Characterization and Quantification of Reversible Redox Sites in Humic Substances

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Cyclic oxidation and reduction reactions using oxygen and palladium with H_2, respectively, of dissolved humic and fulvic acids (HA and FA) and model quinone compounds were used to structurally characterize and quantify the electron-carrying capacity (ECC) of reversible redox sites present in humic substances. This technique was used to examine 8 quinone compounds and 14 HA and FA samples and identified 3 redox sites as a function of their stability against the Pd-catalyzed hydrogenolysis process. Six highly aliphatic HA and FA isolated from landfill leachate did not contain redox sites under any conditions; however, the other HA and FA demonstrated reversible redox properties characterized by a combination of three redox sites. On the basis of the model compound results, it is proposed that one site consists of a non-quinone structure (NQ) and the other two sites have quinone structures. The two quinone sites differ in that one group (Q1) has electron-withdrawing groups adjacent to the quinone functional group while the second group (Q2) contains either no substituents near the quinone or has nearby electron-donating groups with additional substitutents hindering hydrogenolysis through steric interactions. The reversible ECC of NQ sites ranged from 25 to 265 μequiv e^- transferred/g HA or FA, representing 21−56% of the total ECC of the HA and FA when measured with the mildest reducing method (pH 8.0, pure Pd). Q1 redox sites resistant to hydrogenolysis at pH 8.0 using Pd/Al_2O_3 accounted for 13−58% of the total ECC and ranged from 40 to 120 μequiv e^-/ g HA or FA. The most sensitive Q2 reversible redox sites accounted for 8−50% of the total ECC (20−220 μequiv e^-/ g HA or FA). These results directly demonstrate that HA and FA are capable of acting as reversible electron-transfer agents using different functional groups, some of which may not be quinones.

Introduction

Dissolved organic matter (DOM) and humic substances (HS) can act as electron-transfer agents that facilitate the reduction of chlorinated aliphatic compounds (*1*−*4*), substituted nitrobenzenes (*5*), and oxidized metal ions such as Fe(III) (*6*), Cr(VI) (*7*−*9*), and U(*VI*) (*9*). Furthermore, during anaerobic microbial respiration, dissolved HS can act as either an electron acceptor (*10*−*15*) or as an electron donor (*16*, *17*). In environmental systems, HS can exist in multiple redox states; fulvic acids were found in an oxidized state at the photic zone of Lake Fryxell, Antarctica, and in a reduced state at the deeper bottomwaters (*18*). The electron-carrying capacity (ECC) of HS is measured as the number of the mole equivalent of electrons transferred from a donor to an acceptor per gram of HS and varies among HS samples (*10*, *11*, *19*−*21*). This variation among different HS is not surprising since the chemical structure of HS is dependent upon its source materials and the environmental conditions under which it was produced. However, the discrepancy in ECC values reported for the similar HS samples when tested with different analytical techniques (chemical oxidation, electrochemical, and microbial reduction/oxidation) is unexpected. For example, the ECC values estimated by the I_2 oxidation method for International Humic Substance Society (IHSS) soil humic acid (HA) and Suwanee river HA (*20*) and for peat HA (*21*) by electrochemical reduction are 1−3 orders of magnitude higher than the ECC values reported in microbial studies using similar HA samples (*10*, *11*, *19*, *22*). These differences may result from an inconsistency among the analytical techniques with respect to their sensitivity to the HS redox groups as well as the fact that the kinetic rates of redox reactions may be overestimated and the HA redox systems have not been allowed to come to equilibrium prior to analytical measurement (*21*). The uncertainty of the exact identity of the redox functional groups and their ECC in HS precludes further understanding of the thermodynamic and kinetic aspects of environmental redox processes involving HS. Although quinones are known to be a predominant HS redox functional group (*11*, *23*, *24*), little is known regarding the structure of these quinone groups and whether non-quinone redox functional groups exist within HS. In this study, an analytical technique based on $Pd-H_2$ catalytic reduction is used to quantify HS redox sites. The primary objectives of this study were (1) to structurally characterize redox functional groups in HS and (2) to quantify the ECC reversibility of these redox functional groups.

