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Dissolution of zippeite via bacterial sulfate reduction.

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ABSTRACT

Microbial reduction of U(VI) from solid uranyl phases, forming nanocrystalline uraninite (UO_2) colloids, could promote dispersal and transportation of U in porous media. Under alternating redox conditions in the subsurface, bacterial reductive dissolution may promote U diffusion through recrystallization of U(VI) crystalline phases to finer-grained U(IV) particles, which would tend to solubilize upon encountering oxidizing conditions. Well-characterized synthetic uranyl U(VI) sulfate minerals were used as potential terminal electron acceptors in laboratory cultures of *Desulfovibrio desulfuricans*. This research assessed the microbial respirative bioavailability of two zippeite group phases with different physicochemical properties and ion substitution. Solid phase characterization using X-ray absorption spectroscopy, field emission scanning electron microscopy and transmission electron microscopy of mineral substrates before and after microbial exposure was performed to observe changes in oxidation and secondary mineralization products formed during microbial reduction. The greatest shift of 1.5 eV to lower energies was observed in the Na-zippeite sulfur enriched conditions, with accompanying evidence of bacterial uraninite nucleation.

CO-AUTHORSHIP STATEMENT

The following thesis contains material from a manuscript that will be submitted in the near future. The manuscript entitled, "Dissolution of zippeite via bacterial sulfate reduction", is co-authored by A.E. Forsyth, C. Weisener, P.C. Burns, V. Phoenix, and D.A. Fowle. The author performed laboratory work presented herein. The submitted version of this manuscript appears in Chapter 2.

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STATEMENT OF ORIGINALITY

I certify that the thesis herein is a product of my own work, except as denoted in co-authorship. All other work from other people is acknowledged.

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Å	-	Angstrom
AgCl	-	Silver Chloride
$CaCl_2 \cdot 6H_2O$	-	Calcium Dichloride Hexahydrate
DIRB	-	Dissimilatory Iron Reducing Bacteria
DMRB	-	Dissimilatory Metal Reducing Bacteria
DSMZ	-	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Resource Centre for Biological Material)
DUR	-	Dissimilatory Uranium Reduction
Eh	-	Redox Potential
EPA	-	Environmental Protection Act
eV	-	Electron Volts
EXAFS	-	Extended X-ray Absorption Fine Structure
FESEM	-	Field Emission Scanning Electron Microscopy
IC	-	Ion Chromatography
ICP-MS	-	Inductively Coupled Plasma-Mass Spectroscopy
K_2HPO_4	-	Potassium Phosphate
K_2SO_4	-	Potassium Sulfate
keV	-	Kilo-electron Volts
MCL	-	Maximum concentration limit
MΩ	-	Mega-ohm
$MgSO_4 \cdot 7H_2O$	-	Magnesium Sulfate Heptahydrate

NH_4Cl	-	Ammonium Chloride
NaOH	-	Sodium Hydroxide
Na_2SO_4	-	Sodium Sulfate
O_2	-	Oxygen
pH	-	- log $[H^+]$
SEM	-	Scanning Electron Microscopy
SRB	-	Sulfate Reducing Bacteria
TEA	-	Terminal Electron Acceptor
TEM	-	Transmission Electron Microscopy
TMAO	-	Trimethylamine N-oxide
UO_2	-	Uranium dioxide / Uraninite
µg	-	Microgram
µm	-	Micrometre
XAFS	-	X-ray Absorption Fine Structure
XANES	-	X-ray Absorption Near Edge Structure
XAS	-	X-ray Absorption Spectroscopy

CHAPTER 1 INTRODUCTION

1.1 Overview

Uranium (U) occurs in the Earth's crust and surficial environments in variety of oxidation states and three isotopes: U-235 (0.71%), U-238 (99.28%), and U-234 (approximately 0.0054%). Uranium abundance in geological environments ranges from 1.2 μg/g in sedimentary rocks to 120 μg/g in phosphate rocks (Langmuir, 1997). Concentrations in seawater average around 3 μg/L and continental surface waters contain 0.1 to 500 μg/L U (Shock and Murphy, 1999). The naturally radioactive actinide has been actively utilized as a fuel in nuclear fission processes, and as a result, it has been an actively mined and exploited resource. However, the mining, milling and processing of U ore and the long-term disposal of spent nuclear fuel has become an abiding global environmental and political issue.

1.2 Background

1.2.1 Uranium Geochemistry

The geochemical cycling of U is highly complex although significant progress has been made in recent years in elucidating the various pathways (Langmuir, 1978; Fayek and Kyser, 1999; Murphy and Shock, 1999). In general, subsurface U mobility is controlled by adsorption to mineral materials and dissolution/precipitation of uranium solids (Abdelouas et al., 2000; Barnett et al., 2000; Fredrickson et al, 2000; Liu et al., 2004), which in turn is impacted by its oxidation state. Of the four oxidation states (3+, 4+, 5+, 6+), the two most common are U^{4+} and U^{6+} (U(IV) and U(VI)) (Murphy and Shock, 1999).

The formation of uranium ore minerals such as uraninite (UO_2), coffinite ($USiO_4 \cdot nH_20$), and brannerite ((U, Ca, Y, Ce)(Ti, $Fe)_2O_6$) occurs as fluids rich in oxidized aqueous complexes of uranium come into contact with reducing environments, such as organic-rich sandstones or a fault containing basinal brines. The formation of uranium minerals is intimately related to the geochemical element cycles within their respective geochemical environment, and thus they exhibit incredible structural and chemical diversity. Uranium ore deposits are classified based on geological setting, including: igneous plutonic and volcanic associations, metamorphic associations, and sediment/sedimentary basin associations (Finch and Murakami, 1999). The largest, most enriched uranium deposits, which are of the unconformity type, are preserved in poorly consolidated sedimentary basin sequences. The second most important economic deposits are of the sandstone type (Fayek and Kyser, 1999; Finch and Murakami, 1999; Plant et al., 1999).

As a uranium deposit develops and matures, its paragenesis generally begins via the infiltration of meteoric water. Typically the oxidation and corrosion of uraninite provides opportunity for mobilization of uranium by transforming insoluble U(IV) into readily soluble U(VI) uranyl species, UO_2^{2+}. Uraninite is altered via oxidative fluids, which leads to the formation of secondary uranyl minerals such as uranyl oxide hydrates, including vandendriesscheite, becquerelite, fourmarierite, and masuyite, surrounded by a rind of corrosion products. This corrosion rind was first described by Frondel (1956) as "gummite", varying in thickness from less than a millimetre up to a few centimetres. The average mineralogical zonation of the rind, as summarized by Frondel (1956), is as follows:

Zone 1: (core) dark brown to black uraninite, usually small veins or inclusions of uranyl oxides and uranyl silicates, and may be hydrated.

Zone 2: Pb-uranyl oxide hydrates, alkaline-earth uranyl oxide hydrates, schoepite.

Zone 3: uranyl silicates and, less frequently, uranyl phosphates.

As the groundwater leaches out uranium, the uranyl oxide hydrate phases are replaced by soddyite (uranyl silicate) and curite (uranyl oxide hydrate). The dissolution and replacement of the uranyl oxide hydrates by uranyl silicates and carbonates is ubiquitous (Finch, 1994). Pseudomorphs after uraninite may be leached away, often with the formation of uranyl phosphate minerals such as autunite, phosphoruranylite, parsonite, and uranyl silicates such as uranophane and beta-uranophane in cracks and fractures in the matrix rock (Frondel, 1956; Frondel, 1958; Finch and Ewing, 1992; Finch, 1994; Finch and Murakami, 1999).

Uranyl sulfate minerals, the topic of this thesis, are an example of secondary minerals typically occurring in the oxidized zone of uraninite deposits; sodium-zippeite is typical of the sandstone type and zippeite is more typical of vein occurrences (Frondel et al., 1976). Uranyl sulfates also occur in uranium mine tailings where high concentrations of sulfate (via sulfide oxidation or acid processing) lead to precipitation of these phases. Beyond these locales, uranyl sulfate minerals are found at long-term disposal sites such as Yucca Mountain in Nevada, where typical host rocks contain sulfates and the disposal containers are made of sulfur-bearing steel (DOE PNNL-6415, 2004). Their formation occurs only when sulfides are being oxidized, releasing dissolved sulfate to groundwater that can complex with UO_2^{2+} to form stable uranyl sulfate complexes in solution.

