Quinone Moieties Act as Electron Acceptors in the Reduction of Humic Substances by Humics-Reducing Microorganisms

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The possibility that quinones serve as electron-accepting moieties when microorganisms transfer electrons to humic substances was investigated. Humic substances from a variety of environments had the ability to accept electrons from a humics-reducing microorganism, *Geobacter metallireducens*. A lignaceous carbon source for the humic substances was not necessary in order for the humic substances to act as electron acceptors. Humic substances extracted from soils typically had a greater electron-accepting capacity than humic substances extracted from aquatic sediments, and sediment humic substances had more electron-accepting capacity than dissolved aquatic humic substances. Electron spin resonance measurements at pH 10 demonstrated that humic substances that had a higher electron-accepting capacity also had a higher free radical content than did humic substances with a lower electron-accepting capacity. The ESR spectra were consistent with semiquinones as the main organic radicals. Microbial reduction of humic substances resulted in an increase in semiquinones that was in proportion to the electron-accepting capacity of the humic substances. These results provide direct evidence that organic radicals in humic substances, which are primarily quinone groups, are reduced when humics-reducing microorganisms transfer electrons to humic substances.

Introduction

Humic substances are present in soil, water, and sediments within the biosphere and account for a large portion of organic carbon on the earth's surface and in the oceans. These macromolecular substances are derived from plant, algal, and microbial material (*1*). Soil humic substances are primarily derived from the degradation of litter fall from vascular plants. In aquatic systems, humic substances may be derived from plant degradation and transported from the
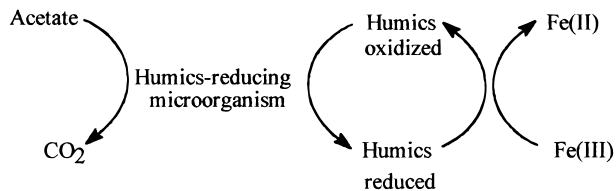
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FIGURE 1. Proposed electron mediator role of humic substances in the reduction of Fe(III) (from ref *2*).

watershed or produced in situ from degradation of algal and microbial biomass. Consequently, humic substances have different chemical properties depending on the chemical characteristics of the precursor organic carbon, the transformations the organic carbon has undergone, and the environment of the isolated humic substances.

Humic substances are generally considered recalcitrant under anaerobic conditions (*1*); however a significant biological role involving humic substances has been discovered. Recent studies have suggested that humic substances may be dynamically involved in carbon and electron flow in anaerobic environments (*2, 3*). Fe(III)-reducing microorganisms can transfer electrons derived from the oxidation of organic compounds and/or H_2 to humic substances (*2–4*). This electron transfer yields energy to support growth. The microbially reduced humic substances can then abiotically transfer electrons to iron(III) oxides. Thus, in anaerobic soils and sediments, humic substances may serve to shuttle electrons from the surface of Fe(III)-reducing microorganisms to iron(III) oxides (Figure 1). This electron shuttling permits Fe(III)-reducing microorganisms to indirectly reduce iron(III) oxides faster than Fe(III) is reduced in the absence of humic substances because it alleviates the need for Fe(III)-reducing microorganisms to come into direct physical contact with iron(III) oxides in order to reduce them. Thus, humics-mediated electron shuttling may enhance rates of organic matter oxidation coupled to the reduction of Fe-(III) in soils and sediments as evidenced by greatly stimulated anaerobic degradation of benzene in sediments from the Fe(III)-reducing zone of a petroleum-contaminated aquifer (*5*).

It has been speculated that quinone moieties are the important electron-accepting groups for microbial reduction of humic substances (*2–4*). This suggestion was based on several lines of circumstantial evidence. First, the presence of quinones within humic substances has been confirmed using nuclear magnetic resonance (NMR) (*6*). Second, quinones have previously been suggested to be involved in abiotic humics-mediated electron transfer from reduced sulfur and iron species to chlorinated and nitroaromatic compounds (*7–9*). The results from these previous experiments were consistent with quinone moieties as the dominant electron acceptor within humic substances. Furthermore, anthraquinone-2,6-disulfonate (AQDS), which has been used as a humic analogue in studies on abiotic humics-mediated electron transfer (*2, 8*), stimulates microbial Fe(III) reduction in a manner similar to humic substances (*2*). Fe(III)-reducing microorganisms can conserve energy to support growth via electron transport to AQDS. The reduced product, anthrahydroquinone-2,6-disulfonate (AHQDS), can abiotically transfer two electrons to Fe(III) with the regeneration of AQDS. When microorganisms are isolated from soils and sediments with AQDS as the electron acceptor, the AQDS microorganisms invariably have the ability to transfer electrons to humic substances.
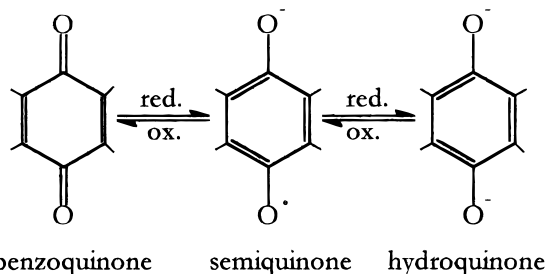
FIGURE 2. Quinone model compound. The semiquinone species contains an unpaired electron.