Materials and Methods

Samples. Suwannee river fulvic acid (SRFA) and humic acid (SRHA), Elliot soil humic acid (SHA), Summit Hill soil humic acid (SHHA), Leonardite humic acid (LHA), and peat humic acid (PHA) and peat fulvic acid (PFA) were purchased from the IHSS. Aldrich humic acid (AHA) was purchased from Aldrich and used as received. Norman landfill humic acid (NLHA) and fulvic acid (NLFA), South East landfill humic acid (SEHA) and fulvic acid (SEFA), and East Oak landfill humic acid (EOHA) and fulvic acid (EOFA) were isolated from municipal landfill leachate (*25*, *26*). Model quinone compounds anthraquinone-2-carboxylate (AQC); 2,6-dihydroxyanthraquinone (AQOH), anthraquinone-2,6-disulfonate (AQDS), 5-hydroxy-1,4-naphthoquinone (juglone), 2-hydroxy-1,4-naphthoquinone (lawsone), 2-methyl-5-hydroxy-1,4-naphthoquinone (plumbagin), 1,4-naphthoquinone (1,-4NQ), and 1,4-naphthoquinone-2-sulfonate (NQS) were obtained from Sigma or Aldrich and used as received.

Redox Cycling Experiments. The reversibility of HA and FA redox sites was measured by repeating sequential reduction and oxidation steps with the same sample. Oxidation consisted of bubbling air into the sample solution

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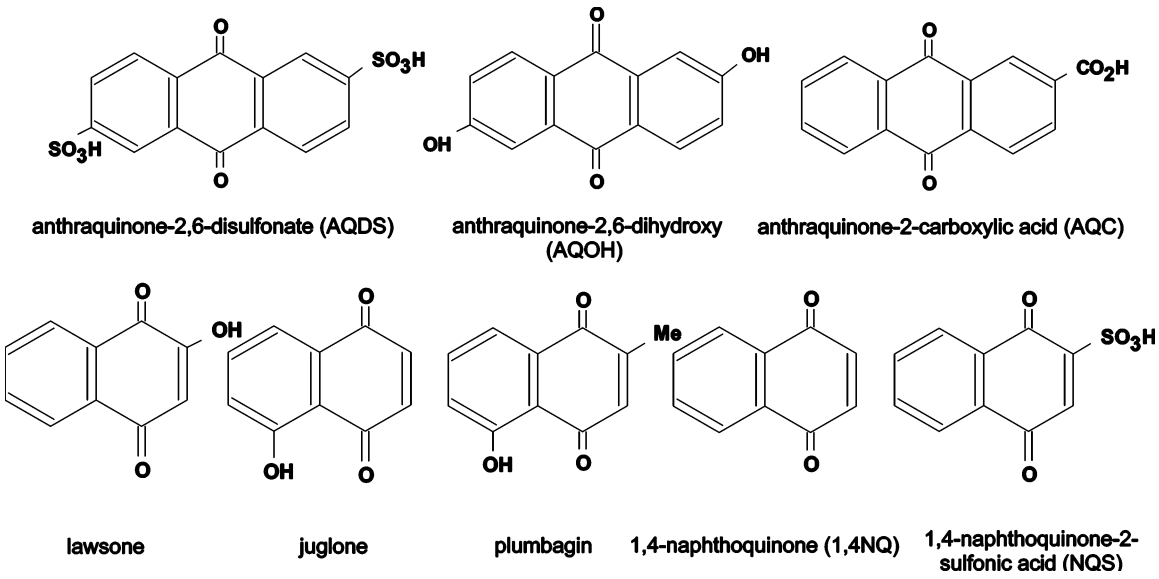

FIGURE 1. Chemical structures of model quinone compounds used in this study.

