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The Effects of Conformational Changes on the Native Fluorescence of Aqueous Humic Materials

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Authors' contributions

This work was carried out by author LS under supervision by author RVW. Both authors have read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To elucidate the effect of induced conformational changes on the native fluorescence of aqueous humic materials.

Study Design: The conformation of dissolved humates was changed by adjustment of a variety of environmental factors and the resulting fluorescence emission, excited at 240nm was monitored in the 300-465nm range.

Place and Duration of Study: Department of Chemistry, University of Idaho, Moscow, ID, USA; April to August, 2012

Methodology: The fluorescence spectra of a number of humic and fulvic acids in different solution environments were measured with a photon counting fluorimeter. Attention was focused on the emission range centered on 400 nm and the intensity of the peak observed in this region was interpreted in terms of conformational changes.

Results: The addition of a multivalent cations produced distinct changes in the native fluorescence of dissolved humic materials that had otherwise broad and featureless emission spectra. A series of divalent cations were found to produce these emission changes. Microaggregation brought about by alternative causes, such as changes in pH, concentration, and solvent, produced similar outcomes. Chain length and rigidity of the humic polyanions also had significant effects.

Conclusion: The appearance and variation of a 400-nm emission peak was rationalized by invoking the formation of pseudomicellar structures that incorporated the emitting entity and provided limited access to water.

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Keywords: Aggregation; polyanions; photolysis; molecular flexibility.

1. INTRODUCTION

Humic substances, the decay products of the total biota in the environment, tend to have highly aromatic cores containing numerous fluorophores [1]. The abundance and similarity of these entities lead to broad, relatively featureless spectra that are of limited analytical utility. For this reason, fluorimetric investigations of humates involving strongly emitting probes are often more fruitful than those relying on native emissions [2]. One technique that does use the native fluorescence to good effect is total fluorescence spectroscopy in which excitation-emission matrices are generated that provide fingerprint-like identification for mixtures of fluorophores [3].

Close inspection of single-scan native fluorescence of humates, however, reveals that certain spectral features of aqueous humates are related in interesting ways to the solution conditions of the humic biopolymers. These responses are based on conformational changes in the materials and aggregates that form as a consequence of environmental influences [4]. The present communication deals with emission changes that occur because of such conformational variations.

The conformations of humic polyanions in aqueous solution have been studied extensively [4]. It is generally agreed that the highly diverse humic solutes progressively aggregate in response to solution parameters such as pH, ionic strength and presence of multiply charged cations, temperature, and concentration [5]. One of the more interesting features of this process is the formation of humic pseudomicelles, [6,7] which can be visualized as microscopic units that, while lacking the ordered structure of a "regular" surfactant micelle, do provide an internal microenvironment that can accommodate hydrophobic co-solutes. The presence of di- and trivalent cations in solution is especially favorable for the formation of humic pseudomicelles, since these ions can bind to functional groups (especially carboxylates) on different parts of the humic structure, thereby connecting them into a coherent entity. It has been shown that this process is accompanied by a reduction in particle size [8].

2. EXPERIMENTAL DETAILS

2.1 Chemicals

All chemicals were of analytical grade. MgCl_2 and NaCl was purchased from Fisher Scientific. $[(\text{CH}_3)_3\text{NCH}_2\text{CH}_2\text{N}(\text{CH}_3)_3]^{2+}$ ("N2"), europium chloride, and holmium chloride were purchased from Sigma Aldrich. $\text{Sr}(\text{NO}_3)_2$, CaCl_2 , $\text{Tb}(\text{NO}_3)_3$, and $\text{La}(\text{Cl}_3)\cdot 6\text{H}_2\text{O}$ were obtained from J.T. Baker Chemical Co. EM Industries, Pfautz and Bauer, Ind and Strem Chem respectively. All reagents were used as received. Standard humic and fulvic acids were purchased from the International Humic Substances Society (IHSS, St. Paul MN 55108) and used as received.

2.2 Isolation and Dissolution of Humates

Latah Co Silt Loam Humic Acid (LSLHA) was isolated from Latah Co Silt Loam (Argiaquic Xeric Argialbolls) according to the procedure provided by IHSS [9] which involves two cycles of HCl treatment for the removal of acid-soluble material; extraction of the residue with 0.1M

NaOH; deashing with 0.1M HCl/0.3M HF; purification by dialysis and isolation by freeze drying.

