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Novel Electrochemical Approach to Assess the Redox Properties of Humic Substances

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Two electrochemical methods to assess the redox properties of humic substances (HS) are presented: direct electrochemical reduction (DER) on glassy carbon working electrodes (WE) and mediated electrochemical reduction (MER) and oxidation (MEO) using organic radicals to facilitate electron transfer between HS and the WE. DER allows for continuous monitoring of electron and proton transfer to HS by chronocoulometry and automated acid titration, respectively, and of changes in bulk HS redox potential E_h. Leonardite Humic Acid (LHA) showed an H^+/e^- ratio of unity and a decrease in potential from $E_h = +0.18$ to -0.23 V upon transfer of 822 $\mu mol_{e^-}\ g_{LHA}^{-1}$ at pH 7, consistent with quinones as major redox-active functional moieties in LHA. MER and MEO quantitatively detected electrons in LHA samples that were prereduced by DER to different extents. MER and MEO therefore accurately quantify the redox state of HS. Cyclic DER and O_2-reoxidation revealed that electron transfer to LHA was largely reversible. However, LHA contained a small pool of moieties that were not reoxidized, likely due to endergonic first electron transfer to O_2. Electron accepting capacities of 13 different HS, determined by MER, strongly correlated with their C/H ratios and aromaticities and with previously published values, which, however, were a factor of 3 smaller due to methodological limitations.

Introduction

Humic substances (HS), including humic and fulvic acids, are redox-active natural organic macromolecules that are ubiquitous in soils and sediments and predominate in the subsurface of peatlands and bogs. The redox-activity of HS has been primarily ascribed to quinone-hydroquinone moieties (*1*−*5*), although complexed metals, in particular iron, may also play a role. In environments with changing redox conditions, such as capillary fringes of soils, HS may act as redox buffers by accepting electrons from microbial respiration under anoxic condition (*6*−*8*), and, upon reaeration, by donating electrons to oxygen. In peatlands and bogs, this process is believed to significantly decrease methanogenesis (*8, 9*). In addition to acting as redox buffers, HS mediate biogeochemical redox reactions, including the transfer of electrons from microorganisms to poorly accessible mineral phases, such as Fe^{3+}-oxides (*6, 7, 10*). HS also mediate electron transfer from abiotic reductants (e.g., H_2S) to organic pollutants (e.g., halogenated hydrocarbons and nitroaromatics) (*11*−*13*). The role of HS as redox buffers and mediators has drawn considerable interest in their redox properties, including the reversibility of electron transfer to and from HS and their electron acceptor (EAC) and donor (EDC) capacities (i.e., the moles of electrons that can be transferred to or withdrawn from HS at a given potential, E_h).

Some previous methods to determine EAC have used H_2S and Zn^0 as reductants (*14, 15*). Following HS reduction, the oxidation products $S_2O_3^{2-}$ and Zn^{2+} were quantified and set equal to EAC. Other studies determined the EAC indirectly by measuring the difference in EDCs of a prereduced and a nontreated HS. Prereduction was achieved either microbially (*Geobacter metallireducens*), chemically (H_2 in the presence of Pt or Pd, H_2S, Zn^0), or electrochemically (bulk electrolysis at a Pt working electrode) (*2, 12, 16, 17*). The EDCs were determined by quantifying the amount of Fe^{2+} formed by the reduction of added Fe^{3+} complexes (most often Fe^{3+}-citrate or hexacyanoferrate) by HS. This experimental approach has also been used to determine changes in the redox states of environmental HS samples, for instance collected along redox gradients (*8*).

Although widely used, the above methods have some limitations: First, the amount of electrons transferred to or withdrawn from HS was determined only indirectly by quantifying the oxidation or reduction of added chemicals. A direct measurement of transferred electrons is, however, required to improve accuracy and reduce analysis time. Second, electron transfer to and from added chemical oxidants and reductants is often coupled to proton exchange, which resulted in a pH-dependent reduction potential E_h and hence driving force for HS oxidation and reduction. This impedes direct comparisons of the redox properties of HS (e.g., EAC and EDC) if determined at different pH. Ideally, both EAC and EDC should be determined relative to reference states with pH-independent E_h. Third, EDC quantification by reoxidation with Fe^{3+} is slow, requiring hours to days to attain apparent reaction equilibrium (*17*). Yet, reaction times were often not more than 15 min to 1 h (*2, 16*), such that determined values underestimated true equilibrium EDCs. Clearly, methods with faster electron transfer kinetics to and from HS are required. Fourth, the use of chemical reductants may result in side reactions, including complexation of added Fe and Zn by HS or covalent modification of HS (e.g., nucleophilic addition of reduced sulfur species) (*14, 15*).

In this paper, we present a novel electrochemical approach to study the redox properties of HS, which overcomes the limitations of previously used methods. The approach combines two electrochemical methods. The first is constant-potential direct electrochemical reduction (DER) of HS at a glassy carbon (GC) working electrode (WE). The use of GC allowed for a continuous chronocoulometric quantification of the amount of electrons transferred to HS, since background currents from H^+ reduction were negligible. DER served to generate clean HS samples with known redox states, to quantify the ratio of protons to electrons transferred during reduction, and to monitor the resulting decrease in redox potential E_h during the reduction. The second method, mediated electrochemical reduction (MER) and oxidation (MEO), quantified differences in the electron contents between small HS samples by chronocoulometry. Organic radicals mediated the electron transfer between HS and the carbon electrode and established stable, pH-independent redox potentials in the electrochemical cell.

