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Probing the pH Dependent Optical Properties of Aquatic, Terrestrial and Microbial Humic Substances by Sodium Borohydride Reduction

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Abstract

Chemically reducing humic (HA) and fulvic acids (FA) provides insight into spectroscopically identifiable structural moieties generating the optical properties of HA/FA from aquatic, microbial and terrestrial sources. Sodium borohydride reduction provides targeted reduction of carbonyl groups. The contrast between the pH induced optical changes of untreated, reduced and reoxidized HA/FA highlights differences in the quantity, and physicality of structural components generating optical properties associated with HA/FA. Because borohydride reactions alter pH, pH re-adjustment to the original pH is required. Careful titrations of selected HA/FA provided the μmole $H^+ g^{-1}_{HA/FA}$ required to titrate reduced and reoxidized HA/FA from pH 2-11; and the pH dependent spectral slope (S) at low (pH 2-3), neutral (pH 7-7.5) and high (pH 11.0). Molar extinction coefficients (ε) ($Lmg^{-1}cm^{-1}$) at pH 7.6 and 350 nm provide a point of consistency linking intrinsic pH dependent optical properties to the concentration of material used for titrations. Soil derived humic acids differ from aquatic humic acids in the heterogeneity of optically identifiable group as well as the overall concentration of those groups. The microbial source of FA has a limited concentration of homogeneous titratable groups when compared to aquatic FA generated terrestrially. Microbial FA exhibits pH linked optical recovery upon reoxidation when compared to aquatic FA which is consistent with the presence of quinones in microbial FA.

Keywords: humic substances (HS), chromophoric dissolved organic matter (CDOM), pH, borohydride reduction, optical titration, spectral slope (S), fulvic acid, humic acid, fluorescence spectroscopy, uv-vis spectroscopy

1. Introduction

Humic substances (HS) and chromophoric dissolved organic matter (CDOM) are related and dynamic parts of the global carbon cycle (Hernes & Benner, 2003). The unique optical properties of CDOM/HS are integral to their environmental behavior. CDOM/HS have been studied for decades but limited understanding of how their optical properties are generated remains elusive (Coble et al. 2007; Moran et al. 2000). The complexity of their environmental interactions in combination with limited understanding of the structural underpinnings make the chemical and physical behavior of CDOM/HS relevant to a broad range of scientific fields including microbial science, soil science, the study of the fate and transport of metals and anthropological chemicals, and the behavior of other nutrient cycles HS (Koopal et al.2005; De Wit et al. 1993b). In this study, the optical properties were chemically probed using a reductant that specifically targeted carbonyl groups. The optical and potentiometic behavior of the selected model CDOM compounds representing specific ecosystems (soil, fresh water aquatic and marine) were compared before and after reduction in order to ascertain their similarities and differences. Several spectroscopic techniques were used to measure the chemical reductant and pH induced changes to the optical properties including UV-Vis, and fluorescence spectroscopy with the goal of identifying the chemical species involved in generating the unique optical properties universally found in CDOM.

Two established models that provide justification for the experiments conducted. The first model presented is the electronic interaction model which seeks to explain the unique optical properties associated with all CDOM/HS (Ma, 2010). The second model is a combination of the non-ideal competitive absorption model (NICA) and the Donnan gel model (Koopal et al. 2005; De Wit et al. 1993a; De Wit et al. 1993b).

The sample size required to implement the NICA-Donnan model limits its use to materials that can be gathered in large quantities making investigations of many colored dissolved organic samples (CDOM) difficult or prohibitively expensive. Optical titrations of lower concentration samples have been used to successfully model proton binding in Suwannee River Fulvic Acid (SRFA) by the use of pH dependent differential absorbance (ΔA) (Dryer et al., 2008). The NICA-Donnan model describes two distributions of titratable groups centered at pH 4 and pH 7-8 respectively (Koopal et al. 2005; De Wit et al. 1993a; De Wit et al. 1993b).

Model humic compounds (Table 1) derived from a solely microbial source as well as terrestrial sources from both aquatic environments and soil systems exhibit many of the same optical properties despite their dispirit methods, of generation in the environment and source materials (Brown et al., 2004). All show a pH dependent absorbance, exhibit increasing absorbance as wavelength decreases and a loss of absorbance upon borohydride reduction (Ma et al., 2010). A prominent theory linking humic model compounds is the electronic interaction model which highlights their ability to form electronic interactions and charge transfer complexes that extend long wavelength absorbance from source material that does not have absorbance spectra at long wavelengths (Ma et al., 2010). The underlying processes by which charge transfer bands or electronic interactions in CDOM/HS are generated are probed by optical and potentiometic titrations of untreated and borohydride reduced material. Borohydride reduction targets carbonyl functional groups such as aromatic ketones and uinines (Tinnacher & Honeyman, 2007). By removing a moiety suspected of participating in electronic interaction, long wavelength absorbance is partially or entirely lost and fluorescence emission increases and blue shifts (Ma et al., 2010). A direct comparison of divergent sources of fulvic and humic acid model compounds including an aquatic terrestrially derived fulvic acid, Suwannee River fulvic Acid (SRFA) and a microbial source of fulvic acid, Pony Lake fulvic Acid (PLFA) (Brown et al., 2004; McKnight et al., 1994), the soil derived humic acids Elliott humic acid (EHA) and Leonardite humic acid (LHA) and a terrestrially derived aquatic humic acid Suwannee River humic acid (SRHA) (Table 1). Analysis and comparison of untreated and borohydride reduced model humic compounds particularly at short wavelength (< 350) using potentiometric and optical titrations, spectral slope values (S), difference plots (ΔA) (Dryer et al., 2008), fluorescence emission spectra, (Jørgensen et al., 2011), and fluorescence difference spectra (ΔF) (Del Vecchio & Blough, 2004) provide insight into how electronic interactions work universally in the environment as well as how differences that exist between sources humic materials can potentially augment existing knowledge about the fate and transport of humic substances in the environment (Hernes & Ronald Benner, 2002).

