Multi-Wavelength Fluorescence-Quenching Model for Determination of Cu$^{2+}$ Conditional Stability Constants and Ligand Concentrations of Fulvic Acid

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In this work, a multi-wavelength model (MWM) is developed. It uses fluorescence bands in the fulvic acid (FA) spectrum that quench upon binding of inorganic Cu$^{2+}$ to FA. Quenching data at pH values of 5, 6, and 7 are placed in sets, containing fluorescence measures at select wavelengths versus added copper ($C_M$). Intensity data of wavelength set 1 are obtained from 25 nm constant offset synchronous fluorescence spectra (SyF), in which are observed distinct peaks ($\lambda_m = 415$ nm, $\lambda_m = 440$ nm; and $\lambda_m = 496$ nm). Wavelength set 2 intensity data are obtained from the FA fluorescence excitation and emission maxima ($\lambda_m = 335$ nm, $\lambda_m = 450$ nm; and $\lambda_m = 471$ nm, $\lambda_m = 496$ nm). Application of MWM shows that the multi-wavelength data sets characterize ligands of different binding strength ($K_m$) and concentration ($C_M$). Corresponding to pH values of 5, 6, and 7, mean and standard deviation values for wavelength set 1 are log $K_{415/440} = 4.66 (0.12)$, $5.03 (0.12)$, and $5.05 (0.08)$, log $K_{440/471} = 4.93 (0.06)$, $5.27 (0.11)$, and $5.39 (0.09)$, $C_{415/440} = 3.1 (1.5)$, $10.9 (4.5)$, and $7.9 (3.9)$ $\mu$M, $C_{440/471} = 14.3 (3.0)$, $1.7 (0.6)$, and $1.4 (0.5)$ $\mu$M. And for wavelength set 2, log $K_{415/440} = 4.50 (0.05)$, $4.96 (0.27)$, and $5.22 (0.08)$, log $K_{440/471} = 5.02 (0.04)$, $5.42 (0.32)$, and $5.71 (0.09)$, $C_{415/440} = 8.8 (0.5)$, $21.9 (7.9)$, and $18.7 (0.3)$ $\mu$M, $C_{440/471} = 21.0 (2.5)$, $7.17 (1.2)$, and $7.09 (0.3)$ $\mu$M. The ability of the 415/440 nm SyF transect to characterize the main excitation and emission maximum of FA at 335/440 nm is evaluated. Relative low concentration values returned by the model for this transect (415/440 nm) suggest that it is not entirely illustrative of the maximum. The model predictive capability is verified at pH 6 with two fluorescing Cu$^{2+}$ chelating organic compounds, L-tyrosine and salicylic acid. This test confirms that the model is capable of providing good estimates of equilibrium binding parameters from multi-wavelength measurements of a mixed ligand system.

Index Headings: Fulvic acids; Cu(II); Fluorescence quenching; Conditional stability constants; Multi-wavelength model.

INTRODUCTION

Fulvic acids (FA) are complex mixtures of macromolecules found in soils and natural waters.1 Classified as the fraction of organic matter soluble at both high and low pH, FA play an important role in the speciation of copper in aquatic systems.2 Studies show that in its free form, copper (Cu$^{2+}$) can be toxic to aquatic biota.3–6 This toxicity can be moderated or completely eliminated provided that the Cu$^{2+}$ coordinates with FA.5,7 Aside from regulating bioavailability, FA alter fate and transport of Cu$^{2+}$ in groundwater because of their capacity to adsorb to mineral surfaces that partition Cu$^{2+}$.8 Accordingly, accurate determination of complexation strength between dissolved FA and potentially toxic Cu$^{2+}$ as well as organic ligand concentrations in FA is essential.

