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Optical Properties of Humic Substances and CDOM: Effects of Borohydride Reduction

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Treatment of Suwanee River humic (SRHA) and fulvic (SRFA) acids, a commercial lignin (LAC), and a series of solid phase extracts (C18) from the Middle Atlantic Bight (MAB extracts) with sodium borohydride (NaBH_4), a selective reductant of carbonyl-containing compounds including quinones and aromatic ketones, produces a preferential loss of visible absorption ($\geq 50\%$ for SRFA) and substantially enhanced, blue-shifted fluorescence emission (2- to 3-fold increase). Comparison of the results with those obtained from a series of model quinones and hydroquinones demonstrates that these spectral changes cannot be assigned directly to the absorption and emission of visible light by quinones/hydroquinones. Instead, these results are consistent with a charge transfer model in which the visible absorption is due primarily to charge transfer transitions arising among hydroxy- (methoxy-) aromatic donors and carbonyl-containing acceptors. Unlike most of the model hydroquinones, the changes in optical properties of the natural samples following NaBH_4 reduction were largely irreversible in the presence of air and following addition of a Cu^{2+} catalyst, providing tentative evidence that aromatic ketones (or other similar carbonyl-containing structures) may play a more important role than quinones in the optical properties of these materials.

Introduction

The importance of humic substances (HS) and chromophoric dissolved organic matter (CDOM) to aquatic ecosystems has become increasingly evident over the past decade (1–5). Despite their extensive study over the last fifty years however, confusion still reigns over the underlying structural basis of their optical properties and how these may be related to the redox state and photochemical reactivity of these materials. Klapper et al. (6) first observed that under conditions of microbial reduction, the excitation–emission matrix spectra (EEMS) underwent shifts in the position and intensity of the excitation/emission maxima and assigned these changes

directly to quinones (and the corresponding hydroquinones). This assignment was based purely on presumed similarities with the emission properties of anthroquinone-disulphonate and its hydroquinone (whether this was the 1,5- or 2,6-derivative was not specified). Similarly, Cory and McKnight (7) later assigned quinone-like and hydroquinone-like structures directly to fluorescent components acquired by parallel factor analysis (PARAFAC), again based solely on their presumed similarities with the excitation and emission maxima of model quinones/hydroquinones. Neither of these two studies employed a selective means of reduction (either electrochemical or chemical), nor did they provide a quantitative comparison between the fluorescence quantum yields of the HS and model quinones/hydroquinones. Further, possible changes in the absorption spectra of the HS under differing redox conditions were neither investigated nor compared with model quinones/hydroquinones. While a later study by Fimmen et al. (8) indicated that the electrochemical reduction of some HS did produce increased fluorescence intensity, these changes were rather small (10–44%). Moreover, the complete spectral dependence of the absorption and emission was not reported, nor was an estimate of the extent of reduction. Although Macalady and Walton-Day (9) recently questioned Cory and McKnight's (7) assignment of the components acquired from a PARAFAC model to "quinone-like" moieties based on the relative invariance of these components under differing chemically induced redox conditions, Miller et al. (10) suggested that this was simply due to inner filter effects in the study of Macalady and Walton-Day.

In contrast to the above studies that have invoked a direct emission contribution from "quinone-like" moieties (6–8), Del Vecchio and Blough (11, 12) and Boyle et al. (13) provided evidence that the optical properties of terrestrial HS and CDOM cannot arise solely from a simple superposition of numerous independently absorbing and emitting chromophores, but instead that intramolecular electronic interactions between chromophores must also be playing an important role. In this model, the optical properties result in part from intramolecular charge transfer interactions between hydroxy (methoxy)-aromatic electron donors and quinoid (or other) electron acceptors formed through the partial oxidation of lignin (13), and possibly other polymeric hydroxy-aromatic precursors (12). The long-wavelength visible absorption and emission have thus been proposed to originate from charge-transfer transitions, whereas the ultraviolet absorption and near-visible emission have been proposed to arise principally from the aromatic structures known to be a part of lignin (local donor states; 12, 13). Elimination of the acceptor moieties by reduction is thus predicted to produce a preferential loss of the visible absorption due primarily to the loss of the charge-transfer transitions and not from the direct loss of acceptor absorption. Enhanced, blue-shifted emission is also expected, due to emission from local donor states that are no longer quenched because of the removal of acceptors by reduction.

Consistent with the expectations of this model, we show here that treatment of aquatic HS and CDOM with NaBH_4 , a selective reductant of carbonyl-containing compounds including quinones, ketones, and aldehydes (14–16), leads to the preferential loss of visible absorption and to enhanced, blue-shifted emission. By comparing these results with those obtained from a series of model quinones/hydroquinones, we show further that these changes in the absorption and emission spectra cannot be attributed directly to absorption

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and emission by quinones/hydroquinones. Interestingly, these changes in optical properties were not reversed substantially in the presence of O_2 , providing tentative evidence for the irreversible reduction of such species as ketones and aldehydes to alcohols. This result further suggests that these species, particularly aromatic ketones (aldehydes), may play a more significant role than quinones in the optical properties of aquatic HS and CDOM. The results of this study thus provide fundamentally new insights into the structural basis of the optical and photochemical properties of these ubiquitous materials.

