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Article

Synchronous-Scan Fluorescence and the Complexation of Copper (II) Ions by Humic Substances

Ana T. Lombardi* and Wilson F. Jardim

Universidade Estadual de Campinas, Instituto de Química, Depto. Química Analítica, C.P. 6154, 13081-970 Campinas - SP, Brazil

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A fluorescência de substâncias húmicas de origem terrestre foi investigada através da técnica de fluorescência sincronizada, e as amostras mostraram duas bandas principais (λex/λem 380/398, 440/458 nm) quando analisadas sem adição do metal paramagnético cobre. Uma terceira banda (λex/λem 502/520 nm) foi detectada após severa extinção de fluorescência da segunda banda causada pela adição de cobre. Uma outra banda, mais energética (λex/λem 333/351 nm) foi detectada em amostras marinhas, porém ausente do material de origem terrestre. Os efeitos de cobre sobre a intensidade e forma do espectro de fluorescência sincronizado e a utilização desta técnica como marcadores são discutidas.

Two fluorescence bands were detected using synchronous-scan fluorescence on samples of terrestrially derived humic substances (λex/λem 380/398, 440/458 nm) without the addition of copper. A third band (λex/λem 502/520 nm) was detected after severe quenching of the second band (λex/λem 440/458 nm) caused by additions of copper. A more energetic band (λex/λem 333/351 nm) is present in marine DOM, but absent in the terrestrial material. The effects of copper on both the intensity and shape of the synchronous-scan fluorescence spectra and this technique's potential use as a tracer are discussed.

Keywords: *fluorescence, humic substances, quenching*

Introduction

The input of organic matter in the ocean, in both its qualitative and quantitative aspects, is still open for discussion[1]. Considered to be a conservative property[2], the fluorescence of humic substances has been shown to be an efficient tool for monitoring the input from continental waters into the oceans[3]. The fluorescence emission of humic substances or naturally occurring organic materials is defined by two main characteristics: a broad, smooth band together with a variation in the wavelength of maximum emission, while changing the excitation wavelength[4].

Qualitatively, fluorescence emission has been used mostly for the characterization of humic substances of differing origin, since the maximum of emission varies according to the origin of the sample. Usually, a comparison of the maximum of emission for a fixed excitation wavelength is done[4,5,6].

The flexibility and sensitivity of spectral techniques arise from the fact that specific excitation and emission wavelengths may be used to select for different chromophore classes. In this sense, fluorescence is a promising tool for investigations related to the input of terrestrially derived organic materials into the ocean. However, any characteristic to be used as a tracer should be conservative, and the technique employed should be able to detect small differences among the various sources of materials, *i.e.*, terrestrial, estuarine, and marine fluorescent materials.

Presenting more structure and selectivity than the conventional fluorescence emission and excitation techniques, synchronous-scan fluorescence discriminates small differences in water mixing studies. It has been shown[3] that the synchronous-scan fluorescence technique is able to detect differences in waters from various rivers in North Carolina, U.S.A., not possible by fluorescence emission.

* present address: Dpto. Botânica, Universidade Federal de São Carlos, C.P. 676, 13560-905 São Carlos - SP, Brazil; e-mail p-atl@iris.ufscar.br

As presented in Lloyd[7] and Vo-Dinh[8], the synchronous-scan fluorescence spectra present combined properties of both emission and excitation spectra. This leads to an increase in selectivity when multicompounds are to be analyzed, giving rise to more structured spectra. The fluorimetric signal may be considered an excitation spectra with the emitted fluorescent light being registered in a synchronized manner[8].

Among the advantages that can be drawn from the utilization of the synchronous-scan fluorescence as a tracer, the most remarkable is that it is a natural property of the environment. This implies cost reduction and avoids the use of disruptive (and often toxic) methods based on the use of dyes.

In addition to dyes[9], synchronous-scan fluorescence suffers from interferences. The spectra are sensitive to chemical factors such as pH[3,11] and metal ions present in natural aqueous samples[3].

In the case of employing the fluorescence property of naturally occurring organic materials as a tracer, knowledge of how any factor interfers in that fluorescence is required. As presented by Cabaniss and Shuman[3], anything that may alter the shape and/or intensity of the spectral peaks should be accounted for.

