H. Chen & J. E. Kenny, Annals of Environmental Science / 2007, Vol 1, 1-9

A STUDY OF pH EFFECTS ON HUMIC SUBSTANCES USING CHEMOMETRIC ANALYSIS OF EXCITATION-EMISSION MATRICES

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Received May 31, 2006; in final form October 23, 2006; Accepted November 11, 2006

ABSTRACT

Excitation-emission matrices (EEMs) of four humic substances (Aldrich, Fluka, and two humic acids 1S102H and 1S103H from the International Humic Substances Society) were collected as a function of pH over the range pH = 2 to 11 at 20°C. Parallel factor analysis (PARAFAC) was applied to the 3-D data to separate the overlapping components. The Aldrich and Fluka samples showed only two components, each of which had humic-like spectral features. The IHSS samples were best modeled by three-component PARAFAC models, two of the components being very similar to the components found in the Aldrich and Fluka PARAFAC models. The third component in each of the IHSS cases was a protein-like substance with structured emission around 330 nm, which was assigned to tryptophan. For all the humic substances, the relative concentrations of the humic-related components showed strong pH dependence, but the protein-like components showed essentially no pH-dependence in either wavelength or relative concentration. Free tryptophan and a tryptophan-Aldrich humic acid mixture were also studied. Free tryptophan shows strong pH-dependence of emission intensity and wavelength (shifting from 350 nm at pH 2 to 361 nm at pH 11) and the emission is featureless within pH 2~11. All evidence suggests that tryptophan in IHSS samples is shielded or protected from the bulk aqueous environment by association with other moieties in the mixture.

***Key words*:** fluorescence, tryptophan, PARAFAC, pH, humic, EEM

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1. INTRODUCTION

Humic substances (HSs) are high molecular weight, heterogeneous organic materials that are the major constituents of natural organic matter (NOM) in soils and aquatic environments [1-4]. It is widely accepted that there is an abundance of oxygen-containing acidic groups in HSs, e.g., carboxyl groups and hydroxyl groups [5]. HSs acid-base behavior plays an important role in the acid-base balance in natural waters. In addition, complexation of metal cations by HSs is significantly affected by pH [6-9]. It is reported that organic pollutants, e.g., polycyclic aromatic hydrocarbons (PAHs), are bound by HSs and transported in the environment. The interaction of PAHs and HSs is pH-dependent [10]. As a result, study of the pH behavior of HSs is of intrinsic interest.

Fluorescence is widely employed in the characterization of HSs, due to its high sensitivity and low perturbation to the samples. The fluorescence signatures of both natural waters [11] and humic samples show pH-dependence [12, 13]. Excitation-emission matrices (EEMs) are usually collected and displayed in contour plots, from which excitation-emission centers (local maxima) are visualized and utilized for further analysis. It is well known that the optical spectra of HSs of natural waters reflect the complex mixture of organic molecules contained in these samples, and different components are likely to overlap in their emission or excitation spectra. As a result, the centers in the collected EEMs are actually a superposition of fluorescence contributions from multiple components. Analysis based on the excitation-emission centers in the contour plot can give misleading results if all the intensity at a given center is attributed to a single component. To the best of our knowledge, to date none of the reported pH-dependence studies in the literature have used the chemometric approach used here to partition the observed fluorescence in the EEMs into excitation and emission spectra of separate components (or groups of covarying or interacting components, see below). Pullin *et al* utilized rank analysis in pH-dependent synchronous fluorescence spectra of humic samples collected using δ = 20 nm with excitation 300~600 nm, which allowed them to estimate the number of fluorescent "factors" but no emission or excitation spectra of these "factors" were given [12].

Parallel factor analysis (PARAFAC), a multi-dimensional or multi-way mathematical approach with its origin in psychometrics [14], is suited to the complexity of EEMs. For the three-dimensional (3-D) EEMs stacked in order of a certain sample mode (here, pH), PARAFAC assumes that there are several independent components that contribute in each EEM,

and the linear superposition of these components gives the experimentally collected EEMs as follows [15],

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \qquad (1)$$

where x_{ijk} is the intensity of fluorescence for the sample i at emission wavelength j and excitation wavelength k. Parameter a_{if} is directly proportional to the concentration of the component f in the ith sample. A vector b_f of elements b_{jf} (j = 1, 2, 3,…) denotes the emission spectrum of component f, and a vector c_f of elements c_{kf} (k = 1, 2, 3,…) denotes the excitation spectrum of component f. PARAFAC tries to find a proper number of components with proper emission and excitation spectra such that the sum-of-squares of the residue e_{ijk} is minimized. F defines the number of components.

