3-14-2014

Investigating the Role of a Less Uranium Tolerant Strain, Isolated from the Hanford Site Soil, on Uranium Interaction in Polyphosphate Remediation Technology

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INVESTIGATING THE ROLE OF A LESS URANIUM TOLERANT STRAIN, ISOLATED FROM THE HANFORD SITE SOIL, ON URANIUM INTERACTION IN POLYPHOSPHATE REMEDIATION TECHNOLOGY

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL ENGINEERING

by

Paola M. Sepulveda-Medina

2014

To: Dean Amir Mirmiran
College of Engineering and Computing

This thesis, written by Paola M. Sepulveda-Medina, and entitled Investigating the Role of a Less Uranium Tolerant Strain, Isolated from the Hanford Site Soil, on Uranium Interaction in Polyphosphate Remediation Technology, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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Date of Defense: March 14, 2014

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Florida International University, 2014

DEDICATION

This work is dedicated to those closest to me and to the Department of Energy-Florida International University Science and Technology Workforce Development Program along with its mentors and fellows. Without their support and patience this project would not come to fruition.

ACKNOWLEDGMENTS

I would like to acknowledge and thank my committee; Drs Anthony McGoron, Yelena Katsenovich, Chenzhong Li, and Leonel Lagos. And thank Brady and Hope Lee for their patience and detailed discussion on microbiology and Live/Dead assay assessment. I would also like to acknowledge assistance from Vishal Musaramthota, Rakesh Guduru, Rupak Dua, Ravi Gudavalli and the DOE-FIU Science and Technology Workforce Development Program for supporting me and this investigation.

I would also like to thank Drs. P. Sobecky and Robert J. Martinez, University of Alabama, for providing us with *Arthrobacter* sp. strains from the SMCC collection. Funding for this research was provided by US DOE Grant Number: DE-EM0000598.

ABSTRACT OF THE THESIS

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Bacteria are key players in the processes that govern fate and transport of contaminants. Previous assessment showed that the *Arthrobacter oxydans (A.oxydans)* G968 strain has a lower ability to tolerate U(VI) toxicity in bicarbonate-free media compared to other isolate *A.oxydans* G975. The study experimentally investigated several parameters such as the potential of bicarbonate to accelerate U(VI) release from autunite mineral in the presence of a less U(VI) tolerant bacterial strain, in the conditions mimicking Hanford Site subsurface environments. Results showed that despite morphological differences between the two bacterial strains, *A.oxydans* G968 and G975, they are able to dissolute uranium at the same capacity. The effect of both bacterial strands on autunite dissolution reduces as the concentration of bicarbonate increases. AFM and viability studies showed that samples containing bicarbonate are able to acclimate and withstand uranium toxicity. This study provides a better understanding of the bacterial role in polyphosphate remediation technology and interactions between meta-autunite and microbes.

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I. INTRODUCTION

During the cold war era, plutonium producing facilities were erected along the Columbia River. Past practices of waste disposal operations allowed waste discharges to retention basins, trenches, or cribs where the waste percolated into the soil, and eventually to the vadose zone and groundwater. There was a total of 43 million cubic yards of radioactive waste, 130 million cubic yards of contaminated soil and debris, and an estimated 475 million gallons of contaminated water released into the soil (Environmental 2014). This devastating amount of contamination needed controlling. One remediation strategy is the injection of a soluble sodium tripolyphosphate into the contaminated soil and groundwater of the Hanford soil. Tripolyphosphate injected to sequester uranium undergoes hydrolysis in aqueous solutions to orthophosphate forms, which serve as readily available nutrients for the various micro-organisms that thrive under these specific conditions and may even lead to an increase in their growth. The presence of rapidly adapting bacterial populations in sediment could strongly influence the stability of uranyl phosphate minerals and affect migration/dissolution of uranium by dissolution and desorption due to the secretion of protons and various ligands. Therefore, understanding the role of bacteria in phosphate remediation technology and the interactions between meta-autunite and the microbes is very important. Of particular concern, however, is the long-term stability of the sequestered uranium in the subsurface that may undergo subsequent remobilization. That is, the uranium has the potential to migrate within the soil causing contaminants to reach the Columbia River and affecting the water quality. Aqueous carbonate ions present in the soil and ground water are the predominant aqueous species affecting the dissolution of uranium-bearing solids and

facilitating uranium desorption reactions from soil and sediments, thus increasing uranium mobility in soil and sediments. This study is designed to investigate bacteria-U(VI) interactions in the presence of bicarbonate under oxidizing conditions to determine the long-term effectiveness of the tripolyphosphate injection remediation strategy.

The main objectives of this study are to examine the ability of oligotrophic microbial species to influence the dissolution pathways of U(VI) present in the groundwater as stable meta-autunite; investigate the bacteria interactions with uranium using a less U(VI)-tolerant strain and study the potential role of bicarbonate, which is an integral complexing ligand for U(VI) and a major ion in the pore water composition; inspect bacterial surfaces after exposure to U(VI) in the bicarbonate-bearing synthetic groundwater solution via atomic-force microscopy (AFM) and investigate for cell viability; and determine the difference between microbial effect on uranium release from synthetic and natural autunite. Understanding the effect of the aforementioned factors on the behavior of U(VI) in groundwater and sediments can potentially support remedial actions that are implemented in the 300 and 200 Areas of the Hanford Site.

II. BACKGROUND

Hanford Site

The Hanford Site was the first nuclear production facility in the world and served a key role in the nation's defense for over 40 years. Located on the Columbia Plateau of southeastern Washington State, the Hanford Site encompasses 586 square miles and composed of 4 subareas; 100 area, 200 area, 300 area and 1100 area. The site was originally built in 1943 as part of the Manhattan Project for the production of plutonium for the first atomic weapons and remained in production throughout the Cold War. During this time the Manhattan Project was broadened to contain an additional nine nuclear reactors and five large plutonium processing complexes within the Hanford Site, which ultimately produced plutonium for more than 60,000 weapons in the United States nuclear armory. Unfortunately, during the Cold War Era the Department of Energy-Environmental Management still did not exist, thus, the United States did not have regulations or knowledge of environmental protection. So, a vast amount of nuclear waste was generated, stored and disposed of in such ways that led to the contamination of soil and groundwater in several locations across the US (US Department of Energy 2009).

The fabrication of natural and slightly enriched uranium into fuel elements for nuclear reactors in Hanford's 300 Area, and the reprocessing of irradiated fuel in Hanford's 200 Area to obtain plutonium and other useful radioisotopes, has resulted in a wide variety of hazardous waste that contained chemical and radiological constituents. This waste was stored in underground storage tanks (USTs). There are 149 underground single-shell tanks (SST) used to store radioactive mixed waste; sixty seven of these SSTs have been

classified as assumed/confirmed leakers, which have leaked contaminants into the surrounding soil. Approximately 200,000 kg of uranium was released into the ground at the 200 East and 200 West Areas. The liquid waste leakage resulting from tank failure has soaked into the ground at the site and has created multiple radionuclide plumes, which are being monitored and treated to remove contaminants. The hazardous and radioactive contamination found in the groundwater has been reported to contain similar chemicals stored in the tank farm, thus indicating pollutant migration from the vadose zone. The vadose zone extends from the top of the ground surface to the water table.

These leakages influenced the vadose zone sediments by creating a potential source for groundwater contamination and risk to receptors, those who will use these groundwater resources down gradient, through water uptake from contaminated wells or discharge to surface water. Despite extensive remediation efforts initiated in the early 1990s, persistent uranium groundwater plumes identified in multiple locations around the site have persisted for many years. There is a growing concern that elevated uranium concentrations could enter the Columbia River along the shoreline contaminating sediment and aquatic biota. The protection of water resources from contaminated groundwater resulting from operations at the Hanford Site is a key element of the overall Hanford cleanup program.

Remediation Strategies

To find a solution to uranium contamination, several remediation strategies were considered. Pump and treat (P&T) systems are extensively used in groundwater remediation strategies. Pump and treat systems are broadly used to describe any system

that withdraws from or injects into groundwater as part of a remediation approach. P&T systems are primarily used to accomplish two goals: (1) hydraulic containment; to manage migration of contaminated groundwater, ultimately reducing tainted areas. (2) Treatment; to lessen dissolved contamination concentrations within the groundwater so that the aquifers comply with cleanup standards and regulations. These P&T systems regularly require an assessment to test the efficiency of the different approaches in restoring contaminated groundwater to proper standards. The 100 area, located on the banks of the Columbia River contains three main contaminants of concern: strontium, diesel, and chromium. The principal contaminant, hexavalent chromium, is being treated via a Pump and Treat System. Specifically, the system removes groundwater and isolates hexavalent chromium using an ion exchange resin; afterwards, the treated groundwater is injected back to the aquifer. Another strategy used is a permeable reactive barrier; this passive treatment transforms hexavalent chromium into an immobile and nontoxic trivalent form when it passes through a region in the soil treated with sodium dithionite. Unfortunately, there is no current method that is capable of effectively treating strontium-90; scientists are only able to immobilize the contaminant by incorporating apatite (Energy 2013).

The 200 area, composed of 200 East Area, 200 West Area, and 200 North Area, is involved in a great number of cleanup projects and remediation efforts for both solid and liquid wastes. This area served as the main area for removal of plutonium from the fuel rods creating billions of gallons of liquid waste. The improper storage eventually created plumes of contamination that require extensive treatment. The main contaminants of

concern located in the 200 area are uranium, technetium-99, carbon tetrachloride, nitrate, and iodine-129. The main method of remediation for the 200 area utilizes groundwater pumping via extraction wells, this method accesses the contaminant plume for mass removal and treatment. Groundwater pumping is designed to reduce migration of uranium and technetium-99 from 200 west area, while also removing co contaminants of nitrate and carbon tetrachloride (Opalski, McCormick and Hedges 2009). In addition to active remediation treatments, monitoring of various sites within the 200 area have been proposed. The facilities are monitored under Resource Conservation and Recovery Act (RCRA) requirements for dangerous waste constituents and special nuclear and byproduct materials (Lindberg 2011).

Hanford's 300 area was the control center for plutonium production, hundreds of thousands tons of raw uranium was sent to the facilities of this area to be manufactured into fuel assemblies. Similar to the 200 area this location was populated with many experiments producing a high number of contaminated zones. The main contaminants of concern for this area include uranium, trichloroethylene (TCE) and tritium. The 300 area uses a combination of remedial strategies to address contamination; the first method is monitored natural attenuation which is a technique used to monitor the reduction of contaminants through natural attenuation processes such as oxidation or biodegradation. This area also uses enhanced attenuation which speeds up the natural processes described above by adding a binding solution to decrease the movement of contamination within the groundwater; a major example of this technique is the amendment of a polyphosphate injection, which will be described in further detail later. The remediation strategies are

monitored to ensure that contaminants meet appropriate RCRA requirements (DOE and EPA 2013).

Despite which remediation strategy is used the main purpose for these techniques is to preserve the Columbia River, protect human health and aquatic life while offering information to scientists that will improve future remediation projects.

Polyphosphate Amendment

An analysis of the concentration of contaminants along the Hanford site was conducted by the Department of Energy and it was determined that although the uranium concentrations have slowly decreased in the groundwater plume, it still continues to stay above the drinking water standard, which is the ultimate goal. A favorite remediation strategy among scientist is a polyphosphate injection for the stabilization and treatment of uranium in the groundwater. This method of remediation was first proposed to treat the soil of Hanford 300 area and has proved promising for decreasing the concentration of dissolved uranium in contaminated plumes (Wellman, et al. 2007) (Wellman, Icenhower, et al. 2005) . Long- chain sodium polyphosphate compounds ($Na_5P_3O_{10} \cdot nH_2O$) have been proposed as a time released source of phosphate for precipitation of uranium-phosphate minerals due to its long chain. Experimental results have illustrated that injecting a sodium tripolyphosphate amendment into a uranium-rich porous media is capable of immobilizing uranium by producing an insoluble uranyl phosphate mineral such as autunite {X1-2[(UO2)(PO4)]2-1•nH2O}, where X is any monovalent or divalent cation (Vermeul, et al. 2009).

Autunite Mineral

Autunite stability in the subsurface can help to determine the long-term effectiveness of the tripolyphosphate injection remediation strategy (Wellman, et al. 2007). Synthetic Na-autunite, Na $[(UO_2)(PO_4)] \cdot 3H_2O$, and natural Ca meta-autunite, $Ca[(UO_2)(PO_4)]_2 \cdot 3H_2O$, minerals largely limit the mobility of dissolved U(VI) in soils contaminated by actinides and are an extremely important group of uranyl minerals when considering U sequestration. The autunite structure is composed of phosphate tetrahedrons linked to uranium-oxygen groups that form distorted octahedrons. The phosphates and uranium groups lie in sheets that are weakly held together by water molecules (Burns, Miller and Ewing 1996). This structure produces the tabular habit, the one perfect direction of cleavage. Autunite mineral precipitation because of polyphosphate injection was identified as a feasible remediation strategy for sequestering uranium in contaminated groundwater and soil *in situ* at the Hanford Site (Vermeul, et al. 2009) .

Arthrobacter Bacteria

Autunite is a phosphorus-containing mineral. It can attract bacteria to liberate phosphorus, meeting their nutrients requirements and causing U mobilization back into the environment as a result of a breakdown of the mineral structure. Increased microbial activity that influences meta-autunite stability is an important geochemical factor affecting the uranium dissolution and transport in the specific environmental conditions present at the Hanford Site's subsurface. It is important to identify the factors that adversely affect the dissolution of uranium in order to eventually mitigate those factors, thus it is essential to first know the behavior of uranium in the presence of bacteria.

Previously, the significance of bacteria-uranium interactions has been illustrated by focusing on three bacterial strains of *Arthrobacter* sp, isolated from Hanford Site soil (Katsenovich, Carvajal and Guduru, et al. 2012 b). The *Arthrobacter* bacteria are one of the most common groups in soils and are found in large numbers in Hanford soil as well as other subsurface environments contaminated with radionuclides (Balkwill, et al. 1997) (Boylen 1973) (Van Waasbergen, et al. 2000) (Crocker, et al. 2000). Balkwill et al. (1997) reported the predominance of the genus *Arthrobacter* among the culturable aerobic heterotrophic bacteria from the Hanford Site sediments with this group accounting for roughly up to 25% of the subsurface isolates. In addition, *Arthrobacter*-like bacteria were the most prevalent in the highly radioactive sediment samples collected underneath the leaking high-level waste storage tanks and accounted for about one-third of the total soil isolatable bacterial population (Fredrickson et al. 2004). Furthermore, a previous study conducted using the *Arthrobacter oxydans* G975 strain illustrated a bio-enhanced release of U(VI) from natural Ca-autunite in the presence of various concentrations of bicarbonate. G975 was found to be the fastest growing and the most uranium-tolerant strain among the studied microorganisms obtained from the Subsurface Microbial Culture Collection (SMCC) (Katsenovich, Carvajal and Guduru, et al. 2012 b). Bacterial cell walls, proteins and lipids contain functional groups such as carboxyl and phosphate which are able to bind with uranium and are ultimately capable of affecting U(VI) mobility in aqueous systems. This *Arthrobacter* G975 is ubiquitous in subsurface microbial communities and can play a significant role in the dissolution of minerals and the formation of secondary minerals (Katsenovich, Carvajal and Guduru, et al. 2012 b). This research was extended to investigate the stability of autunite mineral in oxidized

conditions pertaining to the arid and semiarid climate typical for the Hanford Site and study the effect of *Arthrobacter oxydans* SMCC G968 strain on the U(VI) release from autunite. This strain was found to be less resistant to U(VI) toxicity. The alteration in surface morphology for G968 was noted at 0.5 ppm of U(VI); in comparison, G975 shows signs of cell inhibition at the much higher concentration of 19 ppm of U(VI). Additionally, the G975 strain accumulated up to 92% of uranium in the studied U(VI) concentration range up to 27 ppm, which is almost triple the value compared to the G968. The results on cell density for G968 determined via hemocytometer showed slightly lower values for the same period compared to G975 (Katsenovich, Carvajal and Wellman, et al. 2012a).

Bicarbonate

Uranium (VI) is a key contaminant of concern at the Hanford Site. Due to the complex chemistry of uranium, a predictive understanding of its mobility in the subsurface is limited. In neutral or basic pH conditions, uranium undergoes hydrolysis in aqueous solutions and can readily complex with a wide variety of ligands. These complexation reactions often result in the formation of mobile aqueous species or precipitation of U-bearing minerals. Environmental factors, such as the specific composition of pore water and groundwater of the Hanford Site have tremendous effect on both uranium and its mineral phases. Additional research is necessary to understand the effect of these factors on the behavior of U(VI) in groundwater and sediments.

