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Chemosphere 63 (2006) 430–439

CHEMOSPHERE

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# Effects of pH and electrolyte concentration on the binding between a humic acid and an oxazine dye

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Received 23 April 2005; received in revised form 12 August 2005; accepted 17 August 2005
Available online 22 November 2005

**Abstract**

The binding between an oxazine dye and a humic acid was studied in aqueous solutions in the pH range 4–10 and in the supporting electrolyte (KCl) range 0.001–0.1 M. A rather simple spectrophotometric method was developed to construct binding isotherms under conditions were traditional centrifugation or filtration methods fail. The use of this method is possible because humic acid molecules have the ability of changing the spectrum of dye molecules, and this ability is used to quantify the isotherms. All binding isotherms have a Langmuirian shape. The amount of bound dye is strongly dependent on the ionic strength and less dependent on the pH of the solution. The binding is rather strong and mainly driven by non-electrostatic forces. Whereas the Langmuir binding constant is independent of the pH and electrolyte concentration, the number of assessable sites in humic acid for binding oxazine increases by increasing pH and decreasing electrolyte concentration. These results can be directly related to the flexibility of humic acid molecules, which can swell at high pH and low ionic strength, increasing consequently the availability of binding sites. The results also indicate that humic substances may strongly affect the mobility and fate of dyes and related pollutants in the environment.


*Keywords:* Humic substances; Dyes; Binding isotherms; Organic pollutants

## 1. Introduction

Dyes are extensively used in photographic, food, textile, printing and cosmetic industries as well as in medical and related biological sciences (Duxbury, 1993). As a consequence of their many applications, dyes are important organic pollutants that mankind are pouring into the environment, and therefore research must be directed towards assessing the extent of dye pollution and finding good waste-water treatments.

Dyes are organic molecules that occur in several forms in aquatic environments. In principle, they can be freely dissolved in water or interacting with the components of soil or sediments, mainly minerals and refractory organic matter. The extent of these interactions is a

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0045-6535/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2005.08.053

key factor that controls the mobility and fate of dyes in the environment. The interaction with mineral surfaces has been relatively well studied (Hähner, 2002; Neumann and Gessner, 2002; Rytwo et al., 2002). However, there is very limited information on the binding between dyes and refractory organic matter (Banerjee et al., 1973; Ohga et al., 1990), in spite of the importance that this interaction could have in the environment.

Refractory organic matter in aqueous media is mainly represented by humic substances (HS), such as humic acids (HA) and fulvic acids (FA) (Sparks, 2003). These substances come from the decomposition of plant and animal tissues, and occur in aquatic environments either as dissolved molecules or coating the surface of mineral particles. They are natural weak polyelectrolytes that are very active in binding ions and molecules. Through this binding, HS may affect greatly the environmental behavior of dyes and other organic pollutants. Koopal et al. (2001) have pointed out that HS have a dual role in this respect. Whereas HS that are dissolved in water or coating suspended mineral particles will tend to increase the mobility of the attached pollutants, HS that are forming part of a soil or sediment matrix will decrease it.

There is a rather clear explanation for the scarcity of data related to the interaction between HS and dyes or other soluble pollutants, as compared to the wealth of information existing with mineral particles. Adsorption on mineral particles can be measured by equilibrating a mineral dispersion with a dye solution followed by a separation of the solid phase by centrifugation or filtration. This separation allows a relatively easy quantification of the free pollutant in the supernatant solution, data that is used to evaluate the extent of the adsorption. On the contrary, since most of the HS are soluble in natural waters at normal pH, they cannot be easily separated from the supernatant, complicating the quantification of the free pollutant in solution. This is why alternative methods were undertaken to avoid this complication. Examples of alternative methods for measuring pollutant–HS interactions are immobilization of HS at the surface of solid particles (Yang and Koopal, 1999; Prado et al., 2003), immobilization of HS in a sol-gel matrix (Laor et al., 2002), use of dialysis membranes (Govi et al., 1996), fluorescence spectroscopy (Laor and Rebhun, 2002), etc.

Dyes have the advantage of showing a strong absorption spectrum in the visible region. Their high extinction coefficients make it possible to detect them at very low concentrations with unsophisticated instruments, such as an UV/VIS spectrophotometer. In addition, the spectrum of a dye usually changes when it is interacting with other molecules or solid surfaces. These combined properties are very useful to determine the nature and extent of this interaction. Thus, UV/VIS spectroscopy was used to study the aggregation of dye molecules (Stork et al., 1972), and their interaction with synthetic polyelectrolytes (Peyratout et al., 2001), DNA molecules (Yamaoka et al., 1982), and clay surfaces (Hähner, 2002; Neumann and Gessner, 2002). Moreover, through these interesting properties, dyes are also regularly used as model molecules to evaluate the behavior of organics in different media (Hähner, 2002; Rytwo et al., 2002) or as probe molecules to explore the properties of different systems (Kayanasundaran, 1987; Avena et al., 2001).