1.2.2 Uranium in the Environment

The release of uranium into the environment is an importunate environmental issue because of both the toxicity of the element and its radioactive nature (Touvinen and Kelly, 1974a,b; Leduc et al., 1997; Suzuki and Banfield, 1999). Releases to the environment include natural weathering reactions, the nuclear fuel cycle which involves the mining, milling and processing of uranium ore to prepare concentrated yellowcake (U_3O_8) (Figure 1), and the ultimate containment of the fuel via the underground tank storage of high-level radioactive wastes from weapons production at sites such as the Hanford Site in Washington. Contamination can lead to concentrations as high as 20 mg/L in tailings pore water (Abdelouas et al., 1999), which is clearly higher than the Environmental Protection Act (EPA) drinking water limit of 0.03 mg/L. Implementing the knowledge of a range of exposure routes and transport mechanisms, various methods for U remediation have been suggested, such as a combination of chemical and biological treatment or phyto-remediation, where *in situ* methods are favoured over the more expensive traditional pump-and-treat technology. The conventional pump-and-treat methods involve the extraction of the contaminated water followed by a treatment process typically involving; ion exchange, reverse osmosis, bioremediation (reduction versus adsorption and accumulation), or chemical precipitation. Newer technology involves the use of zero-valent iron (Fe) permeable barriers in the flow path of a contaminant plume that removes U(VI) by reduction/precipitation and adsorption (Abdelouas et al., 1999). Bioremediation is of key interest worldwide as being more environmentally compatible and more economical, and includes processes such as biosorption, bioaccumulation and bioreduction which may lead to the more cost effective

option of *in situ* remediation. Consequently, any process that leads to the reduction of U(VI) to insoluble U(IV), or U(VI) adsorption onto mineral and organic surfaces, may retard the aqueous transport of uranium and will be of interest to the bioremediation community. Hexavalent uranium may also be sequestered by the formation of relatively insoluble U(VI) mineral phases (e.g. phosphates, vanadates, arsenates). However, if microbial reduction of phases such as uranyl phosphates is possible, the sequestration of uranium in phosphate-amended reactive barriers may be compromised (Fuller and Barger, 2001; Fuller et al., 2002).

1.2.3 Microbial Uranium Reduction

Numerous bacteria have been shown to reduce aqueous phase U(VI), producing amorphous, nano- or microparticulate U(IV) phases (e.g., UO_2), either indirectly (abiotic reduction via H_2S) or directly using their enzymes (enzymatic reduction) (Lovley et al., 1991; Lovley and Phillips, 1992a; Lovley and Phillips, 1992b; Caccavo et al., 1992; Lovley et al., 1993a; Lovley et al., 1993b; Rosello-Mora et al., 1994; Abdelouas et al., 1998; Kieft et al., 1999; Wade and DiChristina, 2000; Fredrickson et al., 2000). Some of the many species of bacteria that have been shown to reduce U(VI) to U(IV) via dissimilatory enzymatic reduction are *Shewanella*, *Geobacter*, *Desulfovibrio*, *Pseudomonas*, *Deinococcus*, *Thermus*, and a halophilic archaea. The dissimilatory reduction of uranium U(VI) (DUR) appears to be mainly incidental reduction by assemblages of bacteria that ultimately affects U speciation in natural sediments (Klinkhammer and Palmer, 1991). For example, marine sediments possess a gradational shift in speciation from oxidized surface sediments to more reducing deeper sediments

where sulfate reducing bacteria (SRB) and dissimilatory iron reducing bacteria (DIRB) dominate (Bonatti et al., 1971; Kadko, 1980; Colley and Thompson, 1985; Cochran et al,. 1986; Honeyman and Santschi, 1988; Wallace et al., 1988; Klinkhammer and Palmer, 1991; Lovley and Phillips, 1992). Moreover, previous studies have shown that both DIRB and SRB can use U(VI) as a terminal electron acceptor (TEA) (Lovley et al., 1991; Caccavo et al., 1992; Lovley and Phillips, 1992; Lovley et al., 1993; Pietzsch et al., 1999). Conversely, bacterial reduction of U(VI) from a solid mineral phase has only been studied by Fredrickson et al. (2000). They utilized a dissimilatory metal reducing bacteria (DMRB) with metaschoepite ($UO_3 \cdot 2H_2O$) and yielded U(IV) phases, including uraninite, as either coatings on the primary U(VI) minerals or disseminated as fine-grained particulates. Yet these particulates (colloids or nanosized phases) could result in uranium mobilization by filtration of U(IV) particulates through aquifer materials (i.e. pore spaces, fractures, etc.), or by advective transport of U(IV) phases on bacteria cell walls or as free particles. Such mobilization could potentially lead to re-oxidation of particles upon contact with oxidizing zones and may lead to the formation of a larger contaminant plume.

The principle behind microbial redox reactions is that the bacteria and their enzymes act as catalysts for the transfer of electrons between an acceptor and a donor (Abdelouas et al., 1999); however, the mechanism for DUR is still unclear. Many studies suggest that enzymes (c-cytochromes) located in the outer sphere of the cell are responsible, where bacteria must be in direct contact with a mineral surface for reduction to occur (Lovley et al., 1991; Lovley and Phillips, 1992; Lovley et al., 1993a; Gaspard et al., 1998; Wielinga et al., 2000; DiChristina et al., 2002; Haas and DiChristina, 2002).

However, recent research suggests that the transfer of electrons via nanowire-type pili located on the surface of the cell, which would lead to larger areas of influence for bacterial cells (Reguera et al., 2005). The other common theory is the abiotic reduction via products of iron and sulfate respiration such as sulfide or hydrogen. Additionally, researchers have proposed the reduction is a one-electron transfer process where U(VI) is reduced forming an unstable U(V) intermediate ($\{UO_2\}^+$) species followed by disproportionation to a stable U(IV) product (Renshaw et al., 2005).

1.3 Conclusions

Based on the observations that SRB can reduce U(VI) and that bacteria can utilize mineral-bound U(VI), it is believed that uranyl minerals, possessing different solubilities and structures, will be preferentially exploited by different types of bacteria. For example, this may be studied by comparing bacterial interactions with minerals possessing slight crystal structure variations such as those shown by members of the extensively studied uranyl sulfate group known as the zippeite group (Frondel, 1956; Vochten et al., 1995; Burns et al., 2003; Brugger et al., 2003). The structure of uranyl minerals is dominated by the approximately-linear, positively-charged uranyl ion $(UO_2)^{2+}$, which occurs in crystal structures co-ordinated into polyhedra of four, five, or six oxygen anions. The zippeite group is composed of uranyl pentagonal bipyramids coordinated into sheets with sulfate tetrahedra linking the uranyl polyhedra. The structural and chemical diversity among group members is a direct result of the cation coordination between the uranyl sulfate sheets (Frondel et al., 1956; Vochten et al., 1995; Burns, 1999; Burns et al., 2003; Brugger et al., 2003).

The objective of this study is to examine the possibility of bacterial dissolution of uranyl minerals, and more specifically, any structural or chemical biases among group members. This research will mark the beginning of an attempt to identify which chemical and/or structural classes of uranyl minerals are most susceptible to microbial respiration, and which are currently thought to remain stable in the subsurface even in the presence of microbes. This investigation is critical to the design of durable reactive barriers that mitigate uranium migration in groundwater, as well as for a better general understanding of the mobility of uranium.

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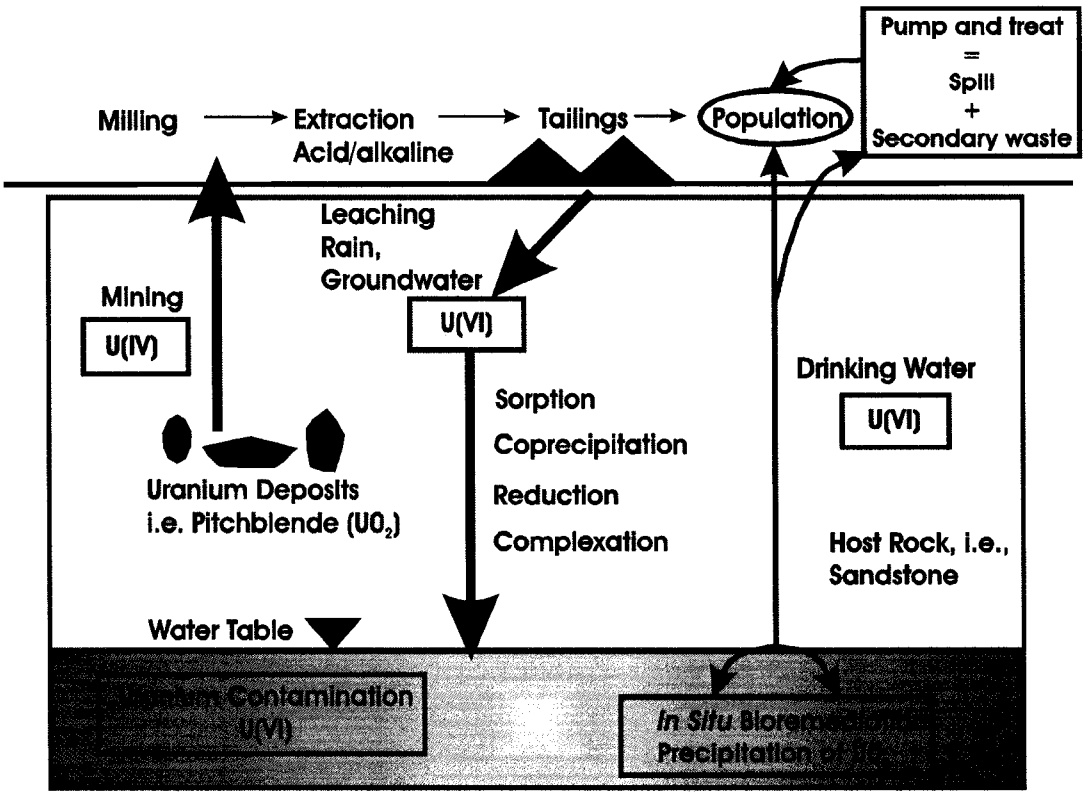

Figure 1: Simplified scheme of human effect on U cycle in nature (adapted from Abdelouas et al. 1999).

