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Natural Humics Impact Uranium Bioreduction and Oxidation

BAOHUA GU,* HUI YAN, PING ZHOU, AND DAVID B. WATSON

Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831

MELORA PARK AND JONATHAN ISTOK

Department of Civil Engineering, Oregon State University, Corvallis, Oregon 97331-4501

Although humic substances occur ubiquitously in soil and groundwater, their effect on the biological reduction of uranium(VI) and subsequent reoxidation of U(IV) is poorly understood. This study investigated the role of humics in enhancing the bioreduction of U(VI) in laboratory kinetic studies, in field push−pull tests, and in the presence or absence of metal ions such as Ca^{2+} and Ni^{2+}, which are known to inhibit the biological reduction of U(VI). Results from laboratory experiments indicate that, under strict anaerobic conditions, the presence of humic materials enhanced the U(VI) reduction rates (up to 10-fold) and alleviated the toxicity effect of Ni^{2+} on microorganisms. Humic acid was found to be more effective than fulvic acid in enhancing the reduction of U(VI). Such an enhancement effect is attributed to the ability of these humics in facilitating electron-transfer reactions and/or in complexing Ca^{2+} and Ni^{2+} ions. Similarly, field push−pull tests demonstrated a substantially increased rate of U(VI) reduction when humic acid was introduced into the site groundwater. However, humics were also found to form complexes with reduced U(IV) and increased the oxidation of U(IV) (when exposed to oxygen) with an oxidation half-life on the order of a few minutes. Both of these processes render uranium soluble and potentially mobile in groundwater, depending on site-specific and dynamic geochemical conditions. Future studies must address the stability and retention of reduced U(IV) under realistic field conditions (e.g., in the presence of dissolved oxygen and low concentrations of complexing organics).

Introduction

Humic substances are ubiquitous in soils, sediments, and natural waters. Humics are a complex mixture of organic compounds and may profoundly affect physical, chemical, and biological reactions in the subsurface (*1*−*6*). They contain both electron-rich and electron-deficient sites; these structural features are believed to be largely responsible for the electron-donating and electron-accepting properties that make humics redox-active and capable of participating in electron-transfer reactions involving redox-sensitive metals such as Fe(III), U(VI), and Cr(VI) (*3*, *7*−*10*). Such redox properties of humics have recently drawn considerable interest because humic materials may facilitate and therefore enhance the electron transfer from microbial metabolisms to contaminant metals or radionuclides that are known to exist at many U.S. Department of Energy (DOE) contaminated sites. Of particular interest is the possibility of humic-mediated bioreduction of contaminants such as U(VI) to less soluble or mobile forms such as uraninite (UO_2) phases. Previous studies have demonstrated that humic substances can act as electron shuttles by accepting electrons from microbial metabolites and then donating them to redox-sensitive metals or metal oxides such as Fe(III) and Fe(III) oxides (*3*, *7*−*13*). *Shawanella alga*, *Geobacter metallireducens*, and *Geobacteraceae* have all been shown to be able to use humics as the terminal electron acceptor and then donate electrons to redox-sensitive contaminant metals. This process regenerates humics to an oxidized form, which can again accept electrons from humic-reducing bacteria. As a result, the reduction rates of these contaminant metals could be enhanced in the presence of humic substances.

However, it is recognized that most of these studies were performed in laboratory batch experiments; the impact of humic materials on the biological reduction and immobilization of uranium under field conditions has not been investigated. In particular, recent studies revealed that the bioreduction of U(VI) could be significantly hindered by the presence of calcium (*14*), toxic metals (such as Ni^{2+}), and nitrate (*15*−*17*) or other electron acceptors such as Fe(III) and Mn(IV) oxides (*18*, *19*). It has been shown that, even in the presence of 0.45 mM Ca^{2+}, the bioreduction rates of U(VI) could be significantly reduced (*14*) because of the formation of calcium−uranyl−carbonate complexes (*20*). Although few studies to date have examined the direct inhibitory effect of Ni^{2+} on U(VI) bioreduction, Ni^{2+} ions are known to be toxic to microorganisms (*21*) and thus are expected to inhibit U(VI) bioreduction as well. The presence of humic substances may play a significant role under these circumstances, and we hypothesized that the addition of humics could increase the bioreduction rates of U(VI) because humics are known to form complexes with metal ions such as Ca^{2+} and Ni^{2+} (*5*, *22*). The formation of calcium− or nickel−humic complexes may prevent or decrease the formation of calcium−uranyl−carbonate complexes and alleviate the toxicity of Ni^{2+} by rendering it less bioavailable. Therefore, this study was aimed to evaluate how humic substances may impact the bioreduction of U(VI) and the oxidation of U(IV) in the presence or absence of Ca^{2+} and Ni^{2+} under varying humic concentrations. Field push−pull tests were performed to evaluate and to demonstrate the feasibility of using humic materials to enhance the bioreduction or immobilization of uranium in groundwater as part of a large-scale field investigation at the DOE Natural and Accelerated Bioremediation Research (NABIR) Field Research Center (FRC) in Oak Ridge, TN (*16*).

