecular fluorescence entities into averaged constants.

CONCLUSIONS

The MSV is developed for fitting fluorescence titration curves. Fluorescence measurements at fixed spectral wavelength positions ($\lambda_{ex} = 335$ nm, $\lambda_{em} = 450$ nm) provide conclusive evidence for at least two Cu^{2+}-complexing, ligand components in the titrated soil FA isolated by Weber and Wilson.[16] Their ability to complex with Cu^{2+} and the site densities they characterize are unique. The difference in MSV-determined stability constants at multisites supports several putative ligand site organizations, among them a site-to-site cooperative interaction and a pH-induced steric hindrance that interferes with Cu^{2+} binding to a fluorescent portion of FA. Estimates of fractional contributions to unquenched fluorescence are convincingly linked to peaks in the SyF and to the preexponential terms used to fit the time-dependent fluorescence decay data obtained for this FA. Mathematically based comparisons of the MSV to its single-component nested version reveal equivalent data fitting. Though, the one-site version is computationally simpler to apply. It is believed that the underlying binding mechanism of this FA is more complex than the simple framework used to derive each model. The multisite Stern− Volmer model inherently assumes a nonlinear relationship between molecular fluorescence and complex. Shielding of binding sites, differences between local molecular binding environments, formation of aggregates, and conformational changes due to ionization state and aspects of Cu^{2+} coordination serve to support this fundamental assumption. In practice, the use of multiwavelength fluorescence measurements, which are more time-consuming to acquire in comparison, may not always be necessary. Finally, the formation of copper hydroxides in solution can be effectively accounted for with MINPRO and integrated with curve-fitting results.

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