DOM
Fluorescence
Estuary
Abiotic parameters
Factorial experiment

MOD
Fluorescence
Estuaire
Paramètres abiotiques
Plan d'expérience factoriel

# Simulation of fluorescence variability in estuaries

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ABSTRACT

Recent studies have revealed differences between the fluorescence spectra of coastal and open seawater. We investigate here the extent to which abiotic parameters such as pH, ionic strength and concentration of dissolved fluorescent matter could be related to this phenomenon. First, pH effect was addressed alone. Second, a $2^3$ factorial experimental design was used to estimate the individual and/or combined contribution of the parameters mentioned above to the spectroscopic characteristics of dissolved organic matter represented here by the Suwannee River Fulvic Acid. High pH variations modify the spectroscopic characteristics of organic matter both qualitatively and quantitatively and those effects seem to be related to the dissociation of the major functional groups (*e.g.* carboxylic and phenolic). General results show that each of the above mentioned parameters affects dissolved fluorescent matter sufficiently to generate slight modifications in its spectral response. These modifications could be related to conformational changes and/or macromolecular weight redistribution of fulvic acid structures. The observations suggest that abiotic parameters could be partially responsible for the spectral differences observed *in situ* and must be taken into account when studying the biogeochemical behaviour of dissolved fluorescent matter through estuaries.

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RÉSUMÉ

## Contribution des paramètres physico-chimiques sur les propriétés de fluorescence de la matière organique dissoute sous conditions estuariennes simulées

Des différences ont été récement observées entre les spectres de fluorescence d'un matériel typiquement continental et d'un matériel marin. Nous étudions ici l'influence des paramètres abiotiques comme le pH, la force ionique et la concentration de matière fluorescente dissoute sur ce phénomène. Dans un premier temps les effets du pH ont été abordés isolement. Deuxièmement, nous avons utilisé une méthode des plans d'expérience du type $2^3$ pour estimer la contribution isolée et/ou combinée des trois paramètres considérés sur les caractéristiques spectroscopiques de la matière organique dissoute représentée ici par l'acide fulvique «Suwannee River». Les variations importantes du pH peuvent influencer qualitativement (position du maximum) et quantitativement (intensité) les spectres de fluorescence de la matière organique, et ces effets

semblent être en relation avec la dissociation des groupes fonctionnels les plus importants (*e. g.* carboxyliques et phénoliques). Les résultats généraux montrent que chacun des paramètres choisis affectent suffisamment la matière fluorescente dissoute pour modifier légèrement sa réponse spectrale. Ces modifications pourraient être associées aux changements de conformation et/ou à la redistribution des masses moléculaires de ces macromolécules. Ces observations suggèrent que les paramètres abiotiques pourraient être partiellement responsables des différences spectrales observées *in situ*, et seraient corrélés au comportement biogéochimique de la matière fluorescente dissoute dans les estuaires.

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## INTRODUCTION

Dissolved organic matter has been the subject of many studies during the last twenty years in marine waters, due to its major contribution to the global carbon budget of the ocean and to major biogeochemical processes such as complexation of metals (Saar and Weber, 1982) and photochemical reactions initiated *via* light absorption by these natural substances (Mopper *et al.*, 1986). Part of this material fluoresces when irradiated with ultraviolet light and this property has been widely used to study biogeochemical cycles involving dissolved organic matter, especially at the continent/ocean interface. Little work has been performed in open marine waters due to the increasing difficulty of measuring fluorescence parameters of samples with very low levels of fluorescence. Most studies performed in general on estuarine or coastal waters are based on samples presenting a significant amount of fluorescent dissolved organic material. Parameters usually considered were only related to the fluorescence intensity measured at a fixed excitation and emission wavelengths. Decreasing linear plots between fluorescence intensity and salinity have been explained as a result of the conservative behaviour of fluorescent organic material during estuarine mixing (Berger *et al.*, 1984).

Recently, a high-resolution and sensitive equipment has enabled us to examine the qualitative aspects of low-fluorescent marine waters. Analyses of the full spectral signature of fluorescence emission of dissolved organic matter have shown that significant differences can be found between spectroscopic characteristics of terrestrial and marine materials (Donard *et al.*, 1989), the most important of these being a blue shift observed between the coastal and marine water fluorescence spectra only when a 3 nm excitation wavelength is used.

Two extreme alternatives could account for the differences observed based on changes of the molecular structure: either a) marine and terrestrial organic matter are originally different, resulting in a different fluorescence signature; or b) fluorescent marine dissolved organic matter results from continentally derived material which is biogeochemically altered during its transport through the estuary. Processes such as photochemistry, complexation with metals, flocculation/precipitation, increase of pH and of ionic strength could be responsible for these transformations. A third alternative could be that the spectral response of the same material is modified by the drastic physico-chemical changes through the estuary without any alteration of its chemical structure.

According to Parker (1968), the fluorescence spectroscopic properties of a given material are strongly dependent on the interaction of the emitting molecules with the solvent molecules and it is known that the physico-chemical characteristics of the aquatic environment change drastically during estuarine mixing. Willey (1984) has observed a slight fluorescence intensity increase in the very low salinity range during early estuarine mixing and attributed this phenomenon to the presence of magnesium.

In this study we have addressed the extent to which abiotic parameters (such as pH, ionic strength and concentration) could alone induce the significant blue-shift on the fluorescence spectra of the same terrestrial organic matter during estuarine mixing.

