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Considering the ionic strength for proper use of 1 or 2-ligands model for static fluorescence quenching or enhancement

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Highlights

- Easy-to-use model for rapid but rigorous analysis of experimental data.
- Consideration of ionic strength which cannot be overlooked in many environmental applications.
- Two ligands are required to correctly model natural organic matter.
- An improvement of the initial Ryan and Weber model.

Abstract

We formally describe a 1- or 2-ligands fluorescence quenching or exhaustion model that takes ionic strength into account. We give ready-to-use formulas, which are easy to implement on a common spreadsheet, to determine complexing capacities and apparent stability constants of fluorescence ligands by adjusting quenching or enhancing experimental curves. The strength of our model is to consider parameters that have rarely taken in account in the literature, resulting in a significant improvement in the quality of the modeling: the charge associated with one or two ligands, and ionic strength. The model predicted fluorescence at various ionic strengths from parameters determined at a given ionic strength. This model is suitable for many applications, such as complexation of dissolved natural organic matter with metal ions, even in sea water, or biologic media.

Keywords: Fluorescence quenching, Modeling, Complexation, Stability constants, Binding capacity, Natural Organic Matter

1 Introduction

Fluorescence quenching or enhancing method is increasingly used in many scientific fields, especially environmental, biological or nanoscience studies, because it is a sensitive, fast, selective, non-destructive, and cheap method to characterize and quantify the interactions between one or more fluorescence ligands and a quencher [1]. Despite its apparent simplicity, this method should be used appropriately to avoid misinterpretation of the data [2,3]. It is necessary to check the inner-filter effect and the type of quenching, most of the time collisional (dynamic) versus binding (static), and in some cases to consider the fluorophore accessibility to the quencher.

Subject to checking its relevance, static fluorescence quenching is a very useful tool to determine complexing capacities and apparent stability constants of fluorescence ligands [1,4]. Changes in fluorescence intensity during fluorescence quenching experiments have been monitored in several ways. The oldest studies considered single excitation/emission wavelength pairs (EEWP) [1], total luminescence [5] or synchronous-scan spectral bands [6]. The development of 3-D fluorescence spectroscopy allowed choosing, from a given excitation-emission fluorescence matrix (EEFM), a number of EEWPs corresponding to the fluorescence peaks or areas of interest quantified by regional integration analysis (RIA) [7] or Parallel Factor Analysis (PARAFAC) data treatment that statistically identify components supposed to be chemically meaningful [8-11].

Whatever method is used, the variation in fluorescence intensity during quenching or enhancement have been used to determine the binding parameters for the metal and the ligands using models usually derived from the Ryan and Weber [1] approach. It considers the formation of a single metal-ligand complex and assumes a linear relationship between the complex concentration and the fluorescence intensity. A single ligand model, however, is insufficient to account for the heterogeneity of binding sites when considering complex compounds such as natural dissolved organic matter (DOM) [12]. The multiwavelength approach as initiated by Luster et al. [4] is a method of choice to account for more than one ligand. Each EEWP, area of interest or PARAFAC component can be considered as a quasiparticle [13] having its own binding parameters with the quencher. Even so, authors had frequently to consider quasiparticles that have two ligands to account for weak and strong complexes or for competition between a non-fluorescent quasiparticle and a fluorescent quasiparticle [4,14]. Multiplying ligands beyond 2 or 3 for a single quasiparticle, however, is not realistic: although it may in some cases be necessary to consider 3 ligands [15], in most cases 2 ligands are sufficient to account for the observations.

When considering one ligand L only, the quasiparticle stability constant K_c and binding capacity [L] can be easily obtained in a common spreadsheet by fitting the experimental quenching curves with a nonlinear solving algorithm, using the mathematical formalism derived from Ryan and Weber [4]. When considering two ligands L_1 and L_2 , the calculation of the free metal concentration requires solving a third order polynomial equation [14] for each point of the simulated quenching curve at each iteration of the curve fitting algorithm. As it is tedious to do with a simple spreadsheet, authors restrict themselves to using only one ligand or develop specific programs, using for example Matlab (MathWorks Inc., Natick, MA) or R software [14-17], which is time-consuming and hard to reproduce on subsequent investigations.