Aqueous carbonate present in the soil and ground water is primary species increasing uranium mobility by affecting the dissolution of actinide and promoting the uranium

desorption reaction from soil (Langmuir 1978). In a calcium-rich environment, the large formation constants of soluble and stable calcium uranyl carbonate complexes, [$Ca_2UO_2(CO_3)_3^0$(aq); $CaUO_2(CO_3)_3^{2-}$], influence the speciation of uranium (Langmuir 1978) (Bernhard, et al. 2001).

III. STATEMENT OF PURPOSE

Research objectives

The main objective of this study is to investigate if *Arthrobacter* strain G968 influences uranium mobility in the subsurface through biogeochemical processes such as biodissolution and study bacteria-uranium interactions in the presence of bicarbonate. This investigation will analyze several parameters such as the potential of bicarbonate to accelerate U(VI) release from autunite mineral in the presence of a less U(VI) tolerant bacterial strain, in the conditions mimicking Hanford site subsurface environments. This study will use Field Emission Scanning Electron Microscopy (FE-SEM), Energy Dispersive X-Ray Spectroscopy (EDS), Atomic Force Microscopy (AFM) and fluorescence microscope to gain a better understanding of the changes of cell morphology and surface compositions after exposure to U(VI) in the presence of bicarbonate ions.

Research Questions

1. How will bicarbonate concentrations affect the release of uranium from autunite?
2. Does *Arthrobacter* bacterial strain G968 influence the release of uranium from autunite mineral over time, and at what capacity when compared to a more U(VI) tolerant strain, G975?
3. Is there a difference in the microbial dissolution of synthetic and natural autunite in the presence of bicarbonate?
4. How are bacterial cells affected when exposed to uranium in the presence of bicarbonate ions?

Hypothesis

Experimental Hypotheses

It is hypothesized that:

1. The presence of bicarbonate increases the release of uranium from autunite compared to bicarbonate-free media.
2. The release of uranyl ion from autunite in the presence of microorganisms in bicarbonate-bearing media is increased compared to the bicarbonate-free control; however, this effect will be diminished at higher bicarbonate concentrations.
3. Uranium release from natural Ca-autunite is expected to be lower than the rate of uranium release from synthetic autunite.
4. Negatively charged uranium carbonate complexes present in the solution are less toxic for bacteria, which increases cells viability.

Importance of Study

This research investigates bacteria-U(VI) interactions under oxidizing conditions in the presence of bicarbonate ions to influence U(VI) release from autunite minerals using a less tolerant to U(VI) *Arthrobacter* strain, G968. In the presence of bicarbonate cells exhibit higher viability in response to uranium toxicity. Active bacteria can decrease the long-term stability of uranyl-bearing precipitates created in soil as a result of remedial actions. The research will promote the understanding of the effect of these factors on the behavior of U(VI) in groundwater and sediments to support remedial actions implemented in the 300 and 200 Areas.

IV. MATERIALS AND METHODS

Arthrobacter Strains and Growth Culture Conditions

A detailed description of where the *Arthrobacter* strains were obtained is described in recent publications (Katsenovich, Carvajal and Wellman, et al. 2012a) (Katsenovich, Carvajal and Guduru, et al. 2012 b). The strains were cultured in 5% PYTG liquid culture media and agar plates consisting of 5 g/L peptone, 5 g/L tryptone, 10 g/L yeast extract, 10 g/L glucose, 0.6 g/L $MgSO_4.7H_2O$, and 0.07 g/L $CaCl_2.2H_2O$. The media was prepared in deionized water (DIW) (Barnstead NANOpure Diamond Life Science (UV/UF), Thermo Scientific), autoclaved at 121^oC and 15 psi for 15 minutes, then allowed to cool before being used.

To account for viable bacteria, a well-mixed homogeneous aliquot (0.01 mL - 0.1 mL) of the suspension from each test vial was uniformly spread on the sterile Petri dishes containing a 5% PTYG growth media mixed with 15 g/L of agar. Inoculated plates were kept inverted in an incubator at 29^oC. Viable microorganisms were calculated from the number of colony-forming units (CFU) found on a specific dilution. In addition, the agar plating was used to provide a quick visual check for contamination and to maintain colonies from each stage of the enrichment for the duration of the experiment. The cell density (cells/mL) was calculated with the help of a glass hemocytometer (Fisher Scientific, Pittsburg, PA). Cell counts in the samples containing uranium employed INCYTO C-Chip disposable hemocytometers. The hemocytometer is a microscope slide with a rectangular indentation, creating a chamber that is engraved with a grid of perpendicular lines. Knowing the area bounded by the lines as well as the depth of the chamber, the cell density in a specific volume of fluid and in a bacterial broth solution

was calculated from a sample, homogenously distributed inside the chamber. Once the average cell count was obtained, it was multiplied by the dilution factor and the volume factor, 10^4, in order to calculate the final concentration of cells per mL.

Uranium (VI) release from Autunite mineral

Two different kinds of bioreactors were used in the bioleaching experiments: (1) Sterile 100 mL glass mixed reactors served as the major bioreactor for initial experimentation. These autunite-containing bioreactors were inoculated with bacterial cells after the autunite equilibrated with the media solution. (2) Sterile culture ware with inserts enclosing bacteria and autunite was kept separated.

Bicarbonate Media Solution Preparation

The media solution to conduct the autunite dissolution experiments was prepared similarly to the growth media without yeast extract due to its high phosphorus content. The media was prepared in deionized water (DIW) (Barnstead NANOpure Diamond Life Science (UV/UF), Thermo Scientific), autoclaved at 121^{o}C, 15 psi for 15 minutes, and cooled to about 30^{o}C. After sterilization, the media was equally distributed between four 200-mL bottles and separately amended to contain 0 mM, 3 mM, 5 mM, and 10 mM of $KHCO_3$. The media was adjusted to pH 7.5 with 0.1 mol/L HCl or NaOH and buffered with 0.02 M 2-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid sodium salt hydrate (HEPES-Na) buffer. Each of the individual four bicarbonate media solutions were filtered-sterilized (0.2 μm) and kept refrigerated until the time of use.

Autunite bioleaching in mixed bioreactors

Natural Ca meta-autunite, $Ca[(UO_2)(PO_4)]_2 \cdot 3H_2O$ obtained from Excalibur Mineral Corporation (Peekskill, New York), was previously characterized using ICP-OES, ICP-MS analyses, X-ray diffraction and SEM/EDS to confirm the mineral composition, structure, and morphology as 98–99% pure autunite (Wellman, Icenhower and Gamerdinger, et al. 2006). The autunite sample was powdered to have a size fraction of 75 to 150 μm or -100 to +200 mesh with a surface area of 0.88 m^2/g as determined by Kr-adsorption BET analysis (Wellman, Icenhower and Gamerdinger, et al. 2006).

Synthesis of Na-autunite, $Na\ [(UO_2)\ (PO_4)] \bullet 3H_2O$, was followed by a modified direct precipitation method described by Gudavalli et al. (R. Gudavalli, Y. Katsenovich, et al. 2013).

In order to test if there is a difference in the microbial dissolution of synthetic and natural autunite in the presence of bicarbonate, a bioleaching experiment in two different kinds of bioreactors was conducted. Synthetic and natural autunite microbial bioleaching experiments were conducted similarly; with 100 mL foam stoppered glass serum bottles containing 50 mL of sterile media buffered with 20 mM HEPES-Na and 91 mg of meta-autunite to provide a U(VI) concentration of 4.4 mmol/L. This concentration was used to compare results with previous data obtained in the dissolution experiments using a high U-tolerant strain, *Arthrobacter* G975 (Katsenovich, Carvajal and Wellman, et al. 2012a). The suspensions were slightly agitated at 60-rpm in an incubator/shaker at 25 ^oC. G968 *Arthrobacter* cells in the amount of 10^6 cells/mL were injected into the reactors after 27 days, giving time for the autunite to reach steady state. During the inoculation, the

reactors kept their sterile foam stoppers to sustain aerobic conditions within the reactors. Abiotic non-carbonate controls were kept without bacterial inoculation to provide a control for the biotic samples.

Autunite bioleaching in culture ware with inserts

To investigate if a direct interaction between the bacteria and the mineral was necessary to result in uranium release from the mineral, a bioleaching experiment in culture ware with inserts was performed. Sterile 6- well cell culture plates with inserts were used in the non-contact bioleaching experiments with natural Ca meta-autunite and bacteria cells kept separately. A 3.2 ml aliquot of sterile media was dispensed in the appropriate well and 2.5 ml inside the insert receptacle. The total volume inside each well added up to 5.7 ml. The culture ware inserts have 0.4 μm cylindrical pores that transverse the membrane and only allow the diffusion of soluble uranium. Ten mg (10 mg) of sterilized autunite powder was added to the bottom of the wells to reach a concentration of 4.4 mmol/L (Katsenovich, Carvajal and Guduru, et al. 2012 b) (Katsenovich, Carvajal and Wellman, et al. 2012a). Abiotic controls were prepared to make a comparison with bacteria bearing wells. Uranium release from autunite was established by taking a 20μL sample from the inserts and processing on the KPA instrument, as described in greater detail in the next section.

Analytical procedures

Every few days, a 0.3 mL sample of the solution was aseptically withdrawn from each bottle, filtered (0.2 μm), and then analyzed for dissolved U(VI) by means of a kinetic phosphorescence analyzer KPA-11 (Chemcheck Instruments, Richland, WA). The

dilution factors for sample analysis were 100 for low concentrations of bicarbonate, and 200 for high concentrations of bicarbonate. Prior to this analysis, sample aliquots were ashed on a hot plate with the addition of concentrated plasma grade nitric acid and hydrogen peroxide solutions (R. Gudavalli, et al. 2013). Wet digestion was continued until a dry white precipitate formed, and then dry ashing was performed in the furnace at 450^{o}C for 15 min. Samples were allowed to cool at room temperature followed by the dissolution of the precipitate by the addition of 1 M nitric acid (HNO_3). Aqueous concentrations of calcium and phosphorus were determined from the digested samples by means of an Optima 7300 ICP-OES (Perkin Elmer). Uranium calibration standards (SPEX certiPrep), blanks and check standards (95-105% recovery) were analyzed for quality control.

SEM/EDS microscopy analysis

Growth Conditions

The *Arthrobacter* strains were aerobically grown to reach confluency in a 15mL polypropylene sterile foam-stoppered centrifuge tubes amended with 4 mL of Na-HEPES buffered culture media at 25˚C. Cell stock suspension was counted using an INCYTO C-chip disposable hemocytometer to establish the number of cells in the media needed to obtain the desired concentration. The tubes were inoculated with approximately log 7.5 bacterial cells/mL. Uranium was injected in the form of uranyl nitrate stock solution to produce the desired concentration of 5ppm and 10 ppm. The samples were prepared at 24 hours to obtain cell count, prepare samples for microscopy analysis via

Atomic Force Microscopy (AFM), Scanning Electron Microscope (SEM) and energy dispersive x-ray spectroscopy (EDS).

Cell Surface Composition Analysis

The purpose of SEM/EDS was to show surface morphology and cell surface composition in the presence of bicarbonate. To prepare the samples for SEM imaging a dehydration process was followed using a sample preservation protocol (Fratesi, et al. 2004) (Araujo, et al. 2003). This process included centrifugation of the G968 bacterial cells at 4000 rpm for 5 min from PTYG media amended with certain concentration of U(VI) and washed twice with deionized water. The pH of the HEPES media was adjusted to 7.2 with concentrated Fisher Scientific nitric acid (HNO_3). The cells were fixed in the 5ml of 2% glutaraldehyde in 0.1M HEPES buffer at pH 7.2 for 2h at 4^oC. The material was removed by centrifugation and washed with 50mM HEPES buffer three times for 10 min. The rinsed cells were then dehydrated in ethanol/ water solutions of 35% (v/v), 70% (v/v), and 90% (v/v) each for 10 min, and two times in 100% (v/v) for 10 min. Dehydrated samples were immersed for 10 min each in 50% and 100% pure hexamethyldisilazane (HMDS) (Pierce Biotechnology, Inc, obtained from Fisher Scientific) followed by 10 min of air-drying to allow liquid to evaporate from a sample. The dehydrated specimens were then kept in the desiccators until the time of SEM/EDS analysis. The dehydrated specimens were mounted on the SEM mounting plates with double sided sticky carbon tape and then coated for one minute with a thin layer of gold to increase conductivity. The cell surface composition of gold-coated samples (PAC-1 PELCO Advanced coater

9500) was analyzed using a SEM-Energy-Dispersive-Spectrometry (SEM-EDS) JEOL System Model 5900LV.

AFM microscopy analysis on bacteria uranium interactions

Sample Preparation for AFM Imaging

The bacterial cells were grown in a 5% PYTG liquid culture media, for two days, consisting of 0.25 g/L peptone, 0.25 g/L tryptone, 0.5 g/L yeast extract, 0.5 g/L glucose, 0.6 g/L $MgSO_4.7H_2O$, 0.07 g/L $CaCl_2.2H_2O$. Media was prepared in deionized water (DIW) (Barnstead NANOpure Diamond Life Science (UV/UF), Thermo Scientific), autoclaved at 121^oC and 15 psi for 15 minutes, then allowed to cool down before being used. Log 7 cells/mL of the bacterial stock solution was incorporated with uranyl nitrate and synthetic groundwater (SGW) media to create individual samples for analysis. The SGW solution contained 5.22 mg/L of KCl, and 520.58 mg/L of HEPES. Phosphorus containing organic media is conducive to produce precipitates and was not included to prevent a potential interference with imaging. The media was prepared in deionized water, autoclaved at 121 ^oC, 15 psi for 15 minutes, and allowed to cool down to about 30 ^oC. Then it was equally distributed between three 250 mL bottles and separately amended to contain 0mM and 5mM $KHCO_3$. The samples that were analyzed included 5mL aliquots composed of: 0ppm of U with 0mM of bicarbonate, 5ppm of U with 0mM and 5mM of bicarbonate, 10ppm of U with 0mM and 5 mM of bicarbonate.

Table 1. Samples containing varying concentrations of uranium in bicarbonate bearing media for imaging analyses

Concentration of Uranyl Nitrate (ppm)	**Concentration of Bicarbonate (mM)**
0	0
5	0
5	5
10	0
10	5

The bacterial samples were centrifuged to create a concentrated sample and washed three times with deionized water from U(VI) and media residuals, and were then immobilized onto the 3-aminopropyltrimethoxysilane coated silicon wafer substrates. A concentrated sample of about 10 µl was dropped onto a silanized silicon wafer. The bacterial cells must be firmly adhered onto a silicon wafer substrate so the sample is immobilized and stabilized during imaging. Samples were air dried until excess moisture was evaporated completely. The substrates were then fixed onto a metallic disc and transferred to an AFM stage for imaging.

Atomic Force Microscopy Instrumentation and Imaging

Atomic Force Microscopy (AFM) was conducted employing a PicoScan AFM (former Molecular Imaging Inc. now Agilent Series 4500 SPM, PicoSPM) for all AFM imaging

analysis. A low force constant (~ 0.2 N/m) Silicon AFM probe tip (NanoAndMore, USA) was utilized with a resonating frequency of 15 kHz. The mode of operation that was exercised was a contact mode. The detailed specifications of the cantilever are 500 μm in length, 30 μm in width, and 2.7 μm in thickness. The tip shape is rotated and the tip radius is <10 nm. The AFM tip is 20-25 degrees along cantilever axis; 25-30 degrees from side and 10 degrees at the apex. In addition, information on the surface topography of cells exposed to U(VI) was obtained, while laboratory conditions were at 25ºC and 55% relative humidity. Along with topographical imaging, a quantitative analysis was conducted; cellular dimensions and force spectroscopy were evaluated. To avoid cell dehydration, the AFM analysis was performed within two hours of samples preparation.

Force Spectroscopy

Forces experienced by the cantilever as it approaches the sample from several microns above the surface can provide information about short-range interactions. As the tip approaches the surface, short-range forces, such as Van der Waals forces can be measured. Once the tip has approached the sample surface, an additional force can be applied to determine viscoelastic properties such as Young's modulus or stiffness for different strain rates or maximum applied forces. Additionally, when the cantilever is retracted away from the surface, adhesion forces or the detach forces can be measured. Adhesion forces are sensitive to modifications in the surface, such as physiological changes on the cellular membrane when exposed to uranium and bicarbonate. The dynamic changes occurring on the bacterial cell membrane before and after exposure to the various concentrations of uranium were evaluated using force spectroscopy analysis.