In contrast to other multivariate analysis approaches, e.g. principal component analysis (PCA), PARAFAC does not have an intrinsic rotational freedom, which means that PARAFAC in principle is capable of identifying the true spectra of the underlying components [15]. Several research groups have demonstrated that PARAFAC can be used successfully in natural waters [16-18]. Recently, Del Vecchio and Blough [19] reported extensive evidence for intramolecular charge transfer (CT) from electron donors (D) to electron acceptors (A) in humic molecules and noted that spectral properties of sets of interacting chromophores will not be simple superpositions of the spectra of the individual chromophores in the absence of interactions. The fluorescent components recovered by PARAFAC may actually represent discrete species, covarying species, or interacting pairs (or sets) of species.

In this work we have applied PARAFAC to three dimensional (3-D) data sets, EEMs of a given HSs stacked in order of pH. We found that the EEMs of a given HS could be well represented by a PARAFAC model with two or three independent components: all four samples showed two different humic-like components whose relative contributions varied with pH; the two reference materials from IHSS also showed a tryptophan-like fluorescence whose relative contribution was independent of pH. In order to clarify such pH-independence, we further investigated the pH-related fluorescence behaviors of two systems, namely free tryptophan and tryptophan mixed with an HS sample.

2. EXPERIMENTAL

The four HSs samples in the present work were Aldrich humic acid salt, Fluka humic acid, and two humic acids 1S102H and 1S103H from the International Humic Substances Society (IHSS). 10mg of each sample was dispersed in 100ml of nanopure water and stirred for 12 hours at approximately 20°C. The resultant mixture was filtered with a 0.45 um nylon membrane (Millipore) to remove the small amount of undissolved particulate. The stock filtrate was stored at 4°C in the dark for no more than 2 weeks prior to usage. Properly diluted solutions were made from the stock solution, if necessary. pH adjustment was conducted by addition of microliters of concentrated NaOH (~5M) or HCl (~6M), and pH was read with a calibrated Corning 430 pH meter.

The UV-visible absorbance measurements were carried out on a Jasco-570 spectrophotometer equipped with a thermostated cell holder at 20 ± 0.1°C. Bandwidths were 5 nm. Double beam mode was employed and nano-pure water (Millipore) in a QG quartz cell (Hellma) of 1 cm pathlength was used as the blank. The maximum absorbance of all samples within wavelength range 225~600 nm was below 1.

EEMs were collected on a Varian Cary Eclipse fluorometer equipped with a thermostated cell holder at 20 ± 0.1°C. Excitation and emission bandwidths were 5 nm. Excitation was scanned from 225 nm through 600 nm with a 5 nm increment. Emission was scanned from 300 nm to 600nm with a 1 nm increment. Due to the absence of fluorescence signals and the presence of strong scattering below emission wavelength 300 nm, a built-in 295 nm long pass filter was selected via software to reduce the scattering. The collected fluorescence was automatically filter corrected. An auto mode was selected in the software for excitation filters. The voltage of a photomultiplier tube (PMT) was set to be high (800 volts) for most experiments to obtain strong signals. A xenon flashlamp pulsed at 80 Hz with 2 μs full width at half maximum (FWHM) was used as the light source. The scan rate of the emission wavelength was 600 nm/min. Each emission wavelength signal was averaged for 0.1 s. A reference detector in this instrument was employed to compensate for fluctuation of the light source.

Free tryptophan (1 μM) EEMs at each pH value were collected at 600 volts of PMT to avoid saturation of the signal. The instrumental emission-dependence bias was corrected using a published approach [20]. Wavelength-dependence of the light source was corrected using 8 g/L of Rhodamine B (RhB) in ethylene glycol [21] in a triangular front face cell as a quantum counter. It is safely assumed that all the

excitation at the cell was totally absorbed by such concentrated RhB and the quantum efficiency of RhB was reported to be excitation independent within 250~600 nm [21]. Work in our laboratory by J. Thomas Brownrigg showed that the useful range of this solution as a quantum counter could be extended down to 225 nm.

The scattering regions, as visualized in the contour graph of each EEM, were removed from the EEM using a MATLAB program written by Hall [18, 22], putting NaN (Not-a-number) to these data-removed regions. The PARAFAC routine was included in a commercial software package PLS Toolbox 3.0.4 provided by Eigenvector Research, Inc. The primary and inner filter effects were corrected using Hall's MATLAB program [22] based on the treatment of MacDonald *et al.* [23]. The beam profile at the sample cell was provided by Varian.

