FOR PUBLICATION**The effect of uranium on ~~the~~ bacterial viability and cell surface morphology
using atomic force microscopy in the presence of bicarbonate ions**

Paola Sepulveda-Medina ^{a, b}, Yelena Katsenovich^{*a}, Vishal Musaramthota^a, Michelle Lee^c,

Brady Lee^c, Rupak Dua^b, Leonel Lagos ^a

^a*Applied Research Center and ^bDepartment of Biomedical Engineering, Florida International University,
10555 W. Flagler Street, Miami, FL 33174, USA*

^c*Pacific Northwest National Laboratory, PO Box 999, K3-62, Richland, WA 99352, USA*

*Corresponding author. E-mail: katsenov@fiu.edu; phone: 305-348-2338; fax: 305-348-5018

Abstract

Past disposal practices at nuclear production facilities have led to the release of liquid waste into the environment creating multiple radionuclide plumes. Microorganisms are known for the ability to interact with radionuclides and impact their mobility in soils and sediments. Gram-positive *Arthrobacter* sp. are one of the most common bacterial groups in soils and are found in large numbers in subsurface environments contaminated with radionuclides. This study

experimentally analyzed changes on the bacteria surface at the nanoscale level after uranium exposure and evaluated the effect of aqueous bicarbonate ions on U(VI) toxicity of a low uranium-tolerant *Arthrobacter oxydans* strain G968 by investigating changes in adhesion forces and cell dimensions via atomic force microscopy (AFM). Experiments were extended to assess cell viability by the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes) and quantitatively illustrate the effect of uranium exposure in the presence of varying concentrations of bicarbonate ions. AFM and viability studies showed that samples containing bicarbonate were able to withstand uranium toxicity and remained viable. Samples containing no bicarbonate exhibited deformed surfaces and a low height profile, which, in conjunction with viability studies, indicated that the cells were not viable.

Keywords: *Arthrobacter* sp.; Aqueous bicarbonate; Toxicity; Atomic force microscopy, Live/dead analysis

1. Introduction

Uranium is one of the most abundant actinide elements found in the environment and is a key contaminant of concern at many US Department of Energy sites, serving a leading role in the nation's defense for over 50 years. Uranium contamination of soil and groundwater is of great environmental concern due to the toxicological properties of the uranyl species. The behavior of uranium and its mobility in the subsurface is affected by various factors such as chemistry of the pore water, groundwater and soil minerals, presence of complex-forming ligands and micro-organisms that thrive under these conditions. Uranium exists in a number of valence states, but under oxidizing conditions, it dominates as a highly soluble and stable uranyl ion, UO_2^{2+} . In neutral or basic pH conditions, uranium undergoes hydrolysis in aqueous solutions and can

readily complex with a wide variety of ligands such as carbonate and phosphate. These complexation reactions often result in the formation of mobile aqueous species or precipitation of U-bearing minerals.

Dissolved inorganic carbon present in soil and groundwater is one of the primary factors controlling uranium aqueous speciation. In oxidized conditions at a $\text{pH} > 4$, uranyl ions interact with carbonate, creating strong neutral and negative soluble anionic complexes such as UO_2CO_3^0 , $\text{UO}_2(\text{CO}_3)_2^{2-}$, and $\text{UO}_2(\text{CO}_3)_3^{4-}$ that dominate aqueous speciation of U(VI) [1]. In Ca-rich soil, calcium-uranyl-carbonate complexes, $\text{Ca}_2\text{UO}_2(\text{CO}_3)_3^0(\text{aq})$ and $\text{CaUO}_2(\text{CO}_3)_3^{2-}$, became the predominant forms of dissolved U(VI) in circumneutral and alkaline pH conditions [2,3]. These uranyl carbonate and calcium uranyl carbonate complexes were identified in contaminated pore water at the Hanford Site, Washington State and have been shown to inhibit the microbial reduction of U(VI) under specific conditions [2,4].

Microbial impact on processes that govern the fate and transport of contaminants in soils and sediments is well established. According to several studies, *Arthrobacter* sp. are considered to be ubiquitous and predominant members of culturable soil microbial communities; they are found in large numbers in Hanford soil as well as other subsurface environments contaminated with heavy metals and radioactive materials [5-9]. *Arthrobacter* sp. are aerobic, chemo-organotrophic, Gram-positive bacteria characterized by a rod-to-coccus morphology change as they enter the stationary phase. These microorganisms are able to survive and reproduce in oligotrophic conditions in the presence of minimal concentrations of organic content [7, 8]. Microbial interaction with toxic metals and radionuclides in aqueous environments is well known and several studies have given insight into uranium tolerance of these microorganisms [10, 11]. In a

previous assessment, *Arthrobacter oxydans* strain 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]. In contrast with G975, *A. oxydans* strain G968 was found to have low resistance to U(VI) toxicity. The alteration in surface morphology for strain G968, with a reduction in cell size and distorted surfaces, was noted at 0.5 mg/L of U(VI); in comparison, strain G975 showed signs of cell inhibition at the much higher concentration of 19 mg/L of U(VI) [13]. Inhibition of bacterial activity was associated with the binding of U(VI) to the cell envelope. The passive binding of uranyl to cell surfaces reduces the fluidity of the cell membrane, limiting nutrient uptake [14]. However, uranyl-carbonate complexes formed in the solution in the presence of bicarbonate do not strongly interact with the negatively charged bacterial surface, which in turn can mitigate U(VI) toxicity on the cells [13, 14].