More direct evidence is required in order to better ascertain whether quinones are important electron-accepting groups in humic substances. Electron transfer to quinones can be expected to lead to an increase in semiquinone radical intermediates as well as hydroquinones (Figure 2). Thus, if quinones are important electron-accepting groups in humic substances, electron transfer to humic substances would be expected to result in an increase in the concentration of the semiquinone species. Furthermore, if quinones are important electron-accepting moieties in humic substances, then it might also be expected that there would be a correlation between the abundance of quinones in various humic substances and the capacity of the humic substances to accept electrons. Quinone content in humic substances can be estimated by measuring the semiquinone radicals with electron spin resonance (ESR) (*10*).

Previous studies have indicated that the organic radicals present in humic substances can be attributed to the presence of quinone groups (*11−13*). Flavin radicals have been considered as a contributor to the presence of organic radicals, although their behavior with respect to spin content and pH is opposite that of humic substances, lignin, tannins, and quinone compounds (*10*). Since lignin is a precursor to humic substances, the ESR characteristics of lignin also support quinone moieties as the primary organic radicals within humic substances.

Therefore, the relationship between the semiquinone organic radical content of humic substances determined by ESR and the electron-accepting capacity of a variety of humic substances was evaluated. The studies were conducted with *Geobacter metallireducens* because *Geobacter* species have been found to be important humic-reducing microorganisms in a variety of sedimentary environments (*4*). The results demonstrate that there is a direct relationship between radical content and electron-accepting capacity and that organic radicals, which are primarily semiquinones, are formed when microorganisms transfer electrons to humic substances. These results suggest that quinones are the important electron acceptors in humic substances for microbial respiration.

Methods

Sources of Humic Substances. International Humic Substance Society (IHSS) humic and fulvic acids from the Suwannee River and several terrestrial humic substances were used in the experiments to obtain a range of reactivity (*14*). Suwannee River humic substances are derived from sphagnum moss and cypress trees (*15*). IHSS terrestrial humic substances used in this study were the following: soil humic acid (S-HA), Leonardite humic acid (L-HA), peat humic acid (P-HA) and Summit Hill humic acid (SH-HA). Fulvic acid isolated from Lake Fryxell (LF-FA), located in an Antarctic desert devoid of vascular plants, was also evaluated (*16*).

Humic substances from freshwater and marine sedimentary environments were isolated and fractionated for this study. Nymph Lake sedimentary humic substances (NL-FA) isolated from Nymph Lake, a subalpine lake located in Rocky Mountain National Park, represented freshwater sedimentary humic substances. Nymph Lake receives a large quantity of terrestrial organic matter from the surrounding watershed. San Diego Bay sedimentary humic substances (SD-FA) represented a source of marine sedimentary humic substances. The Nymph Lake and San Diego Bay sedimentary humic substances were extracted from sediment samples using sodium pyrophosphate (*17*). The initial organic carbon content of the sediments was 28.82% for NL-FA and 0.95% for SD-FA (*17*). To minimize the alteration of the organic matter, a 0.1 N solution of $Na_4P_2O_7$ was buffered to pH 7.0 with HCl. The procedure involved adding 100 mL of sediment to 1 L of the extractant solution, followed by shaking the solution for a period of 24 h at 150 rpm. After 24 h, the solution was dark in color, indicating that a fraction of the organic carbon originally sorbed to the matrix was now dissolved in the solution. The humic substances were then fractionated from the total dissolved carbon by using a modified column chromatography procedure outlined by Thurman and Malcolm (*18*). After collecting the eluate, reconcentrating the eluate on the XAD column, and passing the eluate through a cation-exchange column, the desalted hydrogen-saturated sample was then freeze-dried for preservation. The recovered hydrophobic acid fraction of the extracted organic carbon was 35% for NL-FA and 34% for SD-FA (*17*).

Aromaticity. Aromaticity was measured for the sedimentary humic substances with solid-state carbon-13 nuclear magnetic resonance. The ^{13}C NMR spectra of the other humic substances were previously measured using liquid-state ^{13}C NMR (*16, 19*). Given the wide range of aromaticities of these humic substances, a general comparison among the humic substances is valid despite the slightly different methods.

