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Enhanced microbial reduction of Cr(VI) and U(VI) by different natural organic matter fractions

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Abstract—Although direct microbial reduction of Cr(VI) and U(VI) is known, few studies have examined the kinetics and the underlying mechanisms of the reduction of these contaminants by different natural organic matter (NOM) fractions in the presence or absence of microorganisms. In this study, NOM was found to chemically reduce Cr(VI) at pH 3, but the reduction rates were negligible at pH ~7. The abiotic reduction of U(VI) by NOM was not observed, possibly because of the presence of small amounts of nitrate in the reactant solution. However, all NOM fractions, particularly the soil humic acid (HA), enhanced the bioreduction of Cr(VI) or U(VI) in the presence of *Shewanella putrefaciens* CN32. The reduction rates varied greatly among NOM fractions with different chemical and structural properties: the polyphenolic-rich NOM-PP fraction appeared to be the most reactive in abiotically reducing Cr(VI) at a low pH, but soil HA was more effective in mediating the microbial reduction of Cr(VI) and U(VI) under anaerobic, circumneutral pH conditions. These observations are attributed to an increased solubility and conformational changes of the soil HA with pH and, more importantly, its relatively high contents of polycondensed and conjugated aromatic organic moieties. An important implication of this study is that, depending on chemical and structural properties, different NOM components may play different roles in enhancing the bioreduction of Cr(VI) and U(VI) by microorganisms. Polycondensed aromatic humic materials may be particularly useful in mediating the bioreduction and rapid immobilization of these contaminant metals in soil. *Copyright © 2003 Elsevier Ltd*

1. INTRODUCTION

Natural organic matter (NOM), or humic substances, are known to be redox reactive and therefore capable of reducing redox-sensitive metals such as Fe(III), Cr(VI), Mn(IV), V(V), and Hg(II) (Szilagyi, 1971; Alberts et al., 1974; Sunda and Kieber, 1994; Wittbrodt and Palmer, 1995; Lu et al., 1998; Nakayasu et al., 1999). More recently, NOM was also found to greatly enhance the reduction of Fe(III) metals or metal oxides by a variety of microorganisms (Lovley, 1996; Benz et al., 1998; Coates et al., 1998; Fredrickson et al., 1998; Lovley et al., 1998; Royer et al., 2002b; Chen et al., 2003a). Lovley (1996) postulated that humic substances were likely acting as electron mediators or shuttles between microorganisms and Fe(III) or Fe(III)-oxide minerals. They found that certain anaerobic microorganisms could reduce NOM (as an electron acceptor), which then donates electrons to reduce Fe(III) or Fe(III)-containing minerals to release soluble Fe^{2+}. Iron-reducing microorganisms such as *S. putrefaciens, G. metallireducens, S. alga*, and a variety of fermenting bacteria have all been shown to use humic substances as terminal electron acceptors. By incubating NOM with *S. putrefaciens*, the equivalent Fe(III)-reducing capacity of NOM was reported to range from 0.1 to 0.6 mol/kg (Royer et al., 2002a; Chen et al., 2003a).

An important implication of these studies is the effect of NOM on the potential transport of the metals by either increasing or decreasing their redox states and solubility and thereby causing their mobilization or immobilization in the subsurface soil (Truex et al., 1997; Lovley et al., 1998; Fredrickson et al., 2000; Wildung et al., 2000). For example, under oxic condi-

tions, chromium and uranium are commonly present as CrO_4^{2-} and $UO_2(CO_3)_2^{2-}$ oxyanions (with CO_2 or carbonates) in the environment. These oxidized forms of Cr(VI) and U(VI) are soluble and highly mobile in groundwater because they are poorly sorbed by soil minerals carrying a negative surface charge. On the other hand, the reduced forms of Cr(III) and U(IV) are only sparingly soluble and are strongly sorbed by soil sediments (Puls et al., 1999; Gu et al., 2002). Therefore, of particular interest is the possibility that NOM-mediated reduction of Cr(VI) and U(VI) may lead to more rapid immobilization of these metals in soil and thus the remediation of a contaminated site. Because of the much smaller size of humic molecules as compared to the size of bacteria, humic substances could allow access to locations from which bacteria are excluded due to size or nutrient limitations and therefore transfer the microbial reducing power to contaminants at such isolated locations.

While many studies to date have focused on direct microbial reduction of Cr(VI) and U(VI) (Lovley et al., 1991; Shen et al., 1996; Chen and Hao, 1998; Abdelouas et al., 2000; Fredrickson et al., 2000), few studies have examined the effect of NOM on the enhanced microbial reduction of Cr(VI) or U(VI), as has been observed for the reduction of Fe(III) or Fe(III)-oxide minerals (Lovley, 1996; Royer et al., 2002b; Chen et al., 2003a). In particular, because of the complexity of NOM, the reaction mechanisms and functional groups that participate in metal reduction or electron-transfer reactions are largely unknown. Recent studies have pointed out that the behavior of heterogeneous bulk NOM is not representative of the functional roles of NOM subfractions, which may vary greatly in chemical and structural properties and thus in the reactivities in the natural environment (Gu et al., 1995; Chen et al., 2002). Our main objective in this study was to determine the reaction

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kinetics and effectiveness of various NOM components in the chemical and microbial reduction of Cr(VI) and U(VI) under varying experimental conditions. We used three NOM fractions with significant differences in molecular size, aromaticity, and other structural features for the reduction experiments. These studies allowed us to link the structural and functional properties and the underlying reaction mechanisms of NOM with these contaminant metals in the presence or absence of a specific strain of bacteria, *S. putrefaciens* CN32.

