Final Executive Summary Report-SER Uranium research fund

A column study for enhanced bioremediation of in-situ uranium aquifers with varying levels of total dissolved solids

Principal Investigator: John D. Willford, Lecturer, Asst. ETT, Dept. of Molecular Biology, University of Wyoming, Willford@uwyo.edu

Co-Principal Investigator: Kevin R. Chamberlain, Research Professor, Dept. of Geology and Geophysics, University of Wyoming, kchamber@uwyo.edu

Report written by John Willford and Kevin Chamberlain

June 30, 2015

Table of Contents

Final Technical Report

Abstract

Restoration of in-situ recovered (ISR) uranium aquifers requires the reduction of soluble uranium (VI) back into the insoluble uranium (IV) form. It has been shown that some microorganisms maintain the ability to catalyze this reaction as part of their natural metabolic activity. In a previous study, we demonstrated the efficacy of tryptone at promoting biological reduction within Smith Ranch-Highland (SRH) aquifer sediment and waters. This study intended to further evaluate tryptone as a biostimulant in a column study, which provided continuous flow to better simulate treatment in a well pattern, along with varying stimulation levels and water total dissolved solid (TDS)/soluble uranium levels. At the 2000mg/L level of stimulation, a 99.3% decrease in soluble uranium concentration was observed in the highest TDS/U waters utilized. The 200mg/L level of tryptone also generated positive results in most of the waters with a notable 82.6% decrease in soluble uranium concentration in the medium TDS/U waters. For the columns in which biological reduction of uranium (VI) was suspected, uranium isotopic fraction with significant downward shifts in the $^{238}U/^{235}U$ ratio were again observed along with total carbonate concentration increases, both of which synced quite well with the timing of uranium (VI) reduction. The efficacy of tryptone at supporting microbial growth and subsequent biological reduction of uranium (VI) in the SRH system is evident and provides a positive outlook for biostimulation as part of the SRH restoration scheme.

Introduction

Restoration efforts following in-situ recovery (ISR) of uranium are required by licensing agencies (Wyoming State DEQ and US EPA) and are critical for minimizing the overall environmental impacts of ISR uranium mining operations. Current restoration methods include aquifer sweeps to flush out remaining mining lixiviant and mining byproducts along with chemical treatments to reestablish a reducing environment driving reprecipitation of heavy metals brought into solution during ISR operations. These current methods are costly and often result in large amounts of consumptive water loss, so improved restoration strategies may improve the economics of uranium mining, streamline the mining to restoration process, and further minimize environmental impact.

Uranium exists commonly in two valence states, U(VI) and U(IV). The oxidized U(VI) form is relative soluble and typically the form of dissolved uranium; while the reduced U(IV) form is highly insoluble in most waters (Langmuir, 1978). Natural fluctuations and anthropogenic alterations to the redox state of surrounding fluids control: the transportation of uranium, its deposition in roll-front deposits, in-situ recovery mining, and ultimately mine site restoration. Thereby, altering the redox state of the environment will alter the fate of uranium found within that environment.

Naturally occurring bacteria have been shown to be capable of reducing the valence state of uranium from U(VI) to U(IV), accelerating its precipitation (Cheng *et al.*, 2012; Gorby & Lovley, 1992; Phillips *et al.*, 1995; Uhrie *et al.*, 1996). There is a growing recognition that bacteria may have played an important role in the original uranium roll-front deposition (Boberg, 1981; Cheng *et al.*, 2012), which should allow them to play a similar role in ISR post-mining restoration.

Bioremediation is the use of living organisms to facilitate the clean-up of environmental contamination (USGS, 2011). This restoration strategy is an attractive alternative to physical and chemical restoration treatments as it has the possibility to reduce the time and cost necessary to restore a site, while establishing a final environment with a high likelihood of sustainability. Biostimulation is one form of bioremediation which operates by stimulating the growth of an environment's naturally occurring organisms (often bacteria) by providing them with nutrients to accelerate the remediation (USGS, 2011). In our ISRU case, the bacteria would accelerate the precipitation of uranium and other heavy metals in the mined aquifer. While the biological fixation of uranium and other heavy metals has been demonstrated in numerous laboratory studies (Anderson *et al.*, 2003; Gorby & Lovley, 1992; Hatzinger, 2004; Lovley & Phillips, 1992; N'Guessan *et al.*, 2008; Phillips *et al.*, 1995), the type of biostimulant and its subsequent efficacy appear to differ from site-to-site (e.g. Anderson *et al.*, 2003; Hatzinger, 2004). This is likely due to many factors including but not limited to initial microbial community composition, intrinsic factors of the mine sediment, different levels of traditional remediation strategies applied, and differences in the aquifer water utilized (i.e. uranium level, total dissolved solids level, chemical treatment, etc.) (Luo *et al.*, 2007). Our previous study demonstrated effective reduction of soluble uranium concentration via biostimulation with tryptone utilizing post-mined waters and sediment from the Cameco, Inc. Smith Ranch Highland (SRH) site near Douglas, WY.