This article presents a UV/VIS study of the interaction between an oxazine dye (Oxazine 1, OX) and a soil humic acid. The aims are to quantify the extent of the binding and to characterize their interaction by analyzing the changes of the dye spectrum caused by the presence of the humic acid in solution. Through the quantification of binding isotherms at different pH and ionic strength conditions, binding constants are determined and information about the structural properties of the HA is also obtained. This will allow us to assess the importance of the interaction in determining the mobility of dyes in the environment.

## 2. Experimental

### 2.1. Materials

The HA sample used in this work was taken from an andisol (Boqueixon, A Coruña, Spain) on anfibolite under an Ulex Europaeus vegetation cover. It was extracted and purified following the procedure recommended by the International Humic Substances Society (Swift, 1996; Gondar et al., 2005). The elemental composition of this purified sample is N (4.86%), C (52.57%), H (5.06%), O (37.18%) and S (0.33%). Acid–base potentiometric titration experiments (not shown here) as described by Gondar et al. (2005) have been performed to HA in order to investigate its protonation–deprotonation behavior. They revealed that HA develops pH-dependent negative charges as a consequence of the deprotonation of reactive groups such as carboxylic and phenolic groups. Its charge varied in 0.1 M KCl from −0.8 mmol/g at pH around 4 to −4.5 mmol/g at pH around 10. The charging curves at lower electrolyte concentration had the same shape than that at 0.1 M and were only slightly shifted towards less negative values. It varied in 0.001 M KCl from −0.6 mmol/g at pH 4 to −4.3 mmol/g at pH 10. This is a very common behavior for humic acids (Avena et al., 1999) and reveals their polyelectrolyte nature. Solid state $^{13}C$ nuclear magnetic resonance, as obtained with a Bruker AMX 300 spectrophotometer, indicates that the studied sample contains 29% of alkyl C (0–45 ppm), 38% of O-alkyl C (45–110 ppm), 19% of aromatic and phenolic C (110–160 ppm), 11% of carboxyl C (160–190 ppm) and 3% of carbonyl C (190–240 ppm) (Gondar et al., 2005).

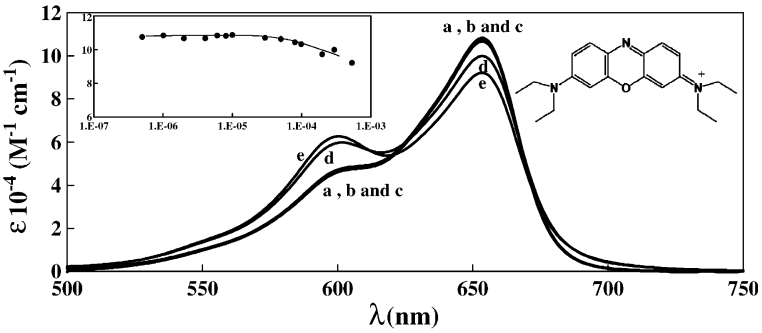


Fig. 1. Spectra of OX in aqueous solution at different concentrations: (a) $4.99 \times 10^{-7}$ M, (b) $4.08 \times 10^{-6}$ M, (c) $1.00 \times 10^{-5}$ M, (d) $2.97 \times 10^{-4}$ M and (e) $5.35 \times 10^{-4}$ M. The left-handed inset shows a $\varepsilon_D$ $10^{-5}$ ($M^{-1}$ $cm^{-1}$) vs. $D$ (M) plot for different OX solutions. The right-handed inset shows the molecular structure of oxazine 1.

The dye used was Oxazine 1 perchlorate (OX), which was obtained from Aldrich (Spain) and used without further treatment. It is a planar monocationic molecule with three connected aromatic rings. Its structural formula is shown in the inset of Fig. 1.

All other chemicals (KCl, HCl, and KOH) were of Merck p.a. quality, and all solutions were prepared with distilled-deionized water. pH 4.00, 7.00 and 10.00 buffers (Crison, Spain) were used to calibrate the glass electrode.

### 2.2. Methods

Preliminary experiments were performed in order to evaluate the association between OX molecules in absence of HA at different pH and ionic strengths. It is well known that in aqueous solutions most cationic dyes are present as monomeric species at low concentrations but undergo dimerization and further aggregation as the concentration increases. This process produces spectral changes that are evidenced by the appearance of new absorption bands, or a shift of previously existing bands in the UV/VIS spectrum. This effect is called metachromatic effect (Schubert and Levine, 1955). In the experiments, OX solutions in the concentration range $4.99 \times 10^{-7}$–$5.35 \times 10^{-4}$ M were prepared at the desired pH (adding HCl or KOH) and ionic strength (adding KCl), and were directly analyzed in the UV/VIS spectrophotometer by measuring their absorption spectra in the 500–750 nm wavelength range. Different Hellma quartz cells having path lengths of either 1 cm, 0.1 cm or 0.01 cm were used for these measurements. KCl solutions of the corresponding concentration and pH were used as blanks.

