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**METAL SPECIATION DETERMINED USING
MULTIRESPONSE FLUORESCENCE**

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

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METAL SPECIATION DETERMINED USING MULTIRESPONSE FLUORESCENCE

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Abstract

Metal fate, transport and toxicity are speciation dependent. Natural organic matter (NOM) is a complex mixture of many potential metal binding sites and it is ubiquitous in the environment. Therefore, a site-specific, non-invasive, sensitive method is required to investigate metal interactions with NOM. This thesis considers the development and application of a multiresponse fluorescence method to determine site-specific binding of Al, Cu and H to model NOM ligands, fulvic acid and isolated NOM samples. Natural organic matter is treated as a mixture of average binding sites. The minimum number of sites along with their excitation and emission wavelengths are determined from deconvolution of fluorescence surfaces using SIMPLISMA (Windig and Guilment, *Anal. Chem.* 1991, 63, 1425-1432). Fluorescence at wavelengths corresponding to these components is measured during metal titration and the multiresponse data are fit to a multi-site speciation model for the metal-NOM system.

This multiresponse fluorescence method is validated in that it recovers known values for Al with two model ligands simultaneously and predicts free copper during a titration of Suwannee River fulvic acid within 0.1 log units when compared to the model of Cabaniss and Schuman (*Geochim. Cosmochim. Acta* 1988, 52, 185-193).

A total of seven different fluorophores were identified in NOM isolated from nine different watersheds in Norway. Each sample contained four to six of the seven identified fluorophores. On average, at pH 4, the fluorophores bind Al with a strength similar to salicylic acid ($\log K' \approx 5.5$), although there are stronger and weaker sites with $\log K' \approx 7$ and $\log K' \approx 3$, respectively.

The speciation results are dependent on the isolation method used to obtain the NOM sample. For the NOM samples, the differences between reverse osmosis and low-pressure-low-temperature evaporative isolates are as large as the differences between sampling sites.

Suwannee River fulvic acid is represented by five fluorescent binding sites for Cu, Al and H binding. The constants for Al and Cu are consistent with salicylic acid-like sites and the H binding can only be explained if diprotic sites are used. Thus, multiresponse fluorescence methods allow discrimination between monoprotic and polyprotic models.

Finally, a protocol is proposed for multiresponse fluorescence determination of metal interactions with NOM.

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1. Introduction

Metal speciation refers to the distribution of a metal among its possible physical and chemical forms. For example, a metal may be free in solution as a solvated ion, or it may be bound to a particular ligand. Speciation information is necessary because the toxicity of metals in the environment is speciation dependent. Furthermore, metal speciation information is needed in order to understand metal fate and transport. Natural organic matter (NOM) is ubiquitous in surface waters, and it is a very important ligand for metals. However, NOM is also a complex heterogenous mixture of potential binding sites; thus, a multi-site specific speciation method is desirable.

Many existing speciation methods are based on uniresponse signals, such as pH measured during acid-base titrations of NOM. The measurement in a uniresponse experiment is a weighted average of effects from all of the metal binding sites. It is reasonable to propose that better results could be achieved by measuring signals corresponding to individual sites, rather than one signal corresponding to all sites. This analysis would involve multiple simultaneous measurements and is termed a multiresponse experiment. Multiresponse methods involve the measurement of multiple sensors during an experiment and fitting all the data simultaneously during data processing. Multiple measurements on a system better constrain the choices of models that can be fit to that system. For example, in a combined spectroscopic and potentiometric acid-base titration, spectroscopic evidence could indicate that the species are diprotic, allowing rejection of a monoprotic model. In the absence of multiresponse data, either a monoprotic or diprotic model could be used to describe the potentiometric data.

An ideal speciation method should also be selective, sensitive and non-invasive.

Fluorescence spectroscopy is a very promising method in all three criteria: different fluorophores can be selected by varying both excitation and emission wavelengths; the measurements are sensitive (~ 1 mg of dissolved carbon/L as a detection limit), and the measurement step minimally perturbs the sample.

This thesis focuses on determining chemical speciation of metals in the presence of NOM. In particular, the amount of free metal and the amount bound to specific sites in NOM is calculated. This can be accomplished by determining conditional stability constants and ligand concentrations for a representative set of metal binding sites in NOM. In order to do this, a new fluorescence methodology is developed and applied to a variety of NOM samples. This thesis presents the development, validation and application of this method. The metals investigated are Al, Cu and H and the NOM samples include model NOM-like ligands, Suwannee River fulvic acid (SRFA) and NOM isolated from a variety of watersheds in Norway.

The objectives of this research are summarized in the next section (1.1). The research involved to meet these objectives is summarized in four papers that are included as appendices to this thesis. These papers will be referred to by their roman numerals, and are listed below along with a statement of the major contribution of each paper.

I. Aluminum Adsorption to Organic Matter Determined using Fluorescence Quenching.

Contribution: Validation of the Ryan-Weber equation for fluorescence enhancement and application to a simple one metal-one ligand system showing wavelength independence of fitting results.

II. Multi-site Aluminum Speciation Determined using Multiresponse Fluorescence Data.

Contribution: Development and application of a generalized Ryan-Weber multi-site multi-sensor approach for Al speciation with fluorescent NOM.

III. Aluminum Interactions with Natural Organic Matter Determined using Multiresponse Fluorescence.

Contribution: Application of SIMPLISMA for spectral deconvolution of NOM fluorescence surfaces and comparison of results to fluorescence of model compounds. Titration of fluorescent components in NOM with Al and fitting using the method developed in Paper-II for a set of NOM samples from the NOM-Typing project.

IV. Metal Binding to Suwannee River Fulvic Acid Determined using Multiresponse Fluorescence

Contribution: Application of fluorescence speciation method, developed in Papers II and III, to Suwannee River fulvic acid complexation of Al, Cu and H. Multi-site multi-metal interpretation of Suwannee River fulvic acid and validation of fluorescence speciation method by demonstrating agreement between predicted $[Cu^{2+}]$ by another independent method.

1.1. Research Objectives

- (1) To achieve a better understanding of the nature of NOM and the metal binding sites in NOM, emphasizing multiresponse observations.
- (2) The development of a site-specific speciation method for metals with NOM using multiresponse fluorescence observations.
- (3) To validate the method for Al/Cu/H with model and reference ligands. Also, for proton binding to combine fluorescence and pH measurements.

- (4) Application of the method for Al-NOM with a wide variety of samples.
- (5) Fluorescence deconvolution (SIMPLISMA) of NOM fluorescence surfaces to determine the nature of fluorophores in NOM
- (6) To demonstrate that multiresponse methods, such as fluorescence, are better than traditional uniresponse methods for metal-NOM speciation studies.

2. Background

2.1 Nature of NOM

Natural organic matter (NOM) occurs as the result of chemical, physical and biological processing of primary organic matter. Primary organic matter results from assimilation of carbon by terrestrial and aquatic vegetation. During decay, this primary organic matter is processed by biological, chemical and physical weathering, and the results are a heterogenous mixture of organic matter that is termed NOM. Natural organic matter is ubiquitous in both aquatic and terrestrial environments. There are components of NOM that are soluble in aqueous solution and components that are insoluble. The work here is concerned primarily with the water soluble portion of NOM.

NOM includes residual plant remains, such as lignin and proteins as well as “new” molecules resulting from chemical and biological processes acting on these raw materials. The result is a distribution of molecule size and functionalities. The largest molecules are classified as humic substances. Their origin can be either polymerization or degradation of primary plant products, but most likely is a mixture of the two processes (Thurman, 1985).

Most relevant to this thesis is the role of NOM in the fate and transport of metals. For

natural or anthropogenic metals NOM contains important ligands that control the speciation of many common metals in solution. There has been a lot of effort to develop methods which characterize NOM and its ability to bind metals. General references such as the books by Senesi and Miano, (1994); Frimmel *et al.*, (1988) and Christman and Gjessing (1983), summarize the state-of-the-art knowledge of NOM. The focus of these references are the macromolecular components called humic and fulvic acids. Overall, the nature of NOM is diverse; it depends on the source as well as formative factors such as hydrology, temperature, redox conditions, pH, and biological processes.

A proposed structure for Suwannee River fulvic acid is shown in Figure 1 (Leenheer *et al.*, 1998). Several potential metal binding sites can be observed in this structure. In particular, hydroxyl and carboxyl sites are present, as well as salicylic-like sites that contain a phenolic group ortho to a carboxylic group. Aluminum and Cu(II) binding occurs mostly at oxygen sites, but Cu(II) can bind at sulfur sites, and Cu(I) is bound very strongly by sulfur sites. Only oxygen sites are shown in the model presented by Leenheer *et al.*, (1998) but other sites (S and N) could occur at lower abundances. The speciation method proposed in this thesis assumes that macromolecules, such as in Figure 1, can be represented as a mixture of simple molecules. For example, in Figure 1, the three aromatic groups could be treated as separate groups to simplify modelling of this complicated molecule. In natural samples, NOM occurs as mixtures of molecules similar to Figure 1, but each is potentially different. An important assumption for modelling as proposed here, is that structural units and binding sites are common between different molecules making up NOM.

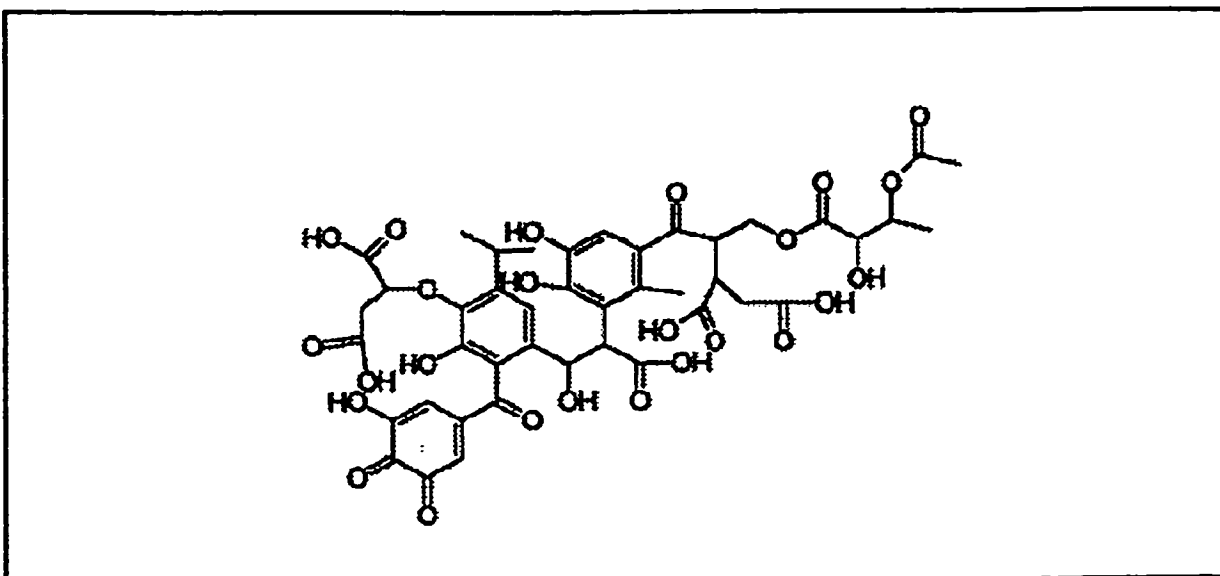


Figure 1: Proposed structure for Suwannee River fulvic acid (Leenheer et al., 1998)

2.2 Fluorescence of NOM

There are several books that discuss the principles and applications of fluorescence. These include the books by Parker (1968), Guilbault (1973) as well Lakowicz (1983).

Luminescence occurs when an excited molecule returns to the ground state by emitting energy in the form of light. A molecule can be excited in different ways. When the molecule is excited by light and the subsequent emission is fast then the process is called fluorescence. Slow light emission is termed phosphorescence. This process can be understood in more detail by considering Figure 2. A molecule, initially in the ground state singlet state (S_0), is excited by the adsorption of light; this is represented by vector a in Figure 2. This light adsorption promotes the molecule to some new excited singlet, (S_2). The molecule does not remain in an excited state but through various pathways returns to the ground state; the possible pathways depend on the nature

of the molecule. If the molecule is non-luminescent then the decay to the ground state involves non-radiative transitions. For example, energy may be lost by collisions while in the excited state.

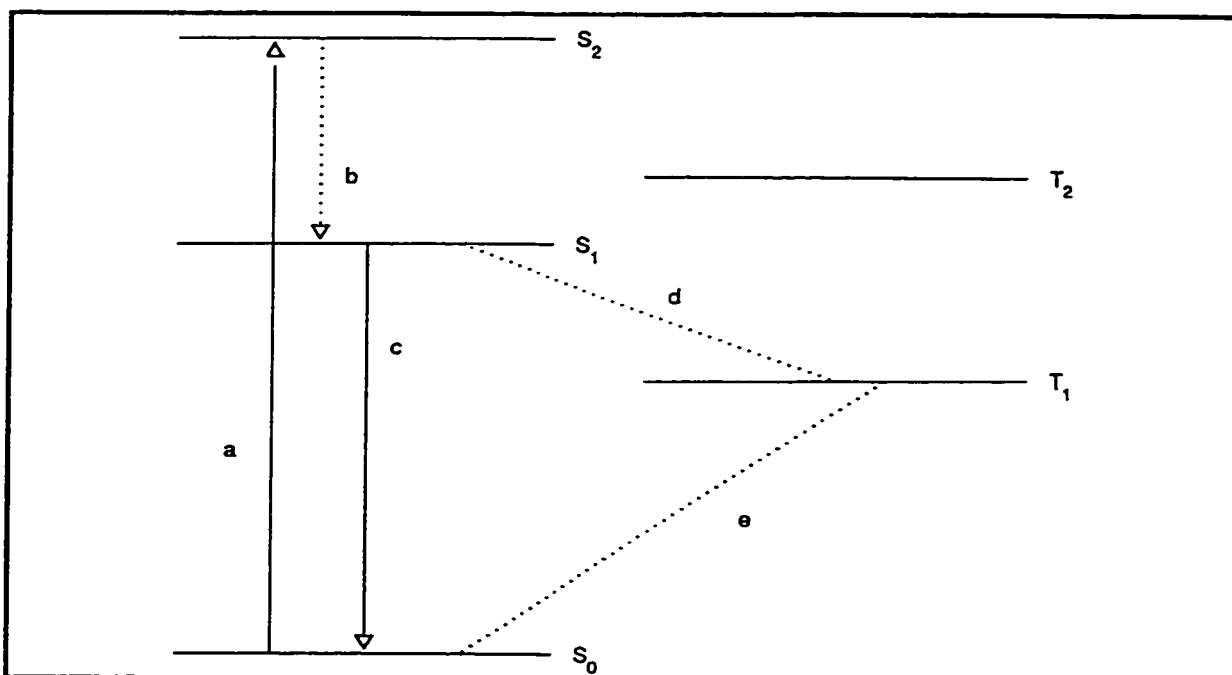


Figure 2: Energy level diagram used to qualitatively explain fluorescence. The electronic energy states are shown as singlet (S) and triplet (T) states. For simplicity the vibrational and rotational energy levels are not shown.

If the molecule is luminescent then the return to the ground state is accomplished by radiative transitions. For fluorescent molecules the molecule will decay to the first excited singlet (S_1) by non-radiative transitions (arrow b in Figure 2) and then emit light with energy S_1-S_0 in returning to the ground state (arrow c in Figure 2). This total process is fast, generally on the order of nanoseconds. Alternatively, phosphorescence will result if the molecule undergoes intersystem crossing to first move to the first excited triplet state (T_1) before decaying to the ground state singlet. This is represented by arrows d and e in Figure 2. There are also higher level triplets (such as T_2) that could be populated before decaying to T_1 . The phosphorescence process

takes longer, possibly on the order of minutes. That is why phosphorescence results in a signal even after the excitation source is turned off.

The excitation energy can promote the molecule to any of its possible excited state singlets. Usually the excitation light is high energy ultraviolet light, and the emitted light is lower energy visible light. The emitted light occurs in the form of a spectrum; for a fixed excitation wavelength this is called an emission spectrum. Similarly, an excitation spectrum is obtained if the emission wavelength is held constant. The discussion above implies that there should only be one sharp line in the emission spectrum, corresponding to the energy of S_1-S_0 . Similarly, there should only be sharp lines in the excitation spectrum corresponding to all of the possible excited states. This is not observed because superimposed on the electronic energy levels given in Figure 2 there are also the vibrational and rotational energy levels which will be populated at ambient temperature so that the electronic states resemble bands rather than discrete lines. The result is the broad fluorescence spectra that are generally observed. A more detailed discussion of this process is given by Parker (1967).

Several emission spectra, obtained at different excitation wavelengths, can be used to form an excitation versus emission fluorescence surface. Paper-II, Figure 4 shows some example fluorescence surfaces for NOM.

As already mentioned, a molecule excited by absorbing light will not necessarily fluoresce. In fact, only certain molecules can fluoresce; others lose their energy through non-radiative transitions. In general, molecules that contain aromatic rings and/or conjugated π -systems are good candidates for fluorescence; making fluorescence a selective technique because relatively few molecules meet these criteria. The selectivity can be further increased by varying both

excitation and emission wavelengths to select individual fluorophores. Provenzano and Sposito (1994) report fluorescence surfaces for pine litter extracts and identify at least three components. Coble *et al.*, (1990) examined the variation of fluorescence with depth for ocean water. They propose at least three components that vary in intensity from the top to the bottom of the 200 m profile. Summaries of excitation and emission wavelengths for NOM are collated in Paper-III Figure 5. This figure is reproduced here as Figure 3.

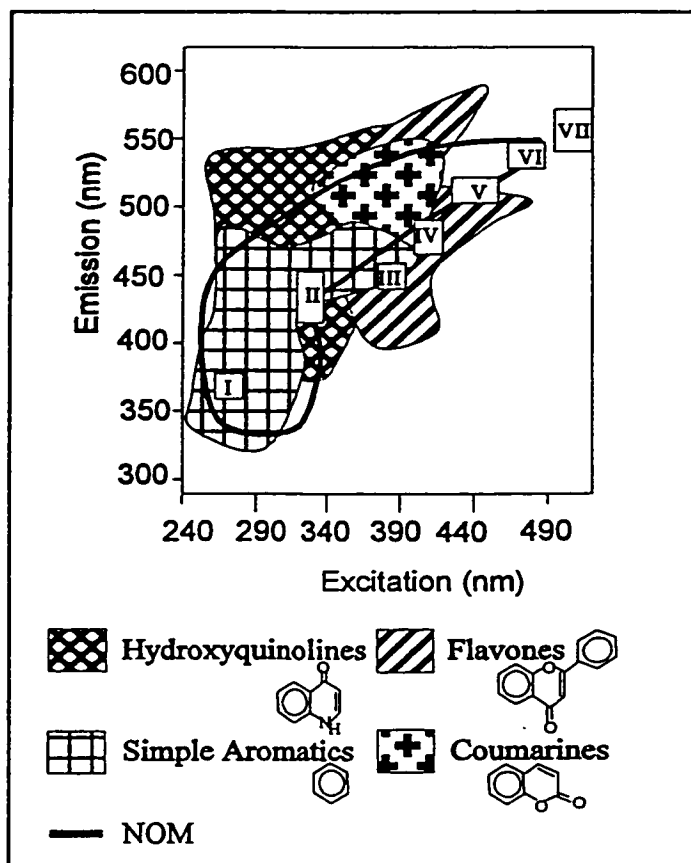


Figure 3: Fluorescence excitation versus emission map for region of NOM fluorescence (area inside solid outline), model compounds (shaded regions) and seven fluorophores identified in Norway NOM (Paper-III)

Fluorescence spectroscopy is a very useful tool for investigation of NOM. Natural organic matter fluoresces at a wide range of excitation and emission wavelengths; the range that

NOM is observed to fluoresce is summarized in Figure 3. Natural organic matter is observed to fluoresce in the range enclosed by the dark line. This was determined by a literature survey of NOM-fluorescence studies. References are provided in Paper-III. Since different NOM has different fluorescence, the groups that fluoresce (fluorophores) must also be different. Figure 3 shows the regions where different model compounds fluoresce. The selected compounds are possible fluorescent building blocks of NOM (Senesi, 1990); they could occur as free molecules or, more likely, as components of macromolecules. Superimposed as roman numerals on Figure 3 are the regions where the components of Norway NOM were observed to fluoresce (Paper-III). The components were determined by spectral deconvolution using SIMPLISMA (Windig and Guilment, 1991). For a complete description of the samples refer to Paper III, and the summary given in Section 3.2 of this thesis.

A proposed structure for Suwannee River fulvic acid, which would fluoresce, is shown in Figure 1. The building-blocks of this structure include three aromatic moieties that would probably fluoresce in the simple aromatic region of Figure 3. More complicated models for NOM would include more complex fluorescent moieties; for example, a United States Geological survey report (U.S. Geological Survey Open-File Report 87-557, 1989) includes a quinone-type structure in one of its proposed models.

There are two effects that metal (M) binding may have on a fluorescent ligand (L). The metal may partially or completely suppress the ligand's fluorescence, or the new species (ML) may fluoresce. In general, paramagnetic species such as Cu^{2+} , tend to quench fluorescence (Paper-IV, Luster *et al.*, 1996, and Ryan and Weber, 1982). Diamagnetic species, such as Al^{3+} , tend to form new fluorescent species, as in the case of aluminum-salicylate (Paper-I and Paper-

II). For Al, the new species are generally more efficient fluorophores, as demonstrated by the large number of fluorescent analytical reagents used for Al determinations (Guilbault 1973).

Metals that quench fluorescence enhance non-radiative transitions as a pathway for the molecule to return to the ground state (see Figure 2 and accompanying discussion). This can be done by dynamic or static processes. Dynamic quenching involves energy loss by collisions between the excited fluorophore and the metal ion, rather than radiative transitions that would result in fluorescence. For static quenching the metal is bound to the fluorophore and the new molecule is such that it is no longer fluorescent, or fluoresces with decreased efficiency.

When fluorescence quenching is observed, the metal ion inhibits the radiative transitions that cause fluorescence emission. Thus, the entire spectrum will be observed to decrease in intensity. This was observed by Yang *et al.*, (1994) for aqueous pine extracts upon addition of Cu. If the bound metal forms a species with different fluorescent properties from the free ligand, then the observed fluorescence will be a combination of spectral contributions from both the ligand and metal-ligand species. Thus, in the case of diamagnetic metals, suppression or enhancement can be observed depending on which part of the fluorescence emission spectrum is monitored. For example, suppression and enhancement are both observed for fulvic acid and Mg (Cabaniss, 1992). Tam and Sposito (1993) observe suppression and enhancement in emission spectra for pine litter extracts when Al is added. Paper-I demonstrates fluorescence enhancement at short wavelengths and fluorescence quenching at long wavelengths for Al with salicylic acid (Paper-I Figure 1). Aluminum is also observed to both quench and enhance fluorescence for river water NOM, as seen in Paper-II, Figure 4. In this figure, the quenched region is labelled four and enhancement is observed at regions 1, 2 and 3.

2.3 Metal Speciation Methods

In natural waters metals can exist in many different species. There are several different ways to measure speciation. Clarke (1994) and Bloom and Erich (1989) give excellent reviews of speciation methods for Al. For metal speciation methods in general the book edited by Kramer and Allen (1988) is recommended.

The ideal speciation method would measure each species by some selective technique such as a unique spectroscopic signature. Given the wide variety of species and the fact that most analytical methods only measure totals, this scheme is seldom used, although measurements of a few select species can be performed. The fluorescence method proposed here, measures signals proportional to each fluorescent ligand.

A less specific speciation method would be to measure fractions of a species such as weakly complexed, strongly complexed, monomeric or polymeric species. A simple example of this scheme is filtration to define particulate versus solution species. A further example is the competitive method of Driscoll (1984) in which signal development for an Al-indicator ligand over time is used to define classes of species. These methods are used more frequently than a specific method; because, it is easier to measure broad groups of similar components, or to physically separate these groups and measure the totals, than it is to selectively measure individual components. In general, these are operationally defined and are valid only as qualitative tools.

The third broad type of speciation method is the modelling approach. In this method, experiments are performed to perturb the system, and parameters are fit to these experimental data. These parameters can then be applied in real situations to predict the distribution of species. Several assumptions are necessary in this approach. The investigator can choose between

thermodynamic or kinetic modelling (or both). This thesis considers only thermodynamic, or equilibrium modelling. For the model experimental system, the investigator selects a speciation model and fits speciation parameters to the measured data. In practice, this approach involves fitting of titration data, for which different options are discussed in Section 2.4.

An important consideration in any speciation method is for the measurement step to not perturb the system, that is, the distribution of species must remain constant during measurements. In most speciation methods a compromise is made so that the system is minimally perturbed. For example, perturbation is inherent in competition methods, because an indicator ligand must be added (Miller and Bruland, 1995; Kramer *et al.*, 1994). Fluorescence methods, however, do not require an indicator ligand. The signal comes from inherent fluorescence of the ligand and is only changed by metal binding.

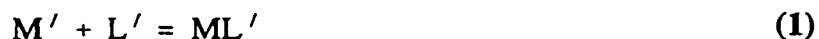
The major drawback of most existing speciation methods is the failure to address NOM as anything more than a “black box”. Many methods treat NOM as a single ligand. It is clear from the discussion in Section 2.1 that NOM is not a single ligand but, in fact, consists of a mixture of complexing sites, so this simplification is generally not valid. Therefore, an advantage of the approach developed in this study is that it allows natural organic matter to be treated as a mixture of sites.

2.4 Fitting of NOM-Metal Titration Data

Natural organic matter is a heterogenous mixture of potential metal binding sites. Metals can bind covalently to functionalities containing O, N and S. Such associations can include mono-metal-mono-ligand associations (ML), or poly-metal-poly-ligand (M_mL_n) binding. In addition, positive metals can associate in a less specific manner by so-called “outer-sphere” complexation

with negative NOM. The amount bound is influenced by the nature of the binding sites and its concentration. Also, this is strongly pH dependent, as protons can compete for available sites and influence both the size and charge of the NOM surface. The nature and strength of metal binding is often investigated by perturbing the metal-ligand system by titration.

There are a wide variety of mathematical methods used to assess metal binding by NOM from titration curves. The methods discussed here are based on the assumption of equilibrium between the metal(s) and ligand(s). The equilibrium assumption is often used in modelling, such as with MINEQL (Westall, *et al.*, 1976), and it is a very useful simplifying assumption to make. Moreover, the equilibrium assumption approximates a wide variety of natural aqueous systems (Stumm and Morgan, 1996). Experimentally, the free metal concentration is usually measured during a titration of total metal. For example, ion-selective electrodes, such as Cu (Cabaniss and Schuman, 1988a) or pH (Brassard *et al.*, 1990) electrodes can be used. Alternatively, a signal corresponding to the ligand can be used, as in the fluorescence approach adopted by Ryan and Weber (1982) and used in this thesis. Other options include other spectroscopic methods, such as infrared spectroscopy (Cabaniss, 1991) and light-scattering (Gamble *et al.*, 1980). Regardless of the experimental measure, the resultant titration data are fit to a speciation model for the NOM-metal system. For experiments performed at fixed pH and ionic strength, results are conditional. In more general experiments, pH and ionic strength are varied to take into account ionic strength effects and competition by protons for available sites. The conditional stability constant indicates how strongly the metal (M) is bound to the ligand (L), according to the following reaction:



and the conditional stability constant is defined as:

$$K' = \frac{[ML']}{[M'][L']} \quad (2)$$

Where $[ML']$ is the total concentration of all ligand-bound metal species, $[L']$ is the total concentration of all free ligand species and $[M']$ is the total concentration of all metal species not bound to the ligand. Activity coefficients are constant for constant ionic strength, so concentrations can be used, rather than activities. The measurement of K' depends on the analytical conditions: ionic strength, temperature, pH, competing metals and competing ligands. For multiple species corresponding to different metals or to different ligand sites, each can be represented by its own conditional stability constant.

For ideal systems, thermodynamic constants that are independent of conditions, can be determined if all the conditions are varied over a wide enough range. Öhman and Sjöberg (1988) favour this approach for natural systems, but it is impossible if the ligand is not accurately defined. Humic substances and natural systems in general are not uniquely defined and a rigorous thermodynamic approach cannot, in general, be applied.

2.5. Mathematical Framework

The methods developed in Papers II and III depend on the theory of multiresponse parameter fitting and on spectral deconvolution using SIMPLISMA. The theory of these two topics is presented below. The section on multiresponse fitting (2.5.1) is a comprehensive, but as simple as possible description of the theory. This has not been done before in the context of metal-ligand equilibria studies. SIMPLISMA is a fairly new technique developed by Windig and

Guilment, (1991). Section 2.5.2 discusses SIMPLISMA with respect to NOM fluorescence surfaces.