There are also very few studies [18,19] that account for ionic strength in the mathematical formulation of fluorescence intensity. Many studies, indeed, were carried out in dilute aqueous media, for which the ionic strength can be neglected. The effect of the ionic strength on the quenching of fluorescence in aqueous solution, however, was underlined nearly 100 years ago [20] and when the studies are conducted in aqueous media with ionic strength greater than 10^{-3} mol L^{-1} , the ionic strength must be considered. For example, in a solution of 10^{-2} mol L^{-1} ionic strength, the activity coefficients calculated with the Guntelberg approximation for divalent and trivalent ions are 0.65 and 0.38, respectively. In a 10^{-1} mol L^{-1} ionic strength solution, they are 0.32 and 0.08, respectively.

Here we give an analytical solving of 1- or 2-ligands fluorescence quenching or enhancement, taking into account the ionic strength and therefore the ligand charges. When considering 2-ligands, the given formulas correspond to a formal resolution of the third order polynomial equation, which allows a simple and fast fitting between calculated and observed data in a common spreadsheet. We give an experimental validation of the theory using a natural organic matter.

2 Methods

2.1 Theory

2.1.1 One ligand

The set of equations that accounts for the complexation of a metal M with a ligand L to give the complex ML is:

$$\begin{cases} \gamma_{ML}[ML] = K_C \gamma_M[M]\gamma_L[L] \\ C_M = [M] + [ML] \\ C_L = [L] + [ML] \end{cases} \quad (1)$$

C_M is the total metal concentration, C_L the complexing capacity of the ligand L, K_C the stability constant and γ_i the activity coefficient of the i specie. The usual Ryan and Weber method neglects the ionic strength, so that the activity coefficients are equal to 1, which results in the following expression of the fluorescence intensity I_f as a function of C_M :

$$I_f = I_{f0} - \frac{(I_{f0} - I_{fR})}{2K_C C_L} \left((K_C (C_M + C_L) + 1) - \sqrt{(K_C (C_M + C_L) + 1)^2 - 4K_C^2 C_M C_L} \right) \quad (2)$$

I_{f0} is the fluorescence intensity at the beginning of the experiment, i.e., with $C_M = 0$; I_{fR} is the fluorescence intensity supposing that all the fluorescent ligands are complexed, i.e., $[ML] = C_L$. In the case of quenching, I_{fR} is the residual fluorescence due to non-complexing fluorophores and to a possible residual fluorescence of the complexed ligands.

When considering the ionic strength I , activity coefficients affect concentrations, and the expression of the fluorescence intensity becomes:

$$I_f = I_{f0} - \frac{(I_{f0} - I_{fR})}{2 K_C \gamma_M \gamma_L C_L} \left((\gamma_{ML} + \gamma_M \gamma_L K_C (C_M + C_L)) - \sqrt{(\gamma_{ML} + \gamma_M \gamma_L K_C (C_M + C_L))^2 - 4 \gamma_M^2 \gamma_L^2 K_C^2 C_M C_L} \right) \quad (3)$$

The activity coefficients, however, vary with I that depends on the charge of the ions in solution z_M , z_{Cl} , z_L and z_{ML} for, respectively, the free metal M, the counterion Cl, the ligand L, the complex ML and eventually an initial ionic strength I_0 due to species supposed not to compete with complexation, as a background electrolyte such as NaCl:

$$I = \frac{1}{2} ([M]z_M^2 + [Cl]z_{Cl}^2 + [L]z_L^2 + [ML]z_{ML}^2) + I_0 \quad (4)$$

The counterion being inactive in the solution, its concentration is given by:

$$[Cl] = C_M \left| \frac{z_M}{z_{Cl}} \right| \quad (5)$$

Combining (3) and (4) it comes:

$$I = \frac{1}{2} \left([M]z_M^2 + C_M \left| \frac{z_M}{z_{Cl}} \right| z_{Cl}^2 + [L]z_L^2 + [ML]z_{ML}^2 \right) + I_0 \quad (6)$$