In the present investigation, we show the effect of a paramagnetic metal (copper) on the synchronous-scan fluorescence spectra of a terrestrially derived humic substance and how this interaction may alter intensity and spectral shape.

Methods

A commercial humic substance (Fluka Chemie, Switzerland, No. 35069989) was used without any further purification.

Fluorescence measurements were performed using a JASCO (model FP 770) spectrofluorometer equipped with a xenon 150W lamp as the light source. The scan velocity was adjusted to 200 nm min^{-1} for both excitation and emission monochromators. All spectra were registered with a 10 nm slit width for the emission monochromator and 5 nm for that of excitation. Measurements were performed on a 1 cm path quartz cuvette.

Synchronous-scan fluorescence spectra were registered from 300 to 600 nm employing a $\delta = 18$ nm. This means that the fluorescence emission was always registered 18 nm ahead of the excitation wavelength. Raman peaks, calculated according to Lloyd[10], should appear at 238 nm, thus they were beyond our working range. Unless otherwise stated, spectra were not blank subtracted.

Using the commonly applied excitation wavelength in fluorescence quenching research[12,13,14], *i.e.*, 350 nm, the fluorescence emission was registered at its maximum (450 nm). Rayleigh scattering was followed at λex 450 nm.

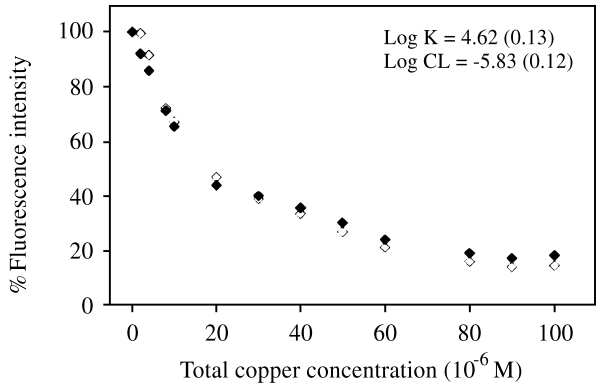

Figure 1. Copper titration curves made of a 10 mg L^{-1} (3 mg L^{-1} TOC) humic substance solution. Each symbol represents one replicate. Ionic strength = 1.5 x 10^{-2} M (KCl), pH = 7.0.

As suggested by Ventry[15], the complexometric titrations were buffered with Hepes (*N*-2-hydroxyethylpiperazine -*N*'-2-ethane-sulfonic acid) at a concentration of 10^{-2} M (Fluka Chimie; ultra-pure), with pH adjusted to 7.00 ± 0.05 (pHM82 - Radiometer, Copenhagen). Whenever necessary, pH was adjusted with a filtered solution of NaOH (Carlo Erba). It has been shown that Hepes presents negligible copper complexing capacity[16].

Ionic strength in the titration cell was adjusted to 1.5 x 10^{-2} M with KCl (Merck, suprapur grade). Three to five min. were maintained as the equilibrium time after each copper addition and fluorescence measurement.

A concentrated copper solution was prepared from the nitrate salt ($Cu(NO_3)_2$, BDH Ltd., U.K.) in Milli-Q water and dilutions were prepared for daily use (10^{-3} M to 10^{-5} M). These were filtered through 0.45 μm membrane filters (Sartorius). All solutions were made in Milli-Q water (Millipore - Bedford, U.S.A.).

The titration curves are shown in Fig. 1. Data treatment was carried out using the nonlinear single binding site model described in Ryan and Weber[12]. Applying a model of multiple binding sites to the present data, we obtained similar values for K, *i.e.*, for two binding sites we obtained $K_1 = K_2$. This result usually applies to standards, and means that one binding site describes the fluorescence quenching data well[17].

Laboratory materials were washed with 3 M HNO_3 and then left in a solution of 0.8 M HNO_3 until use, when they were fully rinsed with Milli-Q water.

After each titration, the quartz cuvettes were rinsed with alcohol (BDH Ltd., Poole, England) and Milli-Q water to remove organic materials, and then with 6 M HNO_3 for metal removal.

After each copper addition, the fluorescence signal was registered by both fluorescence emission and synchronous-scan fluorescence techniques.

