Investigation on Microbial Dissolution of Uranium (VI) from Autunite Mineral – 13421

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ABSTRACT

Precipitating autunite minerals by polyphosphate injection was identified as a feasible remediation strategy for sequestering uranium in contaminated groundwater and soil *in situ* at the Hanford Site. Autunite stability under vadose and saturated zone environmental conditions can help to determine the long-term effectiveness of this remediation strategy. 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. Ubiquitous in subsurface microbial communities, these bacteria can play a significant role in the dissolution of minerals and the formation of secondary minerals. The main objective of this investigation was to study the bacterial interactions under oxidizing conditions with uranium (VI); study the potential role of bicarbonate, which is an integral complexing ligand for U(VI) and a major ion in groundwater compositions; and present data from autunite dissolution experiments using *Arthrobacter* strain G968, a less U(VI)-tolerant strain. Sterile 100 mL glass mixed reactors served as the major bioreactor for initial experimentation. These autunite-containing bioreactors were injected with bacterial cells after the autunite equilibrated with the media solution amended with 0 mM, 3 mM 5 mM and 10 mM concentrations of bicarbonate. 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. Abiotic non-carbonate controls were kept without bacterial inoculation to provide a control for the biotic samples. Samples of the solution were analyzed for dissolved U(VI) by means of kinetic phosphorescence analyzer KPA-11 (Chemcheck Instruments, Richland, WA). Analysis showed that as [HCO_3^-] increases, a diminishing trend on the effect of bacteria on autunite leaching is observed. Viability of cells was conducted after 24 hours of cell incubation with the appropriate uranium and bicarbonate concentration treatment. As expected, the cells started to reduce after day 41 due to the nutritional exhaustion of the media. Moreover, viable bacteria accounted for more than 94% in the presence of 10 mM bicarbonate. Experiments showed that despite differences between the G975 and the G968 bacterial strains resistance to U(VI), in the presence of bicarbonate ions they are able to dissolute uranium from autunite mineral at the same capacity. The effect of both bacterial strains on autunite dissolution is reduced 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^-].

INTRODUCTION

The formation of uranyl phosphate complexes in the soil and groundwater has the ability to control actinide behavior due to their low solubility under pH levels between 6.5 and 7.5 [1]. Autunite and meta-autunite minerals $\{X^{n+}_{3-n}[(UO_2)(PO_4)]_2\, xH_2O\}$ 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 [2]. This structure produces the tabular habit, the one perfect direction of cleavage. Autunite minerals 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 [3]. Autunite stability under vadose and saturated zone environmental conditions can help to determine the long-term effectiveness of this remediation strategy [4].

Aqueous carbonate present in the soil and ground water is a primary species increasing uranium mobility by affecting the dissolution of actinide and promoting the uranium desorption reaction from soil [5]. 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 [5, 6]. 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 et al, 2012b). 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 [7, 8, 9, and 10]. Balkwill et al. [8] 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 [11]. 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) [12]. This bacterial strain is ubiquitous in subsurface microbial communities and can play a significant role in the dissolution of minerals and the formation of secondary minerals [12]. This research was extended to investigate the stability of autunite mineral in oxidized conditions pertaining to 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 the 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 [13].

MATERIALS AND METHODS

Arthrobacter Strains and Growth Culture Conditions

The *Arthrobacter* 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 $CaCl_2.2H_2O$. 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 to about 30^oC 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^{o}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. 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. Having known 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.

Dissolution of U (VI) from Autunite

Sterile 100 mL glass mixed reactors served as the major bioreactor for initial experimentation. These autunite-containing bioreactors were injected with bacterial cells after the autunite equilibrated with the media solution.

The media solution to conduct the autunite dissolution experiments was prepared using 0.25 g/L peptone, 0.25 g/L tryptone, 0.5 g/L glucose, 0.6 g/L $MgSO_4$, and 0.07 g/L $CaCl_2{\cdot}2H_2O$. Due to the high phosphorus content, yeast extract was not included in the media. Media was prepared in deionized water (DIW), autoclaved following the same procedures as *Arthrobacter* strains culturing media. After sterilization, the media was equally distributed between four 200-mL bottles and separately adjusted 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 time of use.