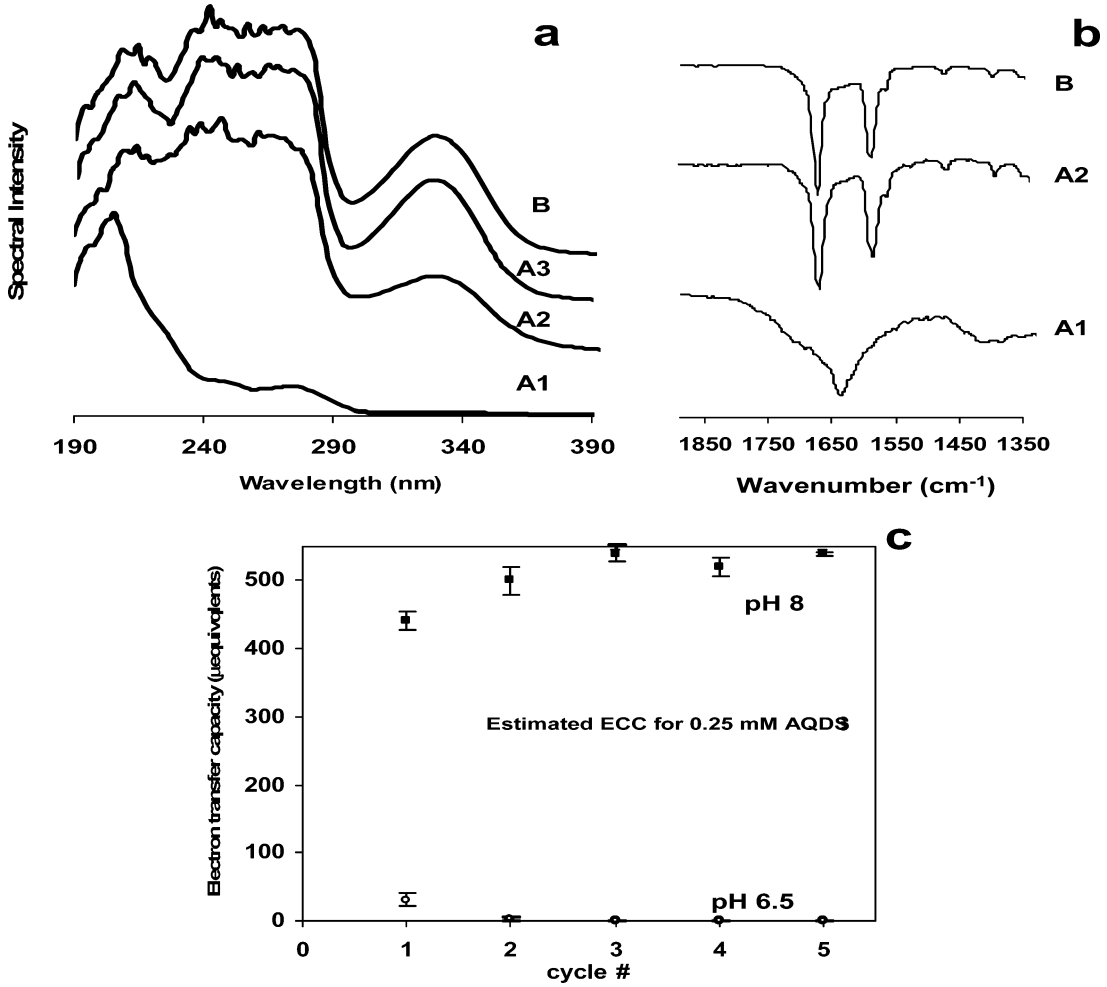

FIGURE 2. Analysis of anthraquinone-2,6-disulfonate (AQDS): (a) UV−vis spectra; (b) FT-IR spectra. Samples include AQDS before reduction (B); after pH 6.5-Pd/Al_2O_3 reduction (A1); after pH 8-Pd/Al_2O_3 reduction (A2); and after pH 8-Pd reduction (A3). (c) ECC of 0.25 mM AQDS measured over five redox cycles of pH 6.5 (open circles) and pH 8 (black squares) both using Pd/Al_2O_3 as catalysts. UV−vis and IR spectra are offset to highlight spectral differences.

while reduction employed a Pd−H_2 catalytic system under one of three conditions. The first and second conditions were conducted at pH 8 with the first condition using pure Pd powder (Aldrich) and the second using Pd supported on Al_2O_3 (Pd/Al_2O_3; Aldrich). The third condition employed Pd/Al_2O_3 at pH 6.5. In the first condition, HA and FA samples and quinone compounds were dissolved in 10 mL of 0.3 M phosphate buffer at the appropriate pH (prepared in an anaerobic chamber using Nanopure water boiled for 15 min followed with 15 min of N_2 bubbling) in a N_2−H_2 (95:5)