In a first set of experiments, the effect of pH alone was addressed. Second, in order to reflect the complexity of the interactions between various natural parameters affecting the fluorescence signature during estuarine mixing, we used a $2^3$ factorial experiment to estimate the combined contribution of the parameters mentioned above to simulate the possible fluorescence spectral changes, of the same fluorescing material, likely to occur during estuarine mixing.

A factorial design of experiments permits simultaneous investigation of the relative importance of different factors and their combinations on the phenomenon studied. When there are n factors to be considered, $2^n$ experiments are necessary to measure the effects of all combinations between them (Cochran and Cox, 1957; Box *et al.*, 1978). Among important factors in estuaries and open sea waters, we have chosen here to study the effect of pH, ionic strength (*e. g.* salinity) and organic matter concentration on the spectroscopic characteristics of fluorescent dissolved organic matter. These factors were chosen because they present an important gradient during estuarine mixing. Because of the complexity of estuarine processes and their combined interactions on the parameters mentioned during seaward transport, the factorial design is very appropriate (Donard and Weber, 1985).

## EXPERIMENTAL SECTION

### Reagents and instrumentation

A Suwannee River Fulvic Acid (SRFA) obtained from the International Humic Substances Society (IHSS) was chosen to represent terrestrial derived material. It is a standardized material and its fluorescence properties are similar to those found in continental waters. Information on chemical composition are available in an IHSS special publication (1986). Working solutions were obtained after convenient dilutions of a 50 mg $dm^{-3}$ stock solution made from the solid product.

The pH adjustments were made with metal-free NaOH and HCl (Prolabo) without further purification. All solutions were prepared with Milli-Q water.

The pH values were recorded with a Tacussel Type TS7 pH-meter equipped with a combined glass/calomel electrode (Tacussel XC211) calibrated prior to the pH measurement with standard buffer solutions of pH 4.65, 7.00 and 9.18.

Absorbance was recorded with a Beckman Acta MV1 spectrophotometer with a 5 cm path length cell.

Fluorescence measurements were recorded on a Perkin Elmer MPF-3 spectrofluorometer. This instrument does not correct spectra with respect to instrumental wavelength dependent functions. Excitation wavelengths were always 313 and 370 nm. Rayleigh and Tyndall intensity was measured with excitation and emission wavelengths fixed at 370 nm.

### Absorbance measurements

A UV-visible absorption spectrum was recorded for each solution. Measurements were from 220 to 550 nm and absorbance intensity values were measured at 313 and 370 nm.

### Fluorescence measurements

For a pure compound, the excitation wavelength of its emission fluorescence spectrum is usually chosen on the basis of the maximum of absorption of its UV-visible spectrum. It has been shown that natural water and fulvic acid absorption spectra do not have a well defined band (Bricaud and Sathyendranath, 1981; Donard *et al.*, 1987; Ewald *et al.*, 1988), but only a monotonous decrease in intensity from the UV to the longer wavelengths. Because of this, the choice of appropriate excitation wavelengths in natural water fluorescence study has been a particular problem.

In the present study the 313 and 370 nm excitation wavelengths were chosen for the following reasons: the 313 nm wavelength corresponds to a mercury emission line and has already been employed by other authors (Stuermer, 1975; Nyquist, 1980); his length has proved to be very sensitive for fluorescent dissolved organic matter analysis (Donard *et al.*, 1989). The 370 nm excitation wavelength (close to the 366 nm mercury line) gives more intense and narrower spectra for humic substances than those obtained with shorter excitation wavelengths.

Limits of recording were from 320 to 600 nm for spectra recorded with a 313 nm excitation wavelength and from 380 to 600 nm for spectra recorded with a 370 nm excitation wavelength. Two parameters were measured on each spectrum: the position of the maximum emission, in nm (qualitative aspect); and the fluorescence intensity, in arbitrary units (quantitative aspect).

According to Ewald *et al.* (1983), residual stray light from the exciting monochromator can modify the fluorescence signal of fulvic and humic acids and shift the position of the maximum of emission. All fluorescence spectra presented in this work were systematically corrected for background scattered light (Ewald *et al.*, 1983) as follows: the Tyndall diffusion band (excitation and emission wavelengths fixed at 370 nm) of each solution was simulated by a non fluorescing solution of glycogen. The fluorescence spectrum of this solution was then subtracted point by point from the raw spectrum (Fig. 1).

Fluorescence intensity measurements were also corrected after normalization with respect to the Raman band intensity recorded with Milli-Q water. Measurements of the position of the maximum and of the intensity were only made on each spectrum after these corrections.

Detailed effect of the pH on the fluorescence emission spectrum were made under nitrogen atmosphere after careful degassing of the solution.

Personal bias may introduce errors in the measurement of the maximum of emission of a spectral band as large as those showed by fulvic acid fluorescence spectra (Fig. 1). These errors may also be enhanced by the correction steps. For the first set of experiments (pH effects alone) we