Uranium-bacteria interactions were rigorously studied to understand the microbial effects that influence the mobility of U(VI) in groundwater, as well as bacterial viability in the presence of U(VI) [15, 16]; however, the mitigation effect of bicarbonate on the viability of cells exposed to U(VI) has not yet been evaluated. The main focus of this investigation was to qualitatively and quantitatively characterize changes on the bacteria surface at the nanoscale level after uranium exposure and evaluate the effect of aqueous bicarbonate on U(VI) toxicity of *A. oxydans* strain G968 by analyzing changes in adhesion forces and cell dimensions via profile plots. In addition, cell viability was assessed by the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes) to quantitatively illustrate the effect of varying concentrations of bicarbonate ions on viability of bacterial cells exposed to uranium. Culturability of *A. oxydans* strain G968 was also determined following exposure to uranium.

2. Materials and methods

2.1. Cell growth and atomic force microscopy (AFM) analysis on bacteria- uranium interactions

2.1.1. *Arthrobacter* strain growth culture conditions

A.oxydans strain G968 previously isolated from Hanford Site soil was obtained from the Subsurface Microbial Culture Collection (SMCC) maintained at Alabama State University. Detailed descriptions of site geology, sample collection procedures, isolation and identification are described in detail in [5, 7]. Bacterial cells of *A.oxydans* strain G968 were grown in a 5% peptone, tryptone, yeast and glucose (PTYG) liquid culture media, 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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 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 min, then allowed to cool before being used. Cells of *A.oxydans* strain G968 were aerobically grown in stoppered 50 mL polypropylene centrifuge tubes amended with 10 mL of 5% PTYG media and kept in an incubator/shaker at 29°C. Bacterial cells were harvested in the late logarithmic phase of growth, washed and experiments were initiated by adding log 7 cells/mL of a bacterial culture to the sterile synthetic groundwater (SGW) solution amended with uranium (Table 1). Prior to inoculation, cell stock suspensions were vortexed for 10 s and counted using an INCYTO C-Chip disposable hemocytometer to determine the number of cells/mL in the suspension, needed to calculate the desired cell concentration in the tubes. Cell densities were also calculated in all samples at the end of the experiment after 24 h exposure to U(VI).

2.1.2. Sample preparation for AFM imaging

The carbon and phosphorus- free synthetic groundwater (SGW) solution prepared in deionized water contained 5.22 mg/L of KCl and 520.58 mg/L of hydroxyethyl piperazine

ethanesulfonic acid (HEPES) with the pH of the solution measured as 7.2. The SGW solution was autoclaved at 121 °C, 15 psi for 15 min and allowed to cool to about 30°C. Sterile SGW was equally distributed between three 250 mL bottles and aseptically amended to contain 0 mM, 5 mM and 10 mM of KHCO_3 , (Table 1). Each of the three individual bicarbonate SGW solutions was filter-sterilized (0.2 μm) and kept refrigerated until the time of use. The composition of sterile bicarbonate and uranium amended SGW is presented in Table 1.

After 24 h, the 5 mL aliquot of bacterial samples kept at 29°C in an incubator/shaker were centrifuged and pellets were washed three times with deionized water to remove U(VI) and media residuals and 10 μL of a concentrated sample was immobilized onto the 3-aminopropyltrimethoxysilane-coated silicon wafer AFM substrates. Bacterial cells must firmly adhere to a silicon wafer substrate so that the sample is immobilized and stabilized during imaging. Samples were air-dried until excess moisture was completely evaporated. Silanized silicon wafer AFM substrates were then fixed onto a metallic disc and transferred to an AFM stage for imaging.

2.1.3. AFM instrumentation and imaging

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 AFM instrument was operated in contact mode. The cantilever was 500 μm in length, 30 μm in width and 2.7 μm in thickness. The tip shape was rotated and the tip radius was <10 nm. The AFM tip was 20-25 degrees along the cantilever axis; 25-30 degrees from the 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 25°C 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 2 h of sample preparation.

2.1.4. Force spectroscopy (FS)

The FS technique has been prominent over the last two decades for measuring various physical and intermolecular forces. These forces are typically measured between the AFM tip and the substrate surface to reveal their interaction behavior. Along with AFM's capability to study surface topography at a nanometer-scale resolution, FS helps to predict changes on bacterial cell surfaces. Adhesion forces are sensitive to modifications in the surface [17] and can be used to characterize the cellular membrane when bacteria are exposed to uranium in the presence of bicarbonate ions. The dynamic changes occurring on the bacterial cell membrane at the atomic level before and after exposure to the various concentrations of uranium in bicarbonate-amended media were evaluated using force spectroscopy analysis. The forces were determined using force distance (FD) curves that were generated from the array of force curves over the selected region. The average adhesion force was obtained from a set of 256 different FD curves in a 5 μm x 5 μm sample surface. This experimental approach enables monitoring of forces at the piconewton/nanonewton scale. Data acquired through FD curves were processed using Scanning Probe Image Processor (SPIP) software by ImageMetrology, Denmark (version 6.2.0) to determine their average adhesion forces.