2. EXPERIMENTAL

2.1. Natural Organic Matter Fractions

The NOM samples used in this study include two subfractions (NOM-PP and NOM-CH) of a total aquatic NOM (hereinafter referred to as GT-NOM) obtained from a wetland pond and a soil humic acid (soil HA) obtained from the International Humic Substances Society (Gu et al., 1995; Chen et al., 2002). The reference soil HA has been studied extensively and was used to represent a high-molecular-weight NOM fraction that is rich in high-molecular-weight, polycondensed aromatic moieties (Senesi et al., 1991; Pullin and Cabaniss, 1995; Chen et al., 2002). The method of fractionation and purification of NOM-PP and NOM-CH has been described in detail elsewhere (Lowe, 1975; Chen et al., 2002). Briefly, this method fractionates the bulk NOM on the basis of its adsorptive behavior on a cross-linked polyvinyl pyrrolidone (PVP) polymer, where components rich in aromatic C=C moieties preferentially adsorb on PVP under acidic conditions, while organic components not adsorbed by PVP are comprised primarily of low-molecular-weight carbohydrates, proteins, amino acids, and uronic acids. Such a fractionation process selectively separates one group of organic compounds from the other and thus provides NOM subfractions with better-defined physical and chemical properties than the bulk NOM. After fractionation, both of the NOM fractions were purified to remove inorganic ions and were freeze-dried before use. The NOM-PP and NOM-CH were found to consist of ~70% and ~20%, respectively, of the bulk NOM. Detailed chemical and spectroscopic characterization of these NOM fractions was given previously (Chen et al., 2002, 2003a, 2003b).

2.2. Reduction of Cr(VI) and U(VI) by NOM

To evaluate the effect of NOM on the chemical reduction of Cr(VI) and U(VI), a series of batch kinetic experiments were performed under anaerobic conditions. For the reduction of Cr(VI), the reaction was initiated by mixing stock solutions of different NOM fractions with Cr(VI) (as K_2CrO_4) in an anaerobic chamber. Stock NOM solutions were prepared by dissolving the freeze-dried NOM fractions in purified Milli-Q water; their total organic carbon (TOC) concentrations were determined by means of a Shimadzu 5000A-TOC analyzer (Shimadzu Co, Japan). Both NOM and Cr(VI) stock solutions were first purged with high-purity N_2 (>99.99%) to remove dissolved oxygen and were left in an anaerobic chamber (97.5% N_2 and 2.5% H_2) for 2–3 d before the initiation of reactions. The pH of samples was adjusted to 3 with dilute HCl, and the final volume was made up to 20 mL in glass vials. The final concentrations of reactants were 0.2 mM Cr(VI) and 100 mg C/L of NOM, respectively. Samples were then agitated on a rotary shaker in the anaerobic chamber, and at specified time intervals, an aliquot of the sample was taken and analyzed immediately for the remaining oxidized form of Cr(VI). The Cr(VI) concentration was measured by a colorimetric method with diphenylcarbazide (DPC) by mixing a 0.5-mL sample with 0.1 mL of a DPC reagent (0.1% in acetone), then adding 2 mL of 0.05 M H_2SO_4 (Wittbrodt and Palmer, 1995, 1996). Ten min after the addition of DPC, the absorbance at 540 nm was measured by means of a Hewlett-Packard 8453 spectrophotometer (Hewlett-Packard, Wilmington, DE). Control samples without added NOM were also prepared and included in each batch of samples. In addition, NOM samples without addition of Cr(VI) were analyzed for background corrections.

For the reduction of U(VI), similar experiments were performed by mixing stock solutions of different NOM fractions with U(VI) [as $UO_2(NO_3)_2$] in 25-mL pressure tubes. The stock solutions of NOM fractions also were purged with N_2 and equilibrated in a glove box before addition of the stock U(VI) solution. The final concentrations of U(VI) and NOM were kept at 100 mg/L (or 0.42 mM UO_2^{2+}) and 100 mg C/L respectively for the kinetic experiment, and the volume was made up to 20 mL. At specified time intervals, an aliquot of a 0.1-mL sample was collected with a syringe, and the U(VI) concentration was measured by the steady-state phosphorescence technique, which is specific for the detection of hexavalent U(VI) because the reduced U(IV) species do not give fluorescence (Kaminski et al., 1981; Brina and Miller, 1992; Gu et al., 2002). In brief, the method involves the addition of 0.1 mL of sample solution into 3 mL of deoxygenated phosphoric acid (10%) in a quartz vial. Phosphoric acid is used to complex U(VI) and to enhance its phosphorescence for sensitive detection. The measured fluorescence intensity is directly proportional to the amount of U(VI) in solution, and the detection limit is better than 0.1 mg U(VI)/L. All measurements were performed with a Fluorolog-3 fluorescence spectrometer equipped with both excitation and emission monochromators (Johin-Yvon-SPEX instruments, New Jersey). A 450-W Xenon arc lamp was used as the excitation source, and the emission spectra were collected from 482 to 555 nm with an excitation wavelength of 280 nm. The peak emission at 515.4 nm was used for the calculation of U(VI) phosphorescence intensity or U(VI) concentration in solution.

