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Modeling the Removal of Uranium U(VI) from Aqueous Solutions in the Presence of Sulfate Reducing Bacteria

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The reduction kinetics of soluble hexavalent uranium (U(VI)) to insoluble tetravalent U(IV) by both a mixed culture of wild-type sulfate-reducing bacteria (SRB) and a pure culture of *Desulfovibrio desulfuricans* (ATCC 7757) were studied at variable cell concentrations. The SRB were grown in chemostats, and the uranium reduction kinetics were evaluated from batch experiments at 21 °C. The initial U(VI) concentration was 1 mM, while the initial cell concentration varied from 0.18 to 1.27 mg dry wt/mL. A modified nongrowth Monod model best fit the data across all cell concentrations. For the mixed culture, average values for the maximum specific reaction rate, k_U, and for the half saturation constant, K_U, are 0.023 mM U(VI)/min·mg cells and 0.25 mM U(VI); for *D. desulfuricans*, k_U is 0.030 mM U(VI)/min·mg SRB and K_U is 0.50 mM U(VI). A lag-time before enzymatic U(VI) reduction occurred was present for systems tested and was inversely correlated to cell concentration. Transmission electron microscopy (TEM) of the cell/precipitate suspension after U(VI) reduction indicated an extracellular amorphous mass of electron dense material. Examination of the cell/precipitate suspension with X-ray Photoelectron Spectroscopy (XPS) indicated the presence of U(IV). The determined rate constants can be used to design an SRB dominated bioreactor for U removal from aqueous solutions.

Introduction

Natural and anthropogenic sources of uranium (U) contamination in surface and groundwaters are widespread. Sources for U input to surface and groundwaters include natural U deposits, mining, milling, and tailing operations and U.S. Department of Energy (DOE) locations. The U.S. Environmental Protection Agency (EPA) has proposed a maximum contaminant level (MCL) for U of 20 μg/L (8.4×10^{-8} M) and a maximum contaminant level goal (MCLG) of 0.0 μg/L under the Safe Drinking Water Act (*1*). Current treatment of contaminated waters for U removal involve one or more of several methods including anion exchange, lime softening, conventional coagulation, and activated alumina. While these methods are effective for removing low concentrations of U from aqueous solution, the amount of radioactive heavy metal waste sludge or brine generated has the potential to be significant. To achieve low effluent concentrations, i.e., ≤20 μg/L, multiple unit processes can be involved (rapid mix, flocculation, sedimentation, filtration, and pH adjustment), large doses of chemicals are needed, and treatment columns need frequent replacement and/or regeneration (*2*−*5*).

Biological treatment of solutions containing uranium offers an alternative to conventional physical/chemical methods. Lovley and Phillips (*6*) described the ability of a pure culture of the obligatory anaerobic sulfate-reducing bacterium (SRB) *Desulfovibrio desulfuricans* (ATCC 29577) to enzymatically reduce soluble U(VI) in an aqueous environment to insoluble U(IV). Other microbial species have been characterized as capable of U(VI) reduction, precipitation, or sorption by cell walls. Organisms capable of enzymatic reduction using U(VI) as their terminal electron acceptor include the iron reducing bacteria *Geobacter metalireducens* (previously GS-15) (*7*), *Shewanella putrefaciens* (*7*), and *Shewanella alga* strain BrY (previously reported as *Shewanella halotolerans* strain BrY) (*8*, *9*). A *Citrobacter* sp. has been shown to accumulate polycrystalline $HUO_2PO_4 \cdot 4H_2O$ on the cell surface as a result of precipitation with enzymatically liberated phosphate at the cell surface (*10*−*13*). Organisms studied for U uptake and or cell surface adsorption include *Pseudomonas* sp. EPS-5028 (*14*) and *Mycobacterium smegmatis* (*15*). There is only one known species of SRB that appears to grow with U(VI) (as well as Cr(VI), Mn(IV), and Fe(III)) as the sole electron acceptor and that is the Gram positive, spore forming *Desulfotomaculum reducens* sp. nov. strain MI-1 (*16*).

Prior to the design of an operational bioreactor, quantification of bioreduction rate coefficients are needed to design a system and select proper operating conditions, such as biomass concentration, in a U(VI) reducing bioreactor. Lovley and Phillips (*6*) grew the SRB cells in batch and showed optimal U(VI) reduction at 35 °C. However, rate constants for U(VI) bioreduction were not determined nor simulated, and the effect of biomass concentration on U(VI) reduction was not explicitly evaluated (*6*). Rate constants normalized to biomass for aqueous U(VI) bioreduction have been determined for the Fe(III) reducing *Shewanella alga* strain BrY at 22 °C under nongrowth conditions (*8*). Ganesh et al. (*17*) explored, but did not model, the effects of organic U(VI) complexes being reduced to U(IV) in the presence of *Desulfovibrio desulfuricans* or *Shewanella alga*. The potential advantages of an SRB dominated treatment scheme include the following: (1) sulfate is available in natural waters to provide energy for the growth of U(VI) reducing SRB and (2) a system based on iron reducing bacteria produces soluble Fe(III) which could oxidize bioreduced insoluble U(IV) back to soluble U(VI) (*6*).

There are at least three possible processes for the removal of U(VI) in the presence of bacterial cells: (1) U(VI) binding to one or more moieties on the whole cell surface; (2) abiotic reduction of U(VI) by SRB produced sulfide in conjunction with precipitation of U(IV) species; and (3) bioreduction of U(VI) to U(IV) via enzymatic dissimilatory metal reduction with U(VI) acting as a terminal electron acceptor. Mohagheghi et al. (*18*) posited that the combined effects of processes 1 and 2 were responsible for U(VI) reduction. Subsequent work by Lovley and Phillips (*6*) demonstrated that process 3, bioreduction, is the dominant mechanism for U(VI) reduction in the presence of SRB. Further studies on *Desulfovibrio vulgaris* (Hildenborough, ATCC 29579) demonstrated that the enzyme responsible for U(VI) reduction is cytochrome c_3, which is one of the enzymes responsible for sulfate reduction (*19*−*21*); it is a periplasmically bound (between