The versatility of this novel approach is demonstrated by evaluation of the reversibility of electron transfer to Leonar-

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dite Humic Acid (LHA) in successive DER and O_2 reoxidation cycles, and by the quantification of the EACs of a broad set of terrestrial and aquatic humic and fulvic acids.

Materials and Methods

Chemicals. Diquat dibromide monohydrate (99.5%, Supelco) (DQ), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (>99%) (ABTS), 2,6-dichlorophenol indophenol sodium salt hydrate (purum. p.a) (DCPIP), starch from wheat, potassium chloride (>99.5%), sodium hydroxide (puriss. p.a.), and hydrochloric acid (puriss. p.a.) were purchased from Fluka (St. Louis, MO). Ethyl viologen dibromide (>99%), 9,10-anthraquinone-2,6-disulfonic acid disodium salt (>98%), and 2-hydroxy-1,4-naphthoquinone (Lawsone) (>97%) were from Sigma-Aldrich (St. Louis, MO), disodium hydrogen phosphate dihydrate (p.a.) was from Merck (Darmstadt, Germany).

All aqueous solutions were prepared with nanopure water (Barnstead NANOpure Diamond Water Purification System).

Humic Substances (HS). HS were used as received. Aldrich Humic Acid was obtained from Sigma-Aldrich (St Louis, MO). All other HS (i.e., Suwannee River Humic Acid Standard II, Suwannee River Fulvic Acid Standard II, Elliott Soil Humic Acid Standard, Eliott Soil Fulvic Acid Standard III, Leonardite Humic Acid Standard, Pahokee Peat Humic Acid Standard, Pahokee Peat Humic Acid Reference, Pahokee Peat Fulvic Acid Standard II, Waskish Peat Humic Acid Reference, Nordic Aquatic Humic Acid Reference, Nordic Aquatic Fulvic Acid Reference, Pony Lake (Antarctica) Fulvic Acid Reference) were from the International Humic Substances Society (IHSS, St. Paul, MN).

Electrochemical Experiments. All electrochemical measurements were conducted in an anoxic glovebox (N_2 atmosphere at 25 ± 1 °C, O_2 < 0.1 ppm). Aqueous solutions were made anoxic by purging with argon for 1 h at 150 °C and for 1 h at room temperature. All solutions used for electrochemical experiments contained 0.1 M KCl as supporting electrolyte. Potentials were measured vs Ag/AgCl but are reported vs SHE.

Direct Electrochemical Reduction (DER) of Leonardite Humic Acid (LHA). Dissolved LHA (2 g_{LHA} L^{-1}) was reduced in a ~0.1 L bulk electrolysis cell. The cell consisted of a glass vessel closed with a Teflon cover, a GC WE (Sigradur G, HTW, Tierhaupten, Germany), an Ag/AgCl reference electrode, and a coiled platinum wire auxiliary electrode (both from Bioanalytical Systems Inc., West Lafayette, IN). The auxiliary electrode was placed in an anodic compartment separated from the cathodic compartment by a glass frit to minimize reoxidation of reduced HA. A CHInstruments 630C instrument (Austin, TX) and an Autolab PG 302 instrument (EcoChemieB.V, Utrecht, The Netherlands) were used to measure currents I (A) and to control potentials at the WE. Unless stated otherwise, DER was carried out at $E_h = -0.59$ V. The transferred amount of electrons, Q_{DER} (μmol_{e^-}), was obtained by chronocoulometry (i.e., integration of the reductive and oxidative currents I over time t (s)).

Two different setups were used to ensure constant pH 7 throughout DER. In the first setup, HS solutions contained 0.1 M phosphate. In the second setup, the HS solution (75 mL, 2 g_{LHA} L^{-1}) was continuously circulated through the electrochemical cell and two flow-through cells (Figure 1a). Increase in solution pH by proton consumption was monitored by a pH electrode in the first flow-through cell and compensated by titration of diluted HCl (18.5 mM) to the electrochemical cell using an automated titrator (751 GPD Titrino, Metrohm, Zofingen, Switzerland). The redox potential E_h was measured by a platinum-ring redox electrode (Metrohm, Zofingen, Switzerland) placed in the second flow-through cell.

Mediated Electrochemical Reduction (MER) and Oxidation (MEO). MER and MEO of HS samples was conducted with the electrochemical equipment described above. However, instead of GC, reticulated vitreous carbon (RVC, Bioanalytical Systems Inc., West Lafayette, IN) was used as WE material to increase the surface area and hence to facilitate electron transfer to and from the mediator. The electrochemical cell was filled with 80 mL of buffer (0.1 M KCl, 0.1 M phosphate, pH 7) and the electrode was equilibrated to the desired potentials (i.e., $E_h = -0.49$ V in MER and $E_h = +0.61$ V in MEO, which were below and above, respectively, the potential range reported for quinones (see Figure S1)). Subsequently the mediators DQ for MER and ABTS for MEO were spiked, resulting in reductive and oxidative currents, respectively. Structural formulas and standard potentials of these mediators are given in Figure 1d. After reattainment of constant background currents, small amounts (<1 mg) of HS samples were spiked to the cells and the transferred amount of electrons was measured by chronocoulometry.