Materials and Methods

Materials. All quinones were acquired from Sigma-Aldrich and most were further purified by sublimation or recrystallization. Suwannee River humic acid (SRHA) and fulvic acid (SRFA) were obtained from the International Humic Substances Society. Alkali-extracted and carboxylated lignin (LAC; Lot 19714 DS), Sephadex G-10 (40–120 μm), and phosphate salts were obtained from Sigma-Aldrich. Sodium borohydride ($NaBH_4$) was from Fisher. Cupric chloride dehydrate (99.5% purity) was obtained from J.T. Baker. Quinine sulfate (QS) was obtained from MC/B. Water was obtained from a Milli-Q Plus purification system (Millipore). Solid-phase C-18 extracts, collected as previously described (13, 17), were obtained from the Delaware Bay (38.81 N, -75.09 W), the continental shelf of Middle Atlantic Bight (MAB) (38.00 N, -74.05 W), and the western boundary of the Gulf Stream (36.40 N, -71.02 W) during a summer cruise (September 2005). Hereafter, these samples are referred to as either the C18 or MAB extracts.

Apparatus. Hewlett-Packard 8452A and Shimadzu UVPC 2401 spectrophotometers were employed to acquire UV–

visible absorption spectra. An Aminco-Bowman AB-2 luminescence spectrometer was employed for the fluorescence measurements (monochromator excitation and emission band-passes set to 4 nm). Fluorescence lifetime measurements of the hydroquinones were performed on an ISS K2 multifrequency phase fluorometer, using previously described data collection and analysis protocols (18).

Optical Measurements. All experiments were conducted using 1-cm quartz cuvettes containing solutions in phosphate buffer (10 mM, pH 7) or in Milli-Q water (neutralized with NaOH to pH 7). Absorption spectra were recorded against the phosphate buffer or Milli-Q water over the range from 190 to 820 nm. Difference absorption spectra (ΔA) were obtained by subtracting the absorption spectrum recorded at time t ($A(t)$) from the original spectrum ($A(0)$). Fluorescence emission spectra were collected over the 280–600 nm excitation range, while the absorbance was kept at ≤ 0.05 – 0.1 OD at the excitation wavelength (12, 13). Difference emission spectra (ΔF) were obtained by subtracting the original emission spectrum, $F(0)$, from the spectrum obtained at time t , $F(t)$. Emission quantum yields were obtained relative to QS as reported previously (12, 13). Molar absorptivities of the quinones were estimated from carefully prepared solutions containing weighed amounts of these compounds; the molar absorptivities of the hydroquinones were estimated from these same solutions following deaeration and reduction with a small quantity of solid $NaBH_4$ or photochemically (see below).

$NaBH_4$ Reduction. Known concentrations of SRHA, SRFA, LAC, and the C18 extracts were transferred to a 1-cm cuvette (3 mL) and sparged with ultrapure N_2 for 10 min. For SRHA, SRFA, and LAC at 50 mg/L, ~ 5 mg of solid $NaBH_4$ was then added to the cuvette and dissolved by bubbling with N_2 , with