3. RESULTS AND DISCUSSION

Two components A and B are recovered for both Aldrich humic salt and Fluka humic acid, as shown in Figures 1 and 2. In both cases, the spectral features and pH dependence of A and B are similar. We shall therefore refer to them as component A and component B, regardless of the sample being discussed. Each should be interpreted as the collective contribution of several (possibly a large number of) compounds with similar excitation and emission spectra and similar pH dependence, rather than a single chemical species. (In the absence of extensive chemical or photochemical interaction, the spectrum of each "component" might appear as the superposition of the spectra of a number of covarying chemical compounds, and as such violate the rules for the appearance of the spectrum of a single chemical species. For example, some of the components reported in the figures of this paper display more anti-stokes fluorescence than typically seen for a single species.

Both of the components show featureless or weakly featured emissions, which is typically characteristic of humic substances at room temperature [24]. Component A contributes significantly more fluorescence to the total signal at all pH than B. Component A has an excitation spectrum that peaks near 230 nm and has an apparent shoulder around 300-320 nm, while its emission spectrum has a broad, flat maximum in the blue, from about 430 to 470 nm. Component B has two distinct excitation maxima, around 280 and 440 nm, and a broad emission peak in the green, around 530 nm. The two-component fitting is apparently appropriate to describe the overall data sets, as shown by the large explained variations (>99%) and core consistency diagnosis [25]. Two-component fitting always gave >99% core consistency, while 3-component fitting usually led to much smaller core consistency, <60%. Besides the core consistency, the model was checked by fitting it several times from random starting positions or initial values [26]. Two-component fitting repeatedly converged to the same solutions, but 3-component fitting was unstable in giving different results, even though high core consistency was sometimes obtained for these results. As a result, the 2-component model is preferred.

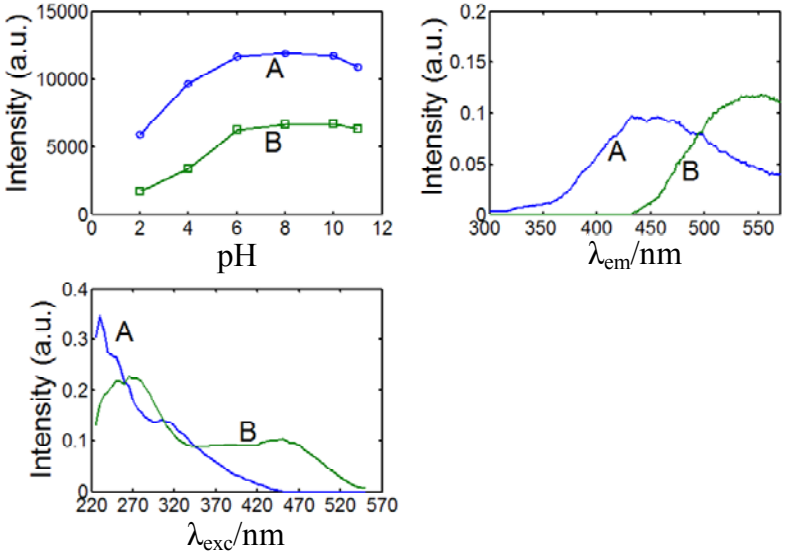

Figure 1 PARAFAC fitting of Aldrich humic acid salt (10 mg/L), showing relative intensities of two components (A and B) as a function of pH, excitation spectra and emission spectra.

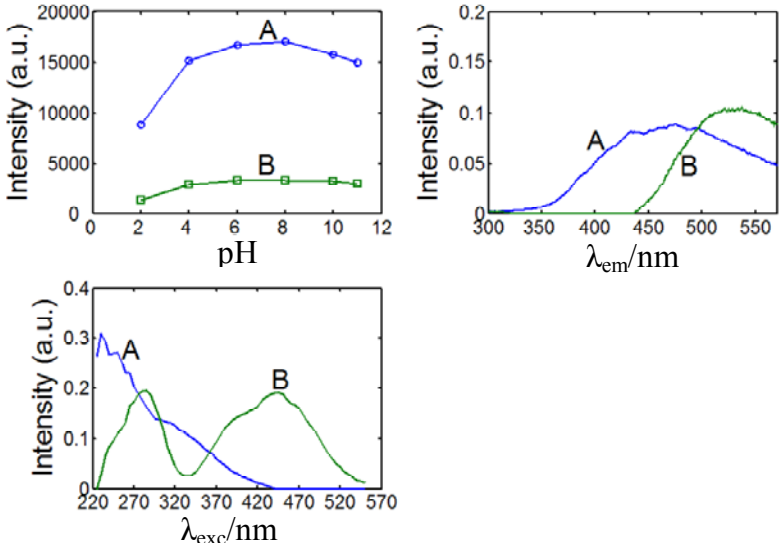

Figure 2 PARAFAC fitting of Fluka humic acid (10 mg/L), showing relative intensities of two components (A and B) as a function of pH, excitation spectra and emission spectra.