Humic acid solutions were prepared in doubly distilled water, treated with a Millipore Milli-Q Reagent water system to a resistivity of at least 16 M Ω cm. The material was brought into solution by adding a minimal amount of base (sodium or ammonium hydroxide) and subsequently adjusting the pH to the desired value with 0.01M HCl. For humate solutions containing magnesium or other metals, the appropriate increments of a 5 μ g/mL of the metal chloride was added to a 10 μ g/mL solution of the humate.

2.3 Fluorescence

Fluorescent spectra was taken with a Fluorolog-3 fluorimeter (Horiba Jobin Yvon, Edison, NJ) with photon counting detection. The samples were excited at 240 nm and the emission spectra were collected in the 300-465nm range. Confirmation measurements were obtained with an SLM-Aminco 8100 Fluorescence Spectrophotometer (Urbana, IL).

3. RESULTS AND DISCUSSION

The fluorescence emission spectrum of a 10 μ g/mL aqueous solution of LSLHA, excited at 245 nm, is shown in Fig. 1. The typical broad emission can be seen to increase gradually in intensity over the 300-465 nm range. The addition of Mg²⁺ to the solution gave rise to general intensity enhancement, as well as a distinct peak centered at 400 nm. Ca²⁺ and a series of divalent lanthanide ions produced similar effects, while monovalent cations such as Na⁺ did not – even at high concentrations. The lanthanides gave an additional spectral feature around 370 nm (not shown here), which can be ascribed to sensitized emission and will not be considered further.

It has been shown [10] that Mg²⁺ and other divalent and trivalent cations cause aqueous humates to undergo progressive intra- and intermolecular aggregation, in which the humic polyanions are drawn into structures that resemble surfactant micelles (“pseudomicelles”). It may be hypothesized that these configuration changes cause humic fluorophores – e.g. the one giving rise to the 400-nm peak – to be placed in a different, less polar, environment and therefore display different emission characteristics. The measurements described here were carried out to clarify this matter.

3.1 Effect of pH

The addition of multivalent cations is the most efficient, but not the only way to induce pseudomicelle formation in dissolved humates. A similar effect can be achieved by reducing the pH of a humic solution; thereby protonating the carboxylate groups of the polyanions [11]. Once these are neutralized sufficiently to minimize coulombic repulsion, the humic chains can fold to form microscopic aggregates with an internal environment that may allow the 400-nm emission peak to appear. The experimental evidence Fig. 2 supported this scenario. It can be seen that the telltale peak at around 400 nm became clearly visible at pH 2, lending further credence to the notion that the sequestration of humic fluorophores in the relatively nonpolar interior of the pseudomicelles gave rise to this emission.

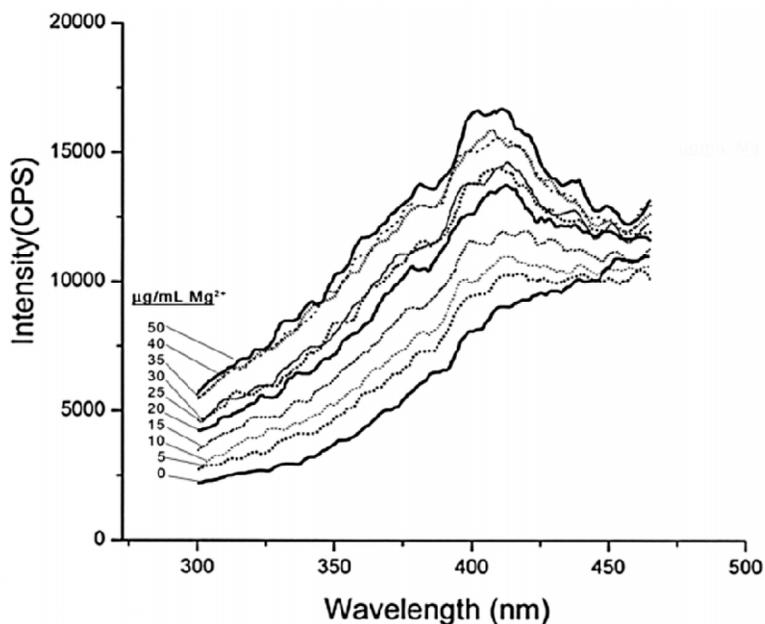


Fig. 1. Effect of different concentrations of Mg²⁺ on the intrinsic fluorescence of LSLHA in the 300-465 nm range; ex 245nm; 10 μg/mL LSLHA