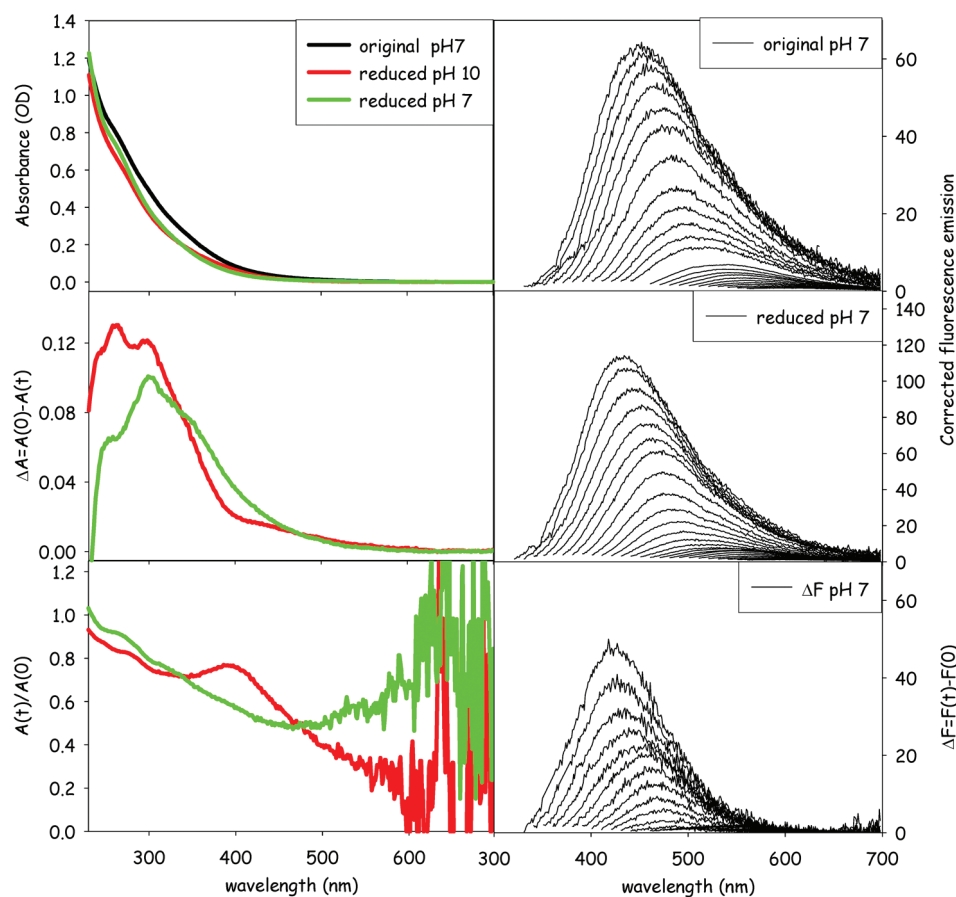


FIGURE 1. Changes in optical properties for SRFA (50 mg/L, pH 7) following $NaBH_4$ reduction (~ 5 mg, 2 h after addition). Note that $\Delta A > 0$ indicates loss of absorbance while $\Delta F > 0$ represents gain of fluorescence signal.

the cuvette then capped under N_2 . For the C18 extracts, the absorbance at 300 nm was matched to that of SRFA (50 mg/L). Higher concentrations of SRHA, SRFA, and LAC (500–1000 mg/L) were reduced under the same conditions, but with a larger amount of $NaBH_4$ (~15–25 mg), similar to the conditions of Tinnacher and Honeyman (15). Samples were treated in the dark, with the optical properties measured prior to and following reduction. The reduction was considered to be complete when no further changes in absorption spectra were observed. Because a substantial excess of $NaBH_4$ was employed in these experiments, the pH rose to ~10 over the course of the reduction and thus the pH was adjusted under air to its original value for comparison with the original spectra. In some cases, a small G-10 column (2.5 × 8 cm, equilibrated with Milli-Q water) was employed to adjust the pH and ensure complete removal of excess $NaBH_4$. To test further the reversibility of the reduction under air, a 600 μM solution of $CuCl_2$ was prepared, with 50- μL aliquots (~10 μM) added to the reduced SRFA at 20-min intervals until the final Cu^{2+} concentration reached ~60 μM .

Benzoquinones and naphthoquinones were dissolved in the phosphate buffer and transferred to a 1-cm cuvette (3 mL). Following sparging with ultrahigh-purity N_2 for 10 min, a small quantity of solid $NaBH_4$ was added to the cuvette (≤ 0.2 mg) and dissolved by bubbling with N_2 . Reduction to the hydroquinones was complete within minutes. Because of the small quantity of $NaBH_4$ employed, no change in pH was observed from the reaction of excess borohydride with water (ref 15; see above). Anthroquinone-2,6-disulphonate and anthroquinone-1,5-disulphonate were reduced photochemically by irradiation at 326 nm (0.4 mW cm^{-2}) of solutions containing a 1:1 volume ratio of the phosphate buffer and 2-propanol under N_2 (19–21). Following photoreduction, introduction of air to these solutions by bubbling regenerated the original spectra quickly, indicating that these quinones were successfully reduced by this method. The reversibility of quinone reduction was examined under both low (≤ 0.2 mg) and high (≥ 5 mg) $NaBH_4$ additions for selected quinones by following the absorption spectra over time after addition of air to the hydroquinones.

Results

HS, LAC, and C18 Extracts. Following treatment with $NaBH_4$, all samples exhibited a significant loss of absorption across the visible and ultraviolet wavelengths and a substantial increase in fluorescence emission intensity in the blue portion of the visible spectrum (Figures 1–3). Reaction of excess $NaBH_4$ with water during the course of the reduction increased the pH to ~10 (15); readjusting the pH to its original value under air resulted in an additional loss of absorption in the visible and a small increase in absorption in the ultraviolet (Figures 1 and 2), but did not largely affect the spectral dependence of the fluorescence emission intensity. Control experiments showed that changes in pH alone could not account for the changes in optical properties (Supporting Information (SI), Figures S1, S2).

At mass ratios of $NaBH_4$ to SRFA of ≥ 5 at pH 7 (or 8), a maximal change in the optical properties was observed, with most of this change occurring within the first 2 h, and with no further change after 24 h (SI, Figure S3). Surprisingly, introduction of O_2 by bubbling with air after complete reduction (24 h) did not produce any significant changes in the optical properties at pH 10 ($\leq 5\%$; SI, Figure S4). Although as noted above, some additional change in absorption was observed with the decrease in pH from 10 to 7 under aerated conditions, no further changes in the optical properties were observed in the presence of air at neutral pH, even over the course of 20 days (SI, Figure S5), consistent with the prior work of Tinnacher and Honeyman (15). Absorption spectra obtained following passage of aerated samples over a small