The intensities of both components increased with pH until pH 8, after which they decreased slightly. The gross shape of the pH dependence suggests that each component is an amphoteric species, e.g. the first conjugate base of a diprotic acid, but the detailed dependence has not yet been modeled. Similar pH-dependence is also reported in the literature for humic materials in natural waters [11,13]. Patel-Sorrentino *et al.* reported that there were two excitation-emission centers, $\lambda_{ex}/\lambda_{em}$ = 245-260 nm/430-460 nm and 325-340 nm/430-460 nm, attributed to "fluorophores A and C", respectively, in the EEMs of Amazon basin waters. A linear relationship between their intensity

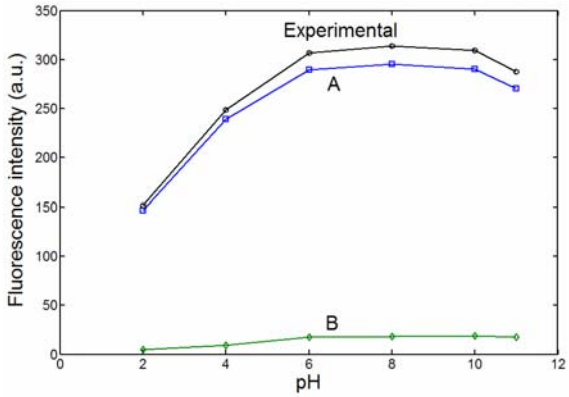

Figure 3 Fluorescence comparison for Aldrich humic acid, excitation: 250 nm, emission: 450 nm.

ratio I_A/I_C and pH (ratio increasing with increasing pH) was found for many different water samples, albeit with varying slopes [11]. No theoretical basis for this relationship was determined. From an inspection of the PARAFAC results for our samples, we note that component A contributes most of the intensity at both centers. Using the results of the model, we calculated the contributions to fluorescence at each center and plotted them for the Aldrich HS in Figures 3 and 4.

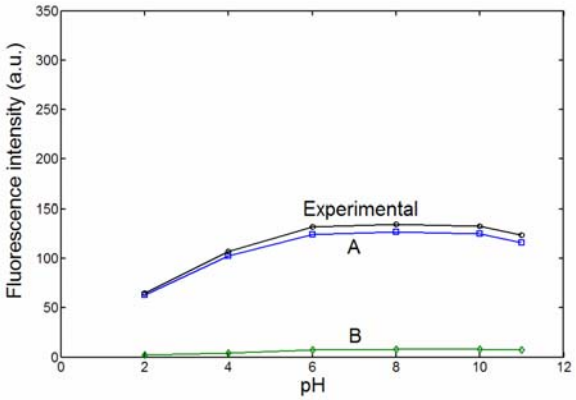

Figure 4 Fluorescence comparison for Aldrich humic acid, excitation: 330 nm, emission: 450 nm.

The ratios of our PARAFAC components A and B as a function of pH are shown for all four HS studied in this work in Figure 5: no simple relationship to pH is evident. Although the humic materials in our study are not the same as in ref. 11, we think it is likely that, given the broad spectral characteristics of humic materials in general, attributing all intensity at a given excitation/pair to a single fluorophore could be

misleading. The PARAFAC analysis provides a more rigorous way of attributing observed fluorescence intensity to individual components in a mixture.

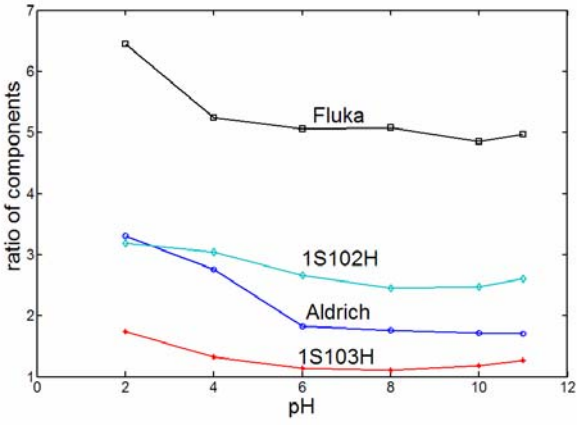

Figure 5 The ratio of component A to component B in all four humic samples as a function of pH, calculated from PARAFAC results.

