Modeling Reduction of Uranium U(VI) under Variable Sulfate Concentrations by Sulfate-Reducing Bacteria

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The kinetics for the reduction of sulfate alone and for concurrent uranium [U(VI)] and sulfate reduction, by mixed and pure cultures of sulfate-reducing bacteria (SRB) at 21 ± 3°C were studied. The mixed culture contained the SRB *Desulfovibrio vulgaris* along with a *Clostridium* sp. determined via 16S ribosomal DNA analysis. The pure culture was *Desulfovibrio desulfuricans* (ATCC 7757). A zero-order model best fit the data for the reduction of sulfate from 0.1 to 10 mM. A lag time occurred below cell concentrations of 0.1 mg (dry weight) of cells/ml. For the mixed culture, average values for the maximum specific reaction rate, V_{max}, ranged from 2.4 ± 0.2 μmol of sulfate/mg (dry weight) of SRB · h^{-1}) at 0.25 mM sulfate to 5.0 ± 1.1 μmol of sulfate/mg (dry weight) of SRB · h^{-1} at 10 mM sulfate (average cell concentration, 0.52 mg [dry weight]/ml). For the pure culture, V_{max} was 1.6 ± 0.2 μmol of sulfate/mg (dry weight) of SRB · h^{-1} at 1 mM sulfate (0.29 mg [dry weight] of cells/ml). When both electron acceptors were present, sulfate reduction remained zero order for both cultures, while uranium reduction was first order, with rate constants of 0.071 ± 0.003 mg (dry weight) of cells/ml · min^{-1} for the mixed culture and 0.137 ± 0.016 mg (dry weight) of cells/ml · min^{-1} (U_0 = 1 mM) for the *D. desulfuricans* culture. Both cultures exhibited a faster rate of uranium reduction in the presence of sulfate and no lag time until the onset of U reduction in contrast to U alone. This kinetics information can be used to design an SRB-dominated biotreatment scheme for the removal of U(VI) from an aqueous source.

Sulfate-reducing bacteria (SRB) are important in the mobility of sulfur in the environment (32, 36). Both higher plants and animals depend upon microbially produced reduced sulfur for acquisition in their own metabolism. SRB use sulfate as an oxidizing agent for the dissimilation of an organic substance (herein, lactate). Several species of SRB have been described for their ability to reduce inorganic aqueous ions in solution. SRB have been shown to metabolize iron [Fe(III)], chromium [Cr(VI)], uranium [U(VI)], manganese [Mn(IV)], and technetium [Tc(VII)], among others (20, 21, 22, 23, 26, 39). Models for the SRB reduction of these metals and radionuclides can be used to develop and design treatment systems employing SRB for bioremediation. For example, bioreduction of soluble hexavalent uranium [U(VI)] to insoluble tetravalent uranium [U(IV)] can be utilized to remove soluble uranium from groundwaters, mine waters, or secondary waste streams.

In addition to SRB, a number of bacterial species have been described as capable of reducing U(VI) to U(IV), including *Geobacter metallireducens* (25) (previously reported as GS-15 [11]), *Shewanella putrefaciens* (25), and *Shewanella alga* strain BrY (previously reported as BrY, or *Shewanella halotolerans* strain BrY [3]). A kinetic study for the reduction of U(VI) has been described for the iron-reducing *S. alga* strain BrY (40) but not for SRB. Before a biotreatment scheme can be designed and implemented, studies must go beyond the identification of novel processes, to quantifying the kinetics of such a process. Because of the metabolic diversity of the SRB, it is important to identify the species involved in the processes studied. Since sulfate reduction rates depend upon the species used and experimental conditions, it is important to characterize sulfate reduction for the mixed culture used in previous

uranium reduction experiments (38) that were performed under repeatable, uniform conditions.

The goals of this study were to characterize the mixed SRB culture previously isolated (38) and to develop models to describe sulfate reduction alone and sulfate reduction with concurrent uranium reduction. Utilizing a mixed cell culture was viewed as an advantage in that in an operational biotreatment scheme, purity of culture in treating large volumes of water will be difficult to maintain. Understanding U(VI) reduction by SRB in a consortium is more likely to be relevant for an operational condition. The nature of the mixed SRB culture was examined via 16S ribosomal DNA (rDNA) analysis. Models for separate sulfate reduction and concurrent sulfate and uranium reduction were fit to data generated by both a mixed and a pure culture of SRB. Sulfate and cell concentrations were varied during these experiments, which were conducted at room temperature, 21 ± 3°C. This is at the upper end of temperatures expected in natural waters that could be treated. Sulfate concentration is expected to be a variable in the treatment of uranium-contaminated waters, and cell concentration is an operational variable for treatment design. These kinetic determinations were made under conditions of insignificant growth (in the absence of nutrients other than a lactate carbon source; the sulfate concentrations present could facilitate growth, but experimental time courses were too short), and under anaerobic batch conditions, to isolate the enzymatic reductive processes of both electron acceptors from those of cellular growth processes. Quantification with modeling of the sulfate reduction rate alone and the uranium reduction rate in the presence of sulfate is important for the design of a treatment system employing SRB to remove metals and radionuclides, likely to be operational under dynamic, nonstatic conditions.

MATERIALS AND METHODS

Bacterial cultures. The mixed culture of SRB was obtained as previously described (38). All chemicals utilized for these studies were reagent grade or

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better and were used without further purification. Continuous cultivation of SRB cells in a chemostat was carried out on an insulated magnetic stirrer in an anaerobic chamber (Bactron II, Sheldon Manufacturing, Cornelius, Oreg.) fed with anaerobic mixed gas (AMG) containing 90% N_2, 5% H_2, and 5% CO_2. The growth medium used was a modified Postgate C medium (36) containing potassium phosphate (mono) at 0.5 g/liter, ammonium chloride at 1.0 g/liter, sodium sulfate at 2.0 g/liter, calcium chloride at 0.06 g/liter, magnesium chloride at 0.06 g/liter, iron sulfate at 0.005 g/liter, sodium citrate at 0.3 g/liter, yeast extract at 0.1 g/liter, and sodium lactate (60% syrup) at 15 ml/liter. Cell concentrations ranged from 0.1 to 0.15 mg (dry weight)/ml of growth medium from the 250- or 500-ml square polycarbonate chemostats with a hydraulic residence time of 8 to 12 h. The average temperature during growth in the chemostat on the chamber stage was 21 ± 3°C. The *Desulfovibrio desulfuricans* (ATCC 7757) culture was grown in continuous fashion with an 8-h hydraulic residence time as described for the mixed culture above. Refrigerated stocks of both cultures were transferred to freshly prepared media approximately every 2 weeks.

To facilitate comparison of the mixed cell culture used in these studies with data by other investigators, the biomass equivalents were determined. By using a Petroff-Hausser counting chamber, the average cell count was 1.5×10^9 cells/ml from a growth chemostat, for an average of 3 mg of cells (wet weight)/ml and 0.148 mg of cells (dry weight)/ml as determined by a standard dry weight analysis (10). The values reported for dry weights of SRB cells per milliliter reflect the cell concentration per milliliter of medium used for the actual batch experimental conditions, not those of growth.

Molecular characterization. For molecular characterization of the mixed cell culture used in this and a previous study (38), the methods of Hugenholtz et al. were followed (12). Bead beating for cellular disruption and DNA extraction was followed by PCR with the primers 8F universal forward and 1492R universal reverse (18). *Taq* polymerase-amplified PCR products were directly inserted into a PCR4-TOPO vector, followed by reaction with One Shot Competent Cells in a TOPO TA Cloning Kit (Invitrogen, Carlsbad, Calif.). Restriction fragment length polymorphism (RFLP) analysis was carried out on a mini-prepped, T3–T7 primer-amplified PCR (Invitrogen) clone colony DNA product, using the restriction enzymes *Hin*PI and *Msp*I (New England Biolabs, Beverly, Mass.). After bead beating, all steps were performed using a 96-well format.