2.1.5. Roughness measurements

The surface of the immobilized bacteria on the Si wafer was also investigated using AFM operated in contact mode as mentioned above. A roughness for bacterial cell surfaces was characterized from a high resolution AFM topography image at different scales. The roughness

value was calculated according to the relative heights of individual pixels in the image [18] over a selected area. The average roughness (R_a) was calculated from the average of the absolute values of profile height deviations from the mean level (Eq.1).

$$R_a = \frac{1}{n} \sum_{j=1}^n |Z_j| \quad (\text{Eq.1})$$

Here, Z is the maximum vertical distance between the highest and lowest data points and n is the number of points scanned.

2.2. Cell viability

2.2.1. Viability analysis via fluorescence imaging and counting

Estimates of bacterial cell wall integrity were made using the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, L-7012) to illustrate the effect of varying concentrations of bicarbonate ions on viability of bacterial cells after exposure to uranium. The Live/Dead assay contains SYTO 9 green fluorescent nucleic acid stain and red fluorescent nucleic acid stain, propidium iodide. SYTO 9 penetrates intact membranes and generally stains all bacteria in a population, regardless if they are alive or dead. Propidium iodide is membrane impermeant and is generally excluded from viable cells with intact membranes. Samples for viability assessment were prepared similarly to samples used for AFM imaging, i.e. grown in SGW media containing uranium and amended with varying concentrations of bicarbonate ions. Two controls were created, one by adding 4 mL of SGW media for the live control and another with 4 mL of 70% isopropyl alcohol (for killed bacteria). All experimental and control samples were then incubated at room temperature for 24 h. In order to stain the samples, 3 μ L of the dye mixture (containing equal parts of SYTO9 and propidium iodide) was added for each mL of the bacterial suspension. Samples were incubated at room temperature in the dark for 15 min and then washed 3 times with SGW to remove the background fluorescence. The stained bacterial

suspension (5 μ l) was then placed on a microscope slide and covered with a cover slip. Prepared slides were then air-dried for 1 h in the dark before being viewed under a fluorescence microscope.

Cells were viewed and counted using a fluorescence microscope at a magnification of 200 x (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. Pictures were taken of the region of interest and an area of 4.32 μ m² was counted from the images (ImageJ software, NIH, Bethesda, MD). The ratio of live cells (green dots) to the sum of live and dead cells (red dots) was calculated according to Eq.2, [19]:

$$\text{Ratio of live cells} = \frac{\text{Number of live cells}}{\text{Number of live cells} + \text{number of dead cells}} \times 100\% \quad \text{Eq. 2}$$

2.2.2. Cell viability using plates

To supplement the 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 whether samples are culturable as well. In preparation for cell viability experimentation, PTYG plates were made using 5% PTYG media and 15 g/L agar. Medium was prepared in deionized water (DIW), autoclaved at 121°C, 15 psi for 15 min and allowed to cool to about 60°C. The PTYG medium was then poured into sterile petri dishes and allowed to solidify overnight in sterile conditions. Samples used for this analysis were the same samples described in section 2.2. To account for viable bacteria, thoroughly mixed samples were subsequently diluted and homogeneous aliquots (0.1 mL) of the suspension from each dilution were uniformly spread on the sterile petri dishes. Inoculated petri dishes were kept for 4 days in the incubator at 29°C. Viable microorganisms were calculated from the number of colony-

forming units (CFU/mL) divided by 0.1 mL plated and multiplied by the specific dilution factor for each dish. Furthermore, plating efficiency was calculated by comparing cell viability before and after U(VI) exposure, which was determined via counts of CFUs.

2.3 Statistics

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

3. Results and discussion

3.1. Cell integrity via the Live/Dead assay

Images of live and dead control samples are presented in Fig.1A and Fig.1B. As for the experimental samples incubated with varying concentrations of uranium in bicarbonate-bearing media, the integrity of the bacterial cell wall was determined after 24 h. Fig.1C-F illustrates the cell integrity assessment via the Live/Dead assay for all samples. Fig.1C is the sample containing 5 mg/L of U(VI) with no bicarbonate; examples of dead cells in this image are located within the red circles. Fig.1D contains 5 mg/L of U(VI) in the presence of bicarbonate and illustrates a larger concentration of “live cells” (green cells with intact membranes) present. Fig.1E and Fig.1F illustrate samples exposed to 10 mg/L of U(VI) with (Fig.1F) and without bicarbonate (Fig.1E). Fig.1E illustrates a larger number of “dead cells” (red cells indicating membrane permeability) when exposed to 10 mg/L uranium only, compared to cells exposed to uranium and bicarbonate (Fig.1F). It is important to note that Fig.1D and Fig.1F 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.