Thus a force spectroscopy analysis was conducted to gain a full understanding of interactions at the atomistic level. The forces are determined using force distance (FD) curves that are generated from the array of force curves over the selected region. The average adhesion force was obtained from an average of 256 different FD curves in a 5 μm x 5 μm sample surface. This experimental approach allows for monitoring of forces on the piconewton/nanonewton scale. These FD curves were processed using Scanning Probe Image Processor (SPIP) software by ImageMetrology, Denmark (version 6.2.0) to determine their average adhesion forces.

Roughness Measurements

The surface of the immobilized bacteria on the Si wafer was also investigated using atomic force microscopy operated in contact mode as mentioned above. The AFM instrument is also capable of measuring surface topography at the atomic scale. Roughness measurements were performed on various points over a selected area. The measured roughness depends on the spatial and vertical resolution of the instrument. The average roughness analysis was determined from the average of the absolute values of the profile heights from the mean level.

Cell Viability

Supplementing AFM analysis, cell viability was assessed by the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes) to quantitatively illustrate how bacterial cells are affected when exposed to uranium in the presence of varying concentrations of bicarbonate ions. The Live/Dead assay contains SYTO 9 green fluorescent nucleic acid stain and red fluorescent nucleic acid stain, propidium iodide. SYTO 9 generally stains

all bacteria in a population, regardless if they are alive or dead; the addition of propidium iodide to the solution causes a reduction of the SYTO 9 stain fluorescence penetrating damaged membranes. Samples used for viability assessment were similar to samples used for AFM imaging: grown in synthetic groundwater media and amended with varying concentrations of bicarbonate ions. Two control samples were created by, one adding 4 mL of SGW media for the live control, and another with 4 mL of 70% isopropyl alcohol (for killed bacteria). The other samples were simply exposed to varying concentrations of uranium and bicarbonate bearing SGW. Samples were then incubated at room temperature over night. In order to stain the samples, 3 μL of the dye mixture (containing equal parts of SYTO9 and propidium iodide) were added for each mL of the bacterial suspension. The samples were then incubated at room temperature in the dark for 15 minutes. Afterwards, the samples were washed 3 times with synthetic groundwater to remove the background fluorescence. 5 μL of the stained bacterial suspension was then placed on a microscope slide and covered with a coverslip. It was then allowed to dry for one hour in the dark before being visualized under a fluorescence microscope.

Fluorescence Imaging and Counting

Cells were visualized using a fluorescence microscope (Olympus IX81, Olympus America Inc., Miami, FL). The emission/excitation wavelength of SYTO9 and propidium iodide are 485/530 nm and 485/630 nm, respectively. A region of interest encompassing an area of 4.32 μm^2 was taken from images (ImageJ software, NIH, Bethesda, MD) and was used to calculate the ratio of live cells (green dots) to the sum of live and dead cells (red dots) and multiplying by 100% (n=4 images).

Cell Viability Using Plates

To supplement Live/Dead fluorescent assay, a cell viability experiment was performed in parallel using plates. Live/Dead analysis may show that samples are viable; however, it is necessary to illustrate if samples are culturable as well. In preparation for cell density viability experimentation, a new sleeve of PTYG plates were made using 5% PTYG media containing 0.25 g/L peptone, 0.25 g/L tryptone, 0.5 g/L glucose, 0.6 g/L $MgSO_4.7H_2O$, and 0.07 g/L $CaCl_2.2H_2O$, 0.5g/L yeast extract and 15g/L agar. Media was prepared in deionized water (DIW), autoclaved at 121^oC, 15 psi for 15 minutes, and allowed to cool to about 30^oC. The PTYG media was then poured into sterile plates and allowed to harden overnight. Samples used for this analysis were the same samples described above under viability assay.

Statistics

Statistical analysis of the results obtained from biodissolution experimentation and cell viability experiments were examined with analysis of variance (ANOVA) statistics. All statistical tests were investigated using a predictive analytical software, SPSS (IBM, Armonk, NY), with significant levels set at $\alpha = 0.05$.

V. RESULTS AND DISCUSSION

Bioleaching of U(VI) from Natural Autunite

The release of aqueous U(VI) over time during the autunite dissolution experiments is presented in Figure 1. U(VI) concentrations in the abiotic control, that is a sample that did not contain bicarbonate or bacteria, reached a steady state over a period of 27 days and reached an average of 0.46± 0.11 micromolar (μM). In bicarbonate-amended reactors, U(VI) release from autunite was strongly enhanced. Prior to strain inoculation, U(VI) concentrations measured at 0.55±0.12 μM, 1.07±0.34 μM, and 44.0±7.16 μM for 3 mM, 5 mM and 10 mM $KHCO_3$, respectively. The steady state maximum concentrations of U(VI) detected were 1.92, 14.4, 30.1, and 44.4 fold higher than the control containing no bicarbonate and no bacteria. Furthermore, after bacteria inoculation, U(VI) concentrations measured in the reactors, increased 3.0 ± 4.16, 23.3 ± 13.8, 19.0 ± 28.0, and 2.00 ± 2.66 fold, respectively, compared to the corresponding bicarbonate-bearing controls at steady state. Statistical analysis indicated very strong evidence that the mean uranium values are dissimilar across the different groups of bicarbonate concentration ($P < 0.05$). Even though there is an increased amount of U(VI) leached out into the solution driven by the presence of bacteria, the effect of bacteria on autunite dissolution is reduced as the concentration of $[HCO_3^-]$ increases. This trend is expected since the thermodynamic stability constants for the interactions between carbonate and both the autunite and the bacteria are higher than the autunite-cell interactions. Since the steady-state U(VI) concentration is higher for larger $[HCO_3^-]$, the increase in soluble U(VI)

concentration induced by bacteria is hindered. Therefore, as [HCO_3^-] increases, a diminishing trend on the effect of bacteria on autunite leaching is observed.

It has been demonstrated that mechanisms of uranium release from autunite minerals is controlled by a surface mediated reaction. Bicarbonate can promote mineral dissolution by binding to surface U(VI) ions causing weaker bonds, which is followed by detachment of the U(VI) species into solution (Sparks 1999). The figure below illustrates the release of uranium (U) from the mineral influenced by the dissolution of U(VI), the red arrow represents the introduction of bacteria into the bioreactors (Figure 1).

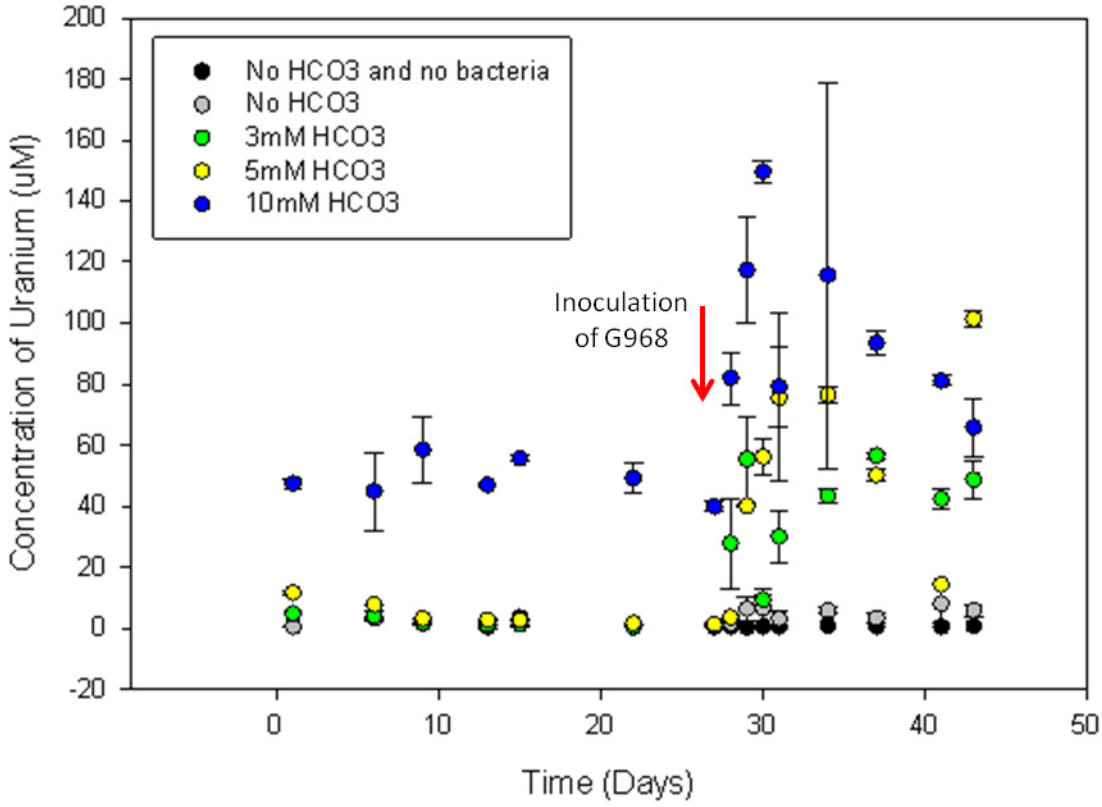


Figure 1. Changes for aqueous U(VI) as a function of time for the natural autunite dissolution experiments inoculated with *Arthrobacter* G968 strain (n=3)

Figure 2 illustrates the aqueous phosphorus release as a function of time from the natural autunite dissolution experiments inoculated with *Arthrobacter* G968 strain. This figure also uses two controls, an abiotic control that contains no bicarbonate and no bacteria, and a biotic control, containing bacteria with no bicarbonate. During the autunite dissolution period without bacteria, phosphorus (P) concentrations were increased compared to the non-carbonate abiotic control. After inoculation, P concentrations in the biotic reactors were found to decrease for all studied bicarbonate concentrations. This was correlated with the exponential growth phase of bacteria that were seeded into the reactors. P is an essential nutrient requirement for bacteria for the synthesis of DNA, ATP, polyphosphates, and cell wall phospholipids. Ivanova et al. found that biocatalytic processes involving simultaneous occurring biosynthetic and chemical reactions greatly enhance the rate of efficiency of phosphorous bioleaching (Ivanova, Bojinova and Nedialkov 2006). Microorganisms are able to dissolve P-bearing minerals by accessing insoluble phosphate bearing minerals through microbial dissolution (Vazques, et al. 2000). Another possibility for the reduction of soluble P concentrations is the formation of calcium phosphates, which are, as predicted by speciation modeling via Visual MINTEQ software (Table 2), supersaturated with respect to the secondary solid phases. The calcium phosphate phases precipitated from media solution and accumulated in the batch reactors.

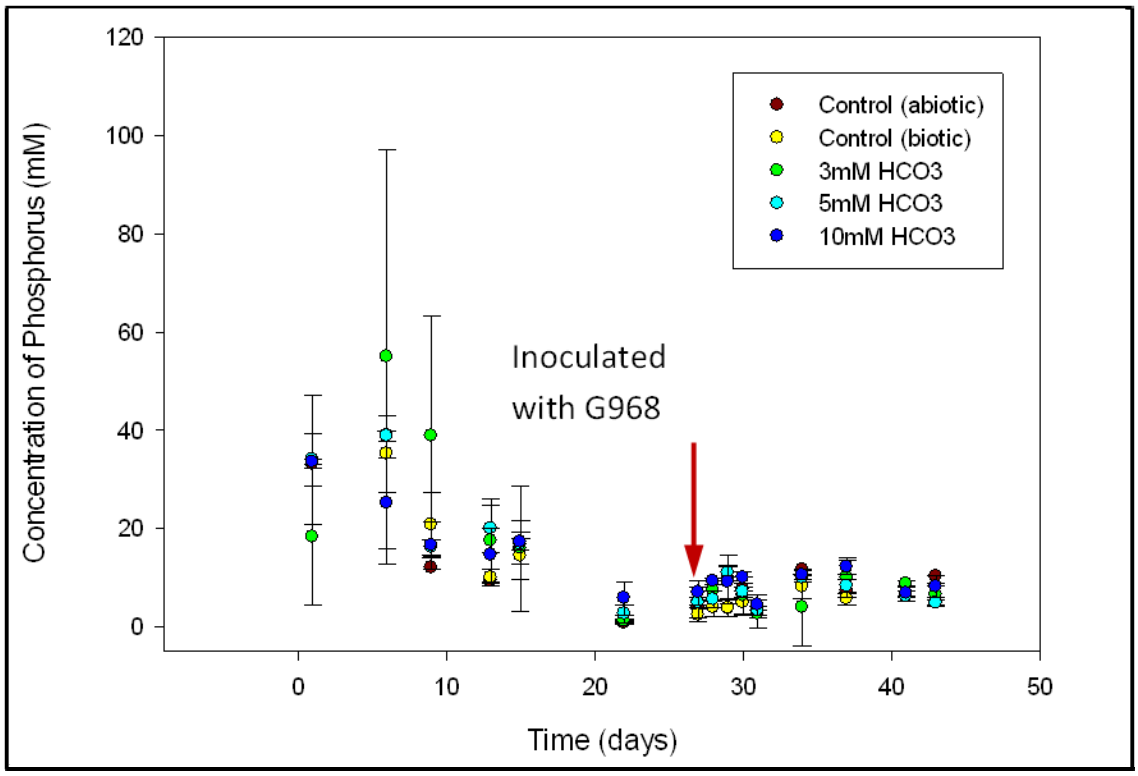


Figure 2. Aqueous phosphorus release as a function of time from the natural autunite dissolution experiments inoculated with *Arthrobacter* G968 strain

Similar to phosphorus, aqueous Ca release over the period of autunite dissolution without bacteria was noted to increase as a function of bicarbonate concentrations, as illustrated in Figure 3. Note that abiotic control refers to samples containing no bicarbonate and no bacteria whereas; the biotic control is the sample containing only bacteria.

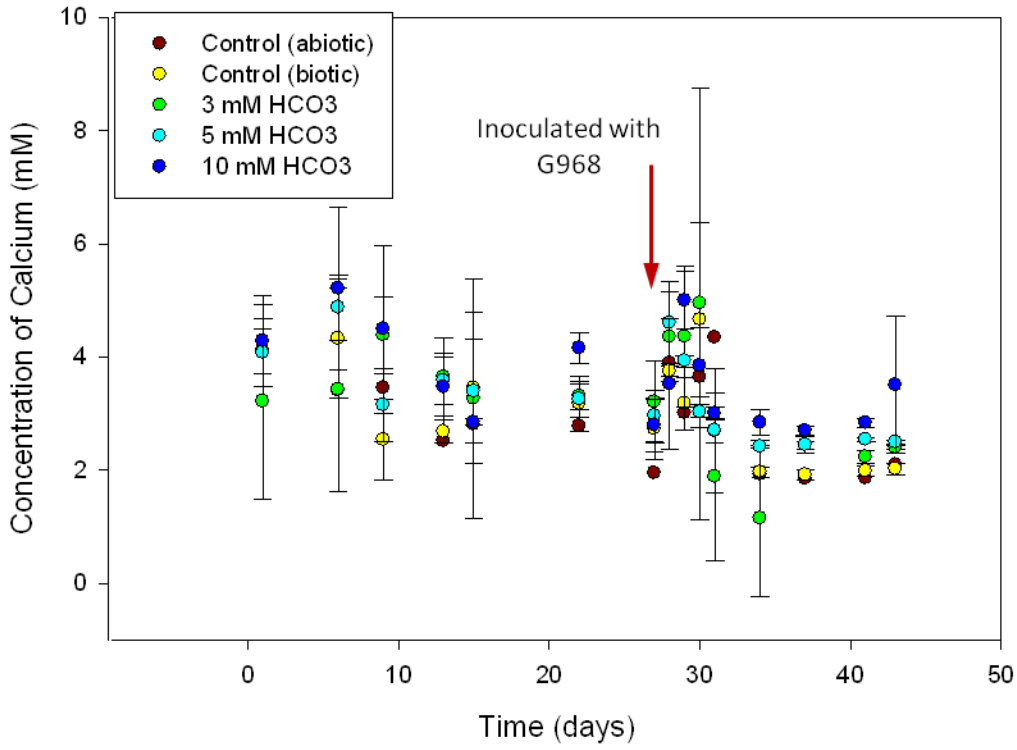


Figure 3. Aqueous Ca release as a function of time for the biotic reactors inoculated with *Arthrobacter* G968 strain

Arthrobacter species has the potential of becoming an important component in the dissolution process while forming secondary minerals. The stability of uranyl- phosphate complexes makes them a strong candidate for the remediation efforts to sequester U in the subsurface. However, in a bicarbonate-rich oligotrophic environment typical for the Hanford Site, autunite mineral has a high liability to dissolution in the presence of bacteria. Autunite, as a phosphorus-containing mineral, can attract bacteria to liberate P to meet their needs for nutrients, which may result in U mobilization into the environment.