Activity coefficients are calculated with the appropriate approximation, the Guntelberg approximation being for example adapted for ionic strength up to 10^{-1} mol L⁻¹.

$$\log(\gamma_i) = -0.509 z_i^2 \left(\frac{\sqrt{I}}{1 + \sqrt{I}} \right) \quad (7)$$

At the start of calculation, I is not known, an approximation is therefore made using the following expression:

$$I \approx \frac{1}{2} \left(C_M z_M^2 + C_M \left| \frac{z_M}{z_{Cl}} \right| z_{Cl}^2 \right) + I_0 \quad (8)$$

The ionic strength I is then recalculated with the obtained concentrations and the procedure is repeated, two iterations being usually sufficient.

From a certain level, the increase in I may result in decomplexation of the metal, so the hypothesis that at the end of the experiment all the ligands are complexed with the metal is no longer valid. The value of I_{fR} therefore corresponds neither to the fluorescence observed at the end of the experiment, nor to the minimum (quenching) or maximum (enhancement) fluorescence I_{fm} observed during the experiment. It might be necessary to assume the value of I_{fR} , this being between 0 and I_{fm} in the case of a quenching. This is illustrated on Fig. 1 where it is shown that, in the case of a high initial ionic strength (here we have taken a ionic strength close to that of seawater), with increasing introduced metal concentration the metal begins to be released from the complex before the residual fluorescence is reached. In most cases, however, the I_{fR} value can be approximated by I_{fm} .

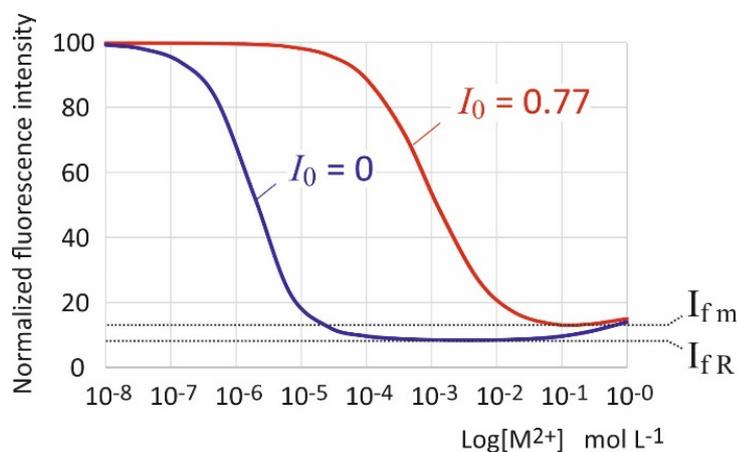


Fig. 1. Modeled fluorescence considering one ligand ($C_L = 1.2 \cdot 10^{-5}$; $K_M = 3.1 \cdot 10^5$), a divalent metal, and two initial ionic strength I_0 .

2.1.3 Two ligands

The set of equation that accounts for the complexation of a metal M with two ligand L_1 and L_2 to give the complexes ML_1 and ML_2 is:

$$\begin{cases} Y_{ML_1}[ML_1] = K_{C1} Y_M[M] Y_{L1}[L_1] \\ Y_{ML_2}[ML_2] = K_{C2} Y_M[M] Y_{L2}[L_2] \\ C_M = [M] + [ML_1] + [ML_2] \\ C_{L1} = [L_1] + [ML_1] \\ C_{L2} = [L_2] + [ML_2] \end{cases} \quad (9)$$

C_{L1} and K_{C1} , C_{L2} and K_{C2} are the complexing capacity and the stability constant of the ligands L_1 and L_2 , respectively. Solving this set of equation, we obtain a third order equation for $[M]$:

$$a[M]^3 + b[M]^2 + c[M] + d = 0 \quad (10)$$

with:

$$\begin{aligned}
a &= \gamma_M^2 \gamma_{L1} \gamma_{L2} K_{C1} K_{C2} \\
b &= \gamma_M (\gamma_{L1} \gamma_{ML2} K_{C1} + \gamma_{L2} \gamma_{ML1} K_{C2}) + \\
&\quad \gamma_M^2 \gamma_{L1} \gamma_{L2} K_{C1} K_{C1} (C_{L1} + C_{L2} - C_M) \\
c &= \gamma_{ML1} \gamma_{ML2} + \gamma_M \gamma_{L1} \gamma_{ML2} K_{C1} (C_{L1} - C_M) + \\
&\quad \gamma_M \gamma_{L2} \gamma_{ML1} K_{C2} (C_{L2} - C_M) \\
d &= - \gamma_{ML1} \gamma_{ML2} C_M
\end{aligned}$$

