The Effect of Bicarbonate on the Microbial Dissolution of Autunite Mineral in the Presence of Gram-Positive Bacteria

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Abstract

Bacteria are key players in the processes that govern fate and transport of contaminants. The uranium release from Na and Ca-autunite by *Arthrobacter oxydans* strain G968 was evaluated in the presence of bicarbonate ions. This bacterium was previously isolated from Hanford Site soil and in earlier prescreening tests demonstrated low tolerance to U(VI) toxicity compared to other *A. oxydans* isolates. Experiments were conducted using glass serum bottles as mixed bioreactors and sterile 6-well cell culture plates with inserts separating bacteria cells from mineral solids. Reactors containing phosphorus-limiting media were amended with bicarbonate ranging between 0-10 mM and meta- autunite solids to provide a U(VI) concentration of 4.4 mmol/L. Results showed that in the presence of bicarbonate, *A. oxydans* G968 was able to enhance the release of U(VI) from Na and Ca autunite at the same capacity as other *A. oxydans* isolates with relatively high tolerance to U(VI). The effect of bacterial strains on autunite dissolution decreases as the concentration of bicarbonate increases. The results illustrate that direct interaction between the bacteria and the mineral is not necessary to result in U(VI) biorelease from autunite. The formation of secondary calcium-phosphate mineral phases on the surface of the mineral during the dissolution can ultimately reduce the natural autunite mineral contact area, which bacterial cells can access. This thereby reduces the concentration of uranium released into the solution. This study provides a better understanding of the interactions between meta-autunite and microbes in conditions mimicking arid and semiarid subsurface environments of western U.S.

Keywords: Autunite mineral, *Arthrobacter sp.*, aqueous bicarbonate, radionuclides, microscopy

1. Introduction

Uranium (VI) is a key contaminant of concern at many former U.S. Departments of Energy nuclear processing facilities and is well known as a health hazard for its toxicity and radioactivity. In neutral or basic pH conditions, uranium undergoes hydrolysis in aqueous solutions and can readily complex with a wide variety of ligands (Grenthe et al., 1992). Common ligands in the environment that form a diverse suite of aqueous uranyl complexes include: hydroxyl, phosphate, carbonate, silicate and organic substances (Lenhart et al., 2000; Davis, 2001). These complexation reactions often result in the formation of mobile aqueous species or precipitation of U-bearing minerals.

Soil properties, groundwater and pore water characteristics have a tremendous effect on uranium speciation and the formation of mineral phases (Sowder et al., 2001). Surveys of the uranium-contaminated areas suggested that carbonate and hydrogen carbonate (known as bicarbonate) are the major ions in the groundwater composition and the pore water is saturated with Ca and Mg-carbonates. Aqueous carbonate is considered one of the major variables affecting the dissolution of actinides from soil and sediments, thus increasing uranium mobility in natural waters (Clark et al., 1995; Zachara et al., 2007). In a bicarbonate-rich environment, carbonate anions are an important complexing agent for U(VI), forming highly soluble and stable uranyl dicarbonate $\text{UO}_2(\text{CO}_3)_2^{2-}$ and uranyl tricarbate $\text{UO}_2(\text{CO}_3)_3^{4-}$ complexes, which became the predominant aqueous species at pH 7.0-8.0 (Langmuir, 1978; Bernhard et al., 2001; Guillaumont et al., 2003; Gorman-Lewis et al., 2008). In the presence of Ca ions, the mobile calcium uranyl carbonate species, $\text{CaUO}_2(\text{CO}_3)_3^{2-}$, influence the speciation of uranium (Clark et al., 1995; Kalmykov and Choppin, 2000; Bernhard et al., 2001).

Uranium readily forms strong and stable complexes with phosphate, promoting the formation of autunite group minerals, $X_{3-n}^{(n)+}[(\text{UO}_2)_2(\text{PO}_4)_2] \cdot x\text{H}_2\text{O}$, that can persist under intense weathering conditions over a long period of time (Giammar D., 2001). One of the remediation strategies to mitigate the mobility of uranium contamination in the subsurface is an injection of sodium tripolyphosphate solutions in water-saturated sediments to form low solubility precipitates, autunite, $\text{Na}[(\text{UO}_2)(\text{PO}_4)] \cdot 3\text{H}_2\text{O}$ (Wellman et al., 2006; Wellman et al., 2007). The autunite mineral group is very diverse and permits the substitution of a wide range of cations and varying degrees of hydration (Burns, 1999). In a calcium-rich soil, typical for arid and semi-arid environments of the western U.S, rapid ion-exchange reactions promote the replacement of sodium for calcium and the formation of calcium-autunite, $\text{Ca}[(\text{UO}_2)(\text{PO}_4)]_2 \cdot 3(\text{H}_2\text{O})$ (Burns, 1999). In these reactions, two sodium cations are exchanged for the calcium ion to maintain the charge balance of the structure; the exchange is also associated with an increase in waters of hydration (Wellman et al., 2005). The solubility constant of the calcium form of autunite, $\log K_{\text{sp}}$, has been measured as -48.36 with 2σ uncertainty values of ± 0.03 (Gorman-Lewis et al., 2009). The associated solubility products are presented in eq. 1.