Reversibility of Electron Transfer to HS using O_2 as Oxidant. DER-reduced LHA was transferred to a gastight serum flask, and reoxidized with excess O_2 by purging the flask with synthetic air for 1 h, followed by 24 h incubation. Subsequently, unreacted O_2 was removed by Ar-purging for 1 h. This reduction−reoxidation cycle was carried out three times, with incubation periods of LHA after purging with synthetic air of 24 h (cycle 1 and 2) and 8 d (cycle 3). After each reduction and oxidation step, an aliquot of the LHA was withdrawn and the redox states were determined by MER and MEO. To determine the reoxidation kinetics of LHA by O_2, 9-mL aliquots of DER-reduced LHA were transferred into stoppered, gastight serum flasks. Using a gastight syringe, 25 mL of the flasks' headspace were withdrawn and replaced with oxygen. The flasks were incubated for different time spans. Oxidation of LHA was quenched by purging the flasks with argon for >1 h to remove unreacted O_2. Changes in EACs of the samples were quantified by MER.

Electron-Accepting Capacities (EAC) of HS. Solutions of a diverse set of HS listed above were prepared in buffer (0.5 g_{HS} L^{-1}; 0.1 M KCl, 0.1 M phosphate, pH 7). The EACs were quantified by MER.

Results and Discussion

Description and Evaluation of Electrochemical Methods. *Electron Transfer, Proton Consumption, and Change in Redox Potential in DER of LHA.* Figure 1b shows the reductive current I during DER of LHA (red line) using acid titration to maintain constant pH 7 (black line). High initial I > 300 μA decreased to (but did not level off at) ~30 μA after 50 h at which DER was stopped. Negligible background currents were measured in the absence of LHA (<1 μA; gray line). This shows that the redox-active moieties in LHA can be directly reduced at GC and that no significant H_2 generation by proton reduction occurred. Hence the electrons transferred during DER could be quantified directly by integration of the LHA reduction current and was $Q_{DER} = 822$ μmol_{e^-} g_{LHA}^{-1} after 50 h. In contrast, the platinum electrode used in refs *12 and 17*, resulted in extensive H_2 production (and potentially catalytic hydrogenation reactions by H_2/Pt), which impaired accurate quantification of the transferred amount of electrons.

The transfer of electrons to LHA during DER resulted in a gradual decrease in solution redox potential from $E_h = +0.18$ to -0.23 V (black line; Figure 1c). E_h decreased only slightly when the same potential was applied to buffer in the absence of LHA (data not shown). The gradual decrease in E_h in combination with the absence of inflection point(s) supports that LHA contains redox-active functional moieties with a wide distribution in redox potentials, falling into the potential range covered by benzo-, naphtha-, and an-

