Final Technical Report, 2014

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Institution: Oregon State University

Title: Microbial Activity and Precipitation at Solution-Solution Mixing Zones in Porous Media – Subsurface Biogeochemical Research

SC Division: SC-23.1

Program Manager: Roland Hirsch

Please briefly (16000 chars or less) summarize your most recent results to date:

This project was conducted collaboratively between co-PIs Rick Colwell, Dorthe Wildenschild and Brian Wood (OSU), Robin Gerlach and Andrew Mitchell (Montana State University) and George Redden (Idaho National Laboratory). The students involved in the research included Megan Kaufman, Chris Neighbor, and Gabe Iltis (OSU) and James Connolly (MSU). Ellen Lauchnor was a postdoctoral fellow at Montana State University. Experimental work was started in August 2009. Conference calls that included the whole project team as well as subsets of the team were conducted frequently (every other week) to coordinate the experimental and modeling efforts. The entire team met for two days in Bozeman, MT in October 2010 to coordinate research and routinely at scientific meetings (e.g., AGU Fall Meeting) whenever several team members were in attendance. The progress attained by OSU investigators and students is summarized here; however, where appropriate, reference is made to collaborative activities that co-=occurred at MSU and INL.

Research Objective:
Microbially accelerated calcite precipitation in aquifers will co-precipitate strontium (Sr) and is a tool for remediation of Sr-90 in contaminated aquifers. The incorporation of the contaminant metal into the calcite occurs naturally at low rates but may be enhanced by the addition of urea to a natural microbial community in an aquifer. Hydrolysis of the urea by microbial urease liberates ammonium and causes an increase in aquifer pH and alkalinity. The alkalinity shifts the bicarbonate equilibrium causing calcium carbonate to precipitate out of solution according the following formulas:

$$\underset{\text{(urea)}}{CO(NH_2)_2} + 2H_20 \rightarrow 2NH_4^+ + CO_3^{-2}$$

$$Ca^{+2} + CO_3^{-2} \rightarrow CaCO_3 \text{ ppt}$$

(see Whiffen et al. 2007. *Geomicrobiol. J.* 24: 417-423)

The goal for this research was to understand how best to add compounds to receptive microbial communities in porous media in order to achieve optimal calcite precipitation in a volumetrically significant space and to understand the physiological health of the cells that are responsible for the calcite precipitation. The specific objectives were to: (1) develop better tools for visually examining biofilms in porous media and calcium carbonate precipitation being mediated by microbes in porous media, and (2) demonstrate the effectiveness of using that tool within a flow cell model system. The overall plan is depicted in Figure 1.

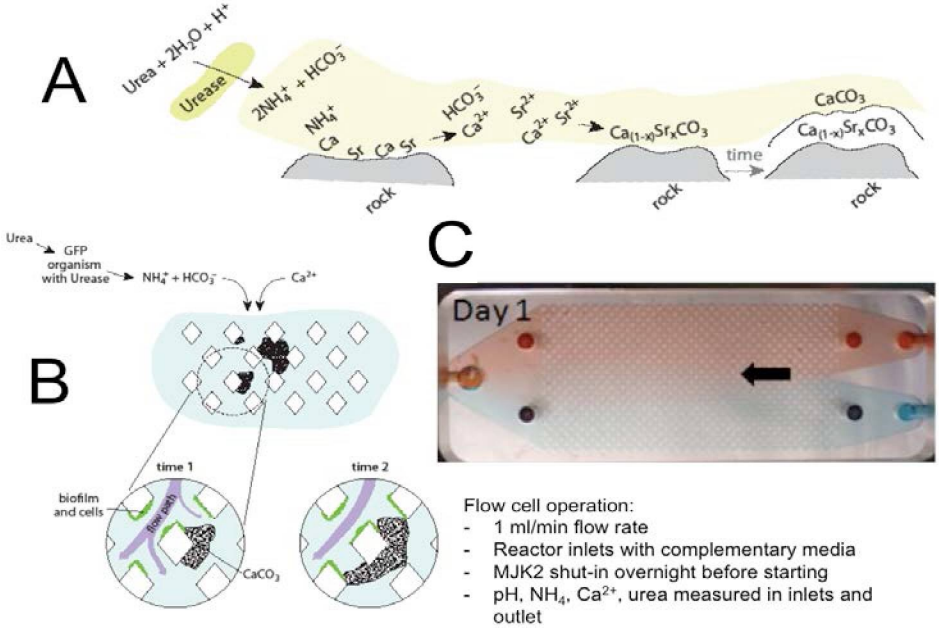

Figure 1. Calcite precipitation/90-Sr removal scheme (A), depiction of related sequential events in a flow cell reactor for studying the process (B), and actual reactor experiment (C).