Along with identifying a proper biostimulant for the environment specific to SRH, our previous microcosm-based study focused on evaluating measurable markers of

biological reducing activity. Isotopic fractionation has been demonstrated to occur differently in biologically-induced systems as compared to abiotic systems (Bopp *et al.*, 2010; Botz *et al.*, 1996). This is largely due to enzymatic selection of reactants having a higher affinity for select isotopes over others, which is not a phenomenon observed in abiotic systems. Our study monitored uranium- and carbon-isotopic stable isotope ratios with the goal of observing a measurable $^{238}U/^{235}U$ ratio shift or $^{13}C/^{12}C$ dissolved inorganic carbon (DIC) ratio shift that correlated with biological reduction of U(VI) to U(IV). The $^{238}U/^{235}U$ ratio shift appears to be more directly associated with U(VI) reduction than the $^{13}C/^{12}C$ shift, which did show a general trend toward ^{12}C utilization. However in measuring DIC, we were also able to quantify total carbonate in the system, which correlated quite well with microbial biomass and suspected microbial metabolic activity (Kern, 1960).

Our laboratory work was designed with the objective of leading to an in-field biostimulation study at the Cameco, Inc. SRH site, which is ongoing as of this writing. However, following the microcosm study and prior to undertaking this field trial, a few key questions existed which required attention. Therefore, the column study reported herein was proposed, which intended to: 1) demonstrate the efficacy of tryptone at promoting the reduction of U(VI) in a continuous flow system, 2) better quantify the amount of tryptone required to promote the reduction of U(VI), 3) better understand where in the entire restoration process that biostimulation may be most beneficial, and 4) further evaluate the measurable metrics and microbial analyses utilized in the microcosm study. The data gathered in the column study have provided necessary insight for planning and understanding the ongoing in-field biostimulation study and should be

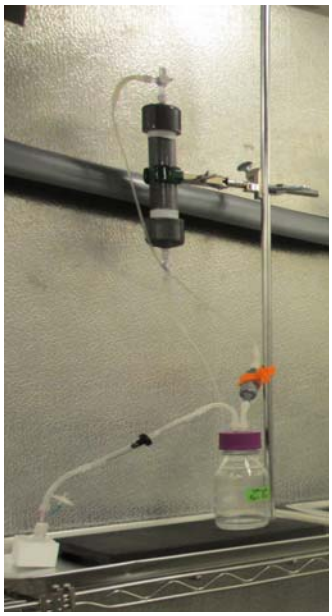
important in future application of biostimulation as part of an ISRU site restoration protocol.

Methods

Column Design and Setup

In an ultra-pure nitrogen headspace hood, columns were prepared using 1" x 6" clear PVC pipe each was packed with 120g of the homogenized aquifer core sample comprised of three discontinuous feet of post-mined aquifer core. Following fill with aquifer solids, both ends of the PVC pipe were wrapped with Teflon tape and capped with PVC end caps. Specific details on end cap contents and overall column construction can be found in the accompanying Final Technical Report. Columns were then hooked into the tubing and attached to a ring stand in a 15^{o}C cold room. Figures 1 and 2 provide a visualization of the final column setup.

Syringe pumps (infusion pump model 220, KD Scientific) were setup with 140mL luer-lock syringes (Covidien) containing the water type needed for each column. Table 1 includes a summary of the starting total dissolved solid (TDS) and soluble uranium concentration of each water type, which will be simply referred to as High, Medium, and Low TDS/U water for the remainder of this report. Table 2 includes a summary of the water type/tryptone stimulation combinations which were utilized to create the 4x4 treatment setup in this study. All syringe pumps, each of which supplied four columns (visible in figure 2), ran at a rate of 1mL/hour throughout this study unless noted otherwise.