Calculations of cells with intact membranes to those with permeable membranes in samples with varying concentrations of uranium and bicarbonate exhibited a ratio of live cells greater than 95%. When making a comparison between bicarbonate-free and bicarbonate-amended samples that contain 10 mg/L of U(VI), the sample with bicarbonate contained a higher ratio of live cells, as noted in Table 1. The total number of cells/mL was determined for all samples at the end of the experiment after 24 h of incubation in the uranium-bearing SGW suspensions prepared with and without bicarbonate ions. Cell densities showed almost no change or a slight decrease from the initial concentration of log 7 cells/mL in bicarbonate-free samples after 24 h of incubation in the SGW media amended with U(VI). In contrast, samples amended with bicarbonate showed a 32-53% increase in cell density similar to U(VI)-free control samples (Table 1).

3.2 Cell viability via plates

Although all samples contain cells with intact membranes indicating viability despite the concentrations of uranium and bicarbonate present, they may be unable to develop into culturable colonies on solid media. Plating efficiency was calculated by comparing cell viability before ($t=0$) and then 24 h after exposure to U(VI), which was determined via counts of colony CFUs/mL. Samples containing 5 mg/L U with 0 mM bicarbonate yielded an *A. oxydans* strain G968 plating efficiency of 1.75% while, with 5 mM bicarbonate, the efficiency increased to 42%. Similarly, samples containing 10 mg/L U with 0 mM bicarbonate had a CFU plating efficiency of 3.25%; with 5 mM bicarbonate, the efficiency increased to 28%. These results illustrate that although the bacterial cells maintained intact cytoplasmic membranes, resulting in viable cells as determined by the Live/Dead analysis, the cells that are exposed to uranium with no bicarbonate show very little growth. This means that the samples containing no bicarbonate

keep the integrity of the membrane, but experience low levels of growing colonies when plated. One possible explanation for these results is that the bacterial cells entered a viable but non-culturable (VBNC) state caused by oxidative stress produced by the uranium or because the cells were inhibited by the uranium at a metabolic level. Likely, inhibition of the cells through oxidative or metabolic stresses may lead to a VBNC state; bacteria entering a VBNC state were initially discussed by [20]. Bacteria enter this state as a feedback response to natural stresses such as starvation or being exposed to toxic substances [21]. These environmental stresses could be lethal if cells do not enter the VBNC state. This state allows for changes in bacterial morphology, size, cell structure and composition. The resistance of microbes in the radioactive environment varies. Gram-negative bacteria are more sensitive than Gram-positive bacteria, which have thicker cell walls that offer more protection [22]. Characterization of the mechanism of inhibition implicated preferential binding of the uranyl ion to oxidative enzymes, inhibiting carbon metabolism [23]. A similar mechanism is likely responsible for the inhibition of growth that was demonstrated for *A.oxydans* strain G968 exposed to uranium with and without bicarbonate.

3.3 Effect of uranium on cell surface morphology using AFM

AFM was employed to monitor changes at the nanoscale level in cell surface topography and adhesion forces after cell exposure to various concentrations of uranium in the presence of bicarbonate. The aim of this task was to present high-resolution AFM images and determine cell dimensions and adhesion forces to illustrate the effect of uranium and bicarbonate on the bacterial surface. Additionally, this method was used 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 low-uranium-tolerant *A.oxydans* strain G968 by analyzing changes in bacterial dimensions via profile plots.

3.3.1. Uranium effect on the microbial cell surface

AFM provided atomic scale resolution images of surface topography of the *A. oxydans* strain G968 in the presence of varying concentrations of uranium in bicarbonate bearing or bicarbonate-free solutions. Additionally, AFM facilitated measurements of cell dimensions, which are explained below. Test samples were treated with varying concentrations of uranium, from 0 mg/L to 10 mg/L. Fig.2A illustrates the control sample with no uranium exposure in which cocci and rod-shaped bacteria were observed to have a relatively smooth surface. Characteristics of the control cells were no different between treatments in uranium-free SGW supplemented with and without bicarbonate. Fig.2B shows bacteria when uranium was added to a concentration of 5 mg/L with no bicarbonate present in the solution and with bicarbonate amended into the solution (Fig.2C). Cells exposed to uranium appear to maintain only a rod-shaped morphology. In contrast, Fig.2C reveals a different cocci-like morphology that appears to be coated with an unknown material, which is still under investigation. Fig.2D and Fig.2E illustrate two samples with a concentration of uranium of 10 mg/L with no bicarbonate (Fig.2D) and bicarbonate (Fig.2E). It is also evident that the sample containing no bicarbonate (Fig.2D) has a distorted bacterial shape due to exposure to uranium. Interestingly, the cells in the sample containing no bicarbonate (Fig.2D) maintained a somewhat rod-shaped morphology. In contrast, in samples amended with bicarbonate (Fig. 2E), only cocci- shaped cells were observed. No precipitation was observed on the bacterial surface of cells treated with 5 mg/L and 10 mg/L of uranium (Fig.2C, Fig.2D). Morphology of *Arthrobacter* species during growth is a distinguishing phenotype for this group of bacteria. During the logarithmic-phase of growth, cells demonstrate

a rod-shaped morphology. Conversely, when cells enter the stationary phase, they revert to the coccus morphology. In general, results from AFM analysis indicate cells exposed to uranium without bicarbonate maintain a rod-shaped morphology, while uranium exposure with bicarbonate allowed for the coccus morphology. The *Arthrobacter* species have also been shown to produce glycogen and trehalose during conditions of nutrient starvation and stress [24]. In combination, these general observations for *Arthrobacter* may provide insight into the morphology demonstrated during the experiment. When *A. oxydans* strain G968 cells were exposed to uranium without bicarbonate, cell growth ceased during exponential growth, leading to the rod-shaped morphology. Alternately, since cells were not completely inhibited by uranium when carbonate was present, the cells were able to proceed to the stationary phase and the associated coccus-like morphology.