Research Progress and Implications

Model Microbes: Progress towards accomplishing the first objective was achieved by constructing two ureolytic, GFP containing microorganisms that could be visualized within a flow cell undergoing ureolysis and subsequent calcium carbonate precipitation. *E. coli* AF504*gfp* and *P. aeruginosa* AH298 (both GFP organisms) were transformed with a pJN105 vector carrying PCR amplified urease genes from *E. coli* DH5α(pURE14.8) ligated at the *PstI* and *SpeI* restriction sites as described earlier (for details, see Kaufman 2011 and Connolly et al. 2013). Sequencing of the pJN105 vector with the urease insert revealed the apparent insertion of two nucleotides into the cloned segment of the *ureFG* genes during the cloning of our ureolytic organism. However, when the sequence was analyzed with an older "base calling" software, there was no difference between the DH5α(pURE14.8) and our insert. Despite this lack of difference, the constructed organisms were both ureolytically active in urea broth tests with the addition of L-arabinose. M. Kaufman's thesis includes the details of how the cloning procedures were conducted to create the model microbes (Kaufman, 2011) and this is shown generally in Figure 2.

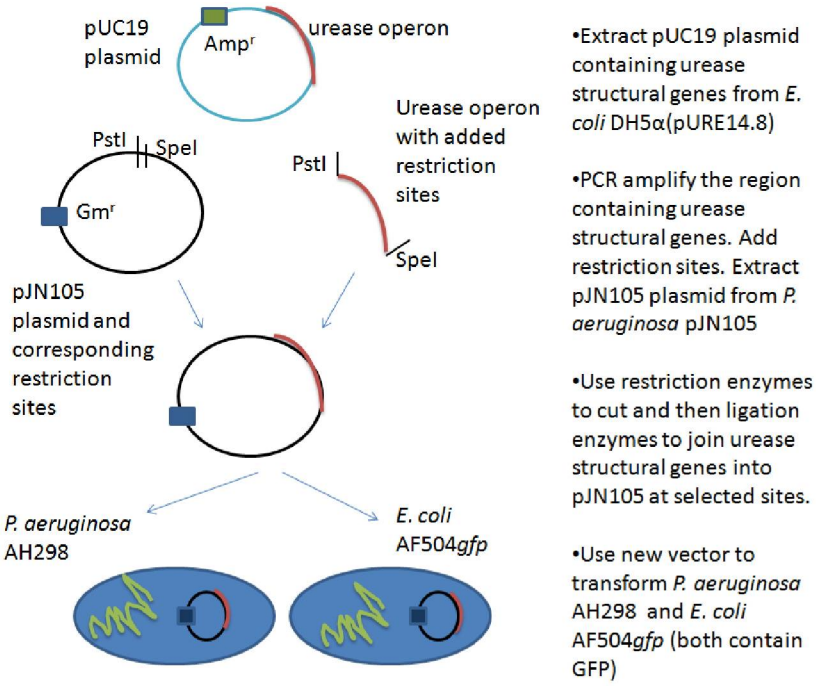

Figure 2. Overview of the cloning procedures used to construct the GFP ureolytic organisms MJK1 (*P. aeruginosa* AH298) and MJK2 (*E. coli* AF504*gfp*). Amp^R refers to an ampicillin resistance marker and Gm^R to a gentamicin resistance marker.

The cloning experiments resulted in a molecular vector with an insert possessing the ability to hydrolyze urea and are shown in Figure 3.