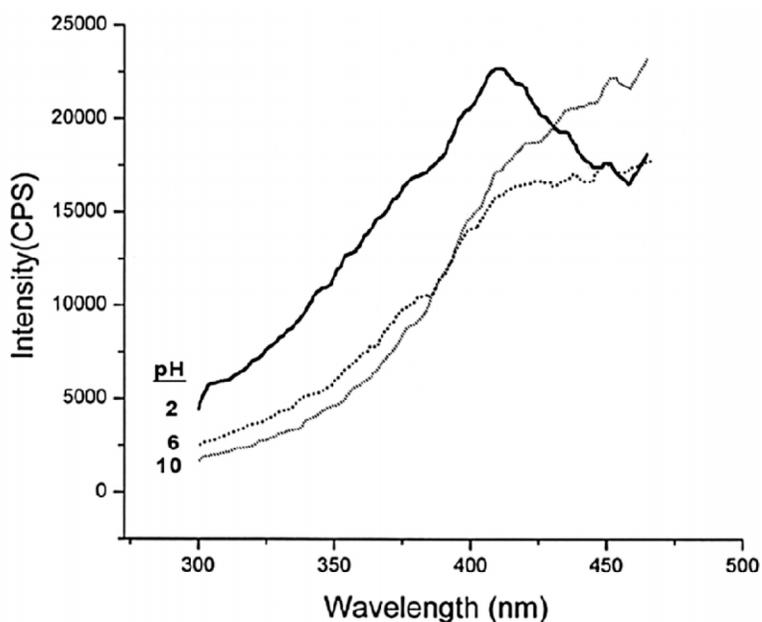


Fig. 2. The effect of pH on the fluorescence of LSLHA (55 μg/mL; ex 245nm)

3.2 Solvent Polarity

In previous work involving humic solutions with an added fluorescent probe [12] it was noted that the sequestration of this probe within the pseudomicelles showed that it resided in a

relatively nonaqueous microenvironment. In the present case, this apparent exclusion of water from the interior of the humic aggregate is also invoked to explain the 400-nm native emission of the humate. It therefore should be expected that the a priori use of a less polar solvent would have a similar effect. To this end, a series of 2-propanol – water mixtures were used as solvents for LSLHA, and the fluorescence characteristics of the humate were monitored in each case. The results Fig. 3. Showed that the 400-nm fluorescence intensities increased with decreasing water content. For reason of scale, the trace for 100% propanol is not shown: it did, in fact, give a peak intensity that was approximately 2.5-fold greater than that of the 80% 2-propanol solvent. These data bear out the contention that water quenches the 400-nm emission and that the exclusion of water from the fluorophore leads to the observed enhancement.

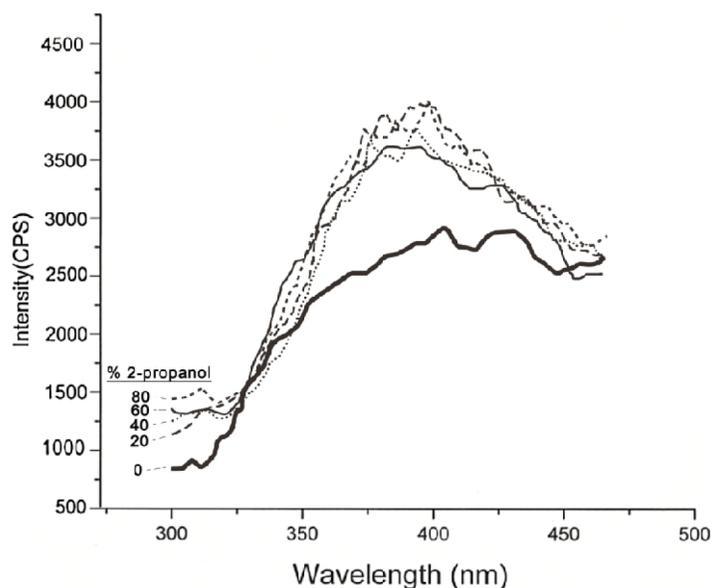


Fig. 3. The effect of solvent composition on the 400-nm fluorescence of LSLHA

3.3 Effects of Molecular Size

Previous work has shown that the formation of humic pseudomicelles is influenced by the molecular sizes of the species involved [13,14]. It is understood that any assembly of humic material is by nature polydisperse, but can display a wide variation in average size. In cases where there is a preponderance of relatively large polymeric material, it should be expected that internal folding and pseudomicelle formation is more likely to occur. With small pieces, on the other hand, folding (“internal aggregation”) cannot happen to the same extent. It has been shown previously that humic materials of which the molecular size has been reduced by photolysis, do not sequester hydrophobic species in aqueous solution in the manner that larger humates do [15]. This was ascribed to the lack of pseudomicelle formation in the photolyzed material.