This equation can be solved by the classical Cardano's method using the discriminant Δ :

$$\Delta = q^2 + \frac{4p^3}{27} \quad (11)$$

with

$$q = \frac{2b^3}{27a^3} - \frac{bc}{3a^2} + \frac{d}{a} \quad \text{and} \quad p = \frac{c}{a} - \frac{b^2}{3a^2}$$

With the possible values of the parameters used in the present chemical problem, Δ is always negative and, amongst the three possible real solutions, the following one only is possible:

$$[M] = \sqrt[3]{-\frac{q}{2} + i \frac{\sqrt{|\Delta|}}{2}} + \sqrt[3]{-\frac{q}{2} - i \frac{\sqrt{|\Delta|}}{2}} - \frac{b}{3a} \quad (12)$$

This value is used with the set of eqs (9) to calculate $[ML_1]$ and $[ML_2]$:

$$[ML_1] = \frac{K_{C1} C_{L1} [M]}{1 + K_{C1} [M]} \quad \text{and} \quad [ML_2] = \frac{K_{C2} C_{L2} [M]}{1 + K_{C2} [M]} \quad (13)$$

The I and γ_i values can be calculated in the same way that for one ligand:

$$\begin{aligned}
I = \frac{1}{2} \left([M] z_M^2 + C_M \left| \frac{z_M}{z_{CI}} \right| z_{CI}^2 + [L_1] z_{L1}^2 + [ML_1] z_{ML1}^2 + \right. \\
\left. [L_2] z_{L2}^2 + [ML_2] z_{ML2}^2 \right) + I_0 \quad (14)
\end{aligned}$$

Calculation of I_f as a function of C_M requires a hypothesis on the ratio k_L of quantum yields Φ_{L1} and Φ_{L2} of ligands L_1 and L_2 :

$$k_L = \frac{\Phi_{L1}}{\Phi_{L2}} \quad (15)$$

I_{f0} and I_f are therefore expressed by:

$$I_{f0} = C_{L1} \Phi_{L1} + C_{L2} \Phi_{L2} + I_{fR} \quad (16)$$

$$I_f = (C_{L1} - [ML_1]) \Phi_{L1} + (C_{L2} - [ML_2]) \Phi_{L2} + I_{fR} \quad (17)$$

The assumption for I_{fR} value is the same than for one ligand. I_f is given by combining eqs (16) and (17):

$$I_f = (I_{f0} - I_{fR}) \frac{[k_L(C_{L1} - [ML_1]) + (C_{L2} - [ML_2])]}{k_L C_{L1} + C_{L2}} + I_{fR} \quad (18)$$

Note that even after verification of the static nature of the fluorescence quenching or enhancement, there remain several uncertainties related to the formulation of the model. In the example given here, the activity coefficients are calculated using the Guntelberg approximation, which may not be suitable for certain media such as sea water. The model also assumes the existence of discrete ligands, which may be an approximation in the case of complex natural media.

2.2 Experimental

2.2.1 Sample Preparation

Validation of the theory was performed using complexation of Cu^{2+} by a dissolved humic acid (HA) at 20 mg L^{-1} , a concentration which is of the order of magnitude of DOM concentration in many natural solutions as soil waters or rivers. The Cu^{2+} stock solution was prepared from a CuCl_2 powder and the HA stock solution from a commercial humic acid (HA) powder (Alfa Aesar, VWR). The tested Cu^{2+} concentrations were 10^{-9} , 10^{-6} , $4 \cdot 10^{-6}$, 10^{-5} , $4 \cdot 10^{-5}$, 10^{-4} , $4 \cdot 10^{-4}$, 10^{-3} and $4 \cdot 10^{-3} \text{ mol L}^{-1}$, the highest concentrations being low enough to avoid precipitation of $\text{Cu}(\text{OH})_2$ at the working pH. As the fluorescence intensity of humic substances is highly sensitive to pH [21], the pH of the solutions was adjusted to pH 5.3 using NaOH and HCl solutions, that were preferred to usual buffers in order to avoid any impact on the fluorescence signal [22]. The ionic strength (I) was adjusted to 0.01, 0.1 and 0.5 mol L^{-1} using NaCl. All reagents were trace metal grade.