CHAPTER 2 SOLID PHASE URANYL REDUCTION VIA SRB

2.1 Introduction

Uranium continues to be an element of concern in both surface and subsurface water at numerous uranium mines, mills and processing sites around the world (Abdelouas et al., 1999). Similarly the vadose zone, groundwater and discharge sites near commercial fuel and weapons manufacturing and production plants have shown signs of significant uranium transport and accumulation (DOE, 2004). All current techniques for remediation involve some degree of concern for their economic viability, long-term stability and effectiveness of the treatment. *In situ* technologies, such as *in situ* redox manipulation and bioremediation, tend to be cheaper and easier to implement; however, the production of nanoparticulate or colloidal U(IV) phases could result in reoxidation of transported particles in the vadose and oxic saturated zones.

Uranium aqueous geochemistry is highly complex and is dependent on a variety of factors including the redox conditions, pH and temperature of the system (Shock et al., 1997). The mobility of the two most common oxidation states of uranium, U(IV) and U(VI), are greatly disparate. Once reduced, U(IV) is widely considered to be immobile, whereas highly mobile U(VI) is heavily influenced by complexation with various organic and inorganic ligands, sorption onto mineral surfaces, precipitation of uranium minerals, the presence and activity of bacteria, as well as coprecipitation into a variety of other minerals (Klinkhammer and Palmer, 1991; Shock et al., 1997).

Various bacteria have been shown to reduce aqueous phase U(VI) producing amorphous, nanosized or microparticulate U(IV) phases (i.e. uraninite) (Lovley et al., 1991; Lovley and Phillips, 1992; Caccavo et al., 1992; Lovley, 1993; Lovley et al.,

1993a; Lovley et al., 1993c; Rosello-Mora et al., 1994; Abdelouas et al., 1998; Gaspard et al., 1998; Kieft et al., 1999; Fredrickson et al., 2000; Wade and DiChristina, 2000; Wielinga et al., 2000; Haas and DiChristina, 2002). Uranium speciation in natural sediments appears to be a result of an association of dissimilatory uranium reducing (DUR) microorganisms (Klinkhammer and Palmer, 1991). Although few bacteria can grow using U(VI) as a terminal electron acceptor (TEA), it is commonly regarded that the enzymes (e.g. reductases or cytochromes) responsible within the electron-transport chain in dissimilatory iron-reducing bacteria (DIRB) and sulfate-reducing bacteria (SRB) are sufficiently nondiscriminatory that U(VI) may substitute for other TEAs (e.g. Fe(III), Mn(IV), various sulfur species) (Lovley et al., 1991; Lovley et al., 1993a; Pietzsche et al., 1999). Among those bacteria that can grow using U(VI) as sole TEA, U(VI) and Fe(III) can compete, depending upon their speciation and relative concentration (Wielinga et al., 2000). As such, dissimilatory Fe(III) and Mn(IV) reduction by DIRB appears to be linked to outer-membrane expression of terminal reductases joined to trans-membrane and intracellular electron transport components, supporting a model wherein direct bacterial cell-mineral contact facilitates respiratory reduction of insoluble Fe(III) and Mn(IV) phases (Gaspard et al., 1998; DiChristina et al., 2002). Blakeney et al. (2000) found that gene expression for Fe(III) reductase was the same for Fe(III), Mn (IV), and U(VI). Furthermore, SRB reduce U(VI) via the outer membrane cytochrome c_3 (Lovley et al., 1993a; Payne et al., 2002; Payne et al., 2004). These data support a model where DUR occurs at the outer cell membrane, leading to increased bioavailability of aqueous U(VI) and potentially solid phases. Fredrickson et al. (2002) studied the reduction of schoepite-bound U(VI) and found that *Shewanella putrefaciens* will utilize and reduce mineral-

bound, solid-phase hexavalent uranium. However, this is the only experiment known to examine the bacterial reduction of U(VI) from the solid phases and is shown to yield U(IV) phases, including uraninite, as either coatings on primary U(VI) minerals or disseminated fine-grained particulates. Additionally, the study of solid-phase electron acceptors has shown that sulfate is biologically reactive under reducing conditions to sulfate-reducing bacteria (Karnachuk et al., 2002).

In this study, the interaction of a well-characterized type of sulfate-reducing bacterium (SRB) on the behavior of uranium in solid phase minerals is studied, to address the following principal research questions:

- Do obligate sulfate-reducing bacteria affect the U(VI)-phase reductive alteration for two different uranyl sulfate group members?
- Does the structure or composition of the U(VI) protolith affect the structure, composition and/or growth habits of the U(IV) products?

These questions will be answered via experiments involving *Desulfovibrio desulfuricans* incubated, under conditions likely to be encountered in the subsurface environment, with selected uranyl sulfate minerals. The solution chemistry and characterization of the solid phases resulting from U(VI) reduction will be investigated using a combination of geochemical, mineralogical and spectroscopic techniques.

2.2 Experimental

2.2.1 Mineral Synthesis and Bacterial Growth.

Uranyl sulfate minerals of the zippeite group were produced following the mild hydrothermal synthesis technique developed by Burns et al. (2003) in the Crystal Structure and Environmental Mineralogy Laboratory at the University of Notre Dame (see Table 1 and Figure 2). Solutions of 0.2 M uranyl nitrate ($UO_2(NO_3)_2 \cdot 6H_20$) with either K_2SO_4 or Na_2SO_4 were combined and pH adjusted (NaOH) in 23 or 125 mL Teflon-lined Parr reaction vessels, which were then heated in Fisher Isotemp mechanical convection ovens to 150 °C for 24 hours. The resultant precipitates were suction-filtered using an acetone wash and dried on the filtration paper. The minerals were identified and confirmed using a Bruker D8 Discovery powder X-ray diffractometer (XRD). Vochten et al. (1995) proposed the solubility of zippeite as such (no data for Na-zippeite):

$$K(UO_2)_2SO_4(OH)_3 \cdot H_2O(s) \leftrightarrow K^+ + 2UO_2^{2+} + SO_4^{2-} + 3OH^- + H_2O(l)$$

$$K_{sp} = 10^{-42.60}$$

All mineral products were sieved and the 75-125 μm fraction was selected for experimental use.

A basal medium (minimal nutrients for growth including inorganic salts and a carbon source) for the sulfate-reducing bacteria was used for all experiments to minimize potential chemical interactions. Experimental cultures were incubated in a COY anaerobic chamber (COY Laboratory Products, Grass Lake, MI, USA) containing a N_2:H_2 gas mixture of 95:5. Slight modification of DSMZ medium 63 was used to grow a stock solution under standard anaerobic conditions of *Desulfovibrio desulfuricans* (DSMZ) as follows: 0.5 g/L K_2HPO_4, 1.0 g/L NH_4Cl, 1.0 g/L $NaSO_4$, 0.1 g/L

$CaCl_2 \cdot 6H_2O$, 2.44 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L 60% Na-lactate syrup, 1 g/L yeast extract, 0.001 g/L resazurin, 0.1 g/L Na-thioglycolate, 0.1 g/L ascorbic acid, made to 1 L using ultrapure (18MΩ) water, pH adjusted to 7.8 using NaOH. After 48 hours, the 9 mL experimental batches were inoculated with 1 mL of the stock solution. The concentrations of the mineral phases were between 1 and 5 g/L. Four batches were run simultaneously for each zippeite mineral phase (see Table 2). Each batch was sampled and syringe filtered through 0.45-micron nylon filters at designated time intervals. The remainder of the sample was subdivided for later solid phase determinations. The rationale for sulfate-deficient media was to identify whether or not the sulfate present in the mineral phase was bioavailable.

2.2.2 Sample Analysis.

An Orion pH electrode was used to measure variations in solution pH. Sulfide concentrations were measured using an Orion platinum redox with a Ag/AgCl reference electrode as a proxy for reduction-oxidation conditions. A standard anti-oxidizing buffer was used in a 1:1 ratio for all sulfide measurements.

2.2.2.1 Ion Chromatography. Upon dilution with ultrapure (18MΩ) water, the lactate-acetate and anion concentrations in the supernatant were measured by ion chromatography (IC) using a CD25 conductivity detector (Dionex Corp., CA, USA) with an IonPac AS11-HC separation column and AG11-HC guard column and ASRS-Ultra 4 mm self-regenerating suppressor.

2.2.2.2 ICP-MS. The samples were analyzed by inductively coupled plasma-mass spectroscopy (ICP-MS) to obtain total uranium concentrations. The samples were

acidified with high purity nitric acid and diluted with high purity internal standards prior to analysis (Be, In, Tl).

2.2.3 Solid Phase Characterization.

2.2.3.1 Microscopy. Solid phase characterization via field emission secondary electron microscopy (FESEM) and transmission electron microscopy (TEM) was used to distinguish the microbial spatial associations and secondary biomineralization products. Sample preparation for all microscopy analyses was performed under anaerobic conditions in the COY anaerobic chamber.