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 [14]. 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 determined by Kr-adsorption BET analysis (Wellman et al. 2006). Autunite microbial bioleaching experiments were conducted 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. The suspensions were slightly agitated at 60-rpm in an incubator/shaker at 25 ^{o}C. G968 *Arthrobacter* cells in the amount of 10^6 cells/mL were injected into the reactors after 27 days, giving time for the autunite dissolution to reach steady state. During the inoculation, reactors kept their sterile foam stoppers to sustain aerobic conditions within the reactors. Abiotic carbonate-bearing controls were kept without bacterial inoculation to compare with biotic samples.

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 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. 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 down at room temperature followed by the dissolution of the precipitate by the addition of 1 M nitric acid (HNO_3). Uranium calibration standards (SPEX certiPrep), blanks and check standards (95-105% recovery) were analyzed for quality control.

Statistical Analysis

The biodissolution experiment was conducted in triplicate to obtain descriptive statistical analyses such as mean, standard deviation and confidence interval of the mean. Uranium calibration standards (SPEX certiPrep), blanks, and check standards (95-105% recovery) were analyzed for quality control before each experiment utilizing the KPA to check on variability of the machine.

RESULTS AND DISCUSSION

Dissolution of U(VI) from 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 did not contain bicarbonate equilibrated over a period of 27 days and reached an average of 0.46± 0.1137 μm. In bicarbonate-amended reactors, U(VI) release from autunite was strongly enhanced. Prior to strain inoculation, U(VI) concentrations measured at 0.5532±0.1231 μm, 1.0715±0.3371 μm, and 43.9839±7.1645 μm for 3 mM, 5 mM and 10 mM $KHCO_3$, respectively. The uranyl release from autunite in the studied bicarbonate concentrations range prior to G968 inoculation was increased by a factor of 1.2±1.08, 2.3±2.96, and 95.6±63.00 compared to the no-bicarbonate control, respectively. After bacteria inoculation, U(VI) measured in the reactors increased 8.9±23.34, 101.0±108.97, 72.1±123.86, and 2.3±8.65 fold, respectively, compared to the corresponding bicarbonate-bearing controls at steady state. 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. 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.