Natural Autunite Bioleaching in Culture Ware with Inserts

An investigation with natural autunite mineral separated from bacteria was conducted using culture ware with inserts. The release of aqueous U(VI) over time during the autunite leaching in non-contact experiments is represented in Figure 4. The steady state maximum concentrations of U(VI) detected were 3.57, 1.34, and 1.41; fold higher than the abiotic control without the bicarbonate amendment. Furthermore, after bacteria inoculation, U(VI) concentrations measured in the reactors increased 3.96 ± 1.17, 2.79±1.62, and 2.82±3.75, fold for 0mM, 3 mM, and5 mM, respectively.

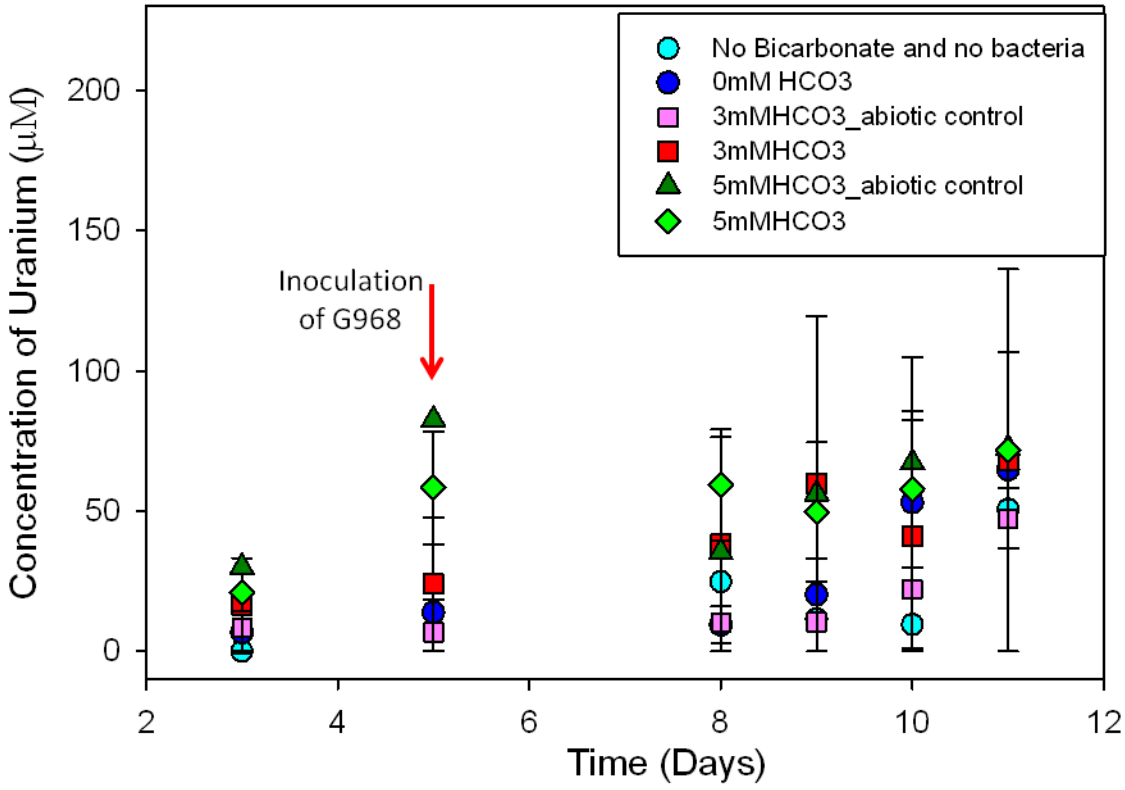


Figure 4. Changes for aqueous U(VI) as a function of time for the non-contact natural autunite dissolution insert experiments inoculated with *Arthrobacter* G968 strain

There is a slight increase in the dissolution of uranium as bicarbonate increases; however, this is not as pronounced when compared to the dissolution experiment (where bacteria and mineral have direct contact with one another). Statistical analysis indicated strong evidence that the means of uranium concentrations are different among different levels of bicarbonate ($P< 0.05$). These measures were not optimum conditions for bacterial growth and proliferation. However, results do illustrate that direct interaction between bacteria and mineral is not necessary to result in U(VI) biorelease from autunite.

Bioleaching of U(VI) from Synthetic Autunite

The release of aqueous U(VI) over time during the synthetic autunite dissolution experiment is presented in Figure 5. The bioreactors were injected with G968 *Arthrobacter* cells after 29 days, giving time for the synthetic autunite to reach steady state. The steady state maximum concentrations of U(VI) detected were 2.09, 8.83, 52.3, 88.4 fold higher than the abiotic control without the bicarbonate amendment. Furthermore, after bacteria inoculation, U(VI) concentrations measured in the reactors, increased 1.13± 0.47, 2.51± 0.53, 1.65± 1.45, and 1.00± 1.82 fold for 0mM, 3 mM, 5 mM and 10 mM $KHCO_3$, respectively. Statistical analysis suggests strong evidence that the mean of uranium concentrations are different among varying concentrations of bicarbonate ($P< 0.05$). From the figure it can be inferred that the effect of bacteria on the synthetic autunite dissolution is reduced as the concentration of bicarbonate increases, despite the increased amount of U(VI) leached influenced by the presence of bacteria. Since the steady-state U(VI) concentration is higher for larger [HCO_3^-], the increase in

soluble U(VI) concentration induced by bacteria is dwarfed. Therefore, as [HCO_3^-] increases, a diminishing trend on the effect of bacteria on autunite leaching is observed. This is very similar to previous experimentation conducted with natural autunite, only U(VI) concentrations observed were slightly higher.

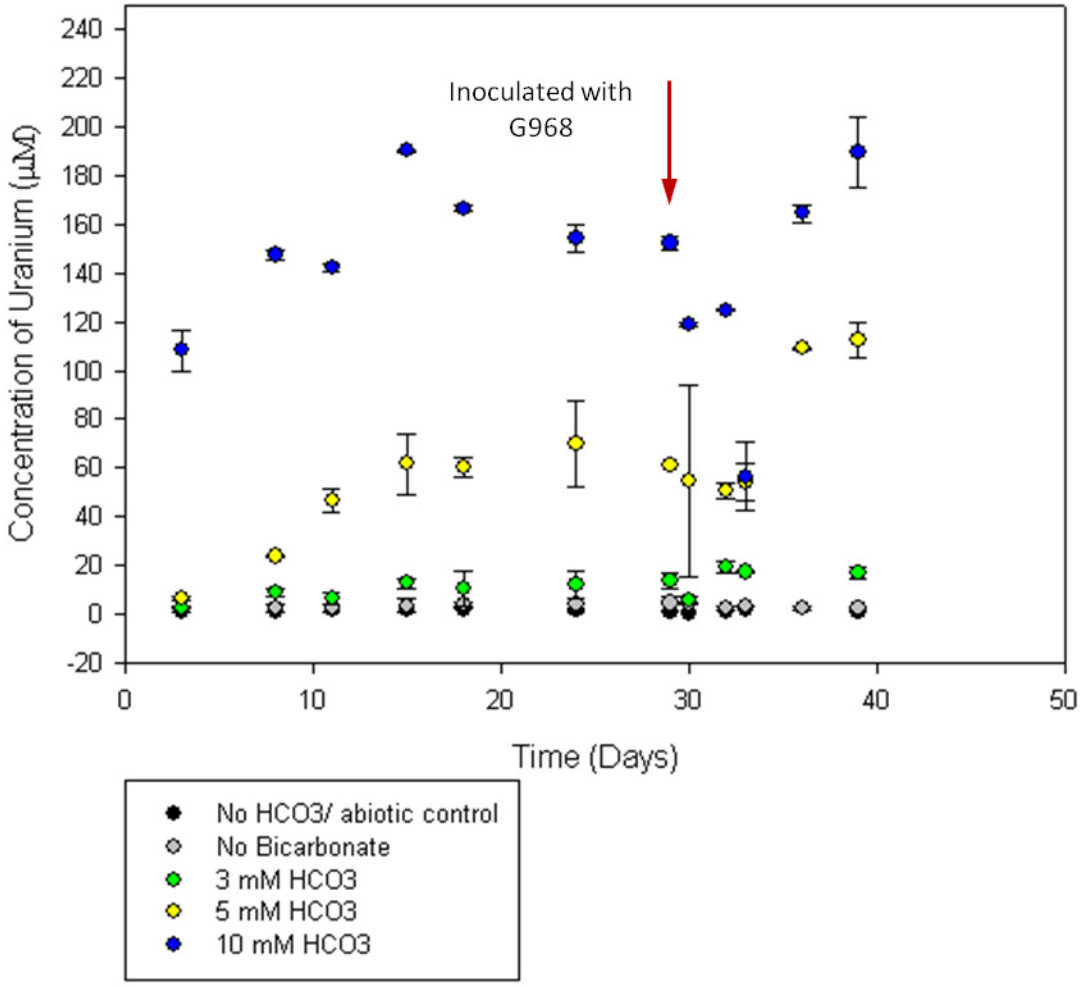


Figure 5. Changes for aqueous U(VI) as a function of time for the synthetic autunite dissolution experiments inoculated with *Arthrobacter* G968 strain

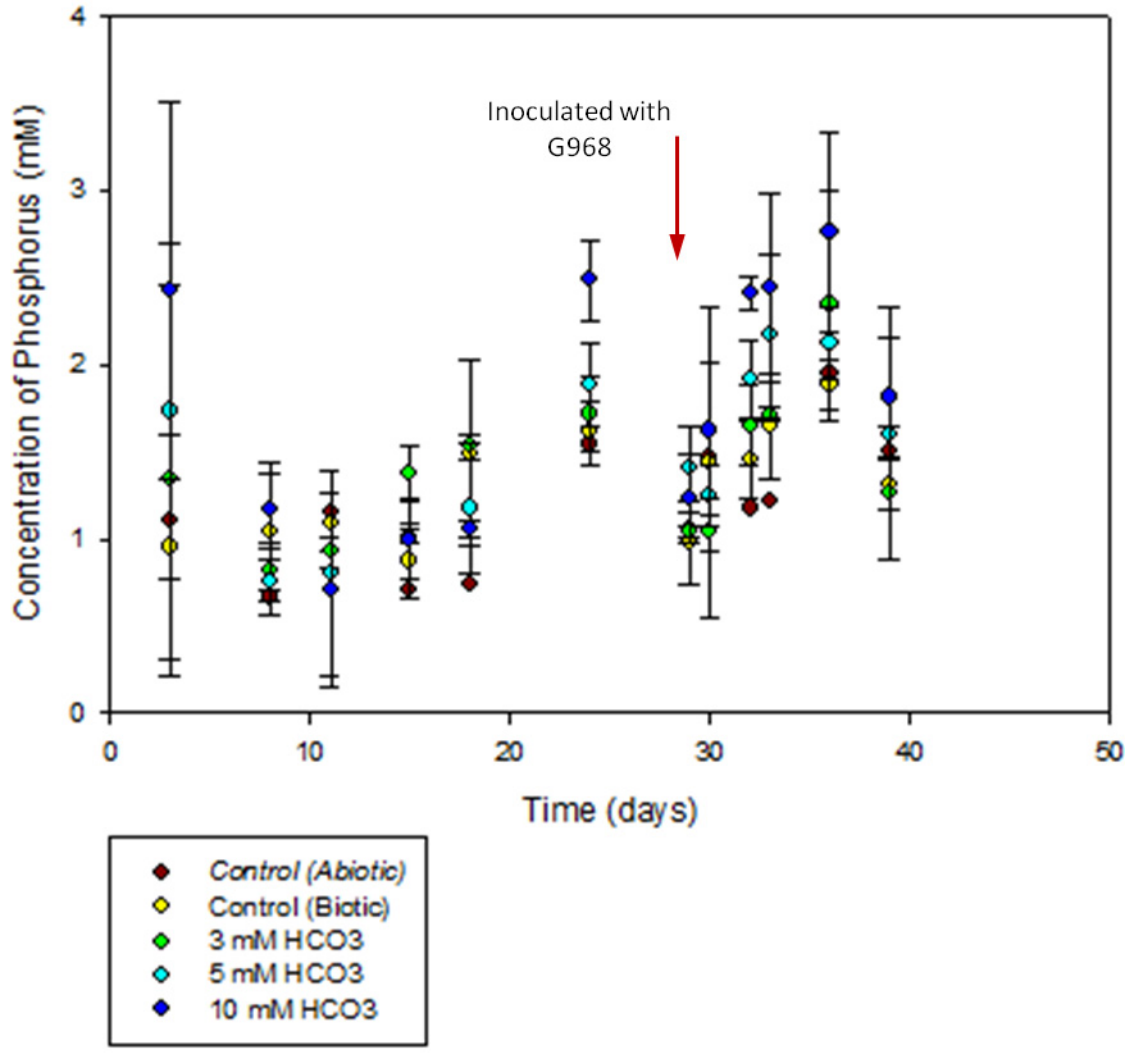


Figure 6. Aqueous phosphorus release as a function of time from the synthetic autunite dissolution experiments inoculated with *Arthrobacter* G968 strain

Phosphorus (P) concentrations in the biotic reactors were found to increase slightly for all studied bicarbonate concentrations. An opposite phenomena found when compared to natural autunite; where the formation of calcium phosphate phases control the transition of P from the soluble phase into the particulate and reduce soluble P concentrations in the reactors.

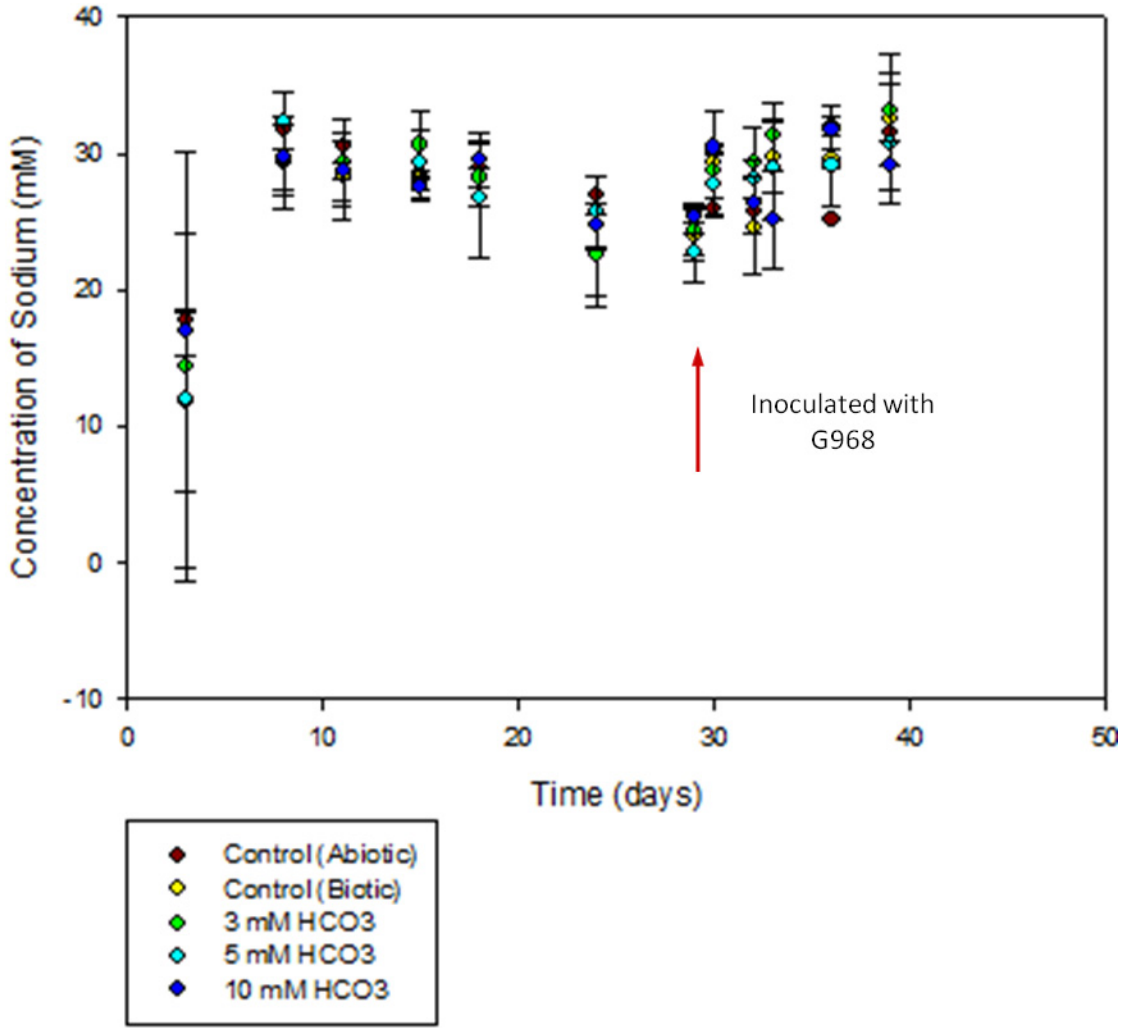


Figure 7. Aqueous Na release as a function of time from the synthetic autunite dissolution experiments inoculated with *Arthrobacter* G968 strain

During the autunite dissolution period without bacteria, sodium concentrations were increased compared to the non-carbonate abiotic control. After inoculation, sodium concentrations in the biotic reactors were found to increase for all studied bicarbonate concentrations. Sodium concentrations in the bioreactor followed a similar trend compared to U(VI) and phosphorus concentrations; although the concentration slightly

decreased in some cases with the introduction of bacteria, the Na concentration slowly started to increase towards the end of experimentation.

