

Soil Humic Acid Decreases Biological Uranium(VI) Reduction by *Shewanella putrefaciens* CN32

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ABSTRACT

Biological reduction of uranium(VI) by *Shewanella putrefaciens* CN32 was investigated in the presence of soil humic acid (SHA). Experiments were performed under resting cell conditions with uranyl acetate as the electron acceptor and sodium lactate as the electron donor in a NaHCO₃ or PIPES/NaHCO₃ buffer. SHA significantly decreased the final extent of U(VI) bioreduction (100% uranium bioreduced without humics vs. 50% uranium bioreduced with humics). Dissolved (0.2- μ m filter) U(VI) was measured by kinetic phosphorescence analysis where samples were kept anoxic during one measurement, and split samples were oxidized and remeasured. The difference between the anoxic and oxidized measurements was operationally defined as “U(IV) < 0.2- μ m.” U(IV) < 0.2- μ m was detected only in samples containing SHA suggesting that soluble U(IV)–SHA complexes were formed or SHA facilitated the transport of U^{IV}O₂(s) nanoparticles through the filter. Identical experiments conducted with nitrate instead of U(VI) showed that SHA interrupted electron transport to nitrate, and may interact similarly with U(VI). These results suggest that humic materials may influence U immobilization by decreasing U(VI) reduction and enhancing the solubility of U(IV).

Key words: uranium reduction; uranium complexation; humics; *Shewanella*; XAFS

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INTRODUCTION

URANIUM EXTRACTION and processing has led to significant soil and groundwater contamination at mining sites and U.S. Department of Energy facilities. Depending on solution chemistry, U(VI) often exists as mobile anionic uranyl-carbonate complexes (Langmuir, 1978; Grenthe *et al.*, 1992). Biological reduction of soluble U(VI) to a sparingly soluble form of U(IV) [e.g., uraninite $\text{U}^{\text{IV}}\text{O}_2(\text{s})$] has been proposed as a remediation strategy (Lovley, 1993). A variety of dissimilatory metal-reducing bacteria (DMRB) and sulfate-reducing bacteria can catalyze this reaction under anoxic conditions (e.g., Gorby and Lovley, 1992; Truex *et al.*, 1997; Spear *et al.*, 1999; Liu *et al.*, 2002).

The impact of humic materials on the bioreduction of soluble U(VI) is not well understood. For example, if a DMRB preferentially uses humic materials instead of U(VI) as its electron acceptor, then U(VI) bioreduction could be inhibited. However, if the humic materials act as effective electron shuttles, then no inhibition would be observed and, depending on the different reaction rates and the solution chemistry, enhancement may occur (Gu and Chen, 2003; Gu *et al.*, 2005). Another possibility is that humic materials may complex U(VI) (Moulin *et al.*, 1992; Higgs *et al.*, 1993; Lenhart *et al.*, 2000), decrease bioavailability and inhibit bioreduction. Finally, humic materials may also complex U(IV) (Li *et al.*, 1980; Zeh *et al.*, 1997), which could interfere with U(IV) precipitation and facilitate U(IV) transport. The objective of this study was to examine the effect of humic acid on the bioreduction of U(VI).

EXPERIMENTAL PROTOCOLS

Shewanella putrefaciens strain CN32 (referred to as CN32) was grown aerobically on tryptic soy broth without dextrose at 20°C on an orbital shaker (100 rpm) (Royer *et al.*, 2002). Cells were harvested by centrifugation ($4900 \times g$, 10 min, 20°C) from a 16-h-old culture and washed three times in 30 mM NaHCO_3 or 10 mM 1,4-piperazinediethanesulfonic acid (PIPES, Sigma, St. Louis, MO) + 30 mM NaHCO_3 (pH 7.0) with the final wash made with deoxygenated solution. Standard anaerobic techniques were used for the preparation of all media and experiments. Cell pellets were resuspended in anoxic 30 mM NaHCO_3 or 10 mM PIPES + 30 mM NaHCO_3 buffer (referred to as NaHCO_3 and PIPES/ NaHCO_3 buffer, respectively) and cell density was determined by absorbance at 420 nm.