2.2.3.1.1 FESEM. Samples were positioned on carbon tape on SEM sample stubs. FESEM micrographs were attained using a FEI field emission secondary electron microscope equipped with an energy dispersive spectrometer (EDS) detector, operating at a 5 kV primary voltage to optimize surface morphology features.

2.2.3.1.2 TEM. Bacteria/mineral composites were diluted with ultrapure water to a fine suspension and then 15 μL droplets were placed onto Formvar® and carbon-coated copper TEM grids. Excess water was blotted off with filter paper. Bacteria-mineral associations and mineral characterization was determined using a Philips EM 400T TEM operating at 100 kV. Mineral compositions were determined using an EDAX Sapphire EDS, whereas selected area electron diffraction (SAED) was used to determine mineral structure. The *d*-spacings and diffraction patterns determined from SAED and EDS compositional data were used together to identify mineral phases.

2.2.3.2 XANES. In order to determine the valence state of uranium, a subset of samples was collected and analyzed using X-ray absorption near-edge structures (XANES). Samples were prepared and placed on double-sided Kapton tape in the COY anaerobic chamber to ensure no further oxidation and transported to the Advanced Photon Source (APS), Argonne National Laboratory, Argonne, Illinois, in vacuum-sealed vessels. The XANES data were collected at the APS on beamline 13BM-GSECARS using a Si (111) monochromator. An energy range from 7-70 keV that covers the range of U *L3* edge (17166 eV) was used for our sample characterization. Experimental data was processed using *ATHENA* software (Newville, 2001). The detector used was a 13-element germanium detector. The scans were collected from 100-50 eV before the edge to 200-600 eV above the edge. All spectra were processed by subtracting the pre-edge and post-edge backgrounds, and normalizing the step height to 1. Spectra were acquired at 0.3 to 2 eV step intervals over a 200 eV range relative to the 17166 eV energy. The XANES spectra were processed by averaging data scans collected with background removal to isolate the fine structure scattering component and Fourier transformations of the scattering curve. The extended X-ray absorption fine structure (EXAFS) spectra were Fourier transformed using an unsmoothed window over the k range from 2 to 13 $Å^{-1}$. The XAFS scattering curve was weighted by k^3 (k is the electron wave vector) during the background removal and prior to the Fourier transformation to enhance weak scattering oscillations.

2.3 Results and Discussion

2.3.1 Analytical Aqueous Chemistry.

In an effort to understand the aqueous chemistry of the zippeite mineral - *D. desulfuricans* reduction batch experiments were performed at a circum-neutral pH of 6.5-7.6 and data collection occurred at specific time intervals. Two different treatments were utilized to elucidate the relationship between sulfate and bacteria. One in which an excess of aqueous sulfate was added to the initial media, and the other in which a trace amount of aqueous sulfate was added during inoculation. The lactate-acetate concentrations are plotted for K-zippeite in sulfate-poor media (see Figure 3). The lactate concentration decreases steadily until approximately 72 hours where the acetate concentration crosses over in an increasing trend. This pattern of decreasing lactate concentrations coincident with the production of acetate is consistent with bacterial oxidation of lactate. However, there is a discrepancy in the mass balance of lactate to acetate in comparison to the initial concentration of lactate of 17.8 mM to the IC data of a total of 0.4 mM. Therefore, the lactate data should be treated as merely an indicator of the occurrence of bacterial oxidation.

Total sulfate concentrations in the sulfur enriched biogenic media show an initial decrease from 14.3 mM to 9.5 mM after approximately 72 hours (see Figure 4a and 4b). The samples under sulfur poor biogenic conditions show the same trend of decreasing concentration, however the initial concentration of 1.5 mM increases to 1.9 mM after 24 hours, then decreases and remains around 0.1 mM after 72 hours. Both sets of experimental controls show no systematic decrease in sulfate concentrations. These data show that the initial inoculation concentrations are reduced within 72 hours. As well,

since no additional spikes in sulfate are observed, it can be assumed that sulfate released from the mineral is subsequently reduced.

An increasing sulfide trend that is indicative of a reducing environment (see Figure 4a and 4b). These reducing conditions are created by microbial reduction of sulfate to sulfide, as seen in Mohagheghi et al. (1984). Sulfide concentrations for the sulfur-enriched biogenic media show the largest increase where concentrations rise from 0.02 to 1.02 mM after 72 hours, then decrease to 0.28 mM after 336 hours. Under sulfur-poor biogenic conditions, we observe markedly lower concentrations of sulfide, but still note a similar peak trend appearing after 72 hours, equilibrating thereafter. Both sets of control experiments show little to no sulfide production, indicating that the reduction of sulfate, via reduction to sulfide, is strictly a bacterial process in our experimental conditions.

Dissolved uranium concentrations (see Figure 5) illustrate that uranium is readily released into solution by the bacteria in the presence of excess sulfate in solution. Concentrations of uranyl species vary from 1.02 μM at 12 hours to 0.20 μM after 24 hours. Beyond 24 hours, a linear increase is evident where the final concentration of 4.74 μM is reached at 360 hours. In contrast, in the sulfur-poor biogenic media, the uranium does not release as rapidly with concentrations ranging from 0.02 to 0.42 μM. According to Yee et al. (2004), the ionic strength of the solution plays a major role in the reduction process. Since electrostatic sorption of bacteria onto charged surfaces is highly sensitive to electrolyte concentration, solutions with a high ionic strength will compress the electric field of the bacterial cell, thereby decreasing the electrostatic attraction between the charged bacterial surface and the zippeite mineral. Since the presence of

such high concentrations of sulfate in solution creates an environment of high ionic strength, the release of uranium into solution would further increase the ionic strength. It would be expected that this situation would retard bacteria-mineral interaction and the subsequent reduction of any uranium in solution.

Phosphate concentrations (see Figure 6) illustrate a preliminary assessment of phosphate behavior in a sulfate-reducing system. Concentrations of phosphate in the sulfur-enriched biogenic samples oscillate within the first 45 hours from 81.5 to 64.0 to 89.5 mM, and then generally decrease due to the use of phosphate in the production of ATP. The sulfur-poor biogenic samples show an initial increase in phosphate concentrations to a peak at 147.1 mM after 72 hours, and then decrease steadily to 65 mM.

The 72-hour correlation observed in all of the aqueous chemistry data could be a result of a long lag phase in the bacterial growth process. Research performed by Mohagheghi et al (1984) describes a two-stage process for the growth of sulfate-bacteria where the first stage (0 to 80 hrs) is characterized by a rapid rate of sulfate removal. The second stage (> 80 hrs) is characterized by a decrease in the rate of sulfate removal. This growth cycle is consistent with the results shown in this experiment, where there is a rapid rate of sulfate removal beginning around 72 hours. A two-step reduction process may also exacerbate the lag, where initially the bacteria directly reduce aqueous sulfate and then, once the most readily accessible electron acceptor source is depleted, the bacteria would reduce the mineral-bound sulfate as its electron acceptor source. The bacterial reduction process would involve the following reactions:

$$CH_3CH(OH)COOH + 3H_2O = 3CO_2 + 12H^+ + 12e^-$$

$$SO_4^{2-} + 9H^+ + 8e^- = HS^- + 4H_2O$$

$$UO_2^{2+} + e^- \leftrightarrow UO_2^+ \quad \Delta_r G = -8.471 \text{ kJ/mol (Grenthe et al., 1992)}$$

$$UO_2(s) \quad \Delta_f G = -1031.83 \text{ kJ/mol (Grenthe et al., 1992)}$$

The apparent sulfur dichotomy (i.e. sulfur-enriched versus sulfur-poor) may provide some insight into the mechanism of DUR. The mobilization of sulfate in sulfate-poor media appears to be directly related to the bacteria's ability to forage for terminal electron acceptors from the mineral surface, with no release of uranyl species to solution. These observations support the findings of Spear et al. (2000) in that the rates of reduction for sulfate and U(VI) are disproportionate, including a lag time in uranium reduction in the absence of sulfate. Conversely, sulfur-enriched solutions showed sulfate reduction with time; however, the overall mass balance between the amount of sulfate reduced and sulfide produced is not evident. Furthermore, the persistence (and increase in concentration) of aqueous uranyl species over the course of the experiments implies that uranyl complexes are being formed effectively sequestering the U(VI) phases from reduction. This also coincides with a slight increase in pH.