Electron Spin Resonance Measurements at pH 10. Electron spin resonance (ESR) was used to measure the organic radical content of humic substances. This approach has previously been used as an indicator for the quinone content in humic substances (*10*). The ESR spectra were obtained with a Bruker ESP300E spectrometer. With the first ESR measurements, humic substances were dissolved in a 0.1 N NaOH solution to obtain a 1.0% w/v concentration. A flatcell was then immediately filled with the solution and placed into the ESR cavity. The concentration of organic radicals was measured as a function of time in the basic solution at room temperature to obtain a stable concentration of organic radicals. The ESR instrument was operated with the following parameters: microwave frequency 9.69 GHz, microwave power 0.998 mW, modulation frequency 100 kHz, conversion time 10.24 ms, and time constant 1.28 ms. Signal intensity varied linearly with the square root of the microwave power ($R^2 = 0.9991$), indicating that power saturation was avoided under the operating conditions of the instrument. 3-Carbamoyl-2,2,5,5,-tetramethyl-3-pyrrolin-1-yloxy, a compound containing a nitroxyl free radical, was used as the standard for ESR. The spectra were electronically integrated on the Bruker ESP300E instrument. Replicate samples ($n = 3$) were measured to determine standard deviations.

Electron-Accepting Capacity. To determine the electron-accepting capacity of humic substances, washed cell suspensions of *G. metallireducens* (0.8 mg/mL protein) in 30 mM bicarbonate buffer were incubated with 2 g/L humic substances and 10 mM acetate as an electron donor at 30 °C under anoxic conditions (*2*). Protein measurement was performed with the Folin phenol reagent (*20*). A 2-h incubation period was chosen from a previous study where a steady-state electron-accepting capacity was reached for Aldrich humic acids (*2*). After the 2-h incubation period in the dark, the cells were removed anaerobically by filtration. The filtrate was incubated with 50 mM Fe(III) for 15 min. The time period of incubation was previously determined by
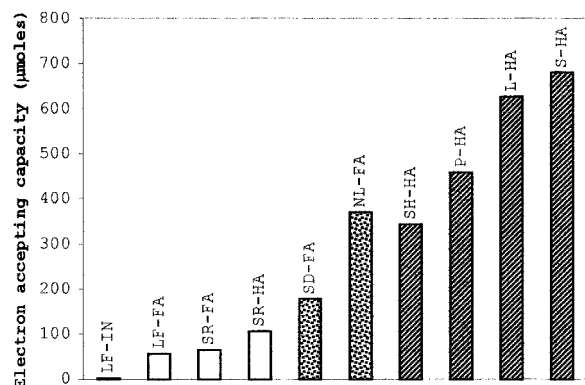
FIGURE 3. Electron-accepting capacities for humic substances measured using the method of Lovley et al. (2) (n = 3). Key: soils, slanted lines; sediment, polka dots; aquatic, white.
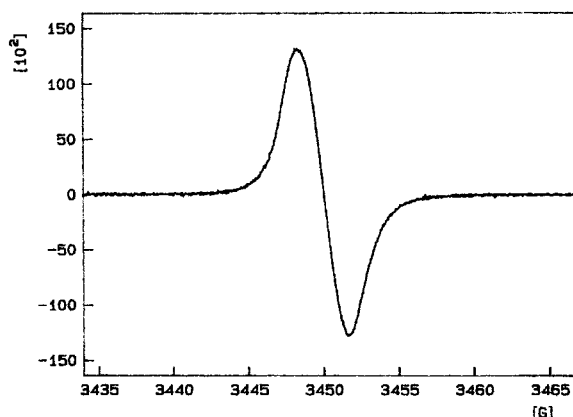

FIGURE 4. ESR spectra for Nymph Lake sedimentary humic substances. The spectra's single peak devoid of hyperfine splitting was indicative of all of the ESR spectra, including AQDS. The instrument's offset was +5.21 G (measured using a gaussmeter probe).

incubating reduced humic substances with Fe(III) for up to 1 h. A time period of 15 min was shown to be sufficient (2). Triplicate measurements of Fe(II) were determined using the ferrozine assay. The amount of Fe(II) reduced was the electron-accepting capacity of the humic substances.

Change in Organic Radical Content after Microbial Reduction. The change in organic radical content of the humic substances was measured before and after microbial incubation. Initially, humic substances were dissolved at a concentration of 2 g/L in an anoxic bicarbonate solution containing acetate. An aliquot was removed to measure the initial concentration of organic radicals. *G. metallireducens* (0.8 mg/mL protein) was then added to the solution containing humic substances. After a period of 2 h, the cells were filtered anaerobically from the solution, and an aliquot was removed to measure the organic radical content. ESR flatcells were filled in an anaerobic glovebag to maintain anoxic conditions. ESR instrumental parameters were identical to the measurement of organic radicals in the basic solution. The increase in organic radicals was then reported as the difference in the organic radicals before and after microbial incubation. Replicate spectra (n = 5) of the same sample were measured to determine the standard deviations.