The interaction between OX and HA was measured by titrating an OX solution with a stock HA solution (concentration about 2.1 g/l) and recording the UV/VIS spectrum after each titrant addition. For this, 100 ml of a $8 \times 10^{-6}$ M OX solution having the desired ionic strength and pH were placed in a thermostated glass reaction vessel, stirred with a magnetic bar and bubbled with $N_2$. Two millilitres of the solution were withdrawn with an automatic pipette and placed into the 1-cm spectrophotometer's quartz cell. Once the spectrum of the dye was recorded, the solution was reincorporated to the reaction vessel and a small measured volume (usually between 0.05 ml and 0.5 ml) of the concentrated stock HA solution having the same pH and KCl concentration was added. The resulting OX–HA solution was left to equilibrate for 1–2 min (time necessary to achieve good mixing and constancy in spectral reading) and a new aliquot was withdrawn to proceed with a new spectrum recording. This procedure (HA addition and spectrum recording) was repeated until the HA concentration was around 140 mg/l in 0.001 M KCl solutions, 160 mg/l in 0.01 M KCl solutions and 250 mg/l in 0.1 M KCl solutions. The pH of the OX–HA solution was continuously measured during the experiments. No significant changes in it were observed ($\pm$0.05 pH units).

Experiments were performed at pH 4.0, 6.0, 8.0 and 10.0 and at KCl concentrations 0.1 M, 0.01 M and 0.001 M. The pH was measured with a Crison 2002 pH-meter equipped with a Radiometer GK2401C (Ag/AgCl reference) combined glass electrode. Absorption spectra were recorder with a JASCO V-530 UV/VIS spectrophotometer. The working temperature was always $25.0 \pm 0.1$ °C.

### 2.3. Data analysis

The method employed in this work to measure interaction between OX and HA allows to perform a whole titration run at a fixed total OX concentration (the titrated solution) and variable HA concentration (the titrant). This method is similar to that reported by Tatikolov and Costa (2004) for the study of the interaction between polymethine dyes and human serum

albumin, although the analysis of data is somewhat different. Since HA is formed by polymeric molecules with many functional groups that can attach OX molecules (see Section 4), the following equilibrium is postulated to occur between OX molecules and HA groups:

$$-\mathrm{S} + \mathrm{D} \rightleftarrows -\mathrm{SD} \quad (1)$$

where −S is a certain site or region in the HA molecule for binding the dye, D is the dye molecule in solution and −SD represents the dye molecule attached to the −S site.

Before starting the titration, only monomeric D species is present in the solution. As the titration proceeds, the equilibrium is shifted to the formation of −SD species, and eventually almost all the dye becomes attached to HA sites and only a negligible amount remains as free dye in the solution. Therefore, the initial spectrum corresponds to that of pure monomeric OX, and the rest of the spectra correspond to those of OX–HA solutions. Since under our experimental conditions the absorbance of HA is relatively low in the wavelength range investigated as compared to that of OX, the spectra of the OX–HA solutions could be safely corrected by subtracting the spectra of the corresponding pure HA solutions. This correction allows focusing the analyses in the spectrum of OX, either in absence or presence of HA. Therefore, the corrected spectra represent the contribution of two absorbing species: monomeric OX in aqueous solution (D) and OX attached to HA functional groups (−SD). This two-component system can be easily solved at any working condition if the spectra of the pure components are known. The solution is as follows:

The total absorbance, $A$, is the sum of the absorbances of both components:

$$A = \varepsilon_{\mathrm{D}} b[\mathrm{D}] + \varepsilon_{-\mathrm{SD}} b[-\mathrm{SD}] \quad (2)$$

where $\varepsilon_{\mathrm{D}}$ and $\varepsilon_{-\mathrm{SD}}$ are respectively the extinction coefficients of the free dye in solution and of the bound dye at the studied wavelength, $b$ is the path length of the quartz cell, and [D] and [−SD] are respectively the concentrations (mol/l) of free and bound dye. In addition, because the experiment is performed at a constant total dye concentration, $[\mathrm{D}]_0$, the following equality holds for a whole titration run:

$$[\mathrm{D}]_0 = [\mathrm{D}] + [-\mathrm{SD}] \quad (3)$$

By combining Eqs. (2) and (3), and rearranging:

$$[\mathrm{D}] = \frac{A - \varepsilon_{-\mathrm{SD}} b[\mathrm{D}]_0}{\varepsilon_{\mathrm{D}} b - \varepsilon_{-\mathrm{SD}} b} \quad (4)$$

from which the concentration of free dye in solution can be known at any point of the titration, provided $A$, $[\mathrm{D}]_0$, $b$, $\varepsilon_{\mathrm{D}}$ and $\varepsilon_{-\mathrm{SD}}$ are known.