2.2.2 Instrumentation and Experimental Setup

Fluorescence measurements were performed using a Hitachi model F-4500 spectrophotometer. The absence of inner filter effect was assessed by checking the proportionality of the fluorescence intensity with a sample dilution factor. A 3D excitation–emission fluorescence matrix (EEFM) was obtained for each batch solution. The excitation and emission windows were set to 5 and 10 nm, respectively. The excitation and emission wavelengths ranged from 200 nm to 500 nm and 250 nm to 600 nm, respectively, with a 5 nm step for both at a scanning speed of $2400 \text{ nm} \cdot \text{min}^{-1}$. The photomultiplier was set to 700 V.

The HA used showed the two characteristic peaks A and C usually observed in dissolved humic matter [23], at excitation-emission pairs (λ_{ex} , λ_{em}) around (260, 450) and (450, 515) nm, respectively (Fig. 1). We used the variation in intensity of the maximum of peak A for the quantification of fluorescence quenching with increasing concentrations of Cu^{2+} .

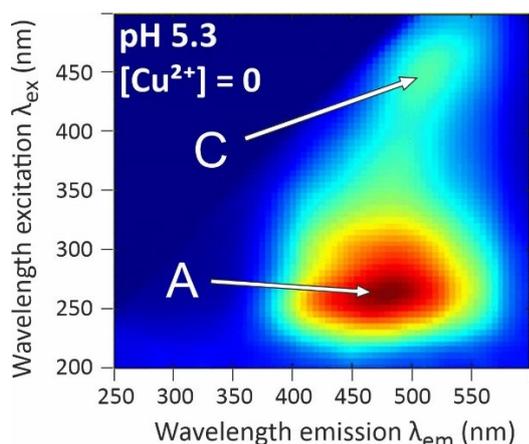


Fig. 2. Excitation–emission fluorescence matrix (EEFM) of the humic acid solution at pH 5.3.

2.2.3 Implementation in a spreadsheet

The implementation on common spreadsheets of the formulas developed above does not present any difficulties, since they support calculations with complex numbers. For the 2-ligands model, the entry data are I_{f0} , I_{fR} , Z_M , Z_{C1} , Z_{L1} , Z_{L2} and the (C_M, I_f) values of the experimental measurement points. The parameters to be adjusted by a non-linear solver are K_{C1} , C_{L1} , K_{C2} , C_{L2} and k_L ; the value to be minimized by the solver is the sum of squared error $(I_{obs} - I_{calc})^2$. Note that depending on the problem or the likelihood of the results, the value of k_L can be fixed and the values of Z_{L1} and Z_{L2} can be adjusted by the solver. An example of an implemented spreadsheet for the 2-ligands model is given as supporting information.