Results

Electron-Accepting Capacity of the Reduced Form of Humic Substances. Previous studies have quantified the electron-accepting capacity for a number of humic substances, but these were primarily from terrestrial sources or aquatic environments with a heavy terrestrial input (2). Evaluation of humic substances from aquatic environments indicated that they also could accept electrons from the humics-reducing microorganism, *G. metallireducens* (Figure 3). The electron-accepting capacity of dissolved aquatic humic substances was consistently less than that of the humic substances from aquatic sediments, which in turn had lower electron-accepting capacities than the humic substances extracted from soils. The difference in electron-accepting capacity cannot be attributed solely to the precursors in humic substance formation. This is shown by the result that dissolved fulvic acids derived from microbial material in Lake Fryxell had a similar electron-accepting capacity to Suwannee River fulvic acids, which originate from terrestrial organic matter. Biogeochemical transformations of aquatic humic substances may influence the electron-accepting capacity. The fulvic acids from the water column of Lake Fryxell, which have diffused from anoxic sediments into a productive oxic zone of the water column, had 25 times the electron-accepting capacity as dissolved aquatic fulvic acids recovered from the sediment interstitial water (16).

ESR of Humic Substances in a Basic Solution. Organic radicals were detected by ESR at pH 10 for all of the humic

substances evaluated. At pH 10, benzoquinone moieties appear to be reduced to produce semiquinone moieties. Functional groups within the humic molecules may be acting as the reducing agent (10). Another possible mechanism for the high concentrations of organic radicals at pH 10 is that OH^- may react with the benzoquinone species to produce the semiquinone species (10).

All of the spectra for humic substances were devoid of hyperfine splitting (Figure 4). The ESR spectrum in Figure 4 is representative the spectra for the various humic substances in this study, with respect to line shape. The spectroscopic splitting constants, g, were in the range from 2.0037 to 2.0048. This range agrees with previously reported g-values for humic substances and is consistent with semiquinones being the primary organic radicals (10). To determine the stable organic radical content, the concentration of the organic radicals was measured as a function of time dissolved in the basic solution.

The time course measurements for the IHSS soil humic acid (S-HA), San Diego Bay fulvic acid (SD-FA), Suwannee River fulvic acid (SR-FA), and Lake Fryxell fulvic acid (LF-FA) are presented in Figure 5. For the terrestrial humic substances (S-HA as well as L-HA, SH-HA, and P-HA), the organic radical concentration increased 320% until the concentration of organic radicals (spins/g) reached a plateau after approximately 400 min. From 400 to 1400 min, the increase in organic radical concentration was 12%. For the S-HA time course, instrument availability allowed for the large amount of data points collected as compared to the time courses for the other humic substances. For the sedimentary humic substances (SD-FA as well as NL-FA), the change of the organic radical concentration over time was not as great as the terrestrial humic substances. In the first 14 min after dissolving the humic substances, the increase in organic radicals was 35%. This initial increase then lead to an 8% increase in organic radical content from 14 to 1400 min.