With this observation in mind, the fluorescence response of a photolyzed LSLHA solution to the addition of Mg^{2+} was monitored. Fig. 4 shows that the cation produced a general increase of intensity of the broad fluorescence band centered around 420 nm, but that the telltale peak at 400 nm was not present. This fluorescence behavior was virtually identical to

that of Minnesota Peat fulvic acid (not shown), which, being a fulvate, is a material of smaller molecular size that also lacks the ability to fold up into pseudomicellar structures. This observation adds yet more weight to the view that this conformational arrangement is the direct cause of the 400-nm peak.

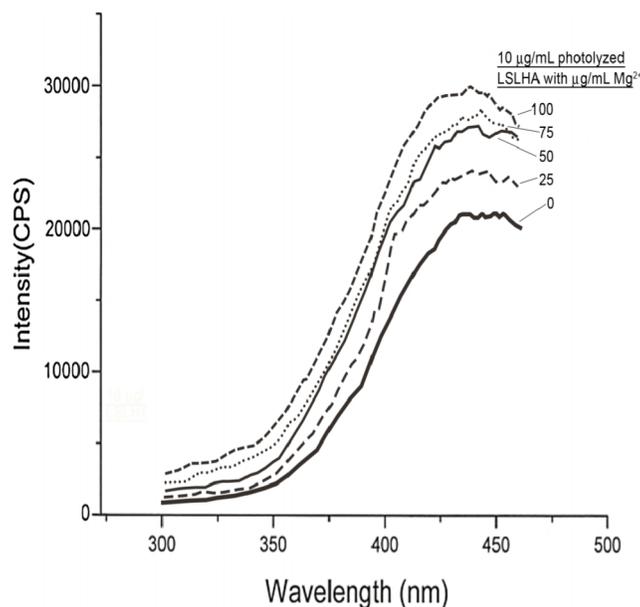


Fig. 4. Fluorescence of photolyzed LSLHA at different Mg^{2+} concentrations

3.4 Effect of Concentration

It has been shown that increasing the concentration of aqueous humates also leads to progressive particle growth, including a pseudomicellar stage [7]. In view of this phenomenon, the 400-nm emission peak was monitored as a function of humic concentration, and the results are shown in Fig. 5.

While the development of the 400-nm peak was rather weak in this instance, it can be seen that the emission peak at this wavelength became more distinct at higher concentrations. It should be noted that the overall fluorescence intensity decreased with concentration from about 40 $\mu\text{g/mL}$ onward because of an increasing inner filter effect.

3.5 Large Cations

To evaluate the influence of cation size on humic fluorescence enhancement, a large organic cation, $[(\text{CH}_3)_3\text{NCH}_2\text{CH}_2\text{N}(\text{CH}_3)_3]^{2+}$ was used in place of Mg^{2+} . It was found that this large ion, at a concentration of 50 $\mu\text{g/mL}$, also enhanced the humic emission at 400 nm. This suggests that nonmetal divalent cations could undergo coulombic interactions with multiple anionic charges on the humic polymer. This, in turn, resulted in a similar pseudomicellar contraction as was observed with multivalent metal ions. In addition, the alkyl moiety of these large cations appeared to be accommodated within the humic structure, enhancing its micellar character.