Autunite is a phosphorus-containing mineral; bacteria can liberate phosphorus, meeting their nutrient requirements and causing U mobilization back into the environment as a result of a breakdown of the mineral structure (Smeaton et al., 2008). The presence of indigenous bacterial populations that rapidly adapt to environmental conditions in soil and sediment can strongly influence the stability of uranyl phosphate phases. These bacteria can affect the dissolution and U(VI) complexation reactions due to the secretion of protons, various ligands and organic acids in their immediate habitats. In addition, 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 (Fredrickson et al., 2004; Beazly et al., 2007). Therefore, understanding the role of bacteria on the stability of uranyl phosphates minerals is very important.

A number of studies have been carried out with a variety of the bacterial species in relation to their interaction with radionuclides in groundwater (Lloyd and Renshaw, 2005; Nedelkova et al., 2007). As previously described, the significance of bacteria-uranium interactions has been illustrated by focusing on three Gram-positive *Arthrobacter* species isolated from Hanford Site, which is the former the United State plutonium producing facilities, located along the Columbia River in eastern Washington State (Katsenovich et al., 2012 b).

Arthrobacter is one of the most common groups of bacteria 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). According to Balkwill et al., (1997), the microbial ecology of vadose zone sediments contaminated with high U(VI) at the U.S. DOE Hanford Site exhibits a high frequency of *Arthrobacter* species. In addition, *Arthrobacter*-like bacteria were the most prevalent in the highly radioactive sediment samples collected underneath leaking high-level waste storage tanks and accounted for about one-third of the total isolatable bacterial population from vadose zone soils (Fredrickson et al., 2004). Furthermore, a prior study conducted using the *A.oxydans* strain G975 illustrated a bio-enhanced release of U(VI) from natural Ca-autunite in the presence of various concentrations of bicarbonate (Katsenovich et al., 2012a). The current research was performed to investigate the effect of *Arthrobacter oxydans* strain G968 obtained from the SMCC on stability of the autunite mineral in carbonate-rich oxidized conditions typical for the

western U.S. This strain was found to possess low resistance to U(VI) toxicity in bicarbonate-free media solutions (Sepúlveda et al., 2013).

The microbial effect of strains that possess low tolerance to U(VI) toxicity on the dissolution of U(VI)-bearing minerals have not been evaluated in publications. Additional research is necessary to understand the effect of bacterial cells with low resistance to U(VI) on the biodissolution of autunite in bicarbonate-amended media solutions mimicking subsurface conditions typical for arid and semi-arid environments of the western U.S. The objective of this research was (i) to evaluate the microbial dissolution of Na and Ca autunite in bicarbonate-amended solutions inoculated with *Arthrobacter oxydans* strain G968, that possesses a low tolerance to U(VI) toxicity, (ii) to compare the results of autunite dissolution with data obtained in previous tests using uranium-resistant subsurface isolate *Arthrobacter oxydans* strain G975, and (iii) to determine whether bacterial tolerance to uranium can affect autunite biodissolution in the presence of bicarbonate.

2. Materials and Methods

2.1 Arthrobacter Strains and Culture Conditions

Environmental sources and isolation of the Gram-positive aerobic *Arthrobacter oxydans* strain G968 used for the research was described in recent publications (Katsenovich et al., 2012a; Katsenovich et al., 2012 b). Identification of the *A. oxydans* strains was previously confirmed by 16S rDNA phylogeny (Balkwill et al., 1997; Van Waasbergen et al., 2000) and detailed descriptions of sample collection procedures and methods of bacterial isolation are available from Van Waasbergen et al. (2000). The bacterial culture was grown in 5% peptone, tryptone, yeast extract, glucose (PTYG) 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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.07 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The media was prepared in deionized water (DIW) (Barnstead NANOpure Diamond Life Science (UV/UF), Thermo Scientific), autoclaved at 121°C and 15 psi for 15 minutes, then allowed to cool before being used. Bacterial cells were harvested in the late logarithmic phase of growth and washed; the cell stock suspensions were counted using a glass hemocytometer (Fisher Scientific, Pittsburg, PA) or INCYTO C-Chip disposable hemocytometer (SKC America). 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. The number of cells/mL in the stock suspension was used to calculate the desired volume of cell solution to inoculate the experimental bottles.