The Suwannee River humic substances (SR-FA as well as SR-HA) had a different pattern of change in organic radical concentration over time. A maximum organic radical concentration was reached for SR-FA after 12 min. The initial increase in this period was 38%. After the initial 12 min period, the organic radical concentration decreased 70% over 1400 min. Similarl to the SR-FA, Lake Fryxell fulvic acids had an initial increase of 108% in the organic radical concentration after 74 min. At this point, the organic radical content decreased 32% (8.0×10^{16} spins/g) over the next 1300 min. On the basis of the time course measurements,
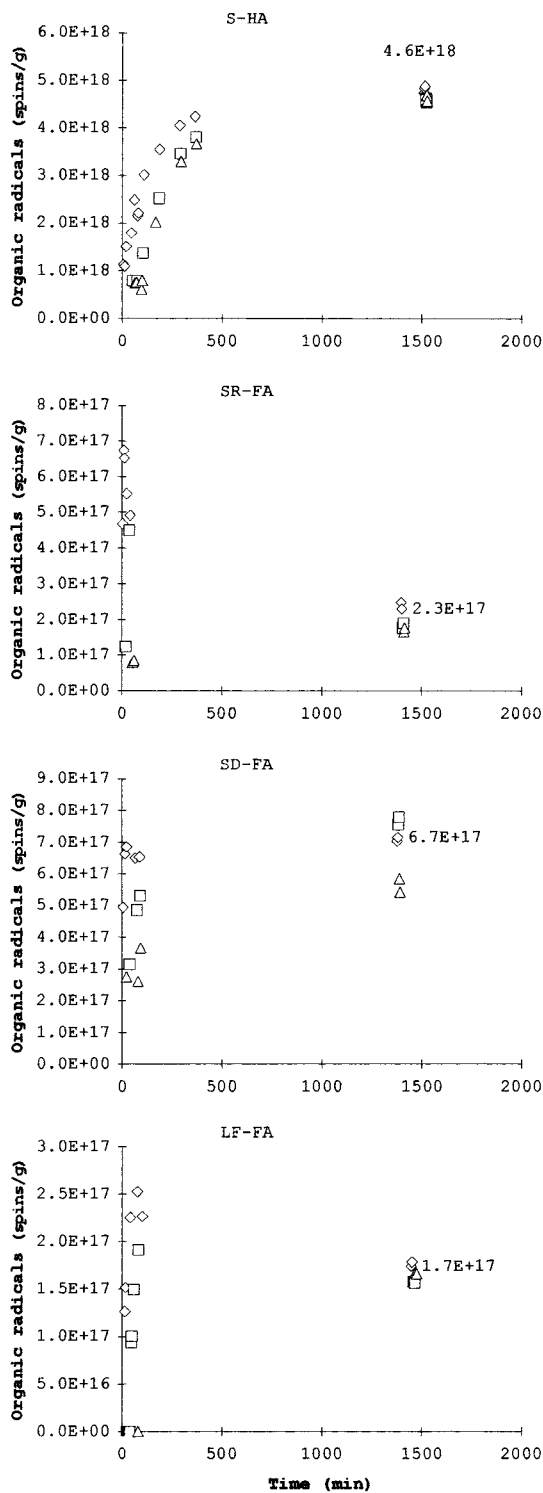
FIGURE 5. IHSS Summit Hill, San Diego, and Suwannee River fulvic and Lake Fryxell humic substances time courses (S-HA, SD-FA, SR-FA, LF-FA). These plots show the change in organic radicals (spins/g) over time. Each symbol represents a measurement made at a specific time of the same replicate.
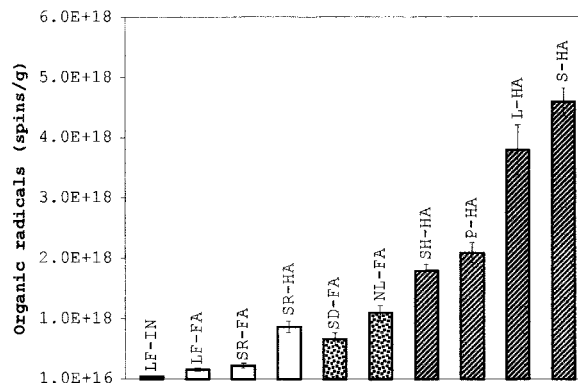

FIGURE 6. Stable concentrations of organic radicals measured by ESR. Error bars indicate ± 1 standard deviation. Key: slanted lines, soil; polka dots, sediments; white, aquatic humic substances.
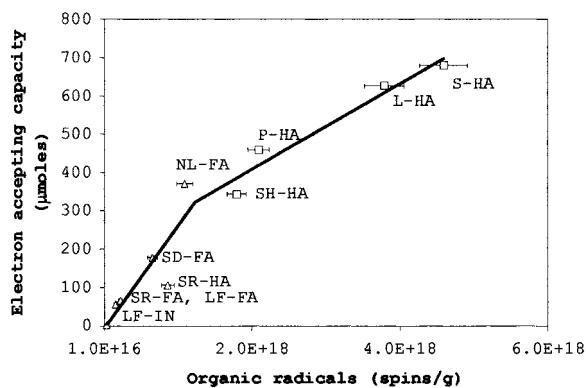

FIGURE 7. Electron-accepting capacity and organic radical concentration for humic substances from a variety of environments. Error bars indicate ± 1 standard deviation. Key: △, aquatic and sedimentary humic substances, □, soil and coal humic substances.

stable organic radical contents were measured after 1400 min.

The organic radical concentration of the humic substances followed the same general trend as the electron-accepting capacity. The terrestrial humic substances had the highest organic radical content, followed by the sedimentary humic substances, and then the aquatic humic substances (Figure 6). Direct comparison between the organic radical concen-

tration and the electron-accepting capacity indicated that the humic substances isolated from aquatic environments exhibited a greater increase in electron-accepting capacity per increase in organic radical content than did the terrestrial humic substances, as indicated by the change in slope in Figure 7. This relationship was expected because the easily reducible organic radicals, which are primarily quinones, are hypothesized to be the electron-accepting moiety.