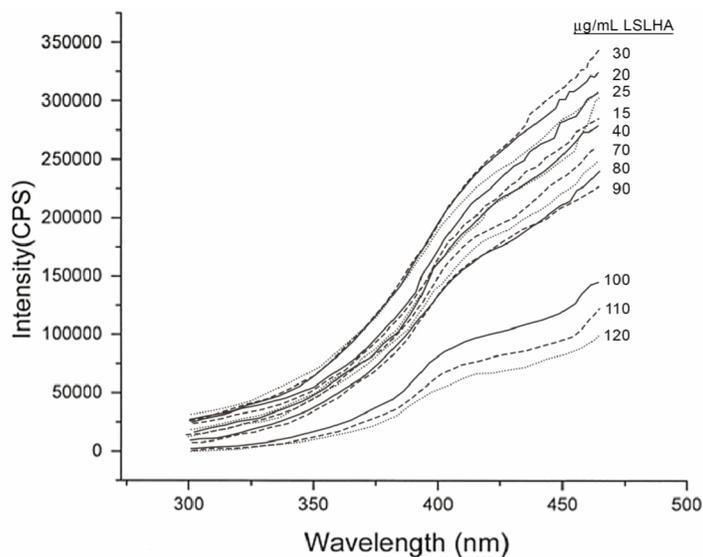


Fig. 5. Family of emission spectra showing the effect of humate concentration on fluorescence

3.6 Temperature

The emission intensity of fluorescent solutions generally decreases with temperature as the thermal motion of fluorophores increases nonradiative deactivation of the excited state [16]. Fig. 6 shows that this was the case with the 400-nm emission peak of an LSLHA solution treated with Mg^{2+} .

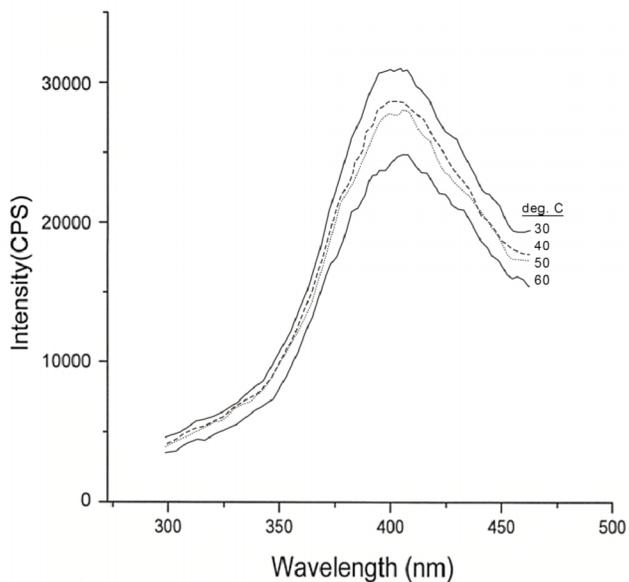


Fig. 6. The effect of temperature on the 400-nm emission of LSLHA in the presence of Mg^{2+}

It is interesting to note, however, that while the intensity decreases upon heating, the peak does not appear to lose its shape. This suggests that thermal agitation in the 30-60°C temperature range, did not lead to significant disaggregation.

3.7 Different Humates

While the mechanism of humic pseudomicelle formation is not entirely understood, it is reasonable to assume that it, as suggested above, involves the folding of polymeric chains and the incorporation of smaller entities in micro aggregates of roughly micellar size [15]. In view of this, the flexibility of the humic chains should be a factor, since it influences their ability to fold and twist into the proper configuration. It is therefore instructive to compare the fluorescence behavior of LSLHA, which comprises relatively flexible polymer chains, with that of other humates with both similar and different structure. In this context, the fluorescence response of the IHSS standards Summit Hill HA and Peat HA to Mg^{2+} was tested. These materials have aggregation properties similar to LSLHA [17] and it was found that the 400-nm emission peak also appeared when Mg^{2+} was added (spectra not shown). Leonardite humic acid (LHA), on the other hand, is a good example of a stiffer, less flexible, humate. Its quantitative ^{13}C NMR spectrum shown in ref [18]. Contains a large aromatic peak at 127 ppm, indicating that it is more aromatic and coal-like in nature than other humates. This structure, containing multiple fused rings, cannot be deformed easily. The spectra shown in Fig. 7 are consistent with these structural constraints. The 400-nm emission peak could be generated, but to get a comparable effect with LHA it took about ten times as much Mg^{2+} as it did with LSLHA. These points to less easy intramolecular rearrangements of the stiffer LHA polyanions, requiring more cationic anchors to create pseudomicellar aggregates.