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TABLE 1. Media Compositions Used in These Kinetic Studies[a]

constituent	isolation medium	growth medium	expt medium	wash	uranium stock
potassium phosphate (mono)		0.5			
potassium phosphate (dibasic)	0.5				
ammonium chloride	1.0	1.0			
sodium sulfate	1.0	2.0			
calcium chloride	0.07	0.06			
magnesium chloride	2.0	0.06			
iron sulfate	0.1	0.005			
sodium citrate		0.3			
yeast extract	1.0	0.1			
ethanol (100%)	4.5 mL/L				
agar	15.0				
sodium lactate (60%)		15 mL/L	1.9 mL/L		
mercaptoacetic acid	0.1				
ascorbic acid	0.1				
resazurin (0.1% soln)	1.0 mL/L				
sodium bicarbonate			2.0	2.5	2.5
uranyl acetate					4.241

[a] Unless noted, all values are given in g/L and mixed in deionized water. With the exception of the U media stock, all media are sterilized by autoclave at 121 °C and 1 kg/cm^2 pressure. The isolation medium is pH 7.5, the others are pH 7.0, and the U stock is made up in pH 7.0 wash media. The isolation medium has ethanol added post-autoclaving.

the outer surface of the cytoplasmic membrane and the inner surface of the lipopolysaccharide layer of Gram negative bacteria), electron-transfer protein (*22*−*24*). Factors that can affect U(VI) bioreduction are competition between electron acceptors and U speciation in solution. The effect of uranyl carbonato species in solution on uranium enzymatic bioreduction has been previously reported (*25*−*27*). Lovley and Phillips (*6*) considered the competition between electron acceptors with the reduction of U(VI) in the presence of sulfate. Ganesh et al. (*17*) considered the reduction of U(VI) with organic ligands.

In this paper we describe the kinetics of U(VI) reduction to U(IV) at room temperature (21 °C) under anaerobic, nongrowth, batch conditions, by both a mixed culture of chemostat grown wild-type SRB and a pure culture of *Desulfovibrio desulfuricans* (ATCC 7757). Modeling the kinetics of this anaerobic dissimilatory metal reduction with subsequent biomineralization is required for the development of biotreatment design schemes capable of removing uranium from a contaminated water using SRB. Biotreatment designs will require continuous growth of bacteria optimized for growth at ambient water temperatures. A wild-type organism was utilized in this study because pure cultures of microorganisms stocked in culture collections can loose their ability to adapt to ever changing environmental conditions via DNA mutation or transposon addition of an important environmental trait (*20*, *28*). Growth of SRB at temperatures lower than growth optimum (e.g., 35 °C) are important for future treatment schemes since most mine or groundwaters are significantly cooler (e.g. 10−20 °C). The whole cell SRB enzymatic biomineralization process is an attractive alternative for biotreatment scale-up because of the ease of growth of SRB on surfaces, rapid enzymatic bioreduction, the potential for minimal media supplementation to an U(VI) containing feedwater, and a wide range of useful operating temperature.

Experimental Section

Bacterial Culture. A mixed culture of sulfate reducing bacteria was obtained by an anaerobic agar deep tube method with dilutions (*20*) from a mud sample taken from a bioreactor used for the treatment of acid mine drainage. Media composition was a modified Postgate E (*20*) containing two reducing agents, with ethanol as the carbon source (Table 1). All chemicals utilized for these studies were reagent grade or better and used without further purification. Verification of anaerobic conditions in the agar deep tube was via a resazurin indicator moving from pink to clear or opaque agar color indicating an $E_h \leq -51$ mV (*20*). Serial dilutions of a 1 g mud sample were made in sterile phosphate buffered saline from 10^{-2} through 10^{-8} g/L concentrations. Once colony growth was indicated by the formation of iron sulfide (black) in the deep tubes, the tube of interest was split open aseptically, and the colonies removed and mixed with an autoclaved modified Postgate C (*20*) (Table 1) medium. Sodium citrate was present in this medium as a chelator to minimize the formation of iron sulfides from the production of biogenic sulfides by the SRB. Initially, cells were grown on ethanol in batch, followed by uninterrupted cultivation in a chemostat fed with lactate containing modified Postgate C (Table 1). The mixed SRB culture's carbon source was changed to lactate to increase cell yield and density (*29*).

Continuous cultivation of the mixed cell culture in a chemostat was carried out on an insulated magnetic stirrer in an anaerobic chamber (Bactron II, Sheldon Manufacturing) fed with anaerobic mixed gas (AMG) containing 90% N_2, 5% H_2, and 5% CO_2. Yield from the chemostat averaged 0.10−0.15 mg dry wt cells/mL growth media. The chemostat hydraulic residence time ranged from 15 to 25 h through the use of either 250 or 500 mL square polycarbonate bottles. Average temperature during growth in the chemostat on the chamber stage was 21 °C ± 3 °C.

Desulfovibrio desulfuricans (ATCC 7757) cells were grown in modified Postgate C medium (Table 1) at 21 °C ± 3 °C in the anaerobic chamber. The *D. desulfuricans* culture was also grown in a chemostat with an 8 h hydraulic residence time as described for the mixed culture above. Refrigerated stocks of both cultures were transferred to fresh media approximately every two weeks.

Microscopy. Samples were prepared for transmission electron microscopy (TEM) by transferring a drop of cell/precipitate suspension to a carbon and Formvar coated EM copper grid that had been freshly glow-discharged. Excess sample was blotted off of the grid, and the grid was allowed to air-dry. The cells were imaged in a JEOL 100C electron microscope operating at 100 kV. Cell suspensions from the growth culture and from U(VI) reduction experiments were examined. Cell suspension samples from experiments with an initial U(VI) concentration of 1 mM $U_{natural}$ (as uranyl acetate) were taken at 3 h of reaction time and maintained in anaerobic 15 mL screw cap vials.