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°C. 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.

2.2 Uranium (VI) release from Autunite mineral

Two different kinds of bioreactors were used in the bioleaching experiments: (1) Sterile 100 mL serum bottles were used as mixed bioreactors for initial experimentation. These autunite-containing bioreactors were inoculated with the desired volume of bacterial cell solution after the autunite equilibrated with the bicarbonate-amended media solutions and the dissolution of autunite was close to its solubility. (2) Sterile 6-well cell culture plates with inserts (BD

Falcon™) were used to keep bacteria and autunite separated during non-contact dissolution experiments.

2.2.1 Bicarbonate Media Solution Preparation

Autunite dissolution experiments were performed in a medium that was similar to the growth media, but did not contain 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°C, 15 psi for 15 minutes, and cooled to about 30°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 KHCO₃. Potassium bicarbonate was used to prevent interference with Na measurements released from Na-autunite during dissolution of the mineral. The media to conduct the autunite dissolution experiments was adjusted to pH 7.5 with 0.1M HCl or NaOH and buffered with 0.02 M 2-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid sodium salt hydrate (HEPES-Na) buffer. At this pH, the major fraction of dissolved inorganic carbon is present in the form of HCO₃⁻. Each of the four individual bicarbonate media solutions were filter-sterilized (0.2 µm) and kept refrigerated until the time of use.

2.2.2 Autunite biodissolution in mixed bioreactors

Natural Ca meta-autunite, Ca[(UO₂)(PO₄)]₂·3H₂O 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 et al., 2006). The autunite sample was powdered and sieved to have a size fraction of 75 to 150 µm or -100 to +200 mesh with a surface area of 0.88 m²/g as determined by Kr-adsorption BET analysis (Wellman et al., 2006).

Synthesis of Na-autunite, $\text{Na}[(\text{UO}_2)(\text{PO}_4)] \cdot 3\text{H}_2\text{O}$, was followed by a modified direct precipitation method described by several authors (Wellman et al., 2005, Gudavalli et al., 2013a).

In order to test if there is a difference in the microbial dissolution of synthetic and natural autunite in the presence of bicarbonate, a biodissolution experiment in two different kinds of bioreactors was conducted. Synthetic and natural autunite microbial biodissolution experiments were conducted similarly using 100-mL foam-stoppered serum bottles prepared in triplicate for each bicarbonate concentration tested. Each bottle contained 50 mL of sterile bicarbonate-bearing 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 U-tolerant *Arthrobacter* strain G975 (Katsenovich et al., 2012a). The suspensions were slightly agitated at 60-rpm in an incubator/shaker at 25°C. Autunite and bicarbonate solutions were allowed to equilibrate with the solution to the point where the concentration of uranium reaches a plateau. *Arthrobacter* strain G968 cells at a cell density of 10^6 cells/mL were injected into the reactors after 27 days. A similar experimental approach to investigate biodissolution of U-bearing minerals in the presence of a dissimilatory metal-reducing bacterium (DMRB), *Shewanella oneidensis*, was discussed by Liu et al., (2006). Abiotic non-carbonate controls were used during testing. After addition of bacteria, the experiment was continued for 20 days.

2.2.3 Autunite biodissolution in culture ware with inserts

To investigate whether direct contact 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 (BD Falcon™) were used in the