Once [D] is known, [−SD] can be calculated from Eq. (3). In addition, the amount of dye bound per unit mass of HA, $D_{\mathrm{HA}}$, can be calculated by dividing [−SD] by the concentration of HA (g/l) at every titration point. Therefore, binding isotherms can be constructed by plotting $D_{\mathrm{HA}}$ as a function of [D].

The binding was analyzed in terms of the Langmuir isotherm:

$$D_{\mathrm{HA}} = \frac{K_{\mathrm{L}} N_{\mathrm{S}} [\mathrm{D}]}{1 + \mathrm{K}_{\mathrm{L}} [\mathrm{D}]} \quad (5)$$

where $K_{\mathrm{L}}$ is the Langmuir constant for process 1 and $N_{\mathrm{S}}$ is the number of −S sites in HA available for binding the dye. $K_{\mathrm{L}}$ quantifies the affinity of the sites for the attaching molecule. For the binding between charged molecules or between a charged molecule and a charged surface this affinity is normally the result of an electrostatic affinity, that quantifies the long-range electrostatic attraction or repulsion, and an intrinsic (or chemical, or non-electrostatic) affinity that quantifies other type of interactions, such as chemical bonds, van der Walls forces, or other non-electrostatic interactions. As stated above, $N_{\mathrm{S}}$ quantifies the number of sites that are available or assessable for binding. In solid surfaces, such as mineral surfaces, it usually takes a constant value and is independent of the solution conditions. In flexible molecules such as polyelectrolytes, it may change by changing conditions such as pH and ionic strength as a consequence of the structural and conformational changes that the polyelectrolyte may suffer.

Any wavelength could be used in principle to perform the data analysis described above. However, the error is minimized by working at a wavelength where the addition of HA produces the largest changes in the absorption spectrum of the dye. This is why 653 nm, which corresponds to the absorption maximum of monomeric OX in solution, was selected as the working wavelength in most of the cases. Other wavelengths were also used in order to show the applicability and goodness of the method.

## 3. Results

### 3.1. Aggregation of OX in absence of HA

Fig. 1 shows the spectra of several OX solutions in the concentration range $4.99 \times 10^{-7}$–$5.35 \times 10^{-4}$ M. At $4.99 \times 10^{-7}$ M, $4.08 \times 10^{-6}$ M and $1.00 \times 10^{-5}$ M all solutions show the same spectrum, with an absorption band at 653 nm and a shoulder at 600 nm. This spectrum is characteristic of monomeric oxazine 1 (Berberan-Santos et al., 2000). At $2.97 \times 10^{-4}$ M and $5.35 \times 10^{-4}$ M the spectrum changes: there is a decrease in the absorbance at 653 nm and a new band at around 600 nm appears. This metachromatic effect indicates that at these relatively high concentrations dimers and/or higher aggregates are formed (Schubert and Levine, 1955; Pal and Schubert, 1963). A plot of $\varepsilon_{\mathrm{D}}$ vs. OX

concentration (inset of Fig. 1) with data obtained at 653 nm shows that $\varepsilon_D$ begins to decrease at concentrations higher than $3 \times 10^{-5}$ M. Therefore, below this concentration, OX is in its monomeric form; above it, dimers or higher aggregates are formed.

### 3.2. Interaction between HA and OX

Fig. 2 shows the spectra of a $8 \times 10^{-6}$ M OX solution to which different amounts of HA were added. The presence of HA modifies the spectrum of OX. As HA concentration increases, the absorbance of OX decreases at wavelengths below 671 nm and increases above it. This wavelength represents an isosbestic point.

Although not shown here, plots of A vs. HA concentration at different wavelengths revealed that the spectrum of OX did not change linearly with HA concentration. Changes were relatively important at low HA concentrations and very small at high HA concentrations. A limiting spectrum was eventually reached at high HA/OX concentration ratios. In fact, in the case of the example shown in Fig. 2, the spectrum at 256.5 mg/l HA can be already considered as the limiting one. Spectra at higher concentrations coincided with this one.