After determination of nonidentical banding patterns of PCR-amplified positive clones on an RFLP gel, the amplified DNA fragments were sent to the Forsythe Dental Center in Boston, Mass., for sequence analysis. Purified DNA from PCR was sequenced using an ABI prism cycle-sequencing kit (dRhodamine Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS; Perkin-Elmer). The manufacturer's protocol was followed. Sequencing was performed using an ABI 377 DNA sequencer. DNA sequences were identified by using the BLAST (basic local alignment search tool) server of the National Center for Biotechnology Information over the World Wide Web (1). The 16S rRNA gene sequences were manually aligned with other sequences by using the Ribosomal Database Project (27; http://www.cme.msu.edu/RDP/) and the ARB database (http://pop.mikro.biologie.tu-muenchen.de/pub/ARB/) taxonomic listings. Percent identity was calculated by using the Lane mask (17) with no right correction.

Kinetics studies. As described elsewhere, a method was developed to examine the enzymatic reduction kinetic of U(VI) to U(IV) by SRB using the radionuclide ^{233}U as a tracer (38). For sulfate reduction experiments, this method, in combination with the methods of Ingvorsen and colleagues (13, 14), was applied by substituting $Na_2{}^{35}SO_4$ as the radionuclide tracer. Briefly, a selected amount of the SRB cell mass (0.2 to 1.3 mg [dry weight]/ml per experiment) was obtained from a growth chemostat, washed in a sulfate-free sodium bicarbonate (2.5 g/liter) buffer, and then suspended anaerobically in a sterile medium (10 mM lactate–20 mM sodium bicarbonate) with a small magnetic stir bar, in sterile 30-ml polycarbonate septum flasks sealed with Teflon-lined butyl rubber stoppers (38). The headspace in these sealed reaction vessels contained ~10 ml of the anaerobic chamber's AMG. The turbid culture was in contact with the buffered carbon source/electron donor for ≤15 min prior to the addition of sulfate. Sulfate was added from a 10 mM stock solution spiked with $Na_2{}^{35}SO_4$ as a tracer, typically 25 μl of a 45,000-dpm/ml stock $Na_2{}^{35}SO_4$ solution (purchased from Isotope Products Laboratory, Burbank, Calif.). The Na_2SO_4 electron acceptor was mixed with the $Na_2{}^{35}SO_4$ spike in a syringe and fed to the cells by injection through the septum. For experiments with both electron acceptors, U(VI) was added as uranyl acetate, $UO_2(CH_3COO)_2 \cdot 2H_2O$, from a 10 mM stock solution spiked with $^{233}U(VI)$, typically 200 μl of a 23,670-dpm/ml stock $^{233}U(VI)$ solution (purchased from Isotope Products Laboratory). The U(VI) electron acceptor was mixed with the $^{233}U(VI)$ spike in a syringe and fed to the cells by the same method as the sulfate. The injection of electron acceptors set the time at $t = 0$ and marked the start of the kinetic experiment. The initial pH of these kinetics experiments was 7.2 ± 0.2.

The anaerobic polycarbonate flasks were stirred on insulated magnetic stir motors at ambient room conditions (21 ± 3°C). Samples (1 1/2 ml) were removed by syringe at times of interest. The removed aliquot of the solution was then placed in a 1.5-ml polystyrene microcentrifuge tube containing zinc acetate to give a final concentration of 6 mM (100 μl of a 0.1 M solution), capped tightly, and spun at $16,000 \times g$ for 3 min. The zinc acetate immediately preserves produced ^{35}S sulfides (14), forming a precipitating $Zn^{35}S$. Zn is not known to complex with sulfate in solution, and this was experimentally validated. Almost 1.5 ml (cell suspension less pellet volume [≈50 μl]) of supernatant solution was

collected by pipette and added to 20-ml plastic scintillation vials containing 10 ml of Ultima Gold scintillation cocktail (Packard Instrument, Meriden, Conn.). The cell pellet was suspended in 0.5 ml of deionized water and transferred to a scintillation vial, and 1 ml of deionized water was added for volume equalization.

The mass balance of uranium and/or sulfate was checked at least three times per experiment. The soluble and the precipitated isotope in one of duplicate samples were separated and prepared as described, and the other sample was blended directly with scintillation cocktail. Over the course of an experiment, some sulfides accumulated in the anaerobic headspace of the reaction vessel, a portion of which contain ^{35}S. To account for this mass, any remaining cells in media at the end of an experiment were removed via syringe. Six milliliters of 2% (wt/vol) zinc acetate was then added through the septum to precipitate the gaseous sulfides, which were then removed in 2-ml aliquots, blended with scintillation cocktail, and counted. This additional sulfide activity was added to the total activity, and balanced the mass between the beginning and the end of the experiment. Vials were analyzed on either a model 1600TR or a model 2500TR Packard Tri-Carb Liquid Scintillation Analyzer for 10 min/vial. Separation of activity was easily accomplished by taking advantage of the β emission of ^{35}S at 167 keV and the α emission of ^{233}U at 4.824 and 4.783 MeV. This allows for tracking of the uranium and sulfate reduction in the pellet and supernatant samples. Typical counting errors were 5% or less. Sulfide determinations were made using a method developed by Updegraff and Wren (41) with 0.01 M silver nitrate as a titrant.

A test was conducted to determine how much sulfate adsorbs to the walls of the experimental reaction vessel, i.e., the Teflon-coated butyl rubber-sealed 30-ml polycarbonate septum flask. Using $Na_2{}^{35}SO_4$ as a tracer for sodium sulfate sorption, we found that 4.7% of the sulfate adsorbs to the walls of the vessel over 4 h. Using the same method, sulfate sorption to the walls of 100-ml glass serum bottles was found to be 9.5%. Spear et al. (38) found that 15% of the initial U concentration sorbed to the walls of traditional glass serum bottles, and that was reduced to 4% in polycarbonate. Because of these adsorptive effects, glass vessels were not used in these studies.

Control experiments were performed by the same method with the addition of 10 mM sodium molybdate, a sulfate analog (36) for the enzyme cytochrome c_3, one of the enzymes responsible for the reduction of both sulfate and uranium (21) prior to the addition of Na_2SO_4 or uranyl acetate. In the presence of the sodium molybdate, no sulfate or uranium reduction by the SRB was observed. Data analysis and kinetic modeling were conducted with the data from the scintillation method on Microsoft EXCEL spreadsheets.

RESULTS

16S rRNA sequence. Cells from the isolated mixed culture initially appeared to be all of vibrio shape, all stained gram negative, and exhibited an active production of sulfide. With time (months), small gram-positive rods were observed and spores were periodically present. At any one time the gram-positive species represented 0 to 10% of the total cells present. For these reasons molecular characterization was needed to define the culture. The decision to sequence 500 bp of the 16S rRNA gene fragments from clones of this mixed culture was made because of differences in banding patterns on RFLP gels. Two hundred twenty clones were subjected to RFLP analysis, and there appeared to be two distinct banding patterns, with one pattern far more prevalent than the other, by a ratio of 10:1. The most prevalent pattern was found to be 99% identical to that of a *Desulfovibrio vulgaris* strain (PT-2; accession number M98496 as described by Kane et al. [16]). The other pattern was found to be similar to that of a unique, low-G+C, gram-positive, anaerobic genus (94% identical to *Clostridium butyricum* over 500 bp considered; accession number M59085 as described by C. R. Woese, D. Yang, and L. Mandelco [unpublished data]). Sequences were manually aligned with similar sequences using the BLAST server. RFLP analysis performed on the culture 1 year earlier yielded the same patterns, indicating no change in culture constituents. The SRB culture was initially isolated by picking one colony from an agar deep tube method (36) at a high dilution; with time, the culture came to be contaminated by the spore-forming *Clostridium* sp. This was viewed as an advantage because such a contaminant would likely come to be present in a bioremediation treatment scheme. This mixed cell culture was stable over a period of 5 years and showed little variability.