2.3. Microbial Reduction of Cr(VI) and U(VI)

The effects of different NOM fractions on the microbial reduction of Cr(VI) and U(VI) were studied in the presence of *S. putrefaciens* strain CN32. This bacterial strain was selected because of its its capability of using humic substances as either electron acceptors or donors (Royer et al., 2002a; 2002b; Chen et al., 2003a). *S. putrefaciens* CN32 cells were initially provided by Dr. D. Boone at Portland State University from his Subsurface Microbial Culture Collection, Western Branch, and were routinely cultured aerobically in trypticase soy broth without dextrose at ambient temperature (~22°C). The stock cultures were maintained by freezing in 40% glycerol at −70°C in our laboratory.

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 a 50 mM 1,4-piperazinediethanesulfonic acid (PIPES) medium at pH 6.8 supplemented with 30 μM phosphate. The final wash was made with the same PIPES-phosphate buffer solution, except that the medium was purged with 97.5% N_2 and 2.5% H_2 to remove dissolved O_2 and kept under anaerobic conditions in a glove box. The cells were resuspended in the buffer solution and transferred to vials for their reactions with NOM and Cr(VI) in the anaerobic chamber. The cell density was estimated by the absorbance at 420 nm, and the cell numbers were calibrated by direct counting using 4′,6-diamidino-2-phenylindole staining and epifluorescence microscopy (Chen et al., 2003a).

The microbial reduction of Cr(VI) in the presence of NOM was then performed in acid-washed glass vials containing the PIPES buffer solution. A "nongrowth" condition (Royer et al., 2002b) or a near-constant cell concentration was maintained during the reaction by excluding assimilable-nitrogen sources using H_2 as the sole electron donor and Cr(VI) as the electron acceptor. Such experimental conditions allowed us to evaluate the effects of different NOM fractions as electron mediators on the reduction of Cr(VI). Washed bacterial cells were added to the reactor to obtain a final concentration of 10^7 cells/mL, as estimated by the relationship between the absorbance at 420 nm and direct counts of washed cell suspensions of the culture. The initial added NOM concentration ranged from 0 to 200 mg C/L for each NOM fraction, and the Cr(VI) concentration (as K_2CrO_4) was 0.2 mM. Samples were shaken gently in an anaerobic chamber at room temperature. At preselected time intervals during the reaction, a 0.5-mL aliquot of sample was taken and analyzed for remaining Cr(VI) by the DPC method as described earlier. Controls consisting of identical treatments either without microbial cells or without NOM fractions were evaluated along with samples. All experiments were carried out in triplicate.

Similar experiments were performed for the microbial reduction of U(VI). However, preliminary experiments indicated that the bioreduction of U(VI) under similar conditions used for Cr(VI) was slow, and
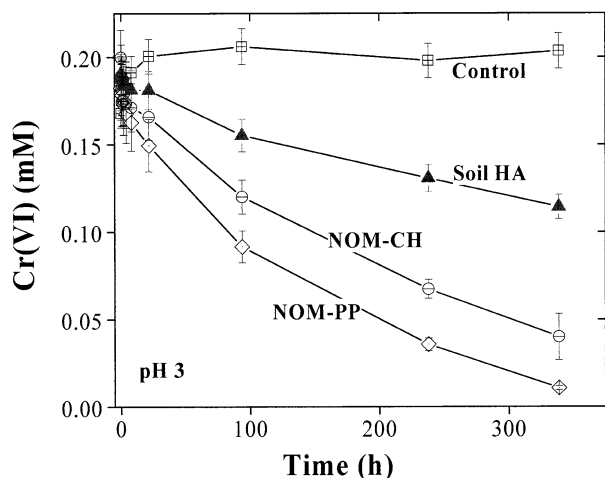
Fig. 1. Effects of NOM fractions on Cr(VI) reduction under abiotic conditions at pH 3. The initial NOM concentration was 100 mg C/L.
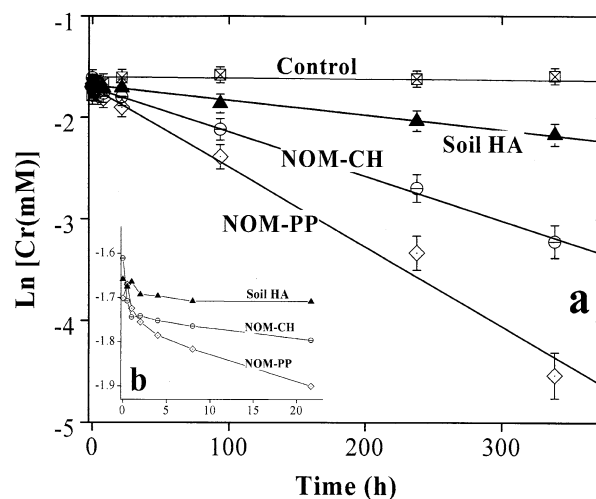

Fig. 2. (a) Logarithmic plot of Cr(VI) concentration against reaction time by three NOM fractions under abiotic conditions as shown in Figure 1 (pH 3). The inset (b) shows the reduction kinetics within the first 24 h. The initial NOM concentration was 100 mg C/L, and Cr(VI) concentration was 0.2 mM.