Materials and Methods

Humic Substances. Three humic samples were used for the present study: soil humic acid (or soil HA), obtained from the International Humic Substances Society (IHSS), and forest soil humic and fulvic acids (namely, FRC HA and FRC FA), isolated from the background surface soil at the DOE FRC site. The soil HA was used as received. The isolation and purification procedures for the FRC HA and FRC FA were similar to those described by IHSS with modifications (*10*). In brief, the FRC soil was extracted with 0.1 M NaOH under N_2 atmosphere, and the extract was acidified with HCl to a pH between 1 and 2 so that humic acid precipitated out. The supernatant (FRC FA) was collected after centrifugation and

* Corresponding author phone: 865-574-7286; fax: 865-576-8543; e-mail: gub1@ornl.gov.

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concentrated using a Speed-Vac concentrator. It was further purified by passing it through a cation-exchange resin (H^+ form, Dowex HCR-W2) to remove metal ions. The precipitated FRC HA was purified by redissolving it in 0.1 M NaOH and then equilibrating with 6 M HCl overnight. After the supernatant solution was decanted, the HA precipitates were washed with 0.1 M HCl/0.3 M HF solution in a plastic container overnight at room temperature. These steps are necessary to dissolve and remove residual complexed metal and aluminosilicates in the FRC HA sample. Finally, both FRC HA and FRC FA were freeze-dried and stored in a desiccator before use.

Laboratory Microcosm Studies of U(VI) Reduction. Biological reduction of U(VI) was performed in the presence of *Shewanella putrefaciens* CN32 bacterial strain in a 30 mM $NaHCO_3$ buffer solution supplemented with 10 mM sodium lactate, as reported previously (*9*). This bacterial strain was selected because it is capable of using humic substances as either electron acceptors or donors (*8*−*10*, *12*). The cells were routinely cultured aerobically in trypticase soy broth without dextrose at ambient temperature (~22 °C), and the stock cultures were maintained by freezing in 40% glycerol at −70 °C. Bacterial cells used for the inoculation were harvested from a 16-h culture suspension by centrifugation at 4000 rcf for 20 min at 4 °C. The cells were washed three times in the bicarbonate buffer solution, which had been previously purged with an 80% N_2 and 20% CO_2 mixture for about 30 min to give a final pH of ~7.5. After centrifugation, the cells were resuspended in the buffer solution and used immediately for the U(VI) bioreduction experiment. Fresh cells were prepared for every batch of the experiment, and the cell density was estimated by direct counting using 4′,6-diamidino-2-phenylindole staining and epifluorescence microscopy (*10*).

The bioreduction experiment was then initiated by transferring the washed cell suspension into acid-washed glass pressure tubes containing various reactant solutions in the bicarbonate buffer in an anaerobic glovebox (with ~97.5% N_2 and ~2.5% H_2). The final cell concentrations in the reactant solution ranged from 1.25×10^8 to 2.5×10^8 cells/mL. The initial added U(VI) concentration was ~0.4 mM (prepared from a uranyl acetate stock solution) and kept constant throughout the experiment. In a separate experiment, uranyl nitrate was used in order to study the effect of nitrate on the rate of U(VI) reduction. Humic materials, including soil HA, FRC HA, and FRC FA, were added in order to evaluate their effects on enhancing U(VI) bioreduction, particularly in the presence of Ca^{2+} or Ni^{2+} ions (*14*, *15*). The added Ca^{2+} and Ni^{2+} concentrations were 0.5 and 0.2 mM, respectively, as commonly observed at contaminated DOE sites (*23*). Three humic materials at concentration levels of 10 and 100 mg C/L were used because our previous studies have shown that different fractions of humic substances exhibit different enhancement effects because of their differences in chemical and structural characteristics (*9*). Total sample volume was 20 mL, and each sample tube was closed with a thick butyl rubber stopper and crimp-sealed with an aluminum cap. Duplicate samples were prepared and equilibrated on a rotary shaker in the glovebox at ambient temperature. At preselected time intervals during the reaction, a 0.5-mL aliquot of sample was taken with an 1-mL syringe, and a subsample (0.1 mL) was diluted with 10 mL of deoxygenated phosphoric acid (10%) for the phosphorescence analysis of remaining hexavalent U(VI). The sample was not filtered because the reduced U(IV) does not interfere with the analysis of U(VI), as described previously (*9*). Control samples consisting of identical treatments but without Ca^{2+}, Ni^{2+}, or humic materials were prepared and analyzed along with the treated samples. Additionally, selected samples in the presence of humics (without Ca^{2+} or Ni^{2+}) were filtered through a 0.2-μm syringe filter to remove bacterial cells and precipitated U(IV) solids, if any. These filtered samples were removed from the anaerobic chamber and subjected to reoxidation to determine if the reduced U(IV) may form complexes with humics that are soluble and thus remain in the filtrate samples.

TABLE 1. Geochemical Properties of the Groundwater from DP-01 and GW835 Wells

parameter	DP-01	GW835
pH	6.6	6.4
uranium (μM)	0.3	4.9
calcium (mM)	4.3	3.5
magnesium (mM)	1.0	1.1
iron (μM)	4.4	4.5
potassium (μM)	48	124
nitrate (mM)	1.5	1.2
sulfate (mM)	0.8	0.8
chloride (mM)	0.9	0.6

[a] DP-01 was used for field push−pull tests, whereas the amended GW835 groundwater (~200 L) was used as a source of injection water.