Figures 1 & 2. Pictures of one column and sampling bottle setup along with a view of a four column block attached to a single syringe pump.

Water Sample	**TDS**	**Soluble U**
P-104 (Low)	0.444 g/L	0.5 mg/L
P-089 (Medium)	1.228 g/L	1.5 mg/L
P-245 (High)	1.952 g/L	7.3 mg/L

Table 1. Initial reported value for TDS and soluble U for each water type. If should be of note that the soluble U values were determined to be somewhat higher after the equilibration period in the columns.

		Tryptone Level			
		No Add	**20mg/L**	**200mg/L**	**2000mg/L**
Water Type	**Deionized**	DI No Add	DI 20	DI 200	DI 2000
	Low TSD/U	Low No Add	Low 20	Low 200	Low 2000
	Medium TDS/U	Med No Add	Med 20	Med 200	Med 2000
	High TDS/U	High No Add	High 20	High 200	High 2000

Table 2. The 4x4 design of the column study and the subsequent 16 water type/tryptone level columns. The 2000mg/L level of tryptone was the concentration of biostimulation utilized in the microcosm study.

Column Water Flow

Following initial setup, each column underwent a five week equilibration period to normalize column conditions to those provided by the water samples. During this equilibration period, no tryptone was added to any column and therefore, the samples taken during this time period are noted as negative numbers (day -35 to day -7) with the

first addition of tryptone being denoted as day 0 in the study. During this period, column pore volume was also determined utilizing a bromide tracer procedure. The columns showed a range of pore volumes from 42.41mL to 48.21mL with an average column pore volume of 44.41mL. Additionally, no evidence of short-circuiting in any column was observed.

Upon completion of the five week equilibration period tryptone was added to sample waters during the syringe fill procedure to final concentrations of 20mg/L, 200mg/L, and 2000mg/L (the concentration utilized in the microcosm study) along with a no tryptone negative control. Tryptone biostimulation was conducted from day 0 until day 269. At day 269, all waters were replaced with upgradient water collected from monitoring well 420 (M420) to evaluate the remobilization potential of any reduced uranium in the columns. For this portion of the study, flow rates were slowed to 0.5mL/hour for nearly eight weeks and then to 0.05mL/hour for the final four weeks (competing the study at day 354) to better simulate slower flow of upgradient water. During this upgradient water period, no tryptone stimulation was utilized.

Sampling and Processing

While all column waters were collected during this study, they were split into two portions during each sample week. The first portion contained the flow through waters up to day five of each week. This portion was moved into sterile 90mL containers and frozen at -80°C or -20°C to be held for PLFA analyses.

The second portion included flow through waters for the last two days of each sample week. This ~48mL sample was processed by filtration and acid preservation to be analyzed for soluble uranium and selenium concentrations (water chemistry),

uranium-isotope ratios, carbon-isotope ratios (no acid preservation), and microbial freezedowns (no filtration or acid preservation).

Following completion of all water flow, the intracolumnar sediment was collected in an argon headspace hood, which maintained the anaerobic nature of these materials. The sediment was split into an influent half and an effluent half to allow for elucidation of differences across the length of the column without minimizing sample size potential.

Results and Discussion

Uranium Reduction

Soluble uranium concentration decreased generally in the 200mg/L and 2000mg/L tryptone stimulation columns. In the High2000, Med2000, Low2000, Med200, and Low200 columns, a consistent significant decrease was observed following the initial decrease (day 42 for 2000mg/L and day 112 for 200mg/L) events. The High200 column saw a significant decrease at day 112, but it was followed by a rebound in soluble uranium concentration to initial levels. No significant reductions as compared to the No Add controls were observed at the 20mg/L treatment levels over the course of this study. All of the measured uranium concentrations are included in figure 3 organized by tryptone stimulation level. To highlight the most notable positive results at both the 2000mg/L and 200mg/L tryptone stimulation levels: a) in the High2000 column, a 99.3% decrease relative to the starting concentration measured in the equilibration period was observed and b) in the Medium200 column, an 82.6% decrease relative to the starting concentration measured in the equilibration period was observed.