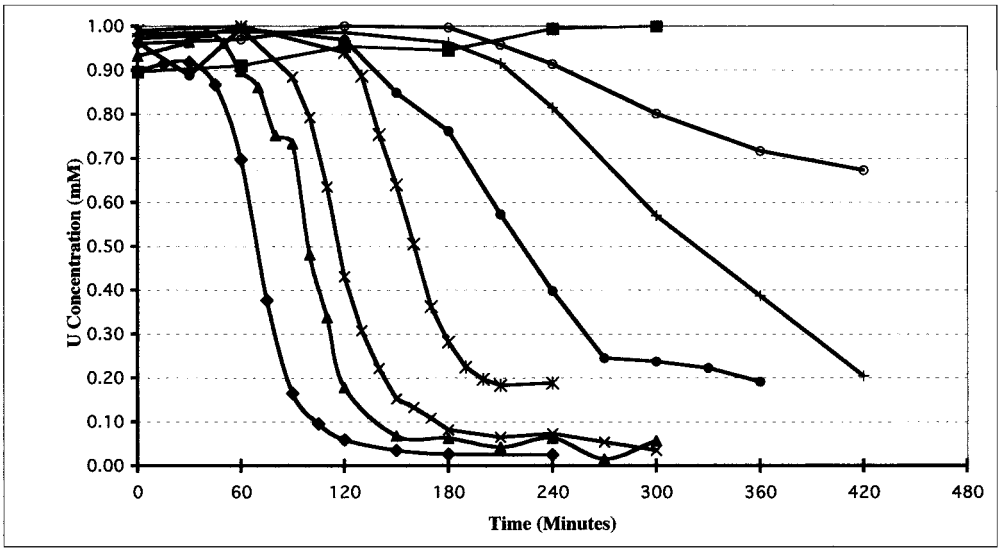

FIGURE 1. Dissolved U(VI) concentration as a function of time for a series of experiments at different cell concentrations. The data points represented for each cell concentration series represent an average of at least three data points for three separate experiments at the time points represented. Each data series cell concentration is represented in mg dry wt cells/mL: (◆) 1.27, (■) 0.32 control experiment, (▲) 0.81, (×) 0.46, (✱) 0.33, (●) 0.29, (+) 0.22, and (○) 0.18.

Spectroscopy. The cell/precipitate phase of a U(VI) reduction experiment was examined with X-ray photoelectron spectroscopy (XPS) by J. Douglas Farr at Los Alamos National Laboratory, NM. At 3.5 h after initiation of the U bioreduction experiment (cell concentration, X = 1 mg dry wt/mL, U = 1 mM), 4 mL of the SRB cell/precipitate/media was removed from the polycarbonate flask with a 3 cm^3/21 gauge syringe and filtered aerobically through a 0.2 μm Nucleopore polycarbonate filter. The Nucleopore filter apparatus with filter was then quickly placed (≤1 min) in an anaerobic chamber, where the filter was removed, placed into a 15 mL conical tube, and anaerobically sealed (90% N_2, 5% CO_2, and 5% H_2). The tubes were shipped to Los Alamos where the two sample tubes were then opened and the filters analyzed using XPS. Measurement was performed as described by Francis et al. (*30*) to analyze for the U oxidation state.

Cell Growth and Preparation. SRB cells were collected from the growth chemostat, transferred to 15 mL centrifuge tubes, and decanted in the anaerobic chamber. Centrifugation to concentrate the cells was performed at 1160*g* for 5 min. After centrifugation of the cells in growth media, the supernatant liquid was poured off, and the cells were washed once in one-third the growth volume (5 mL) of deoxygenated 2.5 g/L sodium bicarbonate buffer (autoclave sterile, pH 7.0) (*6*, *8*). Following a second spin and supernatant liquid removal via pour-off, the residual fluid was removed anaerobically from the tube with a Pasteur pipet, leaving just the SRB cell pellet. The tubes were then weighed on an analytical balance. A wet weight cell mass was obtained and converted to a dry weight cell mass with a dry weight analysis multiplication factor (*31*). Dry weight cell analyses (*31*) were performed monthly on both the pure and mixed cultures. Weighed cell pellets were suspended in 1 mL of sterile, deoxygenated experiment media (Table 1) and subjected to kinetic studies.

Kinetic Studies. A selected amount of the chemostat grown SRB cell mass (cell pellets, 0.2−1.3 mg dry wt/mL) was suspended anaerobically in pH 7.2 sterile experiment media (Table 1) with a small magnetic stir bar, in sterile polycarbonate septum flasks (a 30 mL square polycarbonate bottle with a 1 cm hole cut in the top of the screw cap) sealed with Teflon lined butyl rubber stoppers between the bottle and the screw cap. The U(VI) was added within 15 min as uranyl acetate, $UO_2(CH_3COO)_2{\cdot}2H_2O$, from a 10 mM stock solution (Table 1). The uranyl acetate was mixed with $^{233}U(VI)$ as a yield tracer (typically 200 μL of a 23 670 dpm/mL stock $^{233}U(VI)$ solution; ^{233}U from Isotope Products Laboratory) in a syringe and fed to the cells by injection through the septum with a 3 cm^3/21 gauge syringe.

The anaerobic polycarbonate flasks were stirred on insulated magnetic stir motors at ambient room condition (21 °C ± 3 °C). The 1.5 mL samples were removed via a 3 cm^3/21 gauge syringe at times of interest. The removed aliquot of solution was then placed in a 1.5 mL polystyrene microcentrifuge tube and spun at 12 000 rpm for 3 min. Supernatant solution (≈1.5 mL) was collected by pipet and added to 20 mL plastic scintillation vials containing 10 mL of Ultima Gold scintillation cocktail (Packard). The cell pellet and 1.5 mL of deionized water were added to the same type of vial described above. The mass balance of uranium was checked at least three times per experiment, comparing supernatant liquid activity + pellet activity with total activity. For total activity of samples taken at the same time, the sample was blended directly with scintillation cocktail. Scintillation counting was performed on either a Model 1600TR or a Model 2500TR Packard Tri-Carb Liquid Scintillation Analyzer for 10 min/vial. Typical counting errors were 2% or less. Selected experiments were also performed using SRB cells in experiment media with the addition of the uranyl acetate only. Subsequent analysis was conducted with a kinetic phosphorescence analyzer (KPA) (*8*). Verification of the accuracy of the scintillation counting method was made by comparing data to experiments done with $U_{natural}$ analyzed with KPA as done elsewhere (*6*, *8*).

All experiments were performed at least in triplicate. Negative, i.e., no reduction, control experiments were performed by the same method with the addition of 10 mM sodium molybdate, a known blocking agent of the enzyme cytochrome c_3, prior to the addition of U(VI). Data analysis and nonlinear model fitting was conducted on Microsoft EXCEL spreadsheets with the use of the SOLVER function.