Comparing Natural autunite with Synthetic autunite

One major objective of the study was to investigate if *Arthrobacter* strain G968 influences uranium mobility through biodissolution. There is a question, however, if there was a difference between the microbial dissolution of synthetic autunite and natural autunite in the presence of bicarbonate. A previous study comparing the parameters for dissolution of natural and synthetic autunite in the presence of aqueous bicarbonate ions (R. Gudavalli, Y. Katsenovich, et al. 2013) concluded that the rate of uranium release from natural autunite is higher than from synthetic autunite.

Major factors that contribute to a higher rate of uranium release from natural autunite are the formation of uranyl-carbonate and calcium-uranyl-carbonate species. These complexes act as a driving force for uranyl detachment from mineral surfaces. Furthermore, natural autunite also forms a secondary Ca-P hydroxyapatite and uranyl phosphate minerals, which also serve as a driving force for the detachment of phosphate and calcium. However, the opposite phenomenon was noted in the experimental studies conducted for this project. Several factors could have contributed to such conflicting results. Gudavalli et al. (2013) ran experimentation via a single pass flow through (SPFT) apparatus designed to limit the accumulation of reaction products in the reactor. However, my experimentation was conducted in batch bioreactors that were agitated at 100rpm in the incubator-shaker over more than 30 days. This leaves the possibility of accumulation or precipitation in the bioreactors secondary solid phases predicted by speciation modeling (Table 2 and Table 3). The formation of secondary uranyl-phosphate

and calcium-phosphate mineral phases on the surface of the mineral can ultimately reduce the natural autunite mineral contact area, which bacterial cells can access. It thereby reduces the concentration of uranium released into the solution and detectable via the Kinetic Phosphorescence Analyzer (KPA) instrument. The dissolution of synthetic autunite produces only uranyl-phosphate secondary phases.

Table 2. Mineral saturation indices predicted by speciation modeling via Visual MINTEQ software based on solution compositions from dissolution of natural autunite in 5% PTG media amended with 20mM Na-HEPES and varying bicarbonate concentrations

Minerals	Saturation Indices			
	No Bicarbonate	3 mM HCO3	5 mM HCO3	10 mM HCO3
(UO2)3(PO4)2(s)	10.168	10.125	10.054	9.699
Autunite	9.545	9.202	8.879	8.132
Ca3(PO4)2 (am1)	0.819	Under Saturated	Under Saturated	Under Saturated
Ca3(PO4)2 (am2)	3.569	2.626	1.797	0.266
Ca3(PO4)2 (beta)	4.239	3.296	2.467	0.936
Ca4H(PO4)3:3H2O(s)	5.281	4.037	2.938	0.911
CaHPO4(s)	1.287	0.986	0.716	0.22
CaHPO4:2H2O(s)	1.007	0.706	0.436	Under Saturated
H-Autunite	2.255	2.253	2.217	2.01
Hydroxyapatite	12.959	11.373	9.987	7.42
Schoepite	1.054	1.013	0.978	0.83
UO2(OH)2 (beta)	0.832	0.791	0.757	0.609
UO2HPO4(s)	1.387	1.386	1.368	1.265

Table 3. Mineral saturation indices based on solution compositions from dissolution of synthetic autunite in 5% PTG media amended with 20mM Na-HEPES and varying bicarbonate concentrations

Minerals	Saturation Indices			
	No Bicarbonate	3 mM HCO3	5 mM HCO3	10 mM HCO3
(UO2)3(PO4)2(s)	9.557	9.313	8.885	5.668
K-Autunite	N/A	11.522	11.618	10.035
Na-Autunite	10.41	10.199	9.852	7.666

Comparison of *Arthrobacter* G975 with a less uranium tolerant strain, G968

In an assessment of the resistance of Hanford Site *Arthrobacter* isolates to uranium (VI) exposure, (Katsenovich, Carvajal and Guduru, et al. 2012 b), *Arthrobacter* G975 bacterial strain proved to remain viable in the presence of 0.5 ppm of U(VI). After a week, the number of viable colonies was comparable to the control without U(VI). Furthermore, G975 exhibited the highest tolerance towards U(VI) relative to the other *Arthrobacter* strains and remained viable in the presence of 9.5- 19 ppm of U(VI). A series of tests illustrated that G975 was the fastest growing and the most uranium tolerant strain and can accumulate more than 90% of uranium due to its distinctive surface structure (Katsenovich, Carvajal and Guduru, et al. 2012 b). In order to oversee the changes on the bacterial surface at the nanoscale, AFM analysis was performed and results showed an unusually irregular and wrinkled surface on the bacterial cell G975. Although precipitate was present on all *Arthrobacter* strains, the amount aggregated on the irregular surface of G975 surpassed the rest. The surface morphology of this strain supports a higher accessibility for the formation of the uranium precipitates. Experiments suggested that

despite these morphological differences between the two bacterial strains, uranium biorelease in both cases was noted at the same capacity in the presence of bicarbonate. The effect of both bacterial strands on autunite dissolution reduces as the concentration of bicarbonate increases, while the increase in soluble U(VI) concentration induced by G968 and G975 is dwarfed for larger [HCO_3^-]. It can be explained by the stronger stability constants for the uranyl carbonate complexes than for the uranyl-bacterial surface complex reactions (Gorman-Lewis, Elias and Fein 2005). Furthermore, during the autunite dissolution period without bacteria, P concentrations were increased compared to the non-carbonate abiotic control. After inoculation, P concentrations in the biotic reactors were found to decrease for all studied bicarbonate concentrations. Similar to U(VI), aqueous Ca release over the period of autunite dissolution without bacteria was noted to increase as a function of bicarbonate concentrations for both *Arthrobacter* strains.

Conducting the various biodissolution experiments answers several research questions. Data illustrated that the presence of bacteria in the bicarbonate-amended solutions enhances the release of U(VI). Autunite as a phosphorus containing mineral can attract bacteria to solubilize P, meeting their nutrients requirements, which may result in the release of U into the solution. Despite the morphological differences between G968 and G975 and various responses to uranium toxicity, they are able to dissolute uranium at the same capacity in the presence of bicarbonate ions. This bacterial effect should be considered when applying tripolyphosphate technology in the bicarbonate-rich environments typical for western semi-arid areas of the United States.

Imaging Analyses

To analyze the surface morphology of the bacterial surface when exposed to uranium in bicarbonate bearing media several microscopy techniques were used. FE-SEM (JEOL System Model 5900LV) was used to determine the changes in the cell-surface morphology in the autunite dissolution experiments. Wellman et. al., (2007) found that uranium release from autunite mineral is increased as the pH decreases (Wellman, Gunderson, et al. 2007). Also, bacterial cells, as a result of breakdown of organic compounds, can release organic acids and metabolites that may also lower the local pH on the level of the microbe-solid interface ultimately influencing the dissolution of autunite (Knox, et al. 2008). Figure 8 illustrates bacteria attachment to the mineral surface, as previously described; bacteria may lower the pH in the surrounding environment thereby promoting release of uranium from the mineral surface. Although no measurements could be conducted on what occurs on the microbe-solid interfaces, SEM images below show cellular localization and the bacterial attachment to the surface of radioactive natural autunite mineral. Figure 10 and Figure 11 shows images of secondary minerals and bacteria on the autunite surface. The EDS analyses on Figure 10 and Figure 11 help to distinguish between elemental composition of autunite surface and secondary minerals that may have been formed throughout the experiment. The atomic percentage of uranium on the autunite surface is higher at around 50% for Figure 11 whereas the atomic percent of uranium on the bacterial surface is lower at around 0.9-1.7% (Figure 10).

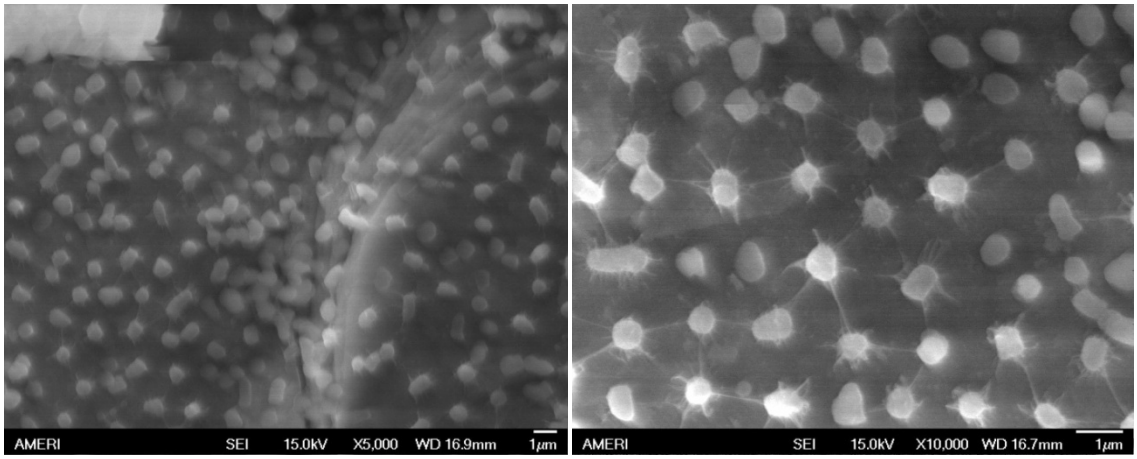
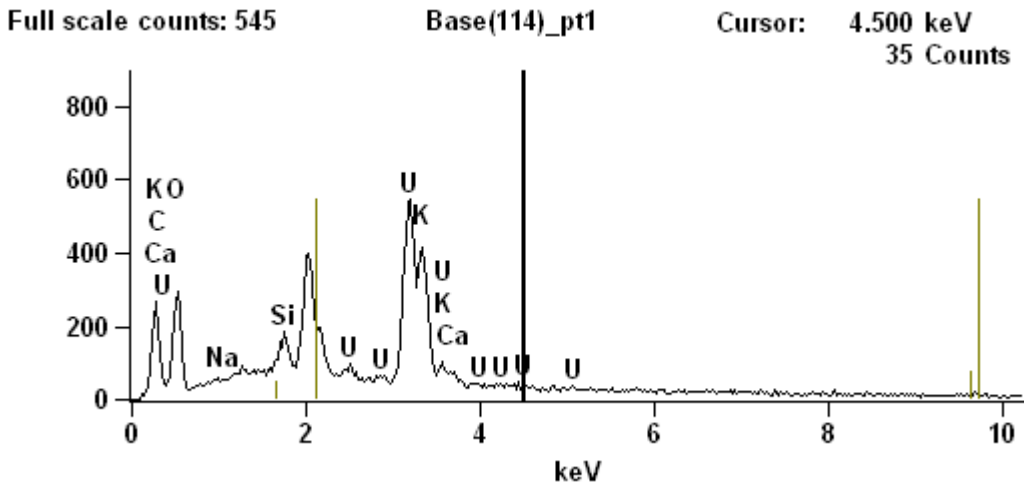


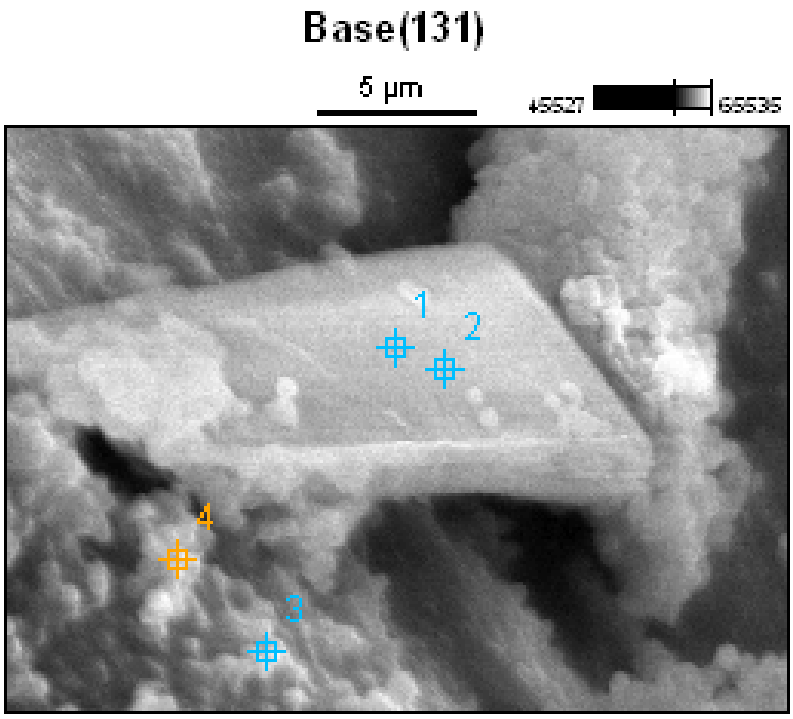
Figure 8. SEM images of G968 grown on autunite surface in the presence of 3mM KHCO3. Clearly illustrating individual bacterial cells attached to the mineral surface



Weight %

	C-K	O-K	Na-K	Si-K	P-K	K-K	Ca-K	Au-L	U-M
Mineral surface	30.19	20.85	0.00	1.19	4.08	1.55	0.73		41.42

Figure 9. EDS analysis of the mineral surface composition (%weight) at 3mM HCO3



	C-K	*N-K*	*O-K*	*Na-K*	*P-K*	*K-K*	*Ca-K*	*U-M*
pt1 **(mineral surface)**	24.1	3.9	29.4	0.08	4.3	0.9	1.9	35.4
pt2 **(mineral surface)**	25.2	2.8	29.6	0.2	4.2	1.3	1.8	35.0
pt3 **(bacterial surface)**	70.9	9.4	17.5	0.1	0.1	0.2	0.03	1.7
pt4 **(bacterial surface)**	67.3	12.9	18.3	0.1	0.2	0.2	0.09	0.9

Figure 10. SEM/EDS analysis of biofilm created by G968 strain on autunite surface and compositional analysis (% weight) for each point at 5 mM HCO3

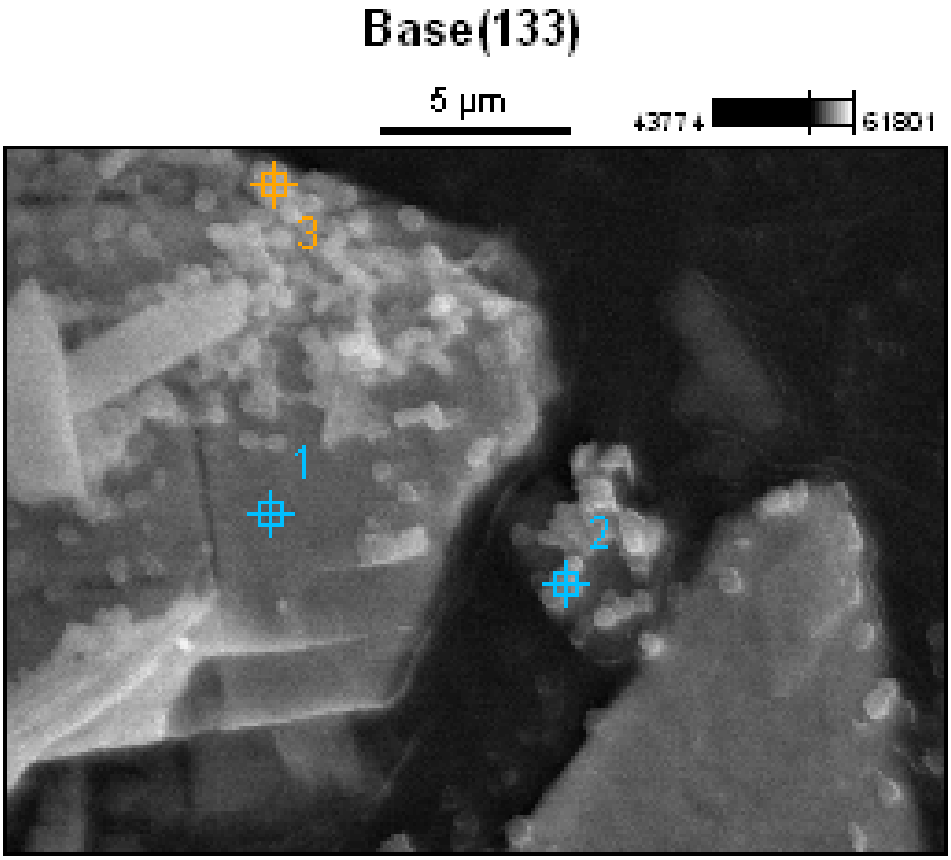


Figure 11. SEM/EDS analysis of secondary minerals and biofilm created by G968 strain on autunite surface and compositional analysis (% weight) for each point at 10 mM HCO3

	C-K	N-K	O-K	Na-K	P-K	K-K	Ca-K	U-M
pt1 **(mineral surface)**	20.4	2.7	18.0	0.00	5.9	2.5	0.2	50.2
pt2 **(biofilm/ secondary minerals)**	62.8	6.9	14.7	0.1	1.3	1.0	0.02	13.2
pt3 **(biofilm/ secondary minerals)**	56.5	7.9	16.6	0.1	1.7	0.6	0.05	16.6

As previously stated the main goal of this imaging analysis was to qualitatively observe what would occur to the mineral surface when exposed to bacteria cultured in varying

concentrations of bicarbonate bearing media. Figure 11 illustrates this phenomenon, showing an accumulation of secondary phases that covers the surface of the mineral, closing active sites.

