Effects of aqueous uranyl speciation on the kinetics of microbial uranium reduction

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Abstract

The ability to predict the success of the microbial reduction of soluble U(VI) to highly insoluble U(IV) as an *in situ* bioremediation strategy is complicated by the wide range of geochemical conditions at contaminated sites and the strong influence of aqueous uranyl speciation on the bioavailability and toxicity of U(VI) to metal-reducing bacteria. To determine the effects of aqueous uranyl speciation on uranium bioreduction kinetics, incubations and viability assays with *Shewanella putrefaciens* strain 200 were conducted over a range of pH and dissolved inorganic carbon (DIC), Ca^{2+} , and Mg^{2+} concentrations. A speciation-dependent kinetic model was developed to reproduce the observed time series of total dissolved uranium concentration over the range of geochemical conditions tested. The kinetic model yielded the highest rate constant for the reduction of uranyl non-carbonate species (i.e., the ‘free’ hydrated uranyl ion, uranyl hydroxides, and other minor uranyl complexes), indicating that they represent the most readily reducible fraction of U(VI) despite being the least abundant uranyl species in solution. The presence of DIC, Ca^{2+} , and Mg^{2+} suppressed the formation of more bioavailable uranyl non-carbonate species and resulted in slower bioreduction rates. At high concentrations of bioavailable U(VI), however, uranium toxicity to *S. putrefaciens* inhibited bioreduction, and viability assays confirmed that the concentration of non-carbonate uranyl species best predicts the degree of toxicity. The effect of uranium toxicity was accounted for by incorporating the free ion activity model of metal toxicity into the bioreduction rate law. Overall, these results demonstrate that, in the absence of competing terminal electron acceptors, uranium bioreduction kinetics can be predicted over a wide range of geochemical conditions based on the bioavailability and toxicity imparted on U(VI) by solution composition. These findings also

imply that the concentration of uranyl non-carbonate species, despite being extremely low, is a determining factor controlling uranium bioreduction at contaminated sites.

1. Introduction

The need for a mechanistic understanding of uranium biogeochemistry is driven by ongoing efforts to control, contain, and predict the fate of this contaminant at nuclear processing facilities, where poor waste disposal practices have resulted in an extensive legacy of uranium contamination (Riley et al., 1992). Speciation (i.e., oxidation state and aqueous complexation) regulates the physical and chemical processes that determine the mobility of uranium in the subsurface. In oxidizing environments, uranium is predominantly under the form of the U(VI) uranyl ion (UO_2^{2+}), which is soluble over a wide range of geochemical conditions (Langmuir, 1978). Uranium(VI) may be reduced abiotically or microbially to the U(IV) uranous ion (U^{4+}), which is highly insoluble and readily precipitates from solution as uraninite (UO_2) (Burgos et al., 2008; Schofield et al., 2008) or other non-uraninite U(IV) compounds (Bernier-Latmani et al., 2010; Boyanov et al., 2011).

Uranium(VI) bioreduction is a promising *in situ* bioremediation strategy which exploits the large differences in solubility among uranium oxidation states with the goal of immobilizing uranium in the subsurface as insoluble U(IV) minerals. Large-scale field studies have demonstrated that U(VI) bioreduction by metal-reducing bacteria is stimulated by injection of organic carbon substrates upstream of the contaminant plume (Anderson et al., 2003; Watson et al., 2013; Williams et al., 2011; Wu et al., 2006b). The broad range of geochemical conditions at uranium-contaminated sites, however, complicates the implementation of U(VI) bioreduction due, in part, to the impact of solution composition on the biogeochemical transformations of uranium. Changes in the physical (e.g., size and charge) and thermodynamic (e.g., redox

potential and solubility) properties of the uranyl ion upon complexation by ligands alter U(VI) reactivity in the subsurface. For instance, the formation of uranyl carbonate and ternary uranyl carbonate complexes decreases uranyl adsorption to mineral surfaces (Nair and Merkel, 2011; Stewart et al., 2010; Waite et al., 1994; Wang et al., 2013), lowers the U(VI)/U(IV) redox potential (Du et al., 2011; Ginder-Vogel et al., 2006), and retards the kinetics of chemical (Behrends and Van Cappellen, 2005; Hua et al., 2006) and biological (Brooks et al., 2003; Sheng and Fein, 2014; Sheng et al., 2011; Ulrich et al., 2011) U(VI) reduction. Following reduction, solution composition (Bernier-Latmani et al., 2010; Boyanov et al., 2011) and the local coordination environment of U^{4+} (Bargar et al., 2013; Latta et al., 2014) affect the formation of U(IV) mineral products and ultimately the long-term stability of uranium against mobilization (Alessi et al., 2012; Cerrato et al., 2013).

The kinetics of microbial U(VI) reduction is highly dependent on aqueous uranyl speciation, and labile uranyl complexes (e.g., the ‘free’ hydrated, hydroxide, and organic uranyl species) have been proposed to represent the bioavailable fraction of U(VI) (Ulrich et al., 2011). Decreased bioreduction rates in the presence of dissolved inorganic carbon (DIC) and Ca^{2+} are attributed to the formation of stable uranyl carbonate and ternary Ca uranyl carbonate complexes which are less thermodynamically favorable for reduction and may be inaccessible to bacteria due to steric hindrance or poor affinity for adsorption to the cell membrane (Brooks et al., 2003; Sheng and Fein, 2014; Sheng et al., 2011; Stewart et al., 2011; Ulrich et al., 2011). Similarly, low molecular weight organic acids (Francis and Dodge, 2008; Haas and Northup, 2004; Sheng et al., 2011; Suzuki et al., 2010) and soil humics (Burgos et al., 2007; Gu et al., 2005) may prevent microbial reductive precipitation of uranium by decreasing the bioavailability of U(VI) (e.g., imparting steric hindrance) and/or increasing the solubility of U(IV).

Although select species of bacteria are able to support cell growth using U(VI) as a terminal electron acceptor (TEA) during anaerobic respiration (Lovley et al., 1991; Sanford et al., 2007; Tebo and Obraztsova, 1998; Wade and DiChristina, 2000), uranium is also toxic to many microorganisms (Konopka et al., 2013; Tapia-Rodriguez et al., 2012). As metal toxicity is a function of aqueous metal speciation rather than the total dissolved metal concentration (Campbell, 1995; Morel, 1983), the toxicity of uranium to aquatic organisms has been linked to the aqueous concentrations of UO_2^{2+} and UO_2OH^+ (Markich et al., 1996; Markich et al., 2000; Trenfield et al., 2011). Ligands such as carbonate (Carvajal et al., 2012; Markich et al., 1996; VanEngelen et al., 2010), phosphate (Fortin et al., 2004), sulfate (Markich et al., 1996), and dissolved organic carbon (DOC) (Trenfield et al., 2011) lessen uranium toxicity by decreasing the concentrations of UO_2^{2+} and UO_2OH^+ , while cations such as Ca^{2+} , Mg^{2+} , and H^+ decrease uranium toxicity by competing with uranyl complexes for biotic ligand binding sites (Charles et al., 2002). Thus far, however, the influence of uranium toxicity on microbial uranium reduction remains poorly defined.

Given the multiple effects of aqueous uranyl speciation, the main objective of this study was to deconvolute the roles of solution composition on the kinetics of U(VI) bioreduction and, hence, provide a unifying framework to account for the effects of bioavailability and toxicity. Uranium(VI) bioreduction incubations and viability assays were conducted with *Shewanella putrefaciens* strain 200 over a broad range of aqueous uranyl speciation conditions, and a speciation-dependent kinetic model for U(VI) bioreduction was developed to reproduce observed time series of total dissolved uranium concentrations. The proposed model is discussed in the context of biostimulation field studies at uranium-contaminated sites and the challenges

associated with transferring knowledge acquired in simple laboratory model systems to real-world applications.

2. Materials and Methods

2.1 Cell cultures

All biotic experiments were conducted with *Shewanella putrefaciens* strain 200 (ATCC 51753), a model uranium-reducing bacteria capable of growth using U(VI) as its sole terminal electron acceptor (TEA) during anaerobic respiration (Wade and DiChristina, 2000). Prior to all biotic experiments, *S. putrefaciens* was grown anaerobically on a defined growth medium (M1) (Myers and Nealson, 1988) modified to decrease the concentrations of NH_4^+ , ΣPO_4^{3-} , and Mg^{2+} to 100 μM , buffered at pH 8.0 with 10 mM HEPES, and amended with 100 μM FeCl_3 . Lactate (15 mM) was supplied as the electron donor and carbon source, and fumarate (60 mM) was supplied as the TEA. Cells were collected during late-log phase by centrifugation and harvested and washed once with growth medium prior to inoculation.