non-contact biodissolution experiments where natural Ca-meta-autunite and bacterial cells were incubated separately. The culture plates inserts have 0.4 μm cylindrical pores that transverse the permeable membrane and only allow the diffusion of small molecules, including soluble uranium and metabolites from *Arthrobacter* strain G968. Ten (10) mg of autunite powder was weighed on paper discs, covered with aluminum foil and then autoclaved at 121°C and 15 psi for 15 minutes. The sterile paper discs with autunite were carefully inserted with a pincette on the bottom of the wells and then insert receptacles were positioned in each corresponding well. Subsequently, the wells were filled with a sterile bicarbonate media solution prepared to be similar to those made for the serum bottle reactors to provide a U(VI) concentration of 4.4 mmol/L (Katsenovich et al., 2012 a). The total volume of the sterile media solution added to each individual well nested with a culture insert receptacle was 5.7 mL . Only the well inserts equipped with 0.45 μm pore size membranes were inoculated with 10^6 cells/mL; this allowed culturing bacterial cells separately from autunite solids. Abiotic controls were prepared to make a comparison with bacteria-bearing wells. Uranium release from autunite was established by taking a 20 μL sample from the inserts and processing on the kinetic phosphorescence analyzer (KPA), as described in greater detail in the next section. The solution volume withdrawn from each well was less than 2.5% of the total solution volume during the entire experiment.

2.2.4 Analytical procedures

Before bacteria inoculation, samples were taken twice a week; after inoculation, samples were taken daily. 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 total solution volume removed from each bottle was less than 5 mL (<10% of total solution volume) during the entire experiment.

The dilution factors for analysis were 100x for samples prepared with low concentrations of bicarbonate (0-3 mM), and 200x for samples prepared with higher concentrations of bicarbonate (5-10 mM). 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 (Gudavalli et al., 2013b). Wet digestion was continued until a dry white precipitate formed, and then dry ashing was performed in the furnace at 450°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 (HNO₃). Aqueous concentrations of sodium, 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. To examine the results that have numerous experimental groups and independent factors, such as studying the effect of U(VI) uptake due to varying concentrations of uranium (VI) in the presence of bicarbonate ions, ANOVA statistics were used. All statistical tests were investigated using a predictive analytical software, SPSS (IBM, Armonk, NY), with significant levels set at $\alpha = 0.05$.

2.2.5 SEM/EDS Analysis of samples

The purpose of scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM/EDS) analysis was to characterize the microstructure of autunite solid phases and bacterial 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 from Fratesi, et al., (2004) and Araujo, et al. (2003). At the end of the experiment samples from bioreactors were transferred to a centrifuge tube and washed twice with deionized water after centrifugation at 4000 rpm for 5 min. Bacteria in the samples were then fixed by treatment with 2%

glutaraldehyde in 0.1M HEPES buffer at pH 7.2 for 2h at 4°C and subsequently 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 microstructure of gold-coated samples (PAC-1 PELCO Advanced coater 9500) was analyzed using a SEM-Energy-Dispersive-Spectrometry (SEM-EDS) JEOL System Model 5900LV at 15kV. The elemental composition was determined via a Noran System Six Model 200SEM Energy Dispersive X-Ray Spectroscopy (EDS).

3. Results and Discussion

3.1 Release of U (VI) from Natural Autunite

The release of aqueous U (VI) over time during the autunite dissolution experiments is presented in Figure 1A, the red arrow indicates when the bioreactors were inoculated with *A. oxygens* strain G968. U(VI) concentrations in the abiotic control (a sample that did not contain bicarbonate or bacteria) reached a steady state over a period of 27 days with an average value of 0.46 ± 0.11 μ M. In bicarbonate-amended reactors, U(VI) release from autunite was strongly enhanced. Prior to strain inoculation, U(VI) concentrations were measured at 0.55 ± 0.12 μ M, 1.07 ± 0.34 μ M, and 44.0 ± 7.16 μ M 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 bacterial

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) released out into the solution driven by the presence of bacteria, the effect of bacteria on autunite dissolution is reduced as the concentration of $[\text{HCO}_3^-]$ increases. Since the steady-state U(VI) concentration is higher for larger $[\text{HCO}_3^-]$, the increase in soluble U(VI) concentration induced by bacteria is hindered. This was expected since the uranium release from autunite is strongly influenced by the increase in bicarbonate concentrations leading to the formation of aqueous uranyl bicarbonate complexes (Gudavalli et al, 2013a, 2013b). It was previously determined that uranium release from autunite minerals is a surface-mediated reaction with the uranium polyhedrons (Wellman et al., 2006; 2007). Bicarbonate can promote mineral dissolution by fast binding to the autunite surface, followed by slow detachment of the U(VI) carbonate species from the surface into solution (Sparks, 1999; Gudavalli et al, 2013a). The formation constants of these ternary uranyl carbonate complexes are higher than for the complexes built between uranyl ion and bacterial surface components (Gorman – Lewis et al. 2005; Haas et al. 2001). Results showed that the steady-state U(VI) concentration increases as a function of increasing bicarbonate, but the increase in soluble U(VI) concentration induced by bacteria is minimized in comparison. Therefore, as $[\text{HCO}_3^-]$ increases, a diminishing trend on the effect of bacteria on autunite dissolution was observed; a similar effect was reported in the autunite dissolution experiments using the A.G975 strain (Katsenovich et al, 2102a).