The presence of an isosbestic point suggests that only two interconverting OX species are contributing to the total absorbance in the spectra of Fig. 2 (Connors, 1987). One of these species is the monomeric form of

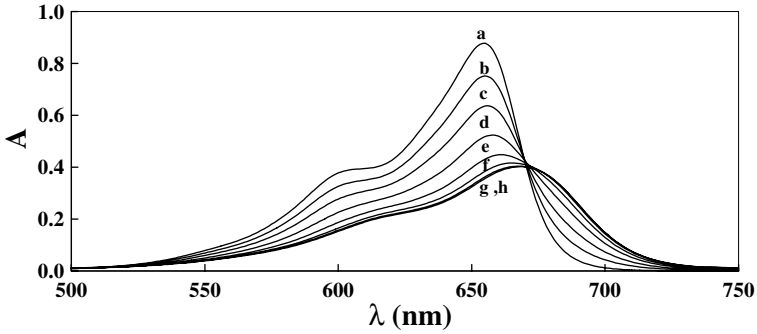


Fig. 2. Absorption spectra of OX ($8.09 \times 10^{-6}$ M) in KCl 0.1 M at pH = 4 at different HA concentrations (mg/l): (a) 0, (b) 17.75, (c) 40.93, (d) 76.54, (e) 126.7, (f) 177.61, (g) 241.32 and (h) 256.55.

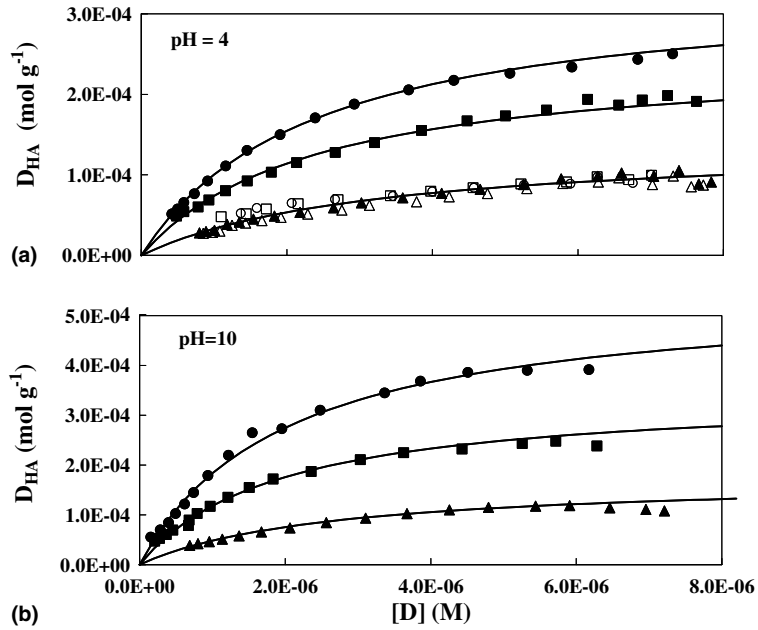


Fig. 3. Binding isotherms at (a) pH 4 and (b) pH 10, and different ionic strengths: (●) 0.001 M, (■) 0.01 M and (▲) 0.1 M. Lines have been drawn according to the Langmuir isotherm and parameters in Table 1. The working $\lambda$ was 653 nm, except for open symbols, which represent data at pH 4 and ionic strength 0.1 M calculated with (○) $\lambda = 710$ nm, (□) $\lambda = 700$ nm, and (△) $\lambda = 600$ nm.

OX in aqueous solution, whose spectrum is the one corresponding to 0 mg/l HA. From this spectrum the value of $\varepsilon_D$ at a given wavelength can be known. The other species is OX interacting with HA, whose spectrum must be the limiting spectrum found at high HA/OX concentration ratios, where the concentration of free OX in solution is negligible. Thus, the value of $\varepsilon_{-SD}$ can also be obtained. The isosbestic point and the limiting spectrum obtained justify the use of a simple equilibrium between OX in solution and OX bound to HA, such as that described by reaction 1, and allow applying equations (2)–(4) in order to solve the two-component system.

### 3.3. Binding isotherms

Fig. 3 shows the effect of the supporting electrolyte concentration on the binding isotherms obtained at two extreme pH values (4 and 10). Symbols correspond to data points whereas lines correspond to the best-fitting Langmuir isotherms calculated by adjusting the parameters $K_L$ and $N_S$. These parameters are listed in Table 1. Four different sets of data points are drawn for pH 4 and 0.1 M KCl concentration. These different sets were obtained by analyzing the experimental data at four different wavelengths (600 nm, 653 nm, 700 nm and 710 nm). The good coincidence of data demonstrates the applicability of the method. All isotherms could be well fitted with the Langmuir equation. Changes in the ionic strength result in important changes in the binding isotherms, which are reflected by an increase in the binding as the ionic strength decreases. Similar electrolyte concentration effects were found by working at pH 6 and 8 (not shown).