2.2 Bioreduction incubations

Uranium(VI) bioreduction batch incubations with *S. putrefaciens* were conducted in duplicate in 100 mL serum bottles containing M1 medium (modified to remove Na_2EDTA , CaCl_2 , and FeCl_3) and continuously mixed with magnetic stir bars (290 rpm). Lactate (15 mM) was provided as the sole electron donor and U(VI) (800 μM uranyl acetate, Spectrum) as the sole TEA. Autoclaved M1 medium was purged with N_2 to remove dissolved oxygen prior to assembling the incubations. The serum bottles were placed inside an anaerobic chamber (5% H_2 , 95% N_2 atmosphere) where U(VI) (800 μM uranyl acetate), DIC (15, 20, 25, 30, or 45 mM NaHCO_3), Ca^{2+} (0.0, 0.5, 1.0, or 4.9 mM CaCl_2), Mg^{2+} (0.0, 1.0, or 5.1 mM MgSO_4), and PIPES buffer (15 mM, adjusted to pH 6.5 ± 0.1 , 7.5 ± 0.2 , or 8.1 ± 0.1) were added from filter sterilized

stock solutions. Overall, 15 incubation conditions were considered. The M1 medium included a background Mg^{2+} concentration of 100 μM in all incubations except those conducted in the presence of Ca^{2+} , which contained no Mg^{2+} . The serum bottles were capped with butyl rubber stoppers and aluminum crimps to avoid exposure to the atmosphere, and all incubations were allowed to equilibrate for approximately one week prior to inoculation. During this time, minor pH adjustments were performed by addition of 1 M NaOH or HCl. Measured initial DIC concentrations were within 13% of predicted values assuming thermodynamic equilibrium (calculations performed with PHREEQC, data not shown), indicating that equilibration was closely achieved prior to inoculation. Incubations were inoculated with an initial cell concentration of approximately 5×10^7 cells/mL, determined using optical density at 600 nm (OD_{600}) and a conversion factor of 2×10^9 cell/mL/Abs. unit.

Small aliquots were collected periodically with a needle and syringe flushed with the anaerobic chamber atmosphere. An unfiltered subsample was processed to measure pH and DIC: pH was measured using a double junction semi-micro pH electrode (Orion) calibrated with three low ionic strength commercial buffers (pH 4, 7, and 10, Fisher Scientific, Inc.), and DIC was measured by a flow injection analysis system with conductivity detection (Analytical Instruments Systems, Inc. LCC100 integrator) (Hall and Aller, 1992). The remaining subsample was filtered (0.22 μm nitrocellulose, Millipore) and used to measure total dissolved uranium by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500a series). Filtered aliquots were diluted in 2% trace metal grade HNO_3 (Fisher Scientific, Inc.) containing 5 ppb holmium and bismuth as internal standards (SPEX CertiPrep) to correct for instrument drift. All total dissolved uranium measurements were conducted in duplicate. Calibration standards were prepared with a certified uranium stock solution (SPEX CertiPrep) in 2% HNO_3 containing the

internal standards and measured approximately every 25-30 samples. Blanks and a calibration standard were measured every 6-7 samples as quality controls.

The averaged, time-corrected data sets from duplicate incubations (discussed in Section 3.1) were used in all kinetic analyses and modeling exercises. Lag phases were defined as the time of inoculation ($t = 0$) up to the last measurement of a total dissolved uranium concentration within 1 standard deviation of the average concentration of previous measurements within the lag phase. The experimental, overall pseudo-first order bioreduction rate constant (k_{obs}) was determined for each experimental condition from the slope of the linearized, integrated equation of total dissolved uranium concentration $[U]$ versus time t (Equation 1, where X is biomass) over the time interval following the lag phase that yielded the largest k_{obs} and conformed to the pseudo-first order rate law (i.e., smallest standard deviation of the linear regression of the data represented using Equation 1). The overall U(VI) bioreduction rate was determined by fitting a linear regression to measured total dissolved uranium concentrations over the same time interval used in the calculation of k_{obs} .

$$\ln \frac{[U]}{[U]_0} = k_{obs}[X]t \quad (1)$$

2.3 Cell viability assays

The effects of uranyl speciation on uranium toxicity to *S. putrefaciens* were determined in viability assays similar to those used to study the effects of uranyl speciation on cell viability during U(VI) biosorption (Carvajal et al., 2012) and bioreduction (Cologgi et al., 2011). The assays were conducted over a range of DIC, Ca^{2+} , and Mg^{2+} concentrations. *S. putrefaciens* was grown anaerobically to late-log phase on fumarate (60 mM) in M1 medium. Cells were harvested by centrifugation and resuspended in 50 mL Falcon tubes under aerobic, non-growth conditions in solutions of uranyl acetate (1 mM), PIPES buffer (20 mM, pH 7.2 ± 0.2), $NaHCO_3$ (10, 30, or

50 mM), and CaCl₂ or MgSO₄ (0, 1, or 5 mM) and mixed on a rotary wheel for 6 hours. Control cells were resuspended in a solution of PIPES buffer (20 mM) and NaHCO₃ (10 mM) without uranyl acetate. Following uranium exposure, cells were harvested by centrifugation, washed twice with M1 medium, and inoculated into anaerobic culture tubes (Chemglass Life Sciences) to an initial OD₆₀₀ of ~0.01 and regrown anaerobically on fumarate in fresh, N₂-purged, uranium-free M1 medium. Cell growth was monitored by OD₆₀₀ (Beckman Coulter DU720 UV/Vis spectrophotometer) and growth curves were conducted in duplicate.

The log-phase region of growth curves was used to calculate survival rates of *S. putrefaciens* following exposure to uranium. This approach assumes that after uranium exposure, only a certain percentage of cells is viable and capable of cell division, which results in a lag phase in the growth curve before exponential growth is observed. A first-order rate law with respect to the concentration of cells (X in Equation 2) was integrated and fitted to the exponential region of the growth curves to obtain a rate constant k for each uranium exposure condition. As the cell concentration at the end of the inherent lag phase (i.e., the lag phase of the control cells not exposed to uranium) represents the concentration of viable cells at the time of inoculation, the rate law (Equation 2) was integrated and used with the rate constant k determined for each uranium exposure condition to calculate the concentration of viable cells at the time of inoculation. The survival rate was then determined for each exposure condition by dividing the calculated concentration of viable cells by the measured initial total concentration of cells.

$$\frac{d[X]}{dt} = k[X] \quad (2)$$

2.4 Speciation-dependent biogeochemical kinetic model

S. putrefaciens couples the oxidation of lactate to acetate and DIC to the reduction of U(VI) to U(IV) (Equation 3), which spontaneously precipitates from solution as biogenic U(IV)

minerals (uraninite in Equation 4) or forms various non-crystalline complexes depending on bacterial strain and solution composition (Bernier-Latmani et al., 2010; Boyanov et al., 2011).