3. Results

3.1 Model Validation

To evaluate the model we compared the parameters C_{L1} , K_{M1} , C_{L2} and K_{M2} calculated for each ionic strength by fitting the 2-ligands model to the experimental points. The ratio of the quantum yields of the L1 and L2 ligands was initially left to be fitted by the model; as the best fit for the 4 ionic strengths showed its value to vary between 0.94 and 1, it was set to 1 for the following. The fitted curves are given in Fig. 3 (plain lines) and the corresponding calculated parameters are given in Fig. 4 (values in circles). The fit of the curves to the data was very satisfactory, with a mean square error equal to 1.2, 0.6, 1.0 and 1.8 for I equal to 0, 0.01, 0.1 and 0.5 mol L⁻¹, respectively. The values of C_{L1} , K_{M1} , C_{L2} and K_{M2} calculated for the different ionic strengths remained close to each other (grey areas on Fig. 4). We used C_{L1} , K_{M1} , C_{L2} and K_{M2} parameters calculated by fitting the experimental data at $I = 0$ mol L⁻¹ to predict the fluorescence quenching at $I = 0.01, 0.1$ and 0.5 mol L⁻¹; the results are given by the dashed- curves in Fig. 3. These curves do not correspond as well to the experimental points as those determined by the direct modeling of these (solid line curves). The prediction, however, turned out to be quite good. The mean square errors between predicted and measured values were 18.6, 6.3 and 3.7 for I equal to 0.01, 0.1 and 0.5 mol L⁻¹, respectively. Surprisingly, the prediction was better at high ionic strength than at low ionic strength.

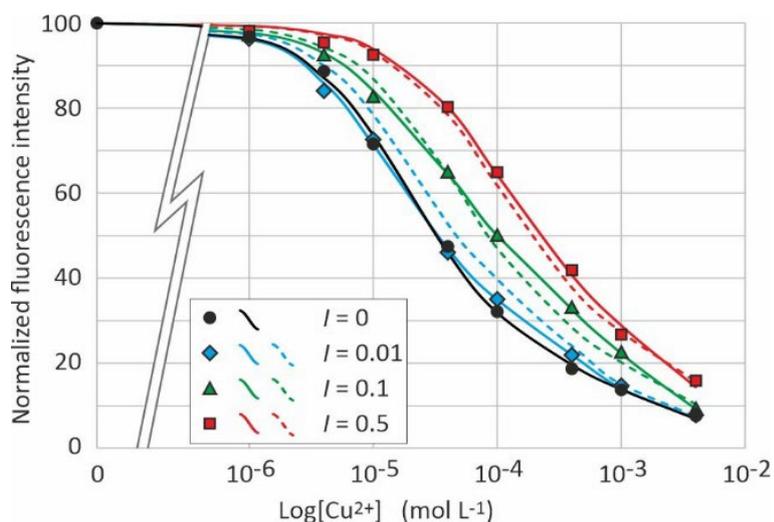


Fig. 3. Humic acid quenching by Cu^{2+} and 2-ligands model results. Symbols: experimental points; plain lines: modeling result using the experimental data at each ionic strength; dashed line: curves predicted by the model by varying the ionic strength and using C_{L1} , K_{M1} , C_{L2} , K_{M2} defined at $I=0$.

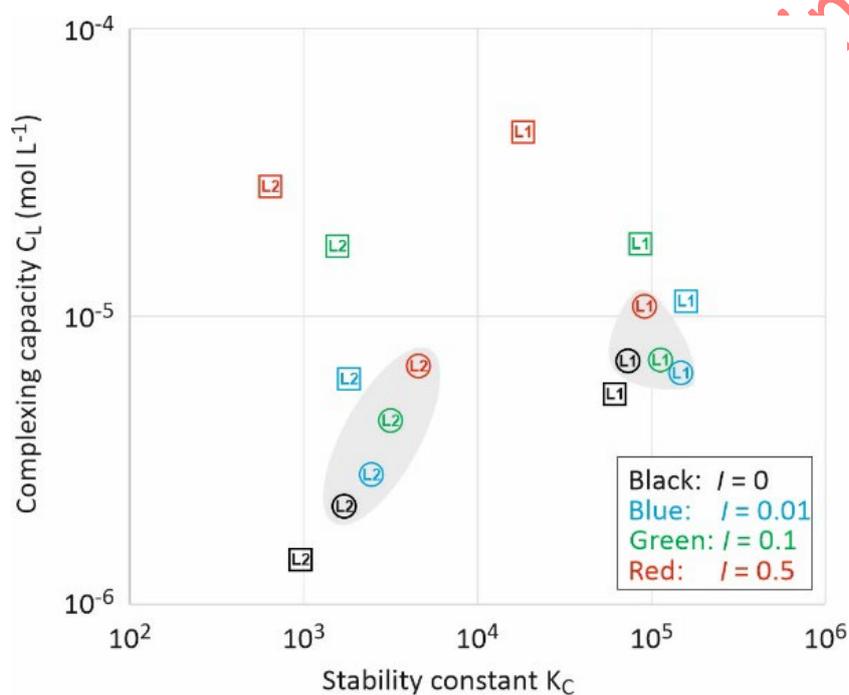


Fig. 4. Complexing capacities and stability constants calculated by fitting experimental data. Modeling with (value in a circle) or without (value in a square) taking into account the ionic strength. L1: ligand 1; L2: ligand 2; I : ionic strength in mol L^{-1} .