Fluorescence spectroscopy is a nondestructive analytical technique that effectively determines Cu$^{2+}$–FA conditional stability constants and ligand concentrations by virtue of the proportionality between signal intensity and the free ligand concentration.9–15 However, use of FA fluorescence for quantitative study of complexation requires that proportionality is independently verified, as is the case for the FA sample used here (isolated by Weber). This verification is made necessary on account of Cook and Langford,16 whose efforts with a Laurentian FA show that it is possible for bound copper to exceed total copper added while implementing a fluorescence approach. This work stands supported by several prior light-scattering and fluorescence studies,17,18 which introduce the possibility of a cooperative effect due to bridged complex (CuL$_2$) and “pseudochelation”. The latter is a delicate association with metal by ligand eventually yielding complex and expected to show a [Cu$^{2+}$] dependence more detailed than a simple linear relationship can describe. Still, fluorescence spectroscopy has several advantages over separation methods used to study Cu$^{2+}$–FA complexation because it is not plagued by loss of material due to adsorption or by disruption of the equilibrium, is capable of high sensitivity, and does not require the adjustment of ionic strength or addition of other reagents for analysis. Fluorescence is also unique among metal speciation methods in that it measures a property of the ligand that changes with complexation rather than measuring free and bound metal.

Synchronous scan fluorescence (SyF) spectroscopy in particular is powerful because of its ability to detect multiple fluorophores in a single spectrum.19 Past work demonstrates that Cu$^{2+}$ induces quenching of multiple FA fluorophores in SyF spectra.20–22 These fluorophores exhibit varying degrees of quenching at independent wavelengths and are thought to be characteristic of different ligand sites. Additional work with FA assigns fluorescence lifetimes to resolved SyF peaks, showing at least two components that bind Cu$^{2+}$ with different strengths.16

Although fluorescence data support the presence of multiple Cu$^{2+}$ binding sites in FA, there are still few multi-site models developed to use fluorescence to evaluate solution equilibria.23,24 And although useful qualitatively, SyF is subject to certain quantitative limitations. The SyF spectrum may sample only a section of a fluorescence peak or might even miss a fluorophore completely, depending on what constant offset ($\Delta \lambda$) value is used.25

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Therefore, because $\Delta \lambda$ is not likely to be optimum for all peaks in any given experiment, it remains uncertain if the SyF spectra of FA are sensitive enough to measure the total possible quenching response due to copper addition. For these reasons, a multi-wavelength model (MWM) is developed to use the fluorescence response at multiple wavelengths to quantify copper binding by FA.

**THEORY**

**Multi-Wavelength Model Development.** The MWM model framework corresponds to the multi-site treatment of Stern–Volmer,\(^26,27\) which assumes that more than one component characterizes fluorescence and withdraws from the pool of added quencher. However, in contrast with the Stern–Volmer multi-site treatment, the MWM does not delineate overlapping fluorescence component fractions. It instead considers them resolved and presumes unity for the fraction of each component contributing to the unquenched fluorescence signal intensity.

As dissolved FA binds Cu\(^{2+}\), quenching of the two fluorophores is exhibited by a static mechanism.\(^29\) Assuming that these fluorophores characterize separate 1:1, Cu\(^{2+}\): ligand binding sites, the equilibria can be expressed with charges omitted for simplicity:

\[
\begin{align*}
M + L_1 & \Leftrightarrow ML_1 \\
M + L_2 & \Leftrightarrow ML_2
\end{align*}
\]

$L_1$ and $L_2$ are the metal-free ligand sites (in all forms, both protonated and unprotonated) corresponding to fluorophores 1 and 2. $ML_1$ and $ML_2$ are the metal-bound species at these sites, and $M$ is the free metal in solution. MWM can be extended to describe metal binding by more than two components by simply including additional terms. In this attempt to better define the Cu–FA system in a mathematical sense, it is possible that the binding sites of FA may not in every case function independently. Induced chelation (bridged complexing) or cooperativity between sites due to metal loading are possible and likely bring about conformational change. With the relationship between fluorescence change and conformational reorganization of FA not yet perfectly understood, exact modeling of this relationship is not yet accomplished but will advance with future work.