The rate law for U(VI) bioreduction is based on Michaelis-Menten kinetics (Equation 5), where k is the rate constant, $[X]$ the concentration of biomass, $[\text{Lac}^-]$ the concentration of electron donor, $[\text{U(VI)}]$ the concentration of U(VI), and K_{Lac} and K_U the half-saturation constants for lactate and U(VI), respectively.

$$\frac{d[\text{U(VI)}]}{dt} = -k[X] \frac{[\text{Lac}^-]}{K_{\text{Lac}} + [\text{Lac}^-]} \frac{[\text{U(VI)}]}{K_U + [\text{U(VI)}]} \quad (5)$$

Equation 5 was further modified to account for aqueous uranyl speciation by assuming that the kinetics of enzymatic electron transfer to U(VI) depend on the type of ligand(s) surrounding U(VI). In addition, as lactate concentration (15 mM) was provided in excess of K_{Lac} (Liu et al., 2001), the Michaelis-Menten term for lactate approaches unity. As a result, the overall rate law under the experimental conditions can be expressed as Equation 6, where i represents an individual uranyl complex and n is the total number of uranyl complexes considered.

$$\frac{d[\text{U(VI)}]}{dt} = -[X] \sum_{i=1}^n \frac{k_i [\text{U(VI)}_i]}{K_{U_i} + [\text{U(VI)}_i]} \quad (6)$$

Due to the large number of uranyl species, Equation 6 was simplified by grouping species based on the type of ligand, as done in previous studies (Stewart et al., 2011; Ulrich et al., 2011). Three groups of uranyl species were considered in our model: non-carbonate, carbonate, and ternary-carbonate (Equation 7). The uranyl non-carbonate species include the ‘free’ hydrated uranyl ion and hydroxide, chloride, sulfate, phosphate, lactate, and acetate uranyl complexes formed in the presence of ligands present in the growth medium. The uranyl carbonate species include

carbonate and hydroxide-carbonate complexes, while the ternary uranyl carbonate species include Ca and Mg uranyl carbonate complexes. Assuming that the uranyl species in each group have comparable chemical properties, each group of uranyl species is assigned a single rate constant and half-saturation constant.

$$\frac{d[U(VI)]}{dt} = -[X] \left(\frac{k_1[U(VI)_{\text{non-CO}_3}]}{K_{U1} + [U(VI)_{\text{non-CO}_3}]} + \frac{k_2[U(VI)_{\text{CO}_3}]}{K_{U2} + [U(VI)_{\text{CO}_3}]} + \frac{k_3[U(VI)_{\text{ternary-CO}_3}]}{K_{U3} + [U(VI)_{\text{ternary-CO}_3}]} \right) \quad (7)$$

Initial modeling efforts with the full Michaelis-Menten terms in Equation 7 revealed that the optimized half-saturation constant concentrations (i.e., K_{U1} , K_{U2} , K_{U3}) were orders of magnitude larger than the corresponding concentrations, such that the Michaelis-Menten terms could be approximated by first order expressions with respect to the concentrations of each group of uranyl species (Equation 8). This approach is consistent with past studies (Liu et al., 2002; Ulrich et al., 2011) and decreases the number of model parameters. Finally, because uranium is toxic to bacteria above a strain-specific threshold level (Katsenovich et al., 2013), an inhibition term was added to the bioreduction rate law to account for the inhibition of anaerobic respiration in the presence of elevated ‘free’ uranyl ion concentrations, in accordance with the free ion activity model of metal toxicity (Campbell, 1995; Morel, 1983). A two-parameter logistic function was used as the concentration-response toxic inhibition factor F_{toxic} (Equation 9), similar to previous approaches describing the toxicity of uranium to aquatic organisms (Markich et al., 1996; Markich et al., 2000). The inhibition factor varies between 0 and 1 as a function of the ‘free’ uranyl ion concentration; the parameters are the concentration of UO_2^{2+} at 50% inhibition IC_{50} , and p which characterizes the slope of the curve at the 50% inhibition inflection point.

$$\frac{d[U(VI)]}{dt} = -F_{\text{toxic}} \cdot [X] \cdot (k_1[U(VI)_{\text{non-CO}_3}] + k_2[U(VI)_{\text{CO}_3}] + k_3[U(VI)_{\text{ternary-CO}_3}]) \quad (8)$$

$$F_{\text{toxic}} = \frac{1}{1 + \left(\frac{[UO_2^{2+}]}{IC_{50}} \right)^p} \quad (9)$$

The biogeochemical kinetic model was fitted to the averaged, time-corrected total dissolved uranium concentration time series obtained after the initial lag phase, beginning at the first measurement used to calculate k_{obs} and the overall U(VI) bioreduction rate. The speciation-dependent bioreduction rate law (Equation 8) was integrated using the geochemical modeling software PHREEQC Version 3.1.1 (USGS, http://wwwbrr.cr.usgs.gov/projects/GWC_coupled). The thermodynamic database in PHREEQC 3.1.1 was updated with the most recently published thermodynamic data for aqueous and solid U(VI) and U(IV) compounds (Dong and Brooks, 2006; Guillaumont et al., 2003; Gustafsson, 2012). All 15 experimental conditions were analyzed together in a single PHREEQC model with the same bioreduction rate law and a single set of parameters. For each experimental condition, the measured initial total dissolved uranium concentration, measured average pH during the bioreduction period, measured initial DIC concentration, and medium composition were provided as model inputs, and the model was run with a time step of 6 hours. PHREEQC executes a Runge-Kutta algorithm to integrate the bioreduction rate law (Equation 8) using the calculated uranyl speciation at each time step, assuming internal thermodynamic equilibrium, and adjusts the solution composition in accordance with the stoichiometry of Equation 3. The solution was further assumed to be in instantaneous equilibrium with amorphous uraninite, that is, U(IV) was allowed to precipitate from solution (Equation 4) when the solubility product of uraninite was exceeded.

The biogeochemical model includes a total of five adjustable parameters: three bioreduction rate constants (i.e., k_1 , k_2 , and k_3 , one for each group of uranyl species) and two parameters for the concentration-response toxic inhibition factor (i.e., p and IC_{50}). The parameter estimation program PEST Version 13.0 (Watermark Numerical Computing <http://www.pesthomepage.org/>) was used to optimize the model parameters to best fit the model-

predicted time series of total dissolved uranium to the measured concentrations using a Gauss-Marquardt-Levenberg nonlinear estimation algorithm. PEST calls PHREEQC which executes the model using initial guesses for the model parameters. Once the first model run is complete, PEST linearizes the relationship between model parameters and model output and solves for a new set of parameters, which PHREEQC uses to execute the model again. This procedure is repeated until the change in goodness of fit between parameter sets drops below a threshold tolerance. The five parameters were optimized to simultaneously maximize the fit between measured and modeled total dissolved uranium concentrations for all 15 experimental conditions. Finally, to compare measured bioreduction rates for each condition with the rates predicted by the model, overall pseudo-first order rate constants k_{obs} were derived from Equation 1 using the time series of total dissolved uranium obtained with the model over the same time period as the experimental data.

3. Results

3.1 Effect of uranyl speciation on U(VI) bioreduction rates

Bioreduction incubations were conducted from pH 6.5 to 8.1 and over a range of concentrations of DIC, Ca^{2+} , and Mg^{2+} , in order to relate aqueous uranyl speciation to uranium bioreduction kinetics (Fig. 1). Uranyl carbonate species dominated uranyl speciation at all pH and DIC conditions without added Ca^{2+} or Mg^{2+} (Table 1): $95 \pm 1\%$ of U(VI) was present as uranyl carbonate species and $5 \pm 1\%$ of U(VI) was present as the ternary Mg uranyl carbonate complex $\text{MgUO}_2(\text{CO}_3)_3^{2-}$ (due to the $100 \mu\text{M}$ Mg^{2+} background in the growth medium). The remaining fraction of U(VI) ($< 0.1\%$) was composed of uranyl non-carbonate species. While the concentration of the most abundant uranyl species was similar across all pH and DIC conditions without added Ca^{2+} or Mg^{2+} , the concentration of the uranyl non-carbonate species, the least

abundant species in solution, spanned four orders of magnitude, ranging from 38 pM (pH 8.1 incubation containing 39 mM DIC) to 562 nM (pH 6.5 incubation containing 23 mM DIC).

As a result of the accompanying changes in aqueous uranyl speciation, the pH and DIC concentrations affected bioreduction rates significantly (Table 1). Uranium bioreduction was negligible in incubations at pH 6.5 (Fig. 1a) which included the highest concentrations of uranyl non-carbonate species (Table 1). Interestingly, in incubations at pH 7.5 (Fig. 1b), the maximum bioreduction rate was observed in the presence of 22 mM DIC whereas bioreduction rates were slower in the incubations containing both higher (35 mM) and lower (13 mM) DIC concentrations (Table 1). In contrast, incubations conducted at pH 8.1 (Fig. 1c) generally exhibited an inverse correlation between the DIC concentration and the bioreduction rate (Table 1). Across all incubation conditions, the pH remained stable (± 0.1 pH units) during the period of bioreduction (data not shown).