TABLE 1. Maximum specific reaction rate coefficients for the mixed cell culture reducing sulfate

Initial sulfate concn (mM)	Initial cell concn (mg [dry wt]/ml)	k_0 (μM SO_4^{2-}/mg [dry wt] of SRB/ml $\cdot$ min^{-1})	V_{max} (μmol SO_4^{2-}/mg [dry wt] of SRB $\cdot$ h^{-1})
0.1	0.07 ± 0.01[a]	31 ± 6	1.9 ± 0.4
0.1	0.10 ± 0.02[a]	51 ± 1	3.1 ± 0.1
0.25	0.57 ± 0.01	40 ± 3	2.4 ± 0.2
1.0	0.56 ± 0.01	33 ± 1	2.0 ± 0.1
10	0.46 ± 0.01	83 ± 19	5.0 ± 1.1
100	0.47 ± 0.01	ND[b]	ND

[a] For 0.1 mM sulfate, the cell concentration is lower because at higher cell concentrations the reduction of the sulfate is too rapid to determine experimentally.

[b] ND, not determined because of insignificant sulfate reduction.

Sulfate reduction kinetics. The reduction of sulfate concentrations ranging from 0.1 to 100 mM was examined for the mixed cell culture. Cell concentrations were similar for all sulfate concentrations except 0.1 mM, where the cell concentration was approximately 1/10 of that in the other sulfate reduction experiments (Table 1). For an initial sulfate concentration (S_0) of 100 mM, there was no measurable reduction over 3 h. For an S_0 of 10 mM, 45% of the sulfate was reduced in 3 h by a similar cell concentration. There was 100% removal of sulfate when S_0 was 1.0, 0.25, or 0.1 mM, as shown in Fig. 1.

Modeling of sulfate reduction. A zero-order model, with respect to sulfate concentration, best fit the experimental data where

$$S = S_0 - k_0 X(t - t_L) \quad (1)$$

and S is the model predicted millimolar concentration of sulfate, S_0 is the initial millimolar concentration of sulfate, k_0 is the maximum specific reaction rate coefficient expressed as the

millimolar concentration of SO_4^{2-} per milligram (dry weight) of cells per milliliter per minute, X is the bacterial cell concentration in milligrams (dry weight) per milliliter, t is time in minutes, and t_L is the lag time until the onset of sulfate reduction. A 30-min lag time was observed only with a low bacterial cell concentration (0.07 mg [dry weight] of cells/ml or lower) and an S_0 of 0.1 mM sulfate, as shown in Fig. 2. For an S_0 of 0.1 mM and an X_0 of 0.1 mg (dry weight) of cells/ml, there was no lag time. Reduction of sulfate alone proceeded without a lag for initial sulfate concentrations of 1 to 10 mM. The zero-order model is a simplification of Michaelis-Menten and Monod type kinetics at high substrate concentrations. Parameter equivalence between models can be given by

$$k_0 \equiv \frac{\mu_m}{Y} \equiv V_{max} \quad (2)$$

where k_0 is defined, μ_m is the Monod maximum specific growth rate constant in units of 1 h^{-1}, Y represents cell yield, expressed as mass of cells in milligrams per milligram of substrate used, and V_{max} is the Michaelis-Menten maximum substrate utilization rate constant, expressed as the millimolar concentration of SO_4^{2-} per milligram (dry weight) of cells per milliliter per minute. Zero-order model fits to sulfate reduction data are shown in Fig. 1, 2, and 3; correlation coefficients, r^2, were ≥0.92.

Figure 4 shows the reduction of 1 mM sulfate by both the mixed and pure cell cultures. The maximum specific rate coefficients are estimated to be the same when normalized to cell mass, where 33 ± 1 μM SO_4^{2-}/mg (dry weight) of SRB/ml $\cdot$ min^{-1}, with an r^2 of 0.99 for the mixed culture, and 26 ± 2 μM SO_4^{2-}/mg (dry weight) of SRB/ml $\cdot$ min^{-1}, with an r^2 of 0.96 (V_{max} = 1.6 μmol of SO_4^{2-}/mg [dry weight] $\cdot$ h^{-1}) for the pure culture, were calculated. The data indicate that *D. desulfuricans* (ATCC 7757) behaves much like *Desulfobacter postgatei* at 21 ± 3°C (13).
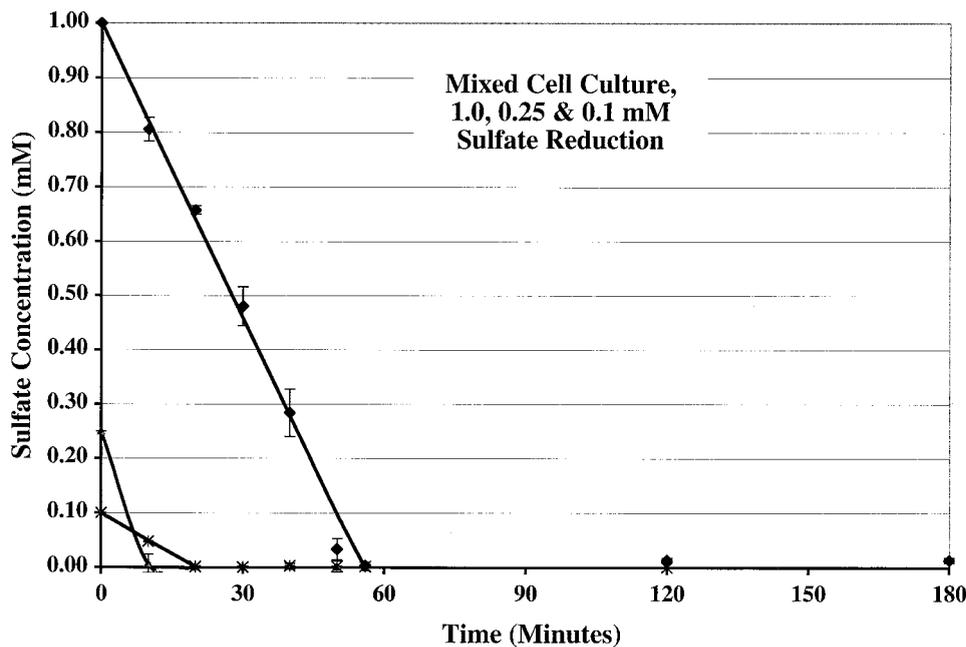

FIG. 1. Time course of sulfate reduction by the mixed cell culture fit with a zero-order model. Model lines are based on the coefficients of Table 1. Each set of data points represents an average of at least two experiments with the same mixed cell culture. ◆, S_0 = 1.0 mM and X_0 = 0.53 mg (dry weight) of cells/ml; ▲, S_0 = 0.25 mM and X_0 = 0.57 mg (dry weight) of cells/ml; ✳, S_0 = 0.1 mM and X_0 = 0.1 mg (dry weight) of cells/ml. Error bars, standard errors.