For fluorophores 1 and 2, 1:1 conditional stability constants ($K_1$ and $K_2$) are introduced:

\[
\begin{align*}
K_1 &= [ML_1]/[M][L_1] \\
K_2 &= [ML_2]/[M][L_2]
\end{align*}
\] (1)

Even if not perfectly ideal, $K_1$ and $K_2$ classify average bulk properties of the ligand site array.\(^9\)

Mass balance equations for both metal and ligand are:

\[
\begin{align*}
C_M &= [M] + [ML_1] + [ML_2] \\
C_{L_1} &= [L_1] + [ML_1] \\
C_{L_2} &= [L_2] + [ML_2]
\end{align*}
\] (3)

$C_M$ is the known total added metal concentration, and $C_{L_1}$ and $C_{L_2}$ are total ligand site concentrations in solution, respectively. Equations 1 and 4 combine to give Eq. 6:

\[
[ML_1] = K_1C_{L_1}[M]/(K_1[M] + 1)
\] (6)

Likewise, Eqs. 2 and 5 combine to give Eq. 7:

\[
[ML_2] = K_2C_{L_2}[M]/(K_2[M] + 1)
\] (7)

The equations associating fluorescence and solution equilibria are as follows:

\[
\begin{align*}
(I_{L_1} - I_1)/(I_{L_1} - I_{RES1}) &= K_1[M]/(1 + K_1[M]) \\
(I_{L_2} - I_2)/(I_{L_2} - I_{RES2}) &= K_2[M]/(1 + K_2[M])
\end{align*}
\] (8)

Equations 8 and 9 were derived with fundamental fluorescence quantum efficiency-concentration relationships developed in Ryan’s original model.\(^29\) $I_1$ and $I_2$ are the fluorescence intensity values measured at two separate excitation–emission wavelengths. We assume that before adding Cu\(^{2+}\), $I_{L_1} = I_1$ and $I_{L_2} = I_2$, $I_{L_1}$ and $I_{L_2}$ are treated as constants to which all other measured intensity values are related. Once quenching terminates, the residual fluorescence intensity parameters ($I_{RES1}$ and $I_{RES2}$) characterize the remaining fluorescence signal at the wavelengths of the set.

Substituting Eqs. 6 and 7 into Eq. 3 yields:

\[
C_M = [M] + K_1C_{L_1}[M]/(K_1[M] + 1) + K_2C_{L_2}[M]/(K_2[M] + 1)
\] (10)

Multiplying through by $([K_1[M] + 1)(K_2[M] + 1)$ gives the following third-order polynomial:

\[
\begin{align*}
K_1K_2[M]^3 + \{K_1K_2(C_{L_1} + C_{L_2} - C_M) + K_1 + K_2\}[M]^2 + \{C_{L_1}K_1 + C_{L_2}K_2 - C_M(K_1 + K_2) + 1\}[M] - C_M = 0
\end{align*}
\] (11)

Equations 8, 9, and 11 predict the fluorescence intensity values $I_1$ and $I_2$ corresponding to a given value of the added metal concentration ($C_M$). We first solve Eq. 11 numerically to find the value of free metal concentration [$M$], then we use Eqs. 8 and 9 to calculate $I_1$ and $I_2$. Of the eight parameters in this model, two ($I_{L_1}$ and $I_{L_2}$) are measured experimentally and six ($K_1$, $K_2$, $C_{L_1}$, $C_{L_2}$, $I_{RES1}$, and $I_{RES2}$) are estimated numerically using the standard least-squares procedure. We measure the values of $I_1$ and $I_2$ corresponding to several different values of $C_M$, and we then choose the values of $K_1$, $K_2$, $C_{L_1}$, $C_{L_2}$, $I_{RES1}$, and $I_{RES2}$ so as 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.

**EXPERIMENTAL**

**Apparatus.** A Farrand Mark 1 spectrofluorometer (Farrand Optical Company Inc., Valhalla, NY) was used to obtain fluorescence data. The instrument was equipped with two grating monochromators with slits set for 5-nm excitation and 10-nm emission bandpass, a 150 watt xenon arc lamp, and a photomultiplier tube. Wavelength and intensity data from the analog output of the instrument were fed to an analog/digital board (Metabyte, Kiethly, Taunton, MA) and processed using several pro-
grams written in BASIC. Data were viewed on a personal computer (Dell Dimension, Dell Computer Corporation, Austin, TX) running GRAMS/386 software (V2.0, Galactic Industries, Salem, NH).