2. Materials

Leonardite Humic acid standard (LHA) (1S104H-S), Elliot Humic acid standard (EHA) (1S102H), Suwannee River humic acid (SRHA) (2S101H), Suwannee River fulvic acid (SRFA) (2S101F) and Pony Lake fulvic acid (PLFA) (1R109F) were obtained from the International Humic Substance Society. Sodium borohydride ($NaBH_4$) was obtained from Sigma-Aldrich. Water was purified to 18 Ω using an Academic Milli-Q water system equipped with a carbon filter. Soil humic acids were filtered with a Nalgene 0.2 μm, 25 mm Nylon disposable syringe filter (catalog no. 195-2520) from Thermo Scientific. Sephadex G-10 was purchased from Sigma-Aldrich. Ultra high purity nitrogen was acquired from Airgas and equipped with an oxygen scrubber purchased from SGE analytical services. Sodium hydroxide (0.100 mole L^{-1} and 0.250 mole L^{-1}) and hydrochloric acid (0.100 mole L^{-1} and 0.250 mole L^{-1}) were obtained from Ricca Chemical Company. Sodium chloride (99.999%) was obtained from Sigma Aldrich.

Quinine sulfate 215ehydrate and potassium hydrogen phthalate were obtained from Fisher Scientific. A Shimadzu UVPC 2401 spectrophotometer was used to acquire UV-visible absorption spectra. Fluorescence measurements were made with an Aminco-Bowman AB-2 luminescence spectrophotometer. A 4 cm band pass was used for excitation and emission. All experiments were conducted using 1 cm quartz cuvette with Milli-Q water adjusted to the appropriate pH.

Humic Acids (HA) were dissolved in a minimum amount of sodium hydroxide, diluted to the desired concentration and pH adjusted using hydrochloric acid (HCl). The pH of each HA standard was adjusted from an average of 12 to the desired initial reduction of pH 7.6 and filtered with a 0.2 um Nalgene Nylon syringe filter (catalog number 195-2520) prior to finial dilution. Fulvic acids (FA) were dissolved in pH adjusted Milli-Q water. The pH was adjusted to the desired pH prior to finial dilution if needed. Fulvic acids were not filtered.

Each standard was purged with UHP nitrogen (N_2) fitted with either a Restek oxygen scrubber (catalog number 20601) or a SGE Analytical Sciences oxygen trap (catalog number 103486) for 30 minutes prior to the addition of the reductant and purged continually with ultra high purity N_2 throughout the time course of the reduction. All reductions of HA/FA were carried out for at least 24 hours and selected reductions were extended to 48 or 72 hours. The samples were reoxidized for 24 hours. Samples were protected from ambient light during reduction and reoxidation. Selected spectra were titrated to the original A(0) pH because addition of borohydride consistently increased the pH of the solution. The pH and absorbance spectrum of each reduction was measured at re-oxidation time points of 10 minutes, 30 minutes, 1 hour and 24 hours. Difference spectra (ΔA) were calculated by subtracting an absorbance spectra at time t (A(t)) from the original spectra (A(0)). Fractional difference spectra were generated by dividing an absorbance spectra at time t (A(t)) by the original spectra A(0). All pH measurements were conducted using an Orion pH meter calibrated at pH 4.00, 7.00 and 10.00 daily. Calibrations were considered acceptable when the correlation coefficient was greater than 0.98.

Fluorescence emission spectra were collected from 280-600 nm excitation range. In order avoid inner filter effects fluorescence measurements were kept between 0.05 and 0.10 OD. Differential emission spectra (ΔF) were calculated by subtracting the original spectra F(0) by subsequently borohydride reduced spectra at time F(t) where t was 24 hours. Quinine sulfate was used to standardize the fluorescence quantum yield measurements according to the method developed by the U.S. Department of Commerce, National Bureau of Standards publication 260-64 Standard Reference Materials: A Fluorescence Standard Reference Material: Quinine Sulfate Dihydrate (Velapoldi & Mielenz, 1980). Fluorescence measurements were made initially at pH 7.60, reduced pH 10.00 and reduced reoxidized pH 7.60.

Samples used for titrations were passed over a Sephadex G-10 column until the pH of the sample was found to be between pH 7.00 and pH 7.60 in order to remove residual borate and facilitate background free titrations. Turmeric paper was used to ensure that no additional borate remained in the effluent (Scott & Webb, 1932). Molar extinction coefficients were determined using Milli-Q water at pH 7.60 and 350 nm. Absorption spectra were recorded from 190 to 820 nm against pH adjusted Milli-Q water. The concentrations of the column effluent from the Sephadex G-10 column were determined using a calibration curve of previously reduced samples at pH 7.60 and 350 nm. The absorptivity (ε) (L $mg^{-1}cm^{-1}$) of the absorbance as a function of concentration was derived according to Beer-Lambert Law (equation 1)

$$A = \varepsilon lc, \quad (1)$$

where A is the absorbance, l is the path length of the cell (cm) and c is the concentration of the sample (mg L^{-1}).

In order to investigate the effect of ionic strength on the optical properties of HS, ionic strengths of 0.01, 0.10 and 1.00 mole L^{-1} NaCl were added to prior to titration of untreated and borohydride reduced samples at concentrations that were optimized for the visible region of the spectrum (1 OD, at 350 nm). Untreated humic and fulvic acid samples were matched to absorbance of their corresponding borohydride reduced and Sephadex cleaned samples. Initial reaction concentrations were chosen in order to generate absorbance spectrum at or below 1 absorbance unit in spectral ranges between 190 and 400 nm and 400 to 820 nm allowing for investigation of the UV and visible ranges of the spectrophotometer. Titrations from pH 3.00 to 11.00 were completed using 0.250 mole L^{-1} sodium hydroxide. Titrations from pH 11.00 to pH 3.00 were completed using 0.250 mole L^{-1} hydrochloric acid. Samples were titrated over the same pH range in order to eliminate hysteresis. Titrant was delivered in 5 μl increments (1.25 μmole aliquots). Untreated Suwannee River humic and fulvic acids (SRHA and SRFA) were titrated using a concentration of 100 mg L^{-1}, the untreated soil humic acids, LHA and EHA were titrated using a concentration of 50 mg L^{-1} and the untreated PLFA was titrated using a concentration of 500 mg L^{-1}.