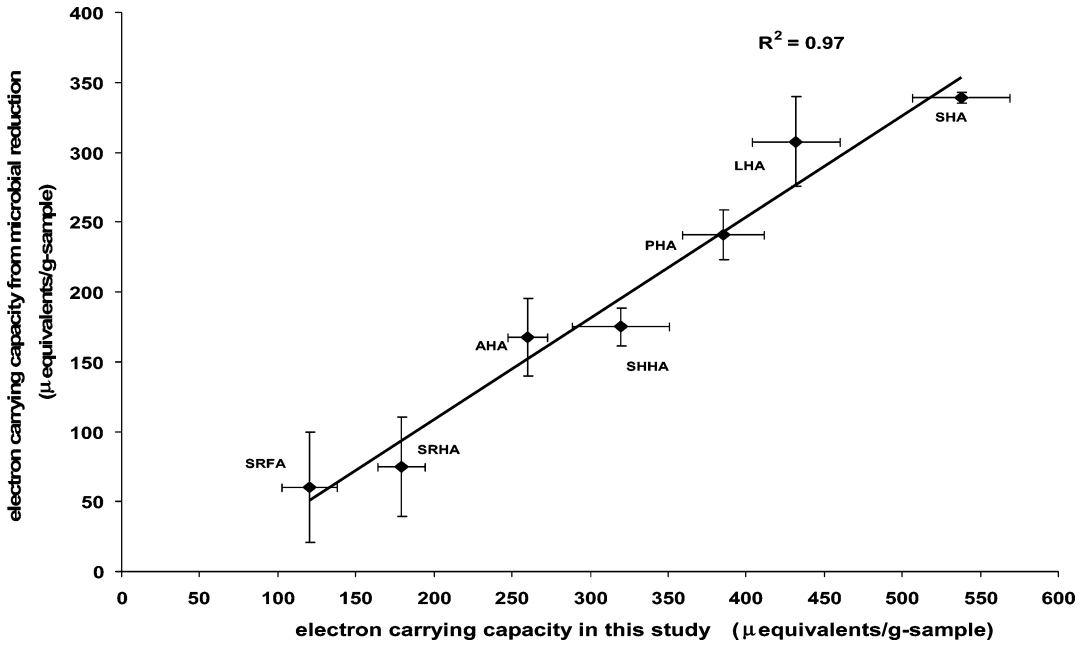

FIGURE 3. Correlation between the ECC of HS samples examined with catalytic reduction techniques (pure Pd: pH 8) in this study and values measured with the microbial reduction method (*10, 11, 19, 22*). Table 1 contains the legend for HS.

anaerobic chamber (Coy Instrument). The HA and FA concentration was 0.5 mg/mL, but quinone concentrations varied as a function of their solubility. Pd powder was added to the solution, which was then sealed in a crimp-top bottle, flushed with H_2, and shaken for 24 h. Afterward, the ECC of a 0.1 mL aliquot filtered with 0.2 μm cellulose acetate membrane (Whatman) was measured as described below. Outside of the anaerobic chamber, the catalyst was removed and the sample was bubbled with air for 30 min. The ECC of a 0.1 mL aliquot of the oxidized sample was measured. The remaining sample was prepared for the reduction step of the next redox cycle by warming to 40 °C, bubbling with N_2 for 30 min, and adding new Pd catalyst before being returned to the anaerobic chamber. For the second and third conditions, all procedures were similar except clean Pd/Al_2O_3 pellets (Aldrich) were utilized and the samples were shaken for 90 min during the reduction step. All samples were run in triplicates. Times specified for the reduction steps were chosen by when a steady-state ECC was reached for both AQDS and AHA.

Dextrose Reduction Measurements. In a N_2-H_2 (95:5) anaerobic chamber, a 2.78 M dextrose solution was anaerobically prepared using a 0.3 M phosphate buffer at pH 8. This solution was used to prepare HA and FA (0.5 mg/mL), AQDS (0.5 mM), juglone (0.05 mM), and NQS (1 mM) solutions. The ECC was measured after 24 h of continuous stirring at room temperature under anaerobic conditions.

ECC Measurements. ECC measurements were conducted in a N_2-H_2 (95:5) anaerobic chamber following the protocol described by Lovely et al.(*10*). Briefly, Fe(III) citrate was mixed with 0.01 mL of the sample to obtain a 10 mM final concentration and then allowed to incubate for 15 min before an aliquot was taken for Fe(II) determination by ferrozine assay (*27*). The Fe(II) concentration was used to calculate the number of mole equivalents of electrons transferred from the sample to Fe(III). The difference between the Fe(II) concentration of the reduced and oxidized samples was defined as the sample ECC.

Spectroscopic Analyses. UV−vis spectra were obtained with a HP 8452 diode array spectrophotometer. FT-IR spectroscopy (Bruker Equinox 55 FTIR) measurements were performed on pressed KBr pellets of AQDS and HA and FA samples (1:50 sample/KBr). Samples were titrated with tetrabutylammonium hydroxide (TBAH) (Aldrich) to pH 10, dialyzed (Spectrum; Spectra/Por), and freeze-dried before FT-IR spectroscopy (*28*).