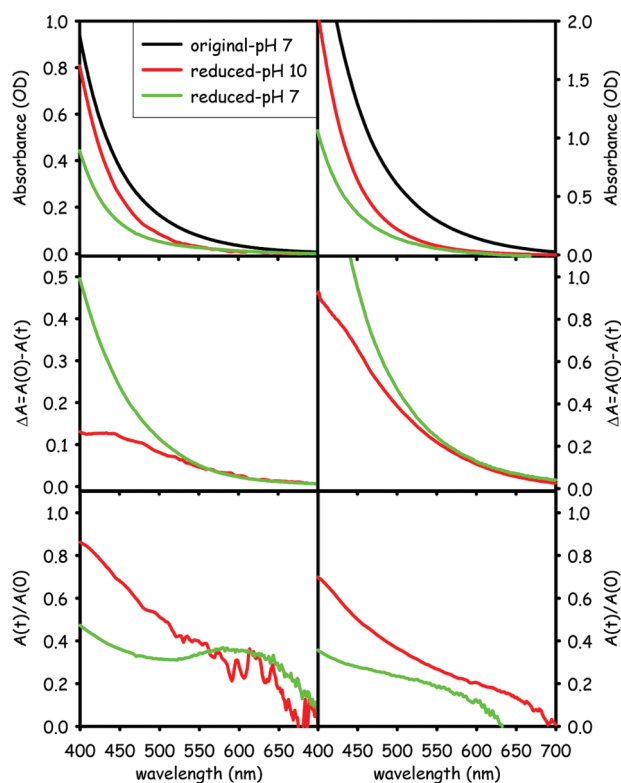


FIGURE 2. Wavelength dependence of absorption prior to (black line) and following (red and green lines) reduction (24 h) of SRFA in Milli-Q water, pH 7. Left panel, 500 mg/L SRFA, ~15 mg of $NaBH_4$. Right panel, 1000 mg/L SRFA, ~25 mg of $NaBH_4$. Note that pH was neutralized to 7 with HCl (left panel) and by passing the samples over a small G10 column (right panel).

G-10 column to adjust the pH to 7 and to ensure removal of excess $NaBH_4$ were not significantly different from those obtained following pH adjustment of aerated samples alone (Figure 2). Further, no changes were observed following addition of micromolar quantities of Cu^{2+} to the aerated solutions at pH 7 (22, 23; SI, Figure S6). These results indicate that the optical changes observed upon $NaBH_4$ reduction are largely irreversible. The ramifications of this observation are discussed below.

Following reduction, all samples exhibited the largest absorption loss in the ultraviolet, peaking at ~300 nm for SRHA, SRFA, LAC, and the Delaware Bay sample, but at slightly shorter wavelengths for the shelf and offshore surface MAB extracts (~280 nm; Figures 1, 3, and 4). However, for all samples, the greatest fractional loss of absorption was observed in the visible, where 50% or more of the original absorption was eliminated by reduction (Figures 1 and 2; SI, Figures S7–S11), as is more easily seen at higher sample concentrations (Figure 2).

For SRHA, SRFA, LAC, and the Delaware Bay C18 extract, emission intensities increased by approximately a factor of 2, while peak maxima shifted to the blue by ~10 to 15 nm (Figure 4; SI, Figures S7–S9). In contrast, the emission intensities of the continental shelf and Gulf Stream C18 extracts increased by approximately a factor of 3, with peak maxima shifting to even shorter wavelengths, ~400 nm (Figure 4; SI, Figures S10, S11). The emission difference spectra (ΔF) were also blue-shifted relative to the original spectra, with the MAB extracts and LAC shifted to shorter wavelengths than SRFA and SRHA (Figure 3).

The fluorescence quantum yields of the reduced samples were substantially enhanced at excitation wavelengths (λ_{ex}) less than ~450 to 475 nm (Figure 4). This enhancement is a result of two factors: (1) the increase in fluorescence