Titrations were performed in a temperature-jacketed titration cell fitted with a cap (Princeton Applied Research, EG & G, Princeton, NJ). Inserted through the top of the cell was a glass combination pH electrode (Ross 8104, Orion, Boston, MA) attached to an autochemistry system (960, Orion). A constant temperature water bath (VWR Scientific, Boston, MA) maintained solution temperature at 25 °C (±2 °C). Transfer of solution from the titration cell to the 10-mm quartz cuvette (NSG Precision Cells, Farmingdale, NY) and reagent additions were made manually by pipette (Brinkmann Instruments Inc., Westbury, NY).

Reagents. The source of FA may very well govern the relationship between Cu²⁺ complexation and quenching. A well-characterized³⁰,³¹ FA isolated from the B₂ horizon of a podzol soil was obtained from Dr. James Weber (Department of Chemistry University of New Hampshire, Durham, NH) and used here. Within the experimental Cₓₘ range, a linear relation between Cu²⁺ complexation and quenching was verified for this FA,⁹ justifying its use in developing the MWM. This linear correlation was also apparent for humic-metal systems of natural waters.¹⁰ For use in this study, all FA solutions were prepared in distilled deionized water (17 MΩ, Barnstead-Nanopure) to prevent interference from other FA-binding cations and filtered through 0.4 μm polycarbonate filters (Costar Corp., Cambridge, MA).

The L-tyrosine (Tyr) (Aldrich Chemical Co., Milwaukee, WI) and salicylic acid (Sal) (Fisher Scientific Co., Pittsburgh, PA) stock solutions were prepared in deionized water made basic with NaOH (1 M), stirred (~2 h), and neutralized with perchloric acid (1 M). A fresh mixture of Sal (70 μM) and Tyr (35 μM), prepared from individual stock solution aliquots, was used for each model compound titration. Appropriate amounts of reagents were added to FA to form model compound solutions to maintain ionic strength (0.1 M, NaClO₄) and pH (from 5 to 7, NaOH and HClO₄) constant.

The Cu²⁺ standard was prepared from reagent grade perchlorate salt and standardized by direct current plasma atomic emission spectrometry (Applied Research Laboratory, Valencia, CA) versus commercial standards.

Fluorescence Titration Procedure. A comprehensive description of the procedure used here was described elsewhere.⁹ Briefly, in a titration cell, the pH of a continuously stirred solution (50 mL) of dissolved FA (15 mg/L) was adjusted to within 0.02 pH units of the desired value. Following an equilibration period (8–10 min), the solution (1 mL) was transferred from the titration cell to the quartz cuvette and transferred back to the cell as a rinse. An aliquot (2 mL) of solution was then delivered to the cuvette, fluorescence measurements at appropriate wavelengths were taken, and the solution was transferred back to the titration cell. A second set of fluorescence measurements of the same wavelengths was taken after a micromolar aliquot of Cu²⁺ was delivered to the titration cell, the pH readjusted, and the solution equilibrated. These steps were repeated until complete titration curves for each wavelength set were generated.

Soil Fulvic Acid Fluorescence Measurements. Excitation and emission spectra as well as ΔN = 25 nm SyF were collected for FA solutions at pH values of 5, 6, and 7. SyF wavelength maxima, λ_N = 415 nm, λ_em = 440 nm (415/440 nm); and λ_N = 471 nm, λ_em = 496 nm (471/496 nm), were utilized. The excitation and emission maxima were observed at λ_N = 335 nm, λ_em = 450 nm (335/450 nm); and λ_N = 471 nm, λ_em = 496 nm (471/496 nm). Measurements were placed into sets (1) wavelength set 1, 415/440 nm and 471/496 nm and (2) wavelength set 2, 335/450 nm and 471/496 nm. Each set was modeled separately with MWM. We assessed the ability of 415/440 nm SyF maximum, a cross-section of the 335/440 nm excitation and emission maximum, to measure quenching and, in turn, binding at the main fluorescence component (335/440 nm).