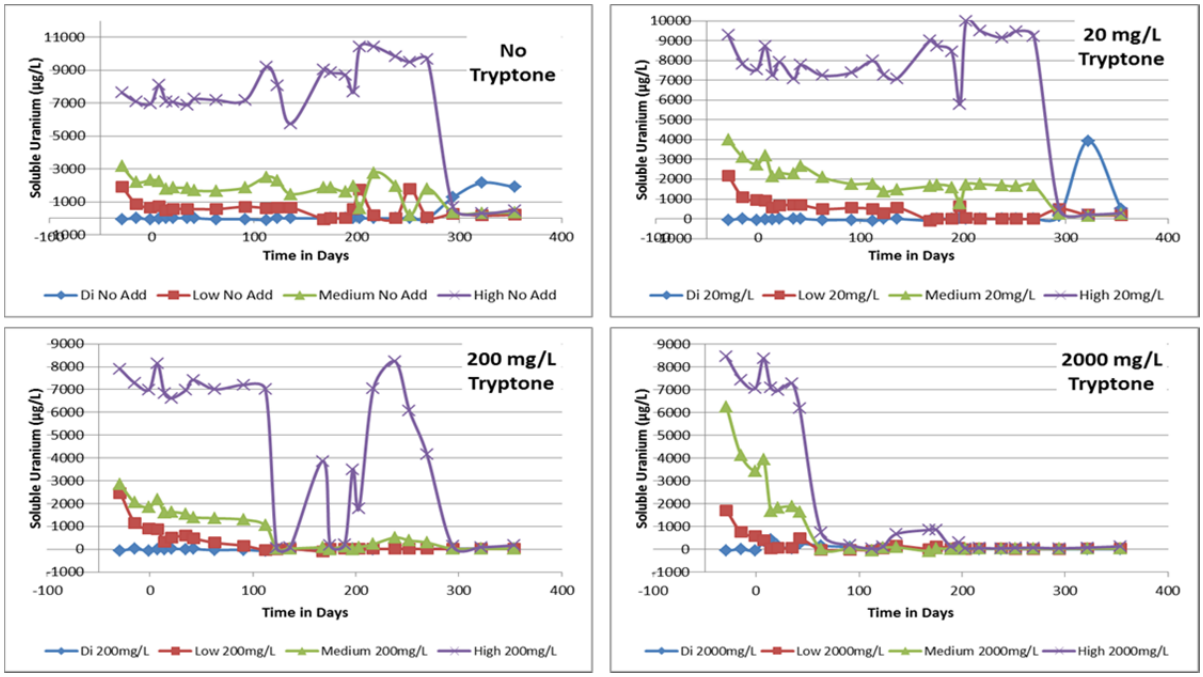
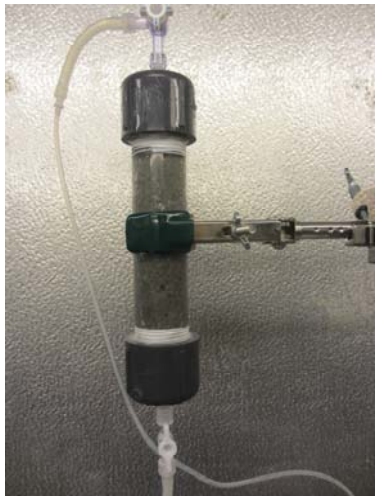


Figure 3. Measured soluble uranium organized by level of tryptone biostimulation for the column study. Noteworthy time points: Equilibration period – Day -35 to day 0, Tryptone stimulation period – Day 0 to day 269, Upgradient water period – Day 269 to day 35.

An altered redox state in the 200mg/L and 2000mg/L tryptone stimulated columns was supported by similar empirically observable traits as in the microcosm study. The most notable was the color change from the starting tannish to a darker blackish color. The noteworthy color change in both the column sediments and in the effluent tubing can be seen in figures 4 &5. The appearance of these black precipitates was first observed at day 42 in the High2000 & Medium2000 columns and at ~day 110 in the Medium200 column. These types of black precipitates often indicate the presence of iron sulfide (FeS) produced from biological reduction of sulfate, which is a regular process of the sulfate-reducing bacteria which are often demonstrated to possess heavy metal reduction capabilities.

Figures 4 & 5. Visualization of the observable differences between columns in which a reducing environment is suspected to have been created (left) versus one in which little reduction has been demonstrated to have taken place (right). Note the color changes of the tubing as well, which was demonstrated to be sediment and not discoloration by wash out during upgradient water treatment.