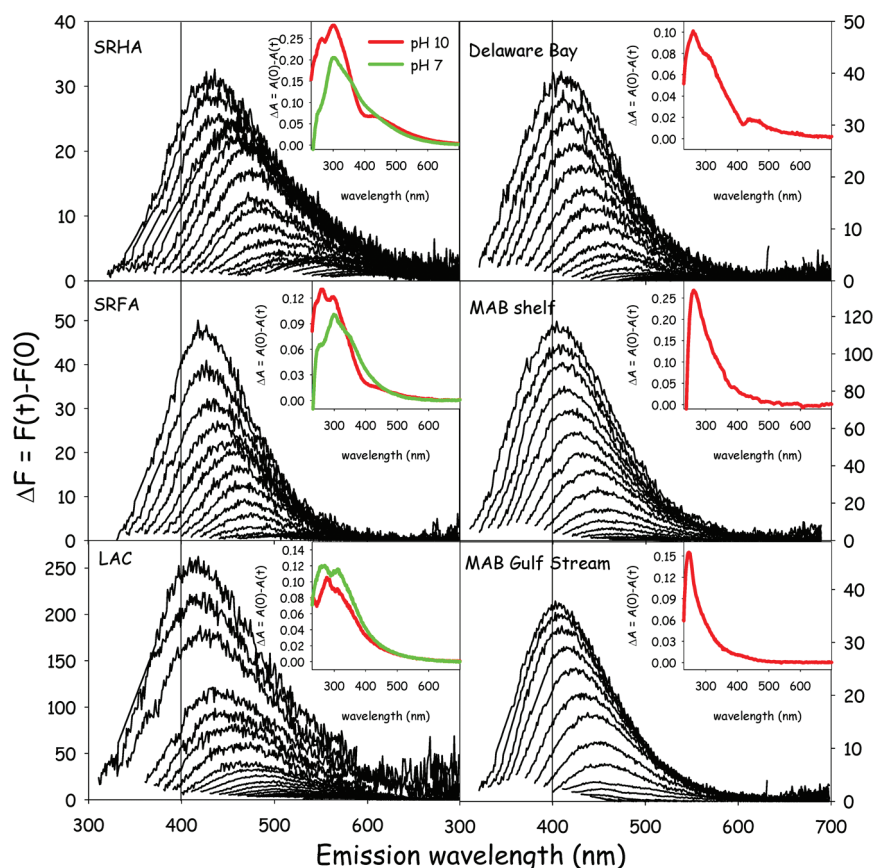


FIGURE 3. Wavelength dependence of ΔF and ΔA (inset) following NaBH_4 reduction (2 h). Note that $\Delta F > 0$ indicates gain in F , while $\Delta A > 0$ corresponds to loss of A . Vertical black lines mark the 400 nm wavelength.

emission at λ_{ex} less than ~ 450 to 475 nm, and (2) the significant decrease in absorption over these same wavelengths (Figures 1, and 3). Because the largest emission increases occurred at the λ_{ex} where the absorption losses were also large, it would appear unlikely that NaBH_4 reduction is producing new, blue-shifted emitting species that absorb in this wavelength range. Instead, as discussed below, it is far more likely that the enhanced emission arises from existing species that are no longer quenched due to the elimination of acceptors by borohydride reduction.

Model Quinones and Hydroquinones. Absorption and emission maxima, fluorescence quantum yields, and where possible, fluorescence lifetimes were acquired for a series of model quinones and their corresponding hydroquinones to test whether quinones or hydroquinones could be directly contributing to the absorption and emission spectra of the natural samples (Table 1; SI, Figures S12–S18). The lowest-lying absorption bands of the *o*- and *p*-benzoquinones were primarily observed in the blue portion of the visible spectrum and were very weak, characteristic of $n \rightarrow \pi^*$ transitions (20, 21). Except for 5,8-dihydroxynaphthoquinone, the lowest-lying absorption bands of the anthroquinones and naphthoquinones were below 400 nm and thus would not contribute to the absorption in the visible (19). The fluorescence quantum yields of *all* quinones examined were either indistinguishable from zero or far smaller than the quantum yields measured for the natural samples (Table 1, Figure 4). Notably, the absence of significant emission from these differing classes of quinones indicates that they cannot be directly contributing to the observed emission from HS and CDOM, in direct contradiction to the previous proposal of Cory and McKnight (7).

Reduction of the quinones to hydroquinones with NaBH_4 shifted the lowest-lying absorption bands of the *o*- and *p*-benzoquinones and the naphthoquinones to substantially

shorter wavelengths, generally to ~ 280 to 290 nm for most of the *o*- and *p*-benzoquinones (Table 1; SI, Figures S12–S18). Only the lowest-lying band of the anthroquinones was shifted to longer wavelengths in the visible following reduction (19; Table 1; SI, Figure S12). Our results indicate (Table 1) that if these types of anthroquinones contributed substantially to the optical properties of terrestrial HS, as implied by Klapper et al. (6) and Cory and McKnight (7), then their reduction should lead to an increase in the visible absorption and enhanced red-shifted emission in direct contradiction to our experimental results (Figures 3 and 4; Table 1). Moreover, the absorption and emission line-shapes and maxima of the hydroquinones (Table 1; SI, Figures S12–S18) are also inconsistent with the proposed assignments of these species to the emission components acquired by PARAFAC (7).

The absorption spectra of the hydroquinones produced by addition of either low (<0.2 mg) or high (25 mg) amounts of NaBH_4 to solutions of methyl-benzoquinone, 2,5-dimethyl-benzoquinone, and duroquinone showed only limited reversion ($<50\%$) to their original spectra over the course of 12 h at neutral pH in the presence of air (SI, Figure S19). For these quinones, absorption spectra characteristic of the hydroquinones were observed under both low and high additions of borohydride, indicating that the minimal reversion was due to slow oxidation kinetics and not to irreversible chemical reaction(s) under high concentrations of borohydride. In contrast, the hydroquinones of 2,6-dimethoxy-benzoquinone, 2,3-dimethoxy-5-methyl-benzoquinone, and 3,5-di-*t*-butyl-1,2-benzoquinone exhibited substantial ($>50\%$) or complete reversion to the quinones under these same conditions (SI, Figure S19). The hydroquinones of the anthroquinones and naphthoquinone were observed generally to oxidize quickly in the presence of air. These results suggest that the oxidation rates of the hydroquinones mirror their