practically no U(VI) bioreduction occurred over a one-week period at about neutral pH conditions. As a result, subsequent experiments were performed in the presence of lactate to determine the roles of NOM in bioreduction of U(VI). Samples were prepared in 25-mL glass pressure tubes containing 1×10^8 cells/mL of CN32 and 0–200 mg C/L of NOM or NOM fractions in 30 mM $NaHCO_3$ buffer (pH 8.1) in an anaerobic chamber (97.5% N_2 and 2.5% H_2). Sodium lactate was added to make a final concentration of 10 mM and a final volume of 10 mL. Note that $NaHCO_3$ buffer and Na-lactate were used here to avoid the complexation and/or precipitation of uranyl with phosphate and PIPES at circumneutral pH conditions, and U(VI) is expected to present primarily as $UO_2(CO_3)_2{}^{2-}$ complexes in solution (Fredrickson et al., 2000). Each pressure tube was then closed with a butyl rubber stopper and crimp-sealed with an aluminum cap. A 0.1-mL U(VI) stock solution [as $UO_2(NO_3)_2$ at 42 mM] was injected into each tube with a syringe, resulting in an initial U(VI) concentration of about 0.42 mM. Duplicate samples were prepared and equilibrated on a rotary shaker at room temperature. At a given time interval, a subsample (0.1 mL) was taken with a needle syringe and diluted 100 times with deoxygenated phosphoric acid (10%) before analysis. Samples were not filtered because the reduced U(IV) species do not interfere with the phosphorescence analysis of hexavalent U(VI), as described earlier. Control samples consisting of identical treatments either without microbial cells or without NOM fractions were also prepared along with samples.

3. RESULTS AND DISCUSSION

3.1. Effects of NOM on the Chemical Reduction of Cr(VI) and U(VI)

The chemical reduction of Cr(VI) by various NOM fractions was observed at a low pH (3) in the absence of microorganisms (Fig. 1). The reaction was relatively slow as compared with the reduction of Fe(III) by NOM (Chen et al., 2003a) and did not reach equilibrium in a 2-week reaction period. Among the three NOM fractions, NOM-PP appeared to be the most effective in reducing Cr(VI), whereas soil HA was the least reactive at pH 3. Approximately 95% of the Cr(VI) was reduced by NOM-PP in 2 weeks, an amount substantially greater than that for either soil HA or the carbohydrate-rich NOM-CH fraction. The remaining Cr(VI) concentrations were 0.01, 0.04, and 0.11 mmol/L after treatment with NOM-PP, NOM-CH, and soil HA, respectively.

An approximately linear relationship was observed between the logarithmic concentrations of Cr(VI) in samples and the reaction time (Fig. 2a). Results suggest that the reduction of Cr(VI) by NOM may be described by a pseudofirst-order reaction kinetics. A close examination of the data, however, indicated that initial reduction of Cr(VI) did not exactly follow either the first-order or the second-order reaction kinetics (within the first 24 h, Fig. 2b inset). These results may suggest that the initial rapid drop in Cr(VI) concentration was likely due to the reactions of certain functional groups in NOM that are much more reactive than the bulk of reaction sites in NOM. This is not surprising considering the multifunctional and multicomponent nature of NOM or its subfractions (Chen et al., 2003a). Indeed, the primary purpose of this study was to investigate the roles of various NOM subcomponents and their structural properties in the reduction of redox-sensitive metals such as Fe(III), Cr(VI), and U(VI), as will be discussed in the following sections. Similar observations have been reported by Wittbrodt and Palmer (1995, 1997), who found that the reduction of Cr(VI) by humic substances commonly displays a nonlinear relation with time and could not be adequately modeled with either the first- or the second-order rate equations.

Nevertheless, by assuming a pseudofirst-order reaction kinetics to compare the reduction of Cr(VI) with that of Fe(III), we found rate constants (k_1) of Cr(VI) reduction by three NOM fractions in the order of NOM-PP (7.9×10^{-3} h^{-1}) > NOM-CH (4.4×10^{-3} h^{-1}) > soil HA (1.5×10^{-3} h^{-1}). These findings are similar to the reduction of Fe(III) by the three NOM fractions, among which NOM-PP was the most effective in reducing ferric Fe(III) at low pH conditions (Table 1) (Chen et al., 2003a). However, the reduction rate of Cr(VI) was nearly an order of magnitude lower than that of Fe(III) despite the fact that about ten times higher initial NOM concentration was used in the reduction of Cr(VI) than in the reduction of Fe(III). The first-order rate constants for the reduction of Fe(III) ranged from 13×10^{-3} h^{-1} to 26×10^{-3} h^{-1} at pH 3.

These observations indicate that the reduction of these met-

Table 1. Effects of different NOM fractions on the abiotic reduction kinetics of Cr(VI) and Fe(III) at pH 3.

	Rate constant, k_I (h^{-1})	
	Cr(VI)	Fe(III)[a]
Soil HA	1.5×10^{-3}	2.6×10^{-2}
NOM-PP	7.9×10^{-3}	4.3×10^{-2}
NOM-CH	4.4×10^{-3}	1.3×10^{-2}

[a] From Chen et al. (2003a) for comparison.