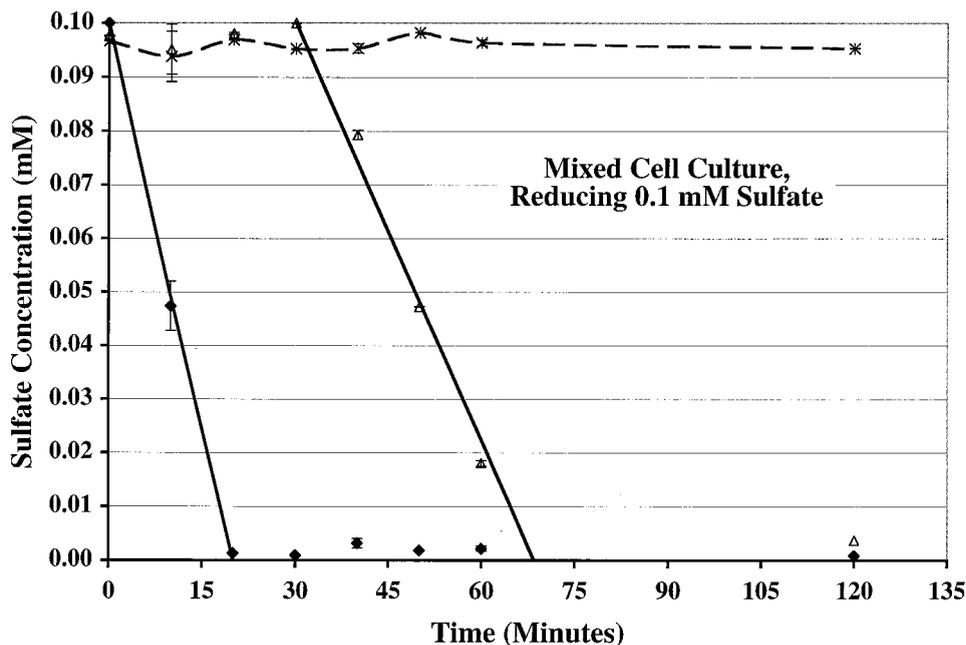FIG. 2. Reduction of 0.1 mM sulfate by low mixed-cell concentrations. ◆, 0.1 mg (dry weight) of cells/ml (no lag time); △, 0.07 mg (dry weight) of cells/ml (30-min lag time); ∗, 0.06 mg (dry weight) of cells/ml (no sulfate reduction). Zero-order model lines are fit through the 0.1- and 0.07-mg (dry weight)/ml data points, representing model values given in Table 1. Each data set represents averages from two experiments. Error bars, standard errors.

Uranium and sulfate reduction. Time courses for the reduction of soluble U(VI) to insoluble U(IV) and for the reduction of soluble sulfate to sulfide for a typical experiment are shown in Fig. 5. Rate constants were determined only for the removal of the U(VI) and sulfate. Uranium reduction was more rapid in the presence of sulfate than in its absence for both the mixed and pure cell cultures. Figure 6 shows data for the reduction of U(VI) alone (38) and for uranium reduction with sulfate [at electron equivalent amounts of U(VI) and sulfate] for the mixed cell culture. Because the reduction of sulfate to sulfide is an 8-electron transfer and the reduction of U(VI) to U(IV) is a 2-electron transfer, the sulfate concentration used was equivalent to 25% of the uranium concentration. A 90-minute lag time for the reduction of U(VI) only was decreased to 5 ±
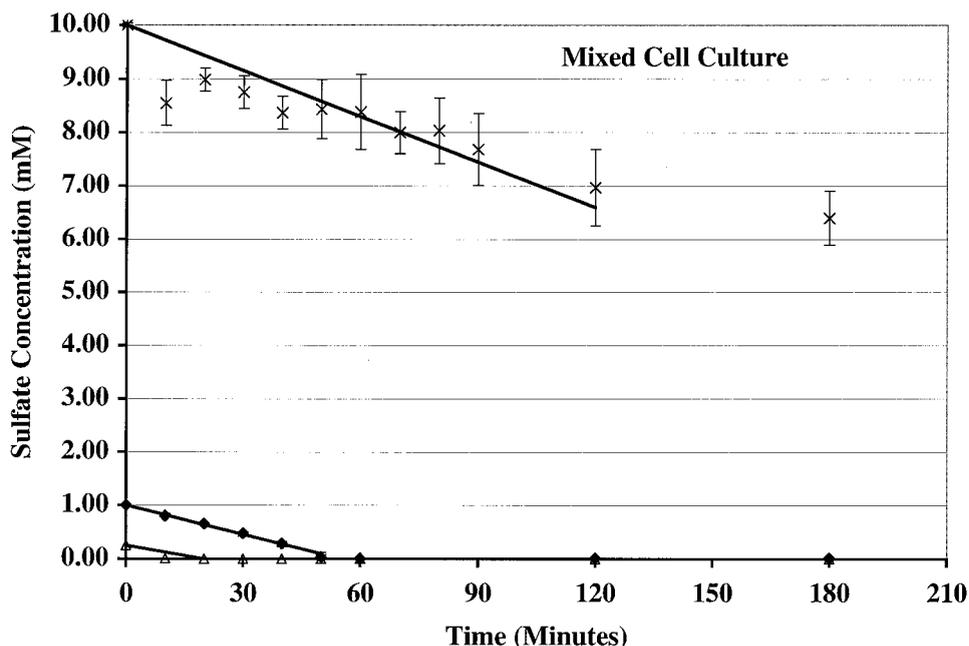

FIG. 3. Model simulations of variable sulfate concentration with approximately equal cell concentrations. ∗, $S_0 = 10$ mM and $X_0 = 0.49$ mg (dry weight) of cells/ml; ◆, $S_0 = 1.0$ mM and $X_0 = 0.53$ mg (dry weight) of cells/ml; △, $S_0 = 0.25$ mM and $X_0 = 0.57$ mg (dry weight) of cells/ml. Error bars, standard errors.
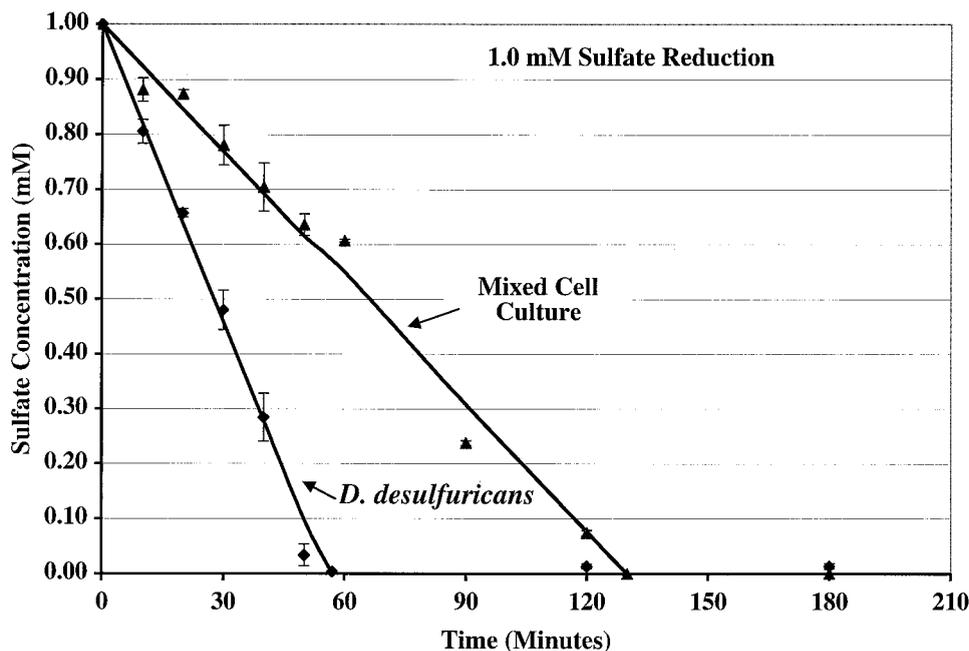
FIG. 4. Reduction of 1 mM sulfate by the mixed cell culture and by the pure culture of *D. desulfuricans*. ◆, 323.0 mg (wet weight) of cell mass, which is equivalent to 0.53 mg (dry weight)/ml of medium used in the batch experiment, for the mixed cell culture; ▲, 298.0 mg (wet weight) of cell mass, which is equivalent to 0.30 mg (dry weight)/ml of medium used in the batch experiment for the pure culture of *D. desulfuricans* (ATCC 7757). Zero-order model lines are fit to the plotted data. Error bars, standard errors.