The addition of Ca^{2+} and Mg^{2+} led to the formation of ternary uranyl carbonate species, which decreased the concentration of carbonate and non-carbonate species and affected uranium bioreduction rates (Table 1): bioreduction rates were inversely proportional to the Ca^{2+} and Mg^{2+} concentrations, though Ca^{2+} led to a larger relative decrease in bioreduction rate than Mg^{2+} for the same concentration (Table 1). An inverse correlation was observed between the lag phase in uranium bioreduction and the concentrations of Ca^{2+} and Mg^{2+} (Figs. 1d and 1e). Incubations containing 1.0 and 4.9 mM Ca^{2+} exhibited shorter lag phases compared to incubations without Ca^{2+} (Fig. 1d). Similarly, the addition of 1.0 and 5.1 mM Mg^{2+} resulted in shorter lag phases compared to incubations conducted in the presence of the background Mg^{2+} concentration (Fig. 1e). The reproducibility of the lag phases in replicate incubations for all conditions was also affected by the concentrations of Ca^{2+} and Mg^{2+} (data not shown): below 0.5 mM Ca^{2+} and Mg^{2+} ,

lag phases were quite variable, differing in length between 1 to 7 days in duplicate incubations, whereas for Ca^{2+} and Mg^{2+} concentrations above 0.5 mM, lag phases were mostly reproducible. When significant inter-duplicate variability in lag phase occurred, the time series data were shifted in time relative to one another until the time intervals of maximum bioreduction in the duplicate experiments coincided. Following time adjustments, the total dissolved uranium measurements from duplicate incubations were averaged and the resulting time series was then used for all subsequent kinetic analyses and modeling exercises. Despite significant variations in the lag phase at low concentrations of Ca^{2+} and Mg^{2+} , bioreduction rates were highly reproducible between duplicate incubations and did not correlate with lag phase duration across all incubation conditions ($R^2 = 0.02$, data not shown). These observations suggest that variability in metabolic performance or adaptation during the lag phase did not affect uranium bioreduction rates significantly.

3.2 Effect of uranyl speciation on cell viability

In viability assays without Ca^{2+} or Mg^{2+} , uranyl carbonate species accounted for >99% of U(VI) while the remaining fraction consisted of uranyl non-carbonate species (i.e., ‘free’ hydrated, hydroxide, and acetate species) (Table 2). Whereas the concentration of uranyl carbonate species was nearly the same across the different DIC concentrations in these assays, the concentration of uranyl non-carbonate species spanned three orders of magnitude, from 42 pM to 46 nM, and correlated inversely with the DIC concentration (Table 2). After 6 hours of exposure to 1 mM uranyl acetate (pH 7.2 ± 0.2) in the presence of 30 and 50 mM DIC, the bacteria recovered rapidly and displayed exponential growth after a relatively brief 5 hour lag phase, similar to control cells not exposed to uranium (Fig. 2a). In the presence of 10 mM DIC,

however, exposure to uranium resulted in a 15 hour lag phase before cell growth was detected (Fig. 2a).

The addition of 1 and 5 mM Ca^{2+} to incubations conducted in the presence of 10 mM DIC led to the formation of ternary Ca uranyl carbonate complexes which dominated uranyl speciation (77% and >99% of U(VI), respectively) and decreased the concentration of carbonate and non-carbonate uranyl species (Table 2). As observed with the increase in DIC concentration, the increase in Ca^{2+} concentration from 1 to 5 mM decreased the lag phase to 14 and 6 hours, respectively, compared to the 15 hours observed in the incubations conducted without Ca^{2+} (Fig. 2b). Addition of Mg^{2+} also altered the speciation of U(VI) via formation of the ternary Mg uranyl carbonate complex, but this complex only accounted for 44% and 78% of U(VI) in the presence of 1 and 5 mM Mg^{2+} , respectively, whereas the remaining fraction of U(VI) consisted predominantly of carbonate species (Table 2). Concentrations of uranyl non-carbonate species were within the same order of magnitude regardless of the Mg^{2+} concentration, in line with the absence of a significant effect of Mg^{2+} additions on the duration of the lag phase (Fig. 2c). The addition of 1 and 5 mM Mg^{2+} with 10 mM DIC yielded only marginally shorter lag phases (45 and 39 hours, respectively) compared to cells exposed to uranium and 10 mM DIC without Mg^{2+} (45 hours).

To compare viability assays across all geochemical conditions, survival rates were calculated from growth curve data and represented as a function of the calculated aqueous uranyl speciation during uranium exposure. A two-parameter logistic function (Equation 9) was fitted to the data, and the strongest negative concentration-response relationships across all viability assay conditions were observed between the survival rate of the bacteria after exposure to uranium and the sum of the concentrations of all uranyl non-carbonate species ($R^2 = 0.97$, Fig. 3) as well as

concentrations of individual uranyl complexes such as the ‘free’ hydrated uranyl ion UO_2^{2+} ($R^2 = 0.98$, Fig. EA1a) and the hydroxide complexes UO_2OH^+ ($R^2 = 0.98$, Fig. EA1b) and $(\text{UO}_2)_2\text{OH}^{3+}$ ($R^2 = 0.98$, Fig. EA1c). Interestingly, a strong positive concentration-response relationship was observed between survival rate and the sum of the concentrations of ternary uranyl carbonate species ($R^2 = 0.97$, Fig. EA1d). In contrast, no significant relationship between survival rate and the sum of the concentrations of uranyl carbonate species was observed ($R^2 = 0.04$, Fig. EA1e).

3.3 Biogeochemical kinetic model

Overall, the optimized speciation-dependent biogeochemical model captured the range of bioreduction rates and reproduced the post-lag phase time series of total dissolved uranium concentration across nearly all incubation conditions (Fig. 1). The notable exceptions are the pH 8.1 incubations containing 1.0 mM Ca^{2+} (Fig. 1d) and 5.2 mM Mg^{2+} (Fig. 1e) for which the modeled time series of total dissolved uranium concentration significantly deviated from measured values. The relatively good correlation between measured and modeled overall pseudo-first order rate constants k_{obs} ($R^2 = 0.69$, Fig. EA2) across a wide range of geochemical conditions supports the conceptual framework of the model, in particular the distribution of the uranyl species into three groups, each represented by a single rate constant. Discrepancies between measured and modeled k_{obs} values generally resulted from underpredicted values by the model, especially for incubations with the largest measured k_{obs} , which also exhibited the largest uncertainties (Fig. EA2).

Unlike the incubations conducted at pH 8.1, bioreduction rates obtained in the incubations conducted at pH 6.5 and 7.5 were not proportional to the concentration of uranyl non-carbonate species (Table 1), and the corresponding time series data could not be reproduced by a first order rate law alone (data not shown). Including the toxic inhibition factor F_{toxic} in the

rate law (Equation 8) greatly improved the model fit and was necessary to reproduce the lack of bioreduction observed in incubations at pH 6.5, as well as the decreased bioreduction rates observed in incubations at pH 7.5 with 13 and 22 mM DIC. By optimizing the kinetic parameters to all 15 experimental conditions simultaneously, the speciation-dependent kinetic model yielded a rate constant of bioreduction for uranyl non-carbonate species that was six orders of magnitude larger than those of uranyl carbonate and ternary uranyl carbonate species (Table 3). The rate constants of bioreduction for the uranyl carbonate and ternary uranyl carbonate species were nearly the same within experimental error.

4. Discussion

4.1 Lag phases

Lag phases of up to 11 days precede the onset of uranium reductive precipitation in the incubations (Fig. 1). Lag phases have been observed in previous uranium bioreduction studies (Brooks et al., 2003; Nyman et al., 2007; Ray et al., 2011; Sani et al., 2006; Sivaswamy et al., 2011; Spear et al., 1999) and may be inversely correlated to biomass concentration (Spear et al., 1999), though such a relationship was not observed in the present incubations. Instead, the duration and inter-duplicate variability of the lag phases correlate inversely with the concentrations of Ca^{2+} and Mg^{2+} , possibly reflecting the limited concentrations of these essential cations in the growth medium. Indeed, preliminary experiments revealed that Mg^{2+} is necessary to sustain the growth of *S. putrefaciens* on fumarate, and for this reason, a background Mg^{2+} concentration of 100 μM was chosen to minimize the duration of the lag phase while avoiding excessive formation of Mg uranyl carbonate complexes.

4.2 Effect of DIC, Ca^{2+} , and Mg^{2+} on U(VI) bioreduction rates

Following the lag phases, uranium bioreduction proceeds at rates highly dependent on the

aqueous uranyl speciation. Increasing concentrations of DIC at pH 8.1 result in the formation of uranyl carbonates, lower concentrations of uranyl non-carbonate species, and slower bioreduction rates (Table 1). These results are consistent with earlier studies showing that DIC inhibits microbial U(VI) reduction (Behrends and Van Cappellen, 2005; Sheng and Fein, 2014; Ulrich et al., 2011), as well as abiotic U(VI) reduction by Fe(II) (Behrends and Van Cappellen, 2005) and $\Sigma\text{H}_2\text{S}$ (Hua et al., 2006). Similarly, increasing concentrations of Ca^{2+} and Mg^{2+} at pH 8.1 decrease bioreduction rates, albeit to different extents, consistent with previous U(VI) bioreduction studies with Ca^{2+} (Brooks et al., 2003; Sheng et al., 2011; Stewart et al., 2011).