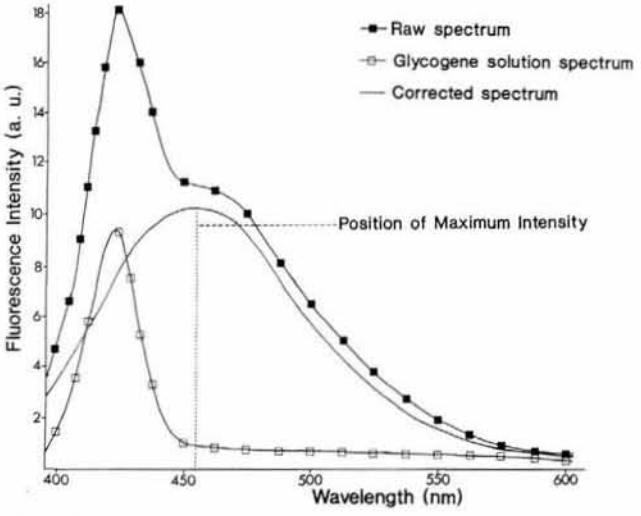


Figure 1

*Emission spectra of a Suwannee river fulvic acid solution (4.5 mg FA $dm^{3}$; 35 g NaCl $dm^{-3}$; pH 8; Exc: 370 nm) before and after correction for Raman and Tyndall scattering.*

Spectres d'émission d'une solution d'acide fulvique Suwannee River (4.5 mg FA $dm^{-3}$; 35 g NaCl $dm^{-3}$; pH 8 ; exc: 370 nm) avant et après corrections tenant compte de la diffusion Raman et de la diffusion Tyndall.

considered a possibility of experimental error of ± 5 nm for each measurement. This experimental error is mainly due to the important glycogen correction needed at high pH values. For the second set of experiments, the degree of reproducibility is defined by the results obtained with four replicate solutions. In this case, increasing accuracy in the data processing and shorter pH range reduced the error to ± 2 nm (Tab. 2).

### Factorial experiment

For the factorial experiment, the parameter levels were chosen to cover the range of variation normally found in estuaries and open sea waters: ionic strength variations were represented by solutions of NaCl of 0, 17.5 and 35 g $dm^{-3}$ arbitrarily designed as low (-), medium (0), and high (+) levels, respectively; organic matter concentrations were simulated by fulvic acid solutions of 1, 4.5 and 8 mg $dm^{-3}$ (-, 0, + levels); pH of solutions were adjusted to give values of 6, 7 and 8 (-, 0, + levels) units of pH (Tab. 1). $2^3$ solutions were prepared using all possible combinations between high- and low-level parameters. Four replicate solutions with parameters at medium level (0) were also prepared for reproducibility. For each solution, fluorescence and absorption spectra were then systematically recorded and spectroscopic parameters measured. Experiments were done in random order to avoid systematic errors such as those due to personal bias or the aging of solutions.

Table 1

*Factorial experiment design parameters definition. IS: ionic strength; FA: fulvic acid concentration in mg $dm^{-3}$.*

Definition des paramètres du plan d'expérience factoriel.IS: force ionique; FA: concentration d'acide fulvique en mg $dm^{-3}$.

| Parameters | Levels of variation | | |
|---|---|---|---|
| | - | 0 | + |
| IS (g of NaCl $dm^{-3}$) | 0 | 17.5 | 35.0 |
| FA (mg $dm^{-3}$) | 1.0 | 4.5 | 8.0 |
| pH | 6.0 | 7.0 | 8.0 |

## RESULTS

### pH against wavelength

The emission spectra of the SRFA solutions were recorded with increasing pH values between 2 and 12, for 313 and 370 nm excitation wavelengths. Figures 2 *a* and *b* show these spectra. Each figure presents two maxima indicating that the pH individually modifies the fluorescence spectra both with respect to intensities and to the position of the maximum of emission. The two maxima for spectra recorded with a 313 nm excitation wavelength (Fig. 2 *a*) are obtained with pH 5 and 8 and those for spectra recorded with a 370 nm excitation wavelength with pH 5 and 9 (Fig. 2 *b*). The variation of 5 nm measured on the position of the maximum for spectra recorded with a 370 nm excitation wavelength is within the experimental error. The most significant modifications with respect to the position of the maximum of emission are observed on spectra recorded with an excitation wavelength of 313 nm: there is a shift of 20 nm (± 5 nm) between the spectrum recorded at pH 5 and that recorded at pH 8. This wavelength has already been proven to be sensitive to modifications in the spectroscopic characteristics of dissolved fluorescent matter (Donard *et al*, 1989).

### Factorial experimental design

Table 2 shows the evolution of spectroscopic parameters recorded during the factorial experiment. The major result obtained is a blue-shift of 9 nm between simulated fresh water (experiment 3) and marine water (experiment 5) conditions, with a 313 nm excitation wavelength.

The first four experiments, which have high fulvic acid concentration, present the highest values of fluorescence and absorbance intensity, as expected. In relation to the position of the maximum of the fluorescence spectra, major differences are observed with spectra recorded with a 313 nm excitation wavelength. The lowest value is 426 ± 2 nm for experiment 5 and the highest value is 436 ± 2 nm for experiment 2, giving a significant difference of 10 nm. For a 370 nm excitation wavelength, this difference is of 7 nm, being the highest value of 457 ± 2 nm for experiment 1 and the lowest value of 450 ± 2 nm for experiments 3, 7 and 8. No noticeable features are found in the Rayleigh intensity set of values.

Table 3 gives the ANOVA results and the significance of parameters of the factorial design for maximum of emission, fluorescence and absorbance intensity and Rayleigh and Tyndall scattering intensity. Underlined (F) values are significant at the 95 % confidence level and reflect the importance of the factor for the spectroscopic parameter considered. The literature F value for 95 % confidence level is 10.13.