2.3.2 Solid Phase Characterization.

Morphological and structural alterations during the experiments were characterized using FESEM, TEM, and X-ray absorption spectroscopy. FESEM micrographs acquired after 144 and 359 hour exposure show etch features characteristic of bacterial contact with minerals (see Figure 7). The edges of the uranyl sulfates appear coated with extracellular polysaccharide (EPS) and demonstrate substantial dissolution textures such as reaction rims, etch pits, secondary crystals, and rounded edges. The

dissolution of minerals via microbial attachment implicates an affinity for the identification of high-energy sites as well as a definite recognition with respect to crystallographic orientation. The presence of reduced uranium (uraninite) is apparent from the TEM data (see Figure 8). After 145 days of exposure, the TEM data shows the complete encapsulation of *D.desulfovibrio* by an electron-opaque mineral phase. This mineral phase was identified using EDS and SAED as a crystalline uranium U(IV) oxide/hydroxide. The diffraction pattern suggests the structure is either hexagonal or cubic with *d*–spacings of 3.6 Å, making cubic uraninite the most likely candidate. Other phases were considered but were rejected on the basis of either compositional or crystallographic data. The size and morphology of the uraninite phase strikingly resembles a cluster of bacteria with rounded ends. The expression of rounded ends involving a crystalline mineral phase signifies the likelihood that the cells function as nucleation sites for the precipitation of uraninite. Furthermore, the formation of electron-opaque nanocrystals on a bacterium in close proximity to the uraninite-encrusted cluster supports the theory of uraninite nucleation on the cell surfaces. Extra-cellular uraninite is also consistent with previous findings where respiratory reductase enzymes or cytochromes are located at the outer cell aspect (Lovley et al., 1993a; Gaspard et al., 1998; DiChristina et al., 2002; Haas and DiChristina, 2002; Payne, 2002). Therefore the bacteria may utilize the sulfate bound in the mineral as a TEA and subsequently reduce the uranium freed from the metabolic process of sulfate reduction. However, Payne et al. (2005) found that a mutant type of *D.desulfuricans* (I2) lacking cytochrome c3 was still able to reduce U(VI) with lactate as the sole electron donor but at only half the rate of the wild type, implying the operation of pathways independent of cytochrome c3 function.

An independent pathway is proposed in the research performed by Reguera et al. (2005), where the study of c-cytochrome-deficient Fe(III)-reducers found that pili on the cell surface can transfer electrons through direct cell-surface contact and effectively serve as biological nanowires.

Synchrotron-based X-ray absorbance spectroscopy was performed to corroborate and document the chemistry during *in situ* morphological alterations. Standards were used to confirm the uranium end member oxidation states i.e. Na-zippeite and zippeite standards represent the most oxidized phases and uraninite represents the most reduced phase. Solid phase composites were analyzed at 24, 144, and 359 hrs using beamline 13-BM-GSECARS at the APS. As the time of exposure increases, an energy shift in U *L3* edge spectra becomes evident. The U *L3* edge spectra (see Figures 9 and 10) show a shift in oxidation state towards a more reduced phase in the samples was apparent for the sulfur-enriched media, however, the shift to lower energies (1.5 eV) was more significant in the Na-zippeite samples with sulfur limiting conditions. After 24 hours of exposure, the sulfur-poor samples show a greater shift for both zippeite and Na-zippeite than sulfur-enriched samples. After 359 hours of exposure, the same zippeite sample without sulfur shows very little to no shift, whereas the greatest shift of 1.5 eV is now seen in the Na-zippeite without sulfur sample. Moreover, after 145 days (see Figure 11), significant reduction occurred in the sulfur-enriched sample. None of the control experiments demonstrated any sign of reduction.

The relationship between bacterial exposure and a shift in energy of uranium, as indicated by FESEM, TEM, and XANES data, implies that *D.desulfuricans* are responsible for reductive dissolution of zippeite group minerals and concomitant

uraninite precipitation. The excess of sulfate appears at first glance to inhibit U(VI) reduction as seen by the increase and persistence of aqueous concentrations of U(VI), however, the greatest shift in oxidation was seen under sulfur-enriched Na-zippeite conditions (3.71 eV after 145 days). Also, it is clear that the zippeite minerals were reduced under sulfur-poor conditions but only produced U(IV) phosphate phases, as shown by TEM images. The observation of uraninite production under sulfur-rich conditions not only supports earlier findings of U(VI) reduction in marine and estuarine environments (Klinkhammer and Palmer, 1991), but also confirms the disparity in reduction rates between sulfate and uranium (Spear et al., 2000). Whether or not one zippeite group member is more readily reduced than the other cannot be concluded from the data presented herein. The apparent extracellular uraninite nucleation and encrustation denotes the function of c-cytochromes and/or pili on the cell surface.

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Table 1: Mineral synthesis parameters for zippeite and Na-zippeite.

Mineral	Solution	pH	T (°C)	t (hrs)
Zippeite	30mL 0.2M uranyl nitrate, 1.975g K_2SO_4	2.5	150	24
Na-zippeite	30mL 0.2M uranyl nitrate, 2.130g Na_2SO_4, NaOH	3.6	150	24

Table 2: Conditions for simultaneous batches (including controls) of bacteria in the presence of uranyl sulfate phase.

Batch	Sulfur Enriched	Sulfur Poor	Bacteria	Mineral
1	+		+	+
2	+			+
3		+	+	+
4		+		+

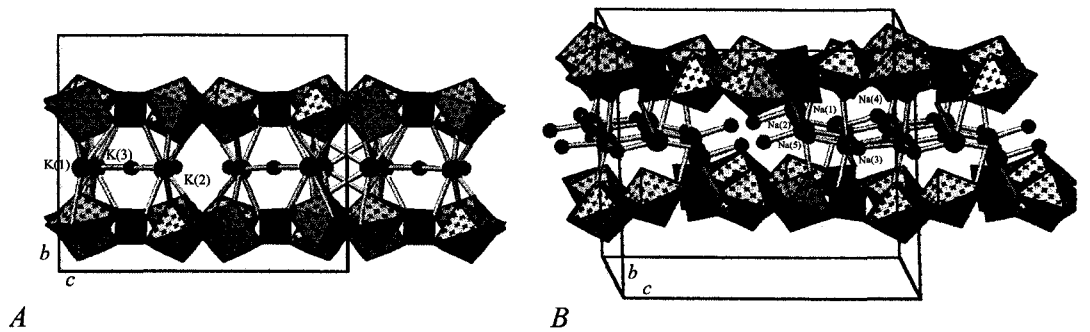


Figure 2: Schematic structural diagrams of A) zippeite and B) Na-zippeite. Yellow pentagonal pyramids represent uranyl groups; blue tetrahedra represent sulfate groups; purple spheres represent hydroxide groups; red spheres represent A) potassium and B) sodium ions (from Burns, 2003).

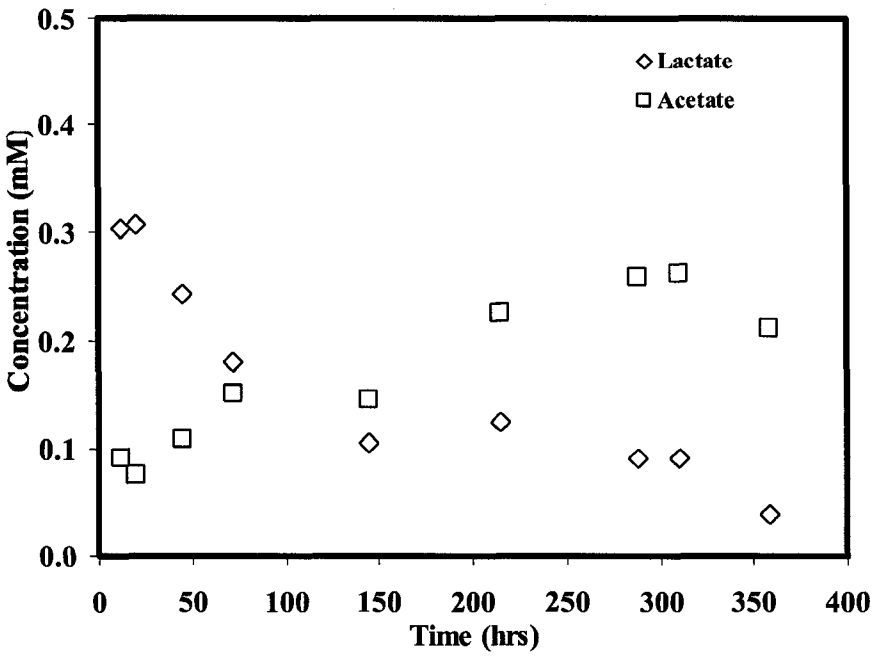


Figure 3: Release of lactate and acetate into solution as a function of time.