2.5.1 Multiresponse Parameter Fitting

Natural organic matter (NOM) is a complex mixture of potential metal-binding sites. In order to address this heterogeneity, it is advantageous to utilize multiple simultaneous sensors during a metal titration of NOM and fit the resultant data to an equilibrium model for the metal-NOM system. Existing fitting methods, such as nonlinear least-squares are only applicable to uniresponse data. The statistical theory to formally address this problem for multiple sensors is called multiresponse parameter fitting. In multiresponse fitting the least-squares criteria is replaced by the determinant criteria. The theory of multiresponse fitting, as applied to chemical kinetic experiments, was developed by Box and Draper (1965), and has been reviewed by Bates and Watts, (1988) and Stewart *et al.*, (1992). Multiresponse fitting has recently been applied to chemical equilibrium calculations for metals with NOM (Papers II-IV, this thesis). The theory of multiresponse fitting as it applies to equilibrium modelling of titration data is summarized below.

For M different kinds of responses (or sensors) and N additions of titrant there is an $N \times M$ matrix, \mathbf{Y} , of measurements. For the i th addition of titrant and j th response, the Y_{ij} element of this matrix can be represented as:

$$Y_{ij} = f_j(x_i, \theta) + \xi_{ij} \quad (3)$$

The first term on the right of Eqn. 3 corresponds to a mathematical model for the system and the second term corresponds to the error, or disturbance term. In the mathematical model of the system, each measurement is a function of the independent variable x_i and a vector of parameters,

θ . The observations in \mathbf{Y} should fit the model within the random experimental noise contained in the $N \times M$ matrix, Ξ , with elements ξ_{ij} .

The errors (ξ_{ij}) can be modelled in many different ways depending on the assumptions about the experimental system. In general, the errors are modelled as random variables with an unbiased multivariate normal probability density with zero mean and a covariance matrix Σ (Stewart and Sørensen, 1981). In error modelling the mean value is often taken as zero, because there is an assumption of no systematic bias in the experiments. The covariance matrix is analogous to the standard deviation in a univariate normal distribution. If it is assumed that measurements for different additions of titrant are independent, but that measurements for the same addition are correlated, then the $M \times M$ covariance matrix relates the different responses. The covariance matrix is symmetric about the diagonal. The diagonal terms are the variances, and the off-diagonal terms are the covariances. If the off-diagonal terms are zero, then the errors are not correlated.

For the i th addition of titrant the row vector of errors:

$\xi_i = [\xi_{i1}, \dots, \xi_{iM}] = [y_{i1} - f_1(x_i, \theta), \dots, y_{iM} - f_M(x_i, \theta)]$, has a normal probability density corresponding to:

$$p(\xi_i | \Sigma) = (2\pi)^{-\frac{M}{2}} |\Sigma|^{-\frac{1}{2}} \exp\left(-\frac{1}{2} \xi_i \Sigma^{-1} \xi_i^T\right) \quad (4)$$

This is the probability of obtaining a value of the measure (y_i) that is different from the “true” value by ξ_i . The overall probability density, for the entire titration, is given by the product of each of the N probabilities. This is called the likelihood function, and it is a measure of the probability

of occurrence of a set of random variables (the errors) that actually did occur, given proposed values for θ and Σ . The goal is to maximize this probability, that is, to maximize the probability that θ and Σ represent the data. Specifically, we want to maximize the probability that the measurements calculated from the parameter estimates correspond to the measurements that were actually observed. To do this we use the maximum likelihood criteria.

The likelihood function is generally expressed as the loglikelihood, which is a sum rather than a product of the probabilities given by Eq. (4). The loglikelihood function (L) for the parameters θ and Σ can be written as (Bates and Watts, 1988):

$$L(\theta, \Sigma) = k + \frac{N}{2} \ln |\Sigma^{-1}| - \frac{\text{tr}(\mathbf{Z}\Sigma^{-1}\mathbf{Z}^T)}{2} \quad (5)$$

where k contains all of the additive constant terms, and \mathbf{Z} is a matrix of residuals calculated as the difference between measured and calculated responses as follows:

$$\mathbf{Z} = \begin{bmatrix} y_{11} - f_1(x_1, \theta) & y_{12} - f_2(x_1, \theta) & \dots & y_{1M} - f_M(x_1, \theta) \\ y_{21} - f_1(x_2, \theta) & y_{22} - f_2(x_2, \theta) & \dots & y_{2M} - f_M(x_2, \theta) \\ \vdots & \vdots & & \vdots \\ y_{N1} - f_1(x_N, \theta) & y_{N2} - f_2(x_N, \theta) & \dots & y_{NM} - f_M(x_N, \theta) \end{bmatrix} \quad (6)$$

To obtain best fit estimates for the parameters in θ , it is necessary to maximize the loglikelihood as a function of θ . The maximum is determined by taking the derivative of Eq. (5) and setting it

equal to zero. This is shown by Bard (1974, p. 296), and the result is a conditional estimate for the covariance matrix:

$$\hat{\Sigma}(\theta) = \frac{\mathbf{Z}^T \mathbf{Z}}{N} \quad (7)$$

If Eq. (7) is substituted back into Eq. (5) then the conditional loglikelihood function is obtained:

$$L(\theta, \hat{\Sigma}(\theta)) = k' - \frac{N}{2} \ln |\mathbf{Z}^T \mathbf{Z}| \quad (8)$$

Equation (8) is maximized when the determinant is minimized. The same criteria was obtained using Bayesian rather than likelihood arguments, (Box and Draper, 1965). In some instances the parameters in Σ are included in the optimization process (Stewart *et al.* 1992), but this can increase the number of optimization parameters dramatically. Also, other conditional estimates of Σ are possible (Stewart *et al.*, 1992), but the determinant criteria still yields the best fit result.

In titration experiments, the independent variable is generally total titrant (metal) added, and the parameters in θ are generally a conditional stability constant (K') and a ligand concentration (L_T) for each proposed binding site. For the case of fitting spectroscopic data, there are additional parameters in θ for converting concentrations into measured responses.

2.5.2 Spectral Deconvolution: the SIMPLISMA Approach

The method used in this study to deconvolute fluorescence surfaces is the SIMPLE to use Interactive Self-modelling Mixture Analysis (SIMPLISMA) developed by Windig and Guilment (1991). A brief discussion of this method follows. Other methods could have been used, such as entropy minimization (Zeng and Garland, 1998), generalized rank annihilation (Wilson *et al.*,

1989), or factor analysis (Dalibart, 1997). However, these techniques are more difficult to use and less intuitive than SIMPLISMA. Future work could include a comparison of different deconvolution techniques.

SIMPLISMA resolves mixture data into pure component spectra and concentrations (% of total), without using prior information about the mixtures. The resolution is performed by identifying pure variables. In a set of fluorescence emission spectra the pure variables are emission wavelengths that have intensity contributions from only one component of the mixture. Pure variables are selected using the following relation:

$$P_j = \frac{\sigma_j}{\mu_j + \alpha} \quad (9)$$

Where p_j is the purity value for the j th variable and μ_j is the mean and σ_j is the standard deviation. Purity values can be represented by a spectrum. Assuming that there is a pure variable in the data set, it will have a maximum value of p_j . The α -term is user defined to give low intensity variables a lower contribution in the purity calculations; this helps to reduce noise. Equation (9) results in a calculation of relative standard deviation for each wavelength, and the variables that change the most are the purest.

Once the pure variables are identified, a set of equations is solved for the spectrum corresponding to each component. The equations are of the form:

$$I_1S_1 + I_2S_2 + \dots + I_NS_N = M_i \quad (10)$$

Where the M_i observed spectrum is a sum of the contributions from each component. The

contributions of each component are determined as the product of I_N , the intensity of the n th pure variable, and S_N is the corresponding spectrum of component N . The intensities and the observed spectra are known, all that remains is to solve for the component spectra. The component spectra are determined by SIMPLISMA using code written in Matlab to solve the matrix equation corresponding to Eq. (10) using unconstrained least squares fitting. The matrix equation can be written as:

$$\mathbf{D} = \mathbf{C}\mathbf{P} \quad (11)$$

Where \mathbf{D} is the $c \times v$ matrix of fluorescence data at c excitation wavelengths with emission spectra in rows v wavelengths long. The matrix \mathbf{C} contains the pure variable intensities, which are linearly proportional to concentration and are determined from the data. \mathbf{C} is $c \times n$ for n pure components and c excitation wavelengths. The unknown matrix \mathbf{P} is $n \times v$ with the pure emission spectrum in the matrix rows. Least-squares methods to solve this type of problem are given in Bates and Watts (1988). Alternatively, this equation can be solved using constrained optimization so that the component spectra must remain positive. Paper III and IV illustrate this method in the deconvolution of measured fluorescence surfaces. Constrained optimization in Matlab was used to keep all parameters positive (Grace, 1992).

3. Results

The original research results of this thesis are presented in the four papers in the appendices. The following sections discuss the body of research as a whole with reference to the individual papers.

3.1 Development of Multiresponse Fluorescence Speciation Method

There is a need for sensitive, site-specific speciation methods to investigate metal-NOM interactions. Fluorescence spectroscopy is such a method. Natural organic matter is fluorescent and changes in that fluorescence during metal binding can be fit to a speciation model for metal-NOM interactions. Ryan and Weber (1982) were the first to quantitatively apply fluorescence quenching for stability constant and binding capacity determination. They measured fluorescence quenching for fulvic acid during a titration with copper at fixed pH. Their data were fit to a simple (1:1) model for Cu binding to one site, and the one site model was assumed to be representative of the fulvic acid. An objective of the research presented here is to generalize the Ryan-Weber equation for multi-site speciation by using multiple wavelength observations. An initial step towards this goal was to validate the Ryan-Weber equation.

The application of the Ryan-Weber equation to metal-NOM interactions, however, has been criticised (Cabaniss and Schuman, 1988b). One criticism by Luster *et al.*, (1996) stated that the results are dependent on wavelength. Several explanations for the wavelength dependence are possible: it could be a mathematical artifact of the fitting method, or it could result from different fluorophores, or mixtures of fluorophores, contributing to the fluorescence at a given wavelength. To determine which explanation dominates, there is a need to first test the Ryan-Weber equation in the simplest of cases; the case of a single metal binding to a single ligand. This was done in Paper-I for Al with the model compound salicylic acid. Experimentally, the fluorescence emission spectrum of salicylic acid was measured during a titration of Al at pH 4. The spectrum was observed to show fluorescence quenching and fluorescence enhancement (Figure 1, Paper-I). The spectrum results from a linear combination of contributions from both H-salicylate and Al-

salicylate fluorescence, where H-salicylate fluoresces less efficiently and its emission peak is at longer wavelengths compared to Al-salicylate. The Ryan-Weber equation was applied to every emission wavelength in the set of emission spectra. The results are shown in Paper-1, Figures 4 and 5, for the conditional stability constant and total ligand concentration versus wavelength. The conditional stability constant is within 0.1 log units from the “true” value and the ligand concentration is overestimated by 5 to 100 %, depending on wavelength; this result is very reasonable for speciation studies. The only wavelengths that give dramatically incorrect results are those where there is large spectral overlap, and the fluorescence changes only marginally with Al additions. The conclusion drawn from these results is that the Ryan-Weber equation is valid for one ligand as long as the changes in fluorescence at the selected wavelength are large relative to experimental noise.

Alternative models to fit fluorescence are also evaluated in Paper-I. The usual Stern-Volmer equation (see Gauthier *et al.*, 1986) was rederived for fluorescence enhancement rather than quenching. In addition, a combined Stern-Volmer-Ryan-Weber equation is proposed. This equation uses simplifying assumptions from the Stern-Volmer equation to rederive the Ryan-Weber equation. Comparison of the different approaches reveals that the usual Ryan-Weber equation is best for one-ligand-one-metal systems.

The Ryan-Weber equation can be applied at multiple wavelengths. This is done in Paper-I for Al with SRFA, and it has also been done by Luster *et al.*, (1996) for Cu and Al with juniper leaf litter extract. In these studies, the multiple wavelengths are assumed to correspond to multiple sites within NOM and each titration curve is fit separately using the Ryan-Weber equation. This approach is not valid because the fluorescent sites are not independent and they all

compete for the available metal. A more correct approach would involve fitting all of the fluorescence data simultaneously. Such a method is presented in Paper-II; it is a multi-wavelength-multi-site generalization of the Ryan-Weber equation.

The derivation of the model for fitting to experimental data is shown in Papers-II, III, and IV. The fitting method is for multiresponse data because fluorescence at a wavelength pair (excitation and emission) is observed for each of the N proposed sites. The experimental observations are fluorescence at N wavelength pairs for M additions of metal titrant at fixed pH. The pH is fixed, thus acidity constants can be ignored (except for H-binding experiments). The data matrix is $m \times n$ with a row for each addition of titrant and a column for each fluorophore. This data matrix Y (from Equation 3), can be simulated by a new matrix F , by assuming that the fluorescence at each wavelength pair is a linear combination of fluorescence from free (L) and bound (ML) ligand. There is a fluorescence observation for each proposed ligand. The distribution of the ligand between free and bound forms is dependent on the speciation parameters, L_T and K' , for each site. Thus, for N sites there are $4N$ parameters: one L_T , one K' , and two linear proportionality constants to convert concentration into fluorescence for each site. The data in Y are fit by calculating F to minimize the determinant criteria of $\det(Z^T Z)$, where $Z = Y - F$. Z is called the residual matrix. The calculations were performed using code written in Matlab.

The method described above was first applied in Paper-II. In this paper, the method is applied to fluorescence enhancement data from an Al titration of salicylic and 2-hydroxy-3-naphthoic acid. There are two fluorescent ligands, thus two sets of observations have to be fit simultaneously (Figure 2, Paper-II). Confidence intervals about the parameter estimates were

determined using Monte-Carlo simulations. Monte-Carlo confidence estimation is discussed by Press *et al.* (1986) and involves 1000 additions of random error to the best-fit data and then fitting the 1000 simulated experiments. The “true” stability constants and ligand concentrations were recovered within one standard deviation of the Monte-Carlo results; thus, the method proposed here is validated for a simple mixture of ligands.

In Paper-II the multi-site generalization of the Ryan-Weber equation is applied to filtered river water NOM. In this paper the choice of wavelength was somewhat arbitrary. The generalized Ryan-Weber method assumes that each wavelength pair corresponds to a different fluorescent site. In Paper-II the sites were identified as regions of the fluorescence surface that increase the most on addition of Al. Paper-III refines the wavelength choice by using spectral deconvolution to suggest a minimum number of sites to describe observed fluorescence. This is accomplished using SIMPLISMA (Windig and Guilment, 1991) and has not previously been performed on NOM fluorescence surfaces. An additional benefit of this approach is that the fluorescence of the fluorophores contributing to NOM fluorescence can be compared to the fluorescence of model compounds (Paper-III).

The multiresponse fluorescence speciation method proposed here now involves measuring a fluorescence surface for the NOM sample as a first step. For the second step SIMPLISMA is applied to the fluorescence surface to identify the number and position of a minimum number of components to describe the surface. These components are now thought of as the “sites”, and titration curves are measured at wavelengths corresponding to each of the sites. The resulting data matrix is then fit to a multi-site speciation model for the system, using the multi-site generalization of the Ryan-Weber equation presented above. All of these steps were applied for

copper, Al and H binding by Suwannee River fulvic acid in Paper-IV. A method protocol is suggested in Section 3.5.

Suwannee River fulvic acid (SRFA) is a reference fulvic acid that has been studied using Cu titrations by Cabaniss and Schuman (1988a). Cabaniss and Schuman develop an empirical model to predict free Cu during titrations of SRFA in the pH range 5 to 8, for total copper between 10^{-7} and 10^{-4} M, and dissolved organic carbon between 1 and 10 mg/ L. Their empirical model was used to validate the multiresponse fluorescence speciation method developed in this thesis. The fluorescence method proposed in this thesis was applied to Cu titration data for five fluorescent sites in SRFA (as determined in Paper-III). The speciation parameters determined from fitting these data were used to predict free Cu and this can be compared to Cabaniss and Shuman's predicted free Cu. The results are shown in Figure 2 Paper-IV. Free copper predicted by the fluorescence method does not differ by more than 0.1 log[Cu²⁺] unit from free Cu predicted by Cabaniss and Schuman (1988a). Thus, the fluorescence method can predict free Cu, even though free Cu is never directly measured during the experiment.

3.2 SIMPLISMA and NOM Fluorescence Surfaces

Fluorescence deconvolution using SIMPLISMA was performed on ten NOM samples (Paper-III). The samples correspond to nine samples from the NOM-Typing project and one sample of Suwannee River fulvic acid. The NOM-Typing project is an international collaborative effort to characterize NOM isolated from watersheds of various characteristics in Norway (Chairperson: E. Gjessing, Agder college, Kristiansand, Norway). Suwannee River fulvic acid is a reference fulvic acid that has been investigated by many researchers by many different methods (Leenheer *et al.*, 1998). The results of fluorescence deconvolution of surfaces corresponding to

these samples are presented in Paper-III. All surfaces were measured at pH 4.36, ionic strength of 0.1 M (KNO₃) and dissolved organic carbon between 5 and 10 mg / L. Results are summarized in Figure-1-Paper-III for SRFA and in Figure-2-Paper-III for the Norway-NOM samples.

The NOM-Typing project sampled eight sites for May, 1996 and one site was re-sampled in October, 1996 for a total of nine samples. The NOM was isolated from the water both by reverse osmosis and by low-pressure-low-temperature evaporation. Site descriptions, isolation method and basic characterization of these samples are given by Gjessing *et al.* (1999). The fluorescence surfaces were only measured for reverse osmosis isolates because Blaser *et al.*, (1999) found no significant differences between these and the low-pressure-low-temperature evaporative isolates.

Overall, the results of Paper-III showed that the broad fluorescence peaks in NOM could be represented by between 4 and 6 fluorophores. These fluorophores can be thought of as averages of many similar fluorescent-sites and they represent the minimum number of fluorescent components necessary to describe the observed surface. The fluorophores can be classified into 7 groups based on excitation and emission wavelength. This classification is shown in Table-1 of Paper-III and is reproduced below with an additional column for possible molecular contributors to the identified fluorescence wavelengths. The possible fluorophores are identified from Figure 3, where the seven fluorophore classes are indicated by their roman numerals. It should be emphasized that these proposed fluorophores are only suggestions and should not be interpreted literally. The data for model compounds reported in the literature was often obtained uncorrected and usually from unidimensional emission or excitation scans; therefore, the wavelengths reported are approximations only. Still, this approximate map serves as a tool to suggest possible

fluorophores contributing to NOM fluorescence at the molecular level.

Table 1: NOM fluorophore classes from NOM-Typing project samples along with possible contributing moieties.

Class	Excitation Range (nm)	Emission Range (nm)	Possible Fluorophores
I	270	390	simple aromatics
II	330-340	410-450	simple aromatics, hydroxyquinolines
III	370-390	440-460	hydroxyquinolines, flavones
IV	400-420	460-480	flavones, coumarines
V	430-450	500-520	flavones
VI	460-480	520-540	unknown
VII	490-500	540-570	unknown

Two of the seven components common among the nine NOM samples (Table 1) cannot be related to literature values for model compounds. This does not imply that there are no model compounds with similar fluorescence to components VI and VII, only that these compounds were not found in the literature search used to generate Figure 3.

Natural organic matter samples can be compared using the SIMPLISMA approach. Without deconvolution each surface looks very much the same with two broad peaks at similar emission (around 420 nm) and excitation (around 250 and 320 nm) wavelengths. After deconvolution the samples can be compared based on the number and the type of fluorophore. The seasonal sample has 4 components in October and 6 components in May. Greater productivity in the spring seems to produce more complicated NOM. In addition, the fall sample showed a unique short wavelength component (I), not observed in other samples. Two NOM

samples from a limed and unlimed catchment were the same except the limed sample had an additional long wavelength component, which is not present in the unlimed sample. There are three samples from the Trehørningen watercourse that change from 5 to 6 and down to 4 components moving downstream. The nature of the fluorophores changes as well, demonstrated by the changing excitation and emission wavelengths. Thus illustrating that the NOM is changing as it moves downstream.

3.3 Application of Method for Al-NOM interactions

The fluorescence method proposed in this thesis is especially good for Al because Al enhances fluorescence very efficiently, as demonstrated by the wide variety of fluorescent analytical reagents for Al (Guilbault, 1973). In addition, Al is difficult to investigate by other speciation methods because many other methods are electrochemical (such as ion-selective electrodes), and Al does not normally display redox chemistry in aqueous solution. Existing methods for Al are mostly limited to “black-box” approaches where NOM is considered as a single ligand. These methods include Driscoll’s fractionation method (1984) and ligand competition methods (Kramer *et al.* 1994). The advantage of using fluorescence to investigate Al speciation is that the method is sensitive to the ligand, not the metal.

The fluorescence method is applied to the NOM-Typing samples in Paper-III. The samples and the nature of their fluorophores are discussed in Section 3.2. Experimentally, the pH was held constant at around 4.3 as the sample was titrated with Al. At each addition of Al the fluorescence corresponding to each proposed component was measured. The resulting multiresponse data were then fit to a site-specific model for Al binding as discussed in Section 3.1. An example fit of data for 4 fluorophores is shown in Figure-3-Paper-III. The results of

fitting both reverse osmosis and low-pressure-low-temperature NOM isolates is shown in Table 2 of Paper-III for all 9 NOM samples and SRFA.

A detailed discussion of the results in Table 2 of Paper-III is given in the text of that paper. The major conclusion is that many fluorescent sites have binding similar to salicylic acid ($\log K' \approx 5.5$ at pH 4.3). Such binding sites correspond to a carboxylic group ortho to an hydroxy group. Weaker sites are also observed ($\log K' \approx 3$) that likely correspond to uni-dentate carboxylic sites without the stabilizing influence of an ortho-hydroxy group. Significantly, the speciation results are as strongly dependent on isolation method as they are on sampling site. Since fluorescence is sensitive enough to detect NOM in raw waters, speciation determinations should be carried out on raw water samples rather than isolates.

3.4 Suwannee River fulvic acid binding of Al, Cu and H

Suwannee River fulvic acid (SRFA) is a reference fulvic acid that has been investigated by many researchers using many methods. Summary work on SRFA is found in a United States Geological Survey report (U.S. Geological Survey Open-File Report 87-557, 1989) and recent papers by Leenheer (1998, 1995).

The fluorescence method proposed here is applicable to any metal that will change the fluorescence of NOM. Fluorescence changes have been observed for Al, Cu, H, Zn, Pb, Mg, Mn, Ni, and Ca (Cabaniss, 1992). A study of Al, Cu and H binding to SRFA was performed as part of this thesis and is included as Paper-IV. The five fluorescent sites for SRFA identified in Paper-III were titrated with Al (Paper-III) at pH 4.36, with H (Smith *et al.*, 1999) and with Cu at pH 6 (Paper-IV). The results of all of these studies are summarized in Paper-IV.

Proton binding experiments were also performed (Smith *et al.* 1999). This paper is not

included as an appendix to this thesis as only part of that paper is relevant to the overall objectives of the work presented here. The relevant parts are included in the description below and in Paper-IV.

For H-binding experiments, free $[H^+]$ is measured using a pH electrode in addition to the 5 fluorescent responses corresponding to 5 proposed binding sites. Thus, 6 responses are fit simultaneously for SFRA-H binding. The raw data are shown in Figure 4.

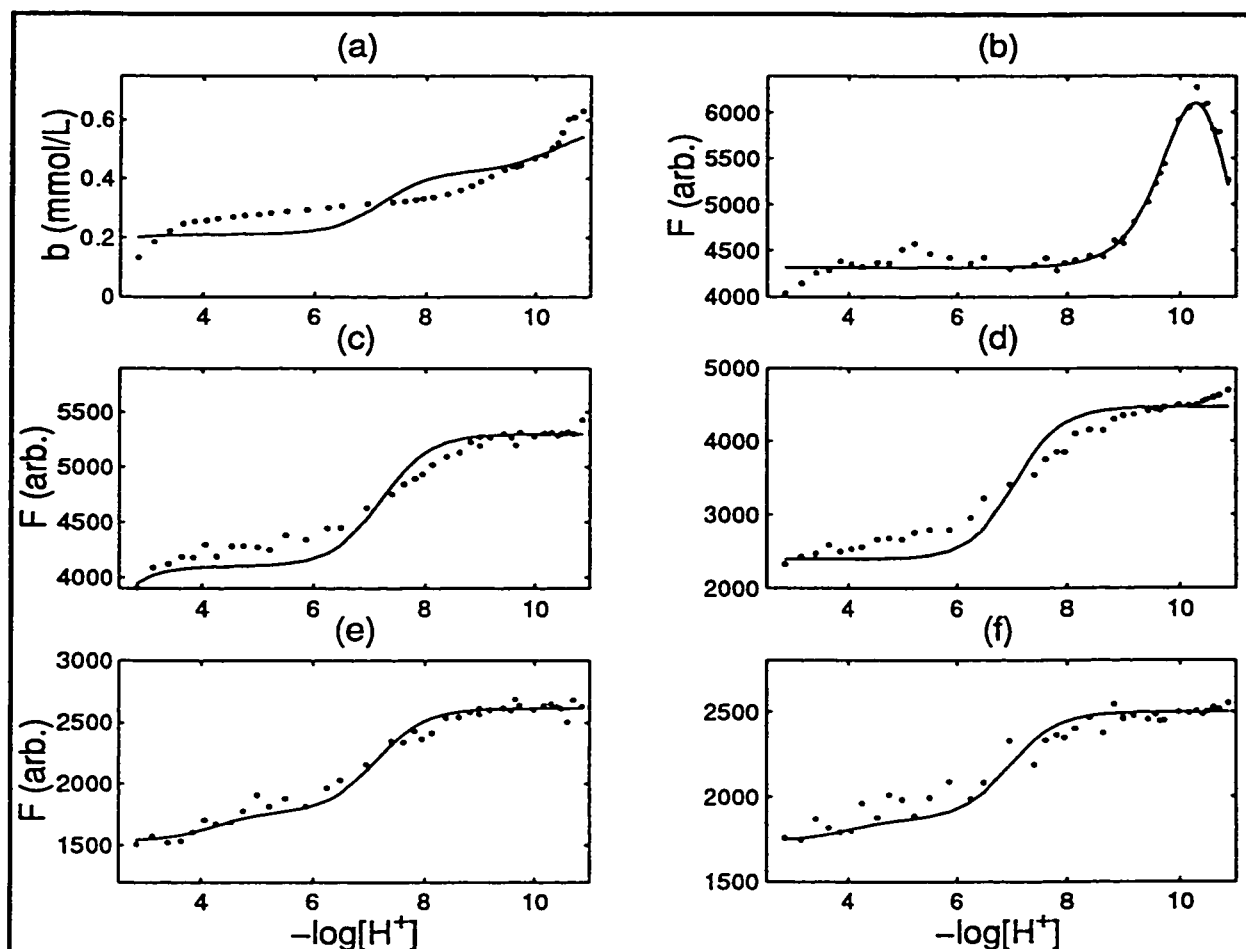


Figure 4: Fluorescence at five sites in SRFA (b to f) and charge excess (a) versus pH during acid-base titration. The dots are the experimental data and the solid lines are the best-fit results for a five fluorescent diprotic ligands model.

Usually, pH data are modelled as a mixture of monoprotic ligands (Brassard *et al.*, 1990). This simple model can represent data for any mixture of n-protic ligands by a mixture of n monoprotic ligands. Before fitting, measured pH values are transformed to the charge balance expression, $C_b - C_a - [OH^-] + [H^+]$, where C_b and C_a correspond to the concentrations of added base and acid respectively. This is summarized in Smith *et al.*, (1999) and Smith and Kramer (1999).

The fluorescence surface deconvolution results place a lower bound on the number of sites at five, whereas other speciation methods have no constraints on the number of sites. Thus, there must be at least 5 sites, although there could be non-fluorescent sites that were not detected by this method. Figure 4 reveals that the fluorescent sites cannot be represented as a mixture of monoprotic acids. There are multiple inflection points (end points), and fluorescence is observed to both decrease and increase. For a specific site, titration of a monoprotic ligand would show one inflection point at the pK_a of the ligand. Fluorescence can only increase or decrease for titration of a monoprotic ligand, because the only potentially fluorescent species are HL and L. If the ligand is diprotic the fluorescence can increase or decrease because there are now three fluorescent species: H_2L , HL and L. Thus, the fluorescent sites in SRFA must at least be diprotic because they all show at least two inflection points, and both increasing and decreasing fluorescence are observed for subplot (b) of Figure 4.

The data in Figure 4 were initially modelled as a mixture of 5 diprotic ligands because this is the minimum required to describe the system. The fitting was performed by using the determinant criteria (Section 2.5.1) with 10 acidity constants (pK_{a1} and pK_{a2} for each ligand), 5 ligand concentrations and 15 fluorescence proportionality constants (three for each ligand corresponding to H_2L , HL and L fluorescence). All 6 responses were fit simultaneously. The

results of this fitting are shown as the solid lines in Figure 4. Inspection of Figure 4 shows that this model is inadequate to describe the data. In particular, there are no strongly acidic ligands to account for the lower end of the charge balance titration curve (Figure 4, subplot (a)).