The steady-state phosphorescence technique was used to determine the U(VI) concentration in samples (*9*). The emission spectra were collected from 482 to 555 nm with an excitation wavelength of 280 nm using a Fluorolog-3 fluorescence spectrometer (Johin-Yvon-SPEX Instruments). The measured peak intensity at about 515 nm is directly proportional to the amount of U(VI) in solution. The detection limit is about 10^{-7} M U(VI).

Reoxidation of Reduced U(IV) in Laboratory Batch Studies. Bioreduced U(IV) was prepared in a similar manner as described above without the addition of Ca^{2+} or Ni^{2+} ions. A relatively high cell concentration (2.5×10^8 cells/mL) and a reaction time of ~72 h were used to ensure a nearly complete reduction of added U(VI). In the absence of humic materials, the reduced U(IV) forms precipitates and can thus be readily separated by carefully decanting the supernatant solution in an anaerobic chamber. The reduced U(IV) precipitates were then transferred out of the glovebox for oxidation studies in either MQ water or 0.03 M $NaHCO_3$ with or without addition of humics. Samples were stirred rigorously in a plastic bottle under oxic conditions (open to air) and sampled at various time intervals to determine the oxidized U(VI) concentration. Additionally, experiments were performed using bioreduced U(IV) in the presence of either FRC HA or FRC FA [with no observed U(IV) precipitates] in order to evaluate the effect of U(IV) complexation with humics on its reoxidation rates. In this case, the humic−U(IV) solution, after incubation, was directly subjected to reoxidation by stirring the sample aerobically, as described earlier.

Field Push−Pull Tests. To further evaluate the effect of humic substances on the rate of U(VI) bioreduction, in situ field push−pull tests (*15*, *16*, *24*) were performed in a shallow monitoring well (DP-01) at the contaminated FRC site. The method involved the injection of amended groundwater, followed by the extraction and analysis of the U(VI) concentration, tracers, and other analytes of interest, as described previously (*11*). Contaminated groundwater from monitoring well GW835 was used as a source injection water, which was supplemented with 80 mM ethanol (as an electron donor), 10 mM $NaHCO_3$ (as a buffer), 1.25 mM NaBr (as a tracer), and 100 mg/L (or ~50 mg C/L) FRC HA. Table 1 lists the general geochemical characteristics of the groundwater from both DP-01 and GW835 wells. Injection volumes were ~200 L, and injected solutions interrogated an aquifer volume of ~800 L. After injection, groundwater samples were collected periodically and analyzed for Br^- and U(VI). Control tests were also performed in the absence of ethanol or FRC HA

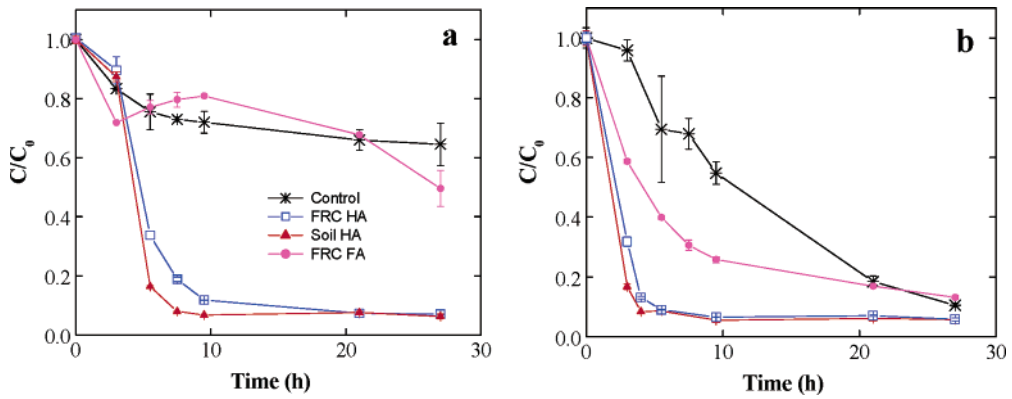

FIGURE 1. Effects of humic substances on the rate of U(VI) bioreduction at CN32 cell concentrations of (a) 1.25 × 10^8 and (b) 2.5 × 10^8 mL^{-1}. The added humic concentration was 100 mg C/L.

TABLE 2. Initial Reduction Rates, *k* (h^{-1}), of U(VI) in the Presence or Absence (Control) of Three Humic Materials at Microbial Cell Concentrations of 1.25 × 10^8 and 2.5 × 10^8 mL^{-1} [a]

cells/mL	control	soil HA	FRC HA	FRC FA
1.25 × 10^8	0.026	0.308	0.210	0.025
2.5 × 10^8	0.079	0.611	0.449	0.153

[a] The estimated *k* values have a ±10% uncertainty.

in adjacent monitoring wells or in the same wells in a series of tests conducted over an approximately 6−9-month period.