5 min for U(VI) in the presence of sulfate for an initial U(VI) concentration (U_0) of 1 mM, an S_0 of 0.25 mM, and an X_0 of ≈0.5 mg (dry weight) of cells/ml.

Modeling of uranium and sulfate reduction. Under all experimental conditions tested, sulfate reduction was best fit by a zero-order model (equation 1), and uranium reduction was best fit by a first-order model. The first-order model fit to the U(VI) reduction data was

$$U = U_0 e^{-k_1 Xt} \quad (3)$$

where U is the model predicted millimolar concentration of uranium, U_0 is the initial millimolar concentration of uranium, k_1 is the first-order rate constant, expressed as milligrams (dry weight) of cells per milliliter per minute, and X and t are as defined above. A linearized form of equation 3 was fit to the data to determine the rate constant k_1,

$$\ln U = \ln U_0 - k_1 Xt \quad (4)$$

with units and terms as defined above. The model fits using average coefficients, and the data are shown in Fig. 7A for the mixed cell culture and in Fig. 7B for *D. desulfuricans* (ATCC 7757). In all cases the models fit the data with coefficients of determination, r^2, of 0.96 or higher. Figure 8 shows the respective model fits for uranium reduction in the presence of a sulfate concentration that might be found in freshwater. Figure 9 shows the uranium reduction kinetics in the presence of a higher, 10 mM sulfate concentration relevant for high-sulfate natural waters containing uranium.

DISCUSSION

Various aspects of the SRB reduction of U(VI) to U(IV) have been studied. Lovley and Phillips (21) found that *D. desulfuricans* was capable of uranium reduction. They later

demonstrated that cytochrome c_3 was an essential component of uranium reduction by *D. desulfuricans* (22). Ganesh et al. (9) considered the SRB reduction of U(VI) in organic complexes. Tebo and Obraztsova (39) identified an SRB capable of growth with U(VI) as an electron acceptor. Spear et al. (38) established the rate constants for uranium reduction by a mixed SRB culture and by *D. desulfuricans*. In all cases U(IV) was precipitated from solution as the mineral uraninite, UO_2. Three studies have considered the reduction of U in the presence of the native sulfate electron acceptor, and that was for a pure culture of *D. desulfuricans* (21, 30, 31, 40a). These studies however, have not modeled the kinetics involved across a range of solution conditions. A few reports have described the kinetics for the enzymatic reduction of sulfate alone under various conditions (8, 13, 14, 21, 28, 29, 33, 34, 37). Ingvorsen and Jørgensen (14) provided kinetics information for four SRB pure cultures at 20°C; Ingvorsen et al. (13) provided the kinetics for both batch and chemostat cultures at 30°C; and Sonne-Hansen et al. (37) provided the kinetics for sulfate reduction by two species of thermophilic SRB at 70°C.

Sulfate reduction. For *Desulfovibrio vulgaris* (Hildenborough), Ingvorsen and Jørgensen (14), found a V_{max} of 1.1 μmol of SO_4^{2-}/mg (dry weight) · h^{-1}. For *D. postgatei*, Ingvorsen et al. obtained a V_{max} of 4.2 μmol of SO_4^{2-}/mg (dry weight) · h^{-1} (13). The *Desulfovibrio* sp. identified in the mixed cell culture for this report has a V_{max} range of 2 to 5 μmol of SO_4^{2-}/mg (dry weight) · h^{-1} (Table 1), similar to the V_{max} for *D. postgatei* and for *D. vulgaris* (Hildenborough) at 21 ± 3°C (13, 14). Vester and Ingvorsen report that 4.1 × 10^{-14} mol of SO_4^{2-}/cell · day^{-1} could be reduced by a pure culture of *Desulfobulbus propionicus* by using a direct cell count method, and a value of 2.43 × 10^{-13} using a T-MPN (tracer most-probable-number) method to calculate cell number (42). The range in this study was 0.73 × 10^{-14} to 1.2 × 10^{-14} mol of SO_4^{2-}/cell ·
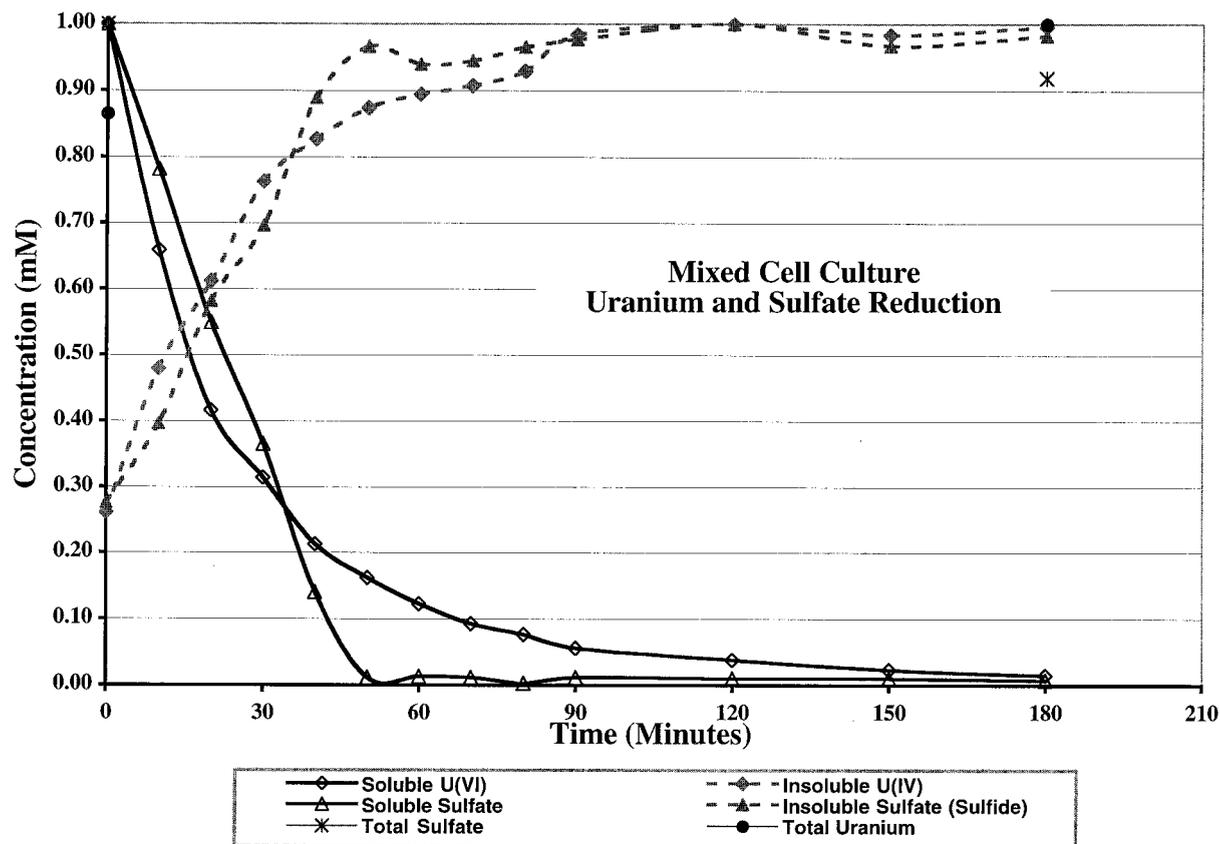
FIG. 5. Time course of 1 mM uranium and sulfate reduction by the mixed cell culture dominated by a *Desulfovibrio* sp. Insoluble U(IV) (as uraninite [38]) was collected in pellet fractions of sample aliquots, and insoluble ^{35}S was collected as insoluble sulfides after reacting with zinc acetate in sample aliquots. Solid circles at time zero and 180 min represent total activity of uranium, ^{233}U [U(VI) plus U(IV)], for mass balance accountability; starbursts at the same time points show total activity for ^{35}S ($^{35}SO_4^{2-}$ plus un-ionized sulfides [$^{35}S^{2-}$]) for mass balance. Data are for one experiment with 0.51 mg (dry weight) of cells/ml.

day^{-1}, consistent with those of Vester and Ingvorsen and others (13, 15).