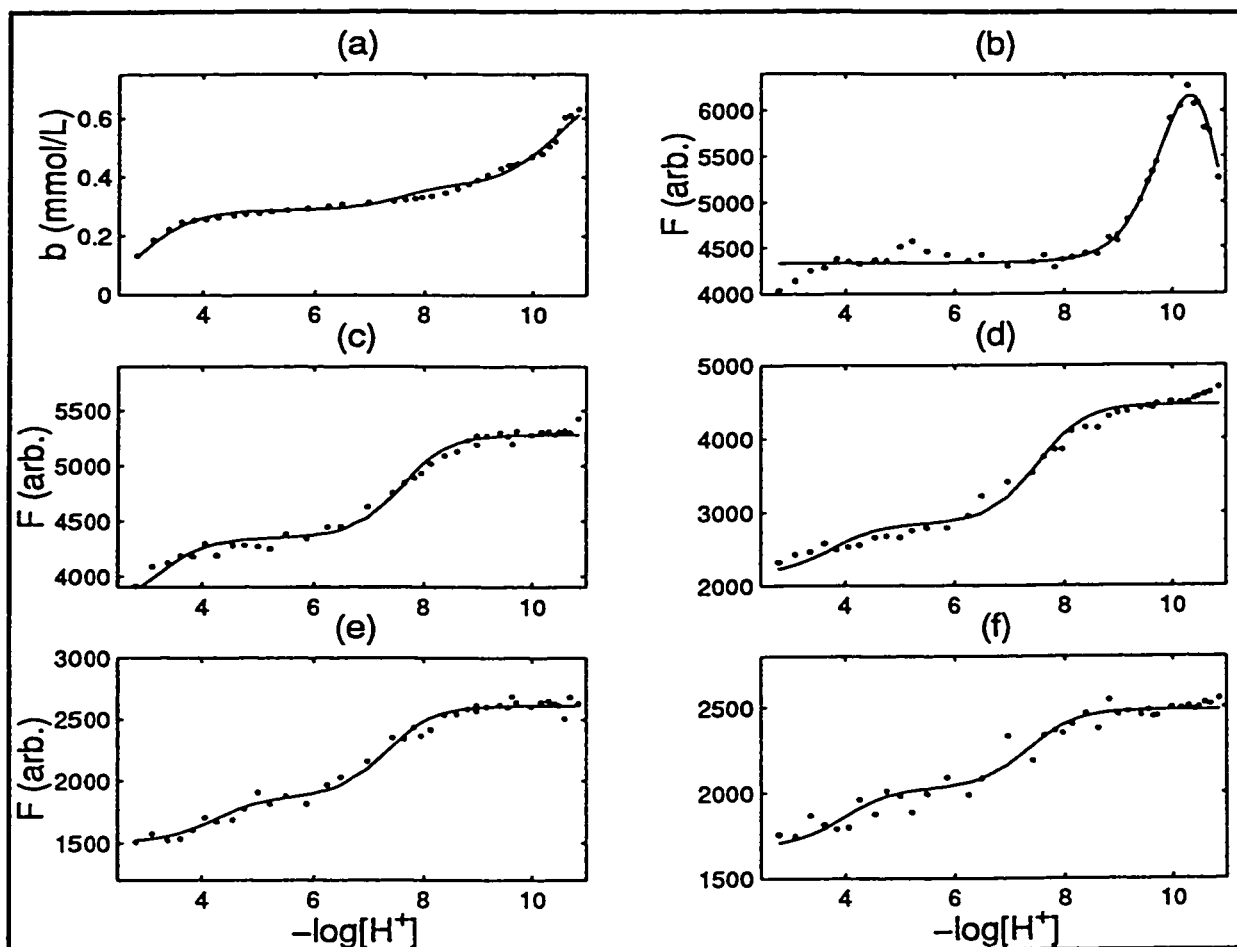


Figure 5: Fluorescence at five sites in SRFA (b to f) and charge excess (a) versus pH during acid-base titration. The dots are the experimental data and the solid lines are the best-fit results for a five fluorescent diprotic ligands model and one non-fluorescent ligand.

The next progression in model complexity was to add a non-fluorescent monoprotic ligand. The results of this fitting are shown in Figure 5. The fit is much improved, and this model

was selected as the best simple model for the measured data. An additional, non-fluorescent, strongly acidic ligand, would not be significant for Al or Cu binding, because such a ligand would weakly bind Al and Cu, as it weakly binds protons. Thus, for Al or Cu binding a model of five fluorescent sites is sufficient; the sixth non-fluorescent site would have negligible influence.

The diprotic nature of the fluorescent sites is consistent with observed Al and Cu binding. A summary of conditional stability constants for Al, Cu and H are shown in Table 1 of Paper-IV and reproduced here as Table 2. The constants for Al and Cu are too high to be accounted for by mono-dentate binding, which would correspond to a monoprotic site, such as a single carboxylic group. Such ligands (like acetic acid $\log K' \approx 3$) only bind metals weakly, and a stabilizing bidentate type ligand is necessary to account for observed binding strength. This is consistent with a diprotic ligand (salicylic acid $\log K' \approx 5$). Binding of Al and Cu at pH 4.36 and 6, respectively yields stability constants around 5 for both metals, which is consistent with salicylic acid constants at the same pH values.

The $\log K'$ s and pK_s s are summarized in Table 2. The first acid dissociation constants are all in the range 3.0 to 4.23, except for one exceptional value of 10.03. The second dissociation constants are between 7.33 and 7.60 with an exceptional value at 11.08. The values in Table 2 are not the same as literature values; Martell and Smith (1987) present step-wise dissociation constants of 2.97 and 12.98 for salicylic acid. As mentioned above, salicylic acid is a good model ligand for Al and Cu binding to SRFA, but it is not consistent with proton binding results. The proton constants with values around 4 likely correspond to carboxylic sites, whereas the constants around 10 are likely phenolic (Perdue, 1985). The intermediate pK_s s around 7 could be carboxylic. There is no generally accepted type of site in humics with a stability constant around

7, although such sites are observed (Smith and Kramer, 1999). The sites at intermediate pK_a values could be attributed to phosphoric sites (Fein *et al.*, 1997).

The concentration of the five sites determined for Al, Cu and H are shown in Figure-3 of Paper-IV and summarized in Table 1 of Paper-IV. The Al site capacities are as much as an order of magnitude lower than those for the same fluorescent site binding of Cu. This could be an effect of increased proton competition for sites, reducing the apparent concentration at pH 4 (Al) versus pH 6 (Cu). The Cu and H sites compare more favourably within the confidence intervals shown. In particular, the total concentrations of Cu and H binding sites are not statistically different, although H confidence intervals permit higher total concentrations. Total Cu sites correspond almost exactly to the best estimate of total carboxylic content (Leenheer *et al.*, 1995). This result is reasonable if Cu binding includes contributions from carboxylic groups. The total H-site concentrations bracket the total H determined by NMR for exchangeable and nonexchangeable protons (Thorn, 1989).

The five fluorescent sites determined for SRFA by SIMPLISMA deconvolution can represent Al, Cu and H binding to SRFA. These sites are bidentate for metals and diprotic for H. The sites do not exactly correspond to salicylic acid because the fluorescence and acidity constants are different. However, salicylic acid-like sites are consistent with Al and Cu binding. The fluorophore types that can account for the observed fluorescence of these sites are tabulated in Table 2 below. These were determined by comparison of the excitation/emission wavelengths for the five sites with the fluorescence map shown in Figure 3.

Table 2: Five Proposed Binding Sites in SRFA for Al, Cu and H

Component	excitation /emission (nm)	model fluorophore	logK' _{Cu}	logK' _{Al}	pK _{a1}	pK _{a2}
1 (II)	330/430	simple aromatics and hydroxyquinolines	4.95	6.89	10	11.1
2 (III)	380/460	simple aromatics and flavones	4.98	5.04	3	7.6
3 (V)	450/500	flavones	5.32	4.9	3.78	7.51
4 (VI)	460/540	unknown	5.59	4.97	4.23	7.33
5 (VII)	500/550	unknown	5.62	5.11	4.02	7.36

Component #1 of SRFA (Table 2) is the most unusual component because it shows very strong Al binding (6.8 versus the more usual 5). In this case, the pK_a values can best be interpreted as phenolic rather than carboxylic.

3.5 Protocol for Multiresponse Multi-site Fluorescence Speciation Method for Metal-NOM Interactions

The aim of this thesis has been to develop and apply a multiresponse fluorescence methodology for metal speciation determinations with NOM. A summary of the method is presented in four steps.

- (1) Measure the fluorescence surface for selected NOM at fixed pH and ionic strength. The ionic strength buffer should not be KNO₃ because it absorbs strongly at 250 nm, but KClO₄ is a good choice because it does not absorb light in the range of NOM excitation or emission. All experiments should be performed at fixed pH; thus, the resultant stability constants are conditional. Total dissolved organic carbon should not exceed approximately 15 mg/L because higher values lead to problems with self adsorption of

excitation and emission light by NOM. The excitation wavelengths should be in the range 250 to 500 nm and the emission wavelengths should be in the range 350 to 600 nm. This is the range that NOM is observed to fluoresce, but the sample should also be tested for fluorophores outside this range.

- (2) Deconvolute the fluorescence surface to determine the number and position of the minimum number of fluorescent components. Before doing this, artifacts at the emission=excitation and emission=2×excitation wavelengths must be removed by spline interpolation. The first artifact is the Rayleigh light scattering line and the second artifact is caused by the excitation monochromator. The artifacts are removed so that the data are in the correct format for input into SIMPLISMA which cannot handle missing observations and would treat the Rayleigh peak as a set of components.
- (3) Measure fluorescence at the excitation and emission wavelength pairs determined in (2) during a titration of NOM by metal at the same pH and ionic strength as in (1).
- (4) Fit data from (3) using the generalized multi-site Ryan-Weber approach presented in Paper-II. The error criteria minimized is the determinant criteria (Section 2.5.1) and the parameters include a conditional stability constant, a ligand concentration for each site and a linear proportionality constant for each ML and L species for converting the calculated concentrations into fluorescence.

4. Conclusions

New contributions of this study are summarized in Section A and B, below. The original contributions of this thesis as a whole are highlighted and specific reference to the relevant paper(s) is indicated. The conclusions are divided into method development and application results. Method development refers to the multiresponse fluorescence method developed to determine site-specific metal speciation at multiple sites within NOM. Application results are conclusions that add new insight into the nature of NOM with respect to fluorescent sites and metal binding.

Conclusions: Scientific contributions of this thesis

A. Method development

- (a) The Ryan-Weber equation is independent of wavelength as long as the fluorescence is from one fluorophore (Paper I).
- (b) The Stern-Volmer equation for fluorescence enhancement was derived and applied to Al-NOM (Paper I).
- (c) A multiresponse fluorescence method for multi-site metal binding to NOM was applied/validated for Al as a modification of the Ryan-Weber equation (Paper II).
- (d) A multiresponse fluorescence method to predict free copper in presence of NOM was validated using SRFA (Paper IV).
- (d) Spectral deconvolution of NOM fluorescence surfaces and comparison of fluorophores in NOM to model compounds (Paper III) was performed. This is done using SIMPLISMA (Windig and Guilment, 1991).
- (e) Part (d) above was used to determine minimum number and wavelengths of fluorescent sites

in NOM, (Paper III) and this was combined with multiresponse parameter fitting for Al (Paper III) and for Cu (Paper IV).

(d) A multiresponse fluorescence and ion-selective electrode (pH electrode) method for H binding to NOM was developed (Paper IV).

B. Nature of NOM

(a) The fluorescence of NOM samples in this study can be represented by 4 to 6 fluorescent components, which many samples have in common (Paper III).

(b) Possible fluorescent building blocks include simple aromatics, hydroquinolines, flavones and coumarines (Paper III).

(c) Fluorescent sites often have strength similar to salicylic acid, but the fluorescence includes all the possible moieties indicated in Paper III.

(d) Isolation method (reverse osmosis versus low-pressure-low-temperature evaporation) influences the nature of NOM potentially as much as sample-site location (Paper III).

(e) Proton binding sites in SRFA must at least be diprotic to explain both pH and fluorescence observations (Paper IV).

(f) The relative concentration of non-fluorescent versus fluorescent carbon in NOM is much higher than previously estimated (Paper IV). Seitz (1981) estimates around 1% of C atoms are fluorescent, but the fitting of copper titration data indicates that up to 100 % of Cu binding sites could be fluorescent. The total concentration of copper sites in SRFA determined in Paper IV is 6.75 $\mu\text{mol/mg}$ of SRFA. The total concentration of carbon, assuming 50 % of the fulvic acid is carbon by weight, is 41.6 $\mu\text{mol/mg}$ of SRFA. Thus, the fluorescent sites are 16.2 % of the carbon sites.

5. Future Work

Further work would include expanding the metals and NOM samples used. The metals could also include Pb, Mn, Zn, Ni, Ca, and As, for example. Natural organic matter samples should include a comparison of raw waters to isolates in order to determine the effects of commonly used isolation methods. Natural organic matters for future investigations could include NOM from source materials, such as aqueous leaf litter extracts for a variety of plant-species and even plankton, as well as NOM from sedimentary materials. Environmentally significant results could be obtained by application of this method to NOM in wastewater effluents. In addition, the use of fluorescence surfaces to characterize NOM samples should be explored further. This would involve careful measurement of natural samples, as well as model compounds to better constrain the types of fluorophores present in NOM.

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Preface to Appendices

Candidate's Contribution to Papers I to IV

For all of the papers the developmental and experimental work was performed by the candidate under the supervision of Dr. James R. Kramer. Initial drafts of all papers were prepared by the candidate and reviewed by Dr. Kramer before submission. Paper III was part of the NOM-Typing project (E. Gjessing, Agder College, Kristiansand, Norway). The samples were obtained and isolated by Gjessing *et al.* (1999).

Appendix I:

Aluminum adsorption to organic matter determined using fluorescence quenching

From: Smith, D.S., and Kramer, J.R., In *Adsorption of Metals by Geomedia: Variables, Mechanisms, and Model Applications*; E. Jenne, Ed.; Academic Press: San Diego, CA, 1998; pp 445-466.

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Chapter 21

Fluorescence Quenching and Aluminum Adsorption to Organic Substances

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Site-specific Al speciation with Suwannee River fulvic acid is determined using total luminescence spectroscopy. There are two fluorescent sites in Suwannee River fulvic acid that interact with Al. One site with a fluorescence excitation wavelength of 250 nm and an emission at 440 nm has a conditional stability constant, $\log K'$, of 5.6 and a concentration of $0.43 \mu\text{mol}\cdot\text{mg}^{-1}$. Another site with an excitation at 330 nm and an emission at 440 nm has a $\log K'$ of 5.1 and a concentration of $0.018 \mu\text{mol}\cdot\text{mg}^{-1}$. The equations used to fit fluorescence data are modified to apply to fluorescence enhancement and reactions with protonated ligand. The mathematical approach is tested with the model ligand salicylic acid, and it is found that for one fluorescent site fitting is independent of whether quenching or enhancement data are used.

I. INTRODUCTION

The toxicity of aqueous Al is speciation dependent (Dobbs *et al.*, 1989; Parker *et al.*, 1989). In particular, the presence of dissolved organic matter (DOM) can reduce Al toxicity (Hue *et al.*, 1986; Kramer *et al.*, 1986; Gjessing *et al.*, 1989). Dissolved organic matter is a complicated mixture (Christman and Gjessing, 1983; Frimmel *et al.*, 1988; Senesi and Miano, 1994) that is very important in Al bind-

ing. In order to properly address the complex heterogeneous nature of metal binding sites within DOM, a sensitive and selective methodology should be used. There are many methods available to speciate Al in the presence of DOM: excellent reviews are given by Bloom and Erich (1989) and Clarke (1994). In general, these methods assume a one to one complex between Al and DOM and cannot easily resolve complexation at discrete sites. Fluorescence measurements have been shown to be able to resolve site-specific Al binding (Patterson *et al.*, 1992). Total luminescence spectroscopy (TLS) is a type of fluorescence measurement that is especially useful in resolving site-specific Al speciation (Yang *et al.*, 1994; Luster *et al.*, 1996). This study uses TLS to investigate Al binding to Suwannee River fulvic acid.

Aluminum binding to DOM has been investigated by various methods (Stevenson and Vance, 1989; Clarke, 1994), including fluorescence spectroscopy (Shotyk and Sposito, 1990; Tam and Sposito, 1993). In general, Al binds to carboxyl and hydroxyl groups, and the binding strength is stronger for bi- and tridentate ligands (Orvig, 1993). Model compounds can be used to represent DOM in studying Al binding (Öhman and Sjöberg, 1988). In fact, Al speciation for several model compounds, such as citrate (Motekaitis and Martell, 1984; Öhman, 1988), catechol (Öhman and Sjöberg, 1983; Motekaitis and Martell, 1984), and salicylic acid (Öhman, 1991), has been determined potentiometrically, but little work, other than L-tyrosine complexation by Cu^{2+} (Ryan and Weber, 1982), has been done with model compounds and speciation as defined by a fluorescence methodology. In this study, the interaction of Al with the model ligand salicylic acid is investigated to further verify the fluorescence methodology, and in particular, the application of the method to fluorescence enhancement measurements in an Al-DOM system.

Dissolved organic matter can be fluorescent (Goldberg and Weiner, 1993). This fluorescence results from fluorescent groups within DOM. These fluorophores may occur as free molecules or as molecular building blocks of macromolecules. Probable fluorophores in DOM include salicylate, catecholate, flavones, chromone derivatives, and quinolines (Senesi, 1990). Fluorescence measurements can be used to characterize DOM (Coble *et al.*, 1990; Belin *et al.*, 1993) because different fluorophores may have different fluorescent properties that can be resolved experimentally; such properties include lifetime (Power *et al.*, 1986; Cook and Langford, 1995), polarization (Lapen and Seitz, 1982; Goldberg and Negomir, 1989; Lakshman *et al.*, 1996), and excitation and emission wavelengths. Traditionally, fluorescence is observed as excitation, emission, or synchronous spectra (Guilbault, 1990), but the dimensionality of fluorescence measurements, and thus its selectivity, can be increased by using TLS (Goldberg and Weiner, 1993). Total luminescence spectroscopy is performed by measuring the entire excitation versus emission fluorescence surface. Fluorescence excitation versus emission surfaces

for DOM can be used to fingerprint samples from different locations (Goldberg and Weiner, 1993; Provenzano and Sposito, 1994) and different fractions within samples (Belin *et al.*, 1993; Campanella *et al.*, 1994). In addition, possible classes of fluorophores can be suggested from TLS studies (Coble *et al.*, 1990; Tam and Sposito, 1993; Provenzano and Sposito, 1994).

The fluorescence of DOM may change upon metal binding to it (Cabaniss, 1992). If the change in fluorescence is assumed to be proportional to the amount of metal bound, then stability constants (Stern and Volmer, 1919) and total ligand concentrations can be determined (Ryan and Weber, 1982). Numerous studies have investigated metal–DOM binding using excitation and/or emission spectra (Ghosh and Schnitzer, 1981; Tam and Sposito, 1993) and synchronous fluorescence spectra (Cabaniss, 1992). Luster *et al.* (1996) emphasize the fact that results obtained from such studies are not necessarily independent of wavelength choice. It is necessary to find a point on the excitation versus emission surface where changes in fluorescence are caused by complexation at only one site. The use of TLS improves confidence in wavelength choices because the effect of the metal across the entire fluorescence surface can be observed and regions of change assigned to each fluorophore (Luster *et al.*, 1994). The result is site-specific speciation (Luster *et al.*, 1994, 1996).

Metal binding sites have been resolved in DOM using TLS: spectra have been reported with and without added metal at fixed pH (Provenzano and Sposito, 1994; Yang, *et al.*, 1994). Recently, this has been taken one step further and TLS was performed during a titration of juniper leaf litter extract with Al (Luster *et al.*, 1994, 1996) and Cu (Luster *et al.*, 1996) at fixed pH. The approach was validated for Al using an equilibrium ion exchange method. The determination of conditional stability constants at different sites was performed using the approach of Ryan and Weber (1982). This involves fitting the fluorescence data from a titration with metal at fixed pH to three parameters in a nonlinear equation. The approach has been criticized in that the three parameters may not be independent (Cabaniss and Shuman, 1988; Ryan and Ventry, 1990), but several of the studies already mentioned have used the equation and obtained physically reasonable results from Al–DOM fluorescence measurements. The method has also been validated using independent speciation measurements (Shotyk and Sposito, 1988; Luster *et al.*, 1996).

In an effort to further validate the Ryan–Weber equation, this study examines fitting results for both a model ligand (salicylic acid) and a reference DOM (Suwannee River fulvic acid) using a one-parameter equation and a two-parameter equation, in addition to the usual three-parameter Ryan–Weber equation. The one-parameter equation is a modification of the Stern–Volmer equation (Stern and Volmer, 1919) that can be applied to fluorescence enhancement measurements; it was originally derived only to apply to fluorescence quenching measurements. The two-parameter equation is a modification of the Ryan–Weber equation using a sim-

plifying assumption to remove one parameter. In addition, the Ryan–Weber equation was originally derived assuming that the free ligand was the fluorescent species, but a modified equation is proposed here, assuming that the fluorescent species is the protonated ligand.

The Ryan–Weber equation is a general equation applicable to positive or negative fluorescence quenching (enhancement), but as Luster *et al.* (1996) point out, fitting results for metal–DOM systems are not independent of wavelength. Salicylic acid data are fit across the entire emission spectra to assess whether the wavelength dependence is an artifact of the mathematical approach. For example, with a single fluorophore, does quenching data yield different results from enhancement data, or is the observed dependence in natural DOM systems due to multiple fluorophores affecting the observed fluorescence at a given point on the fluorescence surface?

II. EQUATIONS TO INTERPRET FLUORESCENCE MEASUREMENTS

There are two effects that metal (M) binding may have on a fluorescent ligand (L). The metal may partially or completely suppress the ligand's fluorescence, or the new species (ML) may be fluorescent itself. In general, if there is an effect, paramagnetic species, such as Cu^{2+} , quench fluorescence (Guilbault, 1990) and diamagnetic species, such as Al^{3+} , form new fluorescent species (Djurdjevic *et al.*, 1995). For Al, the new species are generally a more efficient fluorophore, as demonstrated by the large number of fluorescent analytical reagents used for Al determination (Snell, 1978).

When fluorescence quenching is observed, the metal ion inhibits the radiative transition that causes fluorescence emission. Thus, the entire spectrum will be observed to decrease in intensity (Provenzano and Sposito, 1994). If the bound metal forms a species with different fluorescent properties from the free ligand, then the observed fluorescence will be a combination of the spectral contributions from both the ligand and the metal–ligand species. Thus, in the case of diamagnetic ions, suppression or enhancement can be observed depending on which part of the fluorescence emission spectra is monitored (Cabaniss, 1992; Tam and Sposito, 1993).

The Stern–Volmer (Eq. [1]) and Ryan–Weber (Eq. [2]) equations have been proposed to interpret fluorescence suppression results during a titration of fluorescent ligand with quencher at fixed pH. Equation [1] is a simple linear equation and will yield a conditional stability constant (K'). Equation [2] will also yield a value for K' ; in addition, values for the total ligand concentration (L_T) and the fluorescence intensity when all the ligand is bound to the metal (F_{ML}) are also determined. The fluorescence signal is defined as the fluorescence emission intensity (in arbitrary units) at fixed excitation and fixed emission wavelengths.

The Stern–Volmer equation,

$$\frac{F_i}{F} = 1 + K^*M_T \quad [1]$$

is a linear equation. The conditional stability constant is the slope when the initial fluorescence (F_i) over the fluorescence at any point during the titration (F), is plotted against the total metal concentration (M_T).

Equation [2] is a nonlinear equation in which three parameters must be fit. The parameters are K^* , L_T , and (F_{ML}). The equation can be written

$$F = \left(\frac{F_{ML} - F_i}{2K^*L_T} \right) (KL_T + KM_T + 1 - \sqrt{(KL_T + KM_T + 1)^2 - 4(K^*)^2L_TM_T}) + F_i \quad [2]$$

Equations [1] and [2] both assume that the metal and ligand form a one to one complex according to the reaction



with

$$K^* = \frac{[ML^*]}{[L^*][M^*]} \quad [3]$$

where $[ML^*]$ is the total concentration of all ligand-bound metal species, $[L^*]$ is the total concentration of all free ligand species, and $[M^*]$ is the total concentration of all metal species not bound to the ligand. The conditional constant is only applicable for the experimental conditions at which it is measured. Two important variables affecting the conditional constant are pH and ionic strength. The value of the constant should be independent of total ligand amounts, as Shotyk and Sposito (1990) showed for Al with chestnut leaf litter extract, and total metal concentration unless polynuclear species are forming.

Equations [1] and [2] both assume that fluorescence response increases linearly with fluorophore concentration ($[Fluor]$):

$$F = k[Fluor]. \quad [4]$$

This is a reasonable assumption in dilute solutions (Guilbault, 1990). The constant, k , is specific for each fluorescent species.

The equation derivations differ in that Eq. [1] makes two simplifying assumptions that Eq. [2] does not. These assumptions are:

1. ML species do not fluoresce
2. the total concentration of metal not bound to the ligand ($[M^*]$) is approximately equal to the total metal (M_T)

Equation [2] assumes residual fluorescence from the bound species, and does not assume that M_T is equal to $[M']$.

In order for the parameters measured to be physically meaningful, both equations assume that it is a ground-state association between the metal and the fluorophore that changes the fluorescence. In addition, both equations assume that the initial total metal is negligible.

A. MODIFIED STERN-VOLMER EQUATION

An equation similar to Eq. [1] can be derived for fluorescence enhancement; the assumptions are the same except as further noted.

For reaction (1) the mass balance for ligand can be written

$$L_T = [L'] + [ML'] \quad [5]$$

Dividing both sides of the expression by $[ML']$ and substituting $[L']$ from Eq. [3] yields

$$\frac{L_T}{[ML']} = \frac{1}{[M']K'} + 1 \quad [6]$$

Further, assume that $[M']$ is approximately equal to M_T , as in Eq. [1]; consequently $[L']$ should not change at fixed pH; therefore, the fluorescence change ($F - F_i$) is proportional only to $[ML']$. With the additional assumption that the fluorescence proportionality constants, as shown in Eq. [4], are equal for free and bound ligand species, the equation can finally be written as

$$\frac{F_i}{F - F_i} = \frac{1}{M_T K'} + 1 \quad [7]$$

A plot of F_i over $(F - F_i)$ versus the reciprocal of the total metal concentration will yield a linear plot with slope $1/K'$. The equation is not defined when M_T equals zero, because of division by zero.

B. TWO-PARAMETER RYAN-WEBER EQUATION

The derivation of the modified Ryan-Weber equation is given next. The objective is to derive a two-parameter equation intermediate between the simplicity of Eq. [1] and the complexity of Eq. [2]. The total fluorescence at any point during the titration is a sum of fluorescence contributions from both free and bound ligand:

$$F = k_L[L'] + k_{ML}[ML'] \quad [8]$$

The proportionality constants, k_L and k_{ML} , can be rewritten in terms of the fluorescence response when all the ligand is free (F_i) and when all the ligand is bound (F_{ML}):

$$F = \left(\frac{F_i}{L_T} \right) [L] + \left(\frac{F_{ML}}{L_T} \right) [ML] \quad [9]$$

Using the mass balance expression for the ligand (Eq. [5]) and rearranging yields the following relation:

$$\frac{[ML]}{L_T} = \frac{F_i - F}{F_i - F_{ML}} \quad [10]$$

If the simplifying assumption, M_T equals $[M]$, from Eq. [1] is used then the stability constant can be written

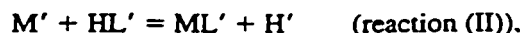
$$K' = \frac{[ML]}{M_T(L_T - [ML])} \quad [11]$$

This expression is then rearranged to solve for the ratio $[ML]$ over L_T and this ratio is set equal to Eq. [10]. The result after rearrangement is a two-parameter (K' and F_{ML}) equation:

$$F = F_i + \left(\frac{K'M_T(F_{ML} - F_i)}{1 + K'M_T} \right) \quad [12]$$

C. ALUMINUM REACTION WITH PROTONATED LIGAND

In the previous equations the reaction between Al and organic ligand is of the form described by reaction (I). In the derivations presented so far it is assumed that the free ligand is the fluorescent species. This is not necessarily a good assumption: in the pH range of these experiments it is likely that the protonated ligand is the fluorescent species. If the reaction is assumed to be of the form



and K' is redefined accordingly, then the three proposed equations can be rewritten. Equation [1] becomes

$$\frac{F_i}{F - F_i} = \frac{[H]}{M_T K'} + 1, \quad [13]$$

Eq. [2] becomes

$$F = \left(\frac{F_{ML} - F_i}{2K'L_T} \right) (KL_T + KM_T + [H]) - \sqrt{(KL_T + KM_T + [H])^2 - 4(K')^2 L_T M_T} + F_i, \quad [14]$$

and Eq. [12] becomes

$$F = F_i + \left(\frac{K'M_T(F_{ML} - F_i)}{[H] + K'M_T} \right) \quad [15]$$

D. SUMMARY OF DERIVED EQUATIONS

There are five equations derived in this study that are modifications of Eq. [1] and Eq. [2]. Equation [1] has been modified to apply to fluorescence enhancement (Eq. [8]) and to the reaction of metal with protonated ligand (Eq. [13]). The three-parameter equation (Eq. [2]) has been modified to apply to reactions with protonated ligand (Eq. [14]) and a two-parameter equation has been derived (Eq. [12]). The two-parameter equation has also been modified for a reaction with HL (Eq. [15]). Note that Eq. [2] and all modifications apply equally well to quenching or enhancement measurements.