Model Compound Fluorescence Measurements. The fluorescence excitation and emission maxima of Sal and Tyr were observed at λ_N = 317 nm, λ_em = 404 nm; and λ_N = 277 nm, λ_em = 296 nm, respectively. Modeling input data were acquired at pH 6 using ΔN = 50 nm SyF. This offset was determined to be optimum for monitoring the Sal and Tyr fluorescence.

Data Treatment. The details of implementation of the MWM curve-fitting operation will be described extensively.³² Briefly, the curve-fitting routine was implemented using a Nelder–Mead simplex algorithm in Matlab (Math Works Inc., Natick, MA).³³ A computer program was designed, in which the parameter values K₁, K₂, C₁₁, C₁₂, l₁₉₁, and lᵦᵡᵠ₂ were chosen so as to minimize the sum of the squares of the differences between experimental intensity values and values predicted by the model. A numerical solution for M was calculated using Eq. 11 and the ROOTS command in Matlab. Only one positive real root exists for Eq. 11.

RESULTS AND DISCUSSION

Fluorescence Titrations of Model Compounds. To assess the predictive capability of the MWM, Sal and Tyr are titrated with Cu²⁺. Although not perfectly representative of the entire complex FA ligand mixture, these compounds exhibit fluorescence behavior and carboxyl and phenolic functionalities similar to the FA sample used here.²⁸ Also, as evidenced by electron spin resonance analysis, the FA used here primarily comprises aromatic moieties in the form of semiquinone-like structures,³⁴ attesting to the refractory nature of FA. Though useful for this work, extension of the chemical properties of these model compounds to typify all FA samples may not be reasonable. Arrays of macromolecular structures in the FA sample likely depend on its source. It should also be clear that these model compounds cannot mimic all binding stoichiometries (such as growth of a metal chelate from independent sites), aggregate forms, and conformational properties of FA, particularly at high metal loadings.

For replicate Cu²⁺ titrations (N = 3) of fluorescent Sal and Tyr, Table I lists the mean conditional stability constant (log K₁ and K₂) and ligand concentration values (C₁₁ and C₁₂) and their experimental standard deviations as predicted by the MWM. These MWM estimates reveal that Cu²⁺ complexes Tyr more strongly than Sal. And
TABLE I. MWM calculated equilibrium parameters for a mixture of the two model compounds.

<table>
<thead>
<tr>
<th>Model compound</th>
<th>Conditional log ( K ) from literature ref. 24</th>
<th>MWM calculated log ( K )</th>
<th>Experimental ( C_L (\mu M) )</th>
<th>MWM calculated ( C_L (\mu M) )</th>
<th>MWM calculated ( I_{RES} (\mu M) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal</td>
<td>3.20</td>
<td>3.76 (±0.14)</td>
<td>70.0 (±6.47)</td>
<td>65.8 (±6.47)</td>
<td>15.9 (±8.6)</td>
</tr>
<tr>
<td>Tyr</td>
<td>4.55</td>
<td>4.79 (±0.16)</td>
<td>35.0 (±6.47)</td>
<td>14.9 (±6.6)</td>
<td>2.4 (±0.5)</td>
</tr>
</tbody>
</table>

*Standard deviations are in parentheses.

consistent with the model framework, a larger percentage of Tyr fluorescence is quenched (data not shown). Closer analysis shows that mean log \( K \) values of Tyr and Sal are within 5 and 20% of their respective theoretical conditional stability constant values, which are calculated using the experimental pH of 6, each species' p\( K_a \), and thermodynamic stability constants.\(^{35}\) The high average MWM-approximated log \( K \) value for the Cu–Sal complex may be due to the moderate fluorescence change caused by the weak complexation of Cu\(^{2+} \) by Sal. As expected, the calculated average value of \( C_L \) for Sal is within 6% of its experimentally fixed concentration, whereas the average calculated \( C_L \) value for Tyr is estimated as approximately half of its experimental value. For the latter result, formation of a 2:1 Tyr–copper complex seems evident.\(^{36}\)