A uranium stock solution was made from uranyl acetate (Polysciences Inc., Warrington, PA) prepared at 1.0

mM in NaHCO_3 or PIPES/ NaHCO_3 buffer, and stored under an 80:20% $\text{N}_2:\text{CO}_2$ atm. Soil humic acid (SHA) was purchased from the International Humic Substances Society (IHSS, St. Paul, MN), and a 1,000 mg L^{-1} SHA stock solution was prepared in NaHCO_3 or PIPES/ NaHCO_3 buffer 1 and stored under an 80:20% $\text{N}_2:\text{CO}_2$ atm. A nitrate stock solution was made from NaNO_3 prepared at 1.0 mM in NaHCO_3 buffer and stored under an 80:20% $\text{N}_2:\text{CO}_2$ atm.

Bioreduction experiments were conducted under resting cell conditions using CN32 (1×10^8 cells mL^{-1}) with ca. 100 or 500 μM uranyl acetate as the sole electron acceptor, 5 mM sodium lactate as the sole electron donor, and 0 or 100 mg L^{-1} SHA. All solutions were prepared in NaHCO_3 or PIPES/ NaHCO_3 buffer depending on the experimental components. Experiments were conducted in serum bottles sealed with thick butyl rubber stoppers and aluminum crimp tops. All preparations were performed in an anaerobic chamber (Coy, Grass Lakes, MI; 95:5% $\text{N}_2:\text{H}_2$ atm). Serum bottles were removed from the anaerobic chamber and incubated at 20–22°C on a shaker table. Abiotic (no-cells) and biotic no-humic controls were prepared in parallel with all experiments, and all systems were prepared in triplicate.

We found that soluble or precipitated U(IV) could pass through a 0.2- μm filter in the presence of SHA, be oxidized before or during measurement in a kinetic phosphorescence analyzer (KPA), and create a significant analytical artifact. We therefore developed a method to measure U(VI) using a KPA under anoxic conditions. In this method, split samples were oxidized and measured by KPA. The difference between the anoxic and oxidized measurements was operationally defined as “U(IV) < 0.2 μm .”

Samples were collected from the serum bottles using sterile needles, filtered (0.2- μm , cellulose acetate), and used to measure U(VI) and pH. Dissolved U(VI) was measured by KPA and U(VI) standards were prepared in the exact matrix of the samples to account for any matrix quenching. “Anoxic” filtrate samples were prepared with anoxic Uraplex reagent inside the anaerobic chamber and transferred into KPA cuvettes (ChemChek, Richland, WA). KPA cuvettes were sealed with a fitted plastic stopper, removed from the anaerobic chamber, and analyzed immediately using the manual mode of the KPA. Sealed KPA cuvettes filled with resazurine maintained anoxic integrity for more than 1 h outside of the chamber. “Oxidized” filtrate samples were removed from the anaerobic chamber, oxidized with a concentration HNO_3 , stored overnight in the presence of air, and analyzed using the KPA autosampler.

Because of the analytical challenges associated with samples containing U(IV) and to validate our operational U(IV) < 0.2- μm procedure, we repeated experiments

where samples were analyzed by U L_{III}-edge X-ray absorption spectroscopy (XAS) at the Advance Photon Source (APS) at Argonne National Laboratory (ANL). XAS measurements were made on filtrate (0.2- μm) samples mounted in holes machined in Plexiglas sample holders. The holes were covered with Kapton film held in place with Kapton tape. All sample preparation was performed in an anaerobic chamber (Coy; 95:5 N₂:H₂ atm %) and all samples were stored in the chamber prior to analysis. Samples mounted in the holders were exposed to the atmosphere for less than 1 min before being mounted for XAS measurements in a free-flowing N₂ environment to limit possible sample oxidation. XAS sample holders maintained anoxic integrity up to 8 h. XAS data were collected at the Materials Research Collaborative Access Team (MR-CAT) sector 10-ID beamline (Segre *et al.*, 2000) at the APS at ANL using a 13-element solid-state detector (Canberra with X1A electronics) in fluorescence mode. Data for X-ray absorption near edge structure (XANES) were collected at the U L_{III}-edge. Reference spectra from a uranyl phosphate standard were measured using scattered X-rays (Cross and Frenkel, 1998). All data sets were accurately aligned in energy using the derivative of the edge of the uranyl phosphate standard. [U(IV)]/[U(VI)] ratios were determined from the XANES spectra by using the energy value at the 0.5 edge step in the normalized absorption data compared to the value for U(IV) (natural UO₂ minerals mixed with SiO₂) and U(VI) (1 mM uranyl acetate solution) standards as previously described (Kelly *et al.*, 2002). The U(IV) and U(VI) standards have absorption energy values (17169.3 and 17174.6 eV, respectively) that are separated by 5.3 eV.