III. EXPERIMENTAL METHOD

Organic ligand solutions were titrated with Al at fixed pH and ionic strength and fluorescence was monitored. In the salicylic acid titrations the Al concentration was in the range $0.7 \mu\text{mol}\cdot\text{L}^{-1}$ to $0.4 \text{mmol}\cdot\text{L}^{-1}$ for a total of 11 solutions. The Suwannee River fulvic acid titration was in the range $0.2 \mu\text{mol}\cdot\text{L}^{-1}$ to $0.06 \text{mmol}\cdot\text{L}^{-1}$ for a total of 5 solutions. The reproducibility error in salicylic acid fluorescence measurements was $<1\%$. The total salicylate concentration was $26.4 \mu\text{mol}\cdot\text{L}^{-1}$ and the total fulvic acid concentration was $8.4 \text{mg}\cdot\text{L}^{-1}$. The organic ligands used were salicylic acid (Fisher Scientific, Fair Lawn, NJ) after recrystallization, and Suwannee River fulvic acid (International Humic Society Standard). Aluminum stock solution ($1.18 \text{mmol}\cdot\text{L}^{-1}$) was made from Al-nitrate (BDH Chemicals, Toronto, Canada) with pH adjusted to 4.0 with sodium hydroxide. The pH after Al addition was adjusted with dilute NaOH or HCl as necessary to achieve a final pH of 4.0. The ionic strength was held fixed at $0.01 \text{mol}\cdot\text{L}^{-1}$ using potassium nitrate.

Solutions were equilibrated for 2 days before fluorescence measurement. This was determined to be long enough for equilibrium because at acidic pH, Al reaction times with salicylic acid (Plankey *et al.*, 1986) and fulvic acid (Plankey and Patterson, 1987; Plankey *et al.*, 1995) are on the order of minutes, and although reactions to form aluminum polynuclear species may occur on the order of days, these species were not expected to occur according to equilibrium calculations using Öhman's constants (1991). Fluorescence excitation versus emission surfaces were measured using a Perkin-Elmer LS-5 spectrophotometer with fixed excitation and emission slits at 5 nm. The size of the surface was limited by the controlling soft-

ware to an excitation range of 240 to 340 nm and an emission range of 350 to 500 nm. Emission spectra were recorded for every 10 nm of excitation. For salicylic acid, the excitation light source was a General Electric 110-855 bulb and a 7-54 Corning glass filter combined with a NiSO_4 solution (Murov, 1973) to achieve a constant excitation wavelength of 300 nm with a bandwidth of about 10 nm. Emission light was collected by a fiber optic probe down the sample axis and detected by an InstaSpecIV charge coupled detector (Oriel Corporation, Stratford, CT).

Nonlinear regression analysis was performed using the simplex function minimization algorithm in Matlab (The Mathworks Inc., South Natick, MA). The sum of the absolute value of the difference between data points and the fit curve was the minimized function. The minimization was set to stop once the difference reached a total relative error of 10^{-4} . Error estimates in the fit parameters were obtained by taking the average and standard deviation of the parameters over the applicable range of the emission spectra.

IV. RESULTS

A. SALICYLIC ACID TITRATIONS

Fluorescence emission spectra from the salicylic acid titration are shown in Figure 1. The curves in region B have predominantly salicylic acid contributions and the curves in region A have a stronger Al-salicylate contribution. Equilibrium calculations, using Öhman's constants (1991), show that at pH 4 the only bound Al species is AlL^+ and, at maximum Al addition, 90% of the total ligand is complexed. Therefore, the observed fluorescence spectra are a combination of AlL^+ and HL^- fluorescence spectra only, where region B is mostly HL^- and region A is mostly AlL^+ . The HL^- spectra have a maximum emission at 410 nm and the AlL^+ spectra have an emission maximum around 380 nm. The curves between region A and region B are a combination of HL^- and AlL^+ spectra. Fluorescent enhancement is observed in the region around 380 nm and fluorescence quenching in the region around 420 nm.

The Stern-Volmer plot for fluorescence quenching and enhancement is shown in Figure 2. The plot for quenching is not linear but the plot for enhancement is linear. The stability constants determined differ by two orders of magnitude: $\log K'$ is 3.2 for quenching versus 5.2 for enhancement. It is interesting to note that a linear plot is observed for enhancement, but the Eq. [7] assumption that the Al is predominantly free is not valid in this case except at very high total Al; when the metal and ligand are one to one, almost all the Al is complexed. Another consequence of the inappropriate assumption is that the intercept is not 1 in the enhancement plot, as it should be according to Eq. [7].

Quenching and enhancement measured at 420 and 380 nm, respectively, are

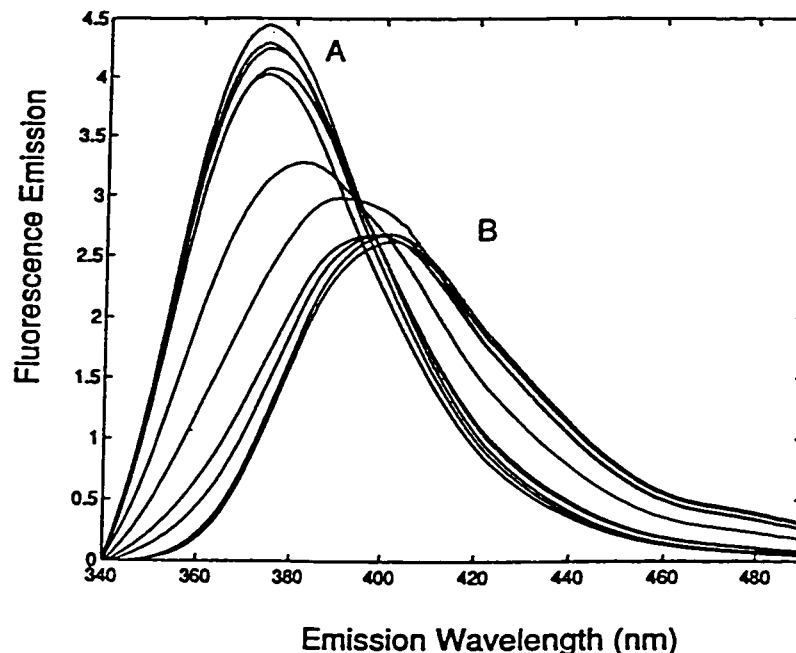


Figure 1 Fluorescence emission spectra, in arbitrary units, for salicylic acid titration with Al at pH 4.0. Region A corresponds to high amounts of total Al, and region B corresponds to low and zero amounts of total Al.

shown in Figure 3, along with fit curves from Eq. [2] and Eq. [14]. It can be seen that both equations fit the data well except in the enhancement case when the observed fluorescence starts to decrease with increasing Al and the fit curve does not. The observed decrease cannot be accounted for using equilibrium calculations; it is possibly the result of collisional deactivation of the fluorescent species in the more concentrated solutions, so-called dynamic quenching (Guilbault, 1990).

The results of parameter fitting of the salicylic acid fluorescence data are shown in Table I. The quenching results are calculated at 420 nm and the enhancement results at 380 nm. For comparison, the literature value of the conditional stability constant calculated from thermodynamic stability constants reported by Öhman (1991) and the actual values for L_T and F_{ML} are also given.

The actual conditional stability constant for reactions (I) and (II) is underestimated by all of the equations except Eq. [2] and Eq. [14] which, within experimental error, recover the actual values. Equations [12] and [15] underestimate K' by almost an order of magnitude, but Eq. [7] and Eq. [13] yield values only 0.3 of a log unit under the true value. For fluorescence enhancement, the calculated F_{ML} is very close to the observed value, but for quenching the calculated value is twice

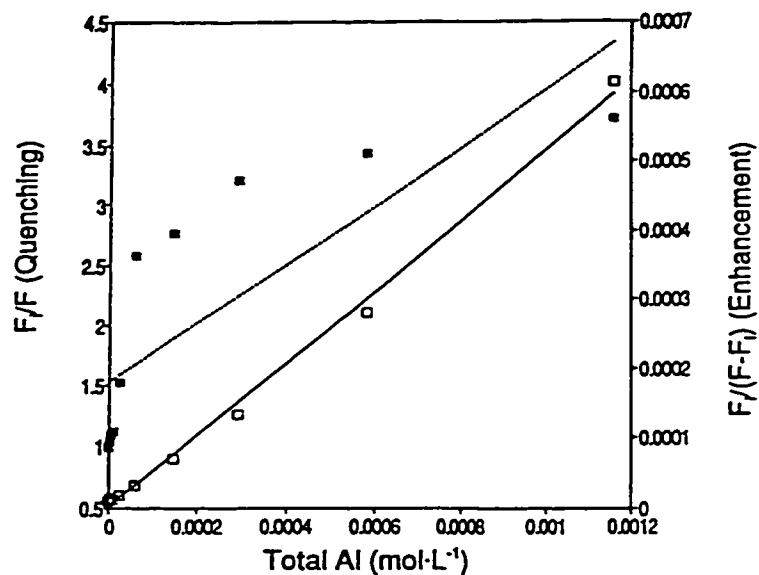


Figure 2 Stern-Volmer plot for fluorescence quenching and enhancement of salicylic acid with Al at pH 4.0. The open squares and the right axis correspond to enhancement data (at 380 nm) and Eq. [7]. The filled squares and the left axis correspond to quenching data (at 420 nm) and Eq. [1]. The enhancement data have been transformed to plot versus Al_T instead of $1/Al_T$.

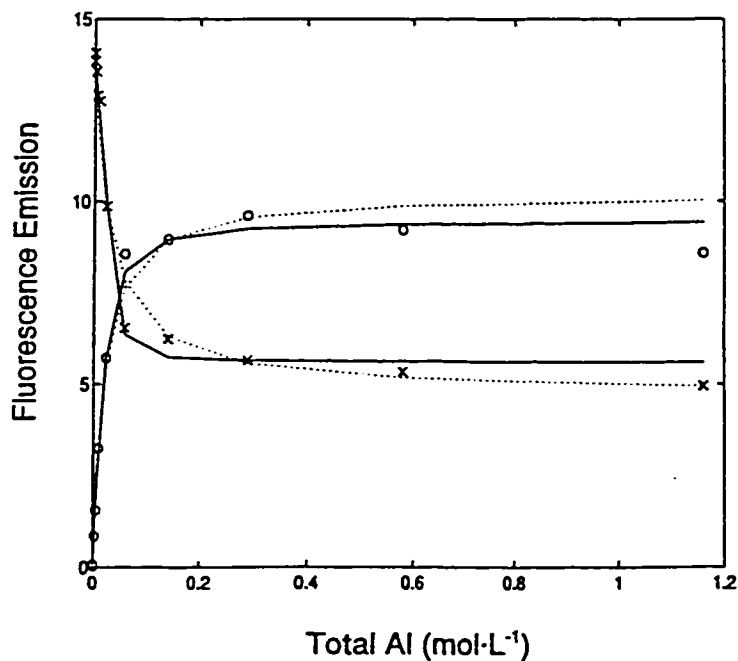


Figure 3 Fluorescence data at 380 nm (O) and 420 nm (X) in the salicylic acid titration with Al at pH 4.0. The solid lines correspond to fitting with Eq. [2] and the dotted lines to fitting with Eq. [14].

Table I
Summary of Parameter Fitting Results from Salicylic Acid Titration

Equation ^a	Reaction (I) log K'	Reaction (II) log K'	F _{ML} (arbitrary units)	L _T ($\mu\text{mol}\cdot\text{L}^{-1}$)
2.14.E	5.4 ± 0.1	2.0 ± 0.1	4.4	22 ± 1
2.14.Q	5.4 ± 0.1	1.4 ± 0.1	0.5	40 ± 1
12.15.E	4.7 ± 0.1	0.5 ± 0.1	4.6	na
12.15.Q	4.6 ± 0.1	0.25 ± 0.1	0.5	na
7.13.E	5.2 ± 0.1	1.1 ± 0.1	na	na
1.Q	3.2 ± 0.9	-0.8 ± 0.9	na	na
Actual	5.5	1.5	4.5 (E), 0.25 (Q)	21.6

^aThe equation numbers refer to equations in the text, and the E or Q refers to enhancement or quenching measurements, respectively.

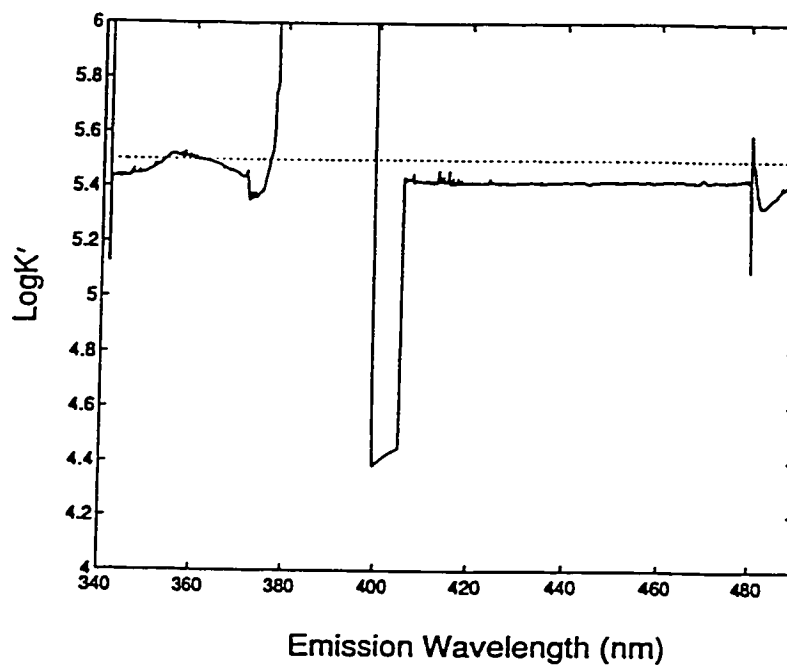


Figure 4 Log K' fitting results across the entire emission spectrum for salicylic acid titration with Al at pH 4.0. The dotted line corresponds to the actual stability constant and the solid line is the result of fitting Eq. [2] to the titration data.

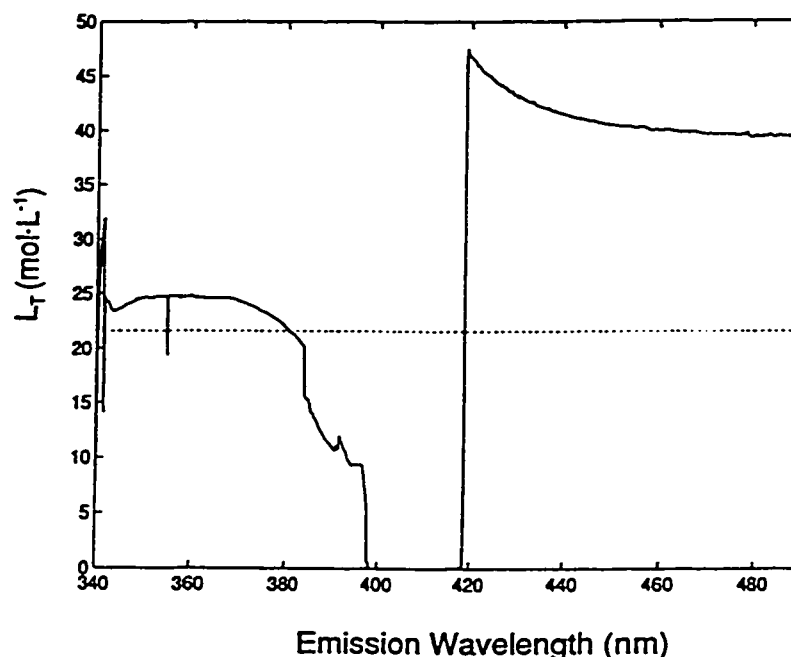


Figure 5 L_T fitting results across the entire emission spectrum for salicylic acid titration with Al at pH 4.0. The dotted line corresponds to the actual ligand concentration and the solid line is the result of fitting Eq. [2] to the titration data.

the observed value. It should be noted that the observed fluorescence is for a solution with 90% of the salicylic acid complexed, but the calculated value assumes that it is all bound. The total ligand concentration determined from fitting enhancement measurements to Eqs. [2] and [14] yields a value, within experimental error, identical to the true value. The same equations overestimate L_T by two times when quenching observations are used.

To access the effect of wavelength choice, parameter fitting can be performed across the entire fluorescence spectrum. For every set of emission measurements the constants can be determined. The results of fitting the three parameters in Eq. [2] across the entire spectrum, along with the actual values, are shown for K' in Figure 4, for L_T in Figure 5, and for F_{ML} in Figure 6. The agreement between the actual values and the fit parameters is very good except in the region, around 400 nm, where the AlL^+ spectra and the HL^- spectra coincide. To the left of 400 nm, enhancement is observed, and to the right, quenching is observed. There is no systematic difference in fitting the quenching or enhancement data except that estimates of L_T from quenching yield values almost twice as large as those for enhancement.

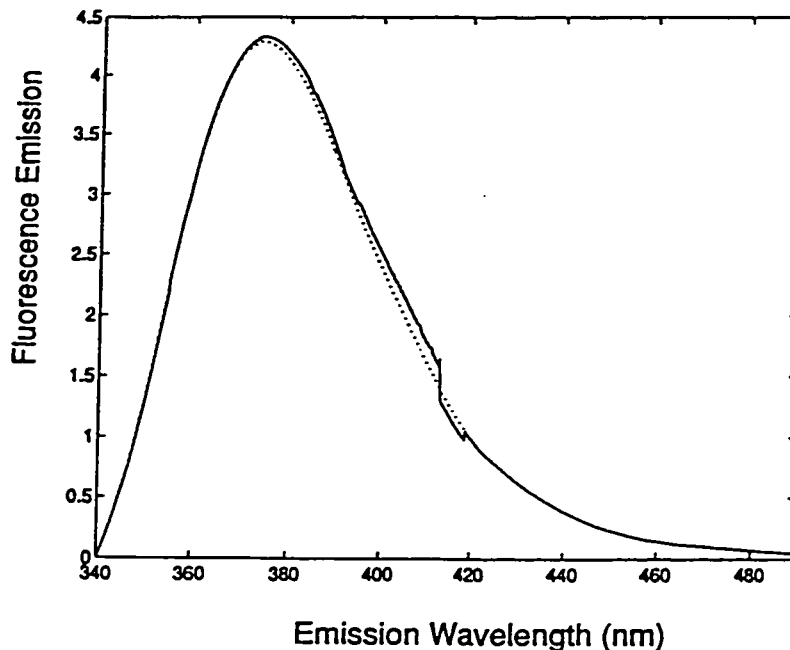


Figure 6 F_{ML} fitting results across the entire emission spectrum for salicylic acid titration with Al at pH 4.0. The dotted line corresponds to the maximum observed fluorescence during the titration and the solid line is the result of fitting Eq. [2] to the titration data.

B. SUWANNEE RIVER FULVIC ACID TITRATION

The excitation versus emission surface for Suwannee River fulvic acid is shown in Figure 7 as a surface plot and as a contour plot. It can be seen that there are two peaks, both around an emission wavelength of 440 nm and with an excitation wavelength of either 255 or 350 nm. The resolution of this plot is only every 10 nm for excitation so the exact location of the peaks on this surface cannot be determined. The surface was determined with higher resolution by Goldberg and Weiner (1994). They concluded that the two peaks are indeed two different fluorophores. The large peak at the 500-nm emission wavelength is an artifact from the instrument that is not reproducible and therefore cannot be background subtracted away. The small peaks around the 320-nm excitation wavelength are the Raman peaks for water.

Fluorescence increased with the addition of Al. Figure 8 shows the difference surface resulting from subtracting the fulvic acid spectrum from the spectrum with $0.059 \text{ mmol}\cdot\text{L}^{-1}$ total Al. There are two peaks, resulting from Al binding, in the

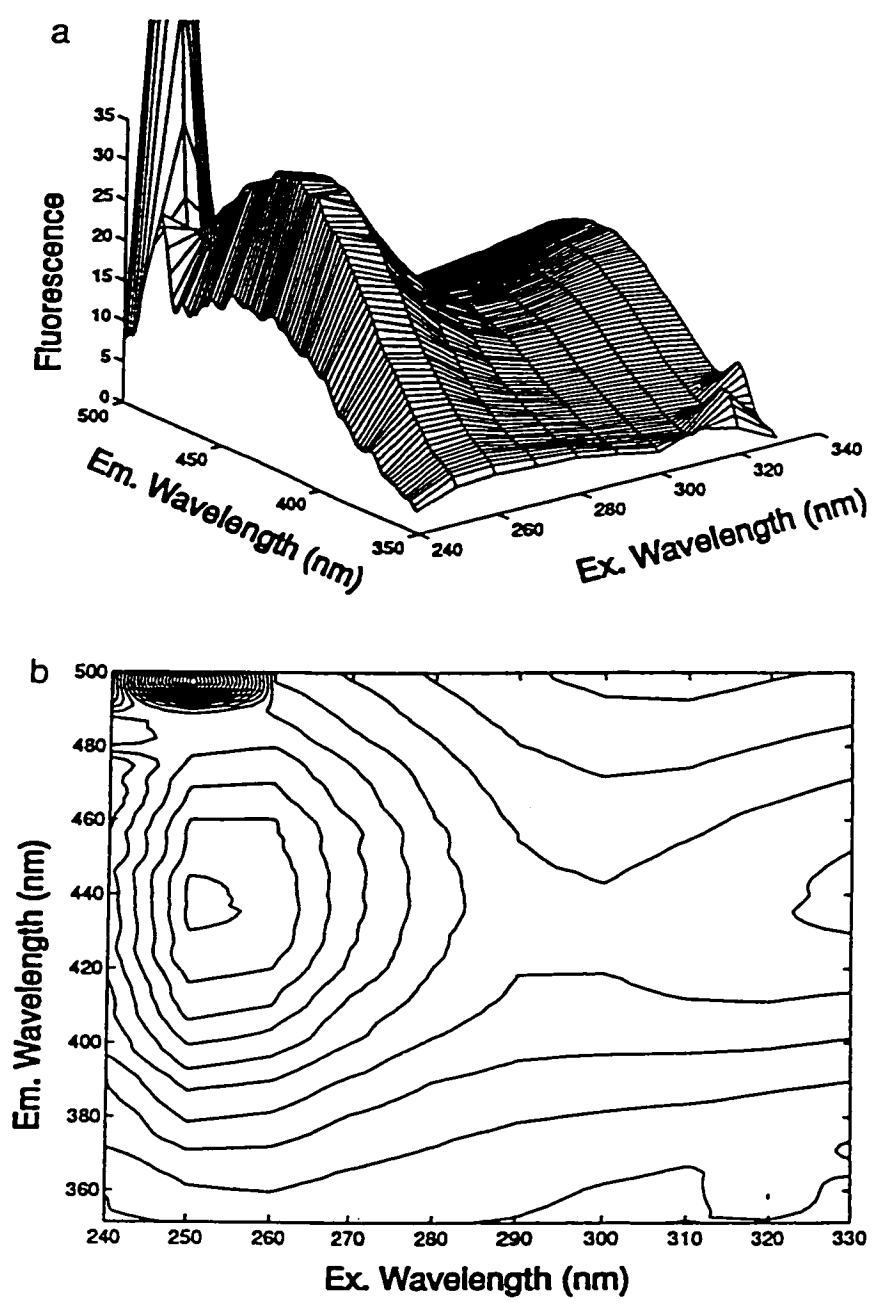


Figure 7 Excitation versus emission surface for Suwannee River fulvic acid at pH 4.0: (a) is a perspective drawing and (b) is a contour map.

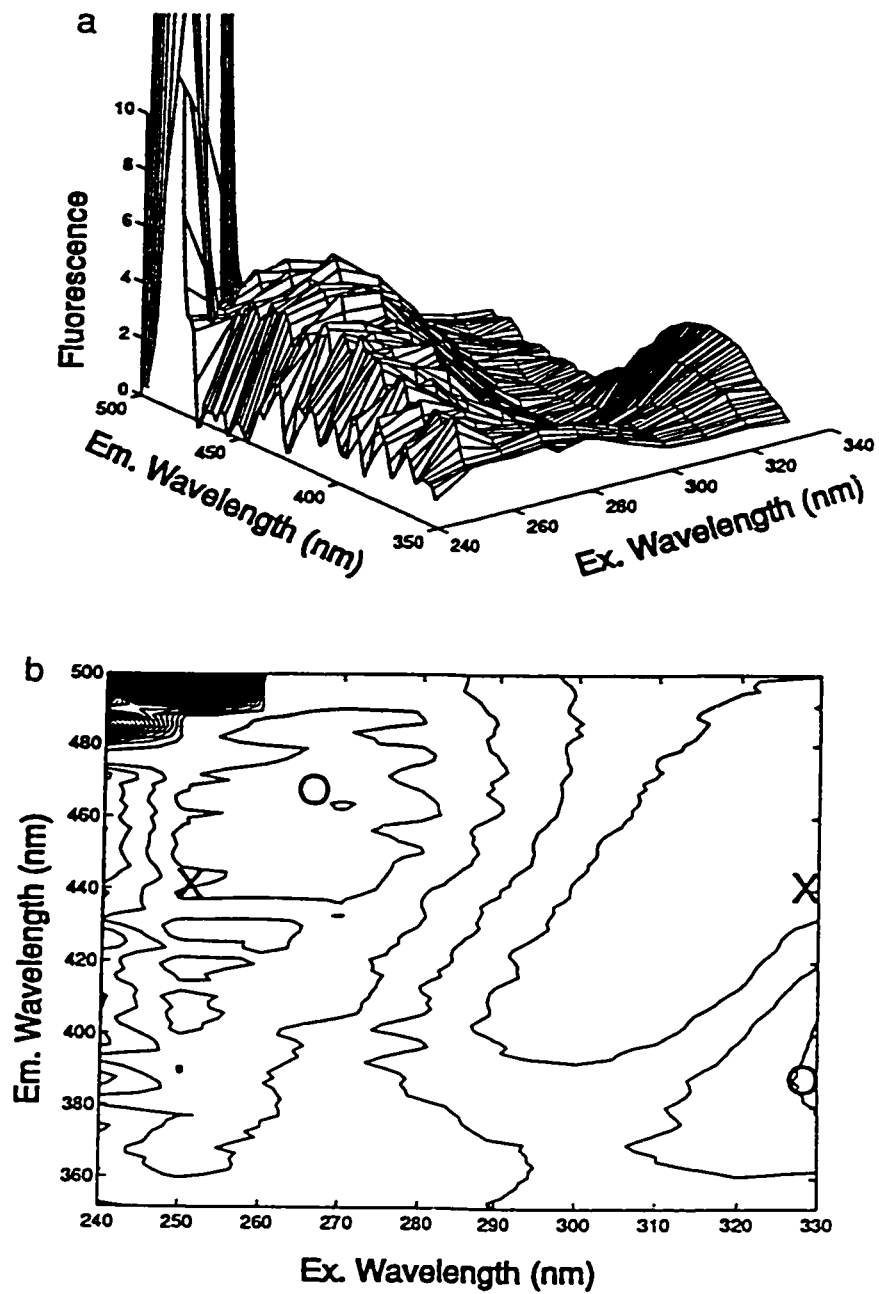


Figure 8 Excitation versus emission difference surface for Suwannee River fulvic acid with Al ($0.06 \text{ mmol}\cdot\text{L}^{-1}$) minus Suwannee River fulvic acid without Al at pH 4.0. Plot (a) is a perspective drawing and plot (b) is a contour map. In (b) the peaks in the fulvic acid spectrum (x) and in the difference surface (o) are indicated.

difference spectrum, one at the 270-nm excitation and 460-nm emission wavelengths, and one at the 330-nm excitation and 380-nm emission wavelengths.