275 The liberation of uranyl ions influences incongruent reactions to release phosphorus and sodium
276 from autunite. Figure 1B illustrates the aqueous phosphorus release as a function of time from
277 the natural autunite dissolution experiments inoculated with *Arthrobacter* strain G968. This
278 figure also shows results for two controls, an abiotic control that contains no bicarbonate and no
279 bacteria, and a biotic control, containing bacteria with no bicarbonate. During the autunite
280 dissolution period without bacteria, phosphorus (P) concentrations increased compared to the
281 non-carbonate abiotic control. After inoculation, P concentrations in the biotic reactors were
282 found to decrease for all studied bicarbonate concentrations. This was correlated with the
283 exponential growth phase of bacteria that were seeded into the reactors. P is one of the major
284 essential macronutrients for biological growth, playing a central role in the cellular activities for
285 the synthesis of DNA, ATP, polyphosphates, and cell wall phospholipids. Within 5 days after
286 inoculation, the total bacterial density showed a significant increase to log 8.77cell/mL and log
287 8.94cell/mL for the bicarbonate-free and reactors amended with 10 mM of HCO_3^- , respectively.
288 The viability of cells was slightly higher for the bicarbonate-amended reactors, 92-99%,
289 compared to bicarbonate-free reactors with values ranging between 86.5-94%. It is known that
290 microorganisms are capable of solubilising phosphate by accessing insoluble phosphate-bearing
291 minerals through microbial dissolution (Vazques, et al. 2000; Goldstein, 1986). The primary
292 mechanisms of P solubilization are H^+ excretion, production of low molecular weight organic
293 acid, and acid phosphatase biosynthesis (Arcand and Schneider, 2006; Behera et al, 2014). As
294 microbial growth consumes phosphorus as a nutrient, there is also likely a reduction in soluble P
295 content in the media solution caused by the formation of insoluble secondary calcium phosphate
296 phases (Figure 1B). Their formation was predicted by speciation modeling via Visual MINTEQ
297 software [v.3.0, maintained by J.Gustafsson at KTH, Sweden, available at

<http://www.lwr.kth.se/English/OurSoftware/vminteq/> updated with the Nuclear Energy Agency's thermodynamic database for uranium (Guillaumont et al. 2003)] (Table 1). The calcium phosphate phases precipitated from the media solution and accumulated in the batch reactors. After bacteria inoculation, the average concentration of phosphorus in the solutions were measured as 4.3 ± 0.66 mM, 7.19 ± 1.75 mM, 6.74 ± 0.85 mM, and 8.57 ± 0.92 mM for the tested 0 mM, 3 mM, 5 mM and 10 mM, respectively. Similar to phosphorus, aqueous Ca release over the period of autunite dissolution without bacteria was noted to increase as a function of bicarbonate concentrations (Figure 1C). Ca concentrations after bacterial inoculation averaged 2.8 ± 0.6 mM, 2.99 ± 1.16 mM, 3.01 ± 0.24 mM, and 3.34 ± 0.34 mM for the tested bicarbonate concentrations, respectively. The data indicate that release of U, P and Ca from Ca-autunite was non-stoichiometric.

The *Arthrobacter* species has the potential of becoming an important component in the dissolution process. Their activities can contribute to the release of uranium via the dissolution of autunite minerals and might result in the formation of secondary calcium phosphate (apatite) phases. The high 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 western U.S., the Ca-autunite mineral has a high liability to dissolution in the presence of bacteria.

3.1.2 U(VI) Release from Natural Autunite Mineral in Sterile Culture Plates with Inserts

Dissolution experiments in which the natural autunite mineral was separated from the bacteria were conducted using sterile culture plates with inserts. The release of aqueous U (VI) over time in non-contact mode is represented in Figure 2. 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 bacterial inoculation, U (VI) concentrations measured in the reactors increased 3.96 ± 1.17 , 2.79 ± 1.62 , and 2.82 ± 3.75 fold for 0 mM, 3 mM, and 5 mM bicarbonate, respectively.