Both thermodynamic and kinetic explanations have been proposed to address the speciation-dependence of uranium bioreduction kinetics (Brooks et al., 2003; Stewart et al., 2011; Ulrich et al., 2011). Existing models primarily focus on the inhibition of bioreduction by Ca^{2+} , which forms ternary Ca uranyl carbonate complexes ($\text{CaUO}_2(\text{CO}_3)_3^{2-}$ and $\text{Ca}_2\text{UO}_2(\text{CO}_3)_3^0$) (Dong and Brooks, 2006) that consequently decrease the concentrations of uranyl carbonate species in solution. Uranyl carbonate complexes generally display higher redox potentials than ternary Ca uranyl carbonate complexes, supporting the hypothesis that the decrease of the most abundant and thermodynamically more favorable species in the presence of Ca^{2+} is responsible for the inhibition of uranium bioreduction (Brooks et al., 2003). The redox potential of the most favorable uranyl carbonate complex, $\text{UO}_2(\text{CO}_3)_3^{4-}$, however, is on the order of 46 mV larger than the most favorable ternary uranyl carbonate complexes, $\text{CaUO}_2(\text{CO}_3)_3^{2-}$ and $\text{MgUO}_2(\text{CO}_3)_3^{2-}$ (Table 4), even in incubations with 4.9 mM Ca^{2+} or 5.2 mM Mg^{2+} , when ternary uranyl carbonate species represent the dominant fraction of U(VI) (Table 1). In addition, the Gibbs free energies of reaction (ΔG_{rxn}) for the reduction of the carbonate and ternary carbonate complexes are all highly favorable and differ by less than 14% (Table 4), consistent with previously

reported findings (Stewart et al., 2011; Ulrich et al., 2011). The small energetic differences between respiration of uranyl carbonates and ternary uranyl carbonates are therefore unlikely to explain the decreases in bioreduction rate in the presence of 4.9 mM Ca^{2+} (14 ± 2 % per mM Ca^{2+}) and 5.2 mM Mg^{2+} (10 ± 2 % per mM Mg^{2+}) (Table 1). Alternatively, ternary Ca uranyl carbonates may be less readily reducible by metal-reducing bacteria as a result of steric hindrance or poor molecular orbital overlap between the U(VI) center and the U(VI) terminal reductase (Sheng et al., 2011; Stewart et al., 2011). A similar explanation may apply to Mg^{2+} , which forms a single ternary carbonate complex with the uranyl ion ($\text{MgUO}_2(\text{CO}_3)_3^{2-}$) (Dong and Brooks, 2008; Geipel et al., 2008). Thus, the formation of ternary uranyl carbonate complexes in the presence of Ca^{2+} and Mg^{2+} may limit uranium bioavailability and prevent electron transfer to U(VI).

4.3 Effect of non-carbonate uranyl species on U(VI) bioreduction rates

Despite the multiple effects of pH and concentrations of DIC, Ca^{2+} , and Mg^{2+} on U(VI) bioreduction rates, the speciation-dependent kinetic model reproduces the bioreduction of uranium as a function of time across nearly all solution compositions tested (Fig. 1). In the model, the bioavailability of uranyl non-carbonate species to *S. putrefaciens* explains the dependence of the bioreduction rates on solution composition. Several uranyl non-carbonate species, including the ‘free’ uranyl ion and some of the hydroxide complexes, have redox potentials comparable to $\text{UO}_2(\text{CO}_3)_3^{4-}$ under the experimental conditions (Table 4), despite consistently representing the least abundant species in solution (Table 1). Simultaneously, the rate constant for the reduction of uranyl non-carbonate species is six orders of magnitude larger than that of the other species (Table 3). Significant differences in bioreduction rate constants among U(VI) species are not unexpected; they are consistent with the recent findings that rate constants for the bioreduction of uranyl hydroxide and organic uranyl species by *S. oneidensis*

strain MR-1 are an order of magnitude larger than those determined for uranyl carbonate species and two orders of magnitude larger than Ca uranyl carbonate species (Ulrich et al., 2011).

Attributing the bioavailability of U(VI) to uranyl non-carbonate species explains the decrease in bioreduction rates with increasing concentrations of DIC in the pH 8.1 incubations, as well as the more significant deceleration of bioreduction in the presence of Ca^{2+} compared to Mg^{2+} (Table 1). The apparent inhibition of uranium bioreduction by Ca^{2+} can be attributed to its ability to significantly decrease the concentration of the more bioavailable uranyl non-carbonate species due to the large formation constants of the two Ca uranyl carbonate complexes ($\text{CaUO}_2(\text{CO}_3)_3^{2-}$, $\beta_{1,1,3} = 27.18$ and $\text{Ca}_2\text{UO}_2(\text{CO}_3)_3^0$, $\beta_{2,1,3} = 30.70$ (Dong and Brooks, 2006)). Simultaneously, the smaller decrease in bioreduction rate in the presence of Mg^{2+} is explained by the lower formation constant of the single Mg uranyl carbonate complex ($\text{MgUO}_2(\text{CO}_3)_3^{2-}$, $\beta_{1,1,3} = 26.11$ (Dong and Brooks, 2006)) and, hence, the smaller decrease in the concentration of uranyl non-carbonate species. Taken together, the kinetic results presented in this study support the hypothesis that the concentration of uranyl non-carbonate species, rather than uranyl carbonate species, controls U(VI) bioreduction rates (Ulrich et al., 2011).

4.4 Uranium toxicity

Regardless of the DIC concentration, uranium bioreduction is negligible in the pH 6.5 incubations (Fig. 1a), even though these incubations exhibit the highest concentrations of bioavailable uranyl non-carbonate species (Table 1). Similarly, bioreduction rates in the pH 7.5 incubations containing 13 and 22 mM DIC are also lower than expected given the calculated aqueous uranyl speciation. As the pH does not significantly affect the kinetics of Fe(III) reduction by *S. putrefaciens* between pH 6.5 and 7.5 (Arnold et al., 1986), the metabolic potential of *S. putrefaciens* (i.e. growth and respiration rate) can be assumed to be the same

across the range of pH conditions employed in the uranium bioreduction incubations. Thus, changes in U(VI) bioreduction kinetics across different pH and medium compositions used in the current study are attributed to changes in uranyl speciation, and any background effects of experimental conditions on cell activity are assumed to be minimal. As a result, uranium toxicity to *S. putrefaciens* is able to explain the inhibition of bioreduction under conditions which U(VI) bioavailability is predicted to be the highest. The viability assays confirm that uranium toxicity to *S. putrefaciens* is also a function of uranyl speciation rather than the total dissolved uranium concentration alone. In other words, uranium toxicity can be predicted by knowing the aqueous uranyl speciation only, whereas knowledge of the total dissolved uranium concentration alone is not sufficient to predict its toxicity. The viability assays further identify the concentration of uranyl non-carbonate species as the best predictor of toxicity across all conditions (Fig. 3), in line with the observed inhibition of bioreduction under incubation conditions containing the highest concentration of uranyl non-carbonate species (Table 1). Under these conditions, accounting for uranium toxicity in the model was required to reproduce the data accurately. Taken together, the combined results of the viability assays and bioreduction incubations therefore indicate that uranium toxicity is primarily responsible for the lack of bioreduction in incubations with the highest concentrations of bioavailable uranyl non-carbonate species. Additionally, the positive concentration-response relationship observed between survival rate and the concentration of ternary uranyl carbonate species (Fig. EA1d) demonstrates that Ca^{2+} prevents uranium toxicity to *S. putrefaciens* by decreasing the concentration of uranyl non-carbonate species via the formation of Ca uranyl carbonate complexes.