Fig. 4 is analogous to Fig. 3 and shows the effects of pH at two extreme ionic strengths (0.001 M and 0.1 M

Table 1
Langmuir isotherm parameters for the interaction between OX and HA

| pH | Log $K_L$ (l mol$^{-1}$) | | | $N_S$ (mol g$^{-1}$) | | |
|---|---|---|---|---|---|---|
| | $10^{-3}$ M KCl | $10^{-2}$ M KCl | $10^{-1}$ M KCl | $10^{-3}$ M KCl | $10^{-2}$ M KCl | $10^{-1}$ M KCl |
| 4 | 5.62 | 5.62 | 5.49 | 3.39E−04 | 2.52E−04 | 1.40E−04 |
| 6 | 5.61 | 5.60 | 5.62 | 3.40E−04 | 2.48E−04 | 1.10E−04 |
| 8 | 5.61 | 5.57 | 5.49 | 4.70E−04 | 2.99E−04 | 1.45E−04 |
| 10 | 5.70 | 5.71 | 5.58 | 5.50E−04 | 3.45E−04 | 1.75E−04 |

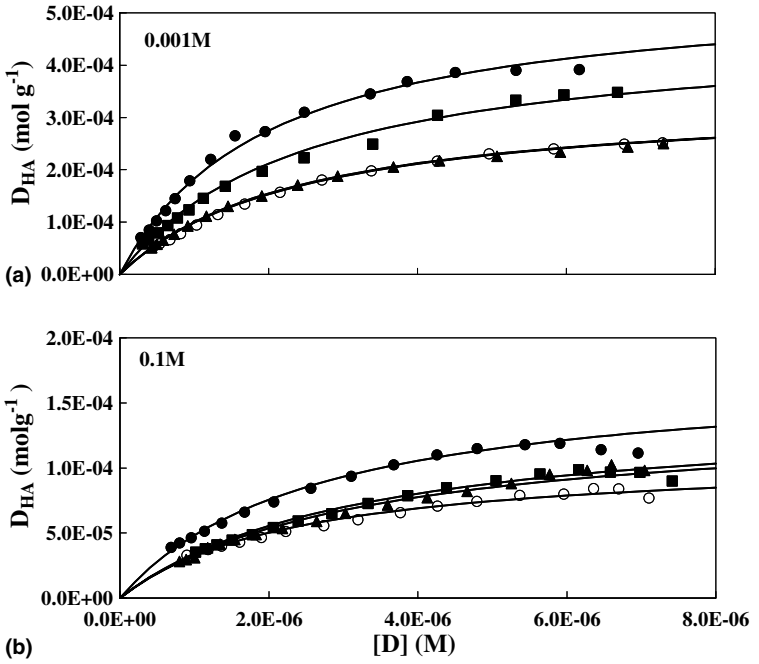


Fig. 4. Binding isotherms in 0.001 M and 0.1 M KCl at different pH: (▲) pH = 4, (○) pH = 6, (■) pH = 8 and (●) pH = 10. Lines have been drawn according to the Langmuir isotherm and parameters in Table 1.

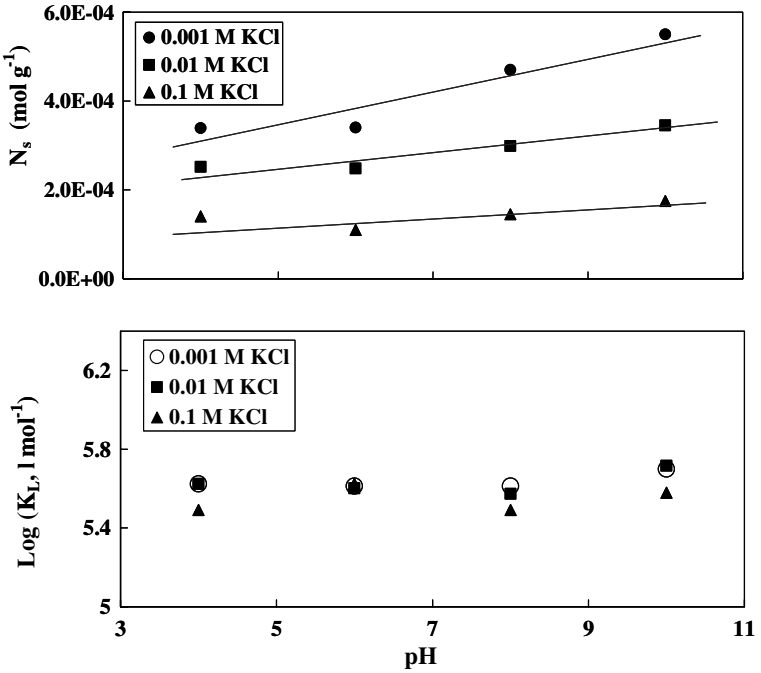


Fig. 5. $N_S$ and Log$K_L$ values as a function of pH at the three studied ionic strengths.

KCl). In 0.1 M KCl the change of pH between 4 and 8 does not result in important changes in the binding isotherms; only an increased bound amount can be observed at pH 10. In 0.001 M KCl the effects of changing the pH are more obvious, especially between pH 6 and 10. Increasing the pH increases the amount of bound OX. An intermediate behavior was found using a 0.01 M KCl solution (not shown). Data in Figs. 3 and 4 exemplify the general trend observed: the attachment of OX to HA depends mainly on the electrolyte concentration and less on the pH.