Borohydride reduced and Sephadex cleaned humic and fulvic samples were titrated at the following concentrations SRFA was titrated using 760 mg L^{-1}, SRHA was titrated using 200 mg L^{-1}, EHA was titrated using 48 mg L^{-1}, LHA was titrated using 75 mg L^{-1}, PLFA was titrated using 260 mg L^{-1}. All titrations were carried out using an initial volume of 3.00 ml and volume adjusted. Each reduced, cleaned and titrated sample was carbon normalized in order to complete the spectral slope calculations.

A second set of titrations were completed at concentrations appropriate for the investigation of the UV region of the spectra (< 350 nm). These titrations were completed at an ionic strength of 0.01 mole L^{-1} NaCl. Difference plots were generated for the both the low concentration UV range titrations and the high concentration visible range titration. The concentrations for the untreated and borohydride reduced and Sephadex cleaned UV range samples respectively were for SRHA 20 and 19 mg L^{-1}, SRFA 26 and 28 mg L^{-1}, PLFA 75 and 30 mg L^{-1}, EHA 10 and 48 mg L^{-1}, LHA 13 and 11 mg L^{-1}.

The wavelength dependence of the specific absorbance coefficient (a^*) (equation 2) at low (pH 3.00), high (pH 11.00) and neutral pH (pH 6.00-7.60) at an initial wavelength 350 nm (equation 2) were used to calculate the spectral slope (S) as in equation 3. Specific absorbance (a^*) were calculated according to equation (2).

$$A^*\ (350\ \text{nm}) = 2.303\ a(\lambda)/(b\ *\ c), \quad (2)$$

where a^*(350 nm) is specific absorbance at 350 nm henceforth referred to as a^*, $a(\lambda)$ = absorbance at a given wavelength, b = 0.01 is the absorbance path length in meters (1 cm cell), C is the total organic carbon in mg carbon L^{-1}. Total organic carbon was determined by high temperature oxidation using a Schimadzu 500A TOC analyzer calibrated using potassium hydrogen phthalate (KHP) at 680 ^{0}C. The spectral slope parameter (S) was obtained by non-linear least squares fitting of the spectra over the range of 290-820 nm to expression (3)

$$a^*(\lambda) = a^*(\lambda_{Ref})e^{-S(\lambda - \lambda Ref)}, \quad (3)$$

where $a^*(\lambda_{Ref})$ is the specific absorption coefficient at the reference wavelength of 350 nm. Spectral slope parameterization was completed with a minimum of three replicates at pH 7.60 and 350 nm.

3. Results

Borohydride reduction of soil derived terrestrial material (Elliott and Leonardite humic acids) over a period of 24 hours decreases in absorbance across all wavelengths, but a maximum reduction is seen between 300 and 600 nm (Figure 1, Table 1). The difference and fractional difference maximum loss is 50 % for both Elliott and Leonardite HS. The percent loss of SRHA was consistent with the soil Has (50 %). The maximum fractional and differential fraction loss of SRFA was 70 % (Figure 1, Table 1).

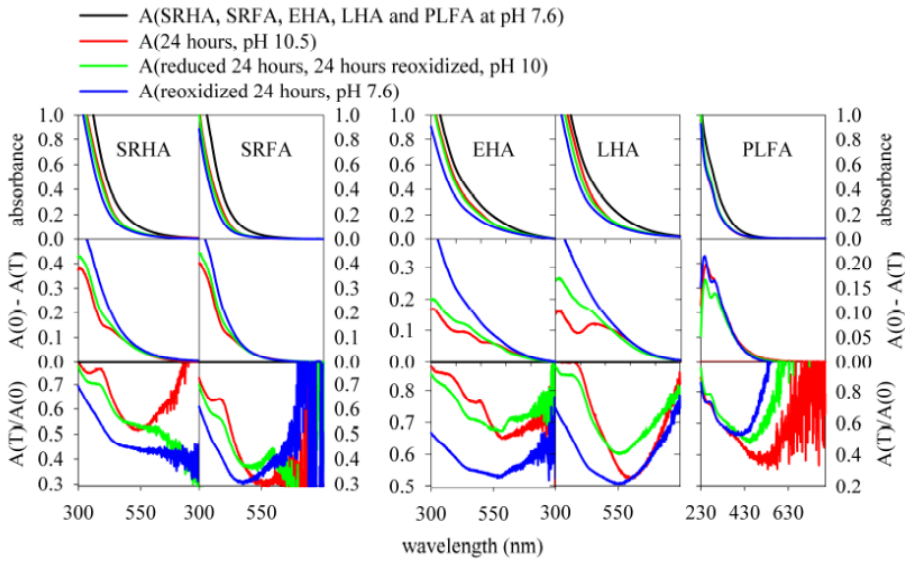

Figure 1. Absorbance, difference A(0) – A(T) and fractional difference A(T)/A(0) of the borohydride reduction and reoxidation of 100 mg L^{-1} Suwannee River humic acid (SRHA), Suwannee River fulvic acid (SRFA), 50 mg L^{-1} Elliott humic acid (EHA), 500 mg L^{-1} Leonardite humic acid (LHA) and 50 mg L^{-1} Pony Lake fulvic acid (PLFA) with 5 mg of sodium borohydride. Reduction and reoxidation were allowed to continue for 24 hours each. The finial pH was adjusted back to the initial pH 7.60

Table 1. Sample descriptions and abbreviations

Sample name	Description	Abbreviation
Suwannee River Humic Acid	Suwannee River, GA USA Drains the Okeefenokee swamp a cypress wetland	SRHA
Suwannee River Fulvic Acid	Suwannee River, GA Drains the Okeefenokee swamp a cypress wetland	SRFA

Elliott Humic Acid	Central plains IO, USA Soil type Mollisol sub order: Aquic Arguidoll- poorly drained prairie soil	EHA
Leonardite Humic Acid	Lignite Coal precursor fossilized plant material SD, USA	LHA
Pony Lake Fulvic Acid	Hypereutrophic pond, Antarctica	PLFA

Table 2. Molar extinction coefficients (ε) (L mg^{-1} cm^{-1}) of untreated and borohydride reduced Suwannee River humic (SRHA) and fulvic (SRFA) acids, Pony Lake fulvic acid (PLFA), Elliott humic acid (EHA) and Leonardite humic acid (LHA). Intercept values are 0.0. A minimum of three replicates were used