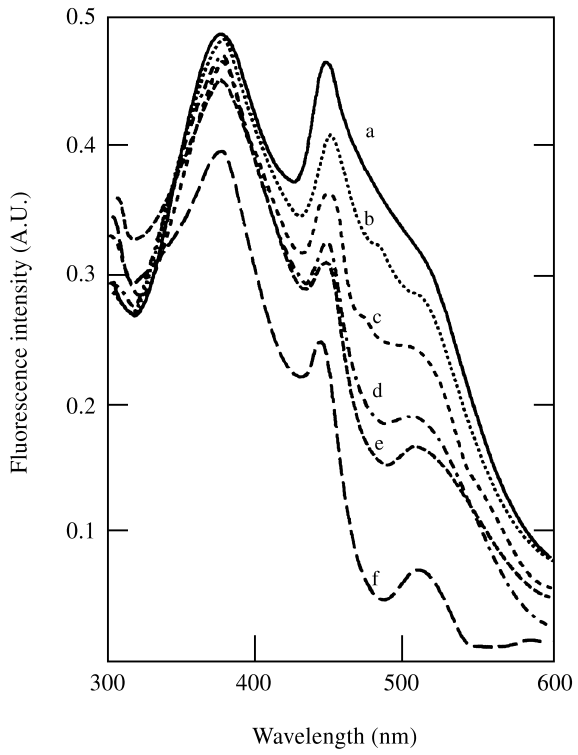

Figure 2. Synchronous-scan fluorescence of a 10 mg L^{-1} humic substance solution. Spectra not blank subtracted. (a) no copper added; (b) 2 x 10^{-6} M copper; (c) 4 x 10^{-6} M copper; (d) 10 x 10^{-6} M copper; (e) 40 x 10^{-6} M copper; (f) 80 x 10^{-6} M copper.

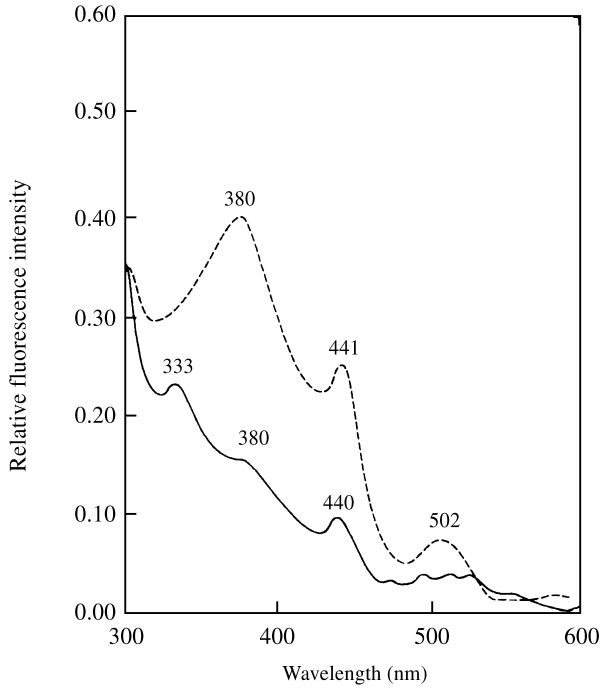

Figure 3. Synchronous-scan fluorescence of a 10 mg L^{-1} (3 mg L^{-1} TOC) humic substance solution with a copper concentration of 80 x 10^{-6} M (dashed line) and marine DOM (10 mg L^{-1} TOC) without copper addition (solid line). Marine DOM spectra was obtained from Lombardi and Jardim[21].

Spectral manipulations were performed on an Apple Macintosh microcomputer.

Total organic carbon (TOC) was measured by high temperature catalytic combustion with infrared detection using a commercial Dorhmann DC-190 carbon analyzer (California, U.S.A.). The calibration curve was made against $C_8H_5KO_4$, and the parameters obtained were: linear regression equation y = 0.99 + 0.912, r = 0.9990; linear range from 1.25 to 75.0 mg L^{-1}.