Cell Viability via Live/Dead Assay

The viability of the cells was observed for twenty-four hours after the bacterial cells were exposed to varying concentrations of uranium in bicarbonate-bearing media. It is important to note that Figure 13 and Figure 15 illustrate a clustering of cells resulting in larger green intensity regions which therefore create the possibility of underestimating the number of live cells in samples containing bicarbonate.

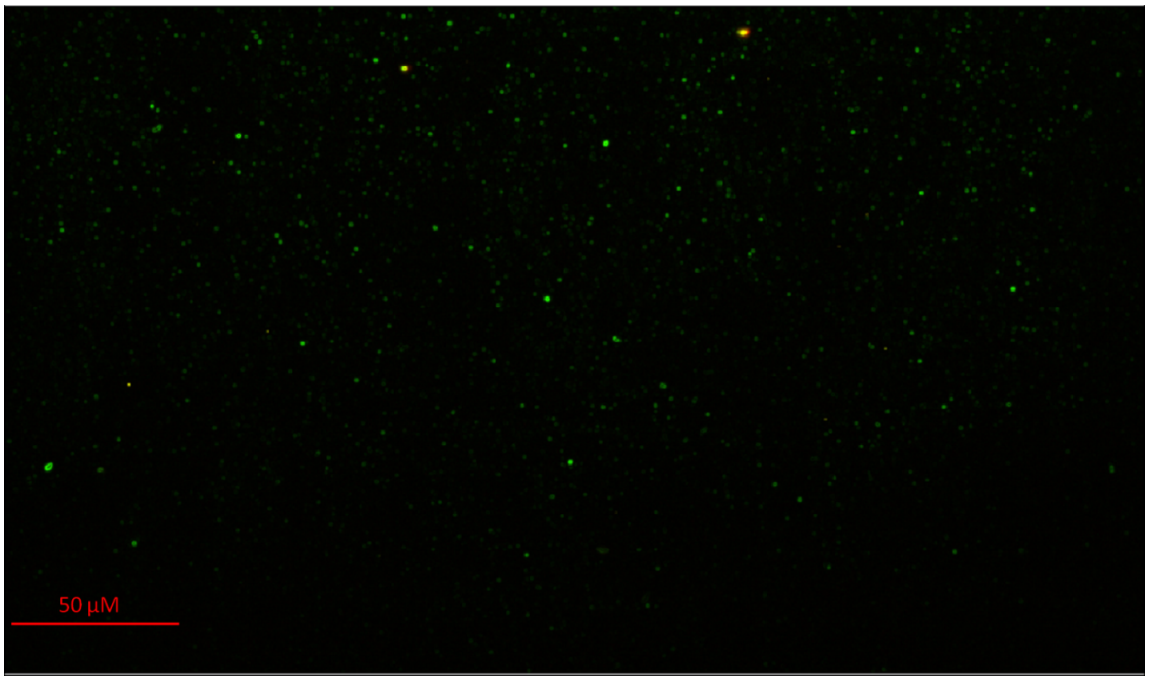


Figure 12. Live Dead assay of sample containing 5ppm of U(VI) with no bicarbonate. This sample illustrates a large concentration of live cells (green dots) with scattered dead cells (orange/yellow dots)

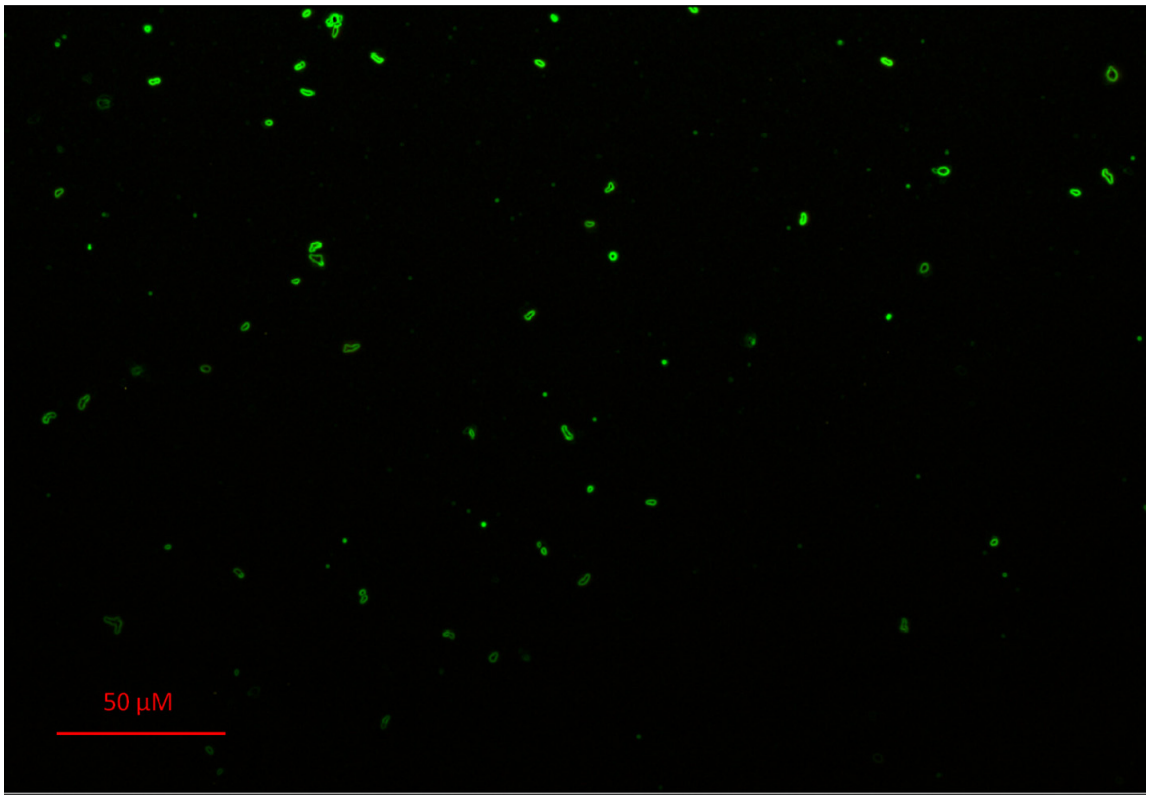


Figure 13. Live Dead assay of sample containing 5ppm of U(VI) with 5mM bicarbonate. This sample illustrates a large concentration of live cells (green dots) with a smaller almost nonexistent concentration of dead cells (orange/yellow dots)

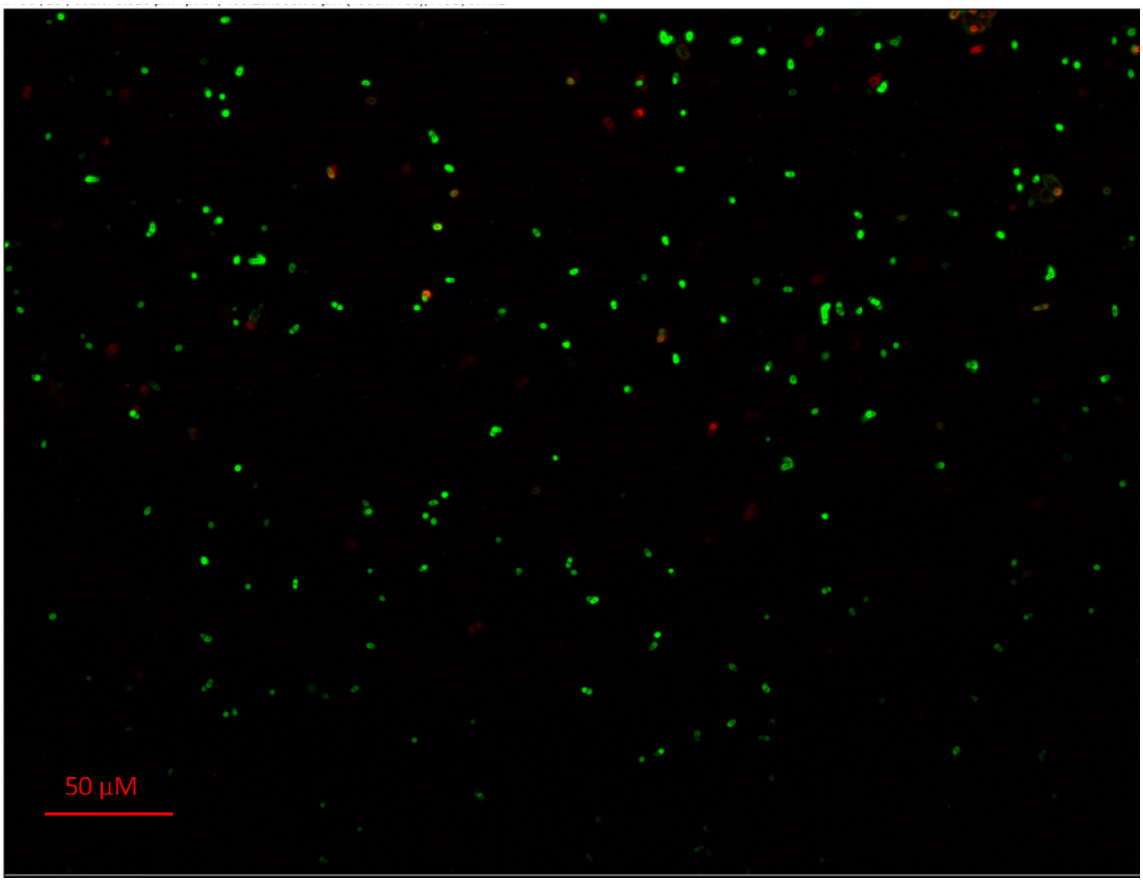


Figure 14. Live Dead assay of sample containing 10ppm of U(VI) with no bicarbonate.

This sample illustrates a higher concentration of dead cells compared to Figure 15.

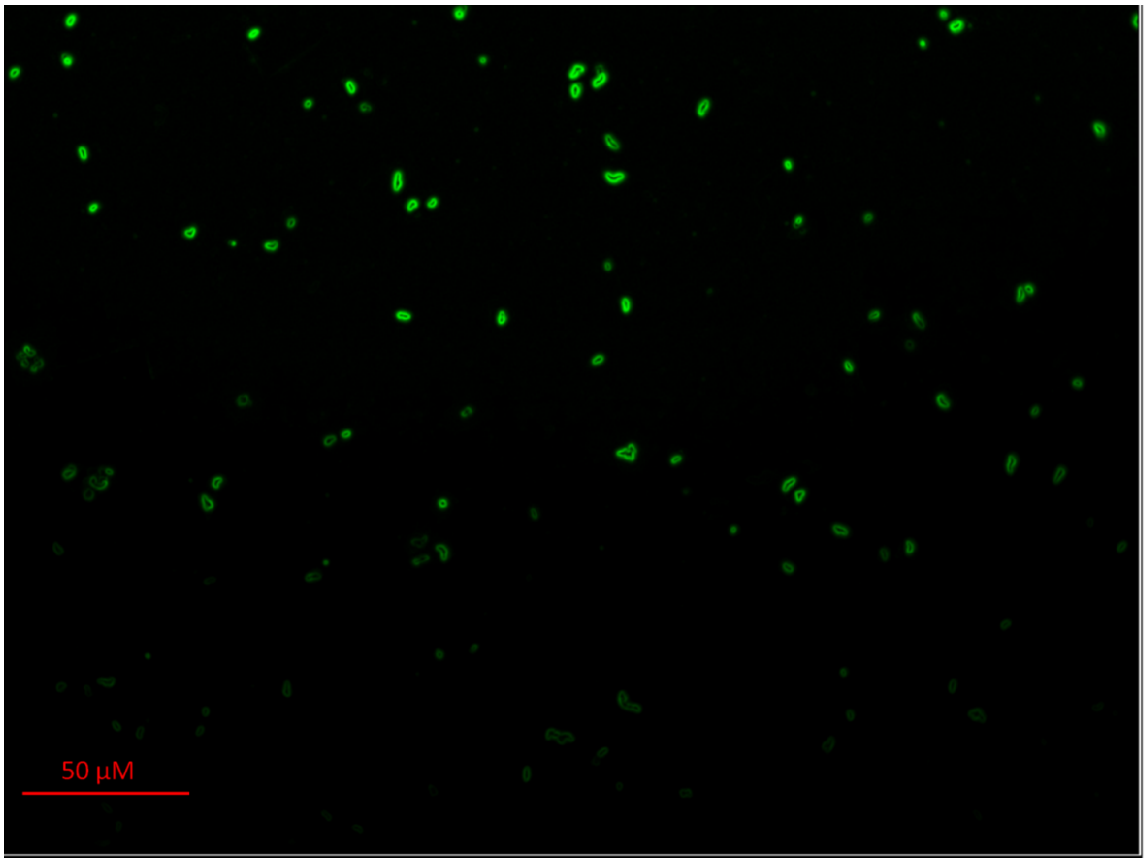


Figure 15. Live Dead assay of sample containing 10ppm of U(VI) with 5mM bicarbonate. This sample illustrates a large concentration of live cells.

When calculating the viability of cells for each sample it has been found that there is not much difference between the varying concentration of uranium and bicarbonate. Each sample exhibited a ratio of live cells greater than 95% and when making a comparison between the sample containing 10ppm of U(VI) with and without bicarbonate it is apparent that the sample containing bicarbonate contained a higher ratio of live cells as seen in

Table 4. Statistical analysis indicated a statistically significant difference between the means of cell viability among the different levels of uranium and bicarbonate concentrations ($P < 0.05$).