The PARAFAC analyses of both 1S102H and 1S103H resulted in best fits using three-component models, consisting of one amino acid-like component (which we will refer to as C) and two humic-like components very similar to the two humic components seen in the Aldrich and Fluka HS above (therefore denoted A and B as well). The results are shown in Figures 6 and 7. For the amino acid-like component, the excitation spectrum shows a small maximum around 280nm and a much larger one at the shortest wavelength used (225 nm); there is structured emission with maxima located around 325 nm and 337 nm. The relative concentrations of the amino acid-related components were pH-independent, while humic-related components showed strong pH-dependence, similar to but more complicated than components A and B in Aldrich and Fluka HSs. The observation of component C in these IHSS samples is not surprising, as it has been reported that biomolecules, e.g., amino acids, usually exist in HSs [3]. A recent report also mentioned that there is a pH-independent amino acid-related fluorescence in natural waters, and the authors ascribed it to tryptophan. However, there was no detailed description or explanation [27].

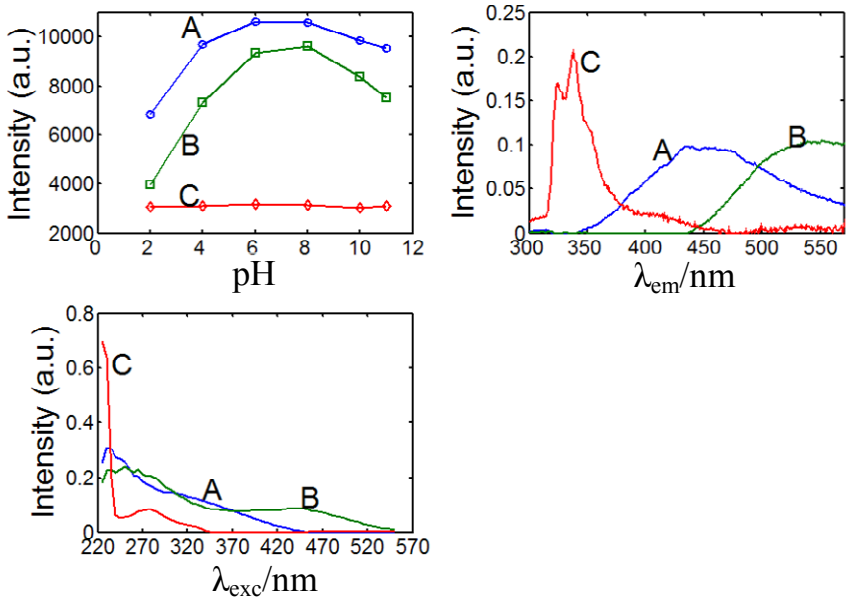

Figure 6 PARAFAC fitting of IHSS humic acid 1S103H (100 mg/L) showing relative intensities of three components (A, B, and C) as a function of pH, excitation spectra and emission spectra

Of the three fluorescent amino acids, phenylalanine can be eliminated from consideration on several counts: it is likely present in lowest concentration; it has the smallest absorbtivity and fluorescence quantum yield and its emission would be blocked by the cutoff filter used [28]. The observed emission is from tyrosine (300 nm) and/or tyrosinate (350 nm). Tyrosine is present in the samples, according to IHSS. But the intensity should migrate from 300 nm to 350 nm as pH increases [28] and it does not (Figure 8). A broad, unstructured tryptophan emission appears typically around 350 nm in water, but it shifts to the blue with increasing structure in protected nonpolar environments. We propose that our IHSS samples contain tryptophan that is protected or shielded from the bulk aqueous environment by complexation with the humic material.

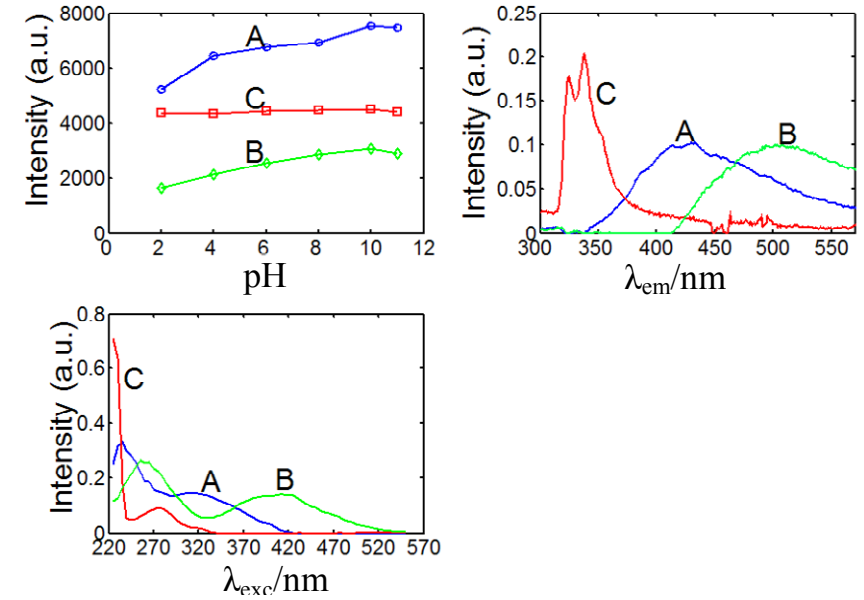

Figure 7 PARAFAC fitting of IHSS humic acid 1S102H (100 mg/L) showing relative intensities of three components (A, B and C) as a function of pH, excitation spectra and emission spectra.