Results and Discussion

Quinone Model Compounds. The effect of pH and catalyst support upon quinone hydrogenation (addition of H across π bonds) and hydrogenolysis (addition of H across σ bonds) and subsequent ECC properties was examined using the eight quinone model compounds listed in Figure 1. If hydrogenolysis occurs during the reduction process, OH functional groups of the reduced compound will be cleaved as H_2O and as a result, quinone moieties will not been detected upon oxidation. Figures S1 (Supporting Information) and 2a illustrate the presence (UV−vis spectra labeled B) or absence of quinone moieties (UV−vis spectra labeled A1), as determined by UV absorbance at 290 nm (A_{290}) (*29*), before and after experiencing reduction−oxidation cycling under each of the three reduction conditions. These results show that all quinone compounds lost quinone moieties, presumably by hydrogenolysis, at pH 6.5 using Pd/Al_2O_3. FT-IR spectra (Figure 2b; UV−vis spectra labeled A1) confirm the loss of quinone moieties in AQDS by the disappearance of the characteristic absorption band for quinones at 1650 cm^{-1} upon reduction at pH 6.5 with Pd/Al_2O_3.

Figures S1 (Supporting Information) and 2b (UV−vis spectra labeled A2) illustrate that at pH 8 and with Pd/Al_2O_3 the quinone moieties of AQDS, AQC, and NQS were unaffected by the reduction process. Furthermore, Figure 2c demonstrates that AQDS was able to function reversibly over five reduction−oxidation cycles at pH 8 and with Pd/Al_2O_3. The quinone moieties in AQDS, AQC, and NQS all have a neighboring electron-withdrawing group (EWG; SO_3^- and COO^-) while the five other quinone compounds which experienced hydrogenolysis at pH 8 with Pd/Al_2O_3 either have an electron-donating groups (EDG; CH_3, O^-) close to the quinone moieties or do not have a functional group present (e.g., 1,4NQ). Therefore, the nearby substitutent played a crucial role in the stability of quinone compounds upon reduction at pH 8 and Pd/Al_2O_3. Substitutent effects can be explained in a similar fashion as in S_N2-type reactions which are characteristic of the hydrogenolytic cleavage catalyzed

TABLE 1. Carboxyl Carbon Contents and ECC of Humic Substances Measured with Three Reduction Systems

			ECC (μequiv/g HS)		
		carboxyl C (%)	pH 6.5-Pd/Al_2O_3[b]	pH 8-Pd/Al_2O_3[b]	pH 8-Pd[b]
Aldrich humic acid	AHA	15[a]	132 ± 8	167 ± 12	260 ± 13
Suwannee River fulvic acid	SRFA	20[a]	25 ± 17	95 ± 7	120 ± 17
Suwannee River humic acid	SRHA	19[a]	100 ± 12	140 ± 18	179 ± 15
Summit Hill humic acid	SHHA	19[a]	143 ± 18	216 ± 22	320 ± 31
Elliot soil humic acid	SHA	18[a]	262 ± 35	349 ± 21	538 ± 31
Peat humic acid	PHA	20[a]	174 ± 10	287 ± 19	385 ± 26
Peat fulvic acid	PFA	28[a]	103 ± 10	183 ± 13	199 ± 19
Leonardite humic acid	LHA	15[a]	117 ± 9	213 ± 15	429 ± 28
Norman landfill humic acid	NHA		0	0	0
Norman landfill fulvic acid	NFA		0	0	0
South East landfill humic acid	SEHA		0	0	0
South East landfill fulvic acid	SEFA		0	0	0
East Oak landfill humic acid	EOHA		0	0	0
East Oak landfill fulvic acid	EAFA		0	0	0

[a] From IHSS. [b] Average values of five redox cycles

by Pd (*30*). In this study, EWGs such as SO_3^- and COO^- decreased the electron density of quinones moieties, making them less nucleophilic and less attractive to electrophilic sites on catalyst surfaces.

Under reducing conditions of pH 8 and with a Pd catalyst without Al_2O_3 support, only juglone and AQOH experienced hydrogenolysis. It is hypothesized that the EDGs present in juglone and AQOH increased the hydrogenolysis rate and that although lawsone and plumbagin have EDGs, the presence of an adjacent functional group impedes coordination of the quinone moieties to reaction sites on the catalyst surface, thereby slowing the rate of hydrogenolysis through steric hindrance.