Results and Discussion

Enhanced U(VI) Bioreduction by Humic Substances. The effect of humic addition on the reduction of U(VI) by *S. putrefaciens* CN32 was first investigated in laboratory studies and is presented in Figure 1. The initial rates of U(VI) reduction were calculated by assuming a pseudo-first-order reaction. Note that a consistently low percentage of residual U(VI) (~5−10%) appeared to remain in the solution phase even after an extended period of reaction in the presence of soil HA and FRC HA (Figure 1). These data points were excluded in calculating the initial reduction rates and were partially explained by the fact that U(VI) was analyzed directly by diluting the unfiltered, whole cell suspension [containing both reduced U(IV) and oxidized U(VI)] in 10% phosphoric acid, as opposed to analysis using filtered samples [to remove precipitated U(IV) solids (*14*, *25*)]. A small percentage of U(IV) could have been reoxidized to U(VI) in the process, because U(IV) is known to be readily oxidized, as will be discussed in detail later. Nonetheless, results indicate that the initial rate of U(VI) reduction was greatly increased in the presence of humics, particularly humic acid (both soil HA and FRC HA) (Table 2). In comparison with the controls (no humics added), the rate increase was nearly 10-fold at a cell concentration of about 1.25 × 10^8 mL^{-1} when soil HA and FRC HA were added. However, the FRC FA showed no significant impact with regard to increasing the rate of U(VI) bioreduction. At a higher cell concentration (2.5 × 10^8 mL^{-1}), U(VI) bioreduction rates also increased about 6−8-fold with the addition of the FRC HA or soil HA, whereas the addition of FRC FA nearly doubled the initial reduction rate. These observations confirm our previous findings that humic substances could play a significant role in enhancing the bioreduction of U(VI), perhaps by acting as electron shuttles or mediators between U(VI) and microbes (*9*). Similarly, other investigators reported that humic substances could enhance the electron-transfer reactions and therefore the bioreduction of Fe(III) or iron oxides by a variety of microorganisms (*3*, *8*, *10*, *12*, *26*). Fredrickson et al. observed that anthraquinone-2,6-disulfonate (AQDS), an analogue of humic substances, could also act as a biological electron shuttle; the bacterially reduced AQDS was found to rapidly reduce U(VI) in the absence of cells (*27*). The fact that U(VI) bioreduction rates varied with different humic materials (e.g., HA versus FA) provided additional evidence that humics may play an important role in such electron-transfer reactions (*9*). As shown in Figure 1 and Table 2, HA (both soil HA and FRC HA) was much more effective than FRC FA in enhancing the reduction of U(VI) by *S. putrefaciens* CN32, and this effect likely can be attributed to its relatively large molecular size and polycondensed aromatic structural features, as reported previously (*9*, *10*). These structural components of HA (such as quinone and hydroquinone moieties) are thought to be particularly important in mediating the electron-transfer reactions because of their capability to either accept or donate electrons. On the other hand, both ^{13}C NMR and FTIR spectroscopic analyses indicate that FA usually contains relatively low molecular weight organic components and low proportions of aromatic compounds compared with HA (*28*). Similar observations have been reported previously in studies of the effect of humics in the microbial reduction of Fe(III) and U(VI) (*9*, *10*). The bioreduction rates of Fe(III) were reported to be nearly an order of magnitude higher in the presence of soil HA than in the presence of two FA fractions consisting of primarily low molecular weight polyphenolic and carbohydrate organic moieties. Humic substances of aquatic origin were also reported to show lower Fe(III)-bioreduction capacity than humic acids obtained from sediments or soils, because these soil humic materials are likely to contain higher molecular weight organic moieties than those from an aquatic environment (*29*).

The reduction rates of U(VI) reported in this study appeared to be about 2−6 times faster than those reported previously (*9*), partly because relatively higher cell concentrations (1.25 to 2.5 × 10^8 mL^{-1}) were used in the present study than in the previous study (1 × 10^8 mL^{-1}). More important, perhaps, is that uranyl acetate was used in the present study because nitrate (used as uranyl nitrate) is believed to have inhibited the bioreduction of U(VI) in our previous studies (*9*). Indeed, the bioreduction of U(VI) occurred quickly when uranyl acetate was used (Figure 1), as opposed to a significantly delayed reduction of U(VI) when uranyl nitrate was used in the reaction. This observation has been attributed to the preferential utilization of nitrate prior to the onset of U(VI) reduction. Senko et al. (*15*) reported similar inhibitory effects of nitrate and denitrification intermediates on U(VI) bioreduction. In addition, they found that some denitrification intermediates were effective at oxidizing U(IV).

However, the reduction rates of U(VI) observed in this study (in the absence of humics) were slower than those

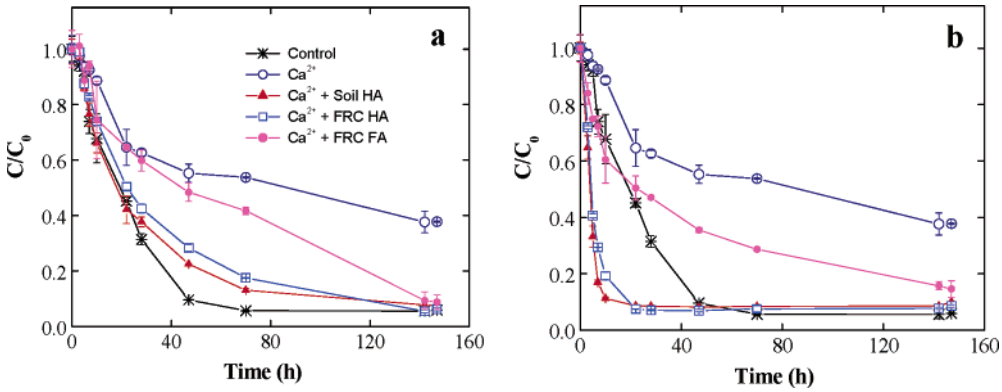

FIGURE 2. U(VI) bioreduction is inhibited by Ca^{2+} ions (0.5 mM) but enhanced by the addition of humic substances at concentrations of (a) 10 and (b) 100 mg C/L, respectively. The CN32 cell concentration was ~1.5 × 10^8 mL^{-1}. The control (in the absence of Ca^{2+} and humics) and the Ca^{2+}-only treatments were plotted in both figures for comparison.