C. FULVIC ACID EXCITATION AT 270 nm

The parameter fitting was performed over the entire set of emission spectra for excitation at 270 nm. The parameters are reported for the regions of maximum fluorescence increase in Table II; because no significant quenching was observed, only enhancement data were interpreted.

The logarithm of the conditional stability constants for reaction with deprotonated ligand, reaction (I), compare well for both Eq. [2] and Eq. [12], 5.7 versus 5.3, respectively, but Eq. [7] yields a value over an order of magnitude smaller. The agreement for the alternate reaction (reaction (II)) is just over an order of magnitude different, 2.6 versus 1.3 for Eq. [14] and Eq. [15], respectively, and is lower (0.4) for Eq. [8]. The fluorescence emission peak and intensity are essentially identical for both equations. The ligand concentration of $3.4 \mu\text{mol}\cdot\text{L}^{-1}$ corresponds to $0.43 \mu\text{mol}\cdot\text{mg}^{-1}$ of fulvic acid.

D. FULVIC ACID EXCITATION AT 330 nm

The parameter fitting was performed over the entire set of emission spectra for excitation at 330 nm. The parameters are reported in Table III, for the region of maximum fluorescence increase; because no significant quenching was observed, only enhancement data were interpreted.

The parameter fitting for the 330-nm excitation wavelength yielded the same values for all parameters within experimental uncertainty. The ligand concentration of $0.15 \mu\text{mol}\cdot\text{L}^{-1}$ corresponds to $0.019 \mu\text{mol}\cdot\text{mg}^{-1}$ of fulvic acid.

Table II
Summary of Results for Excitation at 270 nm
of Suwannee River Fulvic Acid

Equation ^a	Reaction (I) log K'	Reaction (II) log K'	F _{ML} (arbitrary units)	L _T ($\mu\text{mol}\cdot\text{L}^{-1}$)
2.14.E	5.7 ± 0.1	2.6 ± 0.1	23	3.4 ± 0.7
12.15.E	5.3 ± 0.1	1.3 ± 0.1	25	na
7.13.E	4.4 ± 0.9	0.4 ± 0.9	na	na

^aThe equation numbers refer to equations in the text, and E refers to enhancement measurements.

Table III
Summary of Results for Excitation at 330 nm
of Suwannee River Fulvic Acid

Equation ^a	Reaction (I) log K'	Reaction (II) log K'	F _{ML} (arbitrary units)	L _T ($\mu\text{mol}\cdot\text{L}^{-1}$)
2.14.E	5.3 \pm 0.3	1.3 \pm 0.3	17	0.15 \pm 0.05
12.15.E	5.3 \pm 0.3	1.3 \pm 0.3	17	na
7.13.E	4.9 \pm 0.9	0.9 \pm 0.9	na	na

^aThe equation numbers refer to equations in the text, and E refers to enhancement measurements.

V. CONCLUSIONS

The two fluorophores in Suwannee River fulvic acid reported by Goldberg and Weiner (1994) both react with Al, and their concentrations and stability constants can be estimated from TLS performed during a titration with Al at fixed pH. Information about these fluorophores, and salicylic acid for comparison, is reported in Table IV.

The conditional stability constant of both groups is very close to the value for salicylic acid. Therefore, it is likely that the binding sites are like the bidentate, 1-carboxyl-2-hydroxyl group in salicylate. The fluorophore labeled as site 2 is actually similar to salicylic acid in its fluorescent properties as well. The shift in wavelength on adding Al is the same for both salicylic acid and Suwannee River fulvic acid, but the fulvic acid is excited and emits light of 30 nm longer wavelength than free salicylate.

The values determined for log K' in this study, in the range 5.1 to 5.6, compare within a log unit with conditional stability constants obtained in other studies performed around pH 4.0. Pott *et al.* (1985), using a cation exchange method and

Table IV
Summary of Two Al Reactive Sites in Suwannee River Fulvic Acid

Ligand	No Al (ex/em) ^a	With Al (ex/em) ^a	log K' for reaction (I)	L _T ($\mu\text{mol}\cdot\text{mg}^{-1}$)
Site 1	250/440	270/450	5.6	0.43
Site 2	330/440	330/380	5.1	0.018
Salicylic acid	300/410	300/380	5.5	na

^aThe region of the excitation versus emission surface is indicated by the wavelength couple in nanometers.

Aldrich humic acid, obtained a log K' of around 6.8, and studies using bog water and a potentiometric method (Lövgren *et al.*, 1987) in the pH range 3.0 to 4.2 yielded a log K' between 4.1 and 4.7. The TLS study of Luster *et al.* (1996) resolved two sites in a juniper leaf litter extract at pH 5 with log K' values of 5.79 and 8.05. The lower strength site is similar in strength to salicylic acid but the other site is two orders of magnitude stronger than any sites observed in this study. The calculated L_T for Suwannee River fulvic acid can be accounted for within the total carboxyl group content of $6 \mu\text{mol}\cdot\text{mg}^{-1}$ (Leenheer *et al.*, 1995); this corresponds to 7.5% of the total carboxyl content being sites available for Al.

The modified equations derived in this study reinforce the Ryan–Weber equation's validity by obtaining similar values. The modified equations present no advantages over the usual equation, except modification for reaction (II) yields stability constants for reaction with protonated ligand. The Stern–Volmer equation modified for fluorescence enhancement yields K' values surprisingly consistent, within less than an order of magnitude, with those obtained using the Ryan–Weber equation. The usual Stern–Volmer equation applied to fluorescence quenching is not useful in Al–DOM systems because the data are not linear, and therefore do not yield reasonable results.

Fitting the three parameters in the Ryan–Weber equation across the entire emission spectrum for salicylic acid demonstrated that for one fluorophore, the fitting results do not depend on whether quenching or enhancement data are used as long as the fluorescences of the metal–ligand and ligand species do not coincide. It is important in multiple-fluorophore systems that the data for fitting are taken where the fluorescence is only influenced by changes in one site.

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Appendix II:

Multi-site aluminum speciation determined using multiresponse fluorescence data

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Multi-site aluminum speciation with natural organic matter using multiresponse fluorescence data

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Abstract

A revised method for determining conditional binding constants and site concentrations for Al and natural organic matter (NOM) is presented. By using total luminescence spectroscopy and multiresponse parameter estimation, the method is capable of resolving Al complexation at discrete sites within NOM. The method is tested on a mixed model ligand system of salicylic acid and 2-hydroxy-3-naphthoic acid and recovers known values for stability constants and ligand concentrations. The method is also applied to a sample of filtered river water at pH 4.02 and reveals three sites with conditional stability constants with log values of 5.77, 5.56 and 5.81 and standard deviation of 0.09. In addition, for the same three sites the densities are determined to be 0.427, 0.448 and 0.354 $\mu\text{mol mg}^{-1}$ of dissolved organic carbon (DOC), with a standard deviation of 0.14 $\mu\text{mol mg}^{-1}$ of DOC. © 1998 Elsevier Science B.V.

1. Introduction

Aluminum binding to natural organic matter (NOM) is very important in many geochemical systems. For example, Al is mobilized by NOM in the weathering of aluminosilicate rocks [1], and Al toxicity can be reduced by complexation with organic matter [2]. Aluminum toxicity and reactivity is species dependent [3,4]. Unfortunately, experimental determination of Al speciation in natural waters, as reviewed by Clarke et al. [5], is difficult because NOM is a complex mixture of potential binding sites [6,7]. A simple method capable of resolving Al interactions at discrete sites within NOM, is required. This method should also be sensitive and not modify the substrate.

Total luminescence spectroscopy satisfies all of these criteria [8].

Total luminescence spectroscopy is a map of fluorescence versus both excitation and emission wavelengths, and is well suited for site specific speciation because it can be used to resolve different fluorophores within NOM. It has been used to characterize NOM from such diverse sources as: the Black Sea [9], Antarctica water fractions [10], leaf litter extracts, [11,12], coral extracts [13], and Suwannee river fulvic acid [14]. Traditionally, changes in a single wavelength, emission spectra or synchronous fluorescence spectra have been used to investigate metal complexation by NOM. The selectivity of fluorescence measurements are increased by looking at both excitation and emission simultaneously [15]. Examination of the total luminescence surface gives a more complete picture of metal–NOM interactions. Changes in the fluorescence surface on addition of metal have been

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used to characterize metal–NOM species [11], and changes during titrations with Cu and Al at fixed pH have been used to determine binding parameters for those metals with leaf litter extracts [8].

Fluorescence changes, either enhancement or quenching, during a titration of NOM with metal are usually fit to the Ryan–Weber equation [16]. Values for the conditional stability constant and ligand concentration obtained in this manner are only valid for complexation with one ligand, as pointed out by Ryan and Ventry [17]. The equation works well for one ligand–one metal systems, such as Cu and tyrosine [16], or Al and salicylic acid [14], but often gives low values for ligand concentrations when applied to NOM samples [18]. Difficulties in fitting data from NOM titrations to the Ryan–Weber equation probably result by imposing a one component model on a multi-component system.

In order to fit multi-response fluorescence data, previous work [8,14,16], has assumed that the simultaneous metal–ligand systems in NOM are independent. This is not true, because different sites may compete for the available metal. In order to work on complex, heterogenous natural samples, the existing fluorescence methodology has to be modified to take into account multiple ligands. This paper modifies the approach of Ryan and Weber to allow one metal to interact with multiple ligands. In order to perform the fitting operation, it is necessary to use multiple responses and fit them simultaneously to a multi-site speciation model. The theory of fitting multiresponse data is taken from Box and Draper [19], and has recently been reviewed by Stewart et al. [20]. The method proposed here is tested with a system of model ligands and Al. The model ligands are salicylic acid and 2-hydroxy-3-naphthoic acid because they fluoresce and contain an *ortho*-hydroxy benzoic acid group, which is the type of site often invoked to explain metal complexation by NOM [21]. In addition, the validated method is applied to a sample of river NOM and its complexation by Al.

2. Theory

2.1. Multi-site Ryan–Weber equation

The mathematical definition of this problem is analogous to that given by van Stokkum et al. [22],

to fit kinetic parameters to the time evolution of multi-component fluorescence spectra. In this study, equilibrium parameters are used to define the system, and total metal is the independent variable. The fluorescence at any excitation and emission wavelength pair is a function of the concentrations of the fluorescent species. If the fluorescence is assumed to be linear, which is a good assumption in dilute solution [23], then the fluorescence as a function of total metal, M_T , can be written:

$$f(M_T) = \sum_{i=1}^p \sum_{j=1}^m (\epsilon_j C_{ij} + \xi_{ij}) \quad (1)$$

where there are p additions of metal, and m different fluorescent species with proportionality constants ϵ_j and concentration C_{ij} . The term ξ_{ij} includes all of the experimental variation, which is assumed to have a Gaussian distribution. This equation can be rewritten in matrix form as:

$$F = CE^T + \Xi \quad (2)$$

where F is a $p \times q$ matrix containing a column of length p for q different sets of fluorescence observations, C is a $p \times m$ matrix with a column of concentrations for each of the m fluorescent species, and E^T is a $m \times q$ matrix containing a proportionality constant for each species at each set of observation wavelengths. The error is contained in a matrix the same size as F and is given the symbol Ξ . The calculations are simplified by assuming that wavelength pairs can be found, for each site, at which fluorescence depends only on speciation at that site. The fluorescence at site n , of a total of N sites, can now be written:

$$F_n = C_n(\theta)E_n^T + \Xi_n \quad (3)$$

where the matrices have the same meaning and dimensions as in the general form of the equation Eq. (2), except q is now equal to one. The problem then becomes fitting N simultaneous equations of the form given in Eq. (3) to parameters that describe the speciation given in each of the C_n matrices. The parameters are common to all N sets of observations, and are collected in the vector θ . The parameters include N conditional stability constants and N ligand concentrations. The conditional stability constants, K'_n , are defined as follows:

$$K'_n = \frac{[ML'_n]}{[L'_n][M'_n]} \quad (4)$$

where, $[ML'_n]$ is the sum of the concentrations of all metal-bound species at site n , $[L'_n]$ is the sum of the concentrations of free site n , and $[M']$ is the sum of the concentrations of all metal species not bound by any of the N sites. Experiments are performed at fixed pH; therefore, acidity constants can be ignored. For N equal to one and m equal to two, Eq. (3) is the Ryan–Weber equation before substitution of a quadratic expression for $[ML'_n]$.

Following the approach of van Stokkum et al. [22], the QR decomposition of $C(\theta)$ is performed in order to remove the linear parameters contained in E^T :

$$C(\theta) = [Q_1(\theta)Q_2(\theta)] \begin{bmatrix} R_1(\theta) \\ 0 \end{bmatrix} = Q(\theta)R(\theta) \quad (5)$$

where $Q_1(\theta)$ and $Q_2(\theta)$ are $m \times 1$ and $m \times (m-1)$ matrices, respectively, which together form the orthogonal matrix $Q(\theta)$. $R_1(\theta)$ is the upper triangular matrix portion of the matrix $R(\theta)$. Using the QR decomposition of the speciation matrix a residual vector can be calculated [22]. The residuals for each n set of observations of fluorescence are:

$$Z_n(\theta) = Q_{2n}(\theta)Q_{1n}(\theta)^T F_n \quad (6)$$

The N residual vectors are combined into a residual matrix, Z , in such a way that each column of the matrix corresponds to the residuals for one of the N responses. The error, to be minimized, is then defined as $\det(Z^T Z)$, according to the proper Bayesian estimate of error in multi-response systems, as given by Box and Draper [19].

2.2. Additional relationships

The nonlinear parameters in θ are correlated with each other and with the linear parameters contained in E^T . This can lead to problems in fitting; for example, a high proportionality constant can compensate for a low ligand concentration. In fact, simulated data showed that the global minimum often occurred at ligand concentrations ten orders of magnitude lower than the actual values. To avoid this problem, an additional assumption is invoked. If it is assumed that the fluorescence proportionality constants, k_n , for the ML_n species are equal, then only one ligand concentration must be fit and the rest can be determined from the ratios of the end member fluorescence.

The proportionality constants, k_n , are given by the ratio of the end member fluorescence, I_{ML_n} , over the total ligand concentration, L_{Tn} [16]. Thus, for a fitted value of L_{Tn} the other ligand concentrations are determined as:

$$L_{T_n} = L_{T_{r,n}} \left(\frac{I_{ML_n}}{I_{ML_{r,n}}} \right) \quad (7)$$

2.3. Wavelength selection

In order to use Eq. (3) it is necessary to select excitation and emission wavelength pairs at which fluorescence changes are dependent upon speciation changes at only one site. In previous work [14], it was found that as long as changes in fluorescence are significant relative to noise, there is no wavelength dependence in the fitted values using the Ryan–Weber equation for one ligand. Therefore, if regions of the fluorescence surface can be found where only one site fluoresces, then any wavelength pair where significant changes occur, can be used in parameter fitting. In this work, wavelengths were chosen where the largest fluorescence differences occurred upon metal addition and it is assumed that widely different excitation and emission pairs represent different fluorophores.

3. Experimental

3.1. Materials and methods

Two sets of experiments were performed: a model ligand study and a NOM study. The model ligand study consisted of titrating a solution of both salicylic acid and 2-hydroxy-3-naphthoic acid with Al, and the NOM study involved titrating filtered water obtained from the Moose river in northern Wisconsin. In both cases fluorescence was measured versus total added Al.

Initial measurements of the fluorescence surfaces with and without Al were used to determine which wavelength pairs to monitor during titrations. The surfaces were measured by taking emission scans at every 10 nm of excitation. Initial studies indicated that at least two hours were necessary to achieve equilibrium in the model ligand system, therefore the solu-

tions were prepared in a batch manner and measured after equilibration overnight. The NOM sample achieved equilibrium in less than 15 min, so titrations of NOM were performed using a peristaltic pump to introduce the sample to the fluorometer. Titrations were performed at fixed pH and ionic strength. For the model ligands, salicylic acid and 2-hydroxy-3-naphthoic acid, the pH was 4.27 ± 0.02 ; for the river NOM sample the pH was 4.02 ± 0.02 . In both cases the pH was adjusted using dilute nitric acid and sodium hydroxide as necessary. The ionic strength was 0.01 mol l^{-1} , and was buffered using potassium nitrate for the model ligand study. The ionic strength for the NOM study was approximately 0.0001 mol/l calculated from the measured concentrations of the dominant cations (Ca and Mg) in the actual sample. In both studies the samples were contained in a temperature bath at 23°C – 24°C . Total Al was titrated in the range from 0 to $110 \mu\text{mol/l}$ and from 0 to $30 \mu\text{mol l}^{-1}$ for the model ligands and NOM sample respectively.

Salicylic acid was obtained from Fisher Scientific and purified by recrystallization from methanol/water. The naphthoic acid was obtained from Sigma Chemical and used without further purification. The total ligand concentration for salicylic acid was $19.19 \mu\text{mol l}^{-1}$ and for 2-hydroxy-3-naphthoic acid it was $15.73 \mu\text{mol l}^{-1}$. The NOM sample was obtained from the Moose river in northern Wisconsin at the start of summer in 1996, stored at 4°C and analysed within a month of its collection. The sample was filtered in the field using tangential ultrafiltration and a Millipore Prep-Scale TFF 10K nominal molecular weight polyethersulfone cartridge.

The fluorescence was measured using a Perkin-Elmer MPF-44 spectrofluorometer for the model ligand experiments, and a SLM-Aminco SPF-500C spectrofluorometer for the NOM experiments. In order to obtain a signal from the dilute solutions, both the excitation and emission slits were set at 20 nm, improving instrument sensitivity. Spectra are reported uncorrected. Experimental uncertainty in model ligand experiments was determined from three repeat experiments, but for the NOM samples only one experimental run was possible because the sample fluorescence changed over time. The uncertainty for the NOM titration is estimated from the variability about the best fit line.

3.2. Data analysis

Nonlinear regression analysis was performed using code written using Matlab™ (The MathWorks). The Nelder–Mead simplex algorithm was the fitting method used [24]. The fitting operation ended when subsequent steps had a relative difference less than or equal to 0.01% for both the parameter vector and the error value. In applying Eq. (7), it was necessary to step iteratively towards the solution using the previous answer to determine the new ratios of end member fluorescence. This was performed until all of the ratios differed from the previous ones by less than 0.01%. The 0.01% level was selected as the convergence criterion because it is the minimum relative error observed in the fluorescence measurements. In applying the fitting methodology, it does not make a difference which ligand concentration is fit and which is determined using Eq. (7). The required speciation calculations for systems with three or more ligands were performed using the iterative method of Perrin and Sayce [25], which was also programmed within the Matlab environment. For two ligands, the speciation calculations were faster when the system was solved explicitly using a cubic equation.

A pseudo Monte-Carlo method was used to estimate uncertainty, according to the method described by Press et al. [26]. Random error equal to the estimated experimental noise was added to each point in the best fit curve and this new simulated data set was fit to the same parameters. This was repeated 1000 times to get a statistically meaningful distribution of results. For the model ligand data the standard deviation was estimated from repeat measurements to be 0.000025, corresponding to 0.1% of the maximum observed fluorescence. For the NOM data the standard deviation was estimated as 0.015, corresponding to 0.3% of the maximum observed fluorescence.

4. Results and discussion

4.1. Model ligand studies

The fluorescence surface for salicylic acid and 2-hydroxy-3-naphthoic acid, with 100 times excess Al at pH 4.27, is shown in Fig. 1. Speciation calculations indicate that greater than 90% of both ligands were

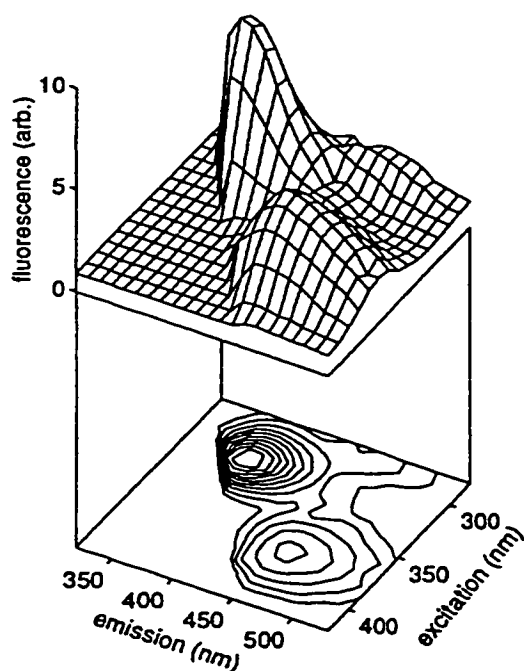


Fig. 1. Fluorescence surface for the Al complexes of salicylic acid and 2-hydroxy-3-naphthoic acid. Aluminum is in excess of the ligands by 100 times and the pH is 4.27.

complexed. This surface shows that the greatest increase for salicylic acid occurs at 317 nm excitation and 375 nm emission. For 2-hydroxy-3-naphthoic acid the maximum is at 372 nm excitation and 455 nm emission. These were selected as the wavelength pairs to monitor during Al titrations. Contributions to the fluorescence at one site from species at the other fluorophore are negligible.

Titration results for the model ligand system, along with the fitted curves, are shown in Fig. 2. The fluorescence increases with addition of Al for both sites.

The curves look like other reported enhancement curves [8], except for the initial S-shaped portion of the curve for 2-hydroxy-3-naphthoic acid. Fitting was performed using N equal to two. For both sets of observations m is equal to two, representing free and bound ligand, and p is equal to 16. The results of Monte-Carlo simulations on these fitted results are shown in histograms in Fig. 3; with true values indicated by highlighted bars. The calculated distribution of parameter values include the true values for all four fitted parameters. The 'true' conditional constants were calculated using thermodynamic constants from Öhman and Sjöberg [27] for salicylic acid and from Martell and Smith [28] for 2-hydroxy-3-naphthoic acid. A summary of these results is shown in Table 1.

The fitting method recovers the ligand concentrations and stability constants well; the true values are essentially within one standard deviation of the Monte-Carlo simulation results for all four parameters. If the traditional Ryan-Weber equation is used to fit the two sets of data independently the parameter values obtained do not recover the true values. The ligand concentrations are overestimated by 7% and 313% for salicylic acid and 2-hydroxy-3-naphthoic acid respectively. The stability constants are underestimated by 45% and overestimated by 298% for salicylic acid and 2-hydroxy-3-naphthoic acid respectively. The curve for 2-hydroxy-3-naphthoic acid is particularly difficult for the Ryan-Weber equation to fit as the equation does not have the flexibility to recover the 'S' shaped portion of the curve that is observed at low total Al.

4.2. River NOM studies

Fig. 4 shows the total luminescence surface for the NOM sample without Al and the enhancement surface

Table 1
Comparison of fitted parameters to true values for model ligand titration

Parameter ^a	'True value'	Experimental			
		Mean	Median	Standard deviation	Range
L_T for SA ($\mu\text{mol l}^{-1}$)	19.19	19.1	19.1	1.5	14.1–22.8
L_T for NA ($\mu\text{mol l}^{-1}$)	15.73	16.5	16.5	1.2	12.2–20.2
$\log K'$ for SA	5.286	5.20	5.20	0.08	4.96–5.51
$\log K'$ for NA	4.724	4.79	4.79	0.07	4.53–5.04

^a SA refers to salicylic acid and NA refers to 2-hydroxy-3-naphthoic acid.

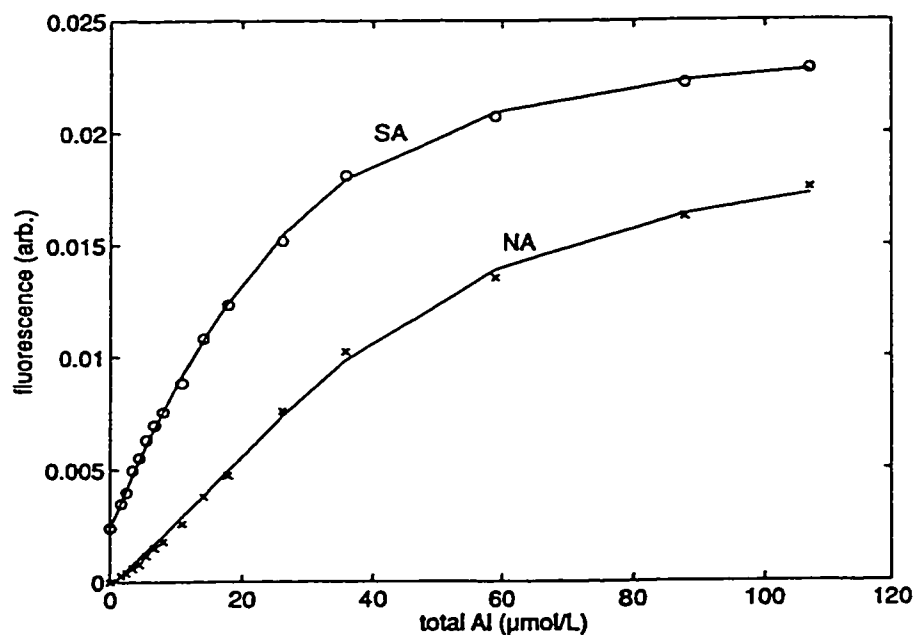


Fig. 2. Titration curve for mixed ligand system with Al. The solid lines represent best fit results and the points represent one set of experimental observations. The X's and O's correspond to responses for 2-hydroxy-3-naphthoic acid (NA) and salicylic acid (SA) respectively.

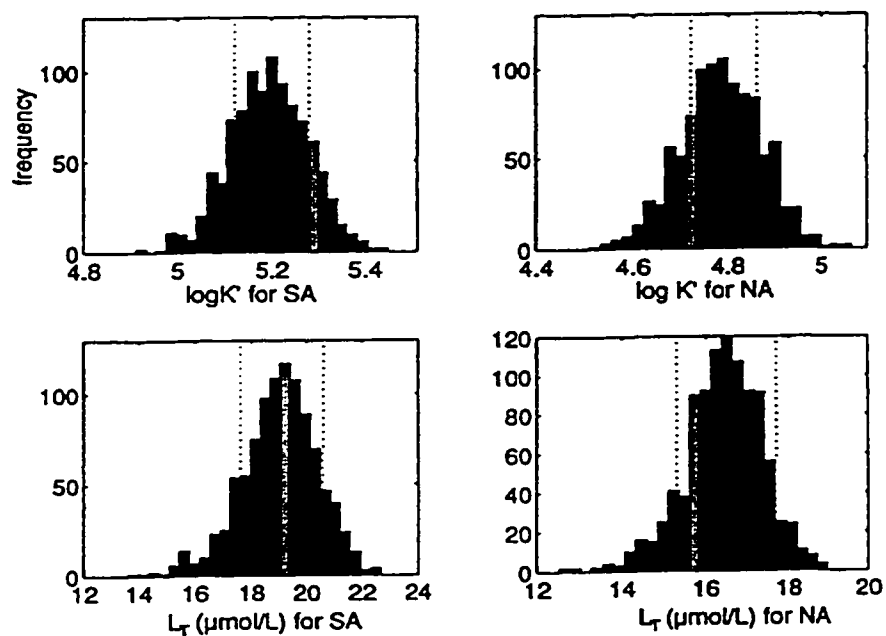


Fig. 3. Histograms showing results of 1000 Monte-Carlo simulations for mixed model ligand titrations with Al. The true values for the four parameters are indicated by highlighted bars, and one standard deviation about the simulation results are indicated by dotted lines. Salicylic acid and 2-hydroxy-3-naphthoic acid are symbolized by SA and NA respectively.