Sample Identification	ε ($Lmg^{-1}cm^{-1}$) untreated pH 7.6, 350 nm, (r^2)		ε ($Lmg^{-1}cm^{-1}$) borohydride reduced for 24 hours, reoxidized for 24 hours, pH 7.6, 350 nm absorbance, (r^2)	
SRFA	0.00786	(0.999)	0.00352	(0.999)
PLFA	0.00356	(0.999)	0.00220	(0.999)
SRHA	0.0114	(0.999)	0.00805	(0.999)
EHA	0.0205	(0.999)	0.0107	(0.999)
LHA	0.0194	(0.994)	0.0137	(0.999)

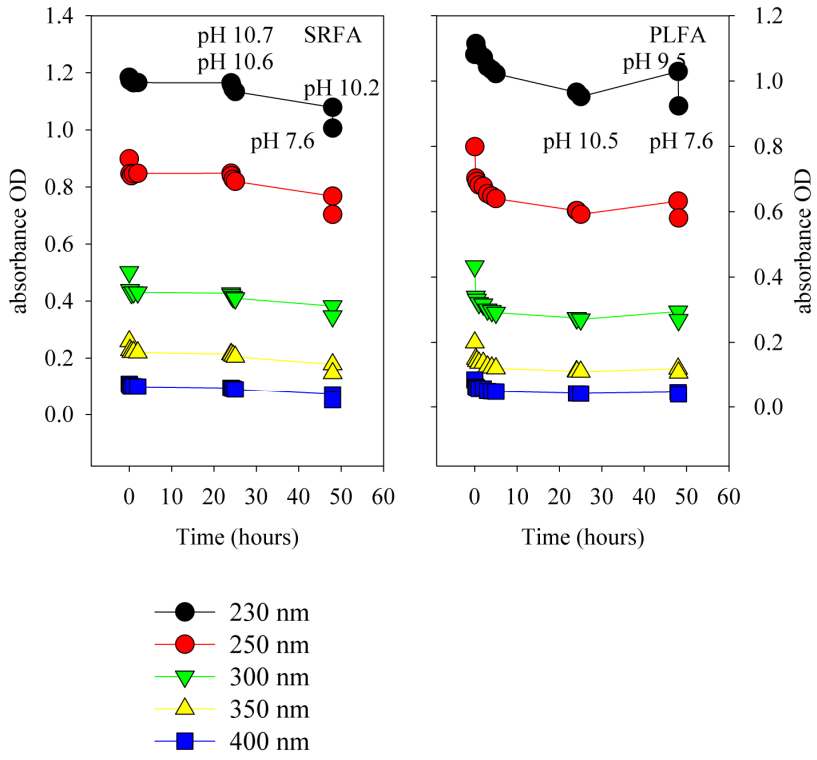

Figure 3. Time dependence of the borohydride reduction and reoxidation of 35 mg L^{-1} Suwannee River fulvic acid (SRFA) and 50 mg L^{-1} Pony Lake fulvic acid (PLFA) over an optical absorbance range 230 to 350 nm. Samples were reduced for 24 hours, reoxidized for 24 hours and titrated back to the initial pH 7.60

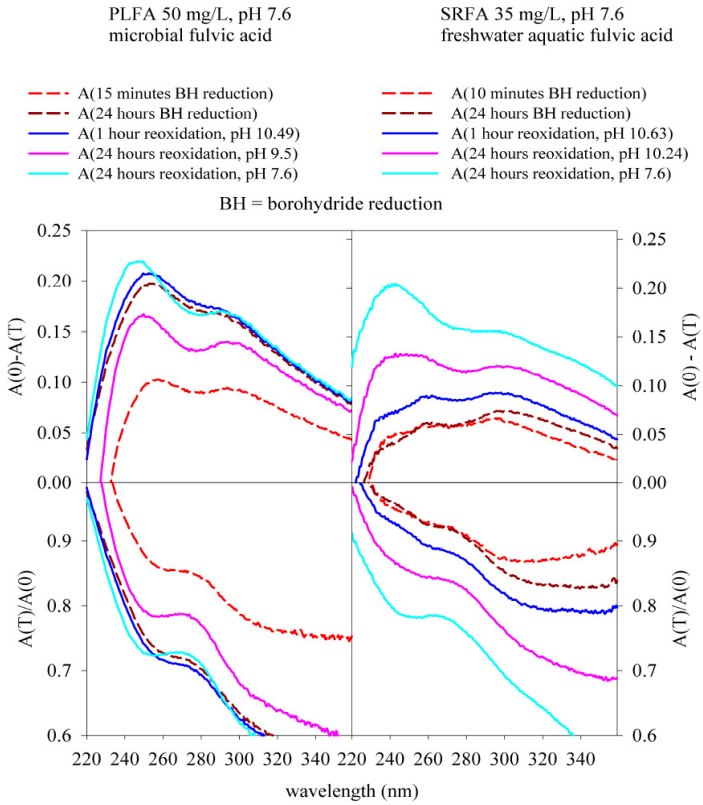

Figure 4. Difference A(0) – A(T) and fractional difference A(T)/A(0) spectra of 50 mg L^{-1} Pony Lake fulvic acid (PLFA) and 35 mg L-1 Suwannee River fulvic acid (SRFA) reduced with 5 mg of sodium borohydride for 24 hours, reoxidized for 24 hours and titrated to the initial pH of 7.60 highlighting differences in reduction and reoxidation behavior in fulvic acids from different source materials