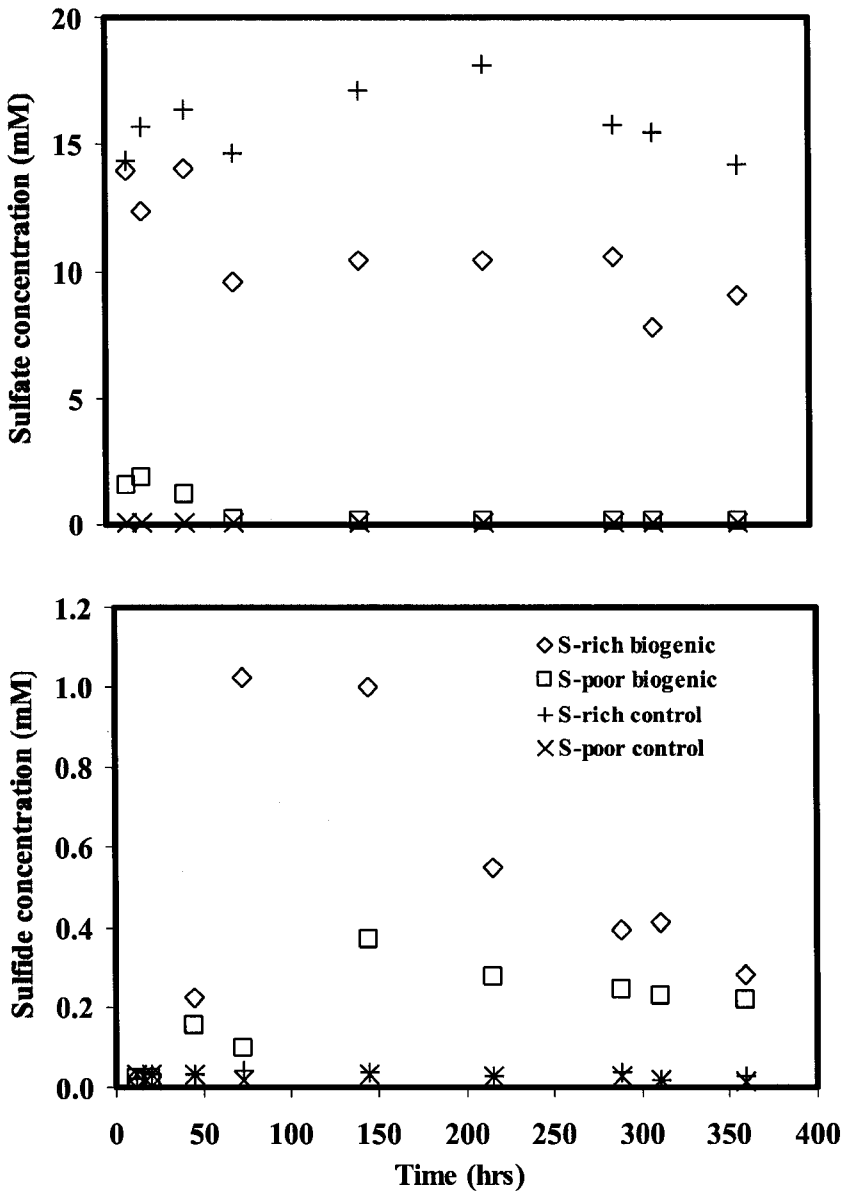


Figure 4a: Aqueous concentrations of sulfate and sulfide as a function of time for Na-zippeite.

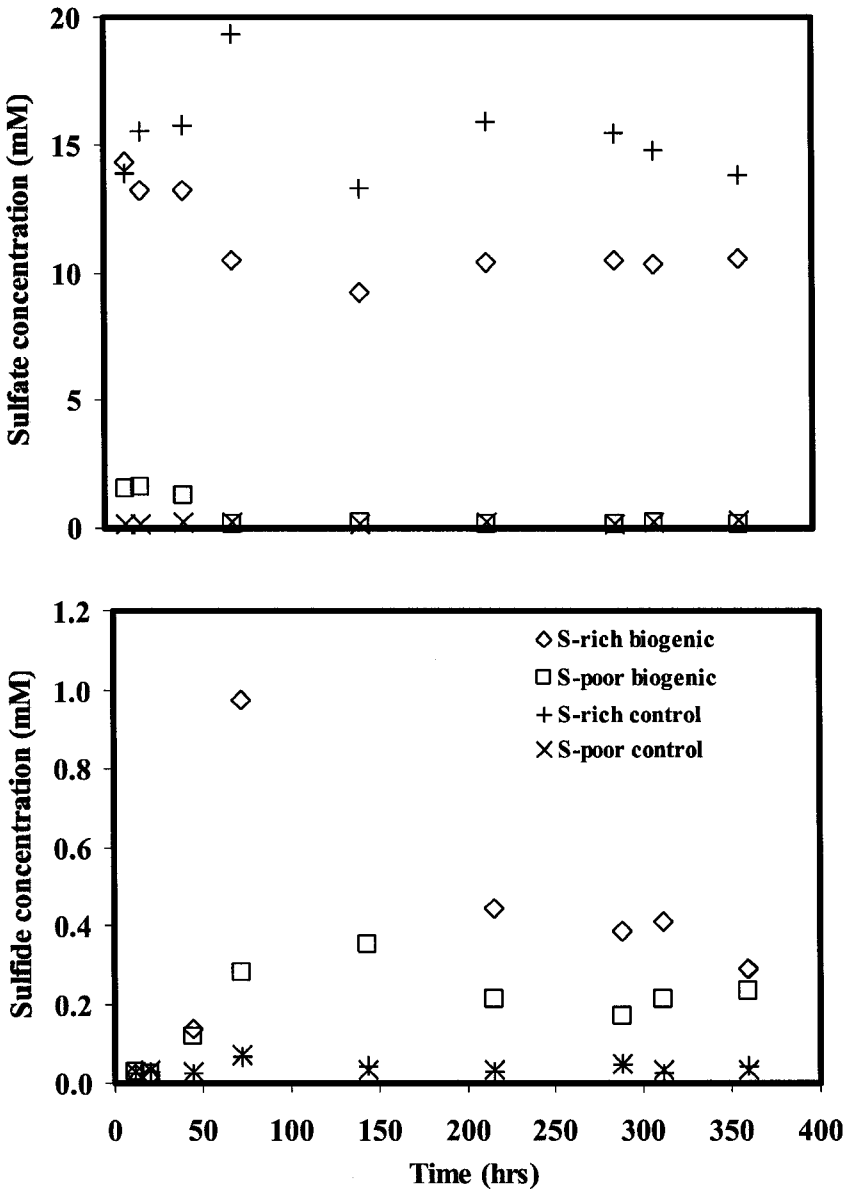


Figure 4b: Aqueous concentrations of sulfate and sulfide as a function of time for zippeite.

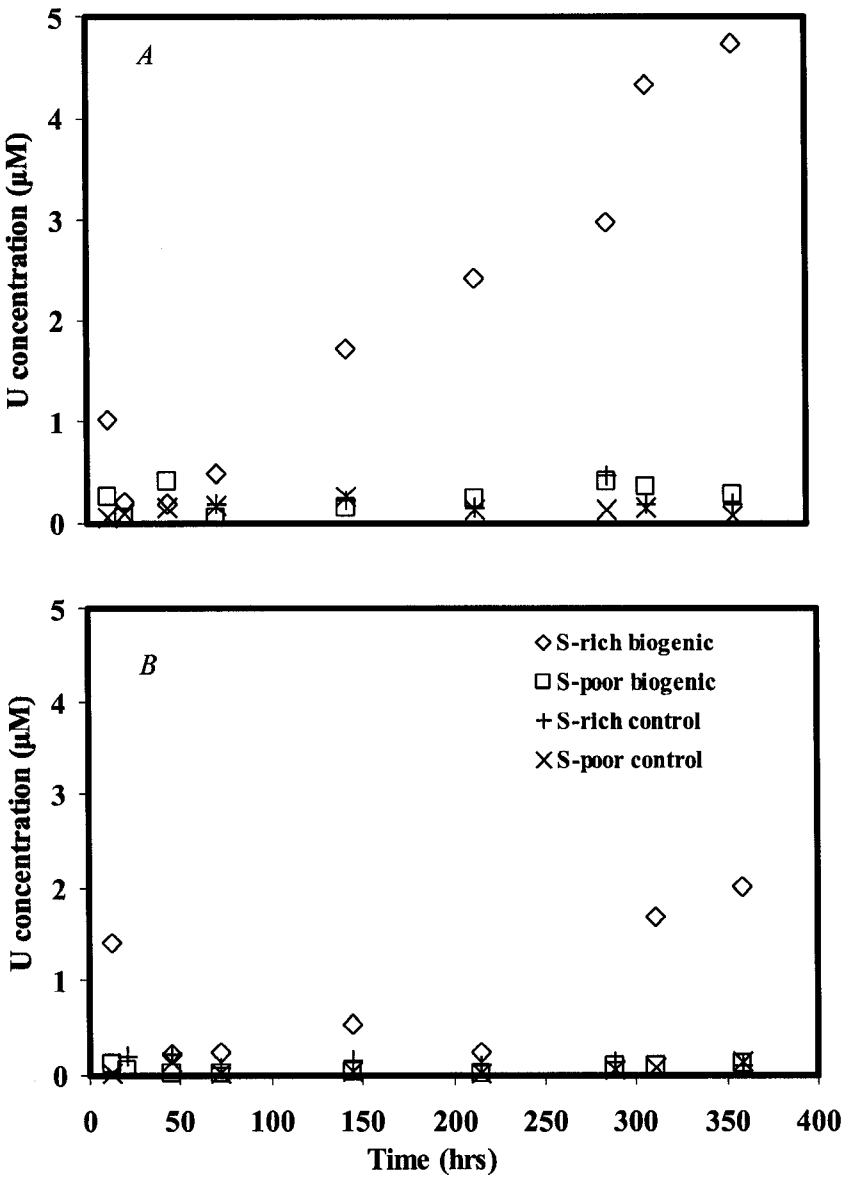


Figure 5: Uranium concentrations for Na-zippeite (A) and zippeite (B).