als depends not only on different NOM components but also on the metals themselves. The reported redox potential of humic materials varied widely, from -0.9 up to $+0.8$ V (Visser, 1964; Helburn and MacCarthy, 1994; Nakayasu et al., 1999; Struyk and Sposito, 2001; Nurmi and Tratnyek, 2002), and could not be directly used to assess their relative effectiveness in reducing these metals. However, CrO_4^{2-} is thermodynamically the preferred electron acceptors as it is a stronger oxidant than Fe(III). The standard redox potential of the Fe(III)/Fe(II) couple is $+0.771$ V as compared with $+1.35$ V for the Cr(VI)/Cr(III) couple under acidic pH conditions (Lide, 2000). Similarly, Liu et al. (2002) recently found that, among various metal ions studied, Cr(VI) consistently showed the slowest bioreduction rate although its calculated free energy changes were the most favorable. It therefore appears that the reactions between Cr(VI) and NOM fractions were kinetically limited and required a higher activation energy than that of Fe(III). Indeed, the reduction of Cr(VI) only accelerates at extremely acidic pH conditions (<3) and/or by heating the samples as is done in the method commonly used for the determination of the maximum quantity of reducing equivalent of NOM by the well-known Walkley-Black method (Walkley and Black, 1934). At pH 3, the anionic $HCrO_4^-$ and cationic Fe^{3+} or $FeOH^{2+}$ are the predominant ionic species in solution (Baes and Mesmer, 1976). Because both NOM and $HCrO_4^-$ are negatively charged, an unfavorable electrostatic interaction may thus result in a high activation energy that is likely to slow down the electron transfer reactions between NOM and $HCrO_4^-$. On the other hand, NOM is known to form strong complexes with Fe^{3+} or $FeOH^{2+}$, and such complexation could have facilitated the electron-transfer reactions and therefore the reduction of Fe(III) by NOM.

Although the reactions of Cr(VI) with three NOM fractions were also performed at about neutral pH conditions, no significant Cr(VI) reduction was observed over a 2-week reaction period (data not shown). These observations are consistent with previous studies showing that the reduction of Cr(VI) by NOM is strongly pH-dependent and proceeds extremely slow at pH ~7 (Wittbrodt and Palmer, 1995; Nakayasu et al., 1999). These observations may be attributed to both the kinetic and thermodynamic factors because of the changes of Cr(VI) speciation in solution (as CrO_4^{2-}) at pH ~7. More importantly perhaps, an increased pH results in a significantly reduced redox potential of the Cr(VI)/Cr(III) couple to -0.13 V at pH above neutral [i.e., $CrO_4^{2-} \rightarrow Cr(OH)_3$] (Lide, 2000). In other words, CrO_4^{2-} becomes a much weaker oxidant at pH 7 than at pH 3.

For U(VI), NOM was found to be ineffective in chemically reducing U(VI) even with the NOM-PP fraction at pH 3 in a three-week reaction period (data not shown). On the basis of our studies with Cr(VI) and Fe(III) (Chen 2003a), we anticipated that NOM would be ineffective in reducing U(VI) as well at about neutral pH conditions. Furthermore, because UO_2^{2+} may undergo hydrolysis and precipitation at pH >5.5 (Gu et al., 2003), no additional abiotic experiments were performed between U(VI) and NOM. However, as will be presented below, the abiotic reduction of U(VI) was performed later in the bicarbonate buffer solution at pH 8.1 and in the presence of lactate as controls. Similarly, no significant reduction of U(VI) was observed within the experimental error. In a study of the redox behavior and complexation between hexavalent actinides and a peat humic acid, Nash et al. (1981) also reported that no reduction of U(VI) was observed in a 2-month reaction period although the humic acid reduced Np(VI) and Pu(VI) to Np(V) and Pu(IV). These studies appear to support our observations that NOM has little or no effect in abiotic reduction of U(VI). However, we may also argue that U(VI) reduction by NOM could have been inhibited by the presence of NO_3^- since $UO_2(NO_3)_2$ was used for the experiment. It is generally known that microbial reduction of U(VI) does not occur until nitrate is consumed as preferred electron acceptors (Abdelouas et al., 1998). Although, to the best of our knowledge, no studies have been reported to suggest a direct abiotic reduction of NO_3^- by NOM, nitrate may nevertheless result in reoxidation of the reduced forms of U(IV) species. Further studies are obviously needed in this respect.

3.2. Effects of NOM on Microbial Reduction of Cr(VI) and U(VI)

Although the reduction of Cr(VI) by NOM has been studied extensively, to our knowledge no studies have examined the role of NOM in the microbial reduction of Cr(VI), particularly in the presence of different NOM subfractions. Numerous studies, however, have reported that humic substances could greatly enhance the microbial reduction of Fe(III) or Fe(III)-oxides by acting as electron shuttles (Lovley, 1996; Benz et al., 1998; Coates et al., 1998; Fredrickson et al., 1998; Lovley et al., 1998; Royer et al., 2002b). To evaluate the potential enhancement of Cr(VI) and U(VI) reduction by NOM, we examined the reduction of Cr(VI) and U(VI) after freshly grown cells (*S. putrefaciens* CN32) were inoculated into the samples containing various NOM fractions at circumneutral pH conditions. As shown in Figure 3, microbial reduction of Cr(VI) occurred rapidly (within several hours), a rate substantially greater than rates observed in the absence of microorganisms (Fig. 1) or in the absence of NOM (but with cells as a control). The enhancement of Cr(VI) reduction was particularly evident in the presence of soil HA; about 50% of Cr(VI) was reduced in 28 h in the presence of *S. putrefaciens* CN32 as opposed to less than 10% Cr(VI) reduction in the absence of these microorganisms (Fig. 1). However, the reduction rates of Cr(VI) decreased over time and did not appear to follow either the first-order or the second-order reaction kinetics, as noted earlier.