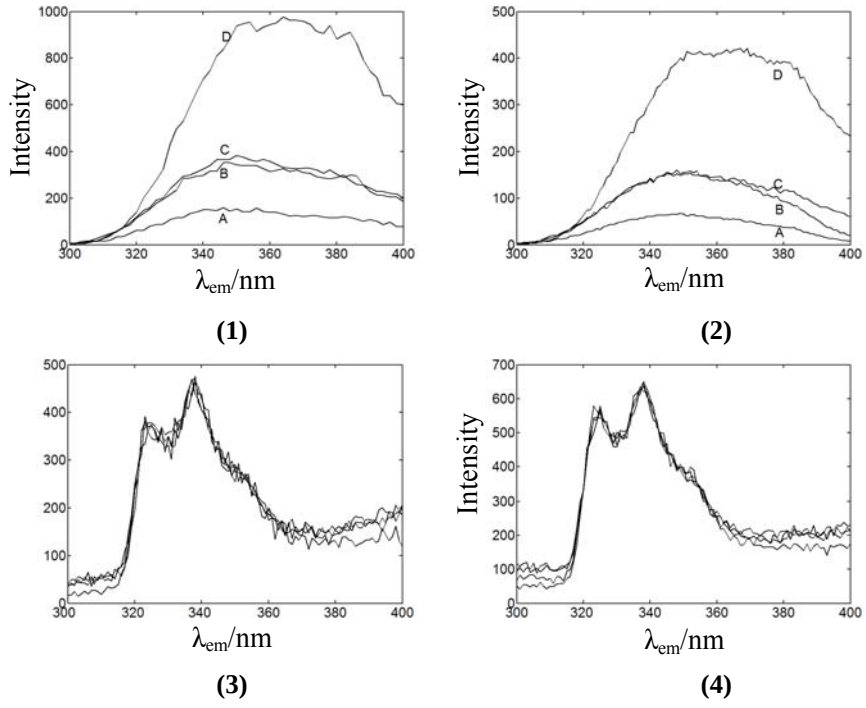

Figure 8 Corrected emission spectra excited by 225 nm at varied pH values in (1) tryptophan (1μM) mixed with Aldrich (10 mg/L); (2) free tryptophan (1μM); (3) 1S103H (100 mg/L); (4) 1S102H (100 mg/L). A: pH 2; B: pH 6; C: pH 8; D: pH 11.

In a non-polar environment, polar solvents, e.g., water molecules, have difficulty in accessing the indole ring of tryptophan. Interactions of solvents with indole rings are believed to affect the fluorescence shift [29]. Tyrosine is present in the sample, according to IHSS [30], but it is reported that the fluorescence spectra of most tryptophan-containing proteins are attributed mainly to tryptophan residues due to the higher absorbtivity of tryptophan than of tyrosine and the energy transfer if proper distance and dipole-dipole orientation criteria are satisfied [28, 31]. 1S102H is Elliott soil humic acid collected at Joliet Army Ammunition Plant near Joliet, Illinois, and 1S103H is obtained from Pahokee peat at the Florida Everglades [30].

These two samples are of different origins, but they show very similar amino acid-related emission [5]. Tyrosine is very likely to fluoresce at 303 nm, regardless of the polarity of the microenvironment around it [32]. A red shift (305-340 nm) of tyrosine in proteins is due to hydrogen-bonding between the phenolic hydroxyl of tyrosine and proton acceptors around tyrosine [32]. It is expected that H^+ would affect the hydrogen bonding, hence shifting tyrosine emission. However, we did not find any shift of the amino acid emission when changing pH values, which means that the amino acid-related component is less likely to be tyrosine.