*FA*

FA (fulvic acid concentration) values are higher than 10.13 for almost all the parameters measured, being extremely high for fluorescence intensity and very high for absorbance intensity. These results confirm that fulvic acid concentration is the most significant factor for fluorescence and absorbance intensities. Fulvic acid concentration also plays a role in the position of the maximum, for both 313 and 370 nm excitation wavelengths. It can be seen in Table 2 that for a 313 nm excitation wavelength, decreasing fulvic acid concentrations generate a slight blue-shift in emission spectra. Solutions 5, 6, 7 and 8 (low fulvic acid concentrations) have the position of maximum emission blue-shifted in comparison to solutions with high and medium fulvic acid concentrations. For the 370 nm excitation wavelength, fulvic acid concentration has little effect.

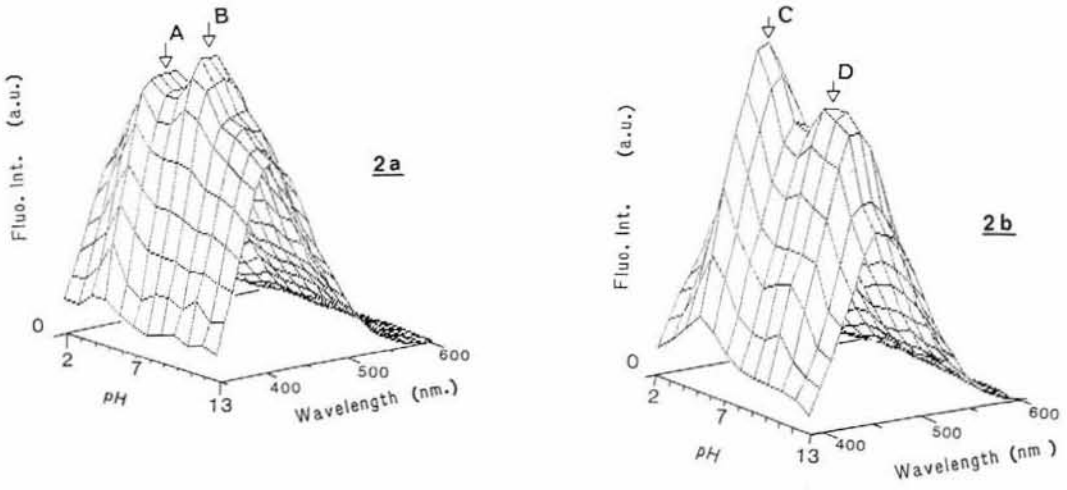


Figure 2

*Three-dimensional plot of fluorescence emission spectra of Suwannee River fulvic acid solutions (4 mg FA dm$^3$) as a function of pH for a 313 nm (2a) and a 370 nm (2b) excitation wavelength, respectively. Spectra are corrected for Raman blank and stray light scattering. Each figure presents two maxima: 2 a) A: wavelength = 440 nm; (F.I.) = 13.5; (pH) = 5; B: wavelength = 420 nm; (F.I.) = 15.2; (pH) = 8; 2 b) C: wavelength = 445 nm; (F.I.) = 17.8; (pH) = 5; D: wavelength = 450 nm; (F.I.) = 15.4; (pH) = 9.*

Représentation en trois dimensions des spectres de fluorescence d'une solution d'acide fulvique Suwannee River (4 mg FA dm$^{-3}$) en fonction du pH pour l'excitation à 313 nm (2 *a*) et à 370 nm (2 *b*). Les spectres ont été corrigés pour la diffusion Raman et pour la diffusion Tyndall. Chaque figure présente deux maximums : 2 a) A: wavelength = 440 nm; (F.I.) = 13.5; (pH) = 5 ; B: wavelength = 420 nm; (F.I.) = 15.2; (pH) = 8 ; 2 b) C: wavelength = 445 nm; (F.I.) = 17.8; (pH) = 5 ; D: wavelength = 450 nm; (F.I.) = 15.4; (pH) = 9

*IS*

IS (ionic strength) has a statistically significant effect on the position of the maximum of spectrum for both excitation wavelengths. Fluorophors excited with 370 nm seem to be relatively more sensitive to ionic strength changes: F = 54.55 for 370 nm against 12.50 for 313 nm (Tab. 3). Table 2 shows that for the four solutions with low fulvic acid concentration, at the same pH conditions (solutions 7 and 5; solutions 8 and 6) , an increase of the ionic strength from 0 (-) to 35g (+) NaCl dm$^{-3}$, generates a slight blue-shift (4 nm) in the position of the maximum of spectra recorded with a 313 nm excitation wavelength. On the other hand, spectra recorded with a 370 nm excitation wavelength are red-shifted (5 and 3 nm, respectively). For the four solutions with high fulvic acid content, changes in the position of the maximum for both excitation wavelength are not significant.

*pH*

pH is only statistically significant with respect to the position of the maximum of spectra recorded with a 370 nm excitation wavelength and to the intensity of spectra recorded with a 313 nm excitation wavelength. Reasons for the nonsignificance of pH in relation to the position of the maximum of spectra recorded with a 313 nm excitation wavelength as well to the intensity of spectra recorded

Table 2

*Factorial experiment results. IS: ionic strength; FA: fulvic acid concentration.*

Résultats de l'expérience factorielle. IS: force ionique; FA: concentration d'acide fulvique.