Results and Discussion

Lag Time. The removal of U(VI) by the mixed SRB culture (cell concentrations varying from 0.2 to 1.3 mg dry wt/mL) from aqueous solutions with an initial U(VI) concentration of 1 mM is presented in Figure 1. Two dominant trends can

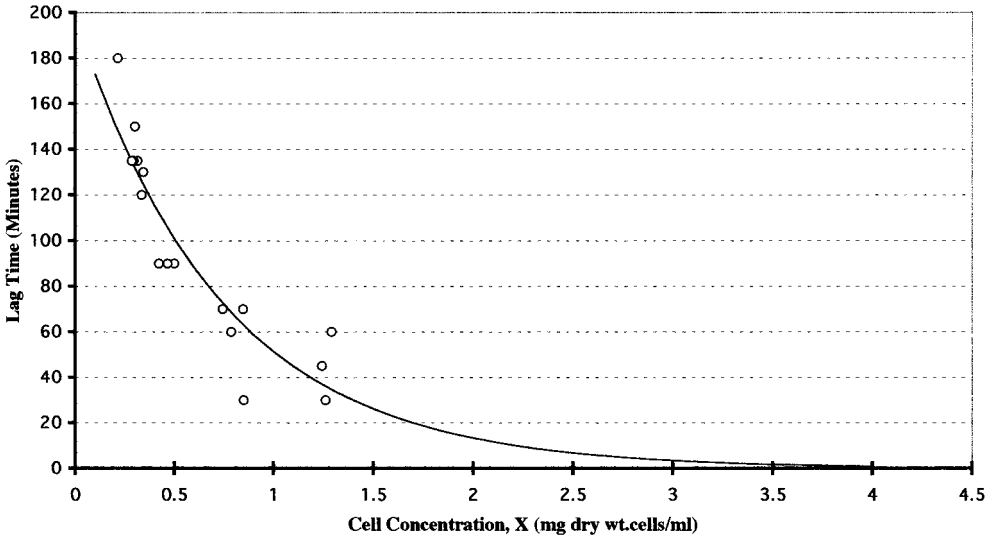

FIGURE 2. Lag time, t_L, as a function of cell concentration, *X*. Equation 1 model line is represented with experimental cell concentrations tested as data points. These cell concentrations plotted on the best fit line yield a value for the time lag which was then used for the inverse modeling. Lag time is predictable and observable for both the mixed and pure SRB cell cultures, though this curve represents the mixed culture only. The highest cell concentrations tested in these experiments utilized 1.3 mg dry wt cells/mL, well within the observable lag time. (o) points represent the time lags observed from the experimental data.

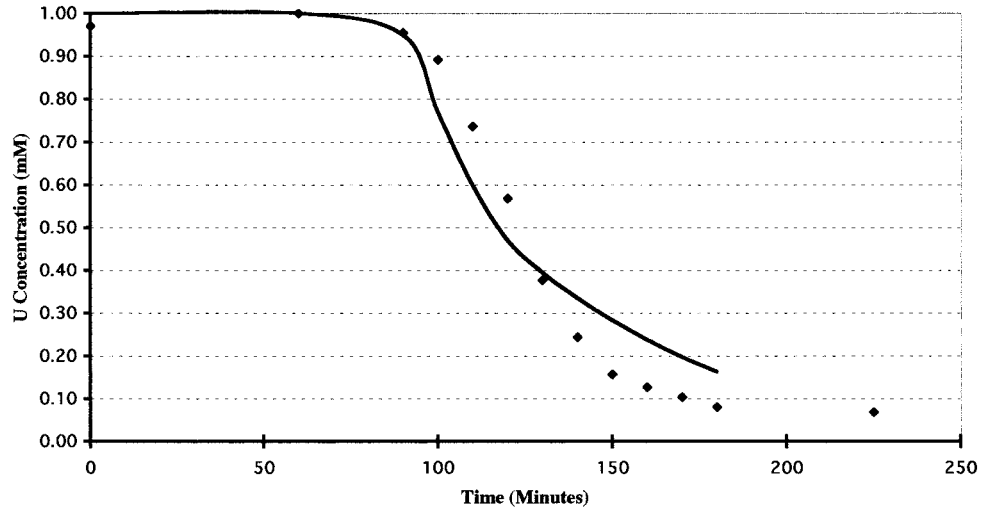

FIGURE 3. Dissolved U(VI) reduction by a pure culture of *Desulfovibrio desulfuricans* (ATCC 7757) reducing 1 mM U(VI). Cell concentration is 0.33 mg dry wt cells/mL with an observed lag time of 90 min. The data points represent an average of two experiments performed at the same U(VI) and *X* concentrations. The model line is a plot of eqs 6 and 7 after having the sum of the errors squared minimally solved on spreadsheets between the experimentally determined data (the data points on graph) and those predicted using three of the five model parameters.

be seen in the data: (1) a lag time (t_L) to the onset of U(VI) reduction was observed to be a function of cell concentration, and (2) the U(VI) reduction rate increases with increasing cell concentration as indicated by the slopes of the lines drawn through the data. The data represented in Figure 1 are averaged data of at least three experimental runs per cell concentration. The lag time is inversely correlated to an exponential function of cell concentration with a coefficient of determination, r^2, of 0.99, and ranges from approximately one-half hour at a cell concentration, *X*, of 1.3 mg dry wt/mL, to 3 h at *X* equal to 0.2 mg dry wt/mL (Figure 2). A first-order model for t_L as a function of *X* best fit the observed lag. The model (eq 1) was fit to the data by linear regression

$$t_L = \beta_L e^{-\alpha X} \quad (1)$$

where, after regression, $\beta_L = 198$ min, $\alpha = 1.35$ mL/mg, and *X* is the SRB cell concentration in mg dry wt cells/mL.