Biogenic uraninite [U^{IV}O₂(s)] particles were imaged by negative staining transmission electron microscopy (TEM). Cells and solids were collected after 48 h from the biotic-no humic NaHCO₃ experiment. Two milliliters of settled cell suspension were fixed in 2 mL of 4.0% glutaraldehyde in the anaerobic chamber, sealed in a serum bottle, and transported to the Penn State EM Facility. A drop of the cell/glutaraldehyde mixture was placed on 200 mesh-copper grids with formvar support film coated with carbon. The mixture was allowed to cure on the grid for 2 min; a drop of uranyl acetate was then placed on the cured sample and wicked off after 30 s. The grids were examined at 80 kV using a JEOL 1200 EXII TEM.

Nitrate (100 μM NaNO₃) bioreduction experiments were conducted in NaHCO₃ buffer with 0 or 100 mg L⁻¹ SHA. Nitrate and nitrite were quantified by ion chromatography with conductivity detection (Dionex DX 100 fitted with an AS-4A column; Dionex Corp., Sunnyvale, CA).

RESULTS AND DISCUSSION

The addition of 100 mg L⁻¹ SHA decreased the rate and extent of bioreduction of U(VI) by CN32 (Fig. 1). In the absence of SHA, U(VI) was removed to below detection limit (<0.1 μM) after 30 h. The kinetics of U(VI) bioreduction was first order with respect to [U(VI)]. Based on anoxic samples collected over the first 22 h, the first-order rate constant was 0.15 h⁻¹ ($R^2 = 0.98$). After accounting for our lower cell concentration (1×10^8 cells mL⁻¹) and temperature (20–22°C), this rate