| Expt | FA | IS | pH | Max. Emission (nm) | | Fluorescence Int. (Arb. Units) | | Rayleigh (Arb. Units) | Absorbance (Arb. Units) x 10$^3$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Exc: 313 nm | Exc: 370 nm | Exc: 313 nm | Exc: 370 nm | Exc: 370 nm | 313 nm | 370 nm |
| 1 | + | + | + | 432 | 457 | 63.1 | 76.1 | 22.0 | 73.2 | 31.0 |
| 2 | + | + | - | 436 | 452 | 65.8 | 75.6 | 15.0 | 78.2 | 31.0 |
| 3 | + | - | - | 435 | 450 | 66.0 | 75.7 | 17.0 | 80.4 | 32.2 |
| 4 | + | - | + | 435 | 455 | 62.7 | 70.6 | 21.5 | 82.4 | 35.2 |
| 5 | - | + | + | 426 | 455 | 9.5 | 10.9 | 17.0 | 6.4 | 1.2 |
| 6 | - | + | - | 428 | 453 | 9.7 | 10.5 | 16.0 | 9.2 | 3.8 |
| 7 | - | - | + | 430 | 450 | 8.7 | 9.8 | 22.0 | 9.8 | 4.8 |
| 8 | - | - | - | 432 | 450 | 9.1 | 10.0 | 16.0 | 11.4 | 5.8 |
| 9 | 0 | 0 | 0 | 432 | 454 | 36.1 | 41.2 | 12.5 | 42.0 | 16.8 |
| 10 | 0 | 0 | 0 | 430 | 453 | 37.6 | 41.5 | 15.0 | 42.8 | 16.8 |
| 11 | 0 | 0 | 0 | 432 | 454 | 36.2 | 40.6 | 13.5 | 32.0 | 11.8 |
| 12 | 0 | 0 | 0 | 432 | 453 | 38.4 | 42.9 | 10.0 | 43.0 | 17.8 |

Table 3

*Statistical significance of parameters. IS: ionic strength; FA: fulvic acid concentration. Underlined values indicate significance at the 95 % confidence level. The literature F value for 95 % confidence level is 10.13.*

Signification statistique des paramètres. IS: force ionique; FA: concentration d'acide fulvique. Les chiffres soulignés indiquent un intervalle de confiance de 95 %. Dans la littérature la valeur F pour un intervalle de confiance de 95% est 10,13.

| Parameter | Max. Emission | | Fluor.Intensity | | Rayleigh | Absorbance | |
|---|---|---|---|---|---|---|---|
| | Exc: 313 nm | Exc: 370 nm | Exc: 313 nm | Exc: 370 nm | Exc: 370 nm | 313 nm | 370 nm |
| F. A. | 60.50 | 13.64 | 4,996.95 | 8,660.64 | 0.57 | 357.35 | 220.85 |
| I. S. | 12.50 | 54.55 | 0.21 | 6.77 | 1.19 | 1.34 | 2.06 |
| pH | 8.00 | 54.55 | 24.45 | 2.15 | 9.68 | 0.25 | 0.01 |
| FA - IS | 4.50 | 6.06 | 1.15 | 2.28 | 0.35 | 0.15 | 0.00 |
| FA - pH | 0.00 | 24.24 | 16.61 | 2.75 | 0.57 | 0.01 | 0.74 |
| IS - pH | 2.00 | 1.52 | 0.37 | 5.45 | 0.18 | 0.31 | 0.36 |
| FA-IS-pH | 2.00 | 1.52 | 0.05 | 3.85 | 1.59 | 0.16 | 0.03 |
| Lack of fit | 0.17 | 4.55 | 0.14 | 2.17 | 18.67 | 1.68 | 1.97 |

*Underlined values indicate significance at the 95 % confidence level. The literature F value for 95 % confidence level is 10.13.*

Les chiffres soulignés indiquent un iuntervalle de confiance de 95 %. Dans la littérature, la valeur F pour un intervalle de confiance de 95 % est 10,13.

with a 370 nm excitation wavelength (opposition of results between set of experiments 1 and 2) are not completely clear, but this result could be biased due to the increasing difficulty of measuring parameters on spectra from very diluted solutions and with very large bands. Table 2 shows that spectra recorded with a 370 nm excitation wavelength are red-shifted with the increase of the pH. In contrast with ionic strength effects, pH effects are only observed in high fulvic acid content solutions (solution 2 to 1: 5 nm, and solution 3 to 4: 5 nm, Tab. 2).

*Combined factors*

Among combined factors the only combination which seems to have statistical significance for fluorescence parameters is the FA-pH pair. Its effects are similar to those generated by pH alone.

One of the most curious findings of this experiment is that the combinations FA-IS , IS-pH and IS-pH-FA are not significant for any of the parameters considered.

Except for fulvic acid concentration, none of the factors affect absorbance intensity for the wavelength chosen.

The "lack of fit", which originates from ANOVA calculations, permits a testing of the curvature of experimental response surfaces. Significance of the "lack of fit" term means that there is not a linear response to the parameter tested (Cochran and Cox, 1957). Rayleigh and Tyndall scattering intensity has a significant "lack of fit", indicating that there is no linearity between pH, IS, and FA increase or decrease and the Rayleigh and Tyndall intensity.

Each of the above mentioned factors and/or physical-chemical effects will be analyzed and discussed separately.