Fig. 5 resumes all the measured data by plotting the fitting parameters $N_S$ and Log$K_L$ as a function of pH and electrolyte concentration. $N_S$ is almost independent of pH in 0.1 M KCl, somewhat dependent in 0.01 M KCl and clearly dependent on pH in 0.001 M KCl. In these two last cases, $N_S$ increases as the pH increases. No significant variations in Log$K_L$ were observed, either by changing the pH or the electrolyte concentration.

## 4. Discussion

### 4.1. Aggregation of OX in absence of HA

Data in Fig. 1 indicates that OX behaves as most cationic dyes do in aqueous solutions, changing the spectra above a certain concentration. These spectral changes are usually explained by using the theory elaborated by Kasha et al. (1965), which is based on a simplified orbital interaction principle and analyses two cases for planar molecules such as OX: (i) spectral changes due to a face-to-face aggregation of molecules, which leads to the formation of the so called H-aggregates, and (ii) spectral changes due to a side-by-side aggregation, which leads to the formation of J-aggregates. According to this theory, the formation of aggregates gives rise to new molecular orbital levels that result in the appearance of a new absorption band at shorter wavelengths in the case of H-aggregates, and at longer wavelengths in the case of J-aggregates (Neumann and Gessner, 2002). The decrease in $\varepsilon_D$ (563 nm) and the appearance of a new absorption band at shorter wavelengths when OX concentration exceeds $3 \times 10^{-5}$ M (Fig. 1) is consistent with and the formation of H-aggregates above this concentration. Similar results were informed by Herkstroeter et al. (1990).

### 4.2. Interaction between HA and OX

Most cationic dyes also suffer spectral changes when interacting with solid surfaces, biopolymers, polyelectrolytes or other molecules as a consequence of structural and/or electronic changes produced to the dye molecule (Rytwo et al., 2002; Tatikolov and Costa, 2004). The type of interaction between OX and the studied HA appears to be similar to that that proposed by Peyratout et al. (2001) to explain the binding between bisindolenyl-

pentamethine (a cationic dye) and polystyrene sulfonate (a negatively charged polyelectrolyte). According to these authors, the direct binding of the dye to the polyelectrolyte disturbs the symmetry of the delocalized π-electron system in the dye, causing a relatively small shift of the absorption bands to longer wavelengths, such as it is observed in Fig. 2. This kind of π-bonding is normally suggested for the interaction between cationic dyes and negatively charged surfaces (Neumann and Gessner, 2002) or molecules (Koopal et al., 2004). HA contains negatively charged groups (carboxylate and phenolate) with a strong affinity for positively charged ions, and thus a direct interaction between these functional groups in HA and OX could be postulated. In addition, the carbon skeleton of HA molecules contains hydrophobic regions due to the presence of aliphatic and aromatic components, which are very active in binding organic molecules, such as carbohydrates, pesticides, dyes, etc. (Sparks, 2003). Thus, it is also possible that OX attaches HA molecules by interacting with their hydrophobic regions.

In many cases, interactions with solid surfaces or other molecules can also promote dye aggregation with the consequent spectral changes (Peyratout et al., 2001; Neumann and Gessner, 2002). However, data in Fig. 2 clearly indicates that HA is not promoting formation of either H-aggregates or J-aggregates since the spectra of OX–HA mixtures do not correspond at all with the spectra of these aggregates (Wang et al., 2000; von Berlepsch et al., 2002).

### 4.3. Binding isotherms

The conditions for the validity of the Langmuir isotherm at a given pH and electrolyte concentration are: (a) established equilibrium between OX in solution and OX bound to HA, (b) homogeneity of binding sites in HA (i.e. all −S sites have the same affinity for OX molecules), and (c) absence of interaction between bound OX molecules. Not all of the above assumptions may hold in practice, even when good fit is obtained. More sophisticated isotherms could be proposed. Yang and Koopal (1999), for example, introduced electrostatics to the Langmuir isotherm in order to take into account the charge development in the HA molecules. In addition, in view of the observation of Avena et al. (1999), who showed that HA behave as flexible entities that can swell or shrink in response to changes in pH and ionic strength, size variations and the consequent changes in the number of assessable sites for binding should also be considered. All these sophistications require the use of extra parameters or functions, such as electric potentials and distribution functions, and most of them cannot be independently determined. Therefore, the simple Langmuir isotherm is used in this work as a description of macroscopic data and as a tool to gain some insights into the binding of OX to HA and into the properties of the studied HA.