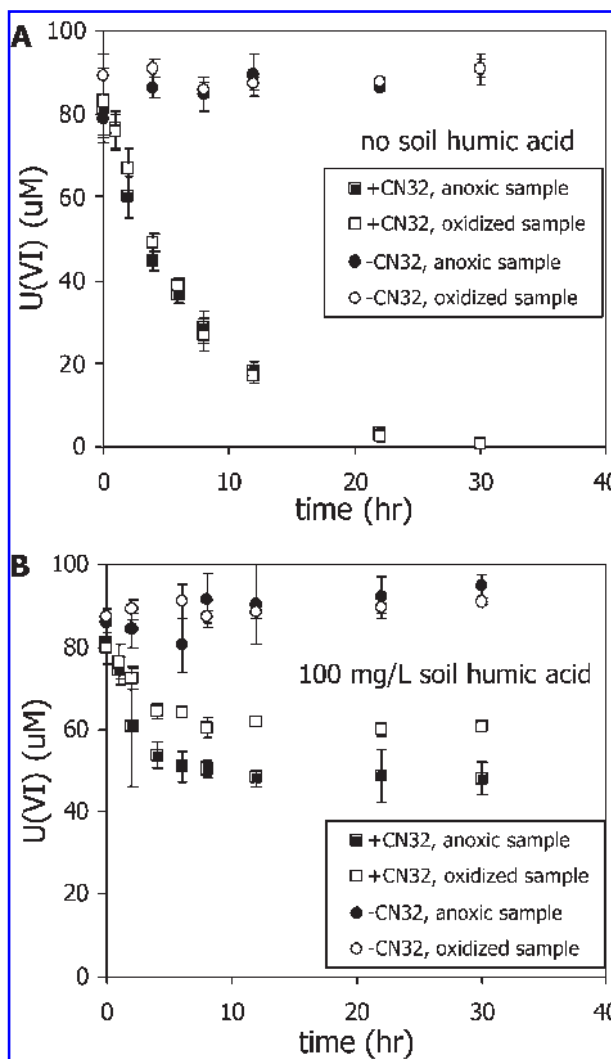


Figure 1. Bioreduction kinetics of 90 μM uranyl acetate in the (A) absence and (B) presence of 100 mg L⁻¹ soil humic acid. All experiments were conducted with 1×10^8 cells mL⁻¹ CN32 and 5 mM Na-lactate in a 30 mM NaHCO₃ buffer under an 80:20% N₂:CO₂ atm, pH 7.0, 20–22°C. Symbols represent mean values of triplicate measurements and error bars represent one standard deviation.

compares favorably with a U(VI) bioreduction rate of 0.60 h^{-1} for CN32 reported by Liu *et al.* (2002). In that study, experiments were conducted with $500 \mu\text{M}$ uranyl chloride, 5 mM Na-lactate, and $2 \times 10^8 \text{ cells mL}^{-1}$ at 30°C in a 30 mM NaHCO_3 buffer.

In the presence of SHA, the initial kinetics of U(VI) bioreduction was first order; however, this rate quickly decreased after 6 h. Based on anoxic samples collected over the first 6 h, the first-order rate constant was 0.11 h^{-1} ($R^2 = 0.94$). SHA had the most significant effect on the extent of bioreduction, where the concentration of U(VI) in the final anoxic samples remained constant at $48.3 \pm 0.2 \mu\text{M}$ U(VI) ($n = 3$) from 12 to 30 h. U(IV) $< 0.2 \mu\text{m}$ was detected only in the presence of SHA. From 6 to 30 h, the U(IV) $< 0.2\text{-}\mu\text{m}$ concentration remained nearly constant at $12.2 \pm 1.6 \mu\text{M}$ ($n = 5$). Based on these average KPA-measured concentrations, the $[\text{U(IV)}]/[\text{U(VI)}]$ ratio was 25% near the end of this experiment.

To confirm the presence of soluble U(IV) in the $0.2\text{-}\mu\text{m}$ filtrates, the $[\text{U(IV)}]/[\text{U(VI)}]$ ratio was measured by XANES in a series of repeat experiments. The redox state of U(IV) $< 0.2 \mu\text{m}$ was confirmed by XANES, showing a net oxidation state intermediate between U(IV) and U(VI) in the biotic-with-SHA filtrate samples (Fig. 2). The XANES data were used to calculate a $[\text{U(IV)}]/[\text{U(VI)}]$ ratio of 50% in these samples. This ratio was reasonably consistent with the ratio measured by KPA, considering the accuracy of the XANES estimates ($\pm 20\%$), the additional several-day incubation period prior to XAS analysis, and possible oxidation effects associated with the “anoxic” KPA measurement. Due to the low concentration of uranium in solution, the specific coordination environment of the U(IV) $< 0.2\text{-}\mu\text{m}$ could not be resolved by extended X-ray absorbance fine structure (EXAFS), for example, to differentiate between $\text{U}^{\text{IV}}\text{O}_2(\text{s})$ nanoparticles and soluble U(IV)–SHA complexes. Additionally, a comparison of U XANES standards to the U(VI) $< 0.2\text{-}\mu\text{m}$ data can not definitively distinguish between UO_2 and U(IV)–SHA complexes.