ECC of Humic Substances. Table 1 lists the average ECC values for 14 HS samples, measured in triplicate and averaged over five reduction−oxidation cycles, for each of the three reduction conditions. Eight samples had measurable ECC values under all three reduction conditions. The six landfill leachate HS samples had no ECC, indicating that they lack functional groups capable of redox reactions such as quinones. These landfill leachate HS are highly aliphatic and have low aromaticity (10−14% aromaticity) (*25*). Figure 3 illustrates that the magnitude of the ECC values for the other eight HS compare favorably ($R^2 = 0.97$) with those measured in ferric reduction reactions by *Geobacter metallireducens* (*16*, *19*, *31*, *32*). However, our ECC values are 1−2 orders of magnitude lower than the oxidation capacity measured by I_2 titration at pH 7 (11 500, 4600, and 3300 μequiv/g HS for SRHA, PHA, and SHA respectively (*20*)). Since the I_2 titration method could not the measure redox reversibility of the sites oxidized by I_2, it was inconclusive whether these sites were actual redox centers. The strong oxidizing power of I_2 might react with electron-rich functional groups not involved in electron-transfer processes.

Unlike the quinone model compounds, eight HS displayed ECC under the pH 6.5-Pd/Al_2O_3 reduction conditions. To confirm that quinone moieties were not responsible for this ECC, HS samples before and after the reduction step were esterified with TBAH and analyzed by FT-IR (*28*). This method removes the spectral interference of the free carboxyl groups from the carbonyl-group absorbance region; esterification of carboxyl groups shifts the IR absorbance from about 1700 cm^{-1} (free carboxyl groups) to 1600 cm^{-1} (esterified carboxyl groups) (*28*). Figure S2 (Supporting Information) illustrates the disappearance of the aromatic ketone peak (resolved at 1650−1670 cm^{-1}) in the FT-IR spectra after treatment with pH 6.5-Pd/Al_2O_3. On the basis of these FT-IR results and the behavior of the quinone model compounds, it is highly unlikely that quinone moieties are responsible for the ECC values reported in HS experiencing the pH 6.5-Pd/Al_2O_3 reduction conditions, therefore these redox moieties will be referred to as non-quinone (NQ) redox sites. In other words, the pH 6.5-Pd/Al_2O_3 selectively destroyed quinone groups so that they were no longer capable of transferring electrons back to the Fe^{3+} citrate probe.

Since thiols and disulfides are capable of reversible redox cycling to sulfoxides (*33*) and because the C−S bond is less susceptible than the C−O bond to hydrogenolytic cleavage (*34*), thiols and disulfides could be responsible for NQ ECC. However, their presence may not account for all the measured NQ ECC. For example, 56−62% of the total sulfur in a muck HS and 26% in a Fayette silt-loam soil HA was present as thiol and disulfide (*35*). Therefore, assuming that approximately 50% of the total sulfur in SHA (total sulfur concentration of 0.4 wt %/wt) is present as either thiol or disulfide that is reversibly oxidized to sulfoxide, this provides an estimated NQ ECC value ranging from 125 μequiv/g HS (2 electron transfer from disulfide to sulfoxide) to 187 μequiv/g HS (3 electron transfer from thiol to sulfoxide); a value approximately 50−70% of NQ ECC measured in SHA (262 μequiv/g HS). Furthermore, Fimmen et al. (*36*) in studies of terrestrially and microbially derived DOM proposed the possibility of nitrogen and sulfur functional groups as redox sites.

Table 1 shows that the ECC of HS samples increased upon using Pd/Al_2O_3 at pH 8 compared with that of pH 6.5. On the basis of the quinone model compound results, it is hypothesized that the additional ECC (beyond that provided by the NQ redox sites) measured under these conditions resulted from quinone moieties with adjacent EWG such as carboxylic acid functional groups. The redox sites responsible for the increased ECC are referred to as Q1 ECC sites. Likewise, the ECC increased further upon using pure Pd as the catalyst at pH 8. This additional increase in ECC is hypothesized to arise from quinone moieties either containing nearby EDG with additional substitutents hindering hydrogenolysis through steric interactions (such as in the case for juglone and plumbagin) or not having any nearby EDG substitutents such as that for the model compound 1,4NQ. The redox sites responsible for the increased ECC under the reduction conditions of pH 8 and Pd are referred to as Q2 ECC sites. Overall, Q1 and Q2 ECC values indicate that quinone moieties in HS exist in at least two different configurations.