Uranium Isotopic Results

Uranium isotopic ratios once again provide evidence of microbially-induced valence reduction of uranium, especially in the Medium200, High200, Medium2000, and High2000 columns. Over the course of the experiment, the $^{238}U/^{235}U$ ratios in these columns' waters moved significantly toward a lower isotopic ratio (Figure 6), suggesting a preferential precipitation of ^{238}U within those columns, which is similar to the microbial affinity for ^{238}U previously reported (Bopp *et al.*, 2010) and observed by our group in the microcosm study. All of these samples also produced ratios outside of the reported naturally-occurring ratio range for the high-uranium silicate mineral zircon (Hiess *et al.*, 2012). This isotopic shift is further evidence for biogenic valence reduction of the uranium in these samples and further supports this as a monitoring metric for field applications. The $^{238}U/^{235}U$ ratio appears to be very sensitive to changes in soluble uranium concentration, including increases which were observed in this study. Our ability to utilize the effluent waters suggests that in a field application of bioremediation,

this isotopic measurement could be made from monitoring well waters, without the need for post-remediation coring.

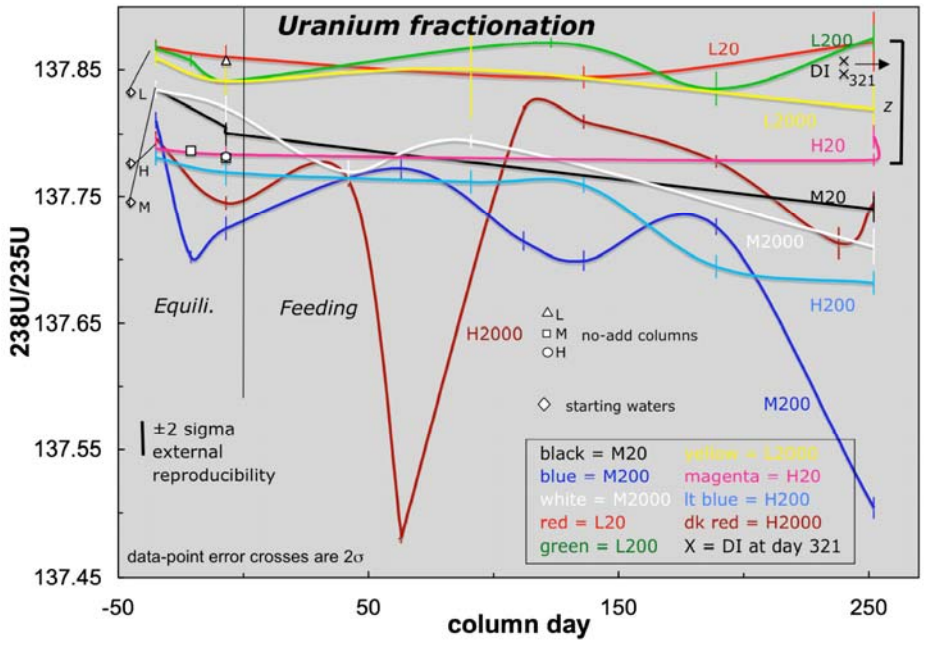


Figure 6. $^{238}U/^{235}U$ ratio values. A designation of a 2 sigma error bar size is included on the left side of the graph. The bracket on the right side of the graph designates the naturally-occurring $^{238}U/^{235}U$ ratio range determined utilizing zircon (Hiess *et al.*, 2012). Abbreviations: Low TDS/U water (L), Medium TDS/U water (M), High TDS/U water (H), Tryptone stimulation level noted by their number in mg/L.

Carbon Isotopic and Carbonate Results

The DIC carbon isotopic results from this column study demonstrated less clear trends than were previously observed in the microcosm study. Overall, a downward trend was observed which appeared to grow stronger with increasing levels of tryptone, suggesting that the DIC in the solution is becoming isotopically lighter as the $^{13}C/^{12}C$ ratio decreases. This suggested preferential use of ^{13}C is different than the general trend observed in the microcosm study. It is important to note that the starting value of $^{13}C/^{12}C$ ratios for all of the waters was between +2 and +14. In the microcosm study, the waters had starting values between -11 and -15 with the highest observed value in the entire study was near -8 which we suspected was due to biological activity. With the

understanding that changes in the $^{13}C/^{12}C$ ratio due to biological activity are due to increased enzyme affinity for one isotope over the other (Botz *et al.*, 1996; Hellings *et al.*, 2000; McLaughlin *et al.*, 2011; Whiticar, 1999), a difference in starting water $^{13}C/^{12}C$ ratio values of this magnitude would significantly impact which enzymes would be most beneficial in a microbial population. More results in these trends need to be observed before we can confidently endorse DIC carbon isotopic ratio analyses for field viability.