TABLE 3. Effects of Ca^{2+}, Ni^{2+}, and the Addition of Humic Materials on the Initial Reduction Rates, *k* (h^{-1}), of U(VI) at Microbial Cell Concentrations of 1.5 × 10^8 mL^{-1} [a]

treatment	*k*, h^{-1}	treatment	*k*, h^{-1}
Ca^{2+} only	0.017	Ni^{2+} only	0.001
Ca^{2+} + low soil HA	0.031	Ni^{2+} + low soil HA	0.002
Ca^{2+} + low FRC HA	0.026	Ni^{2+} + low FRC HA	0.003
Ca^{2+} + low FRC FA	0.017	Ni^{2+} + low FRC FA	0.001
Ca^{2+} + high soil HA	0.224	Ni^{2+} + high soil HA	0.008
Ca^{2+} + high FRC HA	0.167	Ni^{2+} + high FRC HA	0.010
Ca^{2+} + high FRC FA	0.031	Ni^{2+} + high FRC FA	0.005
no Ca^{2+} or humics	0.041	no Ni^{2+} or humics	0.037

[a] Two humic concentration levels were used (low = 10 mg C/L, and high = 100 mg C/L). The estimated *k* values have a ±10% uncertainty.

reported by Liu et al. (*25*), who found that a nearly complete reduction of U(VI) occurred in about 5 h in the presence of 2.5 × 10^9 CN32 cells mL^{-1} at 30 °C (with lactate as an electron donor). This discrepancy may be largely explained by the fact that different experimental conditions (e.g., cell concentration and temperature) were used. The cell concentration used in this study was more than an order of magnitude lower than that used by Liu et al. Additionally, a relatively low incubation temperature (~22 °C) was used in this study, and it may have played a significant role in these reactions. For example, at 30 °C, Brooks et al. (*14*) reported that a nearly complete reduction of U(VI) occurred in about 25 h in the presence of ~(5−8) × 10^7 CN32 cells mL^{-1}. In the study of U(VI) reduction from various organic complexes, Ganesh et al. (*30*) observed that a large percentage of U(VI) could be reduced in ~5 to >60 h by *Desulfovibrio desulfuricans* (~5 × 10^8 cells mL^{-1}) at 32 °C.

Inhibitory Effect of Ca^{2+} and Ni^{2+} and the Role of Humics on U(VI) Bioreduction. The presence of Ca^{2+} or Ni^{2+} ions in solution has been reported to greatly inhibit the bioreduction of U(VI) because of (a) the potential formation of calcium−uranyl−carbonate [$Ca_2UO_2(CO_3)_3$] complexes and (b) the toxicity of Ni^{2+} ions to microorganisms (*14*−*16*). Indeed, laboratory studies indicate that the addition of only 0.5 mM of Ca^{2+} caused a more than 2-fold decrease in the initial rate of U(VI) reduction (Figure 2 and Table 3). The inhibitory effect of Ni^{2+} was even more pronounced with the addition of as little as 0.2 mM Ni^{2+}. It essentially completely inhibited U(VI) reduction, with an estimated reduction rate of only ~0.001 h^{-1} (Figure 3 and Table 3).

However, the addition of humic materials appeared to alleviate the inhibitory effect of Ca^{2+} or Ni^{2+} and thereby increase the rates of U(VI) reduction compared with those observed in the absence of humics (Figures 2 and 3). U(VI) reduction rates also increased as the concentration of added humics increased (Table 3). In the presence of Ca^{2+} and soil HA or FRC HA (10 mg C/L), the reduction rates of U(VI) nearly doubled compared with rates in the presence of (Ca^{2+} + FRC FA) or Ca^{2+} only. Ni^{2+} ions appeared to be particularly inhibitory toward U(VI) reduction; despite the addition of humics (10 mg C/L), the reduction rates of U(VI) were more than an order of magnitude lower than the ones without Ni^{2+} or even with Ca^{2+} added (Table 3). However, compared with those with (Ni^{2+} + FRC FA) or Ni^{2+} added only, the addition of soil HA or FRC HA (10 mg C/L) still doubled or tripled the U(VI) reduction rates. At a high humic concentration (100 mg C/L) and in the presence of Ca^{2+}, the addition of soil HA and FRC HA not only completely restored but also exceeded the U(VI) reduction rate in the absence of Ca^{2+} (0.041 h^{-1}, as a control) (Figure 2b and Table 3). This rate increase was about 4−5 times greater than the control (without Ca^{2+} or humics added). When Ni^{2+} is present, even the addition of a high concentration of humics did not fully recover the reduction rate of U(VI) seen in the absence of Ni^{2+} or humics (0.037 h^{-1}) (Figure 3b). On the other hand, compared with the reduction rate with only Ni^{2+} added, the use of these humic materials increased the reduction rate by about 5−10-fold (Table 3), and even the FRC FA significantly increased the U(VI) reduction rate (Figure 3b).