The relatively high \( I_{RES} \) value for Sal indicates that it is in its free form near the titration end. In marked contrast, the low \( I_{RES} \) value for Tyr suggests that quenching is nearly complete and essentially all the Tyr is bound at high \( C_M \). Examination of the curve-fitting results shows that values of log \( K \) calculated for Sal and Tyr are well within the acceptable uncertainty range of ±0.25 log units. MWM predictions generally agree well with theory and verify that the multi-wavelength measurements of a mixed ligand system may be used to estimate coordination strength and ligand capacity for Cu\(^{2+} \).

**Fluorescence Quenching of Wavelength Set 1.** The \( \Delta \lambda = 25 \) nm SyF of our soil FA contains a 415/440 nm peak, which transects the excitation and emission maximum (335/440 nm), and the 471/496 nm maximum, which make up wavelength set 1. Figure 1 shows the effect at pH 6 of 0–60 \( \mu M \) Cu\(^{2+} \) on the FA SyF spectra (15 mg/L). The narrow 471/496 nm component is more rapidly quenched than the broader, less intense 415/440 nm peak, an indication that these two fluorophores represent ligands that uniquely complex Cu\(^{2+} \). This pattern is observed at all pHs studied. Figure 2 provides the fluorescence titration data at pH 5 at these wavelengths. These data (Fig. 2) (1) verify the difference in the degree of quenching at each wavelength, (2) show that a large proportion of the quenching occurs at high ligand-to-Cu\(^{2+} \) ratios (low Cu\(^{2+} \) loadings) as expected, and (3) show titration data in agreement with the MWM-predicted values (solid lines).

Table II gives MWM-predicted mean and standard deviation values of \( log K_1 \), \( log K_2 \), \( C_{L1} \), \( C_{L2} \), \( I_{RES1} \), and \( I_{RES2} \) from replicate titrations \((N = 3)\) at pH values of 5, 6, and 7. At each pH, \( log K_2 \) (471/496) > \( log K_1 \) (415/440), confirming that the 471/496 nm fluorophore characterizes stronger binding organic ligands. Estimates show that \( log K_1 \) (415/440) and \( log K_2 \) (471/496) increase as a function of pH; however, the increase is negligible from pH 6 to 7. Perhaps ligand sites in our FA are completely deprotonated across this range, thereby lessening the influence of pH on the \( log K_1 \) and \( K_2 \) values. Alternatively, this result may be due to the omission of formed Cu–hydroxy species from model calculations at pH 7, which in effect decreases the [Cu\(^{2+} \)] available to coordinate to ligand sites.

Because \( C_{L1} = [L1] + [ML1] \), model-obtained values...
of $C_{L_1}$ should be equal despite pH. The same is also valid for $C_{L_2}$ (471/496), $C_{L_2} = [L_2] + [ML_2]$. MWM-determined mean values of $C_{L_1}$ and $C_{L_2}$ at pH values of 6 and 7 correspond to within 28 and 18%. Because $C_{L_1}$ (415/440) > $C_{L_2}$ (471/496) at these pH values, a lower concentration of stronger binding ligands is assigned to the 471/496 nm fluorophore. At pH 5, $C_{L_2}$ > $C_{L_1}$, and $I_{RES_1}$ increases by a factor of two when compared with $I_{RES_1}$ values at pH values of 6 and 7. Ryan and Weber\(^a\) had difficulty modeling fluorescence titration data at pH 5 with the same FA used here. A single data set that was treated at this pH provided a relatively high $I_{RES_1}$ of 40.2. This value is nearly identical to the $I_{RES_1}$ of 39.2 ± 1.7 calculated here and suggests that significant levels of molecules are in solution that (1) fluoresce but do not complex, (2) fluoresce, complex, and quench only partially, or (3) fluoresce, complex, and do not undergo quenching. Only type (3) material remains unaccounted for by the model. Curve-fitting results show that $I_{RES_1}$ (415/440) > $I_{RES_2}$ (471/496) at each pH; fluorophore 2 exhibits a higher degree of quenching. At all pH values, the calculated averages of $I_{RES_2}$ (471/496) are similar, indicating that termination of quenching at this peak is independent of pH.