The data output from the UW SIF provided us with ability to calculate carbonate (CO_3) present in each sample. The concentration of carbonate which can be correlated with microbial metabolic activity appeared to sync quite well with reductions soluble uranium concentration once again. Graphs for the High2000, Medium2000, Medium200, and Low200 columns display this trend in figure 7. The highest levels of microbial activity sync well with the highest rate of reduction in soluble uranium concentration. This trend also supports the explanation of biological reduction of uranium and has been observed in both studies by our group, so should transition well to a field-based setting.

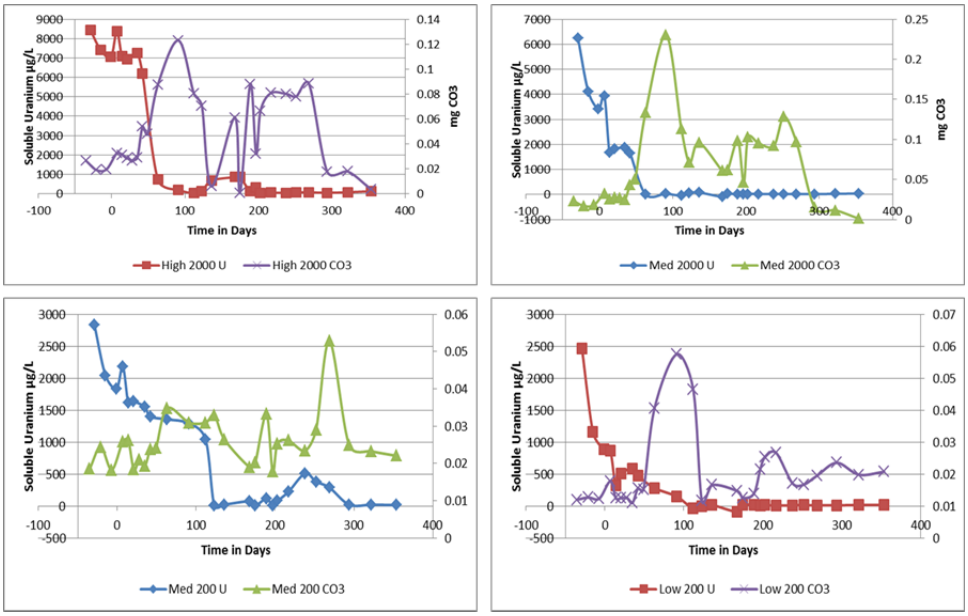


Figure 7. Soluble uranium concentration and carbonate concentration. For all graphs, uranium concentration in µg/L is on the left Y-axis and carbonate concentration in mg is on the right Y-axis.

Microbial Biomass and Community Analyses

To date, many of the phospholipid fatty acid (PLFA) analyses are still in queue to be analyzed. An initial batch which evaluated the ability of the technique to quantify and discriminate the fatty acids from the concentrated water samples, rather than from soil/sediment as previously performed. The data demonstrated suitable recovery of fatty acids for each sample analyzed and demonstrated considerable biomass differences in the effluent water for each tryptone treatment level. These data points appear to demonstrate higher levels of microbial biomass with higher levels of stimulation and uranium (VI) reduction similar to what was observed in the microcosms.

Summary

The data from our column study demonstrate the continued efficacy of tryptone as a biostimulant to promote the reduction of U(VI) in a continuous flow system. However, there appears to be a critical limit of tryptone which needs to be added (somewhere between 20mg/L and 200mg/L) to see general efficacy, with that concentration increasing as uranium/TDS concentrations in the water increase. In terms of further analyzing measurable metrics, uranium isotopic fractionation and carbonate concentration once again sync well with suspected biological uranium (VI) reduction, while carbon isotopic analyses did not present as clear of a correlation or pattern as previously observed.

As noted earlier in this report, the data generated in this column study was utilized to assist in planning a field trial currently being conducted at Smith Ranch-Highland. This study is expected to have a much longer run time as we are treating upwards of 750,000 gallons in each well pattern. The results of this study are also planned to be published in scientific journals once complete.