Figs. 3 and 4 reveal that the attachment of OX to HA depends mainly on the electrolyte concentration and less on the pH (the pH dependency is only important at low electrolyte concentration). Conversely, the development of negatively charged carboxylic and phenolic groups in HA depends mainly on the pH and much less on the electrolyte concentration. On the other hand, at pH 4 the maximum amount of bound OX ranges between 20% and 40% (depending on the ionic strength) of the negatively charged groups in HA, whereas this value ranges between 3% and 16% at pH 10. These data indicate that there is no direct correlation between the number of negatively charged groups in HA and OX binding, and suggest that these groups are not the main binding sites for OX. Thus, an important specific binding to aromatic groups and/or other hydrophobic regions of the HA molecules needs to be postulated.

Although the non-electrostatic binding seems to be important, the electric field around HA molecules surely contributes to the overall binding. The extent of this electrostatic contribution can be estimated with the Boltzman factor $\exp(-F\psi/RT)$, where $F$ is the Faraday constant, $\psi$ is the electric potential at the attaching site, $R$ the gas constant and $T$ the absolute temperature (Sparks, 2003). Even though it is difficult to determine the actual value of $\psi$ in HA, it is usually lower than −0.1 V (Yang and Koopal, 1999). Then, for an extreme value of −0.1 V, the magnitude of the Boltzman factor is around 50, a value that is much lower than $K_L$ values found in this work ($K_L \approx 4 \times 10^5$). This indicates that the non-electrostatic contribution to the overall binding is dominant, fact that seems to be very common for the interaction between humics or other polymers with organic molecules (Yang and Koopal, 1999; Laor and Rebhun, 2002; Tatikolov and Costa, 2004).

The combined effects of changing pH and electrolyte concentration cannot be explained only by a mix of intrinsic and electrostatic interactions. As mentioned above, HA are known to be flexible molecules whose size can change depending on the pH and electrolyte concentration (Avena et al., 1999). At a constant and low ionic strength, HA molecules swell by increasing pH in response to the development of negative charges and the increased repulsive forces between ionized groups in the molecule. At a constant pH, HA molecules shrink by increasing the ionic strength as a consequence of the increased screening of the charges. In 0.1 M electrolyte, the increment in size by increasing the pH is very small or almost inexistent. As the electrolyte concentration decreases the pH effect becomes more evident, and in 0.001 M electrolyte the volume of the molecule at pH 11 may become twice or three times larger than that at pH 4. The shapes of volume vs. pH curves at different electrolyte concentration (Avena et al., 1999) are very

similar to that of the $N_S$ vs. pH curves presented in Fig. 5. These similarities indicate that conformational changes in the HA molecules are also important in determining the binding of OX molecules. The increased swelling of HA molecules by decreasing electrolyte concentration and increasing pH increases the availability of sites for binding but does not change appreciably their affinity for the dye.

$K_L$ values for the interaction between oxazine 1 and the studied HA are relatively high. They are comparable to those corresponding to the adsorption of methylene blue on kaolinite or soils (Avena et al., 2001) and higher than those corresponding to the binding of nitrophenol to HA ($K_L$ around $10^2$, Yang and Koopal, 1999). $K_L$ values are also comparable to those obtained by fitting the initial slope of binding isotherms of polycyclic aromatic hydrocarbons (PAH) on different humic acids (Laor and Rebhun, 2002). This relatively strong interaction suggests that HA and other HS may have an important impact on the transport and bioavailability of dyes. In soils containing high amount of non-soluble organic matter, such as humins, there will be an important retention of dyes. Conversely, in soils containing a high amount of soluble and mobile HS, such as humic acids and especially fulvic acids, there will be an important mobilization of dyes and transport to surface and groundwaters.

## 5. Conclusions

This study shows that UV/VIS spectrophotometry is a simple and valuable method to evaluate the binding between Oxazine 1 and HA in a range of solution conditions were traditional centrifugation or filtration methods cannot be used. This is possible because HA has the ability of changing the absorption spectrum of the dye when both molecules are interacting. The interaction is rather specific and mainly driven by non-electrostatic forces.

The effects of pH and electrolyte concentration on the binding between the dye and HA are strongly related to the conformational changes that HA suffers. Since this polyelectrolyte swells at high pH and low electrolyte concentration, the availability of assessable sites for binding increases, increasing consequently the amount of bound OX.

The relatively strong interaction between OX and HA suggests that HA and other HS may have an important effect on the transport and bioavailability of dyes.

The method presented here opens the possibility of applying it to measure the interaction not only between other dyes and HS but also between other light absorbing contaminants (such as pesticides or PAH) and humic substances.

## Acknowledgments

GPZ thanks Universidad de Santiago de Compostela for a postdoctoral fellowship. Fundings for this work were obtained from CONICET and Fundación Antorchas, Argentina, and from FEDER and Ministerio de Ciencia y Tecnología, Spain.

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