ESR of Microbially Reduced Humic Substances. If electron transfer to humic substances involved reduction of quinones, then it would be expected that microbial reduction of humic substances would increase the radical content of humic substances as quinones are reduced from the benzoquinone state to the semiquinone state and then the hydroquinone state. As evidence of this, organic radicals were not detected before microbial reduction of AQDS, but after microbial reduction 7.4×10^{18} spins/g of organic radicals were detected. To evaluate whether quinones in humic substances were being reduced, the organic radical content of the humic substances was quantified before and after microbial reduction. The ESR measurements were conducted on humic substances in pH 6.7 bicarbonate buffer, which provided suitable conditions for the activity of *G. metallireducens*. The organic radical content at pH 6.7 was significantly lower than at pH 10 (Table 1). For several of the humic substances, the initial radical concentration was below the detection limit of 10^{16} spins/g. After microbial reduction, the organic radical concetration increased for each humic sample (Table 1).

The extent of the increase in radical concentration as the result of microbial reduction of humic substances was related to the electron-accepting capacity of the humic substances
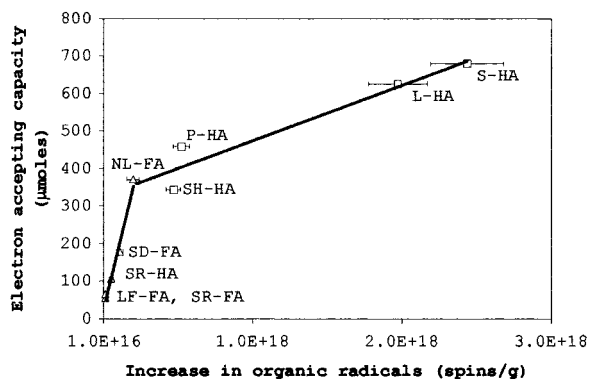
FIGURE 8. Electron-accepting capacity and the change in organic radicals after microbial reduction of humic substances. Error bars indicate $\pm$ 1 standard deviation.
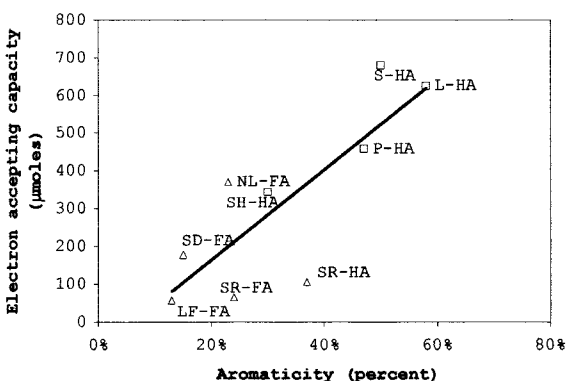

FIGURE 9. Electron-accepting capacity versus aromaticity of the humic substances.

TABLE 1. Organic Radical Concentrations before and after Microbial Incubation

	organic radicals		
sample	oxidized (spins/g)	reduced (spins/g)	increase (spins/g)
Leonardite humic	3.6E+17	2.3E+18	2.0E+18
peat humic	1.7E+17	7.0E+17	5.3E+17
soil humic	7.2E+17	3.2E+18	2.4E+18
Summit Hill humic	2.1E+17	6.9E+17	4.8E+17
Nymph Lake fulvic	0.0E+00	2.1E+17	2.1E+17
San Diego fulvic	0.0E+00	1.2E+17	1.2E+17
Suwannee humic	0.0E+00	6.1E+16	6.1E+16
Suwannee fulvic	0.0E+00	1.1E+16	1.1E+16
Lake Fryxell fulvic	0.0E+00	1.0E+16	1.0E+16
AQDS	0.0E+00	7.4E+18	7.4E+18

(Figure 8). The degree of increase in electron-accepting capacity per increase in radical formation was greater with humic substances from aquatic environments than with humic substances from terrestrial sources. This difference in the response with aquatic and terrestrial humic substances was similar to the pattern observed above with organic radical content at pH 10.

Aromaticity and Electron-Accepting Capacity. Aromaticity of the humic substances and electron-accepting capacity were positively correlated (Figure 9) ($R^2 = 0.66$). Unlike the relationship between electron-accepting capacity and radical content (Figure 7), there was no clear change in slope in the relationship between electron-accepting capacity and aromatic content.

Discussion

Quinone Moieties as Electron Acceptors. Organic radicals, which contain unpaired electrons, can be detected using ESR spectroscopy. As discussed above, organic radicals have properties similar to naturally occurring quinone moieties. ESR experiments on the organic radicals within humic substances, including the effects of pH, oxidation, and UV radiation, are consistent with quinone moieties (*10*). The easily reducible nature of the organic radicals within humic substances supports the hypothesis that quinone moieties are the electron-accepting groups. For example, when AQDS was microbially reduced, the organic radical concentration went from below detection levels to 7×10^{18} spins/g. When oxygen was introduced, the ESR signal disappeared. The organic radicals within humic substances also exhibited this behavior, indicating that quinone moieties may be the primary organic radicals.