3.3.2 Force spectroscopy analysis

It has been proposed that adhesion forces are sensitive to modifications on the bacterial surface [17]; for example, physical changes occur on the cellular membrane when exposed to toxic environments. It was therefore necessary to perform a force spectroscopy analysis to gain full comprehension of the interaction forces between the tip and the sample surface at the piconewton scale level. The results in Table 1 indicate a significant statistical difference in adhesion force values between control samples and those containing varying concentrations of uranium and bicarbonate ($P < 0.05$).

There is an inverse relationship between adhesion forces and the concentration of uranium; as the concentration of uranium increases, adhesion forces decrease. Additionally, when bicarbonate is present within the solution, adhesion forces showed slightly higher values than that of the no-bicarbonate samples, but the difference was not statistically significant ($P =$

0.477). Previous MINTEQ modeling illustrated that the trimetric form of uranium, $(\text{UO}_2)_3(\text{OH})^{5+}$, 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 $\text{UO}_2(\text{CO}_3)_3^{4-}$ species, which are not expected to adhere to the negatively charged bacterial surface [25]. However, at higher uranium concentrations, there is a relatively high extent of adsorption of neutral uranium species on the bacterial surface [25, 26]. The bacterial surface changes associated with U(VI) biosorption lead to the decrease in adhesion values compared to controls.

3.3.3 Roughness analysis

Changes in cell surface roughness of *A. oxydans* strain G968 when exposed to varying concentrations of uranium were analyzed using AFM roughness analysis. Exposure to uranium concentrations ranging from 0 to 10 mg/L was conducted for 24 h prior to imaging. Past research has shown some correlation between increasing roughness values as the concentration of uranium increases [26]. For this particular case, values of roughness were in the range of 25.37 ± 2.31 nm- 26.27 ± 3.73 nm for 5 mg/L of U(VI) and 44.28 ± 6.07 nm- 44 ± 4.58 nm for 10 ppm of U(VI). The values of roughness were not significantly different between the bicarbonate-amended and bicarbonate-free samples for either of the uranium concentrations tested (Table 1). Despite this, however, data indicate that there is a significant statistical difference in roughness values between control samples and those containing varying concentrations of uranium and bicarbonate ($P = 0.006$).

3.3.4. 2D profile analysis of *A. oxydans* strain G968

In order to monitor structural changes in the bacteria with varying levels of uranium concentrations, 2D profiling was conducted on the bacteria. 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. Fig.3A illustrates the height maps of the control sample revealing a maximum height of 250-300 nm. The sample containing 5mg/L of uranium without bicarbonate (Fig.3B) illustrates a profile height of 250 nm-400 nm, whereas when bicarbonate was introduced into the solution (Fig.3C), the height was noted to reduce to a value between 160 and 180 nm. In samples containing 10 mg/L of uranium with and without bicarbonate, the profile heights ranged between 90-160nm and 110-130nm, respectively (Fig.3D, Fig 3E). In addition, profile plots demonstrated smooth cell surfaces in bicarbonate-amended samples (Fig. 3C; 3E); however, bicarbonate-free samples showed cell surface deformation in response to U(VI) toxicity. When comparing the plating method and Live/Dead analysis with AFM assessment, we find that bacteria samples taken from bicarbonate- free solutions have mostly intact membranes, but are not culturable; the cell shape is not altered and remains rod-shaped (Fig.3A, Fig.3B and Fig.3D). In contrast, samples containing bicarbonate have a smaller coccus-shape cellular size with no deformed surfaces, which under the AFM microscope appears similar to U-free control samples. This might be an indication that the cells are alive and have acclimated so as to withstand uranium toxicity. *Arthrobacter* species are known for their ability to change size and shape in response to toxicity from rods to spherical cocci; in this coccusi shape, the bacteria are more resistant to desiccation and starvation [27].

In conclusion, AFM analysis and the Live/Dead fluorescent assay were used to qualitatively and quantitatively illustrate changes in bacterial cell surfaces when exposed to uranium in the presence and absence of bicarbonate. The Live/Dead analysis showed that despite the concentration of uranium and bicarbonate present in the solution, each sample exhibited a ratio

of cells with intact membranes greater than 95%. By performing a cell viability assessment via culture plates, results demonstrated that although the bacterial cells maintained the integrity of intact cytoplasmic membranes, resulting in viable cells as determined by Live/Dead analysis, the cells that were exposed to uranium with no bicarbonate experienced a viable but non-culturable state, that is, they exhibited low numbers of colonies when plated. Force spectroscopy results demonstrated that as the uranium concentration increased in the media, adhesion force parameter decreased. Furthermore, at 5 ppm of U(VI), the height provided from the profile plots revealed that samples containing bicarbonate had a lower profile height compared to bicarbonate-free samples. At U(VI) concentrations tested, samples exposed to uranium with no bicarbonate were mostly viable via the Live/ Dead assay, but exhibited deformed surfaces retaining a rod-shaped morphology, which might be an indication that the cells were viable but non-culturable. In contrast, samples containing bicarbonate exhibited smaller cellular size after a rod- to coccus-shaped transition, with surface morphology replicating the control samples. This data illustrate how cells have become acclimated in order to withstand uranium toxicity.