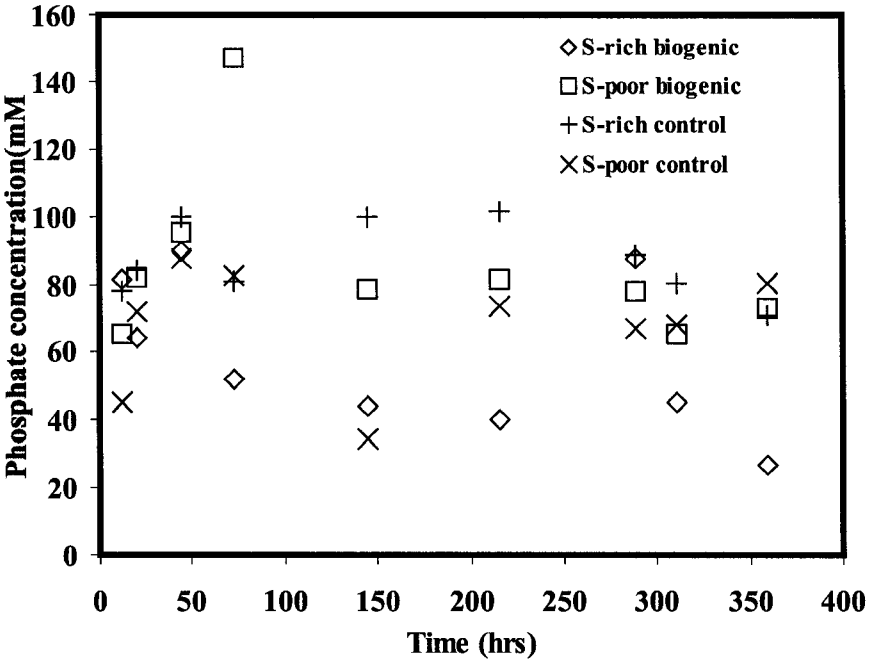


Figure 6: Phosphate concentrations for Na-zippeite.

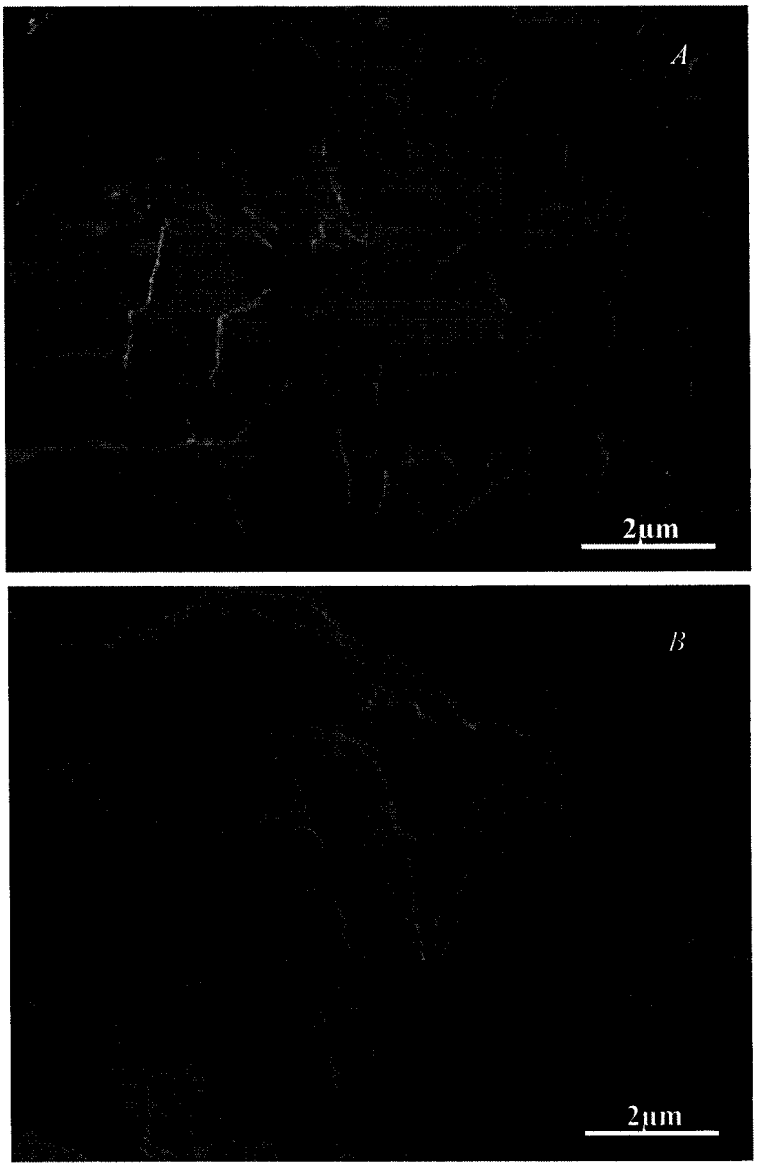
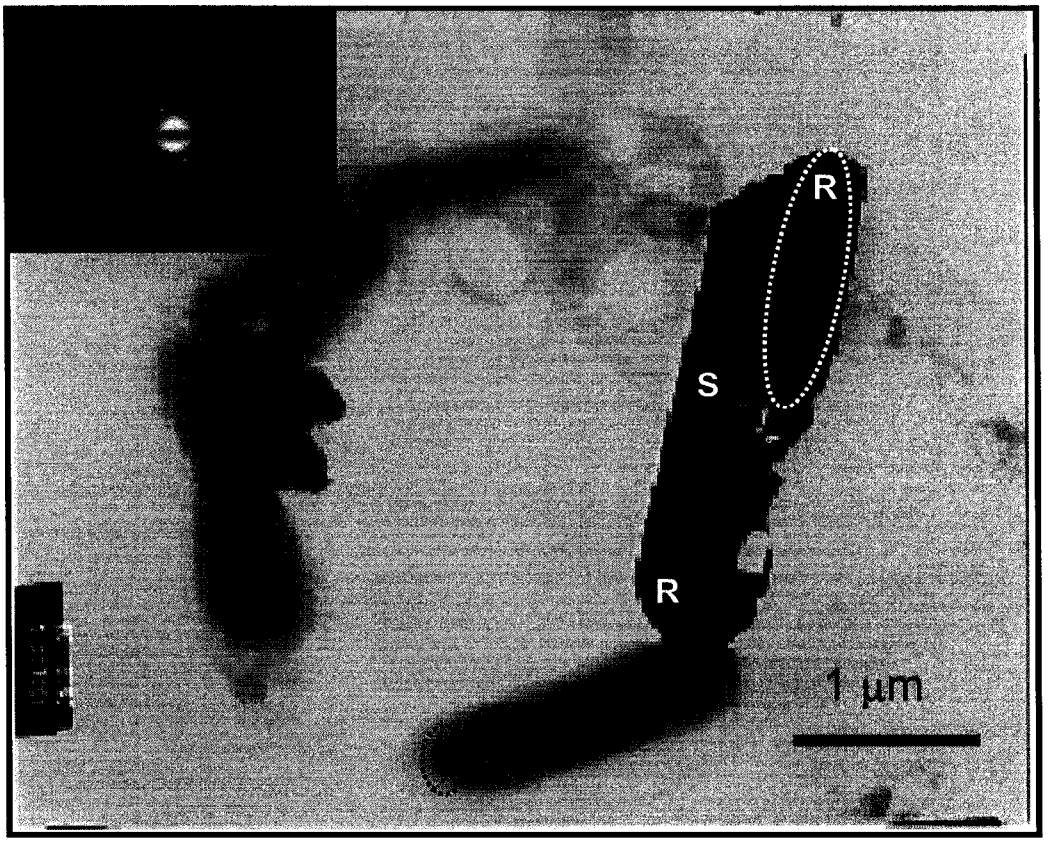


Figure 7: FESEM micrograph of Na-zippeite after: A) 144 hrs of exposure and B) 359 hrs of exposure to sulfur-enriched conditions.



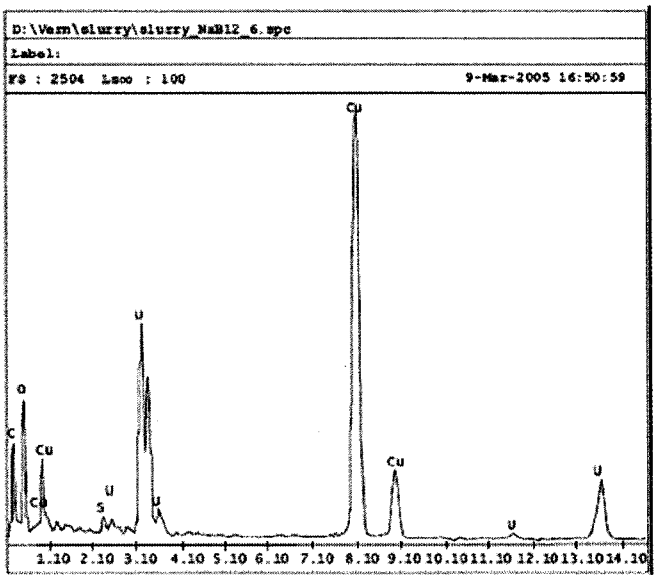


Figure 8: TEM data. Photomicrograph of pure uranium oxide phase encrusting a cluster of bacteria and nanocrystals on the attached bacterium. Corresponding EDS spectrograph and SAED pattern. R symbolizes the rounded end of a bacterium and S represents the location of a septum between two bacteria. The white circle outlines one bacterium and the black circle highlights a nanocrystal of uraninite.