The microbial reduction of U(VI) by NOM fractions was found to be very slow under the same conditions as used for the bioreduction of Cr(VI) with H_2 as a sole electron donor, as indicated earlier. Subsequent experiments were performed in
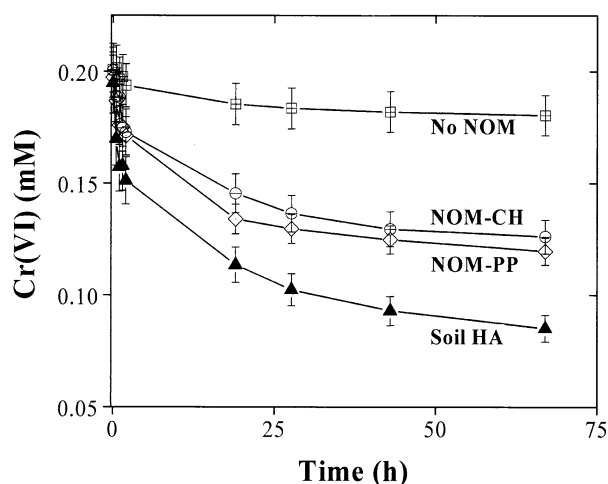
Fig. 3. Effects of NOM fractions on Cr(VI) bioreduction by a bacteria strain of *S. putrefaciens* CN32 in a PIPES buffer solution (pH 6.8). The initial NOM concentration was 100 mg C/L, and the microbial cell concentration was $\sim 10^7$ mL^{-1}.

the presence of lactate and with an increased cell concentration; an increased rate of U(VI) bioreduction was therefore observed by the three NOM fractions (Fig. 4a). The reduction was the most effective in the presence of soil HA and least effective in the presence of the NOM-CH fraction. About 82, 67, and 22% of added U(VI) were reduced in a 24-h period in the presence of soil HA, NOM-PP, and NOM-CH, respectively.

To evaluate if U(VI) was truly reduced to U(IV) species, selected samples were analyzed for U(VI) concentrations by the phosphorescence before and after filtration using 0.2-μm
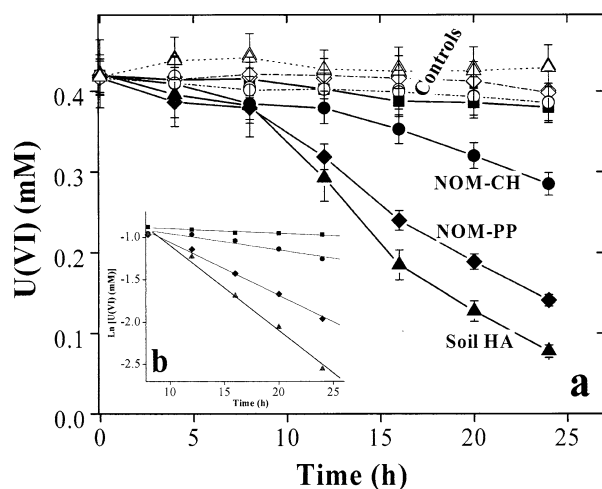

Fig. 4. (a) Effects of NOM fractions on the bioreduction of U(VI) in the presence of *S. putrefaciens* CN32 and Na-lactate in 30 mM bicarbonate buffer solution (pH 8.1). Open symbols (and dashed lines) represent those controls without addition of microbial cells but with three NOM fractions, and the filled squares are for inoculated no-NOM controls. The inset (b) shows the logarithmic plot of the U(VI) reduction within the first 2 h (after subtracting 8 h of the lag time). The initial NOM concentration was 100 mg C/L, and the microbial cell concentration was $\sim 10^8$ mL^{-1}.

syringe filters. As discussed earlier, this analytical method is specific for hexavalent U(VI). If U(VI) is present in solution as sorbed or colloidal species (>0.2 μm), the unfiltered samples should give higher fluorescence signals [or higher U(VI) concentrations] than those of filtered samples under the same experimental conditions. Results between filtered and unfiltered samples generally agreed well, within a $\pm 5\%$ error. It is further pointed out that a decreased U(VI) concentration was not attributed to its sorption onto bacterial cells because, in the bicarbonate buffer solution, negatively charged $UO_2(CO_3)_2{}^{2-}$ was expected to be the dominant species. This conclusion was independently evaluated by analysis of U(VI) concentrations before and after removing bacterial cells by either the filtration or centrifugation techniques.

It is of interest to note that no significant reduction of U(VI) occurred until ~ 8 h of incubation (Fig. 4a). This observation may be partially attributed to the time required for microbes to degrade residual $NO_3{}^-$ in solution and/or for the growth of these microorganisms. As discussed earlier, the bioreduction of U(VI) would be inhibited in the presence of $NO_3{}^-$ in solution (Abdelouas et al., 1998). Therefore, if we ignore the first 8-h lag time, an approximately linear correlation was found between the logarithmic concentration of U(VI) and the reaction time (Fig. 4b, inset). The results suggest a pseudofirst-order reduction kinetics of U(VI) by *S. putrefaciens* CN32 under anaerobic conditions. The estimated rate constants were about 0.1, 0.06, and 0.02 h^{-1} for the microbial reduction of U(VI) in the presence of soil HA, NOM-PP, and NOM-CH, respectively (Table 1). Although these reduction rates appeared to be higher than those observed for the abiotic reduction of Cr(VI) and Fe(III), they could not be directly compared because a much more favorable reducing condition was used for the reduction of U(VI) (i.e., in the presence of lactate and 10^8 cells/mL). In fact, the microbial reduction of Fe(III) was much greater than that of U(VI) even though H$_2$ was used as a sole electron donor in the Fe(III) bioreduction experiments (Chen et al., 2003a).