A lag time was also observed with the pure culture *Desulfovibrio desulfuricans* (ATCC 7757) using the same experimental condition as with the mixed culture system. The pure culture, at *X* equal to 0.33 mg dry wt/mL, had a t_L equal to 90 min (Figure 3). For a similar cell concentration of the mixed SRB cell culture a lag time of 120 min was observed. A cell concentration of 0.58 mg dry wt cells/mL would be required for the mixed culture to show a 90 min lag time to the onset of U(VI) reduction (Figure 2). From this comparison of cultures it can be seen that U(VI) reduction between the mixed and pure cultures indicates a dependence on cell culture type. One possible reason for the different lag times is that the mixed culture contains a different species of SRB. Different species of SRB do not exhibit the same rate of sulfate reduction (*20*) and thus would be expected to exhibit difference in U(VI) reductive behavior. A second possible reason is that the mixed culture may contain a fraction of non-U reducing and/or non-SRB cells that contribute to

biomass only. The determined SRB cell mass used in these experiments would then be overestimated by the total cell mass.

The lag time for U removal is analogous to the lag phase reported for bacterial growth and metabolism whereby innoculum size, change in media, chemostat growth verses batch growth, induction of enzymes, selection of new metabolic capabilities, and an increase in the number of cells able to metabolize a new compound are all factors that can cause and affect a lag (*32*). The growth phase of an innoculum has a strong effect on the length of time of the lag phase. Cultures in exponential growth phase at the time of transfer should have a shorter lag phase than cultures in stationary phase (*33*). The transfer of a small innoculum volume into a large medium volume can cause the outward diffusion of vitamins and cofactors into the bulk medium and reduce the concentration of critical reactants required for a threshold value. This could result in a lag time as the culture reestablishes the necessary internal concentrations (*33*). This is consistent with the observed lag time being a function of cell concentration for both cultures tested here. The cells were transferred from the growth medium in a chemostat to a simpler bicarbonate/lactate/U experiment media in batch for kinetic analysis. Such a transfer between systems could have affected the cells ability to immediately reduce uranium.

Other investigators studying bacterial U reduction have not observed a lag time in U removal (*6*, *8*, *9*, *26*). These studies employed batch growth of the cell cultures followed by batch kinetic studies at 35 °C (*6*, *8*, *9*, *26*). For both cultures tested here, cells were grown continuously at 21 °C, followed by batch kinetic study at 21 °C. The SRB were grown in a chemostat to simulate the growth conditions anticipated for a continuous flow treatment system. Batch kinetic experiments were conducted with the scintillation method to facilitate more rapid data collection. Lower temperature for both growth and kinetic analysis was used to be closer to operational temperatures of a working biotreatment system. Lovley and Phillips (*6*) observed no lag time for U(VI) reduction by SRB for four likely reasons: (1) their systems evaluated high cell concentrations, which would foster rapid reduction; (2) the initial sampling took place after 60−90 min and, thus, did not capture a lag period; (3) the temperature of growth and batch kinetic study was high enough to ensure optimal metabolic activity; and (4) the growth phase of cells used in the kinetic analysis, as mentioned above. For the iron and U(VI) reducing *Shewanella alga* strain BrY, the same four reasons apply. Truex et al. (*8*) used 1 h sampling intervals that also may hide an observable lag. The combination of different growth conditions, different experimental cell concentrations, and different sampling intervals could all contribute to a masking of a lag time. As was done in this study, others (*6*, *8*) have washed cells between growth and batch kinetic study. Cell washing was examined to determine if this altered the U(VI) reducing ability of the mixed SRB cells harvested from the chemostat, i.e., to evaluate if the washing procedure which is part of the experimental protocol may have removed a critical rate-limiting compound or cofactor, resulting in the observed lag time. Cells grown in the CSTR were removed, centrifuged, not washed, and added to U(VI) containing experimental media. Washed verses unwashed cells exhibited the same lag time to quantifiable U(VI) reduction (data not shown).

A series of reinjection experiments was undertaken to determine if a rate limiting compound had been produced in sufficient quantity during the initial lag time. This test was performed to observe if a secondary lag time is needed to reduce additional U(VI). Additional U(VI) was added after 90% of the initial 1 mM U(VI) was reduced at time 150 min

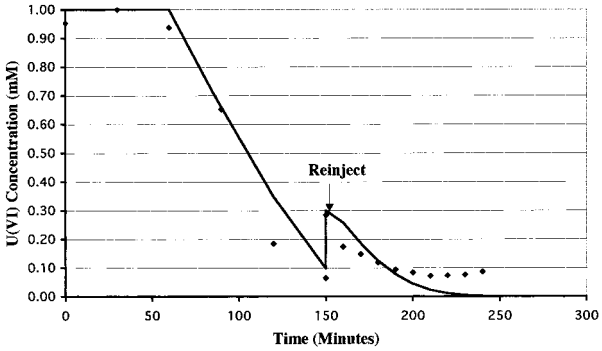

FIGURE 4. Results of U(VI) reinjection after initial U(VI) amount (1 mM) is 90% reduced. Data points represent the reduction of 1 mM uranyl acetate with subsequent reinjection of 0.2 mM uranyl acetate at $t = 150$ min. The model prediction line of eqs 6 and 7 for U(VI) reduction overlays the experimental data points. For modeling, the data regions of peak reduction were broken down into two regions: (1) reduction prior to reinjection and (2) reduction after the 0.2 mM U(VI) reinject. Cell concentration was 0.63 mg dry wt cells/mL with an initial lag time of 60 min.

after the initial 1 mM injection, to return the solution phase U(VI) concentration to 0.3 mM. U(VI) reduction with no lag time was subsequently observed (Figure 4), indicating that the U(VI) reduction mechanism was active during this secondary exposure and no longer rate limiting. This facet is important for continual U(VI) reduction in an SRB biotreatment scheme, where initial exposure followed by secondary, pulse, or continual exposure would be part of the design scheme. For scaling-up this bioreduction process, it is important to evaluate the kinetics relative to experimental operation, conditions, and parameters.

Kinetic Modeling. Nongrowth Monod kinetic models were fit to the data by segregating the data into two regions for model fitting (Figure 5): region 1 is the lag time (t_L) and region 2 is the period of U(VI) reduction.