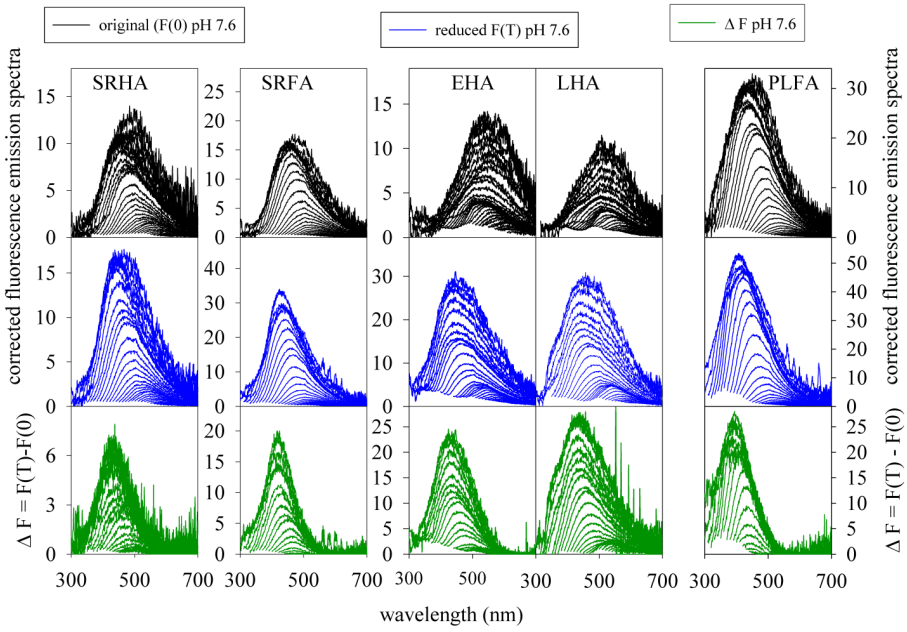

Figure 5. Fluorescence properties of Suwannee River humic acid (SRHA), Suwannee River fulvic acid (SRFA), Elliott humic acid (EHA), Leonardite humic acid (LHA) and Pony Lake fulvic acid (PLFA) prior to and following reduction with sodium borohydride at pH 7.60. ΔF represents the difference before and after reduction

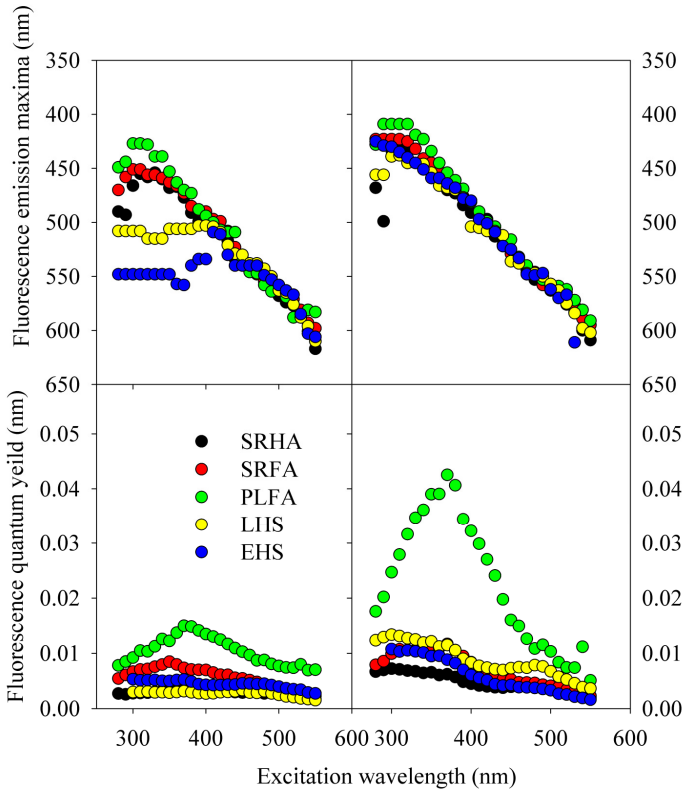

Figure 6. Wavelength dependence of emission maxima (top) and fluorescence quantum yields (bottom) for samples before (right panels) and after (left panels) 24 hours sodium borohydride reduction

Table 3. Reoxidation induced pH changes for Suwannee River fulvic acid (SRFA), Suwannee River humic acid (SRHA), Leonardite humic acid (LHA), Elliott humic acid (EHA), Pony Lake fulvic acid (PLFA)

sample	pH post 24 hour reduction	pH post 24 hour reoxidation	μmole $H^+ g^{-1}_{HA/FA}$
SRFA 100 mg L^{-1} SRFA 71 mg L^{-1}	10.5 10.7	10.0 10.2	0.029 ± 0.012
SRHA 100 mg L^{-1} SRHA 100 mg L^{-1}	10.3 10.0	10.1 9.8	0.098 ± 0.009
LHA 50 mg L^{-1} LHA 50 mg L^{-1}	10.3 10.8	9.5 10.1	0.145 ± 0.120
EHA 50 mg L^{-1} EHA 50 mg L^{-1}	10.5 10.6	9.9 10	0.075 ± 0.012
PLFA 500 mg L^{-1} PLFA 50 mg L^{-1}	11.2 10.7	9.5 10.2	0.026 ± 0.017

Table 4. pH dependence of untreated and borohydride reduced spectral slope values (S) of Suwannee River humic acid (SRHA), Suwannee River fulvic acid (SRFA), Pony Lake fulvic acid (PLFA), Elliott humic acid (EHA) and Leonardite humic acid (LHA)

sample identification	**untreated £ pH, spectral slope (S) (nm^{-1}), r^2**		**borohydride reduced £ pH, spectral slope (S), r^2**	
(organic carbon material) g^{-1}	pH r^2	S	pH r^2	S
SRHA (0.3974)	2.98	0.0138	2.97	0.0145
	0.9982		0.9957	
	7.26	0.0130	7.29	0.0142
	0.9985		0.9973	
	11.03	0.0111	11.05	0.0124
	0.9968		0.9585	
	7.60	0.0125 ± 0.0002	7.60	0.0146 ± 0.0002
SRFA (0.5087)	2.98	0.0153	2.92	0.0166
	0.9971		0.9956	
	7.26	0.0142	7.37	0.0166
	0.9977		0.9978	
	11.04	0.0121	11.04	0.0145
	0.9976		0.9983	
	7.60	0.0147 ± 0.0003	7.60	0.0183 ± 0.0004
PLFA (0.3949)	2.97	0.0153	2.93	0.0139
	0.9964		0.9779	
	6.91	0.0148	7.21	0.0142
	0.9976		0.9808	
	11.04	0.0139	11.04	0.0141
	0.9985		0.9840	
	7.60	0.0151 ± 0.0002	7.60	0.0185 ± 0.0008
EHA (0.4760)	2.96	0.0085	3.06	0.0086
	0.9964		0.9932	
	7.26	0.0073	6.83	0.0088
	0.9972		0.9932	
	11.06	0.0070	11.00	0.0085
	0.9971		0.9935	
	7.60	0.0076 ± 0.0003	7.60	0.0085 ± 0.0002
LHA (0.2501)	2.91	0.0095	2.97	0.0114
	0.9985		0.9972	
	6.84	0.0092	7.71	0.0111
	0.9987		0.9971	
	11.00	0.0082	11.04	0.0103
	0.9995		0.9984	
	7.60	0.0095 ± 0.0004	7.60	0.0105 ± 0.0003

£ a* values were calculated at 350 nm (L(mg org. C)-1m-1).