These observations indicate that the reduction rates are metal specific both under abiotic and microbial reducing conditions in the presence of various NOM fractions. Similarly, in a study of the bioreduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII), Liu et al. (2002) observed the following rate trend: Fe(III) > Co(III) >> U(VI) > Cr(VI) > Tc(VII). Our results (Chen et al., 2003a and this study) appear in general agreement with these observations, except for the reduction of U(VI). Liu et al. (2002) reported a much higher bioreduction rate (~ 0.58 h^{-1}) for U(VI) in the presence of *S. putrefaciens* CN32 and lactate but without NOM. However, it should be noted that about twice as much of microbial cell concentrations were used in their studies, and we also realize that the presence of small amounts of $NO_3{}^-$ in our reactant solution could have been responsible for a slow reduction rate of U(VI) observed in this study. In addition, a relatively high pH condition (8.1) was used in the present study as compared with the conditions (pH ~ 6.8) used by Liu et al. (2002).

The bioreduction of Cr(VI) and U(VI) in the presence of *S. putrefaciens* CN32 was also found to depend on NOM concentrations in the reactant solution (Fig. 5a,b). With an increase in NOM concentration, the microbial reduction of Cr(VI) and U(VI) greatly increased. Among the three NOM fractions, soil HA was again the most effective in mediating the bioreduction
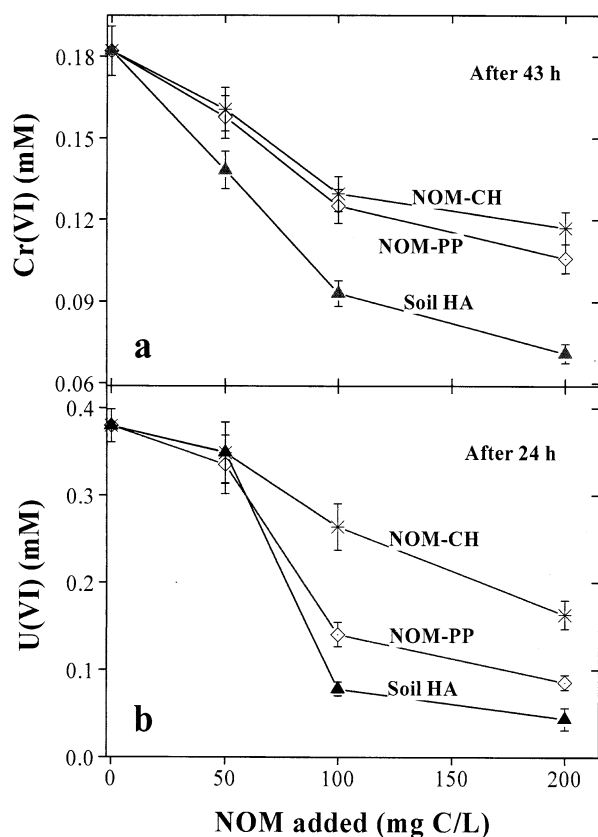
Fig. 5. Effects of added NOM concentration on the bioreduction of (a) Cr(VI) at pH 6.8 (PIPES buffer) and (b) U(VI) at pH 8.1 (NaHCO$_3$ buffer) in the presence of *S. putrefaciens* CN32. Sodium lactate was added in the bioreduction of U(VI). The initial Cr(VI) and U(VI) concentrations were 0.2 and 0.42 mM respectively.

of these two metal species. About 30 and 65% of Cr(VI) were reduced in the presence of 50 and 200 mg C/L soil HA in ~43 h; but only about 20 and 40–47% of Cr(VI) were reduced in the presence of NOM-CH or NOM-PP at the same concentrations and the same experimental conditions. The bioreduction of U(VI) in the presence of lactate increased from ~17% to more than 80% when the soil HA concentration increased from 50 to 100 mg C/L. Further increases in the concentration of soil HA had less effect, probably because of limited amounts of U(VI) added in the initial solution (Fig. 4b).

3.3. NOM Roles and Structural Properties in the Reduction Processes

Data presented in this study suggest different NOM subcomponents play different roles in the reduction of contaminant metals. Both NOM-PP and NOM-CH were found to be more effective than soil HA in chemically reducing Cr(VI) at a low pH (Figs. 1–2). However, soil HA was found to be much more effective than NOM-PP and NOM-CH in reducing Cr(VI) or U(VI) in the presence of microorganisms (Figs. 3–5). These observations could be attributed to the different structural and functional properties of NOM samples as a result of the fractionation process. Similar observations have been reported pre-

viously in studies of the effect of NOM in chemical and microbial reduction of Fe(III) (Chen et al., 2003a). In the absence of microorganisms, the three NOM fractions exhibited significantly different reduction rates for Fe(III); NOM-PP was the most effective. However, the bioreduction rates of Fe(III) by *S. putrefaciens* CN32 were about an order of magnitude higher in the presence of soil HA than in the presence of NOM-PP or NOM-CH.