Model 1. For the $t > t_L$ region, the first model applied was a simple Monod nongrowth model where

$$\frac{dU}{dt} = \left[\frac{-k_U UX}{K_U + U}\right] \tag{2}$$

and k_U is a utilization constant (maximum specific U(VI) reduction rate) in mM U/min·mg SRB, U is the uranium concentration in mM, X is the SRB cell concentration in mg dry wt cells/mL, and K_U is the Monod half-saturation coefficient in mM U. The nongrowth model (eq 2) was fit to the experimental data in the steepest U(VI) reduction range (0.9−0.2 mM), region 2′ of Figure 5; fitting the data well with an $r^2 = 0.9$ or better and yielded values for k_U and K_U. K_U was fixed at the values determined for high X because kinetic analysis of the data suggested it was the same for all experiments, i.e., less scatter was observed at higher cell concentrations. Statistical paired t-tests demonstrated that the model values for K_U for all X's were not from a significantly different population. k_U was fixed at the values determined for high X because kinetic analysis of the data suggested it was asymptotically approaching constant values independent of X with an applied logarithmic fit. Thus, the value of k_U at higher X gives the best estimate of constant k_U. For these reasons, K_U and k_U were fixed for further modeling at the average value obtained from experiments at 1.3 mg dry wt/ml cell concentration to limit degrees of freedom. However, the Monod nongrowth model does not describe the slower rate of initial U(VI) removal. A second model was developed to incorporate the apparent effect of X on k_U as seen from the model 1 fit to the data.

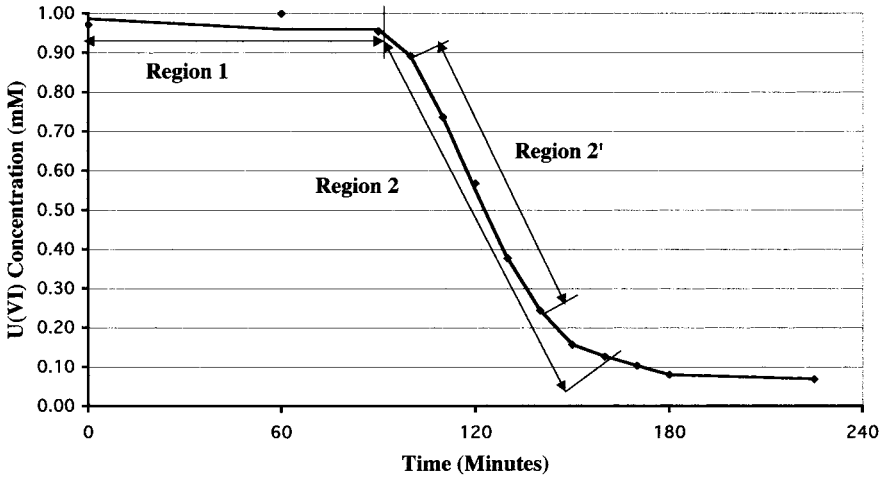

FIGURE 5. Regions of interest on a typical experimental data curve for modeling purposes. Region 1 is the lag time, prior to initiation of U(VI) reduction. Region 2 is when U(VI) reduction commences and is completed. Region 2′ is where modeling of U(VI) reduction is possible by a nongrowth Monod model between 0.9 and 0.2 mM U(VI).

TABLE 2. Kinetic Values Determined from Experimental Data[a]

species	k_r	k_U	K_r	K_U	K_R
mixed culture	0.020 ± 0.002	0.023 ± 0.003	29.7 ± 5.10	0.248 ± 0.075	0.0008 ± 0.0001
D. desulfuricans		0.030 ± 0.0001		0.499 ± 0.0001	

[a] The utilization constant (maximum specific U(VI) reduction rate) k_U are in mM U/min·mg SRB, k_r is a production rate constant for the rate limiting reactant R in mM U/min·mg SRB, while the Monod half-saturation coefficients K_r and K_U have units of mM U, and the Monod half-saturation coefficient for the unknown rate limiting reactant R defined as K_R has units in mM R.

Model 2. To better describe the slower initial rate, a second model was proposed based on a modified Monod nongrowth model that includes the production of a rate-limiting reactant.It was assumed that prior to the onset of observable U(VI) reduction, i.e., the time period of demonstrated lag, an unknown rate-limiting reactant R is produced to a level that then allows for U(VI) reduction. Lovley et al. (*19*) showed that cytochrome c_3 was responsible for U(VI) reduction, but other cofactors and enzymes required for U(VI) reduction have not been defined. Reactant R could be a metal binding protein, a metal reductase enzyme, an inducible enzyme necessary for electron transport, a cell concentration dependent component, or some other biochemical cofactor. At time $t \leq t_L$ no U reduction is in progress and

$$\frac{dU}{dt} = 0, \quad U = U_0, \quad \text{and } R = 0 \tag{3}$$

where U is the uranium concentration in mM, U_0 is the initial U concentration in mM, and R is the rate-limiting reactant in mM. At time $t > t_L$

$$\frac{dU}{dt} = \left[\frac{-k_U UX}{k_U + U}\right]\left\{\frac{R}{K_R + R}\right\} \tag{4}$$

where

$$\frac{dU}{dt} = \frac{k_r UX}{K_r + U} \tag{5}$$

where X is the SRB cell concentration in mg dry wt/ml, k_r and k_U are rate constants (maximum specific U(VI) reduction rate) in mM U/min·mg SRB, K_r and K_U are Monod half-saturation coefficients in mM U, and K_R is a Monod half-saturation coefficient in mM R for the rate-limiting reactant, R. To fit the model with determined experimental data, eqs 4 and 5 are solved for U and R by the following equations

$$U_{n+1} = U_n - \left[\frac{k_U U_n X_n}{K_U + U_n}\right]\left\{\frac{R_n}{K_R + R_n}\right\}(t_{n+1} - t_n) \tag{6}$$

$$R_{n+1} = R_n + \left[\frac{k_r U_n X_n}{K_r + U_n}\right]\{t_{n+1} - t_n\} \tag{7}$$

where n terms represent data at the time point of interest and n+1 terms represent data for the next time point. Minimizing the sum of the errors squared between experimentally determined U concentrations and predicted U concentrations from eq 6 with the Microsoft EXCEL SOLVER function against the three parameters k_r, K_r, and K_R was completed after previously determining best fit values for k_U and K_U from the modeling effort with eq 2. This yields simulated U concentrations that can be compared with experimentally determined concentrations.