Negative staining TEM showed that $\text{UO}_2(\text{s})$ nanoparticles on the order of 20 to 40 nm were produced in the absence of SHA (Fig. 3). No TEM images were collected in the presence of SHA. Interestingly, U(VI) $< 0.2 \mu\text{m}$ was not detected in the absence of SHA, yet TEM images clearly show that particles far smaller than $0.2 \mu\text{m}$ were present. For all U(VI) measurements, the first 5 to 10 drops of filtrate were discarded before sample collection. In the absence of SHA, this filter preparation procedure apparently promoted the formation of a “filter cake” (composed of compacted cells and solids) that was capable of removing particles considerably smaller than $0.2 \mu\text{m}$. Considering that a constant high cell concentra-

tion was used in all experiments, some type of filter cake also had to form in the presence of SHA. Therefore, in the presence of SHA, $\text{UO}_2(\text{s})$ nanoparticles could have been smaller or soluble U(IV)–SHA complexes could have formed.

It is unclear how SHA effects the rate and extent of U(VI) bioreduction but we can speculate on possible interactions. U(VI)–SHA complexation could decrease U(VI) bioavailability or decrease the effective reduction

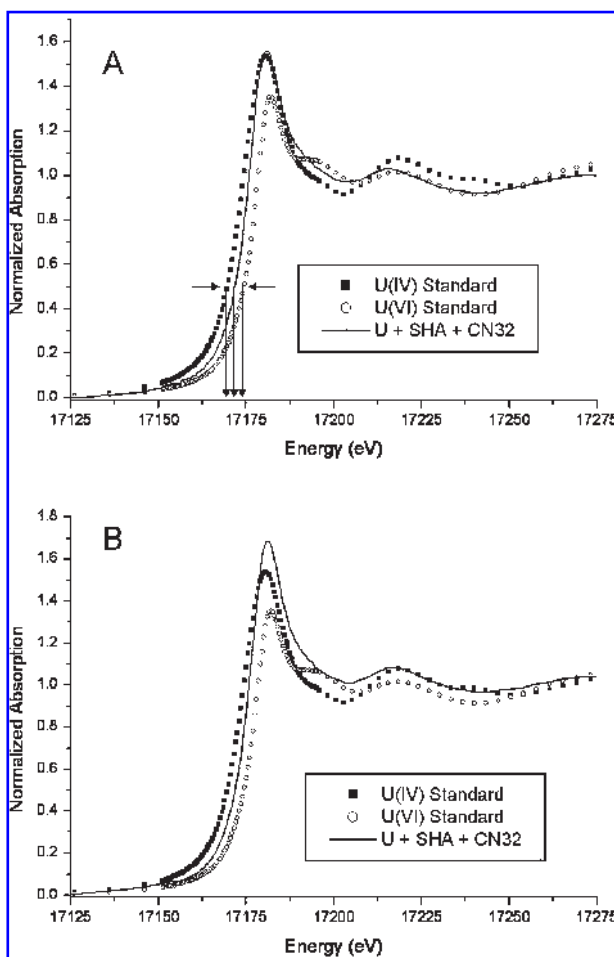


Figure 2. U L_{III}-edge X-ray absorption spectra for $0.2\text{-}\mu\text{m}$ filtrate samples collected after incubation with CN32 and 100 mg L^{-1} soil humic acid (SHA). U(VI) standard is 1 mM uranyl acetate. U(IV) standard is natural UO_2 mixed with SiO_2 as described elsewhere (Kelly *et al.*, 2002). All experiments were conducted with $1 \times 10^8 \text{ cells mL}^{-1}$, 5 mM Na-lactate in a 10 mM PIPES + 30 mM NaHCO_3 buffer under an 80:20% $\text{N}_2:\text{CO}_2$ atm, pH 7.0, (A) $103 \mu\text{M}$ initial uranyl acetate, and (B) $545 \mu\text{M}$ initial uranyl acetate. For (A), after 6 days of incubation, the total U concentration was $53 \mu\text{M}$ (accurate within factor of 2) and the $[\text{U(IV)}]/[\text{U(VI)}]$ ratio was 50% (accurate within factor of 20%). Additional spectra for U(IV) and U(VI) standards and their absorption edge energy values (arrows) are included for comparison purposes.