Uranium toxicity has been shown to inhibit respiratory processes relevant to contaminated sediments, including denitrification (Tapia-Rodriguez et al., 2012), sulfate

reduction (Nyman et al., 2007), and methanogenesis (Tapia-Rodriguez et al., 2012). In addition, the metabolic activity of uranium-reducing microorganisms is inhibited above a threshold concentration of total dissolved uranium (Khijniak et al., 2005; Sani et al., 2006; Wade and DiChristina, 2000), as also observed for other contaminants that undergo biological transformations, such as phenol (Christen et al., 2012), arsenic (Soda et al., 2006), and technetium (Khijniak et al., 2003). As the viability assays were conducted at a single pH, it is impossible to attribute uranium toxicity to *S. putrefaciens* to a specific uranyl complex (Markich et al., 2000). Nonetheless, upon attributing the inhibition of bioreduction to the ‘free’ uranyl ion (Equation 9), in accordance with the free ion activity model of metal toxicity (Campbell, 1995; Morel, 1983), the model is able to reproduce the entire set of bioreduction incubations, including those obtained at pH 6.5 (Fig. 1a) and 7.5 (Fig. 1b) where toxicity is significant. The 50% lethal concentration (LC₅₀) of 8.5±1.9 pM UO₂²⁺ derived from the viability assay concentration-response relationship (Fig. EA1a) and the IC₅₀ determined by the optimized kinetic model (Table 3) are both relatively low compared to the total dissolved uranium concentrations added to the incubations. The LC₅₀ and IC₅₀ values indicate that *S. putrefaciens* strain 200 is highly sensitive to the ‘free’ uranyl ion and may experience uranium toxicity even at low concentrations of total dissolved uranium.

4.5 Mechanism of speciation-dependent U(VI) bioreduction

Existing mechanisms to explain the toxicity of U(VI) to bacteria invoke the interaction of the uranyl ion with the outer membrane, followed by destabilization of membrane integrity (Bencheikh-Latmani and Leckie, 2003); the transport of uranyl into the periplasm or cytoplasm, followed by reduction and intracellular precipitation of U(IV) minerals (Cologgi et al., 2011); the competitive binding of uranyl to an essential enzyme (Pible et al., 2010; VanEngelen et al.,

2011); or the hydrolysis of DNA by intracellular uranyl (Yazzie et al., 2003). Some of these mechanisms resemble those proposed for U(VI) bioreduction by *Shewanella* spp. Uranium(VI) bioreduction is commonly attributed to the interaction of U(VI) with outer membrane cytochromes (Marshall et al., 2006) or with periplasmic reductases that require transport of U(VI) across the outer membrane (Senko et al., 2007). The present incubations reveal that uranium toxicity and uranium bioreduction kinetics are highly correlated with the concentration of uranyl non-carbonate species across a broad range of geochemical conditions, which points to a common mechanism between U(VI) toxicity and U(VI) bioreduction, or at least closely related mechanisms. According to the kinetic model developed in the present study, DIC, Ca^{2+} , and Mg^{2+} decrease uranium bioreduction rates by forming uranyl complexes that are less bioavailable and by simultaneously decreasing the concentration of more bioavailable uranyl non-carbonate species. Thus, bioavailability, rather than thermodynamics alone, accounts for the speciation-dependence of uranium bioreduction kinetics over the wide range of uranyl non-carbonate concentrations covered in this study. Whereas uranyl non-carbonate complexes are small (U-O_{OH} bond length for uranyl hydroxides, ~2.2 Å (Clark et al., 1999)) and generally positively charged, which enhances attraction to the negatively charged outer membrane (Haas et al., 2001; VanEngelen et al., 2010), uranyl carbonate complexes are bulky (bond length of U-O_{distant} in ternary Ca uranyl carbonates, ~4.1 Å (Kelly et al., 2007)) and negatively charged, which may hinder interactions between the U(VI) center and U(VI)-reducing enzymes (Sheng and Fein, 2013, 2014). Furthermore, electrostatic repulsion between the cell surface and uranyl carbonate complexes may interfere with transport across the outer membrane and, hence, prevent U(VI) from interacting with the intracellular metabolic machinery (in the case of toxicity) or periplasmic reductases (in the case of bioreduction).

As uranyl non-carbonate species are consumed by bioreduction, they are replenished via re-equilibration of the solution. The slow release of strongly bound carbonate ligands may therefore limit the rate of bioreduction. This mechanism does not appear to play a significant role in controlling U(VI) bioreduction rates in the present study, as the speciation-dependent model reproduces bioreduction time series across a broad range of uranyl speciation while assuming thermodynamic equilibrium is achieved at each time step. Thus, electron transfer to U(VI), rather than slow equilibration among uranyl complexes, more likely constitutes the rate-determining step in the bioreduction of U(VI). Even though solution composition alters the U(VI)/UO₂(am) redox potential and the Gibbs free energy of reaction (ΔG_{rxn}) of U(VI) respiration (Table 4), the primary role played by solution composition in uranium bioreduction kinetics is through the effect on aqueous uranyl speciation which, in turn, affects the bioavailability of U(VI) to *S. putrefaciens*. The combined effect of bioavailability and toxicity is observed by comparing overall pseudo-first order rate constants k_{obs} to the aqueous speciation of U(VI) at the onset of reduction across all bioreduction conditions (Fig. 4). The k_{obs} for U(VI) bioreduction display a clear increase with increasing concentration of uranyl non-carbonate species (Fig. 4a). Above a concentration of approximately 4 nM, however, the rate constant decreases with a further increase in the concentration of uranyl non-carbonate species due to the onset of uranium toxicity. Simultaneously, the largest uncertainties in measured k_{obs} values, which also coincide with the largest discrepancies between measured and modeled k_{obs} values (Fig. EA2), are found at the onset of uranium toxicity, where U(VI) bioreduction kinetics transitions from being controlled by bioavailability to being controlled by toxicity. In contrast, when represented against the concentration of the uranyl carbonate (Fig. 4b) and ternary uranyl carbonate species (Fig. 4c), almost all the variability of k_{obs} occurs within a very narrow range of concentration,

implying that bioreduction kinetics are largely independent of the concentration of these species. These findings are confirmed by the rate constants predicted by the model for uranyl carbonate and ternary uranyl carbonate species, which are orders of magnitude smaller than the rate constant determined for uranyl non-carbonate species (Table 3).

4.6 Implications for bioremediation

As geochemical conditions at uranium-contaminated sites are diverse, understanding the effects of aqueous uranyl speciation on bioreduction kinetics is crucial for predicting the fate and transport of uranium. Although the bioreduction incubations and viability assays of the present study were conducted at high concentrations of U(VI) relative to most contaminated subsurface environments, the finding that uranium toxicity is controlled by the concentration of uranyl non-carbonate species, rather than by the concentration of total dissolved uranium, suggests that groundwater composition may affect uranium bioreduction rates even at low total U(VI) levels.

At the three Department of Energy (DOE) Integrated Field Research Challenge (IFRC) sites in the United States, where large-scale bioreduction experiments have been conducted (Hanford, WA; Rifle, CO; and Oak Ridge, TN), distinct waste disposal histories and groundwater compositions (Table EA1) affect the implementation of uranium bioreduction as a bioremediation strategy. At all three sites, ternary uranyl carbonate species represent the main fraction of U(VI) above pH 6.5 due to the prevalence of Ca^{2+} and Mg^{2+} (Fig. 5). At the Hanford site (Fig. 5a), a local minimum in bioreduction rate is predicted at pH 8.8, which corresponds to the minimum concentration of bioavailable uranyl non-carbonate species. Below or above this pH, bioreduction rates are predicted to increase, mirroring changes in the concentration of uranyl non-carbonate species. The pH of Hanford groundwater, however, ranges from 7.1 to 8.7 (Zachara et al., 2013) which includes the critical pH window from 7.5 to 8.1 where, according to

the model, bioreduction rates decrease abruptly with decreasing pH even as the concentration of bioavailable uranyl non-carbonate species increases. Over this pH range, uranium toxicity apparently overcomes the increase in bioavailability of U(VI) as a result of the increase in concentration of the ‘free’ uranyl ion (Fig. 5a). Under these conditions, bioreduction rates are highly sensitive to pH, and bioreduction may be promoted by a slight increase in pH, which decreases the concentration of the toxic ‘free’ uranyl ion despite simultaneously decreasing the bioavailability of U(VI). In turn, Rifle groundwater pH varies from 6.6 to 7.4 (Zachara et al., 2013), a range over which bioreduction rates are inhibited by uranium toxicity (Fig. 5b). The model therefore predicts that the pH of Rifle groundwater should be raised to promote bioreduction. Although the kinetic model predicts similar trends in bioreduction rate above pH ~8 at the Hanford (Fig. 5a) and Rifle (Fig. 5b) sites, bioreduction rates at Hanford are predicted to be ~5 times greater due to the larger concentration of bioavailable uranyl non-carbonate species. Finally, as most metal-reducing bacteria are naturally inhibited under low pH conditions (Edwards et al., 2007; Petrie et al., 2003), uranium bioreduction is not feasible in the most contaminated zones of the Oak Ridge site (Fig. 5c) where the groundwater is highly acidic (pH ~3.5) due to the presence of nitric acid in spent uranium wastes. Correspondingly, substantial treatment of the groundwater was found to be necessary to increase pH, decrease Ca^{2+} concentrations, and promote uranium bioreduction at this site (Wu et al., 2006a; Wu et al., 2006b). Interestingly, the model predicts that uranium bioreduction can be extended to a lower pH at the Oak Ridge site (pH 6.8) compared to the Hanford (pH 7.3) and Rifle (pH 7.3) sites due to higher concentrations of DIC, Ca^{2+} , and Mg^{2+} (Table EA1) which decrease the toxicity of uranium and promote bioreduction.