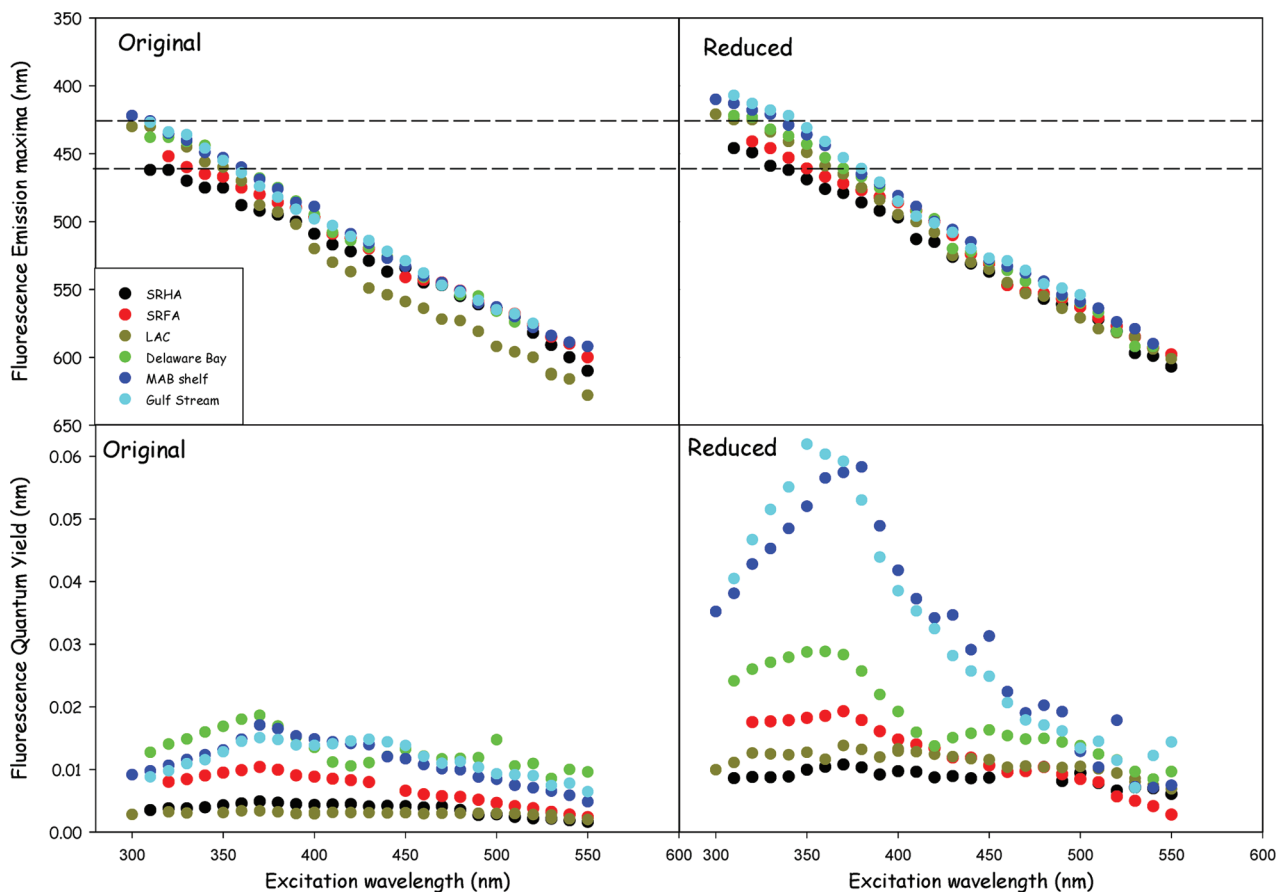


FIGURE 4. Wavelength dependence of emission maxima (top) and fluorescence quantum yields (bottom) for samples prior to (left panels) and following (right panels) NaBH_4 reduction (2 h). Dashed lines bracket the emission maxima prior to reduction.

redox potentials, with more easily oxidized hydroquinones reverting more rapidly to the quinone form.

Discussion

These results are broadly consistent with the charge transfer model previously proposed for the optical properties of HS and CDOM (12, 13) and lignins (13, 24). This model predicts that elimination of electron acceptor groups or moieties (quinoid, aromatic ketone, or carbonyl moieties in other structures) by NaBH_4 reduction will lead to the preferential loss of long-wavelength absorption due to the elimination of the lower energy charge transfer transitions (12, 13; Figures 1–4). The most likely quinoid acceptors within these materials, substituted *p*- and *o*-benzoquinones produced by oxidation of the single-ring aromatics within lignin, exhibit only very weak absorption bands ($n \rightarrow \pi^*$) in the blue portion of the visible, usually at wavelengths much less than 500 nm (Table 1; 20, 21). With the exception of 5,8-dihydroxynaphthoquinone, the anthroquinones and naphthoquinones examined all have their lowest-lying absorption bands in the ultraviolet, and thus could not contribute to the visible absorption.