**Fluorescence Quenching of Wavelength Set 2.** The fluorescence excitation–emission maxima (335/450 nm and 471/496 nm) combine to form wavelength set 2. At these selected wavelengths, fluorescence titration data at pH 5 are given (Fig. 3). The measured quenching provides added support for the presence of individual components with distinct fluorescence and coordination characteristics. Compared with the 335/450 nm maximum, quenching at 471/496 nm advances more rapidly at lower $C_M$ loadings ($C_M < 10 \mu M$). As the titration proceeds ($C_M > 10 \mu M$), the quenching observed at 335/450 nm exceeds that at 471/496 nm. Near the titration end ($C_M > 25 \mu M$), the relative fluorescence at these wavelengths is essentially equal.

The MWM-predicted logarithmic values of $K_1$ (335/450) and $K_2$ (471/496) at wavelength set 2 increase with pH on average (Table III). In addition, log $K_1$ (335/450) < log $K_2$ (471/496) at each pH, verifying that the molecules excited at longer wavelengths bind Cu\(^{2+}\) more strongly. Mean $C_{L_1}$ (335/440) values at pH values of 6 and 7 agree to within 15%, and across the same pH range $C_{L_2}$ (471/496) values agree to within 10% of one another, where $C_{L_1}$ (335/440) > $C_{L_2}$ (471/496). Modeling at the maxima did not correct the reverse trend ($C_{L_2}$ > $C_{L_1}$) at pH 5, which is also observed at wavelength set 1. $I_{RES_1}$ at pH 5 is 2-fold greater than both of the pH 6 and 7 values, which are virtually identical. Data of both wavelength sets show that this increase in the model-estimated value of $I_{RES_1}$ causes a significant decrease in $C_{L_1}$, marking a potential artifact in modeling $C_{L_1}$.

![Figure 3. Wavelength set 2 fluorescence quenching data (○) and model-fitted curves (as solid lines) for the Cu\(^{2+}\) titration of 15 mg/L FA at 0.1 M ionic strength, 25 °C, and pH 5.](image)

**Comparison of Wavelength Sets 1 and 2.** The main features of the data are supported by the model. The fluorescence excitation–emission maxima (335/450 nm and 471/496 nm) combine to form wavelength set 2. At these selected wavelengths, fluorescence titration data at pH 5 are given (Fig. 3). The measured quenching provides added support for the presence of individual components with distinct fluorescence and coordination characteristics. Compared with the 335/450 nm maximum, quenching at 471/496 nm advances more rapidly at lower $C_M$ loadings ($C_M < 10 \mu M$). As the titration proceeds ($C_M > 10 \mu M$), the quenching observed at 335/450 nm exceeds that at 471/496 nm. Near the titration end ($C_M > 25 \mu M$), the relative fluorescence at these wavelengths is essentially equal.

The MWM-predicted logarithmic values of $K_1$ (335/450) and $K_2$ (471/496) at wavelength set 2 increase with pH on average (Table III). In addition, log $K_1$ (335/450) < log $K_2$ (471/496) at each pH, verifying that the molecules excited at longer wavelengths bind Cu\(^{2+}\) more strongly. Mean $C_{L_1}$ (335/440) values at pH values of 6 and 7 agree to within 15%, and across the same pH range $C_{L_2}$ (471/496) values agree to within 10% of one another, where $C_{L_1}$ (335/440) > $C_{L_2}$ (471/496). Modeling at the maxima did not correct the reverse trend ($C_{L_2}$ > $C_{L_1}$) at pH 5, which is also observed at wavelength set 1. $I_{RES_1}$ at pH 5 is 2-fold greater than both of the pH 6 and 7 values, which are virtually identical. Data of both wavelength sets show that this increase in the model-estimated value of $I_{RES_1}$ causes a significant decrease in $C_{L_1}$, marking a potential artifact in modeling $C_{L_1}$.