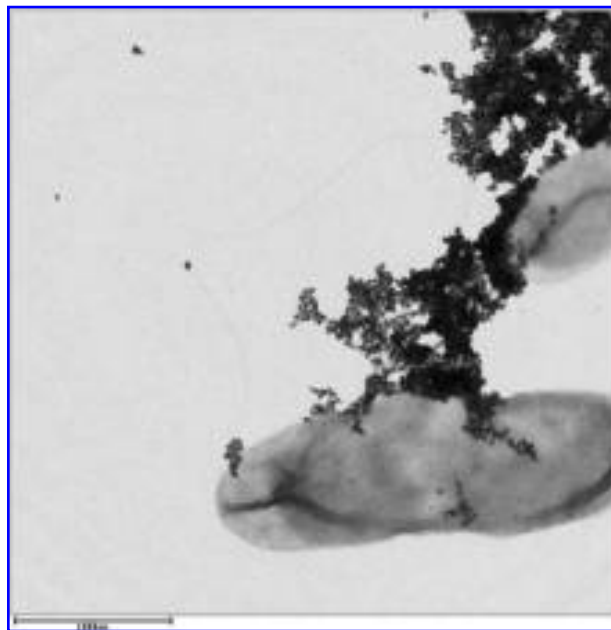


Figure 3. Biogenic uraninite [$\text{UO}_2(\text{s})$] particles imaged by negative staining TEM. The sample was collected after a 48-h incubation with 1×10^8 cells mL^{-1} CN32 and 5 mM Na-lactate in a 30 mM NaHCO_3 buffer under an 80:20% $\text{N}_2:\text{CO}_2$ atm, pH 7.0, 20–22°C.

potential of U(VI) in a manner analogous to calcium-complexed U(VI) (Brooks *et al.*, 2003). However, other soluble U(VI)–ligand complexes are readily bioreducible (Ganesh *et al.*, 1997; Dodge and Francis, 1998; Francis *et al.*, 2002), and the predicted extent of U(VI) complexation by SHA in these experiments is small. The Windermere Humic Aqueous Model (WHAM) with program default constants for U(VI) reactions with humic materials, hydroxide, and carbonate was used to predict that 16% of U(VI) would be complexed by 100 mg L^{-1} humic material for our experimental conditions (Tipping, 1994; CEH, 2001). This does not completely explain why nearly 50% of the U(VI) was not bioreduced by the end of the experiment.

Renshaw *et al.* (2005) recently reported that the DMRB *Geobacter sulfurreducens* reduces U(VI) through an unstable U(V) species, $\{\text{UO}_2^+\}$, and suggested that U(IV) could be produced through the disproportionation of $\{\text{UO}_2^+\}$. If this mechanistic pathway of U(VI) bioreduction is correct, it is possible that humic materials could stabilize $\{\text{UO}_2^+\}$ and interfere with further reduction. Previous XAFS studies of U(V) have found U-Oaxial and U-Oequatorial distances of 1.90 ± 0.02 and 2.50 ± 0.02 Å, respectively (Docrat *et al.*, 1999). Fits to first coordination shell phase components of the XAFS data from samples from this study indicate U-Oaxial distances of

1.74–1.81 Å and U-Oequatorial distances of 2.35–2.38 Å. These interatomic distances are not consistent with the presence of a U(V) phase in our experiments.

SHA could interrupt electron transport to U(VI) and inhibit bioreduction of U(VI). To assess this, a set of experiments were performed with 100 μM nitrate, 5 mM sodium lactate, 1×10^8 cells mL^{-1} CN32 in a 30 mM NaHCO_3 buffer, with 0 or 100 mg L^{-1} SHA (Fig. 4). Importantly, nitrite accumulated to a considerably higher level with SHA, suggesting that SHA interfered with nitrite reduction. The first-order rate constants for nitrate reduction were 0.59 h^{-1} ($R^2 = 0.93$) and 0.67 h^{-1} ($R^2 = 0.97$) in the presence and absence of SHA, respectively,