The modified Monod model was applied to all experimental data sets for the mixed SRB culture to determine individual experimental values for the three constants defined by the model. The values were then averaged and are shown in Table 2. Figure 6 shows 1 mM U(VI) reduction with high, medium, and low mixed SRB cell concentrations, with experimentally individual inverse models imposed over the experimental data points. Statistical comparisons were made between the experimentally determined data and the best fit model defined by the five constants by using coefficients of determination, r^2. In 90% of the experiments, r^2 was 0.91 or better. For the pure SRB culture, data were not generated across a range of cell concentrations as done with the mixed SRB culture. The comparable kinetic values for both the mixed SRB and pure cell cultures for the reduction of 1 mM U(VI) are similar as seen in Table 2.

Truex et al. (*8*) found that Monod based reaction kinetics described uranium reduction by the Fe(III) reducing bacterium *S. alga* strain BrY with initial U(VI) concentrations

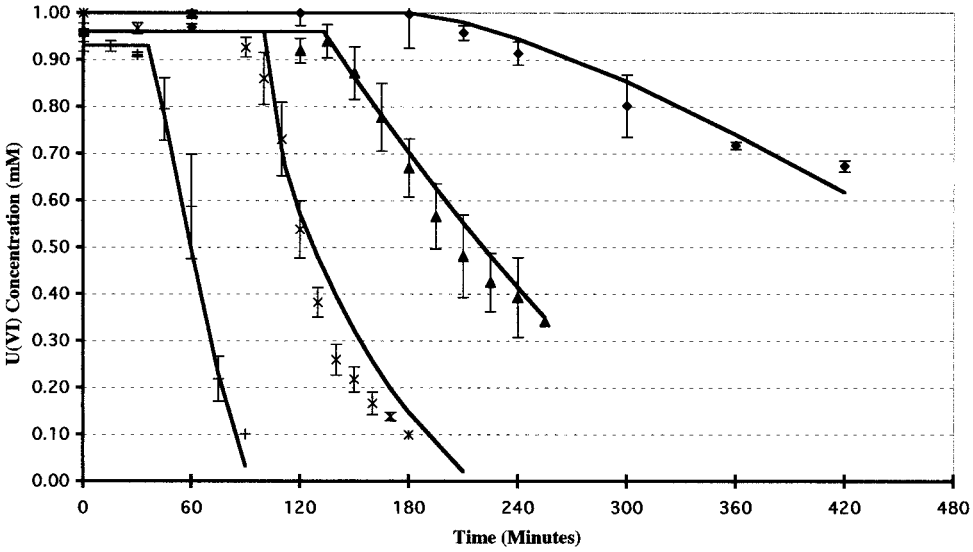

FIGURE 6. Soluble U(VI) experimental data with inverse model fitting. Initial U concentration is equal to 1 mM with varying mixed SRB cell concentration. Data points represent averaged experimental data sets (3); lines represent model predicted outcomes for those points. Modeled lines were determined with three (k_r, K_r, and K_R) of the five constants varying slightly from those reported as each line is fit specifically to these points, not averaged. Each data series cell concentration is in mg dry wt cells/mL: (+) 1.26, (✱) 0.42, (▲) 0.29, and (◆) 0.18. Error bars represent ± standard error.

ranging from 1.7 to 0.01 mM and a biomass of 0.55 mg dry wt cells/mL at 22 °C. A maximum specific U(VI) reduction rate of 2.37 μM U(VI)/mg-biomass-h and a Monod half-saturation constant of 0.132 mM U(VI) were estimated from the data. The Monod half-saturation coefficient, K_U, for the mixed SRB culture from this study, 0.25 mM U(VI), is similar to the half-saturation constant from Truex et al. (*8*) as is the value of 0.5 mM U(VI) for the *D. desulfuricans* in this study. In contrast, the maximum specific U(VI) reduction rate, k_U, for the mixed SRB culture of 1.4×10^3 μM U/mg SRB-h and for the *D. desulfuricans* culture of 1.8×10^3 μM U/mg SRB-hr are 3 orders of magnitude higher than that of *S. alga* strain BrY. Both the wild-type SRB and pure SRB cultures have a faster U(VI) reduction rate than the *S. alga*; therefore, less contact time is required in batch or in a continuous flow system for the removal of U(VI) from a contaminated water.

Lovley and Phillips showed that *D. desulfuricans* could reduce an initial 1 mM U(VI) concentration down to 0.1 mM in 3−4 h (*6*) with an initial biomass concentration of approximately 0.2−1.0 mg dry wt cells/mL (0.5 mg protein/mg dry wt conversion assumed per Bailey and Ollis (*33*)) at 35 °C. A decrease in reaction temperature of 35−20 °C would produce at least a 50% decrease in reaction rate (*33*). Thus, at a comparable temperature of this study, U(VI) reduction by *D. desulfuricans* (ATCC 29577) would be expected to take 6−8 h. For the mixed culture reported here, U(VI) was reduced from 1 to 0.1 mM in approximately 30 min after the lag phase at 21 °C (Figure 1). While the growth conditions used herein produced a lag; they also resulted in rapid kinetics. Additional investigation into operation conditions necessary to minimize the lag for chemostat grown cells is needed to further optimize this process.

Microscopy. Black precipitates were formed over the experimental time and were usually associated with aggregates of bacteria as viewed by phase contrast microscopy at 1250×. The 20−50 μm in size bacteria/precipitate aggregates settle quickly to the bottom of the experimental flask when stirring ceases. Further examination of the bacteria and precipitate by TEM (Figure 7) showed the reduced U by the highly electron-opaque regions observed in multiple images. The electron dense mass of material appears to be associated with the cells extracellularly and appears to be emanating from the periplasmic space of the SRB cells. This

FIGURE 7. TEM photo of the mixed SRB cell culture after approximately 4 h exposure to 1 mM uranyl acetate. Cells appear not to be coated with enzymatically reduced uraninite but rather to produce an amorphous size uraninite particle emitted from the cells. Pieces of these flocs can be large and attached to the cells, as seen in the center; still others can be quite small, broken off, and disassociated with the cells as seen by the additional surrounding electron opaque areas. Only the experimental uranyl acetate concentration produced this image; no additional U was added as is typically performed with TEM cell preparations. Photo is taken at approximately 16 000×. The SRB cells themselves are approximately 1 × 3 μm.

was also observed by Gorby and Lovley (*25*) with GS-15 (*Geobacter metalireducens*) and Lovley and Phillips with *Desulfovibrio desulfuricans* (*6*). The material is amorphous