A lag time until the onset of U(VI) reduction was previously observed for the mixed cell culture used here (38). This lag time was dependent upon cell concentration and ranged from 30 min at a cell concentration of 1.27 mg (dry weight) of cells/ml to 3 h at a low cell concentration of 0.18 mg (dry weight) of cells/ml. A lag time was also present for the pure culture of *D. desulfuricans* (ATCC 7757) that was approximately 30 min less than that of the mixed cell culture for the same cell concentration. Figure 2 shows a similar lag time for sulfate at low cell concentrations. Thus, for the mixed cell culture, a reproducible and predictable lag time until the onset of reduction for both the native electron acceptor and U(VI) is possible. The *D. desulfuricans* (ATCC 7757) culture was not tested at these low cell concentrations for the possibility of a lag time for sulfate reduction.

Ingvorsen et al. (13) observed a sulfate concentration-based threshold, whereby when sulfate concentration decreased low enough in their batch experimental system with both batch- and chemostat-grown cells, no reduction was evident. Figure 2 shows a cell concentration-based threshold, which could be analogous to the sulfate concentration threshold, by which the physiological state of the cells determines the amount of sulfate reduction possible (13).

Concurrent uranium and sulfate reduction. The design of a uranium removal biotreatment system employing SRB requires a knowledge of the individual and concurrent rates of

U(VI) and sulfate reduction. Bioreactor systems can be designed for sequential growth and U(VI) reduction or for concurrent growth and U(VI) reduction, depending upon the system layout and the SRB employed. Rate information is needed to design a growth reactor that integrates into its design the potential for the competitive effects of concurrent U(VI) and SO_4^{2-} reduction in a combined growth and U(VI) reduction system.

The fact that sulfate reduction and uranium reduction were best fit by different models suggests that the rate-limiting step for sulfate and U(VI) reduction is not the same. Sulfate reduction has been hypothesized to occur within the cytoplasmic membrane (35), while uranium reduction has been hypothesized to occur in the periplasmic space (outside of the cytoplasmic membrane) (24). Since these two reductions physically take place in different locations, a difference in the rate-limiting step is feasible even though cytochrome c_3 has been identified as a critical component for both. In addition, the pathway for sulfate reduction involves multiple cytoplasmic enzymes (e.g., adenylyl sulfate reductase [19, 35]) which are probably not used for uranium reduction. One of the enzymatic components that is not common between the two pathways may be rate-limiting for sulfate. Thus, the observation of different rates of sulfate and uranium reduction by the same organism is reasonable. Though the data were not modeled, experimentation performed on a pure culture of *D. vulgaris* (Hildenborough) (ATCC 29579) showed that cytochrome c_3 was the enzyme responsible for U reduction via a first-order process
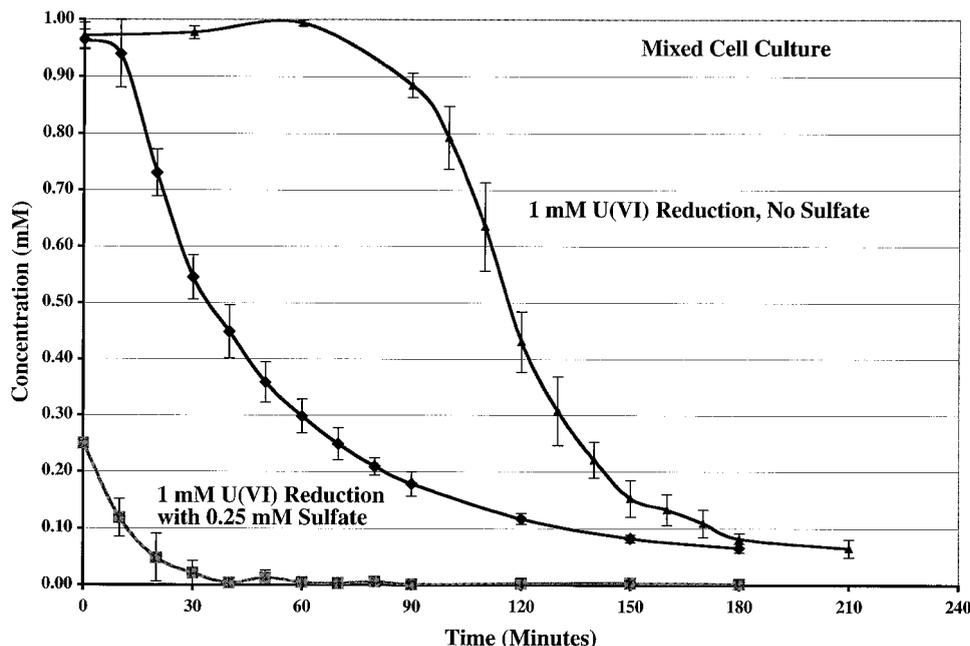
FIG. 6. Time course of uranium and sulfate reduction by the *Desulfovibrio* sp.-dominated mixed culture, with a U_0 of 1.0 mM and an S_0 of 0.25 mM. ▲, U(VI) reduction in the absence of sulfate, with a U_0 of 1.0 mM and an X_0 of 0.46 mg (dry weight) of cells/ml (38); ◆, U(VI) reduction ($U_0 = 1.0$ mM) in the presence of 0.25 mM sulfate; ■, $X_0 = 0.48$ mg (dry weight) of cells/ml. Each data set is an average from three experiments with the mixed cell culture. Error bars, standard errors.

nearly identical to that described for the mixed cell culture here (24).

Since the mixed cell culture contains two species, it was not possible to distinguish the contribution of each to the reduction of uranium. The mixed cell culture described here, however, does contain a *Clostridium* sp., and *Clostridium* is another bacterial genus described as being capable of uranium reduction (5, 6, 7). However, the fraction of biomass associated with the *Clostridium* sp. was no more than 10%. This was observed both by Gram staining of the culture and visualization and by the presence of a 10-to-1 *Desulfovibrio* sp.-to-*Clostridium* sp. banding pattern on a 100-clone RFLP gel. As a genus, *Clostridium* does not dissimilatorily reduce sulfate to sulfide; thus, the sulfate reduction described for the mixed culture is expected to be due to the presence of the *Desulfovibrio* sp. (4). By considering the reduction of both electron acceptors by the pure culture of *D. desulfuricans* (Fig. 7B) under the same experimental conditions as the mixed cell culture (Fig. 7A), a contrast can be made. The rate constant for uranium reduction by the *D. desulfuricans* culture was two to three times higher than that for the mixed cell culture (Table 2), while the rate of sulfate reduction rate was about the same. If both genera were reducing U(VI), the mixed cell culture's kinetics would likely be high, higher than that of the pure culture. In addition, the dry weight of *D. desulfuricans* cells present in the pure culture was 58% of that used for the mixed culture, because experiments were carried out by wet weight cell mass comparisons that were nearly identical (the difference comes from water content and other factors contributing to mass [10]).