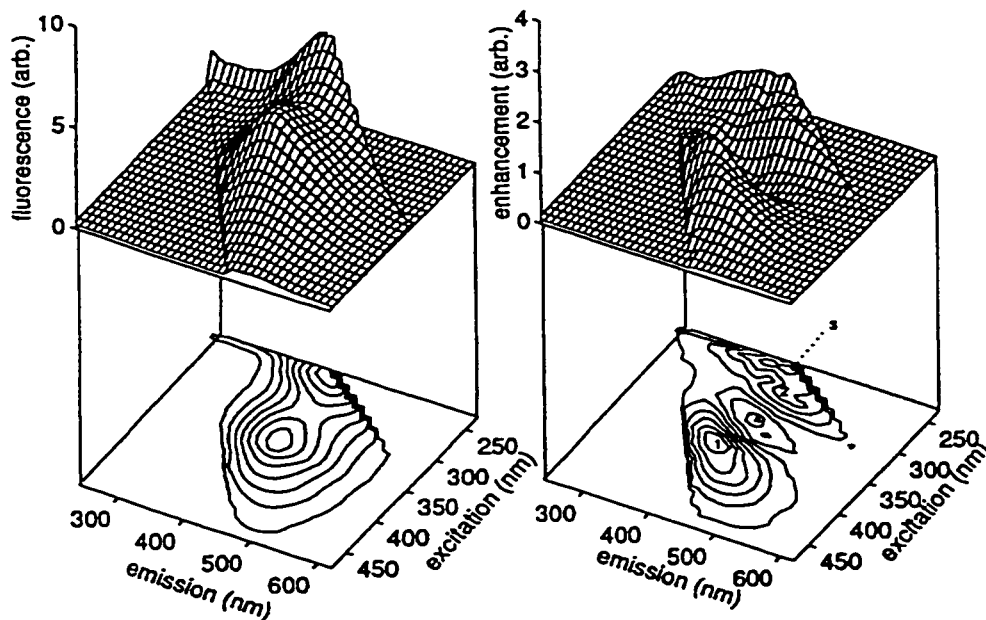


Fig. 4. Moose river fluorescence (a) without Al, (b) enhancement after addition of 0.74 mmol/l total Al at pH 4.02. Enhancement was determined by subtracting the surface without Al from the surface with Al. The four extrema are indicated by numbers.

for addition of $74 \mu\text{mol l}^{-1}$ Al. There are four extrema on the difference plot, obtained by subtracting the surface without Al from the surface with Al. The extrema are labelled 1, 2, 3 and 4 on the contour map. Regions 1, 2, and 3 represent peaks, and region 4 is a valley. The peaks for regions 1, 2 and 3 were selected to represent three distinct sites within the sample, but valley 4 is a result of increases in region 1 and 2 and was therefore not included as a distinct site. The valley is not a result of a distinct site because Al does not quench fluorescence. Observed quenching in Al titrations is a result of new fluorescent Al-species having different spectra from the unbound ligand. These new spectra may have reduced intensity at certain wavelengths, which results in apparent quenching.

The uncomplexed peaks occur at excitation/emission wavelength pairs of 350/420 and 240/425 nm. The peaks in the difference spectra are at 355/420, 270/430 and 240/410 nm. The fluorescence of the free ligand is within the range of values generally observed for humic and fulvic acids [14,29], and two peaks are generally observed in total luminescence surfaces for fulvic acids. There are fewer data values available to compare fluorescence wavelength changes upon Al

binding. A peak at 340/450 observed by Provenzano and Sposito [12] upon addition of Al to pine litter extracts is similar to site 1 fluorescence at 355/420, but Luster et al. [18] report peaks at 420/440 and 515/565 that are not similar to anything observed in this sample.

Titration curves for the sample are shown in Fig. 5. The results of these fitting operations are included in Table 2. The fitting was performed with N equal to three, and for each site m was equal to two and p was equal to 17.

The conditional stability constants for the NOM samples are higher than values observed for salicylic acid and 2-hydroxy-3-naphthoic acid. The lowest stability constant for the NOM has a log value of 5.75, but salicylic acid has a value of 5.02 at the same pH of 4.02, and ionic strength of $0.0001 \text{ mol l}^{-1}$. The values here fall within the range of 4.1–6.8 of $\log K'$ values reported in the literature for Al binding by NOM at pH around four [30,31]. The three stability constants are almost identical, however, making it seem likely that they are similar binding sites, but because the fluorescence is different the functional groups must be attached to different fluorescent moieties. The calculated site densities are similar to each other as well;

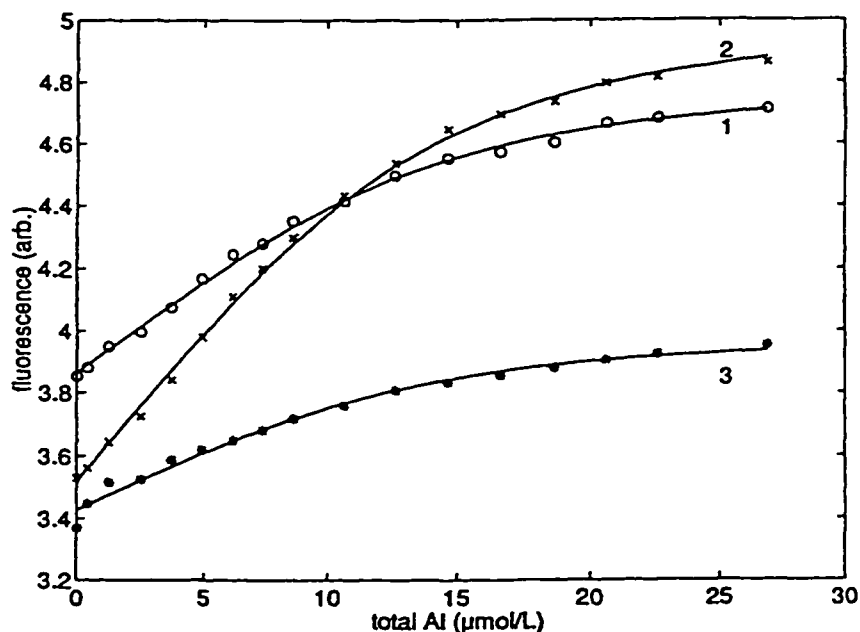


Fig. 5. Titration curves for three fluorescent sites in NOM sample, versus total Al at pH 4.02. The lines indicate best fit results and the symbols represent one set of data. The numbers correspond to the same numbers as in Fig. 4(b), and represent three distinct sites.

this is reasonable based on the fluorescence being similar for each site. In fact, the relative order of the concentrations is the same as the order of the final fluorescence measurements. The dissolved organic carbon for the sample is 10.47 mg l^{-1} and the site densities can be recalculated per mass of organic carbon as 0.427 , 0.448 and $0.354 \text{ } \mu\text{mol mg}^{-1}$ for sites one, two and three respectively. These values can be compared with values for total carboxylic content, which is the likely binding site for Al. The site concentrations determined here can easily be reconciled within the typically reported 1.73–

$3.00 \text{ } \mu\text{mol}$ of total carboxylic content per mg of aquatic humus. [32]

The generalized Ryan–Weber equation, for multiple ligands and multiple observations recovers known values for the model system of salicylic and 2-hydroxy-3-naphthoic acid, and yields reasonable values for an actual sample of NOM. This method has been applied specifically to Al–NOM systems in this paper, but is of general applicability and could be tested for any metal–fluorescent ligand system, such as Cu, Ag or As and NOM, whether quenching or enhancement is observed.

Table 2
Fitted parameters for NOM sample

Parameter ^a	Mean	Median	Standard deviation	Range
$L_{T1} (\text{ } \mu\text{mol l}^{-1})$	4.47	4.48	0.15	4.02–4.84
$L_{T2} (\text{ } \mu\text{mol l}^{-1})$	4.69	4.69	0.15	4.25–5.05
$L_{T3} (\text{ } \mu\text{mol l}^{-1})$	3.71	3.72	0.14	3.29–4.05
$\log K'_1$	5.76	5.76	0.09	5.43–6.07
$\log K'_2$	5.75	5.76	0.09	5.46–6.02
$\log K'_3$	5.79	5.81	0.09	5.59–6.07

^a The subscripts refer to the site number, as indicated in Figs. 4 and 5.

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Appendix III:

**Aluminum interactions with natural organic matter determined using multiresponse
fluorescence**

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FLUORESCENCE ANALYSIS FOR MULTI-SITE ALUMINUM BINDING TO NATURAL ORGANIC MATTER

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Natural organic matter (NOM) samples isolated from different water sources in Norway were compared using their fluorescence properties. Fluorescence surfaces were observed at pH 4.36 and deconvoluted using SIMPLISMA (Windig and Guilment 1991). There were a total of seven different fluorophores observed for these samples and each sampling site had between four and six of the fluorescent components. These components were observed to bind Al during titrations at the same pH. Multiresponse titration curves were fit using the method of Smith and Kramer (1998) and most of the binding strengths are similar to values for Suwannee River fulvic acid ($\log K'$ between 4.8 and 5.5), but there are strong sites ($\log K' = 7$) and weak sites ($\log K'$ between 3 and 4) also observed. Results depended on the isolation method used; reverse osmosis and low pressure evaporation yielded different values but with no consistent trends. ©1999 Elsevier Science Ltd

INTRODUCTION

Natural organic matter (NOM) is an important ligand for metals in aqueous systems. The determination of metal binding constants with NOM is difficult because NOM is a complex, heterogeneous mixture of potential binding sites. Often, metal binding constants are determined by titration of NOM with metal. The result is a titration curve that can be fit to a speciation model. This method is limited in that a single curve is used to determine all of the speciation parameters. The resolution of the method can be improved if multiple titration curves are fit simultaneously. This paper utilizes a multiresponse fluorescence method to determine binding constants for aluminum with NOM samples isolated as part of the NOM-typing project.

Fluorescence is ideally suited for fitting multiple titration curves for metal-NOM interactions. This is because wavelengths can be selected from the excitation vs. emission fluorescence surface that correspond

to single components. The result is component-specific speciation. In previous work (Smith and Kramer 1998; Luster et al. 1996), these wavelengths were arbitrarily selected based on maximum fluorescence change. In this paper, spectral deconvolution is used to define a minimum number of components and their position. This is accomplished using SIMPLISMA (Windig and Guilment 1991), which has the added advantage that it yields spectral information about the components. The binding sites can be compared to model compounds in terms of binding strength as well as excitation and emission wavelengths.

It is important to understand Al complexation by NOM because Al is a potentially toxic metal (Nieboer et al. 1995; Marschner 1995; Cronan and Grigal 1995), but the toxicity of Al is speciation-dependent (Parent et al. 1996). Aluminum is of particular interest because environmental acidification increases the amount of

available Al due to increased solubility of Al minerals at lower pH. In addition, Al is often added as alum (aluminum sulphate) during drinking water treatment, and could still complex with residual NOM.

EXPERIMENTAL METHOD

NOM samples were obtained as part of the international NOM-typing project (Chairman E. Gjessing, Agder College, Norway); both reverse osmosis (RO) and low temperature vacuum evaporative residues (EV) isolates were studied. A description of the sampling sites and basic characteristics of the raw waters as well as the isolates is given by Gjessing et al. (1998). In this paper, the samples will be designated by isolation method (RO or EV) and by sampling site number, where the numbering scheme for the May (1996) samples is as follows: Trehørningen is NOM-1, Hellerudmyra is NOM-2, Aurevann is NOM-3, Maridalsvann is NOM-4, Birkenes is NOM-5, Humex is NOM-6, Gjerstad (limed) is NOM-7, Gjerstad (unlimed) is NOM-8, and for October (1996) Hellerudmyra is referred to as NOM-9. In addition, Suwannee River fulvic acid is referred to as NOM-10. The samples were obtained in powdered form and solutions were diluted to the required volume using MilliQ water ($> 18 \Omega$). Suwannee River fulvic acid was obtained from the International Humic Substance Society which used isolation by adsorption on XAD resins. Aluminum stock solutions were prepared from aluminum nitrate. All glassware was cleaned using a 10% nitric acid bath overnight, followed by repeated rinses with MilliQ water.

The fluorometer used was a Perkin-Elmer MPF-44. The excitation and emission monochrometers were automated and computer-controlled. Data acquisition was computer-controlled and an entire surface could be acquired in 2 h. The fluorometer was operated in ratio mode to correct for variations in excitation source intensity. The excitation and emission slits were both set at 20 nm to increase sensitivity for working with dilute solutions. The sample cell was replaced with a Bausch and Lomb UV-transparent cuvette of sufficient volume (60 mL) to allow titrations to be performed directly in the path of the light. Magnetic stirring was performed throughout the titrations. This setup was found to yield the same results as a traditional quartz cell, but had the added advantage of not needing a pump to introduce the sample to the cuvette. The pH electrode could be placed directly in the sample while fluorescence was measured. A Cole-Palmer glass combination electrode was used for pH measurements.

Initial experiments involved measuring the excitation vs. emission fluorescence surface for the RO samples. This was done at a pH of 4.36 ± 0.03 for each sample; pH was adjusted, after each addition of titrant, with dilute nitric acid or dilute sodium hydroxide as necessary. The ionic strength was set at 0.1 mol/L using potassium nitrate. The isolate concentration was selected to yield a final dissolved organic carbon concentration around 5 to 10 mg C/L. At these concentrations, inner filter effects were negligible, except absorbance of KNO_3 (around 250 nm) was corrected using the method of Tucker et al. (1992). Surfaces were obtained by measuring every 10 nm in both excitation and emission in the range 250-500 for excitation and 380-650 for emission. The fluorescence of the sample with 50 $\mu\text{mol/L}$ additional aluminum was also measured at the same pH.

After the RO fluorescence surfaces were measured, they were deconvoluted using SIMPLISMA as described below. Fluorescence surfaces were not measured or deconvoluted for EV samples because Blaser et al. (1999) found no significant differences between fluorescence surfaces for EV vs. RO samples. Wavelengths were selected corresponding to each component and these wavelengths were monitored during a titration of aluminum at pH 4.36 ± 0.03 . These wavelengths were selected at maximum fluorescence for each component. In general, three repeat measurements were obtained for each addition of Al and at least 10 titration points were recorded in the range of 0 to 100 $\mu\text{mol/L}$ added Al. After each addition of titrant, the pH was adjusted using dilute acid and base as needed. An equilibration time of fifteen minutes was allowed between additions of titrant. Preliminary studies on these samples showed that fluorescent changes after this time are negligible. The titration was halted when the titration curves were observed to be flattening. The initial concentration of aluminum was taken from total aluminum concentrations measured by Gjessing et al. (1999).

DATA ANALYSIS

All data analysis was performed using Matlab™ (The MathWorks, MA, USA). Descriptions are given below for the spectral deconvolution method for fluorescence surfaces and for the multiresponse parameter fitting method used.

Spectral deconvolution

For most fluorophores, the shape of the excitation and emission spectrum is independent of emission and

excitation wavelength, respectively (Guilbault 1973). If this is assumed to be true, then spectral deconvolution can be performed on fluorescence excitation vs. emission surfaces in order to isolate the components. This has in fact been done for mixtures of polycyclic aromatic hydrocarbons (Dalibart 1997) using factor analysis. To the authors' knowledge, this has never been done to NOM fluorescence surfaces before.

Spectral deconvolution was performed using the SIMPLISMA method of Windig and Guilment (1991). This method involves selecting pure variables that are proportional in intensity to only one component of the mixture. Once this is done, a matrix equation is solved to determine the pure spectra and concentrations of each component. In the SIMPLISMA package, this is done using least squares fitting, which can result in negative concentrations. This problem was addressed by modifying the SIMPLISMA code so that constrained non-negative least squares optimization, as implemented by Matlab (Grace 1992), was used to solve the matrix equation.

In order to deconvolute the surfaces, the Rayleigh lines corresponding to scatter at the excitation wavelength and twice the excitation wavelength had to be removed. This was accomplished using a cubic spline fit to replace fluorescence observations within the Rayleigh line. This was done to allow input into SIMPLISMA, because SIMPLISMA can not handle data with missing observations. Interpolated values were never used in parameter fitting.

Multiresponse parameter fitting

The fitting method used was essentially the method of Smith and Kramer (1998), but with some modifications. This method is basically a multiresponse version of the traditional Ryan-Weber equation (Ryan and Weber 1982). The fluorescence at any excitation and emission wavelength pair is a function of the concentrations of the fluorescent species. If the fluorescence as a function of fluorophore concentration is assumed to be linear, which is a good assumption in dilute solution (Guilbault 1973), then the fluorescence at any excitation/emission wavelength pair, as a function of total metal, M_T , can be written:

$$f(M_T) = \sum_{j=1}^m (e_j C_j(M_T)) + \xi \quad (1)$$

for m different fluorescent species with proportionality constant e_j and concentration C_j , where C_j is a function of M_T . The term ξ includes all the experimental variation, which is assumed to have a Gaussian distribution. This equation can be rewritten in matrix form for p additions of metal as:

$$F = CE + \Xi \quad (2)$$

where,

F is a $p \times q$ matrix containing a column of length p for q different sets of fluorescence observations;

C is a $p \times m$ matrix with a column of concentrations for each of the m fluorescent species; and

E is a $m \times q$ matrix containing a proportionality constant for each species at each set of observation wavelengths.

The calculations are simplified by assuming that wavelength pairs can be found, for each component, at which fluorescence depends on the speciation only at that component. These wavelengths are determined using SIMPLISMA as described above. The fluorescence of component n , of a total of N components, can now be written:

$$F_n = C_n(\theta)E_n + \Xi_n \quad (3)$$

where the matrices have the same meaning and dimensions as in the general form of Eq. 2, except now q is equal to one. The problem then becomes fitting N simultaneous equations of the form given in Eq. 3 to parameters that describe the speciation given in each of the C_n matrices. The parameters are common to all N sets of observations and are collected in the vector θ . The parameters include N conditional stability constants (K') and N ligand concentrations (L'_T). In this study, the speciation model consists of a mixture of ligands forming one to one complexes with Al. Experiments are performed at fixed pH so acidity constants can be ignored.

In addition to the nonlinear speciation parameters in θ , there are also two linear parameters for each component. These are contained in the matrix E_n , and these correspond to fluorescence of free and Al-bound ligand. In previous work (Smith and Kramer 1998), the method of van Stokkum et al. (1993) was used to fit these parameters using QR decomposition at each iteration of the search for best nonlinear parameters. QR decomposition is a matrix method used to reduce

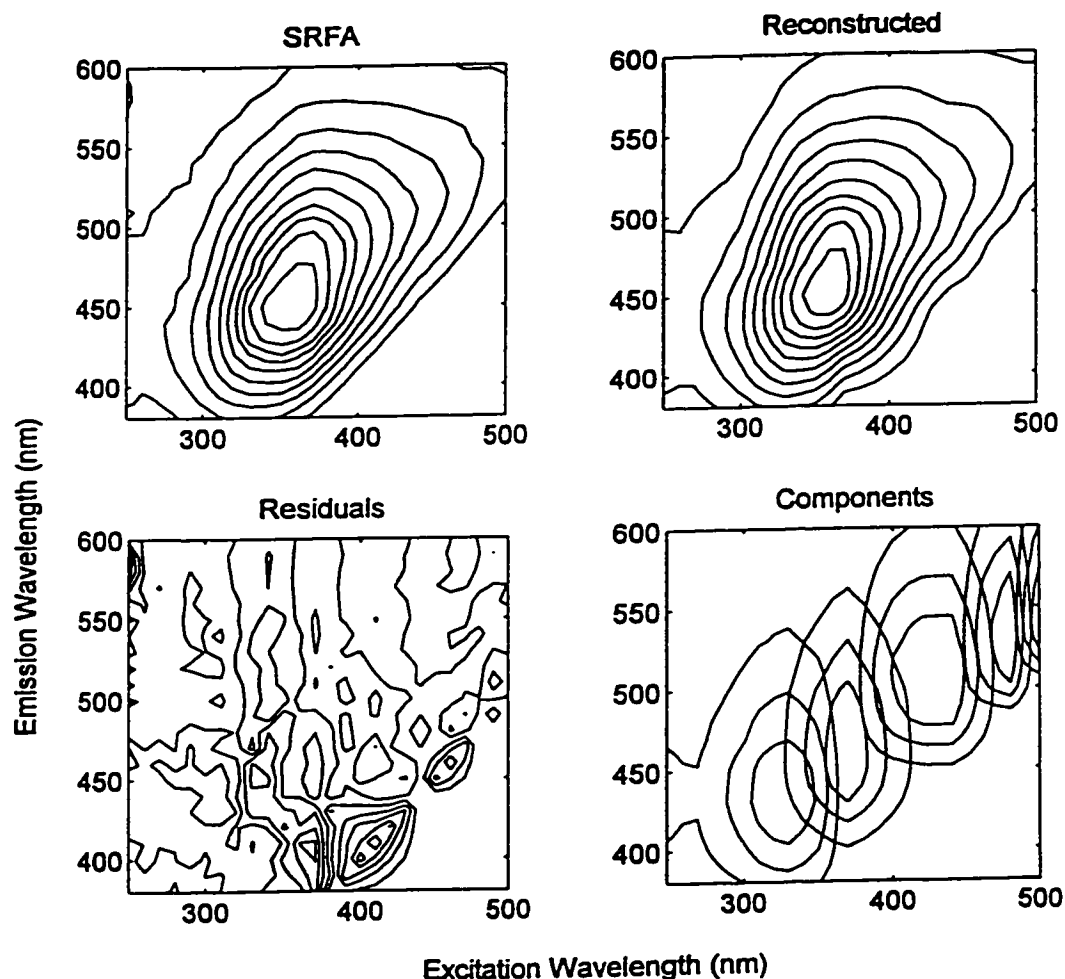


Fig. 1. Example deconvolution of fluorescence excitation vs. emission surface. Includes original data, reconstructed data, components and residuals as contour plots. The contour intervals for the raw data and the reconstructed data are every 10% (0 to 100%). The contour intervals for the components are every 33%. The sample is Suwannee River fulvic acid (SRFA) at pH 4.36 and ionic strength 0.1 mol/L with a concentration of 9.45 mg C/L.

the concentration matrix into two matrices Q and R , which can then be used to calculate the residual matrix. It was found that using this method, although advantageous in that it effectively reduced the number of parameters, could result in negative proportionality constants. In addition, in Smith and Kramer (1998), it was necessary to assume that the fluorescence proportionality constants for all Al-bound fluorophores were equal in order to avoid converging on negligible ligand concentrations. Both of these problems were avoided by fitting the proportionality constants along with the speciation parameters during the optimization. By fitting a linear parameter for the free ligand as well as the bound ligand, the assumption in the original Ryan-Weber equation that initial metal is negligible can be removed and, in fact, the titrations start from the total Al value measured in the original sample.

The objective function is then a function of $4N$ parameters: one K' , one L_T , and two proportionality con-

stants for each component. The error to be minimized is defined as the determinant of $(Z^T Z)$ where Z is a matrix of residuals with one column for each response and one row for each set of experimental observations. Each column corresponds to the difference between the observed F_N and the calculated F_N . This is done according to the proper Bayesian estimate of error in multiresponse systems as originally derived by Box and Draper (1965) and presented by Bates and Watts (1988). As in all nonlinear parameter fitting, good initial parameter estimates are necessary. In the fittings performed here, initial guesses for parameter fitting were determined using the usual Ryan-Weber equation (Ryan and Weber 1982) for each response separately.

The speciation at each iteration is calculated by solving for the roots of the polynomial obtained by expanding the stability constant and mass balance expressions in terms of free metal. The result is a polynomial of degree one greater than the number of ligands;

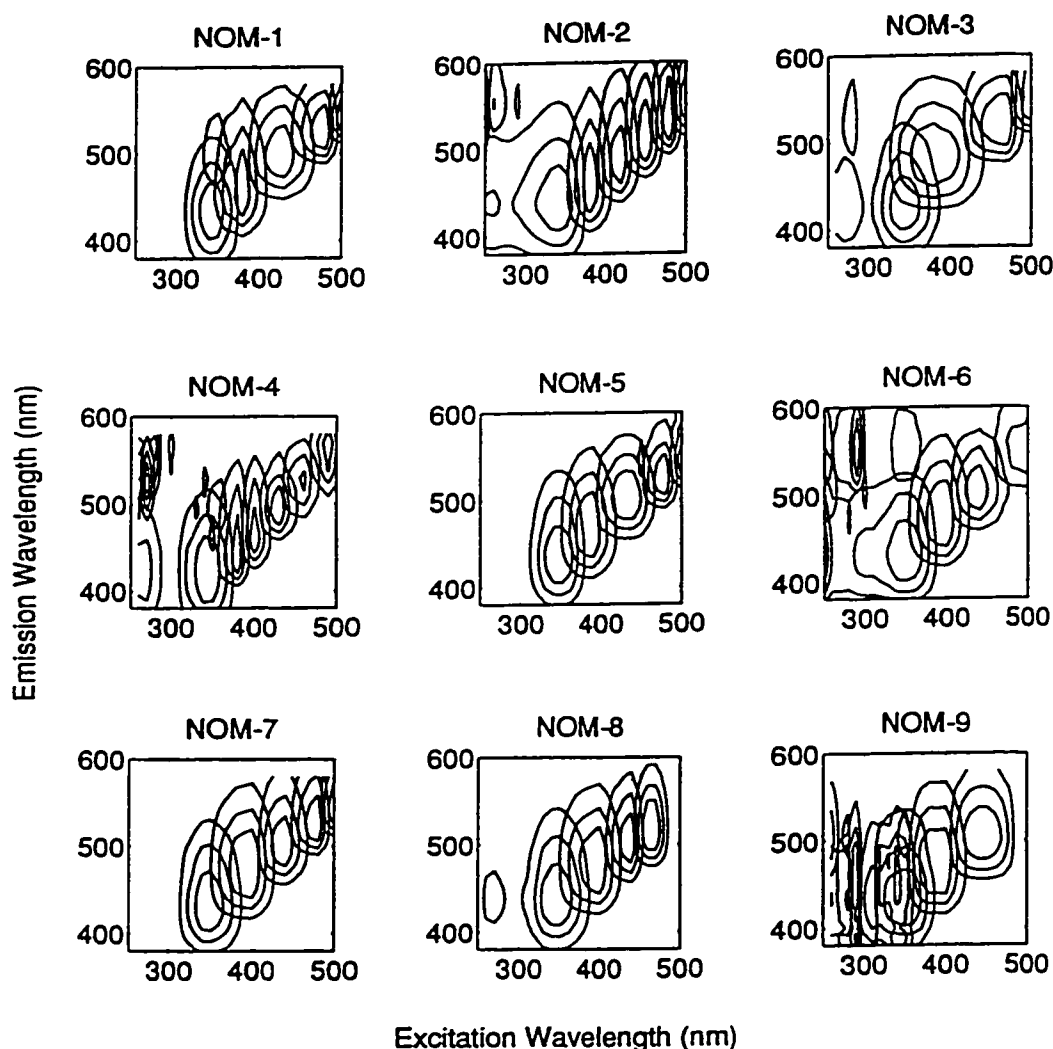


Fig. 2. Contour plots of resolved fluorescent components from NOM RO-isolates. The contour lines indicate equal steps at 33% of the total peak height. The sample numbers correspond to the numbering scheme given in the text.

thus, for more than two ligands, there is no explicit solution to this problem, but Matlab can solve for roots quickly and efficiently. This method was selected for speciation calculations because Newton-Raphson (Benthke 1996) or the fixed point iterative scheme of Perrin and Sayce (1967) were found to be much slower.

The data was tested for linear dependencies using the method of Khuri (1990). This was done to determine if there were multiple responses with information about each component. In all cases, it was found that there were no linear dependencies among the responses. Therefore, SIMPLISMA did not find too many components. Confidence intervals were determined using the method of Kang and Bates (1990), with an approximate Hessian matrix calculated according to the method of Bates and Watts (1988). This method is an approximation but currently is the best one available to assess uncertainty in multiresponse models.

RESULTS AND DISCUSSION

An example result of spectral deconvolution, along with residuals, is shown in Fig. 1 for Suwannee River fulvic acid. The resolved components represent the data well, but there is a trend in the residuals along the Rayleigh scatter line. This is because the data here were obtained by interpolation and not actually measured. The residuals, however, are still less than 5% of the observed values. From plots like this, wavelengths corresponding to each component were selected. The peaks of most components occur where contributions from other components are negligible.

Results of deconvolution of all of the NOM samples are shown in Fig. 2. The number of components varies between 4 and 6 and, in general, the components follow a line about 50 to 80 nm offset from the excitation equals emission line. In addition, the short wavelength

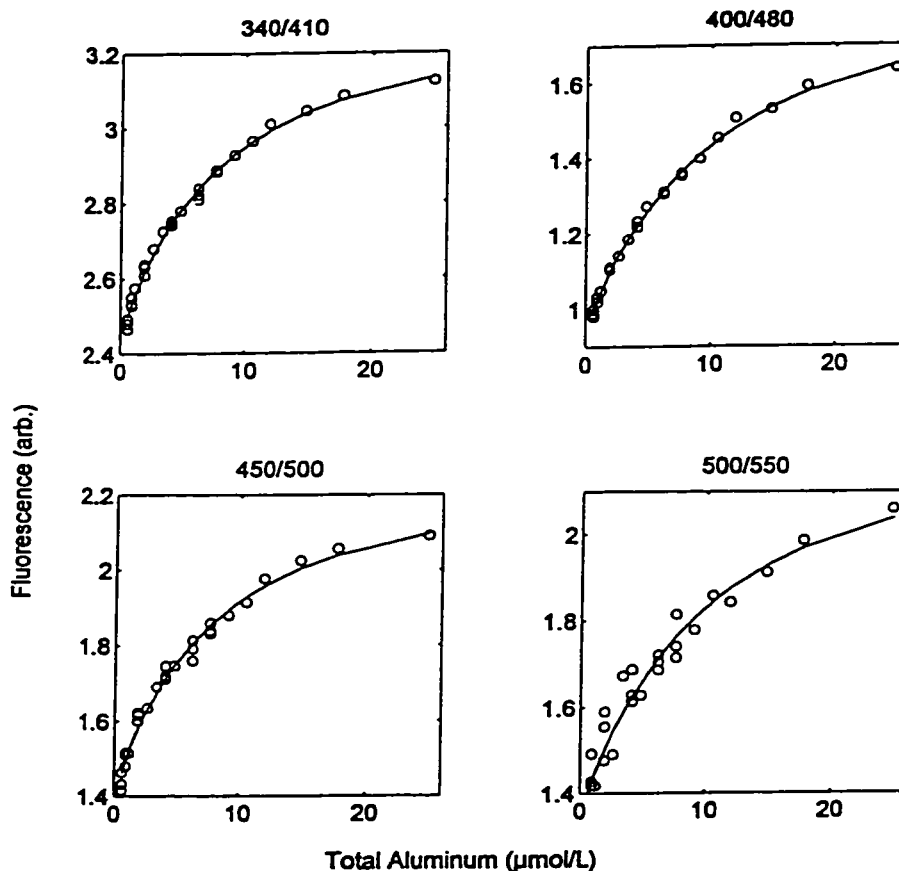


Fig. 3. Fitting results for NOM-3-RO. There are four simultaneous fluorescence responses measured as total aluminum was varied. The responses correspond to the following excitation/emission wavelengths 340/410, 400/480, 450/500, and 500/550.

peak, at excitation 250 and emission 440 nm, that is often designated a unique fluorophore (Goldberg and Weiner 1994) is a second excitation for the fluorophore emitting at around 430 nm. On addition of aluminum, the total fluorescence surfaces were observed to change. Results observed were similar to those reported by Blaser et al. 1999. Upon deconvolutions of the surfaces with added Al, the positions of the components varied only slightly, but the fluorescence intensity increased. This is not surprising because aluminum generally enhances fluorescence (Smith and Kramer 1998; Luster et al. 1996).