Absorbance, difference and fractional difference spectra of PLFA show that borohydride reduction of 500 mg L^{-1} PLFA produces as much as 90 % loss in absorbance with maxima between 400-500 nm (Figure 1, Table 1). Absorbance at longer wavelengths than 500 nm is very close to zero. Long wave length absorbance of untreated and borohydride reduced PLFA were found to be linearly related to concentration at pH 7.6 and 350 nm with molar extinction coefficients ε = 0.00356 L $mg^{-1}cm^{-1}$ and 0.00220 L $mg^{-1}cm^{-1}$ respectively (Table 2). The other humic and fulvic acids, untreated and borohydride reduced exhibit linear absorbance as a function of concentration at 350 nm and pH 7.6 (Table 2). The absorbance of PLFA at long wavelengths is very low, making the use of high concentration (mg L^{-1}) necessary in order to elucidate absorbance trends in this region. The absorbance increases sharply at wavelengths shorter than 350 nm. In order to capture absorbance trends across wavelength 230-800 nm and remain in the linear range of the spectrophotometer, reductions were carried out independently at two concentrations 50 mg L^{-1} from wavelength (230-350 nm) and at 500 mg L^{-1} (350-800 nm). At long wavelengths (350-800) the time dependence of the reduction behaves in a similar manner as do all other humic materials investigated (Figure 2). The majority of the borohydride induced reduction occurs within the first 2 hours of the reduction. Reoxidation over a period of 24 hours, post the 24 hours of borohydride reduction causes further loss of absorbance as seen in the time dependence of the reduction (Figure 3) and absorbance, difference and fractional difference spectra (Figure 1). Concurrently, with reoxidation absorbance loss is a reduction in pH (Table 3). An oxygen dependent reduction in pH was observed in all of the humic and fulvic acids consistent with the presence of 222uinines in the examined humic and fulvic acids (Table 3). An examination of difference and fractional difference absorbance plots and the time dependence of post borohydride reoxidation of Pony Lake and Suwannee River fulvic acids between 230 and 350 nm indicate that PLFA exhibits oxidation induced short wavelength (< 350 nm) absorbance recovery while SRFA and the other terrestrially based humic substances do not (Figures 3 and 4) despite oxidation induced changes (loss) in pH (Table 3). No concurrent, reoxidation induced long wavelength (> 350 nm) absorbance recovery is observed for PLFA or the terrestrially derived humic substances.

Borohydride reduction caused irreversible changes in the fluorescence emission spectra. All reduced spectra were seen to blue shift and increase in intensity. The untreated (pH 7.60) and borohydride reduced (pH 7.60) emission spectra of SRFA and SRHA increase smoothly from long wavelength excitation to short wavelength excitation with the exception of very short wavelength (280-290 nm) which are noisier and shifted to the red (Figure 5). The gain in fluorescence is quantified by $\Delta F = F(T) - F(0)$ recalling that F(T) represents 24 hours of reduction, 24 hours of reoxidation and titration back to the pH of F(0) which was 7.60. SRHA exhibits the smallest gain in fluorescent with an increase of 7 corrected fluorescence units; SRFA has an increase of 20 fluorescence units. Pony Lake fulvic acid (PLFA) doubles in intensity at wavelengths below 500 nm and does not change at wavelengths longer than 520 nm when reduced with sodium borohydride (Figure 5). The emission spectra of both the untreated and borohydride treated PLFA blue shifts uniformly with decreasing excitation wavelength with the exception of the shortest excitation wavelengths from 280-300 nm. Reduction causes a 50 nm blue shift in wavelength maxima of borohydride treated PLFA when compared untreated PLFA. An examination of the ΔF of PLFA shows a gain in fluorescence emission at 300 nm that is not observed in other HA/FA standards. Fluorescence emission spectra and wavelength maxima of untreated terrestrial humic substances Elliott (EHA) and Leonardite (LHA) humic acids differ from SRHA. The fluorescence emission maximum of EHA increases to 575 nm from excitation wavelength 600-440 nm. Fluorescence emission maxima decreases to 550 nm between excitation wavelengths 450-400 nm remaining at fluorescence emission maxima at 550 nm from excitation wavelengths 400-280 nm. Leonardite HS fluorescence emission increases smoothly to 500 nm from excitation wavelengths 600-420 nm. The fluorescence emission remains at 500 nm from excitation 420-280 nm (Figures 5).

Following borohydride reduction (Figures 5) reduced soil derived terrestrial HS (EHA and LHA) double in intensity and blue shift by approximately 100 nm. Upon inspection significant differences can be noted when comparing aquatic to soil derived emission spectra. The untreated soil derived material exhibit high fluorescence emission intensity at long wavelengths (red edge) relative to aquatic samples. Aquatic samples at the red edge recede to the baseline while the terrestrial samples show significant emission under the same optical conditions. Further, upon reduction the blue shift is on the scale of hundreds on nanometers for soil samples as opposed to tens of nanometers found in aquatic samples. Finally, aquatic samples appear to have smooth almost monotonic emission spectra as the excitation spectra increases in energy with the exception of the previously notes short wavelengths (280-300 nm). No sub-features appear in the aquatic samples. This is not the case in the terrestrial samples where a secondary feature can be seen at low excitation wave lengths (Figure 5).