There is a slight increase in the release of uranium as bicarbonate increases; however, this is not as pronounced when compared to the biodissolution experiment in mixed reactors, where bacteria and mineral have direct contact. Statistical analysis indicated strong evidence that the means of uranium concentrations varied among different levels of bicarbonate concentrations ($P < 0.05$). These measures were not optimum conditions for bacterial growth and proliferation. However, results do illustrate that direct interaction between the bacteria and mineral is not necessary to result in U(VI) release from autunite. It was not measured but it is likely that the release of U from autunite in the non-contact experiments was affected by the organic substances produced by bacteria during aerobic metabolism.

3.1.3 U (VI) Release from Synthetic Autunite

The release of aqueous U(VI) over time during the synthetic autunite dissolution experiment is presented in Figure 3A. The bioreactors were inoculated with *Arthrobacter* strain G968 cells after 27 days, giving time for the synthetic autunite to reach steady state with the bicarbonate solutions. The steady state maximum concentrations of U(VI) detected were 2.09, 8.83, 52.3, and 88.4 fold higher than the abiotic control without the bicarbonate amendment. This correlates with the observed increase in the uranium with the rising concentration of bicarbonate solutions, which is explained by the increase of the CO_3^{2-} species in aqueous solutions and the formation of strong and highly soluble U(VI)-carbonate complexes (Gudavalli et al. 2013a). Furthermore, after bacterial 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 0 mM, 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 Figure 3A, 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 in the solution. Similar to previous experimentation conducted with Ca-autunite, as $[\text{HCO}_3^-]$ increases, a diminishing trend on the effect of bacteria on autunite leaching was observed; however, in this case detected U(VI) concentrations were slightly higher.

Phosphorus (P) concentrations in the biotic reactors were found to increase slightly for all studied bicarbonate concentrations (Figure 3B). After bacteria inoculation, the average concentrations of phosphorus in the supernatant solutions were measured as 1.55 ± 0.29 mM, 1.51 ± 0.24 mM, 1.72 ± 0.25 mM, 2.04 ± 0.18 mM for the tested 0 mM, 3 mM, 5 mM and 10 mM, respectively. As suggested by speciation modeling, the dissolution of synthetic Na-autunite doesn't result in the formation of phosphorus-bearing solid phases, and HPO_4^{2-} anions remain in the media solution. Similar to phosphorus concentrations, after bacterial inoculation, sodium concentrations in the biotic reactors were found to increase for all studied bicarbonate concentrations (Figure 3C). Sodium concentrations in the bioreactor followed a similar trend compared to U(VI) and phosphorus concentrations and, by the end of the experiments, increased in average about 10% compared to pre-inoculation levels. Data indicate that the release of elements from synthetic autunite is not stoichiometric.

3.1.4 Comparing Biodissolution of Natural autunite with Synthetic autunite

One of the objectives of the study was to investigate if *Arthrobacter* strain G968 influences uranium release through biodissolution. There is a question, however, if there was a difference between the microbial effect on the dissolution of synthetic autunite and natural autunite in the

presence of bicarbonate. A previous study comparing the kinetic rate law parameters for abiotic dissolution of natural and synthetic autunite in the presence of bicarbonate solutions (Gudavalli et al., 2013b) concluded that the uranium release from natural autunite is higher than from synthetic autunite. Major factors that contribute to a higher uranium release from natural autunite are the formation of uranyl-carbonate, $\text{UO}_2(\text{CO}_3)_3^{4-}$, and calcium-uranyl-carbonate species, $\text{CaUO}_2(\text{CO}_3)_3^{2-}$. These complexes act as a driving force for uranyl release from mineral surfaces. Furthermore, natural autunite also forms secondary Ca-P hydroxyapatite and uranyl phosphate minerals, which also can serve as a driving force for the release of phosphate and calcium. However, the opposite phenomenon was noted in the experiments conducted for this study, where the release of U(VI) from synthetic autunite was slightly higher than U(VI) concentrations obtained in the experiments with natural autunite (Figure 1A and Figure 3A). Several factors could have contributed to such conflicting results. Gudavalli et al. (2013b) ran autunite dissolution experiments via a single pass flow through (SPFT) apparatus designed to limit the accumulation of reaction products in the reactor. This experimentation was conducted in batch bioreactors that were agitated at 100 rpm in the incubator-shaker over more than 30 days. This leaves the possibility of the accumulation or precipitation in the bioreactors of secondary solid phases predicted by speciation modeling (Table 1 and Table 2). The formation of secondary uranyl-phosphate and calcium-phosphate mineral phases on the surface of the mineral can ultimately reduce the natural Ca-autunite mineral contact area, which bacterial cells can access. It thereby reduces the concentration of uranium released into the solution and detectable via the KPA instrument. The dissolution of synthetic Na-autunite produces only uranyl-phosphate secondary phases.