Acknowledgments

Funding for this research was provided by U.S. DOE grant number DE-EM0000598. We would like to thank Dr. Patricia Sobecky and Dr. Robert J. Martinez from the University of Alabama, Tuscaloosa, AL, for providing us with the *Arthrobacter sp.* strains.

References

1. Langmuir, D. Uranium solution- mineral equilibria at low temperatures with application to sedimentary ore deposits. *Geochim Cosmochim Acta* 1978; 42: 547-569.
2. Bernhard G, Geipel G, Reich T, Brendler V, Amayri S, Nitsche H. Uranyl (VI) carbonate complex formation: validation of the $\text{Ca}_2\text{UO}_2(\text{CO}_3)_3(\text{aq.})$ species. *Radiochim. Acta* 2001; 89: 511-518.

3. Kalmykov SN and GR Choppin. Mixed $\text{Ca}^{2+}/\text{UO}_2^{2+}/\text{CO}_3^{2-}$ complex formation at different ionic strengths. *Radiochim. Acta* 2000; 88: 603- 606.
4. Brooks SC, Fredrickson JK, Carroll SL, Kennedy DW, Zachara JM, Plymale AE, Kelly SD, Kemner KM, Fendorf S. Inhibition of bacterial U(VI) reduction by calcium. *Environ Sci Technol* 2003; 37:1850–1858.
5. Balkwill DL, Reeves RH, Drake GR, Reeves JY, Crocker FH, King MB, Boone DR. Phylogenetic characterization of bacteria in the subsurface microbial culture collection. *FEMS Microbiol Rev* 1997; 20: 201-216.
6. Boylen CW. Survival of *Arthrobacter crystallopoietes* during prolonged periods of extreme desiccation. *J Bacteriol* 1973; 113:33-37.
7. Van Waasbergen LG, Balkwill DL, Crocker FH, Bjornstad BN and RV Miller. Genetic diversity among *Arthrobacter* species collected across a heterogeneous series of terrestrial deep-subsurface sediments as determined on the basis of 16S rRNA and recA gene sequences. *Appl Environ Microb* 2000; 66: 3454-3463.
8. Crocker FH, Fredrickson JK, White DC, Ringelberg DB, Balkwill DL. Phlogenetic and physiological diversity of *Arthrobacter* strains isolated from unconsolidated subsurface sediments. *Microbiology* 2000; 146: 1295-1310.
9. Fredrickson JK, Zachara JM, Balkwill D L, Kennedy D, Li SW, Kostandarithes HM, Daly MJ, Romine MF, Brockman FJ. Geomicrobiology of high-level nuclear waste-contaminated vadose sediments at the Hanford Site, Washington State. *Appl. Environ. Microbiol* 2004; 70: 4230-4241.
10. Merroun M L, Geipel G, Nicolai R, Heise K, and S Selenska-Pobell. Complexation of uranium(VI) by three eco-types of *Acidithiobacillus ferrooxidans* studied using time-resolved laser-induced fluorescence spectroscopy and infrared spectroscopy. *Biometals* 2003;16:331–339.
11. Lloyd RJ, Renshaw CJ. Bioremediation of radioactive waste: radionuclide–microbe interactions in laboratory and field-scale studies. *Curr Opin Biotechnol* 2005; 16:254–260
12. Katsenovich YP, Carvajal DA, Guduru R, Lagos LE. Assessment of the Resistance of Hanford Site *Arthrobacter* Isolates to Uranium (VI) Exposure. *Geomicrobiol J* 2012a; 30: 120-130.
13. Katsenovich YP, Carvajal DA, Wellman DM, Lagos LE. Enhanced U(VI) release from autunite mineral by aerobic *Arthrobacter sp.* in the presence of aqueous bicarbonate. *Chem Geol* 2012b; 308-309: 1-9.
14. Carvajal DA, Katsenovich YP, Lagos LE. The effects of aqueous bicarbonate and calcium ions on uranium biosorption by *Arthrobacter G975* strain *Chem Geol* 2012; 330-331: 51–59.
15. Suzuki Y and JF Banfield. Resistance to, and accumulation of, uranium by bacteria from a uranium- contaminated site. *Geomicrobiol J* 2004; 21:113-121.