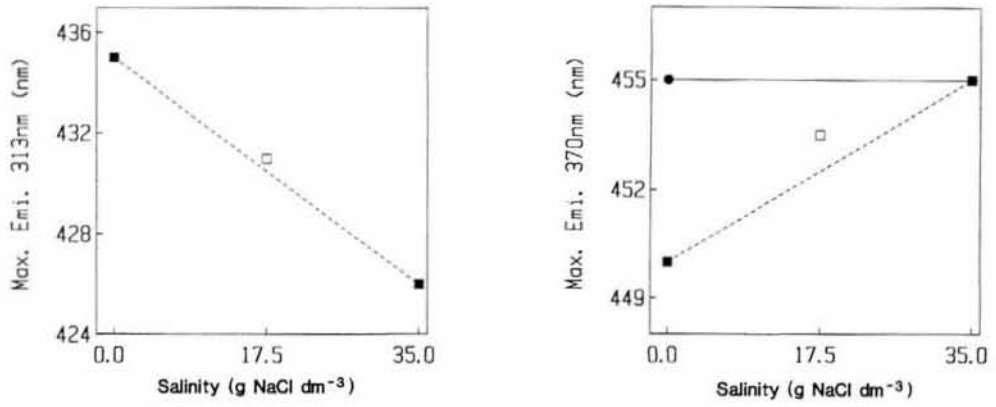


Figure 3

*Variation in the position of the maximum of emission of the fluorescence spectra (3 a, exc.: 313 nm; 3 b, exc.: 370 nm) of the Suwannee River fulvic acid solutions under simulated fresh water, estuarine and seawater conditions (factorial experiment results). 3 a) IS = 0 g NaCl dm$^{-3}$: (■) - pH, + FA IS = 17.5 g NaCl dm$^{-3}$; (❒) 0 pH, 0 FA IS = 35 g NaCl dm$^{-3}$: (■) + pH, - FA; 3 b - IS = 0 g NACl dm$^{-3}$: (■) - pH, + FA; (●) +pH, +FA; IS = 17.5 g NaCl dm$^{-3}$; (❒) 0 pH, 0 FA IS = 35 g NaCl dm$^{-3}$: (■) + pH, - FA.*

Variation de la position du maximum des spectres de fluorescence (3 *a*, exc.: 313 nm; 3 *b*, exc.: 370 nm) de l'acide fulvique Suwannee River dans des solutions simulant les conditions de l'eau douce, de l'eau estuarienne et de l'eau de mer (résultats du plan d'expérience factoriel). 3 a) IS = 0 g NaCl dm$^{-3}$ : (■) - pH, + FA IS = 17.5 g NaCl dm$^{-3}$ : (❒) 0 pH, 0 FA IS = 35 g NaCl dm$^{-3}$ : (■) + pH, - FA ; 3 b) IS = 0 g NACl dm$^{-3}$ : (■) - pH, + FA ; (●) + pH, + FA; IS = 17.5 g NaCl dm$^{-3}$ : (❒) 0 pH, 0 FA ; IS = 35 g NaCl dm$^{-3}$ : (■) + pH, - FA.

*Simulated conditions*

Typical fresh water and seawater conditions are simulated in Table 2 by experiments 3 (pH 6, 0g NaCl $dm^{-3}$ and 8 mg FA $dm^{-3}$) and 5 (pH 8, 35 g NaCl $dm^{-3}$, 1 mg FA $dm^{-3}$) respectively. Spectra recorded with a 313 nm excitation wavelength are blue-shifted by 9 nm from experiment 3 (435 nm) to experiment 5 (426 nm), while those recorded with a 370 nm excitation wavelength are red-shifted by 5 nm (450 nm for experiment 3 and 455 nm for experiment 5). These variations are visualized in Fig. 3 *a* and *b*. In these figures we have also represented the results from experiment 4 which simulates general fresh water conditions at pH 8. For a 313 nm excitation wavelength, results from experiments 3 and 4 are similar (Fig. 3 *a*). The point at 17.5 g NaCl $dm^{-3}$ of salinity corresponds to the arithmetical average of the results from experiments 9, 10, 11 and 12 and represents typical mid-estuarine conditions.

## DISCUSSION

### pH effect alone

Fulvic acids are well known to exhibit typical weak acid polyelectrolyte properties in aqueous solution (Gamble, 1970). The ionization of the functional groups responsible for the fluorescence signal, resulting from changes in pH, alters the molecular geometry in the excited states (Parker, 1968) and this phenomenon could be responsible for the modifications in the intensity and/or position of the maxima observed in Figures 2 *a* and *b*. The fact that the two maxima are not at the same pH values for both excitation wavelengths could indicate that different fluorophors are being excited with these two different wavelengths. We will discuss reasons for the occurrence of these two maxima.

Laane (1982), working with a 365 nm excitation wavelength, has already found similar relationships between fluorescence and pH for estuarine waters. He observed two slopes in the plot of fluorescence intensity versus pH, at pH 3 and 8, and attributed the occurrence of the first slope around pH 8 to the ionization of ammonium and/or phenol groups and the second slope around pH 3 to the ionization of sulphonyl and/or carboxylic acids. Schnitzer and Kahn (1972), using chemical and physical methods of characterization, have shown that humic substances consist of a three-dimensional array of aromatic rings with numerous functional groups of different acid strength and side chains. These peripheral groups are responsible for specific chemical reactivity of the organic matter, and control its geochemical behaviour. By potentiometric acid-base and nonaqueous titrations, other authors (Gamble, 1972; Pobiner, 1983; Andres *et al.*, 1987; Ephraim, 1989) have shown that among these functional groups, two general types of carboxyl groups are present: carboxyl groups which are on the aromatic rings ortho to hydroxilic groups and are quite acidic; and more weakly acidic carboxyl groups, which are not ortho to phenolic groups. The pKa values for hydroxybenzoic acids are as follows: orthohydroxybenzoic acid, 2.97 and 13.40; metahydroxybenzoic acid, 4.06 and 9.92; and parahydroxybenzoic acid, 4.48 and 9.32 (Handbook of Chemistry and Physics, 1969/70). The first values are relative to the dissociation of the carboxylic group and the second to the dissociation of the phenolic group. It is beyond the purpose of this work to specify which of those chemical structures are responsible for the fluorescence signal emitted by fulvic acids; but comparison between these values of pKa and the maxima observed in Figures 2 *a* and *b* indicate that fluorescence parameters are influenced by the pH of dissociation of at least two sets of major functional groups, taking into account the effects of neighbouring groups interaction on the numerical values of these weak acid constants.