Both the mixed and pure cell cultures exhibited lag times of ≥90 min for U(VI) reduction in the absence of sulfate (Fig. 7); in the presence of sulfate these were reduced to near zero. For both cultures, the presence of sulfate aided the reduction of uranium, bringing it to a first-order rate of reduction from a Monod non-growth-based rate with a long lag time (38). However, once the lag phase was over, the amount of time required

to remove 90% of the uranium was about the same. From the kinetics coefficients determined for these two cultures, it appears that the *Clostridium* sp. of the mixed cell culture is not contributing significantly to U(VI) reduction. The rate constants for sulfate reduction were unchanged in the presence and absence of uranium for both cultures.

Further analysis indicates that as the sulfate concentration increases in the medium from 0.25 to 10 mM, the rate of sulfate reduction by the mixed culture doubles. Over the same sulfate concentrations, the mixed culture shows an optimum rate of uranium reduction occurring at a sulfate concentration of 1 mM. The pure culture experiments were done at 1 mM sulfate and uranium concentrations based on the optimum seen for U(VI) removal by the mixed culture.

Lovley and Phillips (21) examined uranium reduction in the presence of sulfate by *D. desulfuricans* (ATCC 29577) in glass serum bottles at 35°C. L-Cysteine was added as a reductant to a bicarbonate-buffered medium for experiments exploring U(VI) reduction in the presence of sulfate, because it yielded higher rates of sulfate reduction. This was not done in our studies. Their results show that for the pure *D. desulfuricans* (ATCC 29577) culture, the presence of sulfate had no significant effect on U(VI) reduction. Our data, for both the mixed and pure *D. desulfuricans* (ATCC 7757) cultures, indicate otherwise, as shown in Fig. 7. The presence of an electron equivalent amount of sulfate, 0.25 mM sulfate, up to 10 mM sulfate (40 times the electron equivalents) removed the lag time for U(VI) reduction and enhanced the overall rate of U(VI) reduction. Lovley and Phillips (21) also suggest that U(VI) reduction did not influence the rate of sulfate reduction by *D. desulfuricans* (ATCC 29577). This was also observed for both the mixed and pure cultures in this study (Tables 1 and 2). Lovley and Phillips showed that *D. desulfuricans* (ATCC 29577) could reduce an initial 0.35 mM U(VI) concentration down to 0.09 mM with concurrent reduction of 2.0 mM sulfate down to 1.1 mM in 4 h, with an initial biomass concentration of
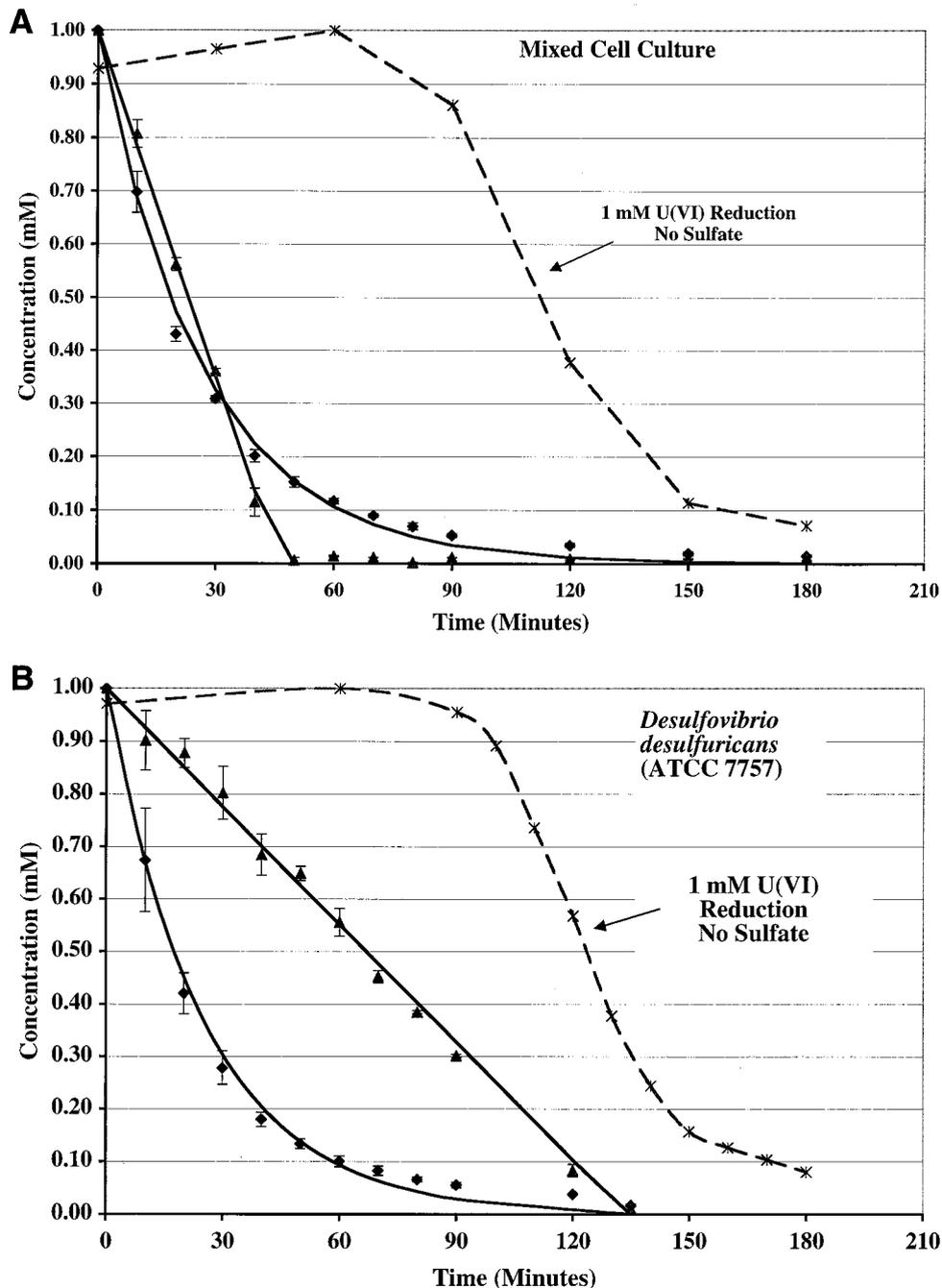
FIG. 7. (A) Time course of concurrent uranium and sulfate reduction for a U_0 of 1.0 mM and an S_0 of 1.0 mM by the mixed cell culture. ▲, sulfate concentration; ◆, U(VI) concentration. Lines represent zero-order and first-order models fit to sulfate and uranium reduction data, respectively. Data points are average values from two separate experiments. Average X_0 = 0.51 mg (dry weight) of cells/ml. ✳, reduction of 1 mM U(VI) alone by 0.50 mg (dry weight) of cells/ml by the same mixed cell culture (38). Error bars, standard errors. (B) Time course of concurrent uranium and sulfate reduction for a U_0 of 1.0 mM and an S_0 of 1.0 mM by the pure culture *D. desulfuricans* (ATCC 7757). Symbols are as described for panel A. Data points are average values from two separate experiments. Average X_0 = 0.29 mg (dry weight) of cells/ml. ✳, reduction of 1 mM U(VI) alone by 0.32 mg (dry weight) of cells/ml (38). A model for this reduction is reported elsewhere (38). Error bars, standard errors.

approximately 0.2 to 1.0 mg (dry weight) of cells/ml (0.5 mg of protein/mg [dry weight] conversion assumed per Bailey and Ollis [2]) at 35°C (18). A decrease in the reaction temperature from 35 to 20°C would produce at least a 50% decrease in the reaction rate (2). Thus, at a temperature comparable to that used in this study, U(VI) and sulfate reduction by *D. desulfuricans* (ATCC 29577) would be expected to take 6 to 8 h. If the

experiment is conducted in glass serum bottles, a 15% sorptive effect of uranium and a 10% sorptive effect for sulfate may be present, though the overall reduction trend is the same. Both cultures utilized in this study showed a higher rate of reduction as described; this, however, may be a function of the cell concentrations used.