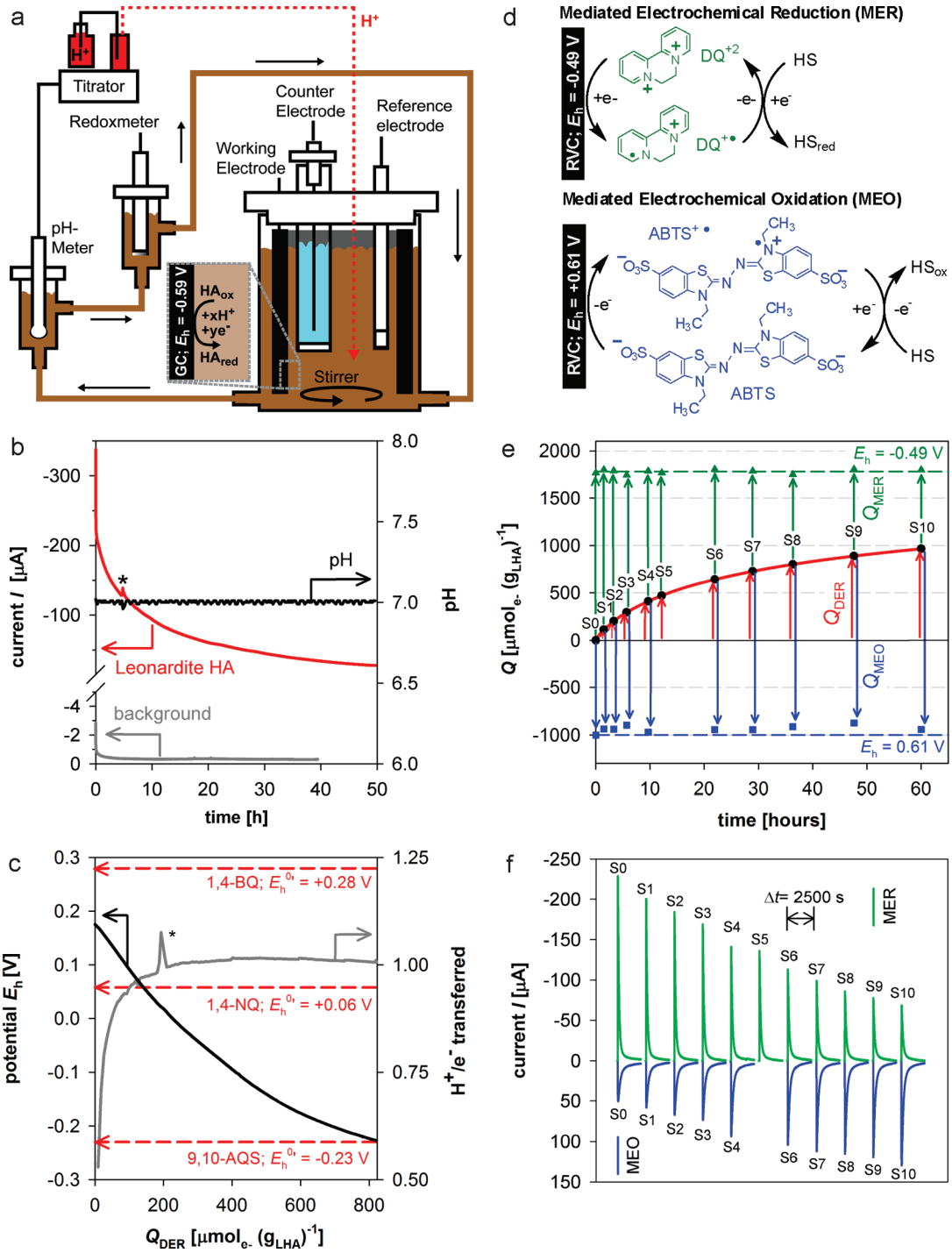

FIGURE 1. (a) Direct electrochemical reduction (DER) of humic acid (HA). The electrochemical cell consisted of a cylindrical glassy carbon (GC) working electrode (applied potential E_h vs SHE), an Ag/AgCl reference electrode, and a platinum-wire counter electrode separated from the main compartment by a glass frit to avoid reoxidation of reduced HA. The HA solution was continuously circulated through two small volume flow-through cells containing a pH electrode and a Pt-ring redox electrode. A constant experimental pH was maintained by automated addition of diluted acid to the electrochemical cell by a titration unit connected to the pH electrode. (b) Reductive current (red trace) and solution pH (black trace) during DER of Leonardie Humic Acid (LHA) at $E_h = -0.59$ V for 50 h. The background current (gray trace) was measured at the same E_h of the WE in the absence of LHA. (c) Decrease in solution redox potential E_h (V vs SHE) (black trace) and ratio of protons to electrons, H^+/e^-, transferred to LHA (gray trace) as a function of the amount of electrons transferred to LHA by DER, Q_{DER}, at pH 7. The standard redox potentials $E_h^{0'}$ of 1,4-benzoquinone (1,4-BQ), 1,4-naphthoquinone (1,4-NQ), and 9,10-anthraquione-2-sulfonate (9,10-AQS) at pH 7 are given as references. The peaks in I, pH, and H^+/e^- (indicated by asterisks *) in panels b and c resulted from short time interruption of the circulation through the flow-through cells. (d) Schematic of mediated electrochemical reduction (MER) at $E_h = -0.49$ V and oxidation (MEO) at $E_h = +0.61$ V using the organic radicals diquat (DQ) and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), respectively, to facilitate electron transfer between the reticulated vitreous carbon (RVC) working electrode and humic substances (HS). (e) Electrons transferred to LHA in DER, Q_{DER} (red trace and arrows) and MEO, Q_{MEO} (green arrows), and electrons withdrawn from LHA in MEO, Q_{MEO} (blue arrows). Samples S0 to S10 were withdrawn from the electrochemical cell during DER and represented LHA standards with known Q_{DER}. (f) Reductive and oxidative current responses in MER (green peaks) and MEO (blue peaks), respectively, for LHA samples S0 to S10. Current peaks were integrated to give transferred charge equivalents, Q_{MER} and Q_{MEO}, represented by arrows in panel e. Sample S5 was only analyzed in MER.

thraquinones (Figure 1c and Figure S1). This is consistent with the hypothesis that quinones are the predominant redox-active moieties in HS. A similar wide range of HS redox potentials was reported in ref *7*. Note that the wide potential distribution of redox-active moieties in LHA and slow electron transfer kinetics at GC provide plausible explanations for the absence of distinct reduction and oxidation waves in cyclic voltammetry measurements of HA (*4, 19*).

The solution potential after 50 h of DER, $E_h = -0.23$ V, was significantly higher than the potential at the WE, $E_h = -0.59$ V. Therefore, LHA had not come to equilibrium with the WE over the course of DER. Plausible explanations for nonequilibrium (i.e., sluggish electron transfer) include passivation of the GC electrode surface due to HA adsorption (*20*) and the macromolecular nature of HA (e.g., slow reduction of redox-sites in the interior of HA colloids) (*21*). The nonequilibrium led to increasing Q_{DER} with increasing reduction time and with decreasing applied potential (shown for LHA in Figure S2a for applied potentials of $E_h = -0.39$, -0.59, and -0.79 V). Slow DER reduction kinetics were linked to HA since the same setup resulted in much faster and quantitative reduction of the model quinone lawsone (Figure S2b). The chosen $E_h = -0.59$ V allowed for DER also at pH < 7 without significant H_2 generation (data not shown).

The molar ratio of titrated protons to transferred electrons increased from 0.6 to 1.0 within the first ~250 $\mu mol_{e^-}\ g_{LHA}^{-1}$ transferred after which it remained constant (Figure 1c, gray trace). The final H^+ to e^- ratio was 1.01. Proton titration was negligible when the potential $E_h = -0.59$ V was applied to buffer in the absence of LHA (data not shown). The H^+ to e^- ratio of unity implies that one H^+ was taken up per e^- transferred to redox-active functional moieties in LHA. This again supports the hypothesis that quinones are the major redox active groups in HS, because most hydroquinones have acidity constants $pK_a > 7$ (*22*). The smaller initial H^+ to e^- ratio likely resulted from internal proton buffering by LHA and/or by the transient formation of semiquinone radical anions, which is not coupled to H^+ uptake (*2, 22*). A detailed analysis of the pH-dependency of the ratio of H^+ to e^- transferred during DER will be presented in a forthcoming paper. DER of LHA solutions in which the pH was buffered by phosphate resulted in very similar current responses (Figure S2a).