Employing the molar absorptivities reported in Table 1 and the electron accepting capacity (EAC) recently reported by Aeschbacher et al. (25) for SRFA ($\sim 600 \mu\text{mole e}^- (\text{g HS})^{-1}$), we can estimate the maximum contribution of the *o*- and *p*-benzoquinones to the absorption loss (ΔA) observed in the visible following NaBH_4 reduction of SRFA (Figure 2). At 500 mg/L SRFA, the EAC reported by Aeschbacher et al. (25) translates to a concentration of $\sim 150 \mu\text{M}$ quinone, based on a two-electron reduction of quinone to hydroquinone. Estimated absorption losses in the visible upon reduction of the model quinones vary from a low of 0.003 for benzo-

quinone at 424 nm to a high of 0.14 at 472 nm for 4-*t*-butyl-5-methoxy-1,2-benzoquinone, corresponding to maximum contributions to the total absorption loss ranging from 0.8% to 78% for benzoquinone at 424 nm and for 4-*t*-butyl-5-methoxy-1,2-benzoquinone at 472 nm, respectively (Figure 2). Only the absorption loss calculated for 5,8-dihydroxynaphthoquinone (0.35 at 514 nm) equals or exceeds the observed absorption loss (Figure 2). Note also that these absorption contributions were calculated assuming that each quinone represented the total quinone concentration of SRFA as estimated from the EAC, although a distribution of quinones would be required to explain the spectral dependence of the visible absorption (Table 1, Figure 2) as well as the redox properties (25, 26). This requirement would further lower the possible contribution of these species to the visible absorption.

Consistent with these calculations, prior work has also shown that the direct loss of absorption from quinones cannot account for the magnitude of the visible absorption loss produced by NaBH_4 reduction of kraft lignin (24). As pointed out above, reduction of the anthroquinones would produce increased visible absorption and enhanced red-shifted emission (Table 1), exactly opposite to the experimental results (Figure 2). Further, the structures of the aromatic ketones thought to be a part of these materials (14), as well as the aromatic ketones used to model the photochemical behavior of these materials (27, 28), do not absorb in the visible.

Thus, we conclude that the broad, preferential loss of absorption in the visible following NaBH_4 reduction is due primarily to the loss of charge transfer transitions and not from the loss of the acceptor absorption. Importantly, the substantial loss of absorption in the visible upon NaBH_4 reduction (Figure 2) further precludes major contributions

TABLE 1. Absorption and Emission Maxima, Molar Absorptivities, Fluorescence Quantum Yields, and Lifetimes of Model Quinones (Q) and Their Corresponding Hydroquinones (HQ)

	compound	Q	ϕ ($\lambda_{\text{em}}, \text{nm}$)	HQ	ϕ ($\lambda_{\text{em}}, \text{nm}$)	HQ τ (ns)
		$\lambda_{\text{abs}} (\text{nm})$ ($\epsilon, \text{M}^{-1} \text{cm}^{-1}$)		$\lambda_{\text{abs}} (\text{nm})$ ($\epsilon, \text{M}^{-1} \text{cm}^{-1}$)		
1	<i>p</i> -benzoquinone	246 (22000) 296 (320) 424 (20)	0	222 (5150) 288 (2690)	0.80 (326)	1.11
2	methyl- <i>p</i> -benzoquinone	250 (20000) 324 (540) 420 (30)	0	288 (3000)	0.28 (325)	0.43
3	2,5-dimethyl- <i>p</i> -benzoquinone	256 (25000) 328 (260) 416 (31)	0	288 (3870)	0.056 (328)	0.075
4	2,6-dimethyl- <i>p</i> -benzoquinone	258 (17000) 332 (280) 424 (30)	0	286 (2400)	0.073 (323)	0.12
5	duroquinone	270 (21000) 352 (2300)	0.0002 (442)	284 (1990)	0.0008 (324)	^a
6	2,5-dimethoxy- <i>p</i> -benzoquinone	194 (11000) 284 (16000) 374 (240)	0	296 (3770)	0.070 (330)	0.11 (74.2%) 2.5 (16.3%) 60 (9.5%)
7	2,6-dimethoxy- <i>p</i> -benzoquinone	290 (18100) 394 (740)	0	282 (3900)	0.0011 (363)	^a
8	2,3-dimethoxy-5-methyl- <i>p</i> -benzoquinone	268 (15300) 408 (750)	0	286 (2900)	0.0022 (391)	^a
9	2,5-dihydroxy- <i>p</i> -benzoquinone	206 (23400) 316 (22500) 500 (120)	0	280 (14300)	0.0004 (369)	^a
10	tetrahydroxy- <i>p</i> -benzoquinone)	292 (1850) 332 (1900) 364 (1970)	0.0004 (498)	296 (1100) 334 (1600) 364 (1880)	0.0007 (466)	^a
11	4- <i>t</i> -butyl-5-methoxy-1,2-benzoquinone	278 (8140) 472 (910)	0	290 (3460)	0.0052 (333)	^a
12	3,5-di- <i>t</i> -butyl-1,2-benzoquinone	260 (1130) 416 (570)	0	282 (760)	0.0027 (323)	^a
13	anthraquinone-2,6-disulphonic acid sodium salt	210 (32000) 258 (48400) 326 (5220)	0.0001 (507)	274 (96800) 388 (7180)	0.0094 (526)	^a
14	anthraquinone-1,5-disulphonic acid sodium salt	258 (32000) 324 (3050)	0	273 (62300) 388 (5900) 446 (4500)	0.055 (591)	3.44
15	1,4-naphthoquinone	252 (16700) 342 (2490)	0.0005 (523)	242 (4800) 320 (1460)	0.062 (438)	^b
16	5,8-dihydroxy-naphthoquinone	212 (14400) 268 (34900) 514 (2330)	0.0052 (631)	224 (8860) 368 (2160)	0.030 (383)	^b

^a Quantum yield is too low to measure lifetime. ^b Reoxidation is too rapid to measure lifetime.

from compound classes that do not undergo reduction with NaBH_4 , which includes some of the most commonly cited classes of compounds purported to be responsible for the visible absorption, namely extended aromatic or highly conjugated structures as well as most heteroaromatic structures.