These observations again suggest that humic substances (especially soil HA and FRC HA) could increase U(VI) bioreduction rates because humic materials may act either as electron shuttles to facilitate electron-transfer reactions or as complexing agents to prevent the formation of $Ca_2UO_2(CO_3)_3$ complexes and to alleviate the toxic effect of Ni^{2+} upon microorganisms. This conclusion was supported by the fact that calcium itself has no known toxic effect, and its inhibitory effect is likely not caused by direct interaction with the microbial cells at the 0.5 mM concentration (*14*). On the other hand, the formation of $Ca_2UO_2(CO_3)_3$ complexes is believed to be largely responsible for the inhibitory effect of Ca^{2+}, because $Ca_2UO_2(CO_3)_3$ complexes are less energetically favorable electron acceptors than free uranyl or uranyl−carbonate ions (*14*). The fact that the addition of soil HA and FRC HA completely restored the reduction rates of U(VI) in the presence of Ca^{2+} could thus be attributed to the complexation reactions of HA with Ca^{2+} ions (*31*, *32*) that inhibited the formation of $Ca_2UO_2(CO_3)_3$ complexes. No data are currently available with respect to the potential formation of $Ni_2UO_2(CO_3)_3$ complexes, but it is unlikely to be a dominant mechanism for observed decreases in U(VI) reduction rates by Ni^{2+}. This argument is based on the fact that Ni^{2+} is known to be toxic to microorganisms (*21*), and the inhibitory effect of Ni^{2+} was much more pronounced (or about an order of magnitude more) than that of Ca^{2+}, even though the added

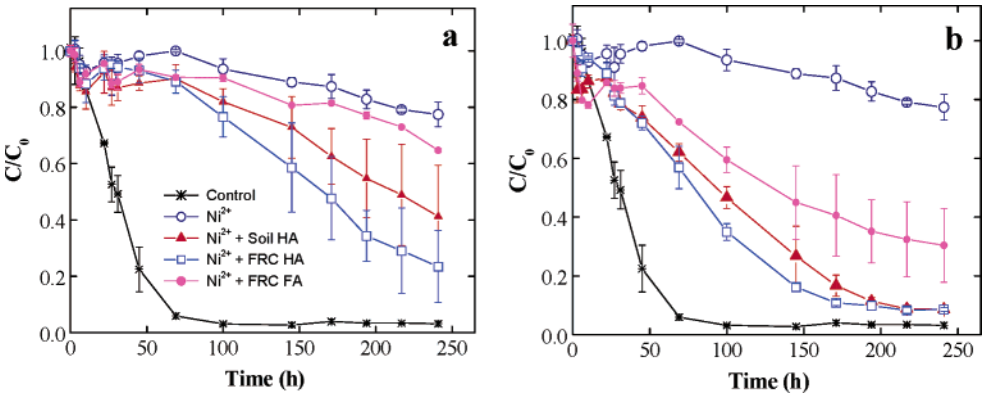

FIGURE 3. U(VI) bioreduction is inhibited by Ni^{2+} ions (0.2 mM) but enhanced by the addition of humic substances at concentrations of (a) 10 and (b) 100 mg C/L, respectively. The CN32 cell concentration was ~1.5×10^8 mL^{-1}. The control (in the absence of Ni^{2+} and humics) and the Ni^{2+}-only treatments were plotted in both figures for comparison.

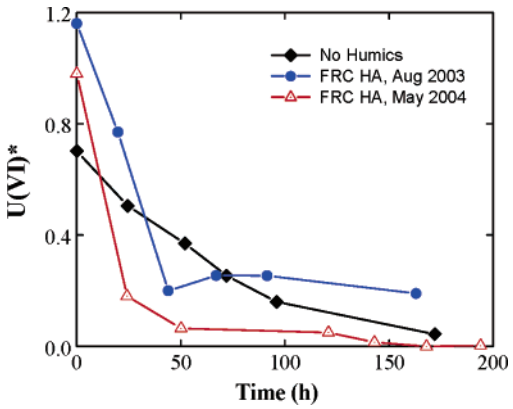

FIGURE 4. Field push−pull tests at the FRC site confirm that the addition of FRC HA enhanced the initial rates of U(VI) reduction or removal. Note that U(VI)* is the dilution-adjusted U(VI) concentration normalized by the injection concentration (*16, 24*).

concentration of Ni^{2+} was only two-fifths that of Ca^{2+} (Table 3). Because humics are known to form stable complexes with Ni^{2+}, they made Ni^{2+} less bioavailable (*5*, *22*), thus alleviating the toxicity of Ni^{2+} to microbes and increasing the bioreduction rate of U(VI). Even FRC FA appeared to be quite effective in increasing the U(VI) bioreduction rate compared with the rate with only Ni^{2+} added (Figure 3b); this is likely a result of the formation of stable complexes between Ni^{2+} and the carboxylic and hydroxyl functional groups in fulvic acids (*5*).