Results and Discussion

The synchronous-scan spectra obtained with the humic substance before and after the addition of copper are shown in Fig. 2. With no metal added (Fig. 2a), the spectrum shows two main bands, *i.e.*, λex/λem 380/398 nm and λex/λem 450/468 nm. These two fluorescence bands represent the fluorescence of the well-known first and second classes of fluorophores[18]. They may be detected separately by conventional emission at λex 350 nm with the maximum emission around 450 nm, and at λex 450 nm with the maximum emission around 520 nm. The alteration of the spectra after the addition of copper may be seen in Figs. 2b-2d.

As copper is added to the solution and fluorescence is dissimilarly quenched, the shape of the synchronous-scan spectra is gradually modified. When total copper concentration reaches the value of 80 x 10^{-6} M, the shape of the synchronous-scan spectrum (Fig. 2f) presents excitation bands similar to those obtained from the synchronous-scan spectrum of marine dissolved organic materials, with the main difference being in the relative intensity values.

The synchronous-scan fluorescence spectra of humic substance (10 mg L^{-1}, approximately 3 mg L^{-1} TOC) plus copper ion at a concentration of 80 x 10^{-6} M, and marine DOM at a concentration of 10 mg L^{-1} TOC without any metal added, are shown in Fig. 3. The presence of fluorescence bands in both marine and terrestrially derived humic substances at λex/λem 380/398 nm, 440/458 nm, and near 502 nm show a certain degree of similarity between these two categories of organic materials which had been hidden before the severe quenching caused by the metal ion.

An important difference between humic substance and marine DOM is the presence of a band at 333 nm (λex/λem 333/351 nm) in the latter, suggested by Coble and co-workers[19] as being due to the presence of proteinaceous materials, *i.e.*, a result of recent biological activity.

Investigating the fluorescence of marine waters, Coble and co-workers[19] classify those presenting λem 420-450 nm as humic substance-type fluorescence and those at λem 300-340 nm as protein-type fluorescence. Also, evidence has been obtained showing that proteins and hu-

mic substances are largely responsible for the fluorescence of marine waters[20]. The authors[20] show that protein is dominant in the upper water column, while the humic substance-type fluorescence is present mostly in deeper waters. This seems to be an important feature for differenciating between the two categories of organic materials.

The preferencial quenching of the band at λex/λem 440/458 nm has revealed a third class of fluorophores (λex/λem 502/520 nm), originally detected as a shoulder (Fig. 2a). A significant quenching (44%) was observed for the second class of fluorophores when copper concentration reached the value of 80 x 10^{-6} M, while only moderate quenching (13%) was observed for the first class.

The interference in both intensity and shape on the synchronous-scan fluorescence caused by copper addition was first detected at 2 x 10^{-6} M in the second class of fluorophores (λex/λem 450/468 nm), while for the first class (λex/λem 380/398 nm), the interference could be quantitatively measured only at a copper concentration of 40 x 10^{-6} M. These results are in partial agreement with those presented by Cabaniss and Shuman[3], in which the authors investigate possible chemical interference (metals and pH) in the synchronous-scan spectra of river waters employing a $\delta\lambda$ = 25 nm. With additions of copper from 0.1 to 10 x 10^{-6} M, the authors report a decrease in the spectral intensity as a whole, but no significant shape alteration, except for one river sample.

A primary advantage related to the use of synchronous-scanning in fluorescence quenching studies is the simultaneous assessment of two classes of fluorophores. If the fluorescence emission of a molecule such as that of humic substances represents the sum of the fluorescence of all fluorophores, the fluorescence signal of the synchronous-scan is more selective, detecting fluorophores which quench at low copper concentration and at high copper concentration only. Thus, different stability constants and ligand concentrations may be obtained after appropriate data treatment.

Conclusion

We have shown how one interference factor, acting alone, may influence fluorescence properties and lead to problems of interpretation. Certainly, many of these factors act simultaneously in nature. If fluorescence is to be used as a tracer and synchronous-scanning as the detecting tool, then the effects of factors which may cause interference should be known. We have illustrated the importance of a paramagnetic metal (copper) in spectral shape alteration.

The synchronous-scan fluorescence of humic substances derived from terrestrial materials presents two main bands (λex/λem 380/398 nm, λex/λem 450/468 nm). A third band (λex/λem 505/523 nm) is detected only after the second fluorescence band is quenched.

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