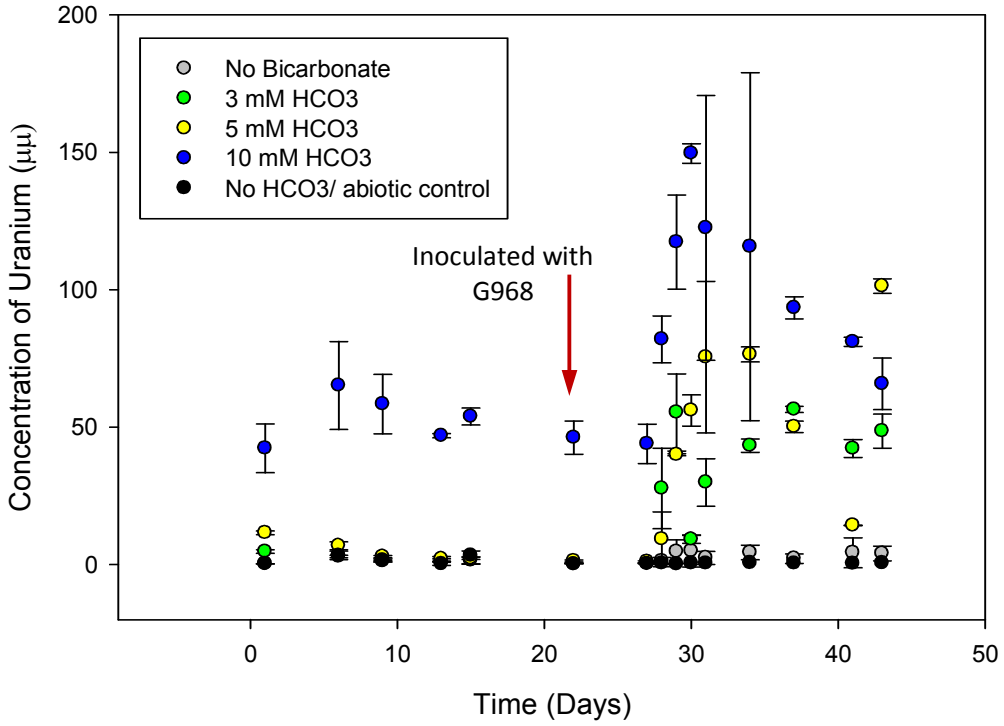

Figure 1. Changes for aqueous U(VI) as a function of time for the natural autunite dissolution experiments inoculated with *Arthrobacter* G968 strain.

Comparison

In an assessment of the resistance of Hanford Site *Arthrobacter* isolates to uranium (VI) exposure, the *Arthrobacter* G975 bacterial strain proved to remain viable in the presence of 0.5 ppm of U(VI) [12]. 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 2012b). After the cells were exposed to various concentrations of uranium, changes on the bacterial surface at the nanoscale level were evaluated via an atomic force microscopy (AFM). As illustrated below, the uranium treated samples contain clear crystalline deposits on the cell surface (Figure 3, Figure 5). Figure 2 and Figure 4 present the deposits-clear bacterial surfaces of the control samples.

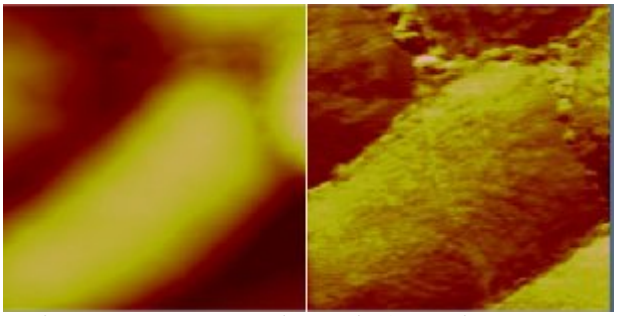

Figure 2. G968 control sample (scan size 10 × 10 µm2). Phase image clearly shows no precipitation on the cell surface.

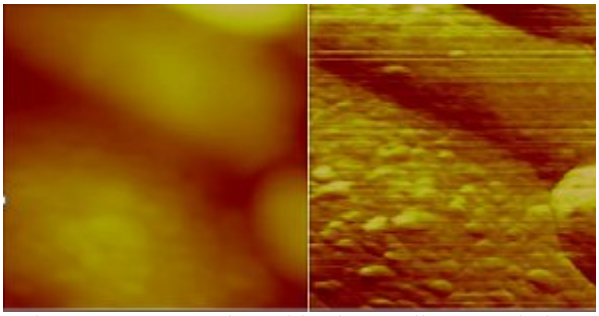

Figure 3. G968 cultured in the media amended with 1ppm of U(VI) (scan size 1.96 × 1.96 µm2). Phase image clearly shows crystalline deposits on the cell surface, which can also be visualized in height image on the left.

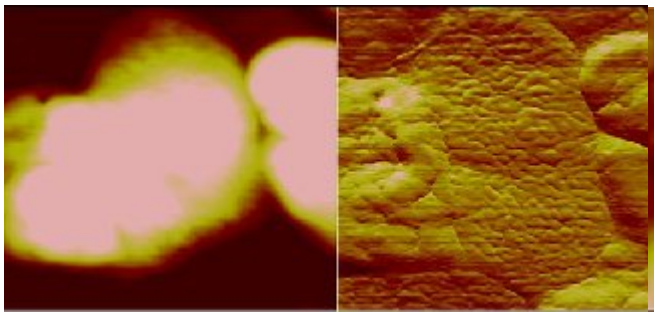

Figure 4. G975 control sample (scan size 2.94 × 2.94 µm2) showing its unusual irregular surface morphology on the right. The topography image on the left (Z range 100 nm) and frictional image (0.3 V) on the right.