16. Merroun ML, Raff J, Rossberg A, Hennig C, Reich T, Selenska-Pobell S. Complexation of uranium by cells and S-layer sheets of *Bacillus sphaericus* JG-A12. *Appl Environ Microbiol* 2005; 71(9): 5532–5543.
17. da Silva L, Ochsner A, Adams R. Handbook of adhesion technology. Springer-Verlag Berlin Heidelberg, 2011.
18. Fung CKM, Seiffert-Sinha K, Lai KWC, Yang R, Panyard D, Zhang J, Xi N, Investigation of human keratinocyte cell adhesion using atomic force microscopy, *Nanomedicine: Nanotechnology, Biology and Medicine* 2010; 6:191-200
19. Dua R, Centeno J, Ramaswamy S. Augmentation of engineered cartilage to bone integration using hydroxyapatite. *J Biomed Res B. Appl Biomater* 2014; 102: 922-32. DOI: 10.1002/jbm.b.33073
20. Colwell RR, Grimes DJ, Nonculturable microorganisms in the environment. ASM Press, Washington D.C, 2000. .
21. Oliver JD, and R Bockian. The viable but nonculturable state and cellular resuscitation. *Microbial Biosystems: New Frontiers, Proceedings of the 8th International Symposium on Microbial Ecology* Bell CR, Brylinsky M, Johnson-Green P (eds) Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 1999;723-730.
22. Chicote E, Garcia AM, Moreno DA, Sarro MI, Lorenzo PI, Montero F. Isolation and identification of bacteria from spent nuclear fuel pools. *J Ind Microbiol Biotechnol* 2005; 32: 155–162. DOI 10.1007/s10295-005-0216-3
23. VanEngelen MR, Szilagyi RK, Gerlach R, Lee BD, Apel WA, Peyton BM. Uranium exerts acute toxicity by binding to pyrroloquinoline quinone cofactor. *Environ Sci Technol* 2010b; 45:937-942.
24. Zevenhuizen LP. Levels of trehalose and glycogen in *Arthrobacter globiformis* under conditions of nutrient starvation and osmotic stress. *Antonie Van Leeuwenhoek* 1992;61:61-68.
25. Carvajal D, Katsenovich Y, Lagos L. The effects of aqueous bicarbonate and calcium ions on uranium biosorption by *Arthrobacter G975* strain. *Chem Geol* 2012; 330-331: 51-59.
26. Gorman – Lewis, D., Elias ,P., Fein, J B., 2005. Adsorption of aqueous uranyl complexes onto *Bacillus Subtilis* cells. *Environ. Sci. Technol.* 39, 4906-4912.
27. O' Loughlin, EJ, Sims, GK and SJ Traina. Biodegradation of 2-methyl, 2-ethyl and 2-hydroxypyridine by an *Arthrobacter sp.* isolated from subsurface sediment. *Biodegradation* 1999; 10: 93-104.

Figure 1

Figure 2

D. 10 mg/L U(VI), 0mM HCO_3^-

E. 10 mg/L U(VI), 5 mM HCO_3^-

Figure 3

Figure 1. Live/Dead assay of sample containing A) live control; B) dead control; C) 5 mg/L of U (VI) with no bicarbonate; D) 5 mg/L of U (VI) with 5 mM bicarbonate; E) 10 mg/L of U (VI) with no bicarbonate; and F) 10 mg/L of U (VI) with 5 mM bicarbonate.

Figure 2. A) G968 control sample (scan size $5 \times 5 \mu\text{m}^2$) illustrating smooth bacterial surface; B) G968 cultured in media amended with a) 5 mg/L U (VI) and 0 mM HCO_3 (scan size $6 \times 6 \mu\text{m}^2$) and C) 5 mg/L of U (VI) and 5 mM bicarbonate, (scan size $5 \times 5 \mu\text{m}^2$). D) G968 cultured in media amended with 10 mg/L U (VI) and 0 mM HCO_3 (scan size $5 \times 5 \mu\text{m}^2$); E) 10 mg/L of U (VI) and 5 mM bicarbonate, (scan size $5 \times 5 \mu\text{m}^2$). For all images the topographic image is on the left and friction image is on the right.

Figure 3. A) G968 control sample (scan size $2.5 \times 2.5 \mu\text{m}^2$) illustrating the height profile plots in X (left) 300 nm and Y (right) direction as 250nm; B) 5 mg/L U (VI) and no bicarbonate. Profile plot illustrates the height X: 400 nm and Y: 250-300 nm; C) 5 mg/L U (VI) and 5 mM bicarbonate. The profile heights illustrate X : 180 nm and Y:160 nm. The effect of uranium could have caused these bacterial cells to shrink revealing rod-to-cocci shape change; D) 10 mg/L U (VI) and 0 mM bicarbonate. The profile heights for this sample X: 110 nm and Y: 130 nm. E) 10 mg/L of U (VI) and 5 mM of bicarbonate. The profile heights for this sample X: 90 nm and Y: 160 nm.