The spring and fall sampling of Helerudmyra (NOM-2 and NOM-9) show differences. The most notable differences are that the spring sample has more components and the fall sample has a short wavelength component, around 290 nm excitation. This indicates that the nature of the organic matter changes seasonally with more complex (more components) organic matter in the spring. NOM-1, 2, and 3, which are along the Trehørningen water course in the order listed, are compared. The nature of the organic matter changes moving from NOM-1 to NOM-3; there are five com-

ponents, then six, and finally just four components. NOM-7 and 8 are from an unlimed and a limed catchment, respectively. These samples are similar, except the unlimed catchment has a fluorophore not observed in the limed catchment.

Multiresponse parameter fitting was performed on simultaneous titration data obtained for each of the resolved components. An example of fitting results is shown in Fig. 3. The calculated and observed responses closely match, and the residuals are distributed randomly about the best fit line. All four responses were fit simultaneously as a function of four stability constants, four ligand concentrations, and eight fluorescence proportionality constants.

The results of parameter fitting for all samples are summarized in Fig. 4 for $\log K'$ and, in Fig. 5, for the total ligand concentrations. The fitted parameters here do not necessarily represent actual thermodynamic binding sites, and the high ionic strength used (0.1) to maintain constant activity coefficients is not representative of the natural samples. The open bars correspond to RO-isolates and the filled bars correspond to EV-isolates. The sample numbers correspond to the

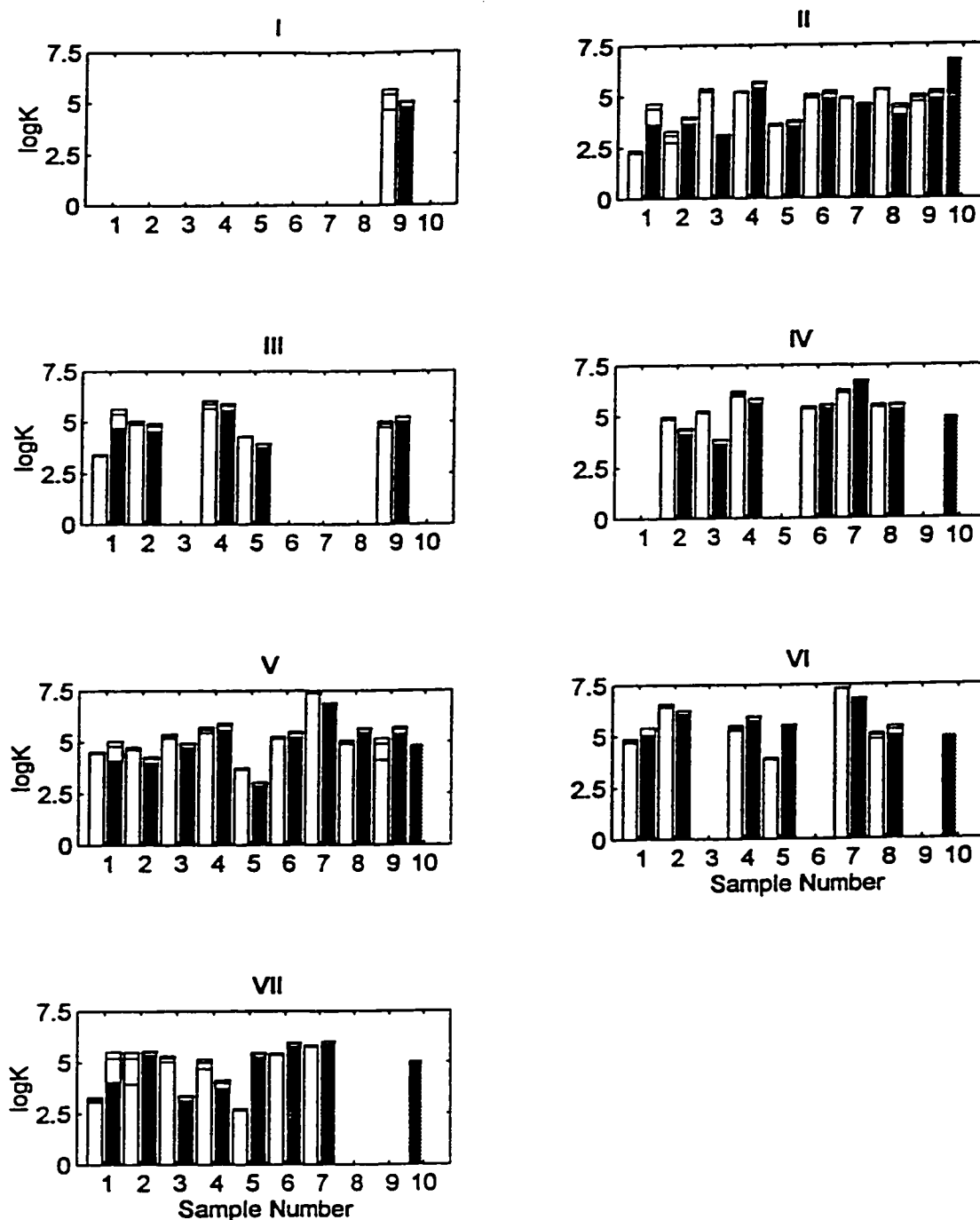


Fig. 4. Stability constants ($\log K'$) vs. NOM sample site for each of seven proposed fluorophores. The RO-isolates are indicated by open bars and the EV-isolates by filled bars. The error bars correspond to approximate 95% confidence regions.

numbering scheme given in the methods section. The standard errors are indicated as error bars. In general, the standard errors on the $\log K'$ values are less than 0.2 of a log unit and the L_T standard errors are less than 5% of the best fit value. The linear fluorescence proportionality constants are not shown because, although they are physically real parameters, they are not comparable between titrations. This is because no external calibra-

tions, using standards, were performed to correct for changes in instrument output on different days.

Overall, most of the constants are around $\log K'$ of 4.5-5.5 with some weaker sites around 2.5-3.5 and some stronger sites near 7. The middle sites can be interpreted as carboxylic sites with an ortho hydroxy group. This corresponds to salicylic acid like sites; at pH 4.36, salicylic acid has a conditional constant of 5.5.

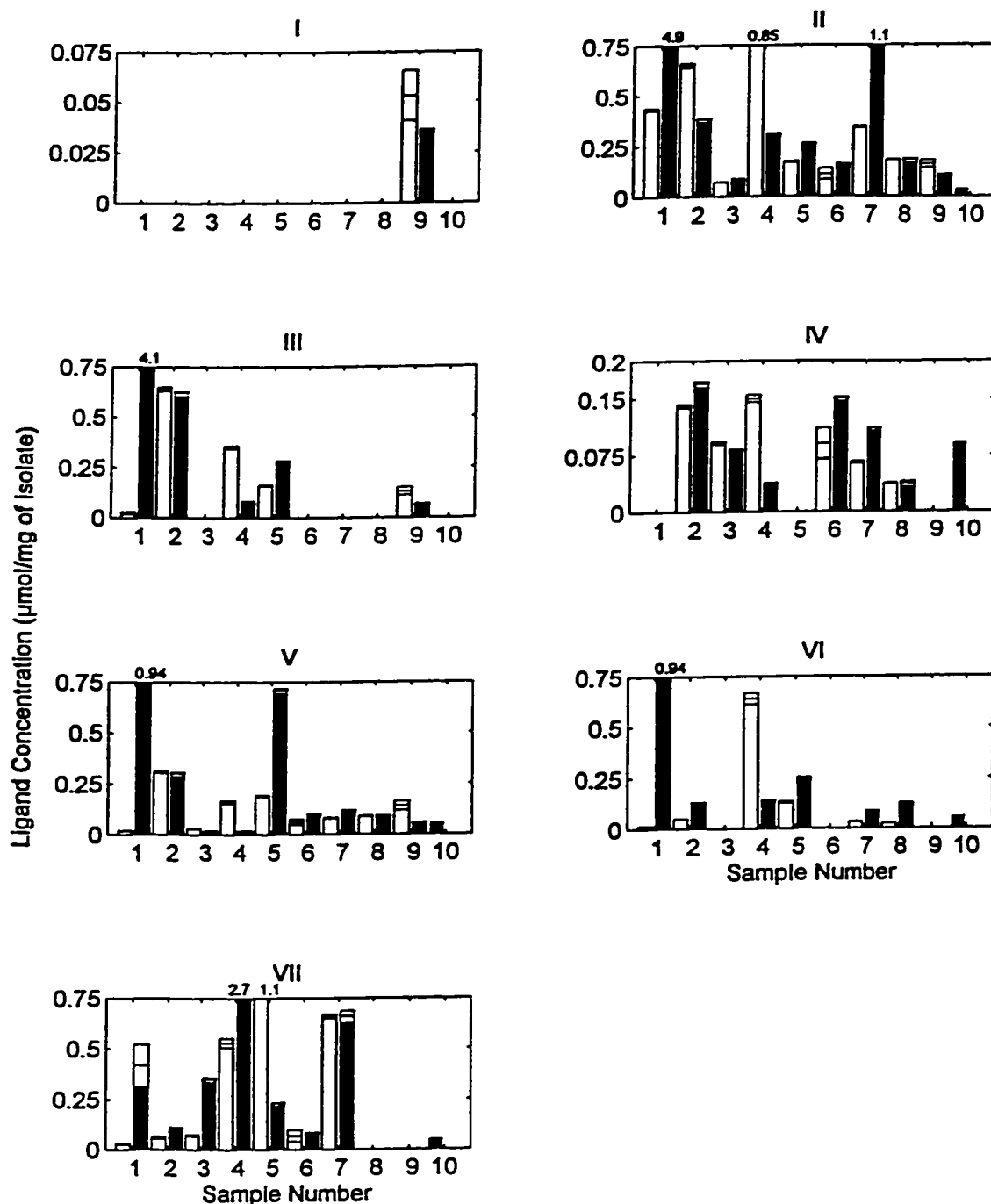


Fig. 5. Ligand concentrations vs. NOM sample site for each of seven proposed fluorophores. The RO-isolates are indicated by open bars and the VE-isolates by filled bars. The error bars correspond to approximate 95% confidence regions.

This is calculated from constants given by Martell et al. (1990). The weaker site could be a single carboxylic site without a stabilizing ortho hydroxyl group. The strongest sites are more difficult to interpret, and, at present, not even a tentative model ligand can be proposed, although similar values have been observed before. In Luster et al. (1996), a $\log K'$ value of 8.5 was observed for Juniper leaf litter extract using fluorescence and the Ryan-Weber equation, and Pott et al.

(1985) observed a value of 6.8 using a cation exchange method and Aldrich humic acid.

From the fluorescence surface, the fluorophores can be classified into seven different classes. Figure 6 shows where model compounds are observed to fluoresce. Superimposed on this is where the observed fluorophore classes fluoresce. The fluorophores are classified according to the excitation-emission groupings in Table 1.

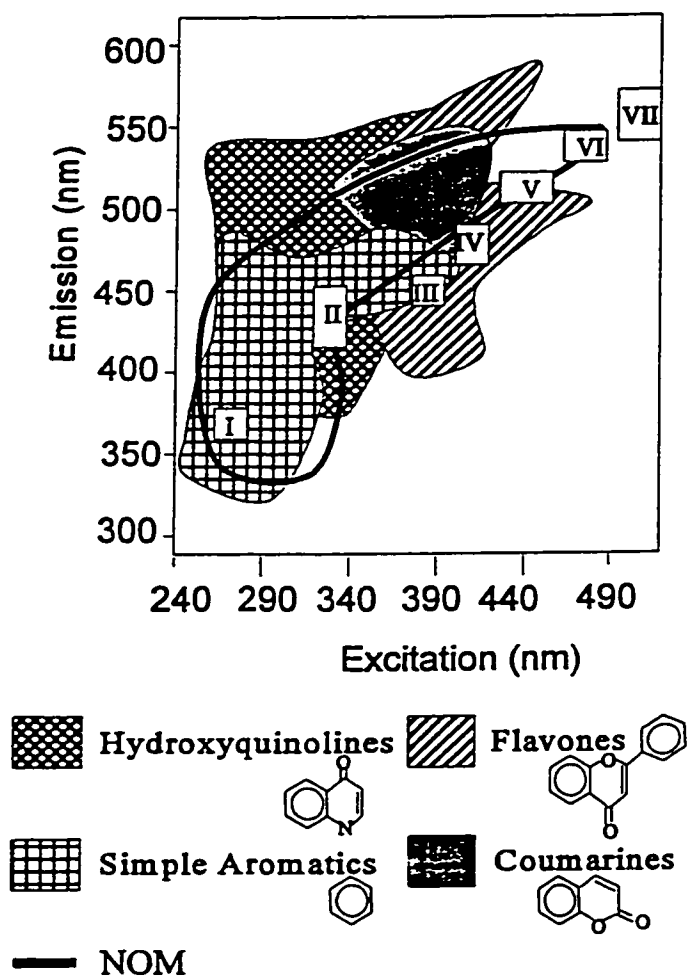


Fig. 6. Regions on an excitation vs. emission fluorescence surface that selected model compounds fluoresce. The regions indicated with boxes represent the seven classes of fluorophores observed in this study. The Roman numerals correspond to the wavelength classes given in Table 1. The solid outline corresponds to the region where NOM is observed to fluoresce from a survey of the literature (references are given in the text).

The data used to generate Fig. 6 came from a variety of sources. Data for hydroxyquinolines came from Williams (1959); data for coumarines and flavones from Wolfbeis (1985); and data for simple aromatic compounds came from Wolfbeis (1985) and Williams (1959). Fluorescence of NOM was compiled from Miano et al. (1988); Coble et al. (1990); Senesi (1990); Shotyky and Sposito (1990); Belin et al. (1993); Tam and Sposito (1993); De Souza Sierra et al. (1994); Provenzano and Sposito (1994); and Luster et al. (1996). The NOM samples include unfractionated water, humic and fulvic acids as well as leaf litter extracts.

Table 1. Classification scheme for fluorophores in NOM.

Class	Excitation range (nm)	Emission range (nm)
I	270	390
II	330-340	410-450
III	370-390	440-460
IV	400-420	460-480
V	430-450	500-520
VI	460-480	520-540
VII	490-500	540-570

The components identified here fall into most of the regions of the map. Component I is in the simple aromatic region. Component II is in both simple aromatics and hydroxyquinoline regions. Component III is in the flavone and simple aromatics region. Component IV is in the flavone and coumarine regions. Component V is in the flavone region. The remaining two components fall outside the range of values observed for these selected model compounds. This is not surprising because of the lack of summary data for model compounds.

Figure 6 is only intended to give ideas of possible fluorophores and should not be interpreted too literally. The values used to generate the figure came from a wide variety of instruments and mostly without corrections. Also, most investigators did not observe full excitation vs. emission surfaces, rather they used one dimensional excitation or emission slices of the surface; thus, the position of the maxima are only approximate. Still, the figure serves to give a broad idea of possible fluorophores in NOM and where they might fluoresce.

Table 2 summarizes the classes of fluorophores along with the observed $\log K'$ and L'_T values found in the NOM samples. The fitting results are somewhat dependent on the isolation method. In particular, NOM-1 and 3 show dramatic differences in best fit parameter values. Stability constants for fluorophores II, III, and VII are 2 orders of magnitude different in both cases, except in NOM-1, the EV sample is higher and, in NOM-3, the RO sample is higher. The RO vs. EV stability constants for the remaining sites agree within an order of magnitude and are generally within 0.5 of a log unit or less.

The binding capacities (Fig. 5) look more variable than the $\log K'$ values, but this is because they are not displayed on a log scale. Generally, the differences are less than 50% with no consistent higher or lower values between RO and EV samples. Again, NOM-1 shows

Table 2. Summary of fluorophores in NOM.

NOM Sample ^a	logK' / L' _T (mmol/mg of isolate) for indicated class of fluorophore						
	I	II	III	IV	V	VI	VII
1-RO		2.3 / 0.43	3.4 / 0.025		4.5 / 0.019	4.8 / 0.014	3.2 / 0.028
1-VE		4.4 / 4.9	5.4 / 4.1		4.8 / 0.94	4.8 / 0.94	5.2 / 0.42
2-RO		3.1 / 0.65	5.0 / 0.64	4.9 / 0.14	4.7 / 0.31	6.5 / 0.049	5.2 / 0.061
2-VE		3.9 / 0.38	4.8 / 0.62	4.3 / 0.17	4.2 / 0.30	6.2 / 0.13	5.5 / 0.11
3-RO		5.3 / 0.071		5.2 / 0.091	5.3 / 0.03		5.2 / 0.072
3-VE		3.1 / 0.089		3.8 / 0.082	4.9 / 0.018		3.3 / 0.35
4-RO		5.2 / 0.85	5.9 / 0.35	6.1 / 0.15	5.6 / 0.16	5.4 / 0.64	5.0 / 0.53
4-VE		5.6 / 0.31	5.8 / 0.082	5.8 / 0.038	5.8 / 0.018	5.9 / 0.14	4.0 / 2.7
5-RO		3.6 / 0.17	4.3 / 0.16		3.7 / 0.19	3.9 / 0.13	2.7 / 1.1
5-VE		3.7 / 0.26	3.9 / 0.28		3.0 / 0.71	5.5 / 0.25	5.4 / 0.23
6-RO		5.0 / 0.11		5.4 / 0.09	5.2 / 0.062		5.4 / 0.070
6-VE		5.1 / 0.16		5.5 / 0.15	5.4 / 0.10		5.9 / 0.086
7-RO		4.9 / 0.34		6.2 / 0.065	7.4 / 0.081	7.3 / 0.032	5.8 / 0.66
7-VE		4.6 / 1.1		6.7 / 0.11	6.9 / 0.12	6.8 / 0.086	6.0 / 1.2
8-RO		5.3 / 0.18		5.5 / 0.037	5.0 / 0.090	5.0 / 0.025	
8-VE		4.4 / 0.54		5.5 / 0.16	5.6 / 0.14	5.3 / 0.12	
9-RO	5.4 / 0.053	4.9 / 0.16	4.9 / 0.13		4.9 / 0.14		
9-VE	5.0 / 0.036	5.1 / 0.11	5.2 / 0.071		5.6 / 0.06		
10		6.8 / 0.041		5.0 / 0.092	4.9 / 0.057	5.0 / 0.057	5.1 / 0.054

^a The number corresponds the NOM-isolate from the NOM-typing project and 10 corresponds to Suwannee River fulvic acid.

the greatest differences, but NOM-3 L'_T values agree better between RO- and EV-isolates than the logK' values.

The seven different fluorophores can be compared between sites as well. The binding strength of each fluorophore is not the same for the different sampling sites. Thus, the fluorescent group might be similar between sites, because the wavelengths are the same, but the attached binding site might be different. In addition, effective binding strength can be reduced by competition with other metals, such as Cu; the constants reported here are conditional. In addition, if the initial concentration of Al saturated strong ligand sites then these would not be observed using this method.

Fluorophore I only occurs in NOM-9; this demonstrates that seasonal differences can be resolved by this method because the same sampling site in the spring (NOM-2) does not show this fluorophore. Fluorophore II occurs in all samples and generally has logK' values around 5 except NOM-5 is weaker (~4) and Suwannee River fulvic acid is stronger (6.8). Fluorophore III occurs in NOM-1, 2, 4, 5, and 9. The logK' values vary around 5±1 and again NOM-5 has the lowest value.

Fluorophore IV occurs in NOM-2, 3, 4, 7, 8, and in Suwannee River fulvic acid. The logK' values vary between 3.8 and 6.7 with NOM-6, 8, and Suwannee River fulvic acid giving similar values around 5 but NOM-7 has a value of 6.8 and NOM-2 and 3 have similar values around 4. Fluorophore V occurs in all samples and has values mostly around 5, except NOM-7 is higher (7.4) and NOM-5 is the weakest (3.0). The rest of the NOM samples are similar to Suwannee River fulvic acid. Fluorophore VI occurs in NOM-1, 2, 4, 5, 7, 8, and in Suwannee River fulvic acid. As in the other classes, NOM-7 is the strongest (7.3) and NOM-5 is the weakest (3.9). The rest of the values are again similar to Suwannee River fulvic acid. Finally, fluorophore VII shows values mostly just above 5 (~5.2) that are similar to Suwannee River fulvic acid. NOM-5 is the weakest (2.7 in RO), except the EV-isolate yields a value of 5.4 which is similar to the rest of the samples. NOM-7 is only slightly stronger than the rest of the samples (5.8 for RO, 6.0 for EV).

Overall, NOM-7 shows stronger binding and NOM-5 shows weaker binding for a given fluorophore. The rest of the sample sites are similar to Suwannee River

fulvic acid, showing binding strengths between 4.8 and 5.5, except fluorophore II in Suwannee River fulvic acid has binding strengths almost 2 orders of magnitude stronger than in the rest of the samples.

CONCLUSIONS

The Norway NOM samples show different fluorescent components, with different binding strengths. There were seven different fluorescent components identified by SIMPLISMA spectral deconvolution. These seven components compare well with the fluorescence of model compounds except two components are outside the region where the model compounds reported here fluoresce. The sampling sites contain between four and six of the identified fluorophores. Seasonal differences can be resolved, as well as differences between limed and unlimed catchments.

The determined binding strengths ($\log K'$) for the fluorophores are generally between 4.8 and 5.5 and agree well with values for the same fluorophores in Suwannee River fulvic acid, except Suwannee River fulvic acid shows stronger binding by almost 2 orders of magnitude in 1 of the fluorophores. There were also weak sites, with $\log K'$ between 3 and 4 as well as stronger sites with $\log K'$ values around 7. Overall, NOM-7 showed the strongest binding constants and NOM-5 showed the weakest stability constants. The isolation method was seen to influence the results; thus, one or both of the isolation methods modify the original raw water samples to some extent.

The method presented here is able to determine differences between NOM samples and yields some qualitative information about the nature of Al binding sites (fluorescence properties) and quantitative information (binding constant and site densities). It is useful to be able to treat the NOM samples as a mixture of a minimum number of fluorescent binding sites (between 4 and 6 for these samples). These sites represent averaging of all of the sites within NOM and, in a broad sense, can allow comparisons between samples.

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Appendix IV:

**Copper binding to Suwannee River fulvic acid determined using multiresponse
fluorescence**

From: Smith, D.S, and Kramer, J.R., to be submitted to *Analytica Chimica Acta*. (1999)

Abstract

Metal binding to Suwannee River fulvic acid (SRFA) is determined using multiresponse fluorescence. The fluorescence surface of SRFA is described by five average fluorescent components, determined by spectral deconvolution using SIMPLISMA (Windig, W.; Guilment, J. *Anal. Chem.* **1991**, *63*, 1425-1432). Fluorescence at excitation and emission wavelengths corresponding to these components was measured during titration with Cu (II). The resultant multiresponse data were then fit to a five site speciation model for Cu-SRFA interactions. The excitation/emission wavelengths identified for the five sites are: 320/425, 370/450, 420/500, 480/540 and 500/550 nm. For the five sites the best fit logarithms of the conditional stability constants at pH 6.02 and ionic strength of 0.1 M (KClO₄) are: 4.95 (0.23), 4.98 (0.23), 5.32 (0.21), 5.59 (0.24), and 5.62 (0.22). The respective concentrations (μmol / mg of SRFA) are 0.83 (0.44), 2.38 (0.56), 2.76 (0.29), 0.74 (0.09), 0.037 (0.005), where the bracket values indicate approximate ±95% confidence intervals. Free copper predicted using these parameters agrees within 0.1 of log unit of predicted free copper from Cabaniss and Shuman's empirical equation (*Geochim. Cosmochim. Acta* **1988**, *52*, 185-193). These values are compared to existing values for Al and H binding by SRFA, for the same five sites, also determined using multi-response fluorescence. The binding of Al, Cu and H are best represented as bidentate/diprotic sites. The conditional stability constants for Al and Cu are consistent with salicylic acid-like binding.

Introduction

Metal fate and transport is strongly influenced by metal speciation. In particular, naturally occurring organic ligands can bind metals in aqueous solution. Natural organic matter (NOM) is a heterogeneous mixture of potential metal binding sites. Within NOM are the macromolecular portions termed humic and fulvic acids. There are many speciation methods to experimentally determine speciation indicators such as concentration of free and bound metal. For example, ligand competition methods (1,2), ion-selective electrodes (3,4), separation techniques such as dialysis membranes (5) and chromatographic methods (6) have been used as for speciation determinations. In general, these methods are limited in that a single type of experimental measurement is utilized to model metal-NOM speciation. This limitation of traditional titration experiments allows for many different models to yield equally good fits to the measured data. If all that is desired is predictive parameters for variables, such as free metal, then any model that correctly represents the data is adequate, but if site-specific speciation is required then more information is necessary to constrain the model imposed on the system. This is possible using multiresponse fluorescence metal titrations of NOM.

Fluorescence enhancement/quenching has been used to determine conditional stability constants (K') and site densities (L_T) for fluorescent natural organic matter. Initial quantitative efforts in this regard were performed by Ryan and Weber (7) and resulted in the well known Ryan-Weber equation. This method has been employed by measuring fluorescence quenching to investigate binding of Cu to fulvic acid (7-9). The method was also applied to fluorescent enhancement for Al binding (10). Luster et al. (8) utilized a fluorescence method at multiple wavelengths on an excitation versus emission surface for juniper leaf litter extract complexation of

Al and Cu. Implicit in this approach was the assumption that the fluorescent sites acted independently in metal binding. In general, binding sites will actually compete for available metal. Smith and Kramer (11) take competition into account in their development of a generalized multi-site Ryan-Weber equation, which was validated for NOM speciation with Al. This approach utilizes multiresponse parameter estimation to simultaneously treat all proposed binding sites.

In a further refinement, Smith and Kramer (12) proposed the use of spectral deconvolution of fluorescence excitation versus emission surfaces to determine a minimum number of binding sites and wavelengths corresponding to those sites. This paper builds upon this earlier work and uses the method applied to Al binding to Cu binding. In addition, proton binding (13) is assessed. In this paper, we utilize a multiresponse fluorescence method to determine Cu speciation in the presence of Suwannee River fulvic acid (SRFA). This method places spectroscopic constraints on the minimum number of sites necessary to describe Cu-SRFA interactions. In addition, the method is validated by showing its predictive capability for free Cu^{+2} , although $[\text{Cu}^{2+}]$ was not directly measured, and is compared to existing speciation parameters for H and Al at the same fluorescent sites.

Experimental Method

The experimental method for Cu is discussed here. The method for Al is discussed in Smith and Kramer, (11) and the H-method is given by Smith *et al.*, (13). Suwannee River fulvic acid (SRFA) was obtained from the International Humic Substances Society and used without further treatment. Initially, a fluorescence excitation versus emission surface was recorded for SRFA at pH 4.36, 18.90 mg SRFA/L and an ionic strength of 0.1 M (KNO_3). This was done in the excitation range 250-450 nm and the emission range 380 to 680 nm with 10 nm resolution in

both excitation and emission wavelengths. This surface was then deconvoluted using SIMPLISMA (14) to determine a minimum number of fluorescent components necessary to describe SRFA fluorescence. The results of this study are described in Smith and Kramer, (12). Five fluorescent components with maxima at the excitation/emission wavelength pairs: 320/425, 370/450, 420/500, 480/540 and 500/550 nm, were identified. Fluorescence at these five excitation / emission pairs were then monitored during a titration with copper.