Enhanced U(VI) Bioreduction by Humic Substances in Field Studies. The effect of humic addition on the reduction of U(VI) was further evaluated in field push−pull tests at the FRC (*15*, *16*). The normalized and dilution-adjusted concentration of U(VI) decreased more rapidly within the first 2 days in the presence of FRC HA than in the absence of FRC HA (Figure 4). For the push−pull test performed in monitoring well DP-01, the initial U(VI) bioreduction rate increased nearly 10-fold; results were also consistent with duplicate experiments performed about 9 months apart in the same well. This decreased U(VI) concentration in groundwater may be partially attributed to an increased rate of bioreduction or precipitation of U(IV), although processes such as chemical precipitation and retention of U(VI) in soil could not be ruled out. These limited data support the hypothesis that humic substances may act either as electron shuttles or complexing agents for metals and thereby increase the bioreduction of U(VI) in soil and groundwater. Because of their small size (relative to microbial cells), humics could potentially mediate the transfer of the microbial reducing power to toxic metals at such locations where microorganisms are excluded because of size or nutrient limitations (*9*, *10*, *12*, *33*). Our work demonstrates that humic substances could enhance the retention and immobilization of uranium in the FRC soil, although the exact mechanisms of U(VI) removal in groundwater with the addition of humics and electron donors are yet to be determined.

Reoxidation of U(IV) and the Effect of Humics. The reduced U(IV) species are known to be susceptible to oxidation when exposed to oxygen (*34*, *35*) or nitrate and several dentrification intermediates (*15*); however, the effects of humics on the reoxidation rates of U(IV) have not been studied. It is interesting that, although humic substances were found to greatly increase the bioreduction rates of U(VI), as discussed earlier, they were also found to increase the rate of U(IV) reoxidation when humics were present during the bioreduction−reoxidation process (Figure 5a). The calculated rates of U(IV) reoxidation in the presence of humics were about 10−30 h^{-1} if a pseudo-first-order oxidation process is assumed. This is equivalent to an oxidation half-life of less than 5 min. On the other hand, the reoxidation rates in water or in 0.03 M $NaHCO_3$ solution were ~1 h^{-1} with a half-life on the order of ~40 min. This oxidation rate of bioreduced U(IV) was about 1−2 orders of magnitude higher than the bioreduction rates of U(VI) (Tables 2 and 3). As has been suggested by other investigators (*15*, *16*, *34*), it is therefore essential to maintain a strict anaerobic condition in order to stabilize the reduced U(IV) and thus prevent it from reoxidation or solubilization (as uranyl carbonates).

These observations have significant implications because added humics increased rates of both U(VI) bioreduction and U(IV) oxidation. Two possible mechanisms could be responsible for humic-enhanced U(IV) oxidation. First, the reduced U(IV) may have formed complexes with humics during the bioreduction process; it is such complexation reactions that could have prevented the precipitation of the reduced U(IV) and thus increased the rate of U(IV) reoxidation [as compared with the reoxidation of U(IV) solids in water or in 0.03 M $NaHCO_3$] (Figure 5a). This conclusion is supported by the fact that, if humics were added separately to reduced U(IV) solids (obtained by the reduction of U(VI) without humics), the oxidation rates of U(IV) were comparable with those added with water or 0.03 M $NaHCO_3$ (Figure 5b). In this case, the reduced U(IV) and humics were present in separate phases (not complexed initially), and the U(IV) oxidation rate was thus limited by the solubilization of U(IV) solids in the presence of oxygen and carbonates (open to atmosphere). The overall oxidation reaction may be written as (*35*, *36*):

$$UO_2(s) + {}^1/_2O_2 + 2H^+ \rightarrow UO_2^{2+} + H_2O$$

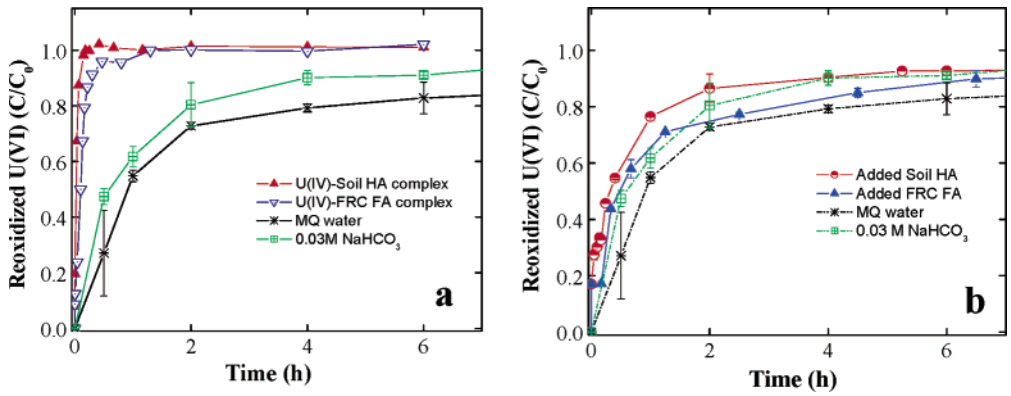

FIGURE 5. Reoxidation kinetics of bioreduced U(IV) in the presence of humics or in 0.03 M $NaHCO_3$ and water. (a) Reduced U(IV) was complexed with humics, obtained by the reduction of U(VI) in the presence of FRC HA or FRC FA; (b) FRC HA or FRC FA were added to U(IV) precipitates, obtained by the reduction of U(VI) in the absence of humics.