3.1.5 The effect of bacterial tolerance to uranium on autunite biodissolution

In an assessment of the resistance of Hanford Site *Arthrobacter* isolates to uranium (VI) exposure (Katsenovich et al., 2012 b), the *Arthrobacter* G968 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). The increase in U(VI) concentration in the media solution highly inhibited G968 cell viability and no growth was noted on petri dishes after cell incubation for 24 h in the media amended with 9.5 ppm of U(VI) (Katsenovich et al., 2012 b). In contrast, a series of tests illustrated that *Arthrobacter* G975 exhibited a higher tolerance towards U(VI) relative to G968 and remained viable in the presence of 9.5- 19 ppm of U(VI) (Katsenovich et al., 2012 b). In order to see the changes on the bacterial surface at the nanoscale, AFM analysis was performed and results showed an unusually irregular and wrinkled surface on the G975 bacterial cell. Although precipitate was present on both *Arthrobacter* strains, the amount aggregated on the irregular surface of G975 surpassed the G968. The surface morphology of G975 strain supports a higher accessibility for the formation of the uranium precipitates. Experiments suggested that despite differences in surface morphology and tolerance to U(VI) between the two bacterial strains, G975 and G968, uranium biorelease from autunite minerals in the presence of bicarbonate ions in both cases was noted at the same capacity. The effect of both bacterial strains on autunite biodissolution decreases as the concentration of bicarbonate increases while the increase in soluble U (VI) concentration induced by bacterial strains is diminished, for larger $[\text{HCO}_3^-]$ (Katsenovich et al, 2012a).

3.2 Imaging Analyses

The main goal of microscopy analysis was to qualitatively characterize autunite mineral surface composition and evaluate changes in the surface morphology in bicarbonate- bearing media

412 inoculated with bacteria *Arthrobacter* strain G968. Figure 4 illustrates the bacterial attachment to
413 the surface of the radioactive natural autunite mineral. After the reactors are inoculated, the cells
414 are present in the liquid media as a suspension. Bacteria colonization of solid surfaces leads to
415 the cells adhesion when bacteria are deposited on the solid surface via special cell surface
416 structures, fibrils, forming links between the cell and the solid surface (van Loosdrecht et al.,
417 1990). As a result of the breakdown of media organic compounds, bacterial cells release protons,
418 organic acids and metabolites into the media solution (Francis, 2000; Knox, et al. 2008). The
419 literature data on the influence of solid surfaces on microbial behavior demonstrate that the
420 proton concentration at the surface is different from the bulk solution. The presence of a solid
421 surface would hinder diffusion of protons away from the bacterium, and as a consequence,
422 adsorbed cells are surrounded by a higher concentration of hydrogen ions as compared with the
423 bulk liquid (Ellwood et al., 1982; Hattori and Hattori, 1963). As previously described, bacteria
424 may lower the pH in the vicinity of the solid surface, thereby promoting release of uranium from
425 the mineral surface. This is consistent with previous research that uranium release from autunite
426 mineral is increased as the pH decreases (Wellman et al., 2007). The bacterial activities coupled
427 with U(VI) complexation with bicarbonate contribute to the biodissolution of autunite minerals.
428 SEM/EDS analysis of secondary minerals and biofilm created by *Arthrobacter* strain G968 on
429 natural autunite surface help to distinguish between the elemental composition of the autunite
430 surface and secondary minerals that may have been formed throughout the experiment. The
431 atomic percentage of uranium on the autunite surface is higher, detected at around 50% (Figure
432 5A); whereas, the atomic percentage of uranium on the bacterial surface is much less, at around
433 0.9-1.7% (Figure 5B). The composition of bacterial cell surfaces also features an elevated
434 content of carbon and nitrogen ranging between 67-71% and 9.4-12.9%, respectively. Further