Table 4. Quantitative Assessment of Percentage Cell Viability in Samples Subjected to Live/Dead Assay (n=4)

Sample Type	**Ratio of Live cells (in percentages)**
5ppm U, 0mM Bicarbonate	98.00 ± 1.02
5ppm U, 5mM Bicarbonate	97.51 ± 0.67
10ppm U, 0mM Bicarbonate	95.59± 1.22
10ppm U, 5mM Bicarbonate	100± 1

Cell Viability via Plates

Although all samples seem viable despite the concentrations of uranium and bicarbonate present they may be unable to develop into culturable colonies in media. Bacteria enter a state known as the "viable but nonculturable (VBNC) state", first coined by Rita Colwell (Xu, et al. 1982). Bacteria would enter this state as a feedback response from natural stresses such as starvation or being exposed to deadly substances (Oliver and Bockian 2000). These environmental stresses could be lethal if cells do not enter the VBNC state. Plating efficiency was calculated by comparing cell viability in the control media before and after U(VI) exposure, which is determined via counts of colony forming units (CFU). Samples containing 5ppm U with 0mM bicarbonate illustrated a G968 plating efficiency

of 1.75%; with 5mM bicarbonate in the solution the efficiency increased to 42%. Similarly, samples containing 10ppm U with 0mM bicarbonate had a CFU plating efficiency of 3.25%, and with 5mM bicarbonate the efficiency increased to 28%. The pH values stayed at 7.5 for all samples. These results illustrate that although the bacterial cells established intact cytoplasmic membranes, resulting in viable cells for the Live/Dead analysis, the cells that are exposed to uranium with no bicarbonate added in the media experience a viable but nonculturable state. Meaning, the samples containing 5 ppm of U(VI) and 0 mM of bicarbonate, and 10ppm of U(VI) and 0mM of bicarbonate keep the integrity of the membrane showing high levels of viability in the Live/Dead fluorescent assay but experienced low levels of colonies when plated. The oligotrophic environment that the Hanford Site contains, offers low levels of nutrients, and organic carbon that is growth limiting within the soil sediments. Because the concentration of carbon is usually low in soil environment, organisms undergo a state of starvation. Bacterial cells are in a resting state of low metabolic activity which describes its inability to produce spores and enters a viable but nonculturable state for long term success. These cells would also maintain an intact cellular membrane to keep the genetic material intact. (Sumner, 1999). Also, Konopka et al. (Konopka, Plymale and Carvajal 2013) found that bacteria suspended in groundwater that lacked added bicarbonate within the Hanford Site had an inhibited metabolic activity. The uranium toxicity to the bacterial cells was reduced, increasing metabolic activity, when increasing amounts of bicarbonate was added to the synthetic groundwater which is a similar experimental setup that we mimicked. It is also interesting to note that when samples were observed under light microscope, the samples containing uranium did not change in number but they looked

impaired and stayed stationary on the slide; while cells that were in bicarbonate amended media increased in total cell number and were actively moving on the slide.

Effect of Uranium on Microbial Surfaces Using Atomic Force Microscopy

Atomic force microscopy (AFM) was employed to monitor changes at the nanoscale level in cell surface topography and adhesion forces after the cells exposure to various concentrations of uranium. The aim of this task was to present high-resolution AFM images and determine cells dimensions and adhesion forces to illustrate the effect of uranium and bicarbonate on the bacterial surface. The results demonstrated the ability of this method to qualitatively and quantitatively characterize changes on the bacterial surface after U exposure and evaluate the effect of bicarbonate ions on U(VI) toxicity of a less uranium tolerant *Arthrobacter* strain, G968 by analyzing changes in bacterial dimensions via profile plots.

Uranium Effect on the Microbial Cell Surface

AFM was used to analyze surface morphology in the presence of varying concentrations of uranium in bicarbonate bearing or bicarbonate-free solutions. The role of bicarbonate is qualitatively shown in the images below; the solutions were treated with varying concentrations of uranium, from 0ppm to 10 ppm. Images revealed changes in bacteria shape and dimensions due to the exposure to uranium.

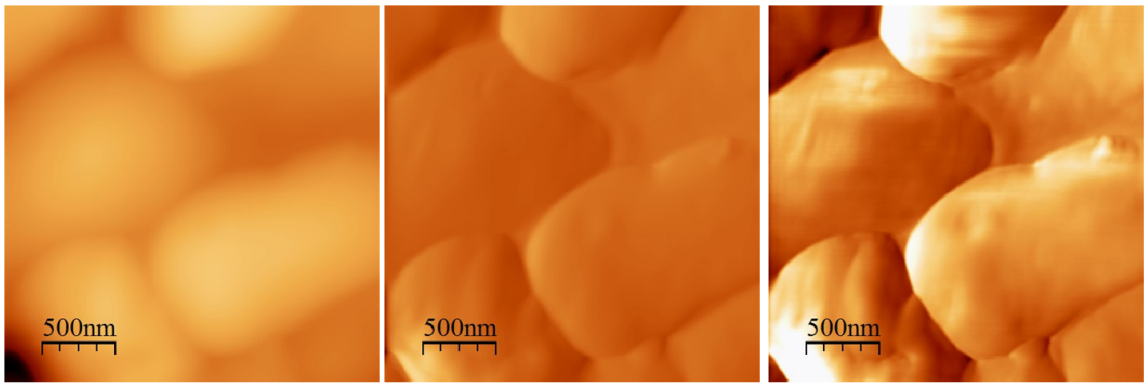


Figure 16. G968 control sample (scan size 2.5 x 2.5 μm^2) illustrating smooth bacterial surface. The topographic image on the left, deflection image in the middle and friction image on the right

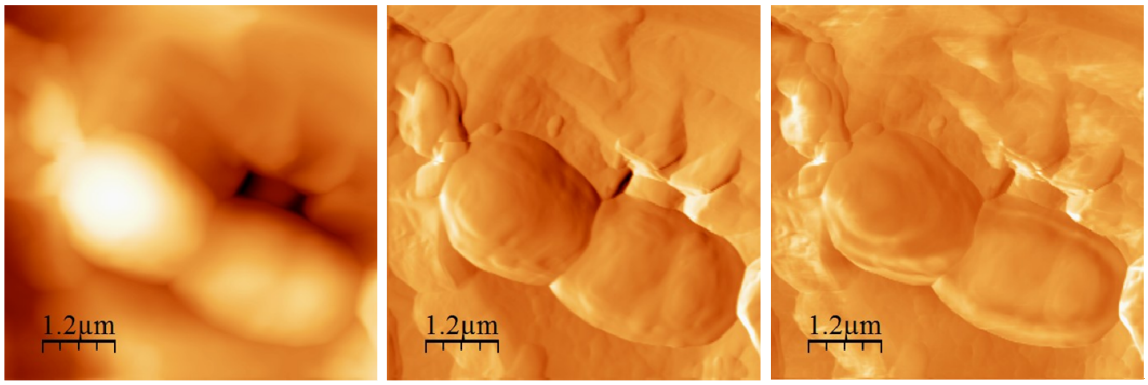


Figure 17. G968 cultured in media amended with 5ppm U(VI) and 0mM HCO3 (scan size 6 x 6 μm^2). The topographic image is on the left, deflection in the middle and friction image on the right.

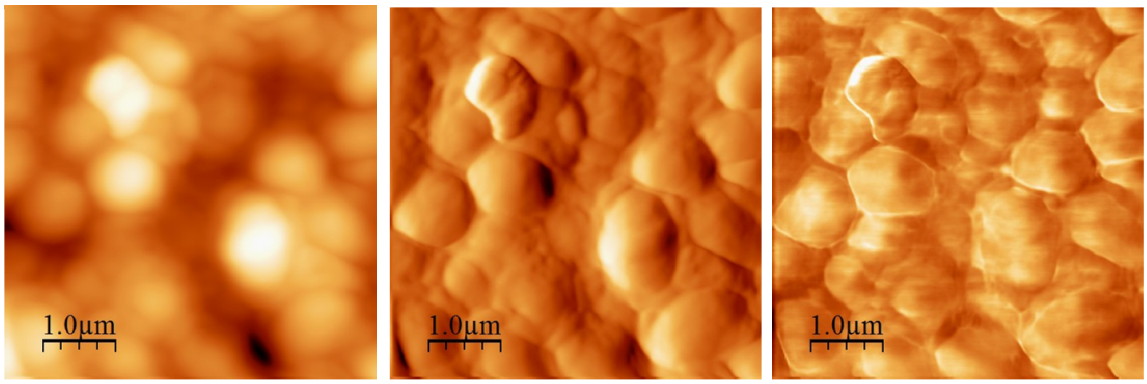


Figure 18. G968 cultured in media containing 5ppm of U(VI) and 5mM bicarbonate, (scan size 5 x 5 μm^2). The topographic image is on the left, deflection image in the middle and friction image on the right

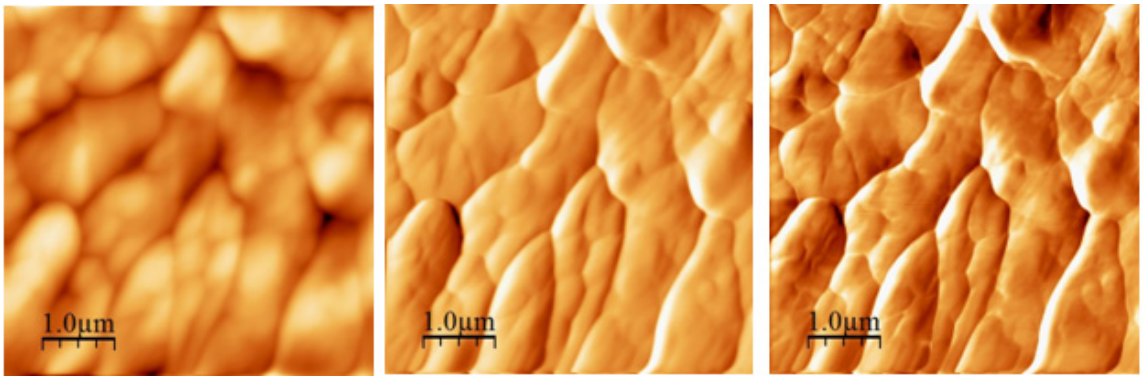


Figure 19. G968 cultured in media amended with 10ppm U(VI) and 0mM HCO3 (scan size 5.0 x 5.0 μm^2). The topography image is on the left, deflection image in the middle and friction image on the right.

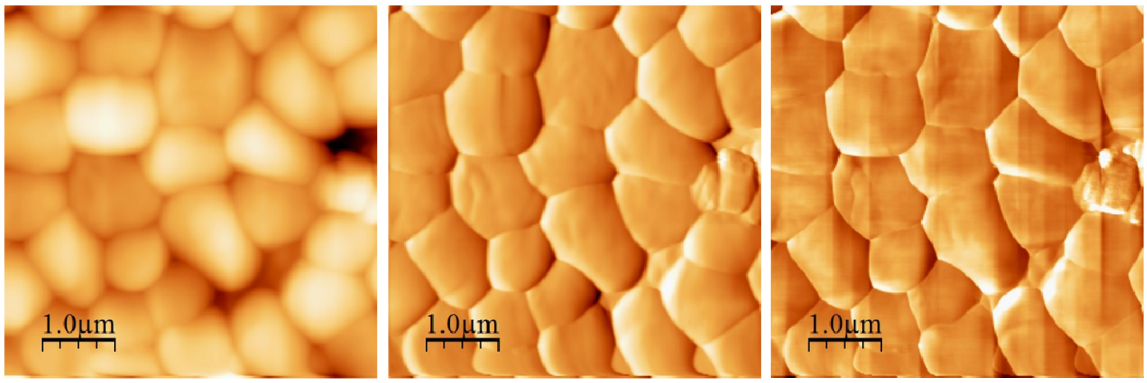


Figure 20. G968 cultured in media amended with 10ppm U(VI) and 5mM HCO3 (scan size 5 x 5 µm2). The topography image is on the left, deflection image in the middle and friction image on the right. These images exhibit no negative effects from uranium exposure

Force Spectroscopy Analysis

It has been proposed that adhesion forces are sensitive to modifications in the surface; for example, physiological changes occur on the cellular membrane when exposed to uranium. So, it was necessary to perform a force spectroscopy analysis to gain a full comprehension of the interaction forces between the tip and the sample surface at the piconewton scale level. The results in Table 5 indicate a significant statistical difference in adhesion force values between control samples and those containing varying concentration of uranium and bicarbonate ($P < 0.05$).

Table 5. Adhesion forces for *Arthrobacter* sp. G968 (n=3)

Uranium concentration (ppm)	**Bicarbonate concentration (mM)**	**Adhesion (nN)**
0	0	11.6 ± 1.68
5	0	7.14 ±0.26
5	5	9.14 ± 4.1
10	0	5.54 ± 4.3
10	5	4.88 ± 2.3

There is an inverse relationship between the adhesion forces and the concentration of uranium; as the concentration of uranium increases the adhesion forces will decrease exponentially. Additionally, when bicarbonate is present within the solution, the adhesion forces showed similar values to that of the control sample when no uranium is present. Adhesion force measures the interaction force between the tip and the sample surface. If something is growing on the surface the attraction is less. Previous MINTEQ modeling illustrated that the trimetric form of uranium, $(UO_2)_3(OH)^{5+}$, is the most positive species that has the most potential to interact or damage negatively charged cell walls. In the presence of bicarbonate, the solution mostly accumulates the negatively charged uranyl carbonate $UO_2(CO_3)_3^{-4}$ species, which are not expected to adhere onto the negatively charged bacterial surface (Carvajal, Katsenovich and Lagos 2012).

Roughness Analysis

Changes in the cells surface roughness in the bacterial strain when exposed to varying concentrations of uranium was analyzed using AFM roughness analysis. Exposure to uranium was conducted 24 hours prior to imaging while the concentrations of uranium ranged from 0ppm to 20 ppm. Past research has shown some correlation between growing roughness values as the concentration of uranium increased (Kazy, D'Souza and Sar 2009). However, for this particular case, values of roughness were in the range of 23.69 and 41.53 nm, and there was no correlation found with respect to roughness and uranium concentrations. Despite this, however, the data indicates that there is a significant statistical difference in roughness values between control samples and those containing varying concentration of uranium and bicarbonate (P = 0.006).

Bacteria cells height maps via AFM

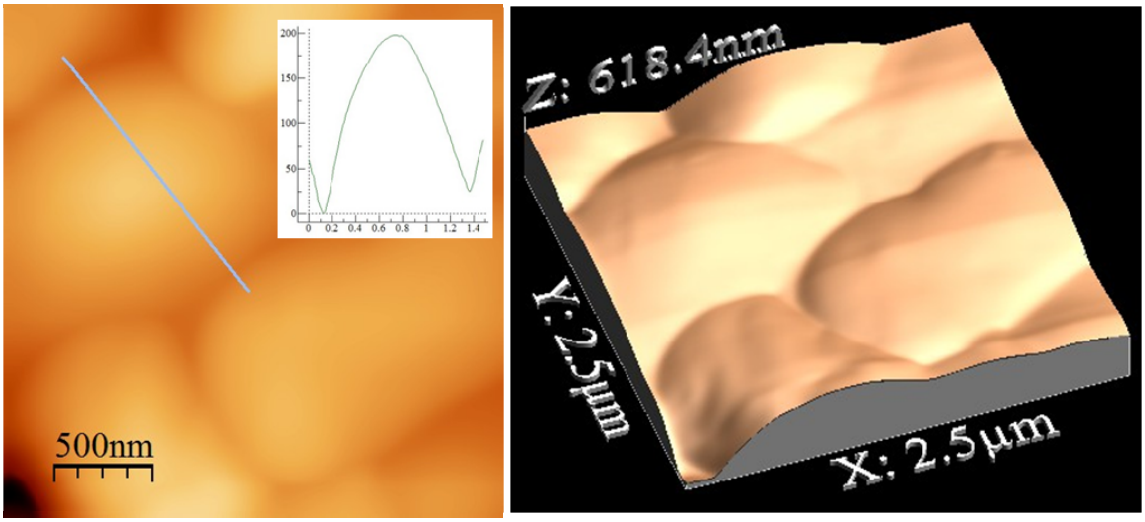


Figure 21. G968 control sample (scan size 2.5 x 2.5 µm2) illustrating a maximum height of 198 nm and length of 1.3 µm. The profile plot is on the left with the 3D topographic image on the right (Z range 618.4 nm)