### Factorial experimental design

*FA effects*

One the most evident findings of this experiment is that solutions with low fulvic acid concentrations have the position of the maximum for a 313 nm excitation wavelength blue-shifted in relation to those with high fulvic acid contents. One possible explanation for this phenomenon is that spectral responses are modified by interactions between neighbouring functional groups of fulvic acid macromolecules in solution. Ghosh and Schnitzer (1980) reported that above a critical concentration (3.5 to 5.0 mg $cm^{-3}$) humic and fulvic acids behave as rigid polymers or spherocolloïdes due to the lack of space to expand their structure. The highest concentration (8 mg $dm^{-3}$) used in the present work is largely below this critical value, indicating that (when FA concentration is the only factor considered), fulvic acid molecules are present in solution as charged flexible linear colloids. At high concentrations, a decrease in the intermolecular distances of these macromolecules could favour interactions such as hydrogen bonding, Van der Waal's forces, and reactions between free radicals, provoking aggregation. These aggregates have red-shifted the position of the maximum in relation to the original structures. Berger (1984) and Ewald *et al.* (1988) working with fulvic acid of microbiological origin have observed a red-shift in the position of the maximum with increasing molecular weight (exc.: 370 nm) after ultrafiltration. The red-shift, at high concentrations, observed in the present work could be attributed to internal quenching of fluorescence resulting from internal energy loss within the FA molecules, whose molecular weight has increased by aggregation. In dilute solutions, intermolecular charge effects cause these macromolecules to repel each other and little aggregates are formed (Swift, 1989). Original structures then present an overall smaller molecular weight distribution leading to a slight blue-shift again. Spectra recorded with a 313 nm excitation wavelength seem to be more affected by FA effects than those recorded with a 370 nm excitation wavelength. We have already mentioned the higher sensitivity of the 313 nm excitation wavelength to detecting eventual qualitative variations in fluorescent organic matter (Donard *et al.*, 1989). The reasons for this

could be that different fluorophors are being excited by these two excitation wavelengths.

*Ionic strength effects*

The statistical significance of IS could be explained in terms of the modifications in the size distribution of fulvic acid molecules in solution caused by intermolecular charge effects. In the following considerations we have again to assume that fluorophors excited at 313 and 370 nm are essentially different. According to Swift (1989), the addition of an electrolyte in a solution of polyelectrolytes generates molecular association. This effect results from the neutralization of the negative charges and the consequent reduction in the intermolecular coulombic repulsion (Rashid, 1985; Swift, 1989). If salt concentration is sufficiently high, precipitation of associated macromolecules can occur. These phenomenon could have spectroscopic opposite effects. Similar to the case of the concentration effects, an increase in molecule size could induce an internal quenching or energy loss of fluorescence within the FA molecules, resulting in a red-shift. On the other hand, the differential removal of longer wavelengths emitting fluorophors by settling will result in increasing the relative concentration of shorter wavelength emitting fluorophors thus leading to an overall blue-shift. Spectra recorded with a 370 nm excitation wavelength seem to be more influenced by the first effect, and spectra recorded with a 313 nm excitation wavelength more by the second. One possibility is that this phenomenon affects only part of fulvic acid macromolecules. Fulvic and humic acids are known to be polydisperse molecules and then to exhibit a gradient of molecular weights in solution (Reuter and Perdue, 1981; Plechanov, 1983), for which differences in the absorbance and fluorescence properties of the various fractions can be found (Ghassemi and Christman, 1968; Levesque, 1972). Components of lower molecular weight fluoresce more intensely per unit of absorbance (Stewart and Wetzel, 1980) and have the emission spectra blue-shifted (Berger, 1984) in comparison to those of highest molecular weight. For Suwannee River fulvate, Beckett *et al.* (1987) found a molecular weight of 860 with a distribution between 300 and up to 10,000. We suggest that the high macromolecular weight structures are relatively more affected by ionic strength changes and would contribute mostly to fluorophors sensitive to 370 nm excitation wavelength. Fluorophors excited with the 313 nm excitation wavelength would rather be related to smaller size distribution components. Table 2 shows that these phenomena are only observed in the solutions with low fulvic acid contents. Reasons for this fact could be due to IS effects are over ranged at high fulvic acid concentrations by the fulvic acid concentration effects themselves.

*pH effects*

Part of the explanation for the shifts observed could once again be related to changes in the macromolecular size distribution of the fulvic acid in solution. De Haan *et al.* (1983) studying pH effects on soil and aquatic fulvic acids have found that an increase in pH increases its molecular weight and size. This effect may be due to the fact that under more alcaline conditions the acidic functional groups ionize, producing negatively-charged sites, repelling each other and maintaining the molecule in a stretched shape (Rashid, 1985). This effect results in the red-shift observed with a 370 nm excitation wavelength. Changes in the position of the maximum of spectra recorded with a 313 nm excitation wavelength are not of statistical importance. At all events, the results once more suggest that different fluorophors are being excited by these two different wavelengths. Influence of pH on the molecular dimensions of fulvic acids is confirmed by the almost significant F value of 9.68 for pH effect on Rayleigh and Tyndall scattering. This photophysical phenomenon results from the increasing size of molecules in solution.