Another prediction of this model is that NaBH_4 reduction will produce enhanced, blue-shifted emission arising from local donor states that are no longer quenched owing to the removal of acceptor groups (13). The enhanced emission that is observed following reduction (Figures 1, 3, and 4) cannot be attributed readily to the hydroquinones produced by reduction of most substituted *p*- and *o*-benzoquinones, or to the alcohols produced by reduction of aromatic ketones; in both cases, reduction eliminates the low-lying $n \rightarrow \pi^*$ transition and shifts the absorption and emission maxima to far shorter wavelengths, generally <300 nm and <350 nm for absorption and emission, respectively (Table 1). Instead, the enhanced emission occurs at excitation and emission wavelengths more in line with the known aromatic structures within lignin (local donor states; 13, 29). Further, as noted above, the enhanced emission occurs at λ_{ex} where the

absorption losses are also large, making it more likely that the enhanced emission arises from existing species that are no longer quenched.

One particularly surprising result of this study is that the optical properties of the reduced samples were not reversed substantially upon aeration, even over extended periods of time and following addition of Cu^{2+} , which should catalyze the air oxidation of any hydroquinones that might be formed (22, 23). It is possible that the significant additional loss of absorption in the visible with decreasing pH under aerated conditions might have hidden some smaller reversible change (Figures 1 and 2). However, it must be recognized that >50 to ~100% (at longer wavelengths) of the visible absorption is lost and not recovered after aeration (Figures 1 and 2), suggesting a major contribution from irreversibly reduced species such as alcohols produced by ketone or aldehyde reduction.

This result is even more surprising in light of recent work on the redox reactions of HS and their reversibility (25, 26). Aeschbacher et al. (25) recently found that the electrochemical reduction of Leonardite humic acid was largely reversible over 24 h with O_2 as the electron acceptor and suggested that

quinone moieties dominate the redox characteristics of HS. Fimmen et al. (8) provided evidence that the electrochemical reduction of several HS produced enhanced emission intensity which was reversed upon exposure to air, but the observed enhancement of intensity was minor compared to that reported here (10–44% as compared to 2- to 3-fold).

We can suggest only three possible explanations for the observed lack of reversibility. First, the initially formed hydroquinones may undergo irreversible secondary reactions under the large excess of borohydride employed. However, our observation that most of the model quinones undergo reversible oxidation under air over the course of 12 h following treatment with either low or high NaBH₄ additions would appear to exclude this possibility (SI, Figures S19, S20).

Second, the hydroquinones are reoxidized but do not readily regenerate charge-transfer contacts because the secondary structure of the material is altered. However, even in this case, some intramolecular quenching of emission might still be expected from the quinones even in the absence of charge transfer contacts.

Third, the hydroquinones are reoxidized but other borohydride-reducible acceptors that are not readily reoxidized by O₂ such as aromatic ketones (14–16, 27, 28) or quinone methides (30) are primarily responsible for the change in the optical properties. Past work by Leenheer et al. (14) has shown that aromatic ketones comprise the majority of the carbonyl-group content in aquatic HS, while Canonica and co-workers (27, 28) and Pos et al. (16) have implicated these substructures in the photochemistry of these materials. Further, the similarity of our reaction conditions and results to those previously reported by Tinaccher and Honeyman (15) provides strong evidence that ketones (aldehydes) are being reduced to alcohols and are not reoxidized under our solution conditions. Interestingly, the very small fluorescence changes observed by Fimmen et al. (8) under conditions selective for the electrochemical reduction of quinones supports the idea that aromatic ketones (or other similar carbonyl-containing structures) may play a more significant role than quinones in the optical properties of these materials. A combination of careful electrochemical (25) and optical measurements (Figures 1–4) applied to a broader range of these materials is clearly needed to provide a more definitive answer to this question.

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Supporting Information Available

Effect of pH alone on absorption and emission spectra (Figures S1, S2), kinetics of reduction (Figure S3), absorption spectra of reduced HS after introduction of air (pH 10) (Figure S4), absorption spectra of reduced HS over long times at neutral pH in the presence of air (Figure S5), absorption spectra of reduced HS following addition of a Cu²⁺ catalyst in the presence of air (Figure S6), effects of borohydride reduction on the complete absorption and emission spectra of SRHA, LAC, and the MAB extracts (Figures S7–S11), complete absorption and emission spectra of the model quinones and hydroquinones (Figures S12–S18), and the reversibility of model hydroquinone oxidation under air (Figures S19, S20). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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