In summary, the results show that DER at GC has several advantages over previously employed reduction methods: DER allows for (i) a direct quantification of transferred electrons during reduction, (ii) simultaneous monitoring of electron and proton transfers, pH, and E_h during reduction, (iii) reduction at different E_h, (Figure S2) and pH (data not shown), and (iv) generation of bulk (mg to g) amounts of "clean" reduced HA samples (devoid of chemical reducing agents such as complexed metal cations or covalently bonded H_2S species) with defined redox states that can be directly used in follow up experiments.

Mediated Electrochemical Reduction and Oxidation (MER and MEO). Spiking of small volumes of prereduced and nontreated LHA samples into electrochemical cells with the WE prepolarized to $E_h = -0.49$ V and +0.61 V, but in the absence of organic electron transfer mediators, resulted in poorly defined reductive and oxidative current responses, respectively (Figure S3), reflecting sluggish direct electron transfer between LHA and the WE. The peaks could not be exactly integrated. Hence, to facilitate the electron transfer, organic electron transfer mediators were employed. The radicals diquat (DQ, first electron reduction potential $E_h^1 = -0.36$ V) and ethylviologen (EV, $E_h^1 = -0.48$ V) (data not shown) were used for MER and ABTS (second electron reduction potential $E_h^2 = +0.68$ V) for MEO (Figure S4) (*23, 24*), because these chemicals fulfilled the following requirements (*25, 26*): (i) fast and reversible heterogeneous electron transfer at the electrode and homogeneous electron transfer with LHA in solution, (ii) pH-independent redox potentials E_h (i.e., organic radicals), (iii) stability at potentials sufficiently low (mediator in MER) respectively high (mediator in MEO) to cover the E_h-range of the redox-active moieties in LHA (Figure S1), and (iv) sufficiently high water solubility. Spiking of DQ to the electrochemical cell with the WE prepolarized to $E_h = -0.49$ V resulted in reductive current peaks due to one-electron reduction of DQ^{2+} to the radical $DQ^{\bullet+}$ (Figures S3a and S4). The transferred amount of electrons obtained from peak integration was 100 ± 5% of the amount expected for an equimolar electron transfer to DQ^{2+}, demonstrating accurate chronocoulometric quantification. Analogously, spiking of ABTS to the electrochemical cell with the WE prepolarized to $E_h = +0.61$ V resulted in oxidative currents due to one-electron oxidation of ABTS to $ABTS^{\bullet+}$ (Figures S3b and S4). Subsequent spiking of LHA samples to both systems resulted in very sharp, well-defined current peaks (Figure S3), demonstrating that DQ and ABTS facilitated the electron transfer between LHA and the WE (Figure 1d).

A series of eleven LHA samples (S0−S10) with increasing Q_{DER} from 0 $\mu mol_{e^-}\ g_{LHA}^{-1}$ (S0) to 966 $\mu mol_{e^-}\ g_{LHA}^{-1}$ (S10) were obtained by DER (Figure 1e). These samples served as standards with known differences in electron contents to test the recovery of DER-transferred electrons by MER and MEO. Figure 1f shows the current responses of samples S0 to S10 in MER (green peaks) and MEO (blue peaks). The height of the current peaks decreased in MER and increased in MEO with increasing prereduction of LHA by DER (i.e., from S0 to S10, Figure 1f). No reductive and oxidative current peaks were detected when background buffer without LHA was spiked. The results of a number of additional control experiments, including MER and MEO of two model quinones, lawsone and AQDS, and of starch, a non redox-active organic polymer, are summarized in Table S1.

The amount of electrons transferred to and from LHA in sample SX (X = 0 to 10) during MER, $Q_{MER}(SX)$, and MEO, $Q_{MEO}(SX)$, were obtained by integration of the respective reductive and oxidative current peaks in Figure 1f. Figure 1e shows that Q_{MER} (green arrows), decreased, while Q_{MEO} (blue arrows) increased with increasing amounts of electrons transferred to LHA during prereduction by DER, Q_{DER} (red arrows). Notably, the same total amount of electrons, $Q_{DER}(SX) + Q_{MER}(SX)$, was transferred to each of the samples. Moreover, the amount of electrons withdrawn by MEO from sample SX, $Q_{MEO}(SX)$, was equal to $Q_{DER}(SX) + Q_{MEO}(S0)$, where sample S0 contained LHA that was not prereduced by DER (Figure 1e). The recovery of Q_{DER} was 104 (±3) % by MER and 90 (±4) % by MEO (determined by regression analysis, see Figure S5 for details). MER and MEO therefore quantitatively detected the electrons that had been transferred to LHA during DER.

These findings have the following implications: (i) Redox-active moieties in HS that were reducible by DER in the absence of mediator were also reduced by DQ in MER. Furthermore, electron transfer to LHA by DER was largely reversible when ABTS was used as oxidant. Reoxidation by ABTS was therefore both kinetically fast and thermodynamically favorable. (ii) Nontreated LHA (sample S0) contained reduced moieties that were oxidized by ABTS. The E_h-data in Figure 1c and S1 suggest that some of these moieties might have been hydroquinones with $E_h^0 > +0.18$ V at pH 7, the potential of untreated LHA inside the glovebox. Note that Q_{MEO} increased with increasing E_h applied to the WE (data not shown). This was likely due to oxidation of phenolic and other oxidizable moieties in LHA, as suggested by MEO of model quinones lawsone and AQDS (Table S1). (iii) Electron transfer to LHA in MER was much faster and more extensive than in DER, even though the potentials of the WE were lower in DER ($E_h = -0.59$ V) than in MER ($E_h = -0.49$ V), thus