Solutions of 13.85 mg of SRFA/L were titrated with copper in the range 0 to 100 μM total added Cu. Copper titrant solutions were prepared at 0.01 and 0.0001 M from $\text{Cu}(\text{NO}_3)_2$. The solution pH was adjusted to be 6.00 ± 0.02 using dilute NaOH or HNO_3 , as required. The ionic strength of the solution was adjusted to 0.1 M with KClO_4 . The temperature was monitored and found to be 25 ± 0.2 °C throughout the titrations. Preliminary experiments indicated that the fluorescence signal stabilized within 10 minutes after Cu addition. Thus, the solution was allowed to equilibrate 15 minutes between fluorescence measurements. Each measurement was followed by the next addition of titrant. Two titrations were performed with replicate fluorescent measurements for each addition of titrant. These two data sets are pooled for a total of 40 data points.

The sample was introduced to the fluorometer via a peristaltic pump to a quartz flow-thru cell for fluorescence measurement. Fluorescence was measured using a 150 W Xe lamp from a Perkin-Elmer MPF-44 as the excitation light source. Emission was detected at 90° using an InstaSpec IV (Oriel Corporation, Stratford, CT) CCD spectrophotometer, configured to collect data at the emission wavelength of interest. The excitation and emission slits were set at 20 nm to maximize instrument sensitivity. All data were gathered and logged using a personal computer.

Data Processing

Data processing was performed using in-house Matlab TM (The MathWorks Inc., South Natick, MA) programs. The method followed essentially that presented by Smith and Kramer in two previous papers (11,12), and is summarized below.

For each fluorescent component the fluorescence can be described as:

$$F_m = k_{L_m} [L_m] + k_{ML_m} [ML_m] \quad (1)$$

for each of the $m=1\dots M$ sites, where L_m corresponds to m th free ligand and ML_m to the m th bound ligand ($M:L_m=1:1$), with fluorescent proportionality constants given by the respective k constant. This treatment assumes that the fluorescence response is linear, which is reasonable in dilute solutions (15).

In order to fit the observed fluorescence to a speciation model the concentrations of L_m and ML_m must be calculated as a function of the conditional stability constants (K') and the total ligand concentrations (L_T). The stability constants for metal associations are written as the association constant (reported as $\log K'$) and for protons as the dissociation constant (reported as $pK'_a = -\log K'_d$). The metal association constants assume a one to one complex stoichiometry. The experiments were performed at fixed pH therefore for metal titrations acidity constants can be ignored and the results are conditional upon pH. In addition, for titration with a given metal the concentration of other metals are assumed to be negligible. The speciation distribution between free and bound for both ligand and metal can be calculated using the $M+1$ mass balance expressions for total metal (M_T) and total ligand (L_{Tm} for $m=1\dots M$):

$$\begin{aligned}
M_T &= [M] + \sum_{m=1}^M [ML_m] \\
L_{T_1} &= [L_1] + [ML_1] \\
&\vdots \\
L_{T_m} &= [L_m] + [ML_m]
\end{aligned}
\tag{2}$$

The above set of equations can be expanded and after substitution of each of the M stability constant expressions the result can be expressed as a polynomial in [M] of degree M+1. For one ligand the result is a quadratic expression, for two ligands, a cubic expression. For one and two ligand systems the results can be solved explicitly, but for >2 ligands the calculations must be performed using numerical techniques for root finding. Once [M] is determined, the result can be substituted back in to determine the concentrations of free and bound ligand. In this paper Matlab™ was used to solve the polynomial expression for [M]. This method is faster than Newton-Raphson or the fixed point iterative scheme of Perrin and Sayce, (16) for determining speciation in these simple systems.

Thus, the fluorescence can be expressed as a function of 4M parameters, K', L_T and a proportionality constant for fluorescence of L and ML for each of the M sites. These parameters are fit using multiresponse parameter fitting, as a function of total metal added (M_T) and the observed fluorescence responses. The theory of multiresponse fitting, as applied to chemical kinetics, was originally developed by Box *et al.*, (17) and has more recently been summarized by Bates and Watts (18) and Stewart *et al.*, (19). In multiresponse fitting, the error criteria to be minimized is det(Z^TZ), where det stands for determinant and superscript T for the matrix transpose. The N×M matrix Z is a matrix of residuals, with a column of “calculated - observed” for each of the N fluorescence responses. This is a multiresponse generalization of least squares

and for one response the result is the usual sum of squares.

For demonstration, the matrix of residuals, \mathbf{Z} , can be shown in full for the i th addition of titrant and the j th fluorescent response for $i=1..N$ and $j=1..M$:

$$\mathbf{Z} = \begin{bmatrix} F_{11}\text{obs}-F_{11}\text{calc} & F_{12}\text{obs}-F_{12}\text{calc} & \dots & F_{1M}\text{obs}-F_{1M}\text{calc} \\ F_{21}\text{obs}-F_{21}\text{calc} & F_{22}\text{obs}-F_{22}\text{calc} & \dots & F_{2M}\text{obs}-F_{2M}\text{calc} \\ \vdots & \vdots & \vdots & \vdots \\ F_{N1}\text{obs}-F_{N1}\text{calc} & F_{N2}\text{obs}-F_{N2}\text{calc} & \dots & F_{NM}\text{obs}-F_{NM}\text{calc} \end{bmatrix} \quad (3)$$

for M fluorescent responses corresponding to M proposed fluorescent sites and for N additions of titrant. The assumptions in using this criteria are that the errors are distributed as a M -dimensional normal distribution, and that there are correlations between responses, but not between additions of metal. In practice, MatlabTM constrained optimization was used (20). The objective function was $\det(\mathbf{Z}^T\mathbf{Z})$, and the parameters were constrained to be positive. In addition, for numerical derivative calculations all parameters, and the objective function, were scaled to be close to a value of one. After convergence, the variables and objective function were scaled back up to their appropriate values. This was done to decrease numerical errors in derivative calculations resulting from up to 10 orders of magnitude difference between the K' and L_T terms.

For Al and Cu titrations standard errors in parameter estimates were determined using the method of Kang and Bates, (21). For H titrations, the charge excess expression, as determined from pH measurements, was included as an additional response. The errors in this case are not random, but are propagated systematically by errors in the pH-electrode calibration. The errors are greatest at the acidic and basic ends of the titration. This is addressed using Monte Carlo methods to determine confidence intervals about parameter estimates by randomly varying

electrode calibration parameters (13).

Results and Discussion

The fluorescence response for each addition of Cu is shown in Figure 1 for all five proposed fluorescent sites. The initial total Cu was calculated from reported Cu concentrations in isolated SRFA (22). Fluorescence is quenched on addition of copper for all five sites and the fluorescence signal begins to level off at high total Cu. The solid lines correspond to best-fit lines determined by fitting all five fluorescence responses simultaneously, as discussed above. The fit on all five signals is very good; the residuals are randomly distributed, and show no trends.

The parameter fitting results for the L_T 's and $\log K$'s are shown in Table 1. The fluorescent proportionality results are not reported, because, although physically meaningful, they are dependent on instrument settings and are only relevant internally to these data and cannot be compared to literature values for model fluorophores. The parameter fitting results are discussed later in relation to Al and H results for SRFA.

The fitted parameters (Table 1) can be used to calculate free Cu at each of the titration points. Figure(2) compares this calculated $[Cu^{2+}]$ to that determined from the semi-empirical relation of Cabaniss and Shuman (4). Cabaniss and Shuman, used Cu titrations of SRFA over a range of pH, ionic strength and total DOC to determine fitting parameters for $[Cu^{2+}]$. They state that their model predicts free Cu within 0.1 pCu units in the pH range 5 to 8, pCu_T 7 to 4 and DOC 1 to 10 mg C/L. Our results fall within 0.1 log units of their results and thus agree with the independent determination of Cabaniss and Shuman.

Figure 2 shows that although free Cu was not measured in this experiment, it can be predicted quite well from the fitted parameters. There is a consistent underestimation around

pCu_T of 6, but even then the difference is less than the error estimate of 0.1 log units. The confidence envelope for this study, however shows that the predicted [Cu²⁺] of Cabaniss and Shuman, is completely within the range predicted by the parameters determined here. The confidence interval around the estimate of [Cu²⁺] was obtained by setting parameters to both extremes of their calculated 95% confidence intervals and calculating [Cu²⁺]. The agreement between free copper predicted by two independent methods improves confidence in the results of both methods; moreover, the agreement reinforces the fluorescent method applied here because free Cu²⁺ is predicted even though it is not directly measured. The results here (Figure 2) show that at the lowest total Cu (II) there is an order of magnitude uncertainty in the free copper, but at the highest total Cu (II) the uncertainty in free copper is less than 0.1 of a log unit. The large uncertainty at low total copper is likely a result of poor confidence interval estimates in the speciation parameters.

Ligand concentrations determined for the five sites with respect to Cu, Al and H are tabulated in Table 1 and shown graphically in Figure 3. The fluorescence deconvolution of SRFA, given in reference (12), imposes a lower bound on the number of sites at five. These five fluorescent sites are proposed to be the same sites observed during titration with any of the three metals. In the presence of Al, Cu and H the total ligand concentration for each site would be equal to the sum of contributions from each bound species and free ligand. For example, $L_T = [AlL^+] + [CuL] + [H_2L] + [HL^-] + [L^2]$. Overall, the concentration determined using Al is less than Cu, but the confidence envelope is tighter. It is likely that the concentration of the sites with respect to Al is probably less, because these experiments were performed at pH 4, not pH 6, and the competition of protons would reduce the apparent site density. The proton ligand

concentrations agree more closely with Cu ligand values, but are still almost an order of magnitude more concentrated at site 1, and an order of magnitude less concentrated at site 4. The total site concentrations are compared in Figure 3f. On this scale, Cu and H-ligand concentrations are fairly close (within 0.2 of a log unit), but again the Al-site concentration is much lower. In reference (13) a sixth monoprotic site is added to accommodate the potentiometric data. This site is monoprotic with a weak affinity for protons ($pK_a < 3$), thus it is not necessary to include in models for Al or Cu binding because it would not significantly influence the binding of these metals.

The bounds shown in Figure 3f are for comparison with results from non-fluorescence literature. The lowest continuous line in (f) corresponds to exchangeable proton concentration and the top continuous line corresponds to nonexchangeable proton concentrations determined by NMR (26). The middle solid continuous line corresponds to the best estimate of total carboxylic content for SRFA of 6.8 $\mu\text{mol/mg}$ of SRFA (23,24) and is almost exactly coincident with the total ligand concentration determined for copper. This is reasonable, because carboxylic sites are often proposed as the binding sites for Cu in humic and fulvic acid (25). The 10.5 and 33.5 $\mu\text{mol/mg}$ of SRFA for exchangeable and nonexchangeable protons respectively, as determined by NMR (26), bracket the confidence range for total fluorescent sites in SRFA with respect to proton binding.

The stability constants and ligand concentrations of this and other studies, are compared in Table 1 and Figure 4. The Al and Cu conditional constants show very similar values, around 5 ± 1 throughout. Note that these are conditional constants and were measured at pH 4 for Al and at pH 6 for Cu. These values correspond within an order of magnitude of the conditional constants for Al and Cu with salicylic acid at pH 4 and 6 respectively. Using thermodynamic stability

constants from Smith and Martell, (27), conditional stability constants for Al and Cu with salicylic acid at pH 4 and 6 are $\log K' = 5.3$ and $\log K' = 5.5$ respectively. The protonation constants, however, do not closely resemble salicylic acid. Reported step-wise acidity constants are 2.73 and 12.98 for salicylic acid (27). These values are outside the analytical window for this experiment (pH 3 to 11), so could not be observed. The values that were observed for ligands 2, 3, 4 and 5 fall around 4 and 7, which could correspond to carboxylic-type sites, although phosphoric sites could also exhibit acidity constants around 7. The first site is unusual in that it has two closely spaced, high, acidity constants around 10. These are likely phenolic protons or amino acid-like sites. There are amino acids with very close acidity constants in the pK_a 9 to 12 range (27). For example, lysine has 9.08 and 10.63 as the last two pK_a values. It is possible to have carboxylic sites with pK_a 's in the range 2 to 8 and for phenolic protons to have pK_a 's in the range 6 to 14 (28). The exact value depends on the chemical environment about the site, and in this discussion electrostatic effects have been ignored.

Error estimates for H^+ binding are probably more realistic than Al and Cu estimates, because they were determined using the Monte Carlo method. For Al and Cu, confidence intervals are approximated by the method of Kang and Bates (21). This method is an approximation because it assumes that the nonlinear response surface can be approximated by a surface corresponding to a linear model. The degree of nonlinearity can be addressed by profile trace plots (18), and work is ongoing to do this for multiresponse fluorescence models.

Metal binding determined here is consistent with a bidentate carboxylic-phenolic site, like salicylic acid. The fluorescence is different for each proposed site though, so the fluorescent backbone of the binding sites are different. Possible molecular building blocks for fluorescent

sites in SRFA are discussed by Smith and Kramer, (12). Using the wavelength ranges for the fluorescence of model-humic compounds presented by Smith and Kramer (12): site 1 corresponds to simple aromatics and hydroxyquinoline type fluorophores, site 2 coincides with both simple aromatic and flavone fluorescence regions and site 3 has fluorescence similar to flavone compounds. The final two sites do not exhibit fluorescence corresponding to any model compounds summarized in reference (12). There is a question whether the proposed fluorescent sites correspond to chemically real moieties, though. The site densities are reasonable compared to literature values, as are the stability constants, and the results can be explained in relation to previous results for Al and H.

This approach assumes that all Cu binding sites fluoresce. Because the total fluorescent site density is the same as the total carboxylic content the usual assumption that only 1 % of the chromophores in fulvic acids are fluorescent (29) should be reassessed. The total copper binding site concentration determined here corresponds to about 16 % of the carbon atoms in SRFA.

Conclusions

Overall multiresponse approaches allow better model discrimination than uniresponse approaches, but due to the heterogeneity of NOM, the results should be interpreted as qualitative-possible average sites, and not as rigorously defined chemical components. An advantage of the method presented here is that a lower bound on the number of sites is obtained by spectral deconvolution of fluorescence excitation versus emission surfaces, and such a bound cannot be imposed in uniresponse experiments, such as with Cu-selective electrodes. In future work, free Cu will be measured as well as the fluorescence responses and thus all six responses can be fit simultaneously in order to further refine model definition. The multiresponse fluorescence

method presented here is validated in that it can predict free copper although this is variable is not directly measured. In addition, an overall multi-metal binding model can be presented for SRFA using the same five fluorescent sites as average sites for Cu, Al and H binding.

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Figure Captions

Figure 1 Fluorescence during titration of copper with SRFA at pH 6.00 and ionic strength of 0.1 M (KClO_4). The excitation/emission wavelengths are 320/425 for (a), 370/450 for (b), 420/500 for (c), 480/540 for (d) and 500/555 for (e). The open circles represent experimental measures, and the solid line corresponds to the best-fit line for the speciation model (Table 1).

Figure 2 Calculated free Cu for the titration of SRFA. The symbols (o) represent calculated results from Cabaniss and Shuman's empirical predictive parameters for free Cu (4). The solid line represents free Cu calculated from parameters determined here (Table 1 and 2). The dotted lines correspond to an estimated 95% confidence envelope about the free Cu estimate for this study.

Figure 3 Ligand concentrations ($\mu\text{mol}/\text{mg}$ of SRFA) determined from Cu, Al and H titrations with fluorescence observations. The fluorescence wavelengths are 320/425 for (a), 370/450 for (b), 420/500 for (c), 480/540 for (d) and 500/555 for (e). Part (f) is the total ligand concentration obtained by summing the results for (a) to (e). The error bars correspond to estimated $\pm 95\%$ confidence intervals.

Figure 4 Conditional association constants for Cu and Al and acidity constants for H determined by titrations with fluorescence responses. The fluorescence wavelengths are 320/425 for (a), 370/450 for (b), 420/500 for (c), 480/540 for (d) and 500/555 for (e). The error bars correspond to estimated $\pm 95\%$ confidence intervals.

Table 1. Conditional stability constants (K') and respective ligand concentrations (L_T - $\mu\text{mol/mg}$) for Cu (pH = 6.0), Al (pH = 4.0) and H for 5 fluorescent components of SRFA at $I=0.1 \text{ KClO}_4 \text{ M}$. Diprotic K' 's are shown for H. Confidence intervals ($\pm 95 \%$) are given in brackets. K' 's are for formation constants for Cu and Al and for dissociation constants for H.

Site		Cu	Al	H	
1	L_T	0.83 (0.44)	0.041 (0.001)	14.59 (7.10)	11.08 (0.32)
	$\log K'$	4.95 (0.23)	6.84 (0.01)	10.03 (0.24)	
2	L_T	2.38 (0.56)	0.093 (0.001)	4.83 (1.32)	7.60 (0.19)
	$\log K'$	4.98 (0.23)	5.04 (0.08)	3.00 (0.79)	
3	L_T	2.76 (0.29)	0.059 (0.001)	0.54 (0.15)	7.51 (0.14)
	$\log K'$	5.32 (0.21)	4.90 (0.08)	3.78 (0.44)	
4	L_T	0.74 (0.09)	0.057 (0.001)	0.15 (0.04)	7.33 (0.32)
	$\log K'$	5.59 (0.24)	4.97 (0.15)	4.23 (1.01)	
5	L_T	0.037 (0.005)	0.054 (0.001)	0.22 (0.10)	7.36 (0.64)
	$\log K'$	5.62 (0.22)	5.11 (0.18)	4.02 (1.23)	

fig 1

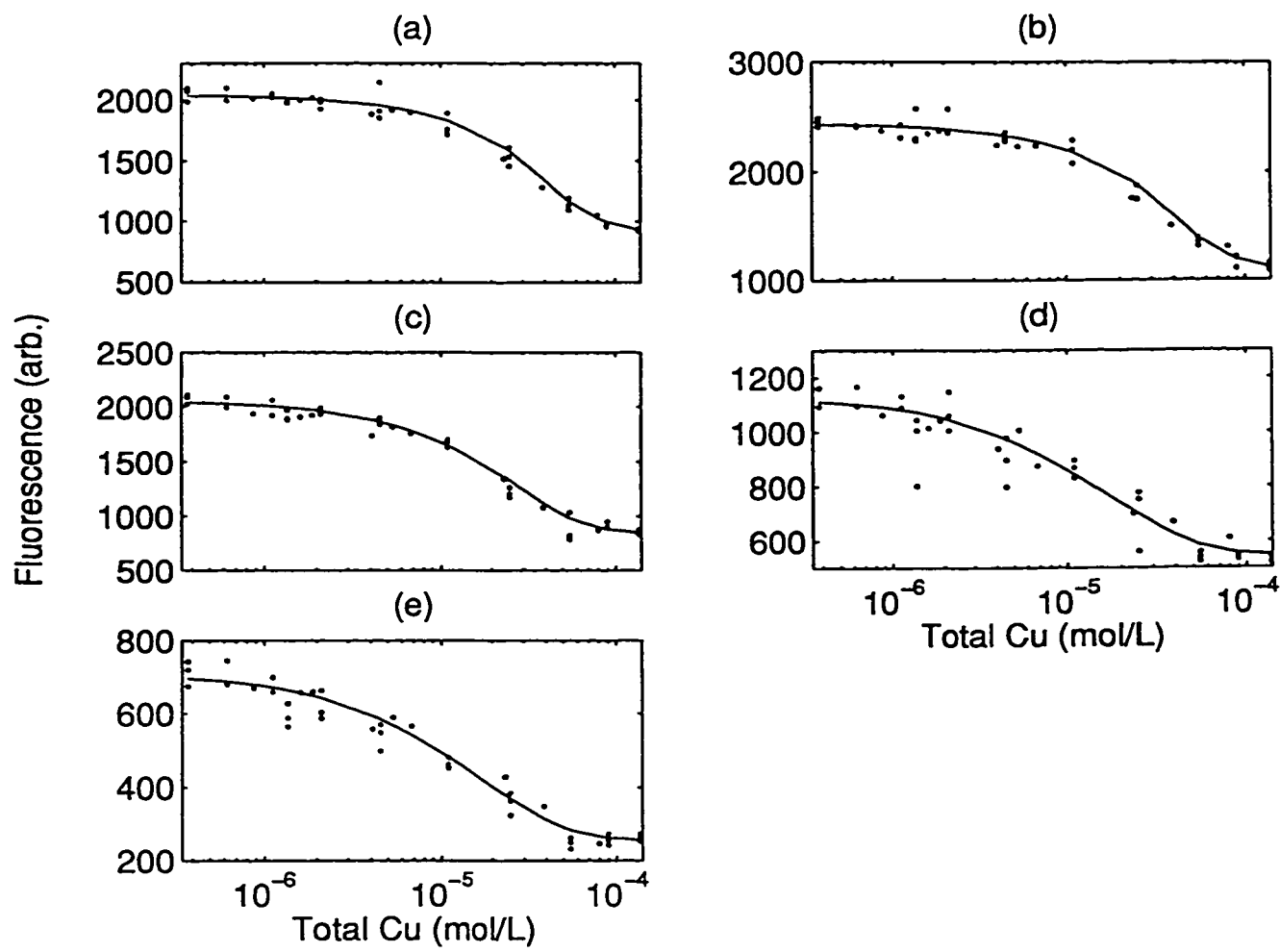


fig 2

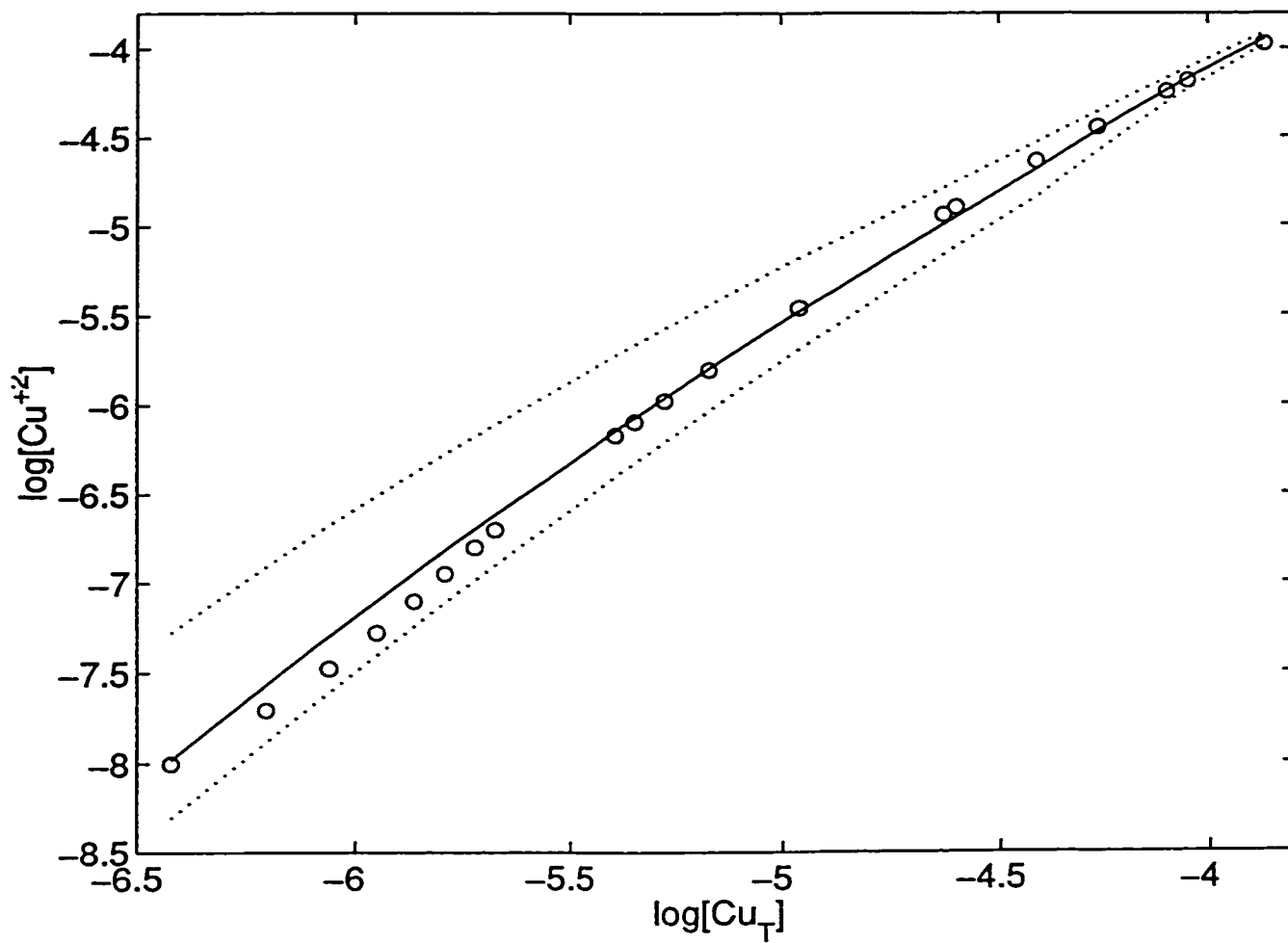


fig 3

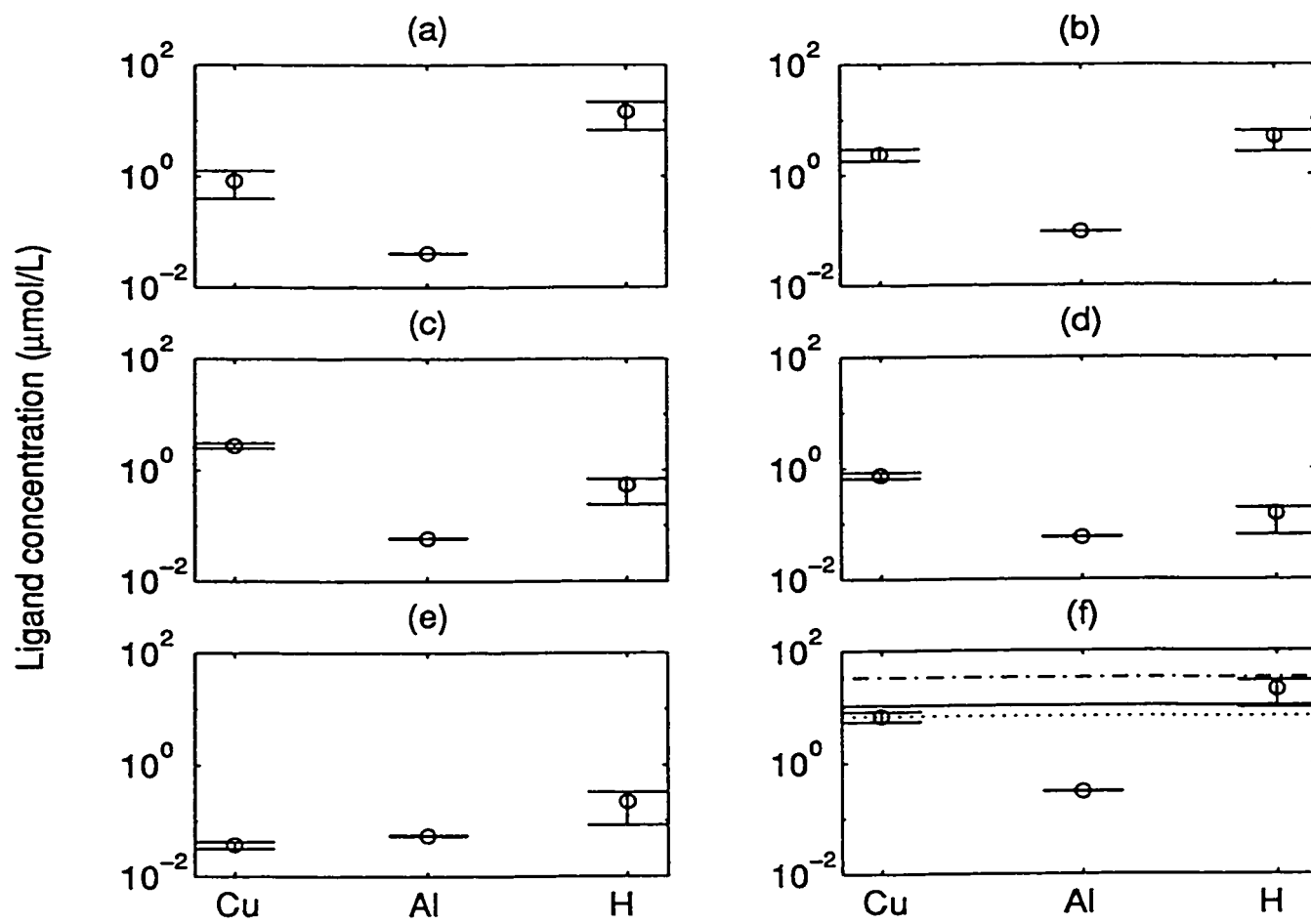


fig 4