617 These results indicate that efforts to increase uranium immobilization via bioreduction by
618 promoting the formation of bioavailable uranyl non-carbonate species may ultimately be limited
619 by the toxicity of uranium to indigenous metal-reducing bacteria. Additionally, these findings
620 may explain, in part, the rebound in dissolved uranium concentration observed after extended
621 periods of biostimulation at uranium contaminated sites (Bargar et al., 2013; Williams et al.,
622 2011). The increase in dissolved uranium coincides with an increase in DIC concentration from
623 enhanced electron donor consumption during sulfate reduction. A large DIC:U ratio favors the
624 formation of uranyl carbonate species and promotes desorption of U(VI) from mineral surfaces
625 (Waite et al., 1994) and oxidation of U(IV) by Fe(III)-oxides (Ginder-Vogel et al., 2006), which
626 increase dissolved uranium concentrations. The results presented in this study indicate that large
627 DIC:U ratios will also decrease the concentration of bioavailable uranyl non-carbonate species
628 and retard U(VI) bioreduction, thereby decreasing the net rate of removal of dissolved uranium
629 from the groundwater.

630 Although the geochemical kinetic model developed in the present study is suitable to
631 describe U(VI) bioreduction rates by a model metal-reducing microorganism across a wide range
632 of geochemical conditions, the challenges associated with implementing this model to predict
633 rates of U(VI) bioreduction by subsurface microbial communities should not be understated.
634 Bioreduction rates presented in Fig. 5 primarily serve to illustrate the importance of taking into
635 account aqueous uranyl speciation in the design and implementation of bioreduction-based
636 remediation strategies. These predicted U(VI) bioreduction rates do not consider the background
637 effect of pH on cell activity as the model does not account for the anticipated decrease in
638 respiration rate outside of the habitable pH range of *S. putrefaciens* (e.g., the model erroneously
639 predicts that U(VI) bioreduction rates increase indefinitely above pH 9). Native microbial

communities at contaminated sites likely display a higher tolerance to uranium than *S. putrefaciens* as a result of prolonged uranium exposure (Mukherjee et al., 2012). Similarly, the pH range of optimal metabolic activity likely varies between sites depending on the resident microbial community, and site-specific model parameterization may be necessary (Jin et al., 2013). Furthermore, efforts to increase the concentration of bioavailable uranyl species may also enhance the toxicity of metal co-contaminants commonly associated with uranium contamination (e.g., technetium, arsenic, vanadium, copper, nickel) (Ganesh et al., 1999), which needs to be considered when modeling U(VI) bioreduction rates. Lastly, while significant attention has been given to the effects of inorganic uranyl speciation on U(VI) bioreduction kinetics (Brooks et al., 2003; Sheng and Fein, 2014; Stewart et al., 2011; Ulrich et al., 2011), the roles of organic ligands (Ganesh et al., 1997; Haas and Northup, 2004) and soil humics (Burgos et al., 2007; Gu et al., 2005) in controlling U(VI) bioavailability, toxicity, and solubility remain poorly defined. Quantifying the contribution of natural organic matter to U(VI) complexation will be essential for the development of speciation dependent reactive-transport models able to reproduce the consequences of large-scale biostimulation on uranium mobility.

5. Conclusions

Uranyl bioavailability appears to be the primary mechanism responsible for controlling the speciation-dependence of U(VI) bioreduction kinetics by *S. putrefaciens* in the absence of additional TEAs. Although uranyl carbonate species are thermodynamically favorable electron acceptors, they are not bioavailable to *S. putrefaciens*, most likely because of steric hindrance or poor molecular orbital overlap with the terminal reductase. Often overlooked, uranyl non-carbonate species are both bioavailable and thermodynamically favorable for reduction. Indeed, their concentration is best able to predict bioreduction rates over a wide range of solution

composition. The results of the present study suggest that any changes in solution composition that significantly alter the concentration of uranyl non-carbonate species should have an impact on uranium bioreduction kinetics. At low concentrations of uranyl non-carbonate species, changes in solution composition that promote their formation increase bioreduction rates. Above a threshold concentration, however, the toxicity of uranyl non-carbonate species to metal-reducing bacteria decreases rates of bioreduction. Changes in solution composition above this threshold that decrease the concentration of uranyl non-carbonate species should therefore diminish uranium toxicity to metal-reducing bacteria and accelerate uranium bioreduction.

Although native microbial communities in uranium-contaminated aquifers are likely to display a higher tolerance to uranium than the model organism used here, a uranium toxicity threshold is expected to exist even for the most tolerant microorganisms. The implementation of uranium bioreduction as a bioremediation strategy therefore depends on the adaptation of the microbial community to uranium exposure at a given contaminated site. These results highlight the complex, counteracting ways in which aqueous uranyl speciation affects the kinetics of uranium bioreduction and demonstrate the importance of incorporating aqueous uranyl speciation into future reactive transport modeling efforts. Understanding the effects of the bioavailability and toxicity of U(VI) in subsurface environments is imperative to develop bioreduction-based bioremediation strategies for uranium immobilization.

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9. Figure Captions

Fig. 1. Averaged, time-corrected total dissolved uranium concentration time series in duplicate bioreduction incubations conducted without added Ca^{2+} and Mg^{2+} at pH a) 6.5, b) 7.5, and c) 8.1 in the presence of various concentrations of DIC, and amended with various concentrations of d) Ca^{2+} ; and e) Mg^{2+} at pH 8.1 in the presence of a constant DIC concentration. Symbols represent measured values and solid lines represent optimized model outputs. Error bars represent duplicate incubations.

Fig. 2. Viability assays of *S. putrefaciens* following 6 hours of exposure to 1 mM U(VI) at pH 7.2 ± 0.2 with various concentrations of DIC, Ca^{2+} , and Mg^{2+} . Shown is cell density as a function of time (growth curves) in the presence of a) 10 mM (■), 30 mM (○), and 50 mM (▲) DIC; b) 10 mM DIC and 0 mM (■), 1 mM (○), and 5 mM (▲) Ca^{2+} ; and c) 10 mM DIC and 0 mM (■), 1 mM (○), and 5 mM (▲) Mg^{2+} . Dashed lines represent cell density as a function of time for control cells not exposed to U(VI). Error bars represent duplicate assays. Cells were grown anaerobically on fumarate as TEA and lactate as electron donor in M1 medium.

Fig. 3. Correlation between survival rate of *S. putrefaciens* and the concentration of the sum of all uranyl non-carbonate species in viability assays of cells exposed to 1 mM U(VI) at pH 7.2 ± 0.2 in the presence of various concentrations of DIC, Ca^{2+} , and Mg^{2+} . A two-parameter

logistic function (Equation 9) was fitted to the data ($R^2 = 0.97$). Nearly identical correlations are observed between survival rate and concentrations of select individual uranyl non-carbonate complexes (e.g. UO_2^{2+} , UO_2OH^+ , $(\text{UO}_2)_2\text{OH}^{3+}$). Standard deviations represent propagated error from fitting the exponential region of growth curves.

Fig. 4. Measured ($\circ$) and modeled ($\bullet$) overall pseudo-first order rate constants k_{obs} versus the sum of the concentrations of a) non-carbonate, b) carbonate, and c) ternary carbonate U(VI) species for all incubation conditions. Error bars represent the standard deviation of the slope of the linear regression used to obtain k_{obs} from averaged duplicate incubations or model output.