The ESR results from this study further indicate that quinone moieties are important electron-accepting groups in humic substances for microbial humic reduction. Several authors have proposed the idea that quinones are important electron-transfer groups in humic substances for both biological and abiotic reduction and oxidation of humic substances. However, previous studies have inferred this from the ability of quinone-containing humic analogues to perform electron transfers similar to those carried out by humic substances. The findings that organic radical content is strongly correlated with the electron-accepting capacity of humic substances and that microbial reduction of humic substances results in a significant increase in organic radical content provides evidence that quinones in humic substances may be involved in electron transfer.

Previous studies focused primarily on the electron-accepting capacity of terrestrial humic substances (*2*) and the ability of humic substances to stimulate microbial activity in terrestrial subsurface environments (*5*). The results presented here demonstrated that humic substances from aquatic environments can also serve as electron acceptors for microbial respiration and that, once reduced, the humic substances can donate electrons to Fe(III).

Importance of Organic Carbon Source for Humic Substances. Quinone moieties may be incorporated into humic substances from degradation of lignin or microbial organic matter. Lignin, which is typically 5−20% of the biomass of plants, can be a major source of quinone compounds in soil humic substances (*21, 22*). Polyphenols synthesized by microorganisms are an alternative source of quinone moieties, especially in environments in which there is little vascular plant input (*22*). Previous studies on microbial electron transfer to humic substances have focused primarily on soil humic acids that have a significant lignin-derived component (*2−4*). The results presented here demonstrate that humic substances from environments without a terrestrial plant input can also serve as electron acceptors in microbial metabolism. This was evident from the finding that fulvic acid isolated from Lake Fryxell located in an Antarctic desert devoid of vascular plants (*23*) was effective in accepting electrons from *G. metallireducens*. Furthermore, San Diego Bay sedimentary humic substances, which have a fluorescence index (ratio of the emission intensities at 450 and 500 nm) indicative of a primarily microbial and algal source (unpublished data), were good electron acceptors (*24*). The ability of humic substances derived solely from microbial biomass to serve as electron acceptors for microbial respiration has implications for metabolism on ancient Earth, as it suggests that microbial humic respiration was possible prior to the evolution of terrestrial plants.

Aromaticity versus Organic Radical Content. The organic radical content has a stronger relationship with electron-accepting capacity than aromaticity. This was demonstrated by the high R^2 value (0.91) between organic

radical content and electron-accepting capacity for all of the humic substances. This result suggests that organic radical content measured with ESR is a better predictor of electron-accepting capacity. The spectroscopic technique used to measure aromaticity, ^{13}C NMR, detects aromatic and unsaturated carbon (25). Since quinone moieties are just one of many aromatic moieties within humic substances, the weaker correlation coefficient with aromaticity and electron-accepting capacity is not surprising because ESR directly measures organic radicals, which are thought to be primarily semiquinone moieties.

Semiquinone Stability. The stability of the semiquinone molecule appears to be enhanced in the more aromatic humic substances as indicated by the ESR time course measurements (Figure 5). When the electron-accepting capacity was plotted against organic radical concentration, a noticeable grouping between the terrestrial and other humic substances was evident (Figure 7). The terrestrial humic substances are all humic acids, which generally have larger molecular weight than fulvic acids (26, 27). Thus, one explanation for the grouping is that the network of aromatic rings within the more aromatic terrestrial humic substances may stabilize the unpaired electrons. For the aquatic fulvic acids, the semiquinone functional group appears to be more transient and the concentration of organic radicals decreases slowly over time, possibly indicating that the semiquinone functional groups are being converted to hydroquinone functional groups. A model suggested by Senesi and Schnitzer can explain this result (10) (Figure 2). The first electron transfer from benzoquinone to semiquinone is fast, resulting in an initial increase in semiquinone functional groups. The second electron transfer to the hydroquinone species is slower, which is indicated by the slow decline of organic radicals. The electrons that are transferred to the terrestrial humic substances during microbial oxidation of acetate may also be more stable, as seen by the stable concentration of organic radicals as a function of time. Thus, the reduced state of these humic substances will be more stable than that of the aquatic humic substances, which slows the rate of electron transfer from reduced terrestrial humic substances to Fe(III) as indicated by the different slope in Figure 7.

Summary. Humic substances from a variety of environments were capable of accepting electrons in anoxic microbial respiration. Organic radicals were shown to be directly reduced by *G. metallireducens*, suggesting that quinone moieties are the primary reducible moiety within humic substances. Thus, humic substances that were traditionally thought to be microbially inert in anoxic environments can participate in microbial reactions.