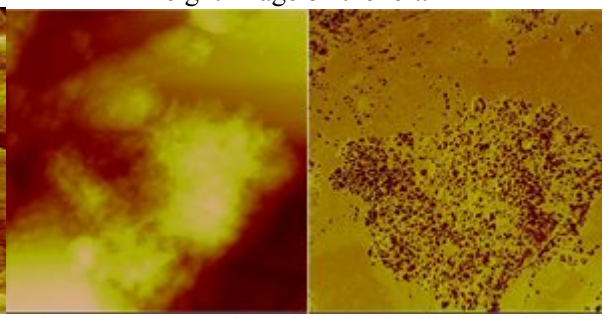

Figure 5. G975 cultured in the media amended with 20ppm of U(VI), (scan size 1.57 × 1.57µm^2). Phase image on the right clearly shows crystalline deposition on the cell surface.

Although precipitate was present on all *Arthrobacter* strains, the amount aggregated on the unusually wrinkled surface of G975 surpassed the rest, which is presented in

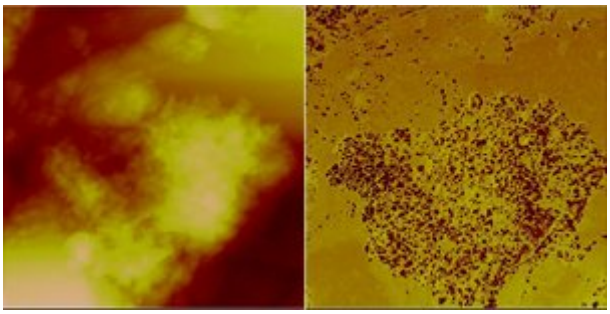

Figure 5. The surface morphology of this strain supports a higher accessibility for the formation of the uranium precipitates.

Despite these morphological differences between the two bacterial strains, they are able to dissolute uranium at the same capacity. The effect of both bacterial strains 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^-].

Cell Viability

The viability of cells was assessed after 24 hours of cell incubation with the appropriate uranium and bicarbonate concentration treatment. Samples of the smallest and largest concentration of bicarbonate were plated and incubated on 5% PTYG hard media and then counted for colony-forming unites (CFU). The number of cells was doubled in the sample containing 10 mM bicarbonate compared to 0 mM bicarbonate. As expected, the cells started to reduce after day 41 due to the nutritional exhaustion of the media. Moreover, viable bacteria accounted for more than 94% in the presence of 10 mM bicarbonate.

CONCLUSION

The effect of bicarbonate on the autunite mineral microbial leaching experiments was evaluated in mixed reactors comprised of autunite powder and media solution. The *Arthrobacter* G968 strain was used in the experiments. The uranium release from autunite prior to the *Arthrobacter* G968 strain inoculation in mixed reactors was increased by a factor between 1.2±1.08 and 95.6±63.00 compared to the no-bicarbonate control. After bacteria inoculation, U(VI) measured in the reactors increased 8.9±23.34 to 2.3±8.65 fold when compared to the corresponding bicarbonate-bearing controls at a steady state. A diminishing trend on the effect of bacteria on autunite leaching was observed as bicarbonate concentrations were increased in the solution. In the future, a Kirby-Bauer Disk-Diffusion technique will be applied to determine the susceptibility of the G968 strain to different concentrations of uranium in sterile Petri dishes. Other future work includes conducting an autunite bioleaching experiment in culture ware with inserts to investigate how the autunite mineral reacts with the bacteria separated from it, and to perform an AFM microscopy analysis on the strain to test the changes in cell surface morphology under the effect of bicarbonate.

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