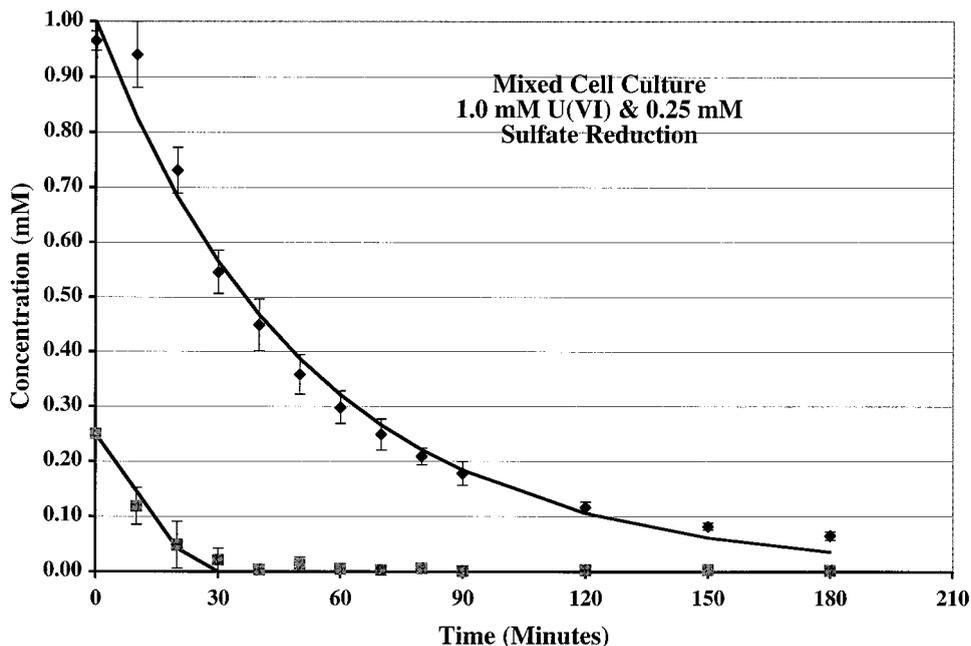For the concurrent reduction of sulfate and uranium by

FIG. 8. Zero- and first-order models applied to the enzymatic reduction of 0.25 mM sulfate and 1 mM uranium by the mixed cell culture, respectively. Each set of data points is an average from three experiments with the same mixed cell culture. Models are fit to plotted data with values given in Table 2. X_0 = 0.48 mg (dry weight) of cells/ml. Error bars, standard errors.

SRB, two reductive processes for U(VI) are possible: enzymatic reduction as described above and chemical reduction by SRB-produced sulfides. Originally, this was thought to be the dominant mechanism, as it is thermodynamically feasible (30, 31). Lovley and Phillips (21) found that the enzymatic reduction was significantly faster than the nonenzymatic, sulfide reduction of U(VI), even in the presence of catalytic SRB cell

surfaces, as the temperature optimum for U(VI) reduction is consistent with enzymatic reduction. Based on Lovley and Phillips' conclusions, we did not test for any sulfide effects in the combined reduction experiments. Considering the relatively short time courses of our experiments, the temperature of our experiments, and the kinetics coefficients described in Table 2, the sulfides produced may have had a role in the fact that the
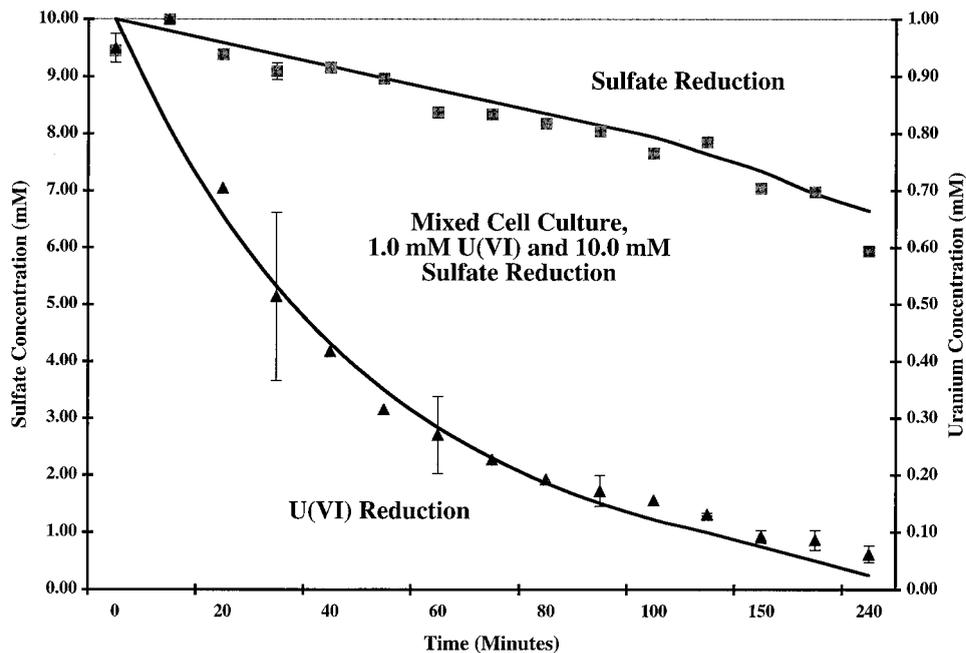

FIG. 9. Zero- and first-order models applied to the enzymatic reduction of 10 mM sulfate and 1 mM uranium by the mixed cell culture, respectively. The average X_0 for the three experiments with the same mixed cell culture represented is 0.46 mg (dry weight) of cells/ml. Error bars, standard errors.

TABLE 2. Zero- and first-order model kinetics coefficients determined for both the mixed and pure cell cultures

Culture	Initial cell concn (mg [dry wt] of cells/ml)	Initial sulfate concn (mM)	Initial uranium concn (mM)	k_0 (μM SO_4^{2-}/ (mg [dry wt] of SRB/ml) $\cdot$ min^{-1})	k_1 (mg [dry wt] of SRB/ml) $\cdot$ min^{-1})
Mixed cell	0.47 ± 0.01	0.25	1.0	20 ± 4	0.041 ± 0.004
	0.50 ± 0.01	1.0	1.0	36 ± 7	0.071 ± 0.003
	0.46 ± 0.01	10	1.0	41 ± 6	0.039 ± 0.009
D. desulfuricans (ATCC 7757)	0.29 ± 0.004	1.0	1.0	26 ± 2	0.137 ± 0.016

lag time seen with reduction of U(VI) only was minimized when sulfate was also present for reduction. This effect, however, is likely to be minor.

Conclusion. For the mixed cell culture, a reproducible and predictable lag time until the onset of reduction for both the native electron acceptor, sulfate and U(VI) is possible. A cell concentration-based threshold until the onset of sulfate reduction can begin was reproducibly found for the mixed cell culture, resulting in a described lag time. This culture exhibited a similar lag time in reducing U(VI) alone, though at a higher cell concentration. Zero- and first-order models best fit the data for the concurrent removal of sulfate and uranium, respectively, suggesting that the rate-limiting step for each electron acceptor's reduction is not the same. These studies were performed at room temperature for both cultures, the upper-end temperature of natural waters. For a bio-based treatment system this is important, as U(VI)-containing waters are naturally cool. For the cultures tested herein, reduction of aqueous U(VI) was enhanced by the presence of aqueous sulfate. The presence of sulfate both minimizes the lag time and increases the overall rate.

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