Literature Cited

(1) McKnight, D.; Aiken, G. Sources and Age of Aquatic Humus. In *Aquatic Humic Substances: Ecology and Biogeochemistry*; Hessen, D., Tranvik, L., Eds.; Springer-Verlag: New York, 1998 (in press).

(2) Lovley, D.; Coates, J.; Blunt-Harris, E.; Phillips, E.; Woodward, J. *Nature* **1996**, *382*, 445.

(3) Lovley, D.; Woodward, J.; Blunt-Harris, E.; Hayes, L.; Phillips, E.; Coates, J. *Acta Hydrochim. Hydrobiol.* **1998**, *26*, 152.

(4) Coates, J.; Ellis, D.; Blunt-Harris, E.; Gaw, C.; Roden, E.; Lovley, D. *Appl. Environ. Microbiol.* **1998**, *64*, 1504.

(5) Lovley, D.; Woodward, J.; Chapelle, F. *Appl. Environ. Microbiol.* **1996**, *62*, 288.

(6) Thorn, K.; Arterburn, J.; Mikita, M. *Environ. Sci. Technol.* **1992**, *26*, 107.

(7) Dunnivant, F.; Schwarzenbach, R.; Macalady, D. *Environ. Sci. Technol.* **1992**, *26*, 2133.

(8) Curtis, G.; Reinhard, M. *Environ. Sci. Technol.* **1994**, *28*, 2393.

(9) Schwarzenbach, R.; Stierli, R.; Lanz, K.; Zeyer, J. *Environ. Sci. Technol.* **1990**, *24*, 1566.

(10) Senesi, N.; Steelink, C. Application of ESR Spectroscopy to the study of humic substances. In *Humic Substances II In Search of Structure*; Hayes, M., MacCarthy, P., Malcolm, R., Swift, R., Eds.; John Wiley and Sons: New York, 1989; pp 373–408.

(11) Wilson, S.; Weber, J. *Anal. Lett.* **1977**, *10*, 75.

(12) Senesi, N.; Schnitzer, M. *Soil Sci.* **1977**, *123*, 224.

(13) Maximov, O.; Glebko, L. *Geoderma* **1974**, *11*, 17.

(14) Cambell, W.; Malcolm, M. *Org. Geochem.* **1985**, *8*, 109.

(15) Malcolm, R.; McKnight, D.; Averett, R. History and description of the Okefenokee Swamp—Origin of the Suwannee River. *Open-File Rep.—U.S. Geol. Surv.* **1989**, *No. 87-557*.

(16) Aiken, G.; McKnight, D.; Harnish, R.; Wershaw, R. *Biogeochemistry* **1996**, *34*, 157.

(17) Scott, D. M.S. Thesis, University of Colorado at Boulder, 1997.

(18) Thurman, E.; Malcolm, R. *Environ. Sci. Technol.* **1981**, *15*, 463.

(19) Thorn, K.; Folan, D.; MacCarthy, P. Characterization of the IHSS standard and reference fulvic and humic acids by solution state carbon-13 and hydrogen-1 NMR. *Water Resour. Invest. (U.S. Geol. Surv.)* **1989**, *No. 89-4196*.

(20) Lowry, D.; Rosenbrough, Q.; Farr, A.; Randall, R. *J. Biol. Chemistry* **1951**, *193*, 265.

(21) Hedges, J.; Clark, W.; Cowie, G. *Limnol. Oceanogr.* **1988**, *33*, 1137.

(22) Stevenson, J. *Humus Chemistry: Genesis, Composition, Reactions*; John Wiley and Sons: New York, 1994.

(23) McKnight, D.; Aiken, G.; Smith, R. *Limnol. Oceanogr.* **1991**, *36*, 998.

(24) McKnight, D.; et al. *Limnol. Oceanogr.* In review.

(25) Malcolm, R. Application of Solid-State ^{13}C NMR Spectroscopy to Geochemical Studies of Humic Substances. In *Humic Substances II In Search of Structure*; Hayes, M., MacCarthy, P., Malcolm, R., Swift, R., Eds.; John Wiley and Sons: New York, 1989; pp 340–372.

(26) Stevenson, F. *Geochemistry of Soil Humic Substances*; Aiken, G., McKnight, D., Wershaw, R., MacCarthy, P., Eds.; John Wiley and Sons: New York, 1985; pp 13–52.

(27) Malcolm, R.. *Geochemistry of Stream Fulvic and Humic Substances*; Aiken, G., McKnight, D., Wershaw, R., MacCarthy, P., Eds.; John Wiley and Sons: New York, 1985; pp 181–209.