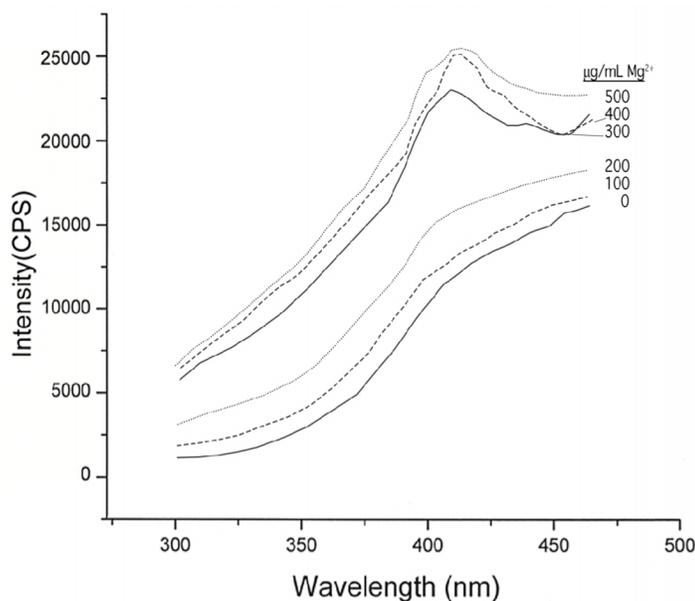


Fig. 7. Fluorescence spectra of LHA at different Mg^{2+} concentrations

3.8 Micellar Solutions

Anon polar, relatively water free, microenvironment in which the 400-nm humic emission peak potentially could be observed can also be created by using a micellar solution of a surfactant [19] and introducing small amounts of LSLHA to it. Incorporation of the humate in the micelles should expose it to a relatively dry environment favorable to the appearance of the 400-nm peak.

The experiment was carried out with a 1.0 mM solution of cetyl trimethyl ammonium bromide (CTAB), a cationic surfactant with a critical micelle concentration of 0.92mM [20]. Small amounts of LSLHA were added drop wise in increments of approximately 0.3 μg . The results are shown in Fig. 8. From which it is clear that the 400-nm emission peak of LSLHA did indeed appear. This provides further confirmation for the necessity of the exclusion of water for this spectral feature to materialize.

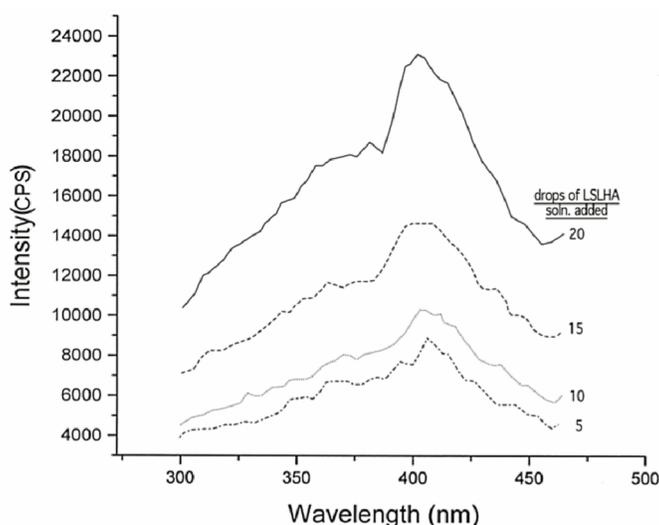


Fig. 8. Fluorescence emission spectra of 50 mL of a 1.0 mM solution of CTAB with 110 $\mu\text{g/mL}$ LSLHA added as indicated. Excitation wavelength 245 nm 245 nm

4. CONCLUSIONS

The evidence presented here suggests that the fluorophore in LSLHA that is responsible for the emission peak at 400 nm is quenched by water, and that microaggregation of the aqueous humate can lead to significant exclusion of this quencher. This, in turn, is consistent with a pseudomicellar model of the humic material, in which the aggregates have a relatively polar exterior and a nonpolar interior. In aqueous solution, the 400-nm peak may be considered a marker for the formation of pseudomicelles. It was observed in different humates, and it may be cautiously proposed that it generally present in these materials. The nature of the fluorophore cannot be pinpointed at this time. Previous work [21] has shown that the ESR signal of quinonic entities that are common in humates change significantly when they aggregate under the influence of Mg^{2+} ions. The fluorescence characteristics of quinones, however, are quite different from the 245/400 nm ex/em wavelengths found here. The abundance of fluorophores in the humates make the unequivocal identification of the emitter responsible for the emission peak discussed here difficult, if not impossible.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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