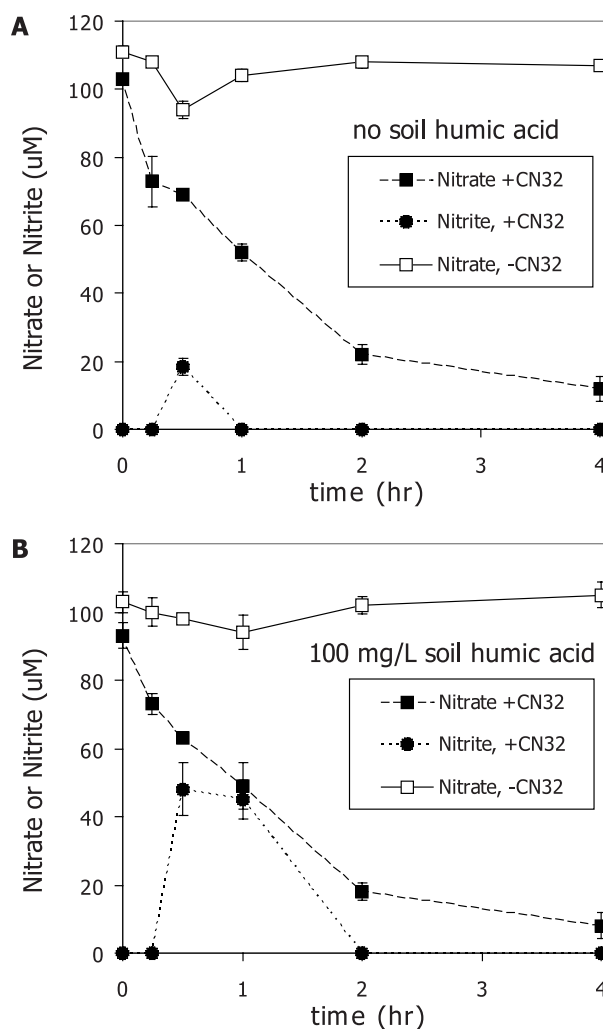


Figure 4. Bioreduction kinetics of ca. 100 μM nitrate in the (A) absence and (B) presence of 100 mg L^{-1} soil humic acid. All experiments were conducted with 1×10^8 cells mL^{-1} CN32 and 5 mM Na-lactate in a 30 mM NaHCO_3 buffer under an 80:20% $\text{N}_2:\text{CO}_2$ atm, pH 7.0, 20–22°C. Symbols represent mean values of triplicate measurements and error bars represent one standard deviation.

suggesting that SHA had a slight effect on nitrate reduction. Wade and DiChristina (2000) have suggested that nitrite and U(VI) reduction pathways in *Shewanella putrefaciens* 200 share a common component that is not necessary for the reduction of Fe(III), Mn(IV), nitrate, or other terminal electron acceptors. The apparent partial inhibition of both U(VI) and nitrite reduction suggests that SHA may interfere with electron transport at this common component in both U(VI) and nitrite reduction.

The bioreduction and immobilization of soluble U(VI) to insoluble U(IV) minerals is a promising strategy for the remediation of uranium-contaminated soil and groundwater. While a mechanistic description is not fully resolved, it appears humic materials could interrupt electron transport to U(VI). The results of this study suggest that humic materials could potentially decrease U(VI) reduction under certain conditions. Furthermore, humic materials could prevent U(IV) precipitation and thus facilitate the transport of U(IV)–humic complexes.

ACKNOWLEDGMENTS

This research was supported by the Natural and Accelerated Bioremediation Research Program (NABIR), Office of Biological and Environmental Research (OBER), Office of Energy Research, U.S. Department of Energy (DOE) through Grant No. DE-FG02-01ER63180 to The Pennsylvania State University. Support for J.M. Senko was also provided by the Penn State Center for Environmental Chemistry and Geochemistry. Use of the MR-CAT sector at the Advance Photon Source (APS) and of the APS was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under contract W-31-109-ENG-38 and the MR-CAT member institutions. Special appreciation is extended to E.J. Oughlin and M. Boyanov for assistance with the U XAFS data collection, and to P.C. Burns and A.J. Locock for the U(VI) and U(IV) XAFS standards.

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