In contrast to the tryptophan-related emission in 1S102H and 1S103H, free tryptophan under the same experimental conditions showed strong pH dependence. Free tryptophan emission shifts from 350 nm at pH 2 to 361 nm at pH 11. The emission shift of tryptophan is attributed to charge transfer between the benzene ring and pyrrole ring in tryptophan [29, 33]. If charge transfer from the pyrrole ring to the benzene ring is enhanced, tryptophan shows red-shifted emission. This mechanism is consistent with our results of free tryptophan at varied pH values. As pH increases, deprotonation of the carboxylic group (R-COOH) increases and more negative charge accumulates around the pyrrole ring, which leads to enhanced charge flow from the pyrrole ring to the benzene ring. As pH decreases, protonation of –NH on the pyrrole ring will decrease the charge transfer from pyrrole to benzene, causing a blue shift of emission. This mechanism, on the other hand, implies that the tryptophan chromophore in 1S102H and 1S103H is strongly protected by humic complexation. As a result, pH change does not affect the tryptophan emission significantly.

It is reported and widely accepted that humic substances can form an intramolecular pseudomicelle structure in which polar or hydrophilic groups point outwards and non-polar or hydrophobic groups are buried inside [34, 35]. We further made a mixture of Aldrich HS (10 and 100 mg/L) with tryptophan (1 μM) and studied the pH-related fluorescence behaviors of such mixtures over the same pH range used above. Tryptophan in Aldrich HS (10 mg/L) still showed strong pH dependence, emission peaks shifting from 361 nm at pH 11 to 350 nm at pH 2. Tryptophan (1 μM) in 100 mg/L Aldrich HS showed very weak fluorescence throughout the pH range 2~11, which probably is due to the strong primary inner-filter effect of Aldrich HS at high concentration. For comparison, the emissions of 1S102H, 1S103H, free tryptophan, and tryptophan mixed with Aldrich HS at varied pH values are shown in Figure 8. It is interesting to note that free tryptophan emission intensity also varied with pH. Free tryptophan was reported to have pH-dependence of emission intensity [36].

These observations imply that 1S102H and 1S103H are less likely to be simple mixtures of tryptophan and humic substances. Instead, it is likely that strong interactions exist between tryptophan residues and the rest of the humic material. The strong interaction between some biomolecules and humic substances was also reported in the literature [3]. The interaction is so strong that it is believed to be virtually impossible to remove those biomolecules from HSs without significantly changing HSs structure [3].

4. ACKNOWLEDGEMENTS

The authors are grateful to Gregory J. Hall for his help in MATLAB programming and PARAFAC analysis. We thank Dr. J. Thomas Brownrigg for his work on Rhodamine B as a quantum counter (unpublished internal research report), and we also thank Qun Gun for his advice in inner-filter correction. This work was supported by NOAA and by Tufts University internal funding.

5. REFERENCES

[1] Chen J, LeBoeuf EJ, Dai S, Gu B. Fluorescence spectroscopic studies of natural organic matter fractions. *Chemosphere*, 2003, 50: 639-647.

[2] Abbt-Braun G, Lankes U, Frimmel FH. Structural characterization of aquatic humic substances - The need for a multiple method approach. *Aquat. Sci*., 2004, 66: 151-170.

[3] Sutton R, Sposito G. Molecular structure in soil humic substances: The new view. *Environ. Sci. & Technol*., 2005, 39: 9009-9015.