observations using microscopy revealed what would occur to the autunite mineral surface when exposed to bicarbonate- bearing media inoculated with bacterial cells of A.G968. Figure 5A illustrates this phenomenon, showing an accumulation of secondary phases that covers the surface of the mineral, obstructing active sites. Some bacteria appeared to be encrusted in secondary minerals, showing slightly lower weight percentages of carbon, phosphorus and nitrogen as 56-63%, 1.3-1.7%, and 6.9-7.9%, respectively, compared to the composition of the bacterial surface. Based on the obtained results, we can hypothesize that a mixture of secondary phases encrusted with bacteria might reduce bacterial access to active sites on the mineral. Alternatively, uranium released during mineral dissolution might not diffuse through the mixture of secondary phases and encrusted bacteria. This study provides a better understanding of the role of indigenous soil bacteria on the stability of autunite precipitates created as a result of the tripolyphosphate remediation technology and investigates the microbial effect on the uranium release from autunite solid phases in bicarbonate rich environments. It can be concluded that the low uranium tolerant strain, *Arthrobacter* strain G968 can accelerate the release of uranium from autunite in the presence of bicarbonate through biodissolution of both natural Ca-autunite and synthetic Na-autunite, in conditions mimicking arid and semiarid subsurface environments of the western U.S. The results of the study provide important insights into key biochemical factors affecting the environmental fate and transport of uranium.

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Figure 1. A) Changes for aqueous U (VI) concentrations as a function of time for the natural autunite dissolution experiments inoculated with *Arthrobacter* G968 strain (n=3). B) Aqueous phosphorus release as a function of time from the natural autunite dissolution experiments inoculated with *Arthrobacter* G968 strain. C) Aqueous Ca release as a function of time for the biotic reactors inoculated with *Arthrobacter* G968 strain. Red arrow indicates time of bioreactors inoculation with G968.

Figure 2. Changes for aqueous U (VI) as a function of time for the non-contact natural autunite dissolution insert experiments inoculated with *Arthrobacter* G968 strain. Red arrow indicates time of bioreactors inoculation with G968.

Figure 3. A) Changes for aqueous U (VI) as a function of time for the synthetic autunite dissolution experiments inoculated with *Arthrobacter* G968 strain. Red arrow indicates time of bioreactors inoculation with G968. B) Aqueous phosphorus release as a function of time from the synthetic autunite dissolution experiments inoculated with *Arthrobacter* G968 strain. C) Aqueous Na release as a function of time from the synthetic autunite dissolution experiments inoculated with *Arthrobacter* G968 strain. Red arrow indicates time of bioreactors inoculation with G968.

Figure 4. SEM images of G968 grown on autunite surface in the presence of HCO_3^- . Clearly illustrating individual bacteria are deposited on the solid surface via special cell surface structures, fibrils; A) Natural autunite, 3mM HCO_3^- . B) Synthetic autunite, 10mM HCO_3^- white arrow shows G968 cells attached to the surface.

Figure 5. SEM/EDS analysis of secondary minerals and biofilm created by G968 strain on natural autunite surface and compositional analysis (% weight) for each point at A) 5 mM HCO_3^- ; B) 10 mM HCO_3^-

Point number	Weight, %							
	<i>C</i>	<i>N</i>	<i>O</i>	<i>Na</i>	<i>P</i>	<i>K</i>	<i>Ca</i>	<i>U</i>
<i>pt1</i> (mineral surface)	20.4	2.7	18.0	0.00	5.9	2.5	0.2	50.2
<i>pt2</i> (biofilm/secondary minerals)	62.8	6.9	14.7	0.1	1.3	1.0	0.02	13.2
<i>pt3</i> (biofilm/secondary minerals)	56.5	7.9	16.6	0.1	1.7	0.6	0.05	16.6

Point number	Weight, %							
	<i>C</i>	<i>N</i>	<i>O</i>	<i>Na</i>	<i>P</i>	<i>K</i>	<i>Ca</i>	<i>U</i>
<i>pt1</i> (mineral surface)	24.1	3.9	29.4	0.08	4.3	0.9	1.9	35.4
<i>pt2</i> (mineral surface)	25.2	2.8	29.6	0.2	4.2	1.3	1.8	35.0
<i>pt3</i> (bacterial surface)	70.9	9.4	17.5	0.1	0.1	0.2	0.03	1.7
<i>pt4</i> (bacterial surface)	67.3	12.9	18.3	0.1	0.2	0.2	0.09	0.9

1. Low-tolerant G968 *Arthrobacter* strain accelerates U(VI) release from autunite in the presence of $[\text{HCO}_3^-]$
2. The effect of bacteria on autunite dissolution is reduced as the concentration of $[\text{HCO}_3^-]$ increases
3. The U(VI) release from natural Ca-autunite is lower than from synthetic Na- autunite
4. A direct interaction between bacteria and mineral is not necessary to result in U(VI) release from autunite