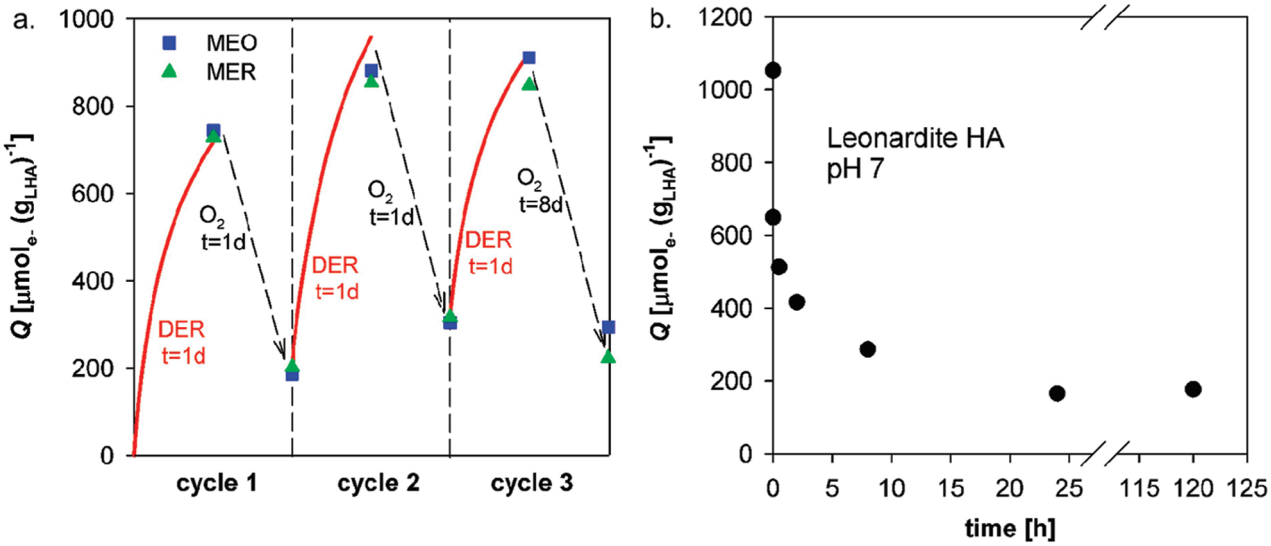

FIGURE 2. (a) Change in the amount of electrons on Leonardite Humic Acid (LHA), *Q*, relative to nontreated LHA during three successive cycles of direct electrochemical reduction (DER) and reoxidation by O_2. Electrons transferred to LHA were directly quantified in DER (red traces). Mediated electrochemical reduction (MER; green triangles) and oxidation (MEO; blue squares) were used to quantify *Q* following DER and O_2 reoxidation and were generally in very good agreement. (b) Reoxidation kinetics of DER-reduced LHA by excess O_2. The amount of electrons on LHA, *Q*, relative to nontreated LHA was quantified by MER.

confirming nonequilibrium conditions of LHA and the WE during DER. (iv) The quantitative recovery confirmed that reductive currents during DER were due to electron transfer to redox-active moieties in LHA and not due to reduction of solution protons (i.e., H_2 generation). (v) MER and MEO are perfectly suited to determine the redox states (i.e., the degree of reduction and oxidation with regard to the reference states of MER and MEO) of HS from small environmental samples. Other redox-active species (e.g., iron) in the sample will also be detected.

Method Applications

Reversibility of Electron Transfer to and from LHA. Redox buffering and electron transfer mediation by HS requires that they contain functional moieties that reversibly accept and donate electrons. Reversibility in successive reduction and O_2-oxidation cycles is of particular interest to studies of redox-dynamics in temporarily anoxic environments. In previous work it was shown for a set of eight HS that the difference in the EDCs of Pd/H_2-reduced and O_2 reoxidized HS to Fe^{3+}-citrate remained more or less constant over five successive reduction and oxidation cycles. This demonstrated that within the range of E_h values considered, each of the tested HS contained a constant pool of functional moieties with reversible electron transfer (*16*). However, because the size of this pool could not be related to the unknown amount of electrons transferred to HS during Pd/H_2 reduction, the degree of electron transfer reversibility in one and over all cycles remained unknown. As demonstrated in the following example, electron transfer reversibility in reduction−oxidation cycles can be accurately determined by combining DER with MEO and MER.

Figure 2a shows the excess amount of electrons, *Q*, on LHA samples relative to nontreated LHA during three DER-reduction (red traces) and O_2-reoxidation (black dashed arrows) cycles. Within each cycle *Q* increased by DER of LHA and decreased by exposure of LHA to excess O_2. Each reduction and reoxidation step therefore regenerated a large fraction of the capacity of LHA to donate and accept electrons, respectively. This reversible fraction underpins that HS act as redox buffers in temporarily anoxic environments. A smaller fraction of DER-transferred electrons was not re-oxidized by O_2 over 1 d in cycles 1 and 2 and 8 d in cycle 3. The size of this recalcitrant fraction was consistently quantified both by MER and MEO and accounted for 28%, 38%, and 32% of *Q* in LHA prior to exposure to O_2 in cycles 1, 2, and 3, respectively.