Since the model quinone compounds juglone and AQOH experienced hydrogenolysis under the pH8-Pd reduction conditions, it was not certain that all quinone redox sites in HS were being measured. Therefore, dextrose was used as a reducing agent with all HS samples. Prior to the HS

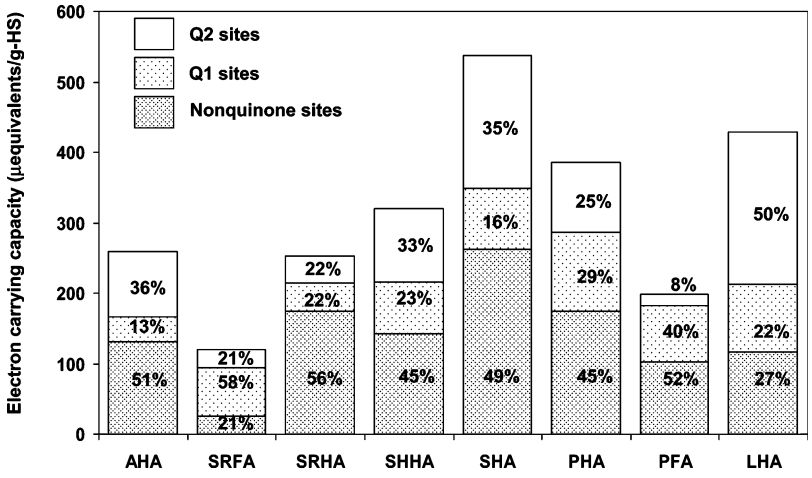

FIGURE 4. Contribution of each redox functional group to ECC of HS samples. ECC was an average of values measured in five redox cycles. Table 1 contains the legend for HS.

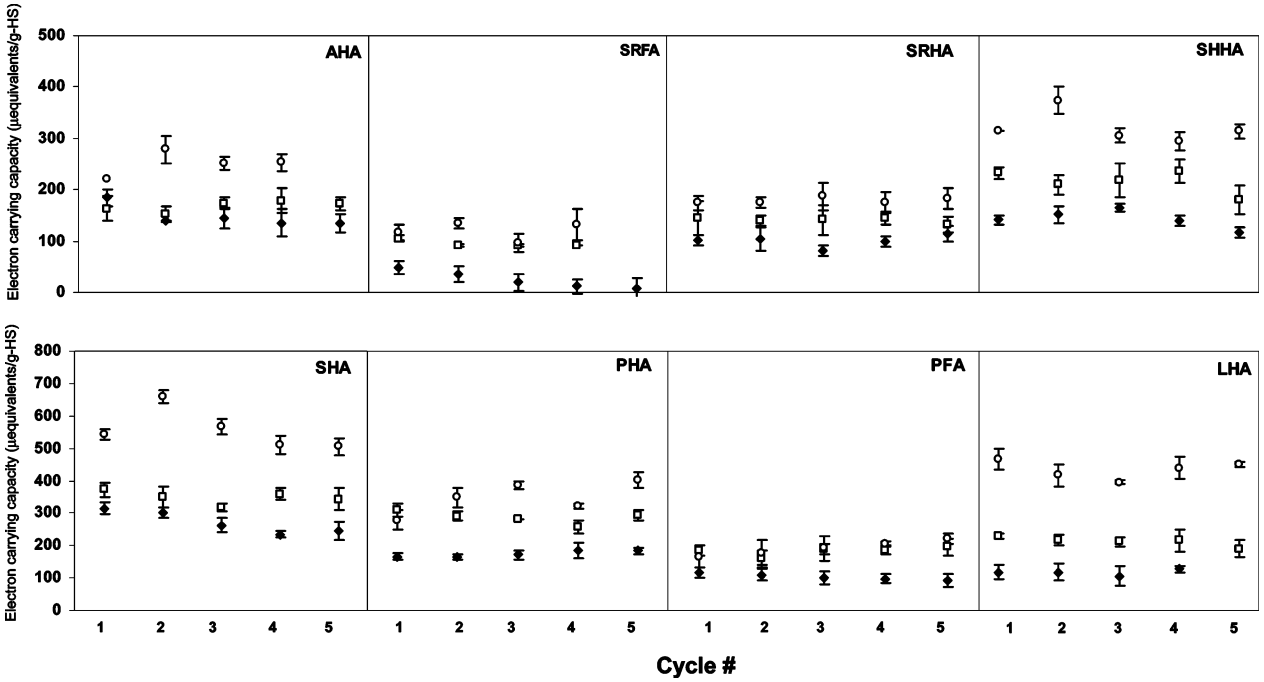

FIGURE 5. ECC of HS samples measured with three reduction systems over five cycles. Key: pH 6.5-Pd/Al_2O_3 (black diamonds), pH 8-Pd/Al_2O_3 (open squares), and pH 8-Pd (open circles). Each data point represents the average ECC value of three replicates at each redox cycle. Table 1 contains the legend for HS.

measurements, AQDS and NQS (quinones with EWG) and juglone were tested. Figure S3 (Supporting Information) shows that all three quinones were fully reduced by the dextrose method and that their quinone moieties remained intact. As shown in Figure S4 (Supporting Information), the ECC of HS samples measured by the dextrose reduction method were comparable to the sum of the ECC of the Q1 and Q2 sites. Therefore, it was concluded that the eight examined HS do not contain a significant amount of quinone moieties with conjugated structures solely containing a hydroxyl group two to three carbon atoms away from the quinone functional group.