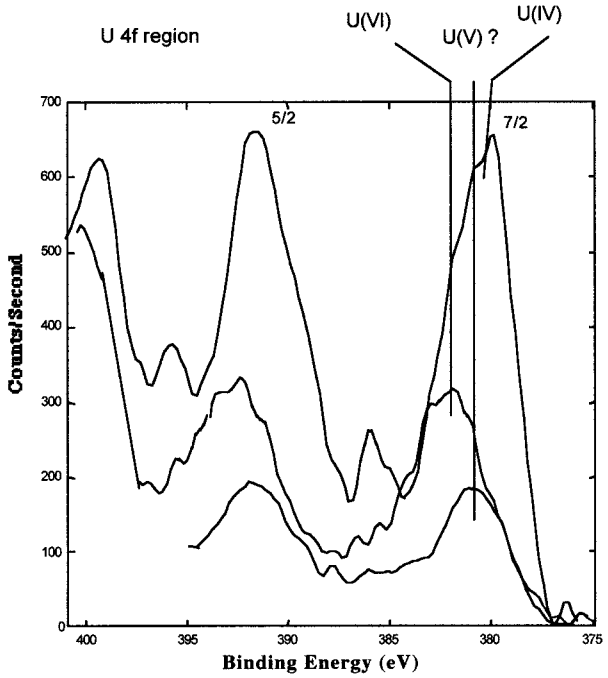

FIGURE 8. X-ray photoelectron spectroscopy obtained from the surfaces of the bacteria/U samples over the nitrogen 1s and uranium 4f regions. Each sample differs from the other, as evidenced by the different binding energy shifts. The peaks are rather broad, indicating a mixture of chemical states for each sample, probably with some overlap between samples. A binding energy of 380.0 eV and shake-up satellite peaks seen about 6 eV above both the $4f_{5/2}$ and $4f_{7/2}$ peaks clearly identify U(IV) as the dominant oxidation state for SRB cells after 3.5 h contact time with U(VI). The binding energy measured for experimental media with U(VI) only, of 382.2 eV is consistent with U(VI). A second 3.5 h SRB cell/U(VI) contact time sample shows binding energies that are intermediate between U(IV) and U(VI), suggesting U(V).

and finely grained, with nanometer sized particles aggregated to form micrometer size particles that can easily disassociate from the cells, as seen by the small aggregates around the cells.

XPS. X-ray photoelectron spectroscopy can identify valence states on a surface, determine atomic ratios of elements on that surface, and identify surface chemical states based upon core level peak shifts from spectra of amorphous or crystalline solids. When combined with spectra of known compounds, substrates, and other control samples, the composition of a surface bound species and the dominant sorption mechanisms can be determined. Examination of the bacteria/precipitate material by XPS indicates that the uranium is predominantly in the form of the mineral uraninite, $UO_{2(S)}$. Figure 8 is a spectral output displaying the dominant uraninite region. The U 4f region's binding energy in eV shows that the bacterially produced precipitate is predominantly composed of U(IV) as UO_2, with some U(VI) present, and less U(V). X-ray diffraction analysis has previously identified U(IV) as uraninite following the enzymatic reduction of U(VI) by SRB (*6*). Francis et al. (*30*) observed U(VI), U(V), and U(IV) extracellularly in a *Clostridium sp.* with XPS and the possible observance of a little described species of U, U(III), existing intracellularly using a synchrotron source and X-ray analyzed near-edge spectroscopy, XANES.

Other Results. The mixed culture of SRB cells used for this study is dominated by a Gram negative motile rod, with the occasional presence of a Gram positive coccus. Initially, the cells appear as small rods, $\approx 0.5 \times 1.0$ μm, and then progress to a longer, curved, or spirella appearance $\approx 1.0 \times 3.0$ μm vibrio shape. An actively growing culture was dominated by a large number of motile cells. Attempts to grow the mixed SRB under anaerobic condition in a CSTR with uranyl acetate as the sole electron acceptor were unsuccessful. Lovley and Phillips found that *D. desulfuricans* (ATCC 29577) would not grow on U(VI) (*6*). Kinetic experiments were carried out in polycarbonate septum flasks after finding that 15% of the U(VI) sorbed to the walls of glass septum flasks after 4 h of contact. Polycarbonate sorption of U(VI) was found to be 4% over the same time period.

Implications of This Research. (1) The kinetic parameters developed herein can be used to design U(VI) reducing bioreactors such as sequencing batch reactors, submerged packed columns, or continuously stirred tank reactors. Issues of cell separation from growth media, carryover of growth media to the U(VI) reducing bioreactor, and cell/precipitate separation have not been addressed. Further investigation to integrate a U(VI) reducing bioreactor into a complete treatment train is needed. (2) U(VI) reduction and lag time are both functions of cell concentration. While high cell concentration appears to mitigate the lag effect, economically achieving high cell concentration in a U(VI) reducing bioreactor is an issue. Further investigation is desirable to optimize the continuous growth of SRB to minimize the lag time. (3) Both the growth of two cultures of SRB tested and U(VI) reduction and precipitation can be carried out at room temperature. The potential for operation at lower temperatures appears possible as increased cell mass was observed in the mixed SRB cultures stored at 4 °C. Further kinetic analysis at temperatures less than 20 °C is needed as mine and groundwaters may have temperatures in the 5−15 °C range. (4) Selecting an SRB culture, either wild-type or pure, over other bacterial genera may be kinetically favorable for the removal of uranium from contaminated waters as indicated by the comparison with the natural iron reducer *S. alga* (*8*). (5) Prolonged exposure of this wild-type SRB culture to U(VI) concentrations as high as 1 mM appear to have no toxic effect on the cells as observed by continuous motility of the cells before, during, and after exposure to uranium. Lovley and Phillips observed no toxicity at concentrations as high as 5 mM U (*6*). Further studies can employ this mixed SRB culture in conjunction with naturally occurring U containing waters or bicarbonately processed U containing soil extractants to observe to what extent U removal is possible as reported for a pure culture of *Desulfovibrio desulfuricans* (*34*, *35*). (6) The parameters determined first using model 1 being fixed and applied in model 2 allow for the thorough evaluation of the bioreduction of U(VI) by two separate SRB cell cultures, herein, which may be applied to other SRB cultures as well as other cultures and/or processes demonstrating a lag time.

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