Fig. 5. Calculated aqueous uranyl speciation (lines) and bioreduction rate ($\bullet$) as a function of pH using background groundwater composition at the a) Hanford, b) Rifle, and c) Oak Ridge US Department of Energy (DOE) Integrated Field Research Challenge (IFRC) sites (Table EA1), assuming a 3 mM acetate injection to stimulate metal-reducing bacteria. Solid vertical lines represent the minimum and maximum measured pH at each site. Bioreduction rates were calculated using the proposed biogeochemical kinetic model and the optimized model parameters (Table 3).

Table 1. Uranium bioreduction incubations: Solution composition, initial aqueous U(VI) speciation assuming thermodynamic equilibrium, and measured overall U(VI) bioreduction rates.

pH	[DIC] ^a (mM)	[Ca ²⁺] (mM)	[Mg ²⁺] (mM)	U(VI) speciation					overall rate ^c (mol/cell/day)
				[UO ₂ ²⁺] (pM)	Σ[non-carb] ^b (nM)	Σ[carb] (μM)	Σ[Ca-carb] (μM)	Σ[Mg-carb] (μM)	
6.5	23±0.2	0.0	0.1	1883	562	686		27.4	1.41±5.63x10 ⁻¹⁷
	36±0.2	0.0	0.1	210	74.2	716		26.6	2.33±3.31x10 ⁻¹⁷
7.5	13±0.3	0.0	0.1	23.9	64.5	366		25.5	2.44±0.46x10 ⁻¹⁵
	22±0.3	0.0	0.1	0.295	2.31	561		28.2	7.02±0.58x10 ⁻¹⁵
	35±1.2	0.0	0.1	0.060	0.404	531		21.6	3.16±0.29x10 ⁻¹⁵
8.1	10±0.8	0.0	0.1	0.089	4.45	523		34.4	6.58±0.48x10 ⁻¹⁵
	23±2.3	0.0	0.1	0.010	0.317	576		27.8	5.00±0.87x10 ⁻¹⁵
	39±0.6	0.0	0.1	0.001	0.038	510		19.4	3.12±0.70x10 ⁻¹⁵
8.1	19±0.1	0.0	0.0	0.004	0.328	525			3.90±0.43x10 ⁻¹⁵
	17±0.4	0.5	0.0	0.005	0.221	199	309		1.68±0.23x10 ⁻¹⁵
	17±1.3	1.0	0.0	0.004	0.206	163	569		1.36±0.11x10 ⁻¹⁵
	17±0.1	4.9	0.0	0.0003	0.010	8.87	720		1.24±0.08x10 ⁻¹⁵
8.1	19±0.1	0.0	0.1	0.024	0.742	535		30.2	1.19±0.28x10 ⁻¹⁴
	18±0.0	0.0	1.1	0.028	0.548	275		191	7.72±1.26x10 ⁻¹⁵
	18±0.1	0.0	5.2	0.019	0.222	138		359	5.74±0.09x10 ⁻¹⁵

^a Shown are the errors associated with measurements of initial DIC concentrations in duplicate incubations.

^b Uranyl non-carbonate species include the ‘free’ hydrated uranyl ion and hydroxide, chloride, sulfate, phosphate, lactate, and acetate uranyl complexes.

^c Shown are the standard deviations of the slopes of the linear regressions used to obtain bioreduction rates from the averaged, duplicate time series of total dissolved uranium concentration. No less than three data points were used to calculate overall U(VI) bioreduction rates.

Table 2. Viability assays: Solution composition, aqueous U(VI) speciation assuming thermodynamic equilibrium, and survival rate.

pH	[DIC] (mM)	[Ca ²⁺] (mM)	[Mg ²⁺] (mM)	U(VI) speciation ^a					Survival rate ^c (percent)
				[UO ₂ ²⁺] (pM)	Σ[non-carb] ^b (nM)	Σ[carb] (μM)	Σ[Ca-carb] (μM)	Σ[Mg-carb] (μM)	
7.2	10	0.0	0.0	210	45.5	937			17.0±1.4
7.3	30	0.0	0.0	1.76	0.488	1000			76.6±24.0
7.5	50	0.0	0.0	0.088	0.042	1000			90.9±16.3
7.2	10	1.0	0.0	80.0	15.5	226	769		13.5±1.1
7.1	10	5.0	0.0	2.98	0.358	4.34	997		71.0±10.0
7.1	10	0.0	0.0	521	65.2	900			0.1±0.1
7.0	10	0.0	1.0	521	50.8	529		421	0.0±0.0
7.0	10	0.0	5.0	298	22.7	219		774	0.4±0.2

^a Total U(VI) is 1 mM for all conditions. Note that concentrations of each group of uranyl species may not sum to 1 mM due to the formation of polymeric uranyl complexes.

^b Uranyl non-carbonate species include the ‘free’ hydrated uranyl ion and hydroxide and acetate uranyl complexes.

^c Standard deviations represent propagated error from fitting the exponential region of growth curves.

Table 3. Optimized rate constants, toxic inhibition factor (F_{toxic}) parameters, and their 95% confidence limits for the speciation-dependent U(VI) bioreduction rate law.

Parameter	Value	95% Confidence limits	
		Lower	Upper
k_1 (non-carbonate) (L/cell/day)	3.42×10^{-6}	1.81×10^{-6}	6.47×10^{-6}
k_2 (carbonate) (L/cell/day)	5.40×10^{-12}	3.79×10^{-12}	7.70×10^{-12}
k_3 (ternary carbonate) (L/cell/day)	1.37×10^{-12}	1.09×10^{-12}	1.71×10^{-12}
IC_{50} (pM UO_2^{2+})	1.02	0.19	5.59
p	1.68	0.95	3.00

Table 4. Solution composition, overall pseudo-first order rate constant (k_{obs}), toxic inhibition factor (F_{toxic}), and calculated thermodynamic parameters for bioreduction incubations over a range of U(VI) bioavailability.

		Bioavailability of U(VI) ^a		
		low	medium	high
pH		8.1	8.1	6.5
[DIC] (mM)		17	18	23
[Mg ²⁺] (mM)		0.0	5.2	0.1
[Ca ²⁺] (mM)		4.9	0.0	0.0
Measured k_{obs} (L/cell/day)		$2.05 \pm 0.08 \times 10^{-12}$	$1.50 \pm 0.05 \times 10^{-11}$	$4.23 \pm 4.10 \times 10^{-14}$
Toxic inhibition factor (F_{toxic}) ^b		1.00	1.00	3.14×10^{-6}
UO ₂ ²⁺	E_h (mV) ^c	-237	-184	-36
	ΔG_{rxn} (kJ/mol) ^d	-124	-136	-153
UO ₂ OH ⁺	E_h (mV) ^c	-245	-191	-43
	ΔG_{rxn} (kJ/mol) ^d	-121	-133	-151
(UO ₂) ₂ OH ³⁺	E_h (mV) ^c	-236	-182	-35
	ΔG_{rxn} (kJ/mol) ^d	-124	-137	-154
UO ₂ (CO ₃) ₃ ⁴⁻	E_h (mV) ^c	-224	-166	-37
	ΔG_{rxn} (kJ/mol) ^d	-129	-143	-153
CaUO ₂ (CO ₃) ₃ ²⁻	E_h (mV) ^c	-271		
	ΔG_{rxn} (kJ/mol) ^d	-111		
MgUO ₂ (CO ₃) ₃ ²⁻	E_h (mV) ^c		-211	-81
	ΔG_{rxn} (kJ/mol) ^d		-126	-136

^a Assuming uranyl non-carbonate species represent the bioavailable fraction of U(VI)

^b Calculated using Equation 9 with the concentration of UO₂²⁺ (Table 1) and optimized values of IC_{50} and p (Table 3)

^c Calculated for the U(VI)/UO₂(am) half-reaction redox couple with the Nernst equation using solution composition and calculated concentrations of each uranyl complex.

^d Calculated for the reduction of each uranyl species to UO₂(am) coupled with lactate oxidation to acetate and bicarbonate using solution composition and the calculated concentration of each uranyl complex. Values are reported per mol lactate.

Figureg

Figure 1 - color

Figure 1 - bw

Figure 2

Figure 3

Figure 4

Figure 5 - color

Figure 5 - bw