Under abiotic conditions, the fact that soil HA appeared to be less reactive with Cr(VI) than NOM-PP or NOM-CH at a low pH may be partially attributed to its lower solubility and conformational changes at low pHs. Unlike NOM-PP or NOM-CH (both of which are soluble in acid), soil HA macromolecules may become coiled or aggregated, with fewer reactive sites, at pH below 3 and thus were ineffective in reducing Cr(VI). Quinone, hydroquinone, phenolic, and ketonic functional groups in NOM have been postulated to be responsible for the reduction of redox-sensitive metals such as Fe(III) (Chen et al., 2003a). Our investigations of structural and functional groups in NOM through ^{13}C-nuclear magnetic resonance, infrared, and UV-visible spectroscopy revealed that the contents of phenolic functional groups in the three NOM fractions decrease in the order NOM-PP > soil HA > NOM-CH (Chen et al., 2002). However, the contents of aromatic functional groups and the UV molar absorptivity decrease in the order soil HA > NOM-PP > NOM-CH, which also holds true for a summation of total amounts of aromatic and phenolic functional groups. Scott et al. (1998) considered that quinone moieties as major components in NOM directly responsible for electron transfer reactions through the stable free radical intermediates detected by electron-spin resonance (ESR) spectroscopy. However, Struyk and Sposito (2001) questioned quinones being the exclusive agents of electron transfer because their theoretical calculations indicate that the measured free radical concentrations may account for only a small fraction of the electrons that could be transferred from NOM to reduce Fe(III). The normalized ESR spin counts of the three NOM fractions used in this study are in the order NOM-PP (2.6×10^{18} spins/g C) > soil HA (2.4×10^{18} spins/g C) >> NOM-CH (not detected) (Chen et al., 2002). This order seems consistent with the observed effectiveness of NOM-PP in reducing Cr(VI) at a low pH. However, it failed to explain the ineffectiveness of soil HA in reducing Cr(VI) for reasons noted above.

Previous studies also have indicated that low-molecular-weight fulvic acids are more effective than high-molecular-weight humic acids in chemically reducing Cr(VI) (Wittbrodt and Palmer, 1995, 1997; Nakayasu et al., 1999). Nakayasu et al. (1999) suggested that this may be related to the redox potential of these organic materials; those low-molecular-weight organic compounds have a low oxidation potential and are thus more readily oxidized by Cr(VI) than high-molecular-weight humic acids. Ironically, Cr(VI) is a strong oxidant at low pH conditions, and it could also cause the breakdown of some organic compounds (Nelson and Sommers, 1982), particularly the NOM-CH fraction, which is enriched with low-molecular-weight organic materials such as carbohydrates, uronic acids, and other compounds (Lowe, 1975; Chen et al., 2002). This may offer a partial explanation on the effectiveness of NOM-CH in chemically reducing Cr(VI) (Fig. 1), although this fraction was depleted of aromatic or phenolic organic

compounds. Similarly, Sunda and Kieber (1994) reported the breakdown of humic substances by chemical oxidation with Mn(IV), which yielded low-molecular-weight organic substrates.

A most striking observation of this study was that soil HA became the most effective in reducing Cr(VI) and U(VI) in the presence of microorganisms at circumneutral pH conditions (Figs. 3–5). The effectiveness of soil HA may be partially explained by the fact that, with an increase in solution pH, soil HA became more reactive with Cr(VI) or U(VI) because of its increased solubility and conformational changes as mentioned earlier. Under these conditions, soil HA acts more favorably than NOM-PP or NOM-CH as electron shuttles, and the result may be correlated to its relatively high molecular sizes and polycondensed and conjugated aromatic components, as reported previously (Chen et al., 2003a). In comparison with NOM-PP, these conjugated and polycondensed aromatic organic moieties may be among the most important components responsible for Cr(VI) or U(VI) reduction at circumneutral pH conditions (Chen et al., 2003b). This argument is supported by the fact that soil HA gives intense fluorescence at high wavelengths (or a red shift at >460 nm), suggesting its relatively high content of polycondensed and conjugated aromatic structural features (Miano et al., 1988; Senesi, 1990; Chen et al., 2003b). On the other hand, the NOM-PP fraction contains relatively low-molecular-weight organic components and thus low proportions of conjugated aromatic compounds as compared with soil HA. Even though it is also enriched with aromatic and phenolic contents, NOM-PP did not perform as well as soil HA in mediating the bioreduction of Cr(VI) or U(VI). Similar observations were reported for the microbial reduction of Fe(III) in the presence of *S. putrefaciens* CN32 (Chen et al., 2003a). Additionally, Scott et al. (1998) observed that humic substances of aquatic origin generally show lower Fe(III)-bioreduction capacity than humic acids obtained from sediments or soils. Those humic materials isolated from soils or sediments presumably contain higher molecular weight organic compounds than those from the aquatic environment.

4. CONCLUSIONS

Different NOM subcomponents are capable of chemically reducing Cr(VI) at a low pH (3), but the reaction rates decrease dramatically at about neutral pH conditions. Although thermodynamically more favorable, the abiotic reduction of Cr(VI) was found to be much slower than that of Fe(III) at both low and high pH conditions due to the kinetic and thermodynamic factors. The abiotic reduction of U(VI) by NOM was not observed, possibly because of the presence of small amounts of nitrate in the reactant solution. However, all NOM fractions enhanced the bioreduction of Cr(VI) or U(VI) in the presence of *S. putrefaciens* CN32, and the reduction rates also varied greatly among NOM fractions with different chemical and structural properties. Although NOM-PP appeared to be the most reactive in abiotic reduction of Cr(VI) at low pH, soil HA acted much more effectively in mediating the bioreduction of Cr(VI) and U(VI) at circumneutral or slightly alkaline pH conditions. These observations were attributed to an increased solubility and conformational changes of soil HA with pH and, more importantly perhaps, the relatively high proportion of

polycondensed and conjugated aromatic moieties in the humic acid structure.

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