*Combined effects*

The non-significance of combined factors could be explained in terms of competitive phenomena. A parameter might be regarded as nonsignificant either because it has no real interaction in the process or because it has opposite responses with respect to the two other variables. As mentioned above, increasing IS, FA and pH seem to be responsible for the conformational changes in humic substances either by coiling or expanding the macromolecules, or by promoting its aggregation. One could expect the combination between these parameters rather to intensify the effects. In practice, a blue-shift is observed with spectra recorded with a 313 nm excitation wavelength and a red- shift is observed in spectra recorded with a 370 nm excitation wavelength with the increase of the IS from 0 to 35 g NaCl $dm^{-3}$ and a red-shift is observed in spectra recorded with a 370 nm excitation wavelength with the increase of the pH from 6 to 8. Reasons for this have been discussed above. Paradoxically, a blue-shift is observed in spectra recorded with a 313 nm excitation wavelength with the decrease of the fulvic acid concentration from 8 to 1 mg FA $dm^{-3}$. No noticeable effects on 370 nm excitation wavelength spectra are observed in this case. These facts can be interpreted in the sense that factors considered do not behave in a cumulative manner but are rather competitive when experimental conditions are changed from low to high levels. This effect could explain the non-significance of combinations like FA-IS, FA-IS-pH or even FA-pH (with respect to spectra recorded with a 313 nm excitation wavelength). IS and pH, on the other hand, seem to generate the same kind of effect on fluorescence spectra. However the IS-pH pair has no more statistical significance for the parameters considered. One possible reason for this is that ionic strength effects are only observed in dilute solutions while pH effects are observed in concentrated solutions. The overall effect of the couple is then masked by the fulvic acid concentration effect.

The above considerations reflect the complexity of the system. In fact we may resume the general phenomenon as a competition of two basic processes which are separately exemplified by choosing two different wavelengths. Everything seems behave around conformational changes

and macromomecular weight redistribution within the fulvic acid macromolecules.

*Biogeochemical implications*

The spectral shifts observed in 313 and 370 nm excitation wavelength spectra could result from competitive phenomena occurring with dissolved fluorescent matter during estuarine mixing, aggregation and consequent precipitation. Humic substances constitute 50 to 80 % of the organic matter in natural waters (Rashid, 1985) and are considered to be in great measure responsible for their natural fluorescence (Visser, 1983; Ewald *et al.*, 1983). They are heteropolycondensates with molecular weights ranging from a few hundred to several hundred thousand (Rashid, 1985). After analysis of the factorial experiment results, we suggest that the following events concerning the molecular weight distribution of fluorescent dissolved matter occur through estuaries. Simulation changes in ionic strength, pH and concentration during estuarine mixing generate the aggregation and precipitation of the largest macromolecules leaving differentially the smaller ones in solution. Sholkovitz (1976) had already observed flocculation and precipitation of dissolved organic matter during estuarine mixing. These phenomena have opposite effects on fluorescence spectra. The first could account for the red-shift observed in 370 nm excitation wavelength spectra; while the second is responsible for the blue-shift measured on 313 nm excitation wavelength fluorescence spectra. In natural samples, while no systematic red-shift is observed in 370 nm excitation wavelength spectra (even if shifts higher than 5 nm are found from one sample to another) going from fresh water to open sea water (Donard *et al.*, 1989), the blue-shift measured on spectra recorded with a 313 nm excitation wavelength is as large as 30 nm (Donard *et al.*, 1989; de Souza-Sierra *et al.*, 1991). These results suggest that abiotic parameters considered here are only partially responsible for the differences observed. In the environment at high pH, $Ca^{++}$ and $Mg^{++}$ concentrations should enhance the organic matter flocculation processes (Ryan and Weber, 1982; de Souza-Sierra *et al.*, 1990).

## CONCLUSIONS

The results of the present study indicate that pH is an important factor regulating the spectroscopic characteristics of dissolved organic matter both qualitatively and quantitatively. These modifications should be related to the dissociation constant values of major functional groups (carboxylic and phenolic) present in fulvic acid structures. If the pH range is restricted to that found in estuaries and open seawater (6.5 to 8.3), these modifications are relatively less important.

Major results from factorial experiments show that, individually, each of the abiotic parameters considered may induce slight modifications in the organic matter spectral characteristics but they do not behave in a cumulative manner. These modifications seems to be related to conformational changes and/or molecular weight redistribution of fulvic acid structures.

The blue-shift observed in spectra recorded with a 313 nm excitation wavelength during factorial experiments is largely below that measured in natural water samples, indicating that the spectral shift observed between continental and marine waters cannot account only for the changes of the physico-chemical environmental conditions but could also be related to differences in the quality of continental and open sea organic matter and/or important structural modifications during estuarine mixing.

Finally, due to the high variety of chemical and physical factors present in estuaries and which are likely to modify organic matter spectral characteristics, results from factorial experiments which take in account only three abiotic parameters cannot be considered as definitive. However the extension and the type of modifications observed in spectra recorded with a 313 and a 370 nm excitation wavelength when changing abiotic parameter conditions emphasize the critical aspect of the choice of the excitation wavelength in natural waters fluorescence analysis. The study of the estuarine behaviour of fluorescent organic matter should be based on the recording of the full emission spectra and using several excitation wavelengths.

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