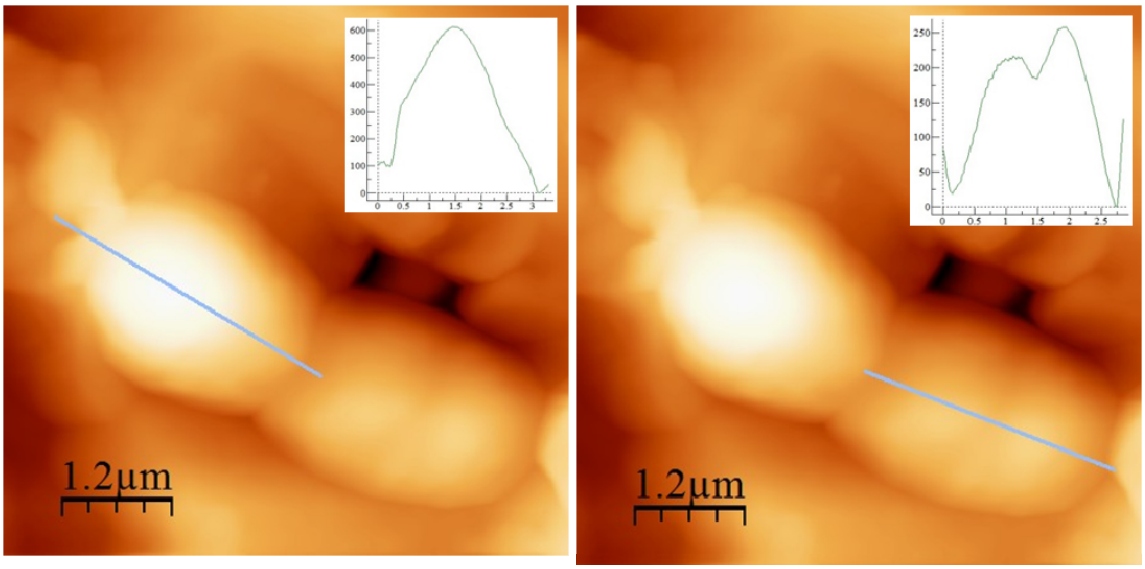


Figure 22. G968 samples amended with 5ppm U(VI) and no bicarbonate. Profile plot on the left illustrates the height of one cell at 600 nm, while the profile height of the image on the right measure 250 nm

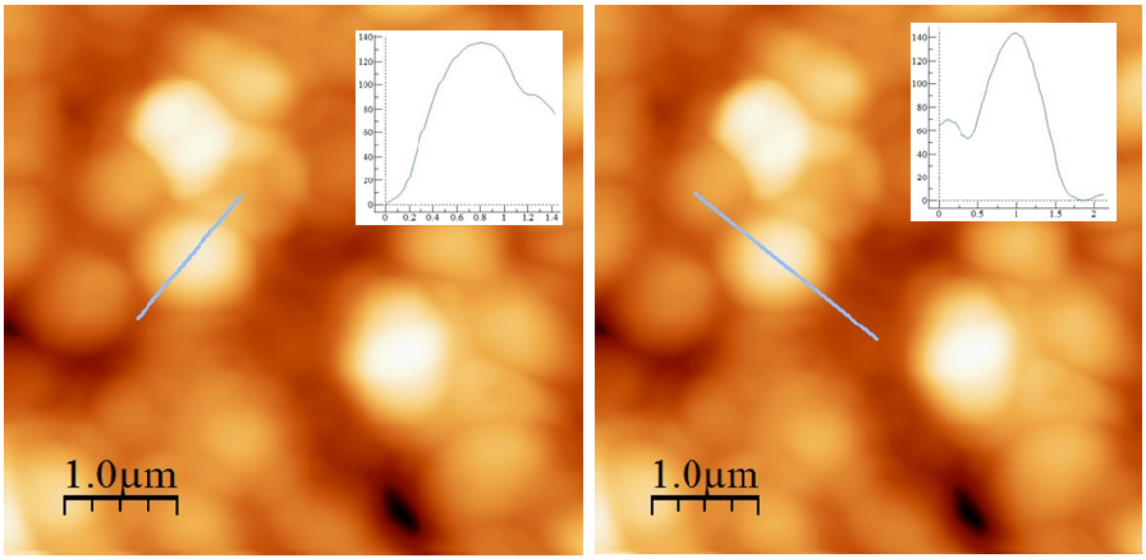


Figure 23. G968 sample amended with 5ppm U(VI) and 5mM bicarbonate. The profile heights for this sample ranges from 135 to 140 nm. The effect of uranium could have caused these bacterial cells to shrink

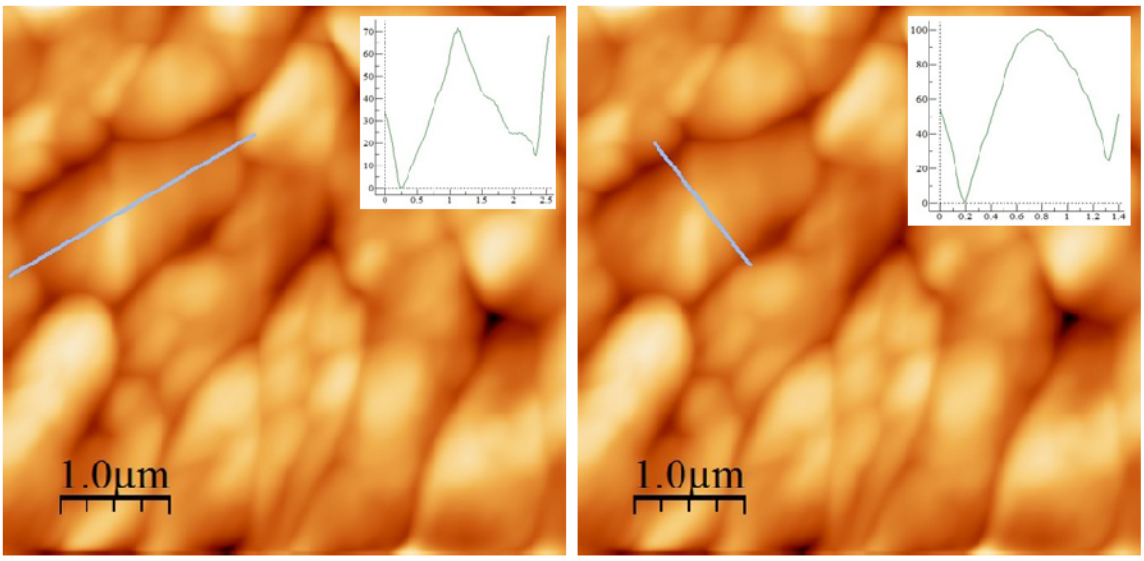


Figure 24. G968 sample amended with 10ppm U(VI) and 0mM bicarbonate. The profile heights for this sample ranges from 70-90nm

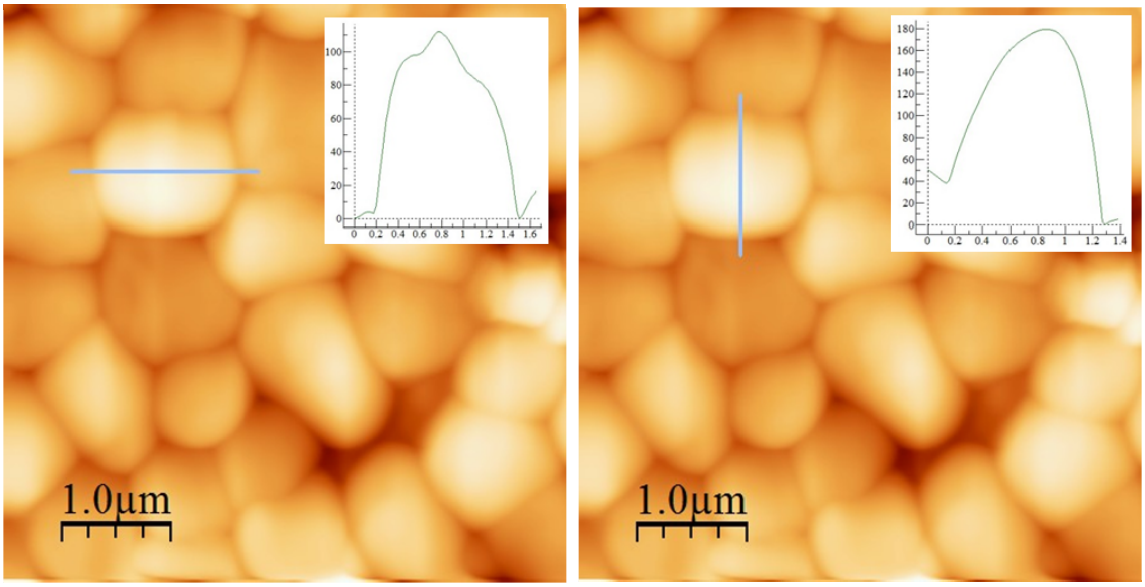


Figure 25. G968 sample amended with 10ppm of U(VI) and 5mM of bicarbonate. The profile heights for the above sample range from 110-180 nm, showing lower values than U-free controls.

The height of a bacterial cell is a good indication of cell response to U toxicity; U-free control samples exhibit a large profile height. When comparing the cells in samples containing 10 ppm of uranium with and without bicarbonate, the samples containing bicarbonate (Figure 25) have an increased height, revealing a smooth surface. When comparing the plating method and Live/Dead analysis with AFM assessment, we find that bacteria samples taken from bicarbonate- free solutions are mostly viable but not culturable; their height is less to uranium-free control sample (

Figure 21, Figure 22, Figure 24). In opposition, samples containing bicarbonate have a reduced height and smaller cellular size with no deformed surface and look identical to U-free controls samples. It might be an indication that the cells are alive and have acclimated to withstand uranium toxicity. Furthermore, *Arthrobacter* has the ability to change size and shape in response to toxicity from a rod to coccus form; in this cocci shape the bacteria are resistant to desiccation and starvation (Banerjee, et al. 2010) (O' Loughlin, Sims and Traina 1999).

VI. CONCLUSIONS

Biodissolution experiments were conducted to test if there was a difference in microbial dissolution of synthetic and natural autunite in the presence of bicarbonate. In natural autunite biodissolution, the steady state maximum concentrations of U(VI) detected were 1.92, 14.4, 30.1, and 44.4 fold higher for 0mM, 3 mM, 5 mM and 10 mM $KHCO_3$ than the abiotic control without the bicarbonate amendment, with the number of samples being 3. Furthermore, after bacteria inoculation, U(VI) concentrations measured in the reactors, increased 3.0 ± 4.16, 23.3 ± 13.8, 19.0 ± 28.0, and 2.00 ± 2.66 fold, for 0mM, 3 mM, 5 mM and 10 mM $KHCO_3$ respectively, compared to the corresponding bicarbonate-bearing controls at steady state. ANOVA Statistical analysis indicated very strong evidence that the mean uranium values are dissimilar across the different groups of bicarbonate concentration ($P < 0.05$). The data suggests that although there is an increased amount of U(VI) leached out into the solution driven by the presence of bacteria, the effect of bacteria on autunite dissolution is reduced as the concentration of $[HCO_3^-]$ increases.

In comparison, the steady state maximum concentrations of U(VI) detected for synthetic autunite dissolution were 2.09, 8.83, 52.3, 88.4 fold for 0mM, 3 mM, 5 mM and 10 mM $KHCO_3$ respectively, higher than the abiotic control without the bicarbonate amendment. After bacteria inoculation, U(VI) concentrations measured in the reactors, increased 1.13± 0.47, 2.51± 0.53, 1.65± 1.45, and 1.00± 1.82 fold for 0mM, 3 mM, 5 mM and 10 mM $KHCO_3$, respectively. Statistical analysis suggests strong evidence that the mean of uranium concentrations are different among varying concentrations of bicarbonate at 0.05 significance levels ($P< 0.05$). Similar to the dissolution of uranium from natural autunite,

the presence of bacteria in bicarbonate-amended media strongly enhances the biorelease of uranium compared to bicarbonate-free media. Comparing the biodissolution of synthetic and natural autunite in the presence of bicarbonate, the uranium release from natural Ca-autunite was found lower than the rate of uranium release from synthetic autunite due to the formation of calcium phosphate secondary solid phases on autunite surface, which reduces bacterial access to active site on the mineral.

To evaluate if a direct interaction between bacteria and mineral was necessary to result in uranium release from mineral; a bioleaching experiment in culture ware with inserts was performed with autunite powder, media solution and G968 bacteria. The steady state maximum concentrations of U(VI) detected were 3.57, 1.34, and 1.41; fold higher than the abiotic control without the bicarbonate amendment. Furthermore, after bacteria inoculation, U(VI) concentrations measured in the reactors increased 3.96 ± 1.17, 2.79±1.62, and 2.82±3.75, fold for 0mM, 3 mM, and 5 mM of bicarbonate, respectively. There is a slight increase in the biodissolution of uranium as bicarbonate increases; however, this is not as pronounced when compared to the biodissolution experiment (where bacteria and mineral have direct contact with one another). Statistical analysis indicated strong evidence that the means of uranium concentrate are different among different levels of bicarbonate ($P< 0.05$). Data suggests that direct interaction between bacteria and mineral is not necessary to result in U(VI) biorelease from autunite.

Making a comparison between *Arthrobacter* G975 and the less uranium tolerant strain, G968, it was found that despite morphological differences between the two bacterial strains, they are able to dissolute uranium at the same capacity in the presence of bicarbonate. The effect of both bacterial strands on autunite dissolution reduces as the

concentration of bicarbonate increases while the increase in soluble U(VI) concentration induced by G968 and G975 is dwarfed, for larger [HCO_3^-].

AFM analysis and Live/Dead fluorescent assay was used to qualitatively and quantitatively illustrate how bacterial cells are affected when exposed to uranium in the presence of bicarbonate bearing media. The Live/Dead analysis showed that despite the concentration of uranium and bicarbonate present in the solution, each sample exhibited a ratio of live cells great than 95%. Although the samples seemed viable, they also showed that they are not culturable. By performing a cell viability assessment via culture plates, results demonstrated that although the bacterial cells established intact cytoplasmic membranes, resulting in viable cells for Live/Dead analysis, the cells that are exposed to uranium with no bicarbonate experienced a viable but nonculturable state, that is, formed few colonies when plated. AFM-adhesion force results demonstrate that as uranium concentration is added to the media, the adhesion force parameter decreases. Furthermore, the height provided from the profile plots revealed that samples containing bicarbonate have a higher profile height, resulting in a much smoother surface. Thus, samples exposed to uranium with no bicarbonate are mostly viable via Live/ Dead assay, but exhibited deformed surfaces and a low height profile, which might be an indication that the cells are not alive. In contrast, samples containing bicarbonate have a reduced height and small cellular size but are alive and have acclimated to withstand uranium toxicity.

It is necessary to identify factors that adversely affect the dissolution of uranium at the Hanford Site, so it is possible to mitigate those factors. This study provides a better understanding of the role of bacteria in the stability of autunite precipitates created as a

result of phosphate remediation technology and investigate the microbial effect on the uranium release from autunite solid phases in bicarbonate rich environments. It can be concluded that the less uranium tolerant strain, *Arthrobacter* strain G968 influences uranium mobility in the subsurface through biodissolution. Furthermore, the presence of bicarbonate is capable of accelerating U(VI) release from both natural Ca-autunite and synthetic Na-autunite in the presence of G968, in conditions mimicking Hanford site subsurface environments. Thus, the effect of bacteria is an important environmental factor that needs to be considered when applying tripolyphosphate amendment to sequester uranium in the bicarbonate-rich environments.

VII. PUBLICATIONS & POSTER PRESENTATIONS

Sepulveda-Medina, P., Katsenovich, Y., Wellman, D., Lagos, L. "Investigation on Microbial Dissolution of Autunite Mineral Using a Less U(VI)-Tolerant Strain, *Arthrobacter oxydans* G968" Manuscript to be submitted to the peer reviewed journal (Chemical Geology).

Sepulveda-Medina, P., Katsenovich, Y., Musaramthota, V., Lee, M., Lee, B., Dua, R., Lagos, L. "A Study of Cell Viability on DOE Hanford Soils Isolates: Effect of U(VI) and Bicarbonate" Manuscript to be submitted to peer reviewed journal (The Science of the Total Environment).

Sepulveda, P. "Investigation on Microbial Dissolution of Uranium (VI) from Autunite Mineral" DOE Fellows' Poster Competition. October, 2012. Miami, Florida.

Sepulveda, P. "Investigation on Microbial Dissolution of Uranium (VI) from Autunite Mineral" DOE Fellows' Poster Competition. October, 2013. Miami, Florida.

Sepulveda, P., Katsenovich, Y, Lagos, L. "A Study of Cell Viability on DOE Hanford Soil Isolates: Effect of U(VI) and Bicarbonate" (poster). Waste Management Symposia. March 2014. Phoenix, Arizona.

Sepulveda, P., Katsenovich, Y. "Investigation on Microbial Dissolution of Uranium(VI) from Autunite Mineral" In the proceedings of the Waste Management Symposia. February, 2013. Phoenix, Arizona.

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