Table 1

Table 1. Viability and adhesion forces results for samples containing varying concentrations of uranium in bicarbonate bearing media

U(VI), mg/L	HCO ₃ ⁻ , mM	Percentage of Viable Cells in Live/Dead Assay (n=4)	Cell viability measured by plate counting, %	Changes in total cell density after 24 h incubation in SGW, %	Cells shape	Cells height value, nm	Adhesion Forces (nN) for <i>Arthrobacter</i> sp. G968 (n=3)	The average roughness (Ra), nm
0	0	100	79.4	42- 66.3	Rod-shaped	250-300	11.62 ±1.68	3.24±2.01
5	0	98.00 ± 1.02	1.75	(-5.7) - +8.7	Deformed rod-like shape	250-400	7.14 ±0.26	25.37±2.31
5	5	97.51 ± 0.67	42	+36.7- +53.0	Cocci-shaped	160-180	9.14 ± 1.2	26.27±3.73
10	0	95.60± 1.22	3.25	(-0.1) - +5.1	Deformed rod-like shape	110-130	4.58± 0.26	44.28±6.07
10	5	98.1± 1	28	+31.8	Cocci-shaped	90-160	4.88 ± 2.3	44±4.58

***Detailed Response to Reviewers**

Reviewer 1	As stated by the authors p14, "characteristics of the control cells were not different between treatments in uranium-free SGW supplemented with and without bicarbonate". In my opinion, it's not enough. The data must be included in the manuscript. This is a very important control to interpret the results.	Thank you for your comment; we included these values in the Table 1.
Specific comments:		
1	Line 77 : « Arthrobacter » instead of "Arthorbacter »	Corrected
2	Section 3.1: this part is still confusing. The authors are mixing contradictory comments here. On one hand, the authors are pointing out "the larger number of dead cells when exposed to uranium only" (line 233) and that "the sample containing bicarbonate contained a higher ratio of live cells" (line 241-242) while on the other hand, no significant difference was observed between the different exposure conditions (line 238-239).	Text on p.11 was modified as follows, "Calculations of cells with intact membranes to those with permeable membranes in samples with varying concentrations of uranium and bicarbonate exhibited a ratio of live cells greater than 95%. When making a comparison between bicarbonate-free and bicarbonate-amended samples that contain 10 mg/L of U(VI), the sample with bicarbonate contained a higher ratio of live cells as noted in Table 1."
3	Section 3.2: what was the cell viability recorded for the uranium-free control? The data described in this section could be included in Table 1.	Cell viability recorded in U-free controls was 79.4%. We included a column in Table 1 to reflect cell viability values measured by plate counting, %.
4	Section 3.3.2: It is stated that the adhesion forces showed "slightly" higher values when bicarbonate is present. Are these differences statistically significant, in particular when the cells are exposed to 10mg/L U?	We ran a t-test and based on the results there is not a statistically significant difference between the input groups (P = 0.477). We modified this sentence as follows, "Additionally, when bicarbonate is present within the solution, the adhesion forces showed slightly higher values to that of the no-bicarbonate samples but the difference is not statistically significant (P = 0.477)."
5	Section 3.3.3: The values of roughness indicated in this section (23.69 and 41.53 nm) do not match with those indicated in Table 1. It is stated that "no correlation was found with respect to roughness and uranium concentrations". In fact, based on the	Thank you for pointing this out. We made corrections in the manuscript starting from line 341, "For this particular case, values of roughness were in the range of 25.37±2.31 nm-26.27±3.73 nm for 5 mg/L of U(VI) and 44.28±6.07 nm- 44±4.58 nm for 10 ppm of U(VI). The values of roughness were not significantly different between the bicarbonate-amended and bicarbonate-free samples for either of

	data given in Table 1, roughness increased with U concentration.	the uranium concentrations tested (Table 1).
6	<p>Section 3.3.4:</p> <p>The height values indicated in the text (line 353) and in Table 1 are different.</p> <p>The height measured for the cells exposed to 10 mg/L U and bicarbonate 10 mM (110-130) is not similar to the uranium-free control (250-300), unlike what is stated in lines 359-360. Same problem in the conclusion section: "samples containing bicarbonate have a higher profile height" (line 379) and "samples exposed to uranium with no bicarbonate (...) exhibited a low height profile" (line 379-381). This is in total contradiction with the data provided. Please explain.</p>	<p>We agree with the comment and made corrections in the text of the manuscript.</p> <p>The height value of the control samples presented in the text and Table 1 is 250-300nm.</p>
7	<p>Figure 1: be careful, the scale bar unit is in μM</p> <p>Table 1: I suggest adding the cell viability measured by plate counting in the Table.</p> <p>column 2: the HCO_3 concentration indicated with 10 mg/L U is 5 in the table and 10 in the mat and met.</p> <p>column 5: "cells shape" instead of "Sells shape"</p>	<p>Corrected the scale bar unit as μm.</p> <p>We agree and added in Table 1 the cell viability values measured by plate counting.</p> <p>The concentration of bicarbonate tested with 5mg/L and 10mg/L of U(VI) was 5mM. Both Table1 and text of the manuscript reflect this concentration correctly.</p> <p>We corrected column 5, as "Cells shape".</p>
Review#2	<p>Following revisions, the report regarding effects of uranium on gram negative <i>A. oxydans</i> strain G968 through live-dead analysis and AFM imaging has improved considerably. The mitigating effects of biocarbonate in the presence of uranium have been further clarified. Additionally, the data better supports the mechanism considered for the response of cells to uranium in the presence of bicarbonate. The report's findings contribute to the scientific community, especially in the fields of</p>	

	environmental contamination and remediation. The manuscript is in acceptable form for the journal.	
--	--	--