Quantitative recovery of DER-transferred electrons by ABTS in MEO at $E_h = +0.61$ V implies that the recalcitrant fraction did not result from truly irreversible electron transfer during LHA reduction. The reduction potential for the half reaction of $O_2 + 4e^- + 4H^+ \leftrightarrow 2\ H_2O$, $E_h^{0\prime} = +0.77$ V at pH 7 (*27*), is much higher than the E_h in MEO and the measured E_h for extensively reduced LHA (Figures 1c and S1), indicating that reoxidation of LHA by O_2 to form water was thermodynamically favorable. In contrast, the first electron reduction potential of O_2 at pH 7, $E_h^1 = -0.16$ V, to form superoxide lies at the lower end of measured E_h for LHA. Notably, the recalcitrant fraction was largely formed during the first DER step and increased only slightly to the second DER step (Figure 2a). This suggests that the oxidation-recalcitrant fraction was composed of (benzo-)quinones with first electron reduction potentials above the potential applied at the working electrode (i.e., moieties that were readily reduced in the first DER step), yet with second electron reduction potentials much higher than the E_h^1 of O_2, resulting in endergonic and hence slow first electron transfer to O_2. This is supported by a highly endergonic free energy of one electron transfer from 1,4-hydroquinone, a model for benzoquinone moieties in LHA, to molecular oxygen of $\Delta G^1 = +64.5$ kJ mol^{-1} at pH 7 (calculations in SI).

Slow and incomplete reoxidation was verified by oxidizing reduced LHA ($Q_{DER} = 1053\ \mu mol_{e^-}\ g_{LHA}^{-1}$) with excess O_2 (Figure 2b). About 50% of Q_{DER} was transferred to O_2 within less than 1 min, consistent with similar findings in ref *10*. This fast initial reoxidation was followed by slower reoxidation (up to 24 h), during which an additional ~35% of Q_{DER} was transferred to O_2. Beyond 24 h, no further reoxidation was detected up to 5 d. The amounts of DER-transferred electrons recalcitrant to O_2-reoxidation in Figure 2a and b were very similar (i.e., 200−300 $\mu mol_{e^-}\ g_{LHA}^{-1}$), albeit the total Q_{DER} was higher in the second than the first experiment. This indicates a constant pool of readily reducible quinones in LHA that form hydroquinones with low first electron oxidation po-

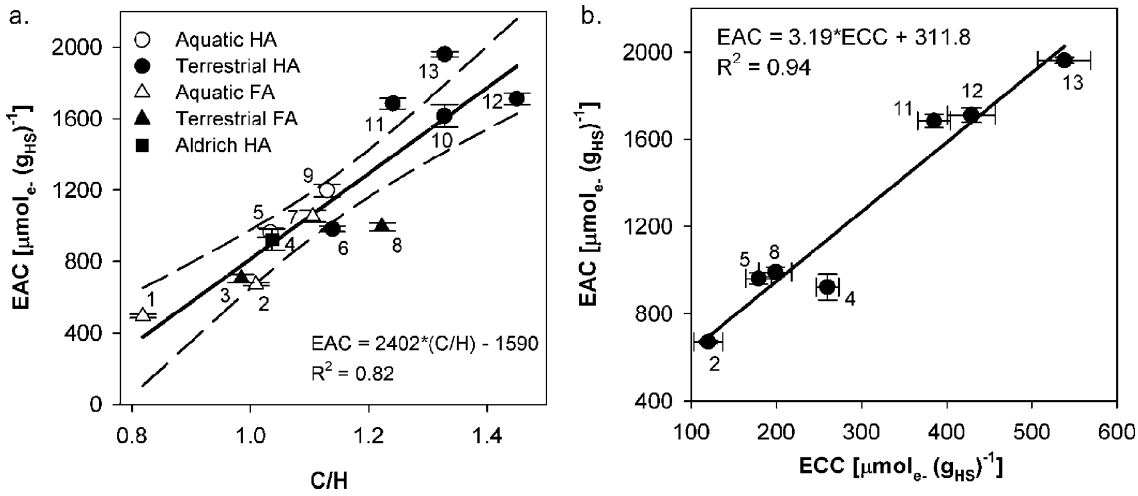

FIGURE 3. (a) Linear correlation of electron accepting capacity (EACs) with the carbon to hydrogen ratio (C/H) of 13 tested humic substances. The EACs were determined by mediated electrochemical reduction (MER) at an electrode potential E_h = −0.49 V vs SHE using diquat as mediator. C/H were determined by the International Humic Substances Society (IHSS; http://ihss.gatech.edu/ihss2/). The solid line represents the linear fit of experimental data and dashed lines correspond to the 95% confidence intervals of the fit. 1: Pony Lake Fulvic Acid Reference; 2: Suwannee River Fulvic Acid Standard; 3: Elliott Soil Fulvic Acid Standard; 4: Aldrich Humic Acid; 5 Suwannee River Humic Acid Standard; 6: Waskish Peat Humic Acid Reference; 7: Nordic Lake Fulvic Acid Reference; 8: Pahokee Peat Fulvic Acid Standard; 9: Nordic Lake Humic Acid Reference; 10: Pahokee Peat Humic Acid Reference; 11: Pahokee Peat Humic Acid Standard; 12: Leonardite Humic Acid Standard; 13: Elliot Soil Humic Acid Standard. (b) Linear correlation of EACs of a subset of tested HS with the electron carrying capacities (ECC) of the same HS taken from ref *16*. The numbers in panel b correspond to numbers in panel a.

tentials. The size of this pool in LHA corresponds well to the size recently reported for a peat humic acid (~220 μmol_{e^-} g^{-1}) (*10*).