[4] Aiken GR, McKnight DM, Wershaw RL,

MacCarthy P. eds. *Humic substances in soil, sediment, and water*. New York: Wiley, 1985.
[5] Yong C, von Wandruszka R. A comparison of aggregation behavior in aqueous humic acids. *Geochem. Trans*., 2001, 2.
[6] Nifant'eva TI, Shkinev VM, Spivakov BY, Burba P. Membrane filtration studies of aquatic humic substances and their metal species: a concise overview. Part 2. Evaluation of conditional stability constants by using ultrafiltration. *Talanta,* 1999, 48: 257-267.
[7] Benegas JC, Porasso RD, van den Hoop MAGT. Proton-metal exchange processes in synthetic and natural polyelectrolyte solution systems. *Colloids Surf. A,* 2003, 224: 107-117.
[8] Gondar D, Iglesias A, López R, Fiol S, Antelo JM, Arce F. Copper binding by peat fulvic and humic acids extracted from two horizons of an ombrotrophic peat bog. *Chemosphere,* 2006, 63: 82-88.
[9] Liu A, Gonzalez RD. Modeling adsorption of copper(II), cadmium(II) and lead(II) on purified humic acid. *Langmuir,* 2000, 16: 3902-3909.
[10] Schlautman MA, Morgan JJ. Effects of aqueous chemistry on the binding of polycyclic aromatic hydrocarbons by dissolved humic materials. *Environ. Sci. Technol*., 1993, 27: 961-969.
[11] Patel-Sorrention N, Mounier S, Benaim JY. Excitation-emission fluorescence matrix to study pH influences on organic matter fluorescence in the Amazon Basin Rivers. *Water Res.,* 2002, 36: 2571-2581.
[12] Pullin MJ, Cabaniss SE. Rank analysis of the pH-dependent synchronous fluorescence spectra of six standard humic substances. *Environ. Sci. Technol*., 1995, 29: 1460-1467.
[13] Fu P, Wu F, Liu C. Fluorescence excitation-emission matrix characterization of a commercial humic acid. *Chin. J. Geochem.,* 2004, 23: 309-318.
[14] Carroll JD, Chang J. Analysis of individual differences in multi-dimensional scaling via an n-way generalization of "Eckart-Young" decomposition. *Psychometrika,* 1970, 35: 283.
[15] Bro R. PARAFAC. Tutorial and applications. *Chemom. Intell. Lab. Syst.,* 1997, 38: 149-171.
[16] Stedmon CA, Markager S, Bro R. Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. *Mar. Chem.,* 2003, 82: 239-254.
[17] Cory RM, McKnight DM. Fluorescence spectroscopy reveals ubiquitous presence of oxidized and reduced quinones in dissolved organic matter. *Environ. Sci. Technol.,* 2005, 39: 8142-8149.
[18] Hall GJ, Clow KE, Kenny JE. Estuarial fingerprinting through multidimensional fluorescence and multivariate analysis. *Environ. Sci. Technol.,* 2005, 39: 7560-7567.
[19] Del Vecchio R, Blough NV. On the origin of the optical properties of humic substances. *Environ. Sci. Technol*., 2004, 38: 3885-3891.
[20] Melhuish WH. Calibration of spectrofluorimeters for measuring corrected emission spectra. *J. Opt. Soc. Am*., 1962, 52: 1256-1258.
[21] Yguerabide J. Fast and accurate method for measuring photon flux in the range 2500-6000 Å. *Rev. Sci. Instrum.,* 1968, 39: 1048-1052.
[22] Hall GJ. *Chemometric characterization and classification of estuarine water through multidimensional fluorescence.* Ph.D. Thesis, Tufts University, Medford, Massachusetts, USA, 2006.
[23] MacDonald BC, Lvin S, Patterson H. Correction of fluorescence inner filter effects and the partitioning of pyrene to dissolved organic carbon. *Anal. Chim. Acta,* 1997, 338: 155-161.
[24] Filippova EM, Fadeev VV, Chubarov VV, Dolenko TA, Glushkov SM. Laser fluorescence spectroscopy as a method for studying humic substances. *Appl. Spectrosc. Rev.,* 2001, 36: 87-117.
[25] Bro R, Kiers HAL. A new efficient method for determining the number of components in PARAFAC models. *J. Chemom*., 2003, 17: 274-286.
[26] Andersen CM, Bro R. Practical aspects of PARAFAC modeling of fluorescence excitation-emission data. *J. Chemom.,* 2003, 17: 200-215.
[27] Lead JR, Momi AD, Goula G, Baker A. Fractionation of freshwater colloids and particles by SPLITT: Analysis by electron microscopy and 3D excitation-emission matrix fluorescence. *Anal. Chem.,* 2006, 78: 3609-3615.
[28] Lakowicz JR. *Principles of fluorescence spectroscopy*, 2nd ed. New York: Kluwer Academic-Plenum Publishers, 1999.
[29] Vivian JT, Callis PR. Mechanisms of tryptophan fluorescence shifts in proteins. *Biophys. J.,* 2001, 80: 2093-2109.
[30] http://www.ihss.gatech.edu/
[31] Mayer LM, Schick LL, Loder III TC. Dissolved protein fluorescence in two marine estuaries. *Mar. Chem.,* 1999, 64: 171-179.
[32] Lee J, Ross RT. Absorption and fluorescence of tyrosine hydrogen-bonded to amide-like ligands.

J. Phys. Chem. B, 1998, 102: 4612-4618.
[33] Dashnau JL, Zelent B, Vanderkooi JM. Tryptophan interaction with gycerol/water and trehalose/sucrose cryosolvents: infrared and fluorescence spectroscopy and *ab initio* calculations. *Biophys. Chem.,* 2005, 114: 71-83.
[34] Engebretson RR, von Wandruszka R. Microorganization in dissolved humic acids. *Environ. Sci. Technol*., 1994, 28: 1934-1941.
[35] von Wandruszka R. The micellar model of humic acid: Evidence from pyrene fluorescence measurements. *Soil Sci*., 1998, 163: 921-930.
[36] Gudgln E, Lopez-Delgado R, Ware WR. Photophysics of tryptophan in H_2O, D_2O, and in nonaqueous solvents. *J. Phys. Chem*., 1983, 87: 1559-1565.

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