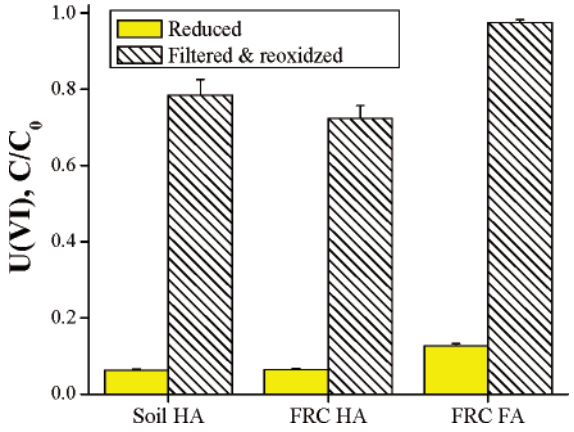

FIGURE 6. After the bioreduction (from Figure 1b), more than 70% of reduced U(IV) in the presence of humics readily passed through 0.2-μm syringe filters and was oxidized to U(VI) in open air.

The formation of U(IV)−humic complexes during bioreduction was evidenced by the fact that >70% of the reduced U(IV) could readily pass through the 0.2-μm syringe filter (or no precipitates formed in the presence of humics), and the U(VI) concentration increased with time and reached its initial concentration when the filtered samples were exposed to air (Figure 6). Such complexation reactions are presumed to occur through the coordination between reduced U(IV) and chelating functional groups of humics such as carboxyls, hydroxyls, and ketones. Using extended X-ray absorption fine-structure spectroscopic analysis, Francis and his colleagues (*37*, *38*) provided direct evidence of the formation of soluble biligand mononuclear U(IV)−citrate complexes—U(IV) formed 8-fold coordination with carboxyl functional groups of citric acid. Similarly, in a study of the reduction of U(VI) from organic complexes by sulfate- and iron-reducing bacteria, Ganesh et al. reported that less than 10−20% of the U(IV) formed precipitates in the presence of organic ligands such as tiron, oxalate, and citrate, which contain carboxyl and hydroxyl chelating functional groups as found in humic substances.

Humic substances can function both as electron donors (during reduction) and electron acceptors (during oxidation), thus increasing the oxidation rate of the complexed U(IV) when it is exposed to oxygen. As stated earlier, humics are naturally occurring, complex mixtures of organic compounds that contain both electron-rich (or electron-donating) and electron-deficient (electron-accepting) moieties, making them redox active (*1*, *9*, *10*, *39*−*43*). The redox potential of each individual organic compound in humics may vary greatly. The reported apparent redox potential of humics ranged from −0.9 up to +0.8 V (*4*, *6*, *43*−*46*). The standard redox potential of U(VI)/U(IV) is well within this range (*34*). In other words, some fractions of humics may act as electron donors during bioreduction under strict anaerobic conditions, whereas other humic components may function actively as electron acceptors during oxidation reactions. This statement is also supported by the fact that humic acids (both FRC HA and soil HA) were found to be much more effective in enhancing the bioreduction of U(VI) than the FRC FA (Figures 1−3 and Tables 2 and 3). This is not surprising because different humic fractions possess different structural features that affect their redox potential and capability in reducing metals such as U(VI) and Fe(III) (*9*, *10*, *28*). In fact, previous studies have also shown that humics, on one hand, could significantly enhance the reduction rate of ferric Fe(III) under anaerobic conditions (*3*, *7*, *10*, *12*), but on the other hand, humics greatly increase the oxidation rate of ferrous Fe(II) when it is complexed with humics under oxic conditions (*47*).

This work thus demonstrates that humics may play dual functional roles in facilitating or enhancing the bioreduction of U(VI) and in increasing the solubilization and reoxidation of U(IV), depending on site-specific geochemical conditions. Although humics could enhance the bioreduction of U(IV) under strict anaerobic conditions, particularly in the presence of Ca^{2+} and Ni^{2+} ions, they can form complexes with reduced U(IV), rendering it soluble and potentially mobile in soil. Moreover, once exposed to oxygen, the humic-complexed U(IV) is readily reoxidized with a half-life on the order of only a few minutes to hours. However, it is pointed out that humic concentrations used in this study (10−100 mg C/L) were much higher than are commonly found in natural soil pore water or groundwater. Whether relatively low concentrations of humic materials might have a similar impact on the complexation, reduction, and oxidation reactions of uranium is yet to be investigated. It is important to realize that, because humic substances occur in soil and groundwater ubiquitously, they present a potential challenge to stabilizing reduced U(IV) in soil. The complexation between U(IV) and humics (as chelates) could occur at low concentrations, depending on the stability of the complexation reaction. Therefore, further studies are warranted, and they must address the long-term stability and retention of reduced U(IV) under realistic field conditions. An improved understanding of the functional roles of humics in soil and groundwater may directly benefit the application of microbially mediated uranium immobilization and stabilization as a remediation strategy at contaminated sites.

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