Literature Cited

Anderson, R. T., Vrionis, H. A., Ortiz-Bernad, I. & other authors (2003). Stimulating the in situ activity of Geobacter species to remove uranium from the groundwater of a uranium-contaminated aquifer. *Appl Environ Microbiol* **69**, 5884-5891.

Boberg, W. (1981). Some Speculations on the Development of Central Wyoming as a Uranium Province. In *Guidebook-1981, 32nd annual field conference-Teton Village, September 20-22, 1981: Energy Resources of Wyoming*, pp. 161-180. Edited by S. Reid & D. Miller. Jackson Hole, WY: Wyoming Geological Association.

Bopp, C. J., Lundstrom, C. C., Johnson, T. M., Sanford, R. A., Long, P. E. & Williams, K. H. (2010). Uranium U-238/U-235 Isotope Ratios as Indicators of Reduction: Results from an in situ Biostimulation Experiment at Rifle, Colorado, USA. *Environ Sci Technol* **44**, 5927-5933.

Botz, R., Pokojski, H. D., Schmitt, M. & Thomm, M. (1996). Carbon isotope fractionation during bacterial methanogenesis by CO2 reduction. *Org Geochem* **25**, 255-262.

Cheng, Y. J., Holman, H. Y. & Lin, Z. (2012). Remediation of Chromium and Uranium Contamination by Microbial Activity. *Elements* **8**, 107-112.

Gorby, Y. A. & Lovley, D. R. (1992). Enzymatic Uranium Precipitation. *Environ Sci Technol* **26**, 205-207.

Hatzinger, P. (2004). Interim Report I: Microcosm Tests, pp. 1-6: Shaw Environmental & Infrastructure, Inc.

Hellings, L., Van den Driessche, K., Baeyens, W., Keppens, E. & Dehairs, F. (2000). Origin and fate of dissolved inorganic carbon in interstitial waters of two freshwater intertidal areas: A case study of the Scheldt Estuary, Belgium. *Biogeochemistry* **51**, 141-160.

Hiess, J., Condon, D. J., McLean, N. & Noble, S. R. (2012). U-238/U-235 Systematics in Terrestrial Uranium-Bearing Minerals. *Science* **335**, 1610-1614.

Kern, D. M. (1960). The hydration of carbon dioxide. *Journal of Chemical Education* **37**, 14.

Langmuir, D. (1978). Uranium Solution-Mineral Equilibria at Low-Temperatures with Applications to Sedimentary Ore-Deposits. *Geochim Cosmochim Acta* **42**, 547-569.

Lovley, D. R. & Phillips, E. J. P. (1992). Bioremediation of Uranium Contamination with Enzymatic Uranium Reduction. *Environ Sci Technol* **26**, 2228-2234.

Luo, J., Weber, F. A., Cirpka, O. A., Wu, W. M., Nyman, J. L., Carley, J., Jardine, P. M., Criddle, C. S. & Kitanidis, P. K. (2007). Modeling in-situ uranium(VI) bioreduction by sulfate-reducing bacteria. *J Contam Hydrol* **92**, 129-148.

McLaughlin, J. F., Frost, C. D. & Sharma, S. (2011). Geochemical analysis of Atlantic Rim water, Carbon County, Wyoming: New applications for characterizing coalbed natural gas reservoirs. *AAPG Bull* **95**, 191-217.

N'Guessan, A. L., Vrionis, H. A., Resch, C. T., Long, P. E. & Lovley, D. R. (2008). Sustained removal of uranium from contaminated groundwater following stimulation of dissimilatory metal reduction. *Environ Sci Technol* **42**, 2999-3004.

Phillips, E. J. P., Landa, E. R. & Lovley, D. R. (1995). Remediation of Uranium Contaminated Soils with Bicarbonate Extraction and Microbial U(Vi) Reduction. *J Indust Microbiol* **14**, 203-207.

Uhrie, J. L., Drever, J. I., Colberg, P. J. S. & Nesbitt, C. C. (1996). In situ immobilization of heavy metals associated with uranium leach mines by bacterial sulfate reduction. *Hydrometallurgy* **43**, 231-239.

USGS (2011). Bioremediation: United States Geological Survey.

Whiticar, M. J. (1999). Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chem Geol* **161**, 291-314.