3.2 Effect of the Ionic Strength

To assess the relevance of taking ionic strength into account, the ligand parameters were adjusted to the experimental results with the 2-ligands model but ignoring the ionic strength by setting all γ_i values to 1 in the model. Results are given in Fig. 4 (values in a square). The values do not differ much from the values calculated taking into account the ionic strength for $I = 0$ and $I = 0.01 \text{ mol L}^{-1}$, they

differ however significantly for $I = 0.1$ and $I = 0.5 \text{ mol L}^{-1}$. Such differences are expected to be higher with a trivalent ion than with the divalent Cu^{2+} used here.

3.3 One Ligand versus Two Ligands

Fig. 5 gives the experimental points obtained for an initial ionic strength equal to 0.5 and the fitted curves obtained by the 2-ligands model and the 1-ligand model. The 2-ligands model provides much better curve fitting than the 1-ligand model. The latter, compared to the 2-ligands model, leads to an underestimation of both the complexing capacity of the fluorophore sites and the complexation constant. Similar conclusions can be made from the data obtained at the other ionic strengths (not shown here).

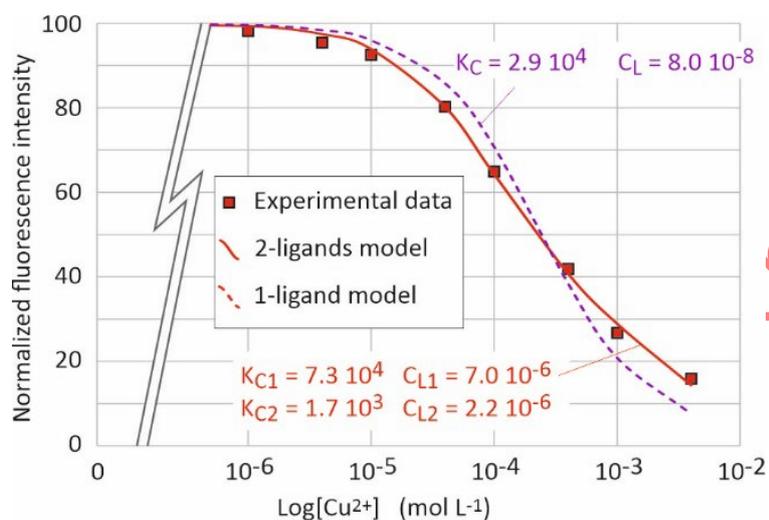


Fig. 5. Humic acid quenching by Cu^{2+} at initial ionic strength $I = 0.5$. Comparison between 1-ligand and 2-ligands model results.

4 Conclusion

The method presented here can be considered as a significant improvement of the initial Ryan and Weber model. It allows a quick and simple adjustment of the fluorescence quenching or enhancement curves, considering one or two ligands and considering the ionic strength. This latter consideration becomes necessary when the ionic strength reaches values higher than $10^{-3} \text{ mol L}^{-1}$ with divalent or trivalent cations. From the parameters determined at a given ionic strength, the model makes it possible to predict the fluorescence at another ionic strength. In the case of two ligands, the ratio of quantum yields can be determined by the model.

The use of this method does not, of course, exempt from precautions such as ensuring the absence of precipitation of solids or competitions between species for complexation. This method can be adapted to any problem which requires the resolution of a cubic equation, such as, for example, a potentiometric titration of a ligand with two pKa.

Supplemental Material

A Supplemental Information file (2-ligand model.xlsx) accompanies this manuscript containing a spreadsheet implemented with the 2-ligands model.

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