Electron Accepting Capacities (EACs) of a Broad Spectrum of Terrestrial and Aquatic HS. The EACs of eight HA and five FA was determined by MER. The EACs ranged from 493 ± 10 μmol_{e^-} g^{-1} for Pony Lake Fulvic Acid Standard to 1961 ± 15 μmol_{e^-} g^{-1} for Eliott Soil Humic Acid Standard (Figure 3a and Table S2). There are two clear trends among the tested HS. First, HA had higher EACs than FA (i.e., 920−1960 μmol_{e^-} g^{-1} compared to 490−1050 μmol_{e^-} g^{-1}, respectively). This is particularly evident when comparing HA and FA extracted from the same source material (No. 2 and 5 or 8 and 11 in Figure 2a). Second, terrestrial HA had higher EACs than aquatic HA. The EACs therefore decreased in the order terrestrial HA > aquatic HA, aquatic FA, terrestrial FA > Pony Lake FA. The latter is of microbial origin (*28*) and therefore devoid of lignin-derived moieties.

The EACs correlated linearly with the C/H elemental ratios of all 13 tested HS (R^2 = 0.82; Figure 3a) and with aromaticity for 11 of the tested HS, for which experimental aromaticity data from ^{13}C NMR was available (R^2 = 0.82; Figure S6). The correlation with aromaticity was stronger than a previously published correlation based on 9 HS (R^2 = 0.66) in ref *2*, in which EACs were determined indirectly by oxidation of nontreated and microbially reduced HS using Fe^{3+}. The good correlations presented herein provide additional evidence that aromatic systems, likely quinone moieties, dominate the redox characteristics of HS. This is supported by calculations showing that the molar concentrations of noncarboxylic and nonphenolic oxygen (μmol_O g_{HS}^{-1}) of the tested HS, which include oxygen in quinone moieties, were much larger than their respective EAC (Table S2). The high EAC of terrestrial HA and the low EAC of aquatic FA, particularly of Pony lake FA, suggests that such quinone groups most likely originate from phenolic moieties in lignin (*2*).

Comparison of Different Methods for Determining EACs. The EACs of 7 of the tested HS linearly correlated (R^2 = 0.94) with their electron carrying capacities (ECC) reported in ref *16* (Figure 3b), where the ECC was defined as the difference in the amount of electrons transferred to Fe^{3+}-citrate from nontreated and from Pd/H_2 prereduced HS within 15 min of reaction. However, since the electron transfer from HS to Fe^{3+}-citrate requires >24 h to reach equilibrium (*17*), reported ECCs likely underestimated equilibrium values. This explains why ECCs were about a factor of 3 smaller than EACs determined by MER (Figure 3b). The linear correlation of EAC and ECC, however, implies that the HS in Figure 3b had similar relative amounts of their EACs that were readily reoxidized by Fe^{3+}-citrate. Thus, even though short time oxidation of extensively reduced HS by Fe^{3+}-citrate resulted in incomplete recovery of reductively transferred electrons, the assay may be used to determine the relative differences in the EACs among HS and to detect changes in the redox states of partially reduced HS.

Similar to reoxidation by Fe^{3+}, the nonradical organic mediator dichlorophenol-indophenol (DCPIP) (Figure S2), when used in MEO at E_h = +0.61 V, recovered 50% of the electrons that had been transferred to LHA samples by DER (samples B0−B4 in Figure S5). Incomplete recovery by DCPIP yet close to complete recovery by ABTS, despite the same electrode potential in MEO, shows that DER-reduced LHA contained moieties that were only slowly oxidized by DCPIP, likely due to slow first electron transfer kinetics. The amount of electrons recovered by DCPIP, however, correlated linearly with the amount of electrons that had been transferred to LHA during DER (Figure S5). DCPIP therefore accurately detected changes in the redox states of partially reduced HA.

Oxidized DCPIP is blue (λ_{max} = 603 nm), while reduced $DCPIPH_2$ is colorless. The reductive decolorization of DCPIP can therefore be used in a spectrophotometric assay to quantify redox changes in HS (method details in SI), extending on an earlier approach using DCPIP to determine sediment redox states (*18*).

Addition of DER-reduced LHA (Q_{DER} = 999 μmol_{e^-} g_{LHA}^{-1}) to DCPIP resulted in a decrease in the absorption at λ_{max} = 603 nm (due to reduction of DCPIP), which was proportional to the amount of LHA added (Figure S7). In contrast, addition of nontreated LHA resulted in little reduction of DCPIP (Figure S7). The calculated amounts of electrons transferred from prereduced and nontreated LHA to DCPIP were 612 and 42

$\mu mol_{e^-}\ g_{LHA}^{-1}$, respectively. This corresponds to a recovery of 57% of Q_{DER}, which agreed well with the recovery in MEO using DCPIP as mediator. Higher recoveries were obtained when the reoxidation was conducted over longer time periods (data not shown). Quantification of HS redox status is faster and more direct by this DCPIP assay than by currently used Fe^{3+}-assays in which complexation of the reduction product Fe^{2+} with ferrozin is required prior to its spectroscopic analysis (*17*). The DCPIP assay is therefore suited for high-throughput analysis of changes in the redox states of large sets of HS samples. Yet, for accurate balancing (i.e., complete recovery) of electrons transferred to and from HS, the use of organic radical mediators in MER and MEO is recommended.

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Supporting Information Available

This material is available free of charge via the Internet at http://pubs.acs.org.

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