Quantum yield of untreated (Figure 6, lower left), borohydride reduced (Figure 6, lower right) show an increase

in quantum yield in the terrestrially derived samples as well as the microbial sample that is at least double upon borohydride reduction at wavelengths below 450 nm reflecting the borohydride reduced loss of absorbance (Figure 1) and simultaneous but variable increase in fluorescence emission (Figure 5). The soil derived humic acids LHA exhibits a feature at 490 nm that corresponds to the secondary feature in the fluorescence emission spectra (Figure 5) that is not completely eliminated by borohydride reduction. The same feature is present in the untreated EHA fluorescence spectra (Figure 5) and eliminated by borohydride reduction. The loss of the underlying structural feature in EHA is reflected by the reduction of quantum yield at wavelengths above 450 nm. The wavelength maxima of fluorescence emission (Figure 6 untreated upper left and borohydride reduced upper right) of PLFA, SRFA and SRHA increase uniformly from low to high wavelength with the exception of very low wavelengths. The untreated soil derived humic acids exhibit a plateau at wavelengths below 420 nm. The fluorescence wavelength maxima of LHA (500 nm) is lower than the wavelength maxima of EHA (550 nm) (Figure 6).

The pH dependence of the spectral slope values for untreated and borohydride reduced, Sephadex G-10 cleaned, terrestrially derived humic acids at concentrations that allow UV (< 350 nm) absorbance spectra and 0.01 mole L^{-1} ionic strength show a consistent pattern of behavior (Table 4). Untreated LHA and SRHA as well as SRFA have lower spectral slope values than do borohydride reduced spectral slope values at all pHs examined (pH 3.00, pH 6-7 and pH 11.00). The untreated terrestrial soil and aquatic samples exhibit less reduction in slope between pH 3.00 and the neutral pH (pH 6-7) then the degree of change found between the neutral pH (pH 6-7) and pH 11.00. Untreated EHA exhibits no difference in the rate of change of the spectral slope value from pH 3.00 to pH 11.00; the spectral slope of untreated EHA decreases as pH increases at a uniform rate. The borohydride reduced spectral slope value of EHA between pH 3.00 and the neutral pH (pH 6-7) increase, but the spectral slope decreases between the neutral pH point and pH 11.00 (Table 4). The borohydride reduced LHA has the same pattern as its untreated counterpart but each point is found at a higher spectral slope value (Table 4). Borohydride reduced SRHA and SRFA show a slight or no decrease respectively in the spectral slope value between pH 3.00 and pH 6-7, and a more pronounced reduction in spectral slope between the neutral pH point and the high pH point (pH 11.00). The neutral pH spectral slope values are consistent with the spectral slope values completed in at pH 7.6 ($n \geq 3$), with no supplemental ionic strength addition, or removal of residual borate (Table 4) with the exception of the two borohydride reduced fulvic acids. These two borohydride reduced samples exhibit a significant difference between the replicate spectral slope values generated at pH 7.6 and 350 nm and the pH dependent spectral slope values. Spectral differences due to changes in ionic strength are presented in Heighton, 2013.

4. Discussion

Spectral slope, optical titration and fluorescence difference spectra (ΔF) indicate that quinones may act as acceptor moieties in PLFA. Quinones do not appear to be an important component of electronic interactions of terrestrially based aquatic or soil derived humic or fulvic acids. The optical properties of terrestrially based sources of humic and fulvic acid have been attributed to partial oxidation of land based plant materials, specifically lignin phenols. Microbial sources of fulvic or humic acids do not contain lignin, hence the generation of long wavelength absorbance cannot be assigned to the same precursor material; instead electronic interactions could be potentially forming in PLFA between secondary amine heterocyclic donors and tertiary amines, heterocyclic moieties, quinones or cyclic ketone acceptors. Amino acid/decomposition products of amino acid and or peptidoglycan decomposition products can form secondary and tertiary amines as well as other heterocyclic moieties (sulfur containing) supplying both donor and acceptor groups able to generate electronic interactions capable of generating long wavelength absorbance which could not be generated independently by the components of the system. The optical properties resulting from electronic interaction between amino acids or heterocyclic aromatic species and quinones could be expected to differ from lignin generated charge transfer bands in several ways.

Historically, marine sources of CDOM have higher spectral slope values (S) than near shore CDOM samples (Helms et al., 2008). If marine sources of CDOM are closely related to or contain a high proportion of bacterial source FA than untreated PLFA should have a high spectral slope when compared to aquatic FA, namely SRFA. The spectral slope value (S) of borohydride reduced material such as SRFA a source of terrestrial humic acid would potentially differ from the spectral slope of borohydride reduced PLFA a microbial source of humic acid due to the difference in the donor moieties in charge transfer bands. If the short wavelength absorbance of PLFA (190-350 nm) can be attributed to largely amino acids/peptioglycan decomposition products producing heterocyclic amines with additional but proportionally less quinone and aromatic ketones than then the spectral slope should be steeper than the spectral slope of SRFA a terrestrial sources of FA. Specifically, the pH

dependent spectral slope values of the microbial source of humic substance should be and are very different from the pH dependent spectral slope values of terrestrially based lignin phenol humic substances. Carboxylic acids from amino acids or other sources are not reduced by borohydride (Cleyden et al., 2001; Tinnacher & Honeyman, 2007). Amines (secondary or tertiary) are not reducible by simple borohydride reduction. The only nitrogen containing compounds easily reducible by borohydride are imides which may be present as they are used in biological systems to produce amino acids from keto acids in an enzymatically driven system (Cleyden et al., 2001) but it is unlikely that they make up a significant fraction of the PLFA structure. SRFA when compared to PLFA would not be enriched with nitrogen but would contain a substantially different group of reducible ketones potentially lacking in PLFA because of the terrestrial plant derived lignin phenol that SRFA and other terrestrially sourced humic material originate from (Fang et al., 2011). Loss in absorbance due to borohydride reduction in terrestrial sources potentially represents higher relative percent carbonyl moieties than that of PLFA but our results indicate that upon borohydride reduction 80% of the long wavelength absorbance of PLFA is lost while only 60 % of the SRFA absorbance is lost (Figure 1). This implies that loss of carbonyl groups in PLFA is seminal to the loss of long wavelength absorbance while SRFA retains some ability to maintain electronic interactions that result in persistence of the long wavelength tail despite borohydride reduction. The ability of SRFA to maintain some electronic interactions may mean that borohydride is physically prevented from reducing carbonyl groups that are sterically hindered or that SRFA has greater diversity of species participating in electronic interactions that can produce long wavelength absorbance and are not borohydride reducible when compared to PLFA.