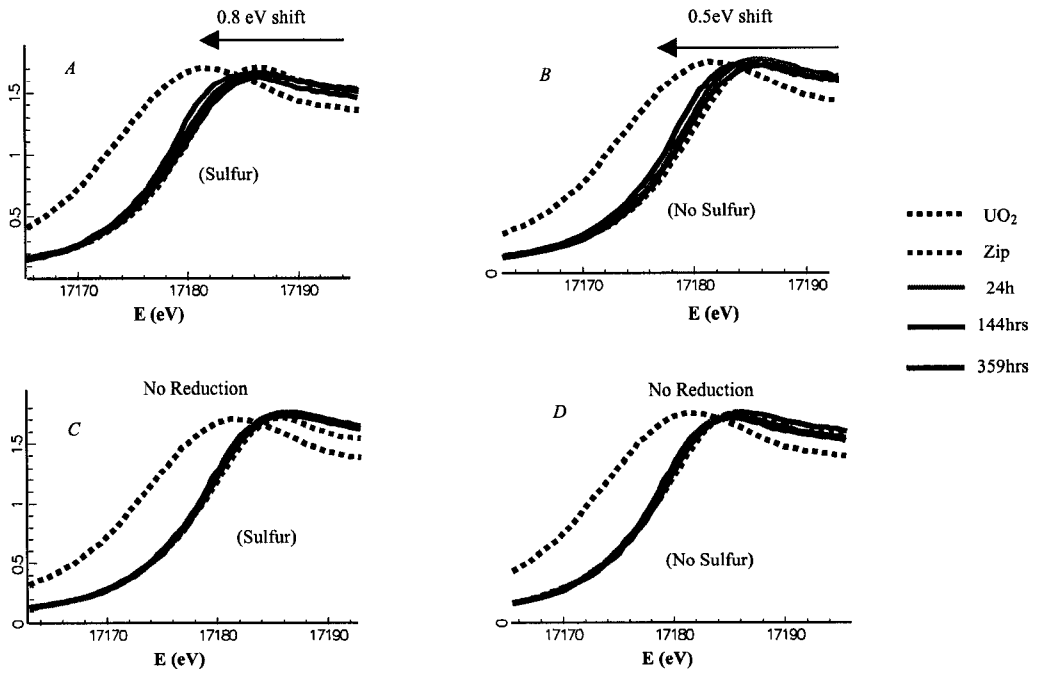


Figure 9: XANES data for zippeite reacted with (A and B) and without (C and D) Desulfovibrio desulfuricans, normalized intensity plotted on the y-axis and energy in electron volts on the x-axis.

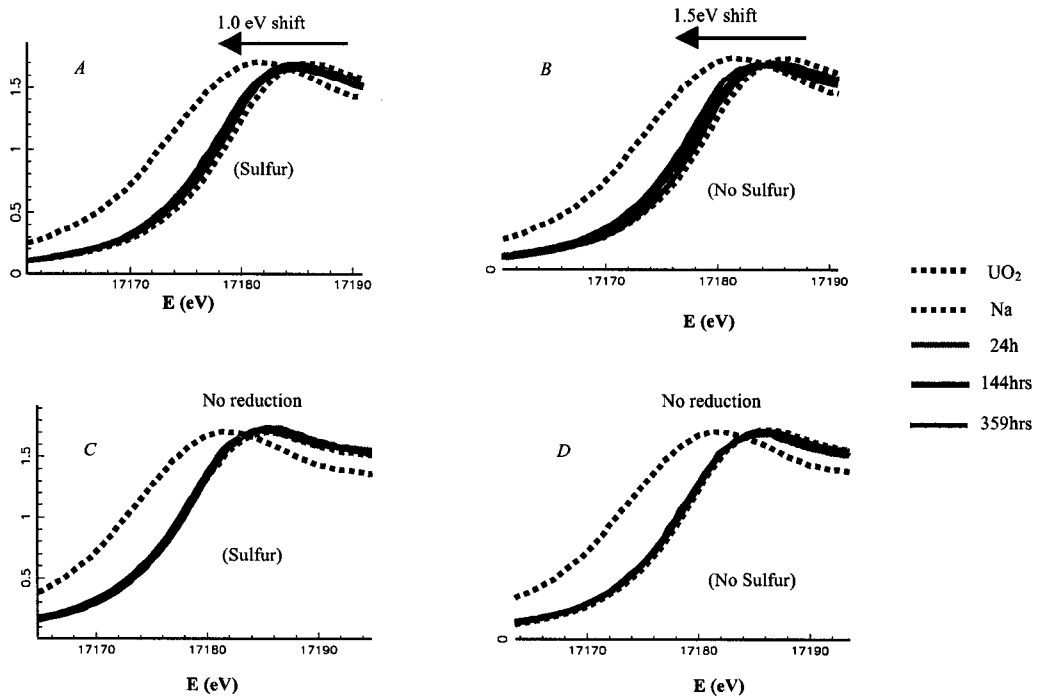


Figure 10: XANES data for Na-zippeite reacted with (A and B) and without (C and D) Desulfovibrio desulfuricans, normalized intensity plotted on the y-axis and energy in electron volts on the x-axis.

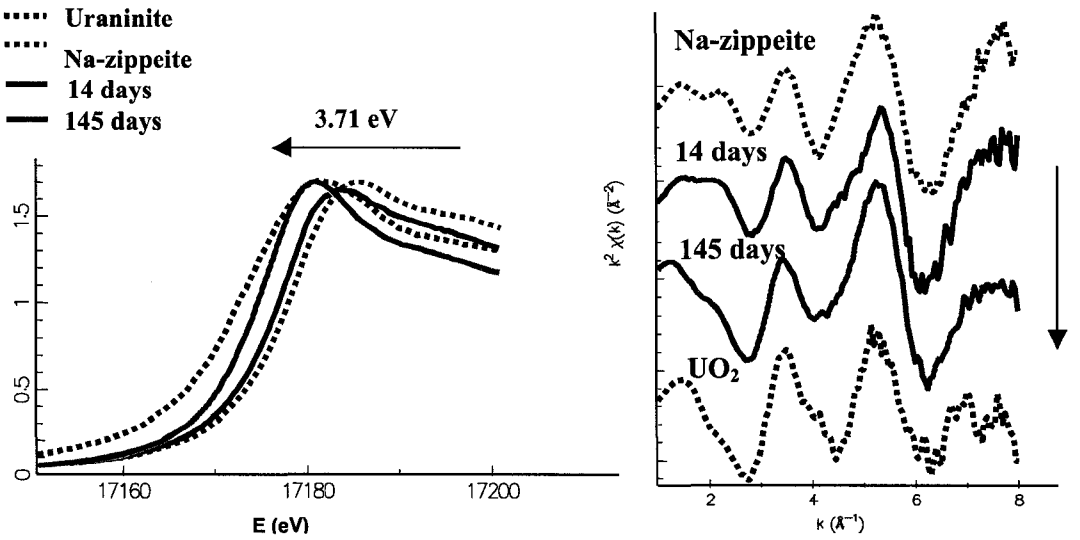


Figure 11: XANES and EXAFS data for Na-zippeite after 145 days of exposure to Desulfovibrio desulfuricans. XANES data plot normalized intensity on the y-axis and electron volts on the x-axis. EXAFS data of a Fourier Transform of χ times k^2 versus k.

CHAPTER 3 CONCLUSIONS

3.1 Conclusion

Evidence presented here supports a conclusion that SRB promote the dissolution of uranium U(VI)-bearing sulphate minerals and results in the precipitation of extra-cellular uraninite on the bacterial cell. Although the two zippeite group minerals utilized in this study differed in ion substitution, they provided slight space group variations, allowing us to examine the effect of structural variations on reductive dissolution. However, both minerals displayed comparable oxidation shifts (0.8 eV and 1.5 eV) as well as uraninite formation and thus no significant structural differentiation can be concluded. While both sulphur-enriched and sulphur-poor conditions produced a shift in oxidation, contrary to the inhibition of reduction via increasing ionic strength, only sulphur-enriched conditions produced uraninite (Yee et al., 2004). The rate of reduction may have been sufficient to minimize the ionic strength produced by the dissolution of the uranyl sulfate mineral. Furthermore, the presence of phosphate in the medium may have affected the amount or degree of reduction, and the possible formation of uranium U(IV) phosphates. It should be noted that the precipitation of extra-cellular uraninite in natural environments could lead to the advective transport and remobilization of the uraninite-coated bacteria and potentially the re-oxidation to soluble U(VI) phases. This is the first study to demonstrate that sulphate-reducing bacteria can enhance the dissolution of uranyl sulphate minerals, and it provides important new insights into the bioavailability and potential mobility of uranium (VI) in mineral assemblages.

3.2 Future direction and research

Studies involving the systematic variations in electron donor concentrations as well as electron acceptor concentrations would complement the dataset herein. Continuous flow-through experiments would more closely represent natural ground water conditions. More detailed solid phase characterization of the U(IV) phosphorus products would aid in understanding the reduction and complexation that occurs. The future of this research is to further examine the potential for structural differentiation, including the study of more uranyl sulfate phases as well as other uranyl species. This line of research would provide valuable insight into our understanding of uranium cycling and remediation strategies.

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