Figure 4 shows that the combined ECC Q1 and Q2 value was responsible for 44−79% of the total ECC of the HS samples. In this study, a good correlation between the combined ECC Q1 and Q2 value with that of the organic radical content (*11*) was observed (R^2 = 0.91; Figure S5, Supporting Information). This supports the hypothesis that Q1 and Q2 sites consist of quinone structures since quinones form semiquinone radicals upon one electron reduction (*37*) and that for several HS, a relationship was observed between the organic radical content and ECC (*11*). Alternatively, a poor relationship (R^2 = 0.23) (data not shown) was found between the organic radical content and the NQ-ECC values, supporting the conclusion that HS also contain redox sites not accounted for by organic radical content.

Figure 4 illustrates that for the two fulvic acid samples, PFA and SRFA, the relative contribution of the Q1 sites to the total ECC was greater than that of the Q2 sites. Q1 sites are those containing EWG such as carboxylic acid functional groups. Table 1 shows that PFA and SRFA have a greater amount of carboxyl functional groups relative to the HAs examined in this study; thus, redox sites in fulvic acids with a quinone structure would have a greater probability of having an adjacent carboxyl substituent. However, plotting carboxyl carbon content versus ECC_{Q1}/ECC_{tot} shows that not much of a trend (R^2 = 0.31) exists for Q1 sites being more abundant in samples with higher carboxylic carbon content (Figure S6a, Supporting Information). In contrast, for Q2 sites, a negative trend occurs between carboxyl carbon content and

ECC_{Q2}/ECC_{tot} (Figure S6b, Supporting Information). These trends underscore the influence that different substitutent groups may have on the redox stability and properties of quinone functional groups in HS.

Reversibility through multiple reduction−oxidation cycles is a key requirement for environmentally sustainable ECC. Figure 5 illustrates that except for the SRFA NQ sites, redox sites (NQ, Q1, and Q2) for all HS were reversible over five reduction−oxidation cycles. The fact that NQ redox sites demonstrated relatively unchanging reversibility for at least five redox cycles under conditions strongly favorable to quinone hydrogenolysis supports the conclusion that NQ sites do not contain a quinone structure. The contribution of NQ sites to the total ECC (the maximum values measured under pH 8-Pd reduction conditions) was substantial, ranging between 21 and 56%. Figure 4 shows that this is particularly true for HA samples (except LHA) where 45−56% of total ECC was attributed to NQ sites.

Environmental Significance

Reduction of HS redox sites using a Pd−H_2 catalytic system at different solution pH values and with different Pd catalytic supports provided information about the chemical structure and ECC of redox sites in various HS. It was revealed that electron-transfer processes in HS are facilitated by multiple redox functional groups that can be divided into three groups: non-quinone redox sites and two different quinone redox sites. This diversity suggests that electron-transfer processes involving HS could occur via several mechanisms besides that of the formation of semiquinone radicals. This point is underscored by the fact that the NQ sites can account for 21−66% of the total ECC in HS. For the Q1 and Q2 quinone redox sites, the finding that they exist with various neighboring substitutents also implies a diverse redox activity. As demonstrated in the microbial reduction of an azo dye (*38*), quinone compounds with different substituents facilitated the reduction reaction with different relative rates that depended largely on the redox potential of the quinone. In a similar manner, substitutents of the Q1 and Q2 redox sites could have a strong influence on the redox potential of each quinone site in the HS and thus be an important factor in determining the overall redox reactivity of HS in the environment.

Acknowledgments

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Supporting Information Available

UV−vis spectra of quinone model compounds after each reduction reaction in Figure S1; FT-IR spectra of SHA, PHA, and SRFA before and after pH 6.5-Pd/Al_2O_3 reduction in Figure S2; UV−vis spectra of AQDS, NQS, and juglone after dextrose reduction in Figure S3; comparison of Q1 + Q2 ECC values with dextrose reduction ECC values for HS in Figure S4; correlation between organic radical content and HS ECC in Figure S5; and correlation between carboxyl carbon content and ECC_{Q1}/ECC_{total} and ECC_{Q2}/ECC_{total} in Figure S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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