Absorbance of PLFA reduced with borohydride and reoxidized would be expected to recover to a greater extent than SRFA upon reoxidation if quinones are enriched when compared to aromatic ketones. Aromatic ketones are present in PLFA but when compared to SRFA and the diverse suite of aromatic ketone acceptors generated by lignin phenols in terrestrial sources of HA/FA the proportion of aromatic ketones in PLFA should expected to be lower. Quinones may be enriched when compared to aromatic ketones as quinones are ubiquitous in microbial systems (Hiraishi et al. 1998; Hiraishi et al. 1989; Liu et al. 2000). Ubiquinone 10 and napthaquinones with multiple isoprene side chains have been found at high concentrations (moles mg^{-1} dried cells) (Hiraishi et al. 1998).

The mechanism generating long wavelength optical absorption bands, electronic interactions, is likely consistent between microbial sources of fulvic acid and terrestrial humic/fulvic substances generated from lignin phenol, but the constituents that act as the donor within the electronic interaction complexes differ based on the availability of source material. Microbial sources (PLFA) do not contain phenolic groups by definition but instead may employ heterocyclic moieties, such as secondary amines as donors and tertiary amines, quinones, aromatic ketones or other unidentified moieties as acceptors. Borohydride reduction of PLFA and the other humic/fulvic acids studied partially or completely remove acceptor moieties (quinones, aromatic ketones) from the charge transfer complex resulting in decreased absorbance, an increased spectral slope coefficient and increased fluorescence emission intensity. The relative amount of quinone moieties participating in electronic interactions of PLFA appears to be higher than found in the other fulvic and humic acids and can be seen in the recovery of absorbance at in the UV range of the absorbance and difference spectra (Figures 3-5, Table 3).

Spectral slope parameterizes an expediential curve and as S increasing the short wavelength curve becomes steeper indicating that borohydride reduction is disrupting charge transfer bands. Donors and or acceptor chemical moieties within the charge transfer model are no longer being quenched resulting in an increase in short wavelength absorbance and a concurrent loss of long wavelength absorbance with a resultant increase in the spectral slope. The spectral slope additionally has a pH dependence that is directly linked to the pka of absorbing species. The pH dependence of the spectral slope of the borohydride reduced humic and fulvic acids of terrestrial origin decrease at high pH but the spectral slope value of PLFA is a consistent value despite changes in pH (Table 4). Clearly, borohydride reduction results in the loss of the long wavelength tail in PLFA but there is no concurrent gain in short wavelength absorbance. This may be a result of how spectral slope values are calculated. The spectral slope is historically parameterized from 290 nm to 820 nm (Green & Blough, 1994). The wavelength maximum of many quinones is at shorter wavelength than 290 nm (Ma et al., 2010). Further many heterocyclic secondary amines have pka values that are close to or above pH 11.00 (Yamauchi & Odani, 1985). This does not definitively implicate secondary amines and quinones formation of electronic interactions in PLFA but it does support the premise.

Quantum yields and wavelength maxima reflect differences between soil and aquatic HS derived from lignin phenol (Figure 6). Two points are evident (1) soil humic substances (LHA) and (EHA) (Figure 5 and 6) have contributions to their fluorescence emission spectra that are not represented in aquatic materials (SRFA) and

(SRHA) (Figures 5 and 6). Borohydride reduction eliminates the long wavelength fluorescence contributor in EHA and halves it in the LHA samples. This loss in long wavelength fluorescence may be an indication of disruption of stacking of black carbon and an inability to regenerate post borohydride reduction physical proximity (stacking) required for the extended conjugation needed to producing increased fluorescence emission at long wavelengths. (2) Untreated soil humic substances exhibit a plateau in fluorescence wavelength maxima that is not seen in other humic substances or observed post borohydride reduction (Figure 6). Speculatively, the soil wavelength maxima trend and the large fluorescence blue shift produced by borohydride reduction may be reflective of the lack of photochemical exposure and/or the relative physical stability afforded by this soil matrix; which may allow some moieties labile to photochemical alteration to be preserved in soil environments. It has been suggested that soil humic material is capable of forming micelles that can provide protection to labile moieties (Sutton & Sposito, 2005). Formation of micelles is unlikely due to the Beer Lambert behavior seen in all HS in this study (Table 2) but other forms of protection such as encapsulation or association with cations or clays is known to provide protection from enzymatic attack (Heighton et al., 2008; Hedges et al. 2000; Baldock & Skjemstad, 2000). The soil humic acids EHA and LHA have a higher percentage of black carbon or conjugated cyclic carbon (Skjemstad et al., 2002). The carbon content of LHA is potentially high when compared to other humic acids as this HA is derived from lignite a precursor of coal. Although, LHA potentially has significant amounts of black carbon, it is likely that titratable quinones shown to be present in the structure of black carbon are not represented as fully as would be in the Mollisol derived EHA because EHA contains more oxygen than does LHA (Mao et al., 2007; Allard & Derenne, 2007). Borohydride reduction of soil HA potentially captures additional types and a higher concentration of reducible species than seen in aquatic sources of lignin derived HA/FA.

Borohydride reduction may cause physical alterations in the macro structure preventing reassembly of optical components not directly affected by the reduction. This is exemplified by the loss of long wavelength fluorescence emission in the HS (LHA and EHA) (Figure 5) and by the short wavelength absorbance recovery (< 350 nm) with no concurrent long wavelength recovery (Figures 1-3). The quinone derived recovery of reoxidized HS is small, much lower than the expected concentration observed by electrochemical means (Aeschbacher et al., 2010) indicating quinones although an important redox buffer may not play a substantial role as a charge transfer acceptor and therefore do not play large role in the optics of terrestrially sourced material (EHA, LHA, SRFA, SRHA). Soil sourced and aquatic sources of HA/FA should fit into a hierarchy of digenesis. The premise is supported by the ranking of spectral slope coefficient values (Table 4), and the identification of chromophores in soil derived humic acids not found in aquatic sources of humic substances (Figures 5).

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