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Table IV. Literature conditional stability constant and ligand concentration values calculated from multi-wavelength fluorescence quenching data of Cu\(^{2+}\)-treated soil FA.

<table>
<thead>
<tr>
<th>pH</th>
<th>log (K_1) (335/440)</th>
<th>log (K_2) (471/496)</th>
<th>(C_{L_1}) (335/440) ((\mu)M)</th>
<th>(C_{L_2}) (471/496) ((\mu)M)</th>
<th>(I_{RES1})</th>
<th>(I_{RES2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.50 (±0.03)</td>
<td>5.02 (±0.04)</td>
<td>8.8 (±0.5)</td>
<td>17.6 (±2.5)</td>
<td>21.0 (±1.5)</td>
<td>21.4 (±1.1)</td>
</tr>
<tr>
<td>6</td>
<td>4.96 (±0.27)</td>
<td>5.42 (±0.32)</td>
<td>21.9 (±7.9)</td>
<td>1.3 (±1.2)</td>
<td>7.17 (±5.6)</td>
<td>15.5 (±3.4)</td>
</tr>
<tr>
<td>7</td>
<td>5.22 (±0.08)</td>
<td>5.71 (±0.09)</td>
<td>18.7 (±0.3)</td>
<td>1.2 (±0.3)</td>
<td>7.09 (±3.7)</td>
<td>19.8 (±3.7)</td>
</tr>
</tbody>
</table>

* Standard deviations are in parentheses.

Titration experiments carried out by these investigators\(^{13,20,21}\) are in some cases orders of magnitude greater than those used in our work, the ligand concentration estimates are normalized to 15 mg/L for ease of comparison.

With the exception of the pH 7 case,\(^{13}\) in which the FA sample is the same as that used in this work, a FA isolated from a Portuguese forest soil is used, and quenching of \(\Delta \lambda = 20 \text{ nm} \) SyF spectra are deconvoluted to obtain signature bands used in modeling groups of Cu\(^{2+}\) binding molecules. In comparing the outcomes at pH 7, it can be seen that the conditional stability constants obtained for wavelength set 2 (Table III) agree well with the work of Seritti et al.\(^{13}\) because the same FA material and wavelengths for collecting quenching data are used in both studies. The ligand concentration values calculated, however, show a significant difference and indicate that the modeling approaches used in these studies, although similar in principle, are not perfectly alike.

The equilibria values determined by Machado, et al.\(^{21}\) significantly vary from those we report (Table II), as the origin of the FA, scanning offset, and modeling and analysis routines used are all different. Estoves da Silva and co-workers\(^{21}\) calculate an added set of equilibria values using the same FA, scanning offset, and modeling protocol employed by Machado et al.\(^{21}\) but implement an evolving factor analysis operation instead. These calculated values are in agreement (Table II) with those obtained here.

CONCLUSION

Evidence of two main fluorescence components in a well-characterized FA isolated from the B\(_2\) horizon of a podzol soil is offered. Among other variables, the wavelength scanning procedure will effect how a fluorophore appears in a spectrum, which organic ligands are excited, and final titrimetric model estimates of solution equilibria. MWM-determined conditional stability constant and ligand concentration values provide features of copper-complexation by natural FA molecules at varying pH values for different multi-wavelength sets. Because quenching at the short wavelength (415/440 nm) peak in the SyF spectra of our FA terminates prior to the titration finish, accurate evaluation of the ligand concentration using this wavelength set proves challenging. This finding may impact investigations for which different wavelength scanning procedures are implemented to model metal complexation and solution equilibria. In summation, fluorescence quenching data in titrimetric form can be collected at representative multi-wavelength sets and used as input to the MWM model for calculation of conditional stability constant and ligand concentration values of FA. In each case, it is expected that the proportionality between.
free ligand concentration and signal is independently verified.

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