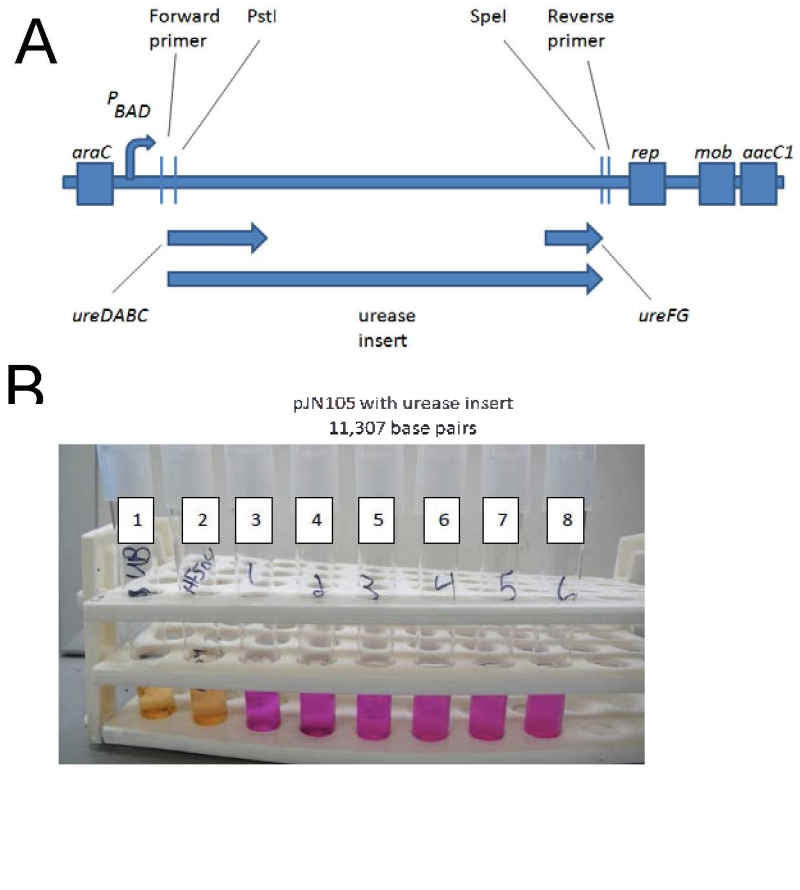

Figure 3. (A) Linearized map of vector pJN105 and insert (cloned fragment of DH5α(pURE14.8)). Arrows *ureDABC* and *ureFG* are previously sequenced sections of *E.coli* DH5α(pURE14.8). Arrow 'urease insert' are the cloned genes transferred into the pJN105. araC P_{BAD} is the promoter, rep encodes trans-acting replication protein, mob encodes the plasmid mobilization functions, and aacC1 imparts Gm^R. (B) Representative urea broth results with pink indicating urea hydrolysis. Results from left are: (1) uninoculated urea broth, (2) negative control AF504*gfp*, (3-8) clones of transformed MJK2.

Experiments in CMM base medium with L-arabinose demonstrated that the transformed cells (strains MJK2 and MJK1) were capable of creating the changes in pH, ammonia, calcium, and urea in the medium and precipitation of calcium carbonate consistent with the presence of active urease. The transformants were appropriately active relative to non-transformed strains. MJK2 performed especially well in flask studies. Addition of supplemental nickel to the medium boosted urease activity, an observation consistent with previous findings that urease activity is enhanced in the presence of higher concentrations of nickel. It is not clear why the MJK2 outperforms the transformed *Pseudomonas* strain (MJK1).

Flow cells: To achieve the second objective we used a 2D porous media flow cell to create parallel flows of complementary solutions. The respective solution would contact through mixing at a location in the flow cell where ureolytic organisms could precipitate calcium carbonate. At other locations, the incomplete nature of either medium by itself would not allow precipitation even if the microbe is present. The flow cell system is shown in Figure 4.

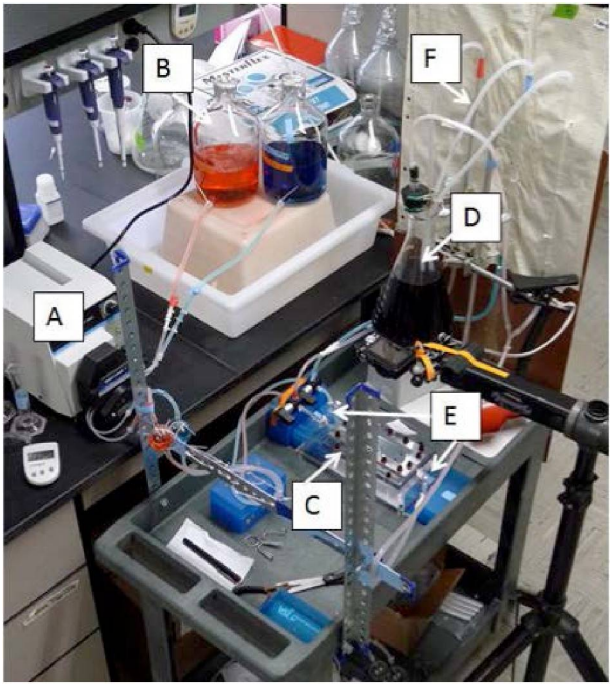

Figure 4. Flow cell system (4x8 cm) with peristaltic pump (A), media reservoirs (B), reactor (C), waste container (D), injection and sample ports (E) and piezometers (F).

We expected that the growth of ureolytic microorganisms and resulting calcium carbonate precipitation would create a wider mixing zone by disruption of the bulk flow direction and introduction of transverse flow paths. The flow cell was transparent to microscopic and macroscopic imaging thus permitting observation of the resulting mixing patterns, localization of cells and precipitation at a pore scale level.

MJK2 was established in the flow cell and calcium carbonate precipitation resulted. Visual inspection and photographs showed evidence of precipitation mainly in the mixing zone of the two parallel flows of complementary media (Figure 5). Furthermore, a widening of the mixing zone over time with the presence and activity of the ureolytic organism in the flow cell was apparent as early as day three and was even more evident on day four. Flow cell operated under the following conditions:

- 1 ml/min flow rate
- Reactor inlets with complementary media

- MJK2 shut-in overnight before starting
- pH, NH_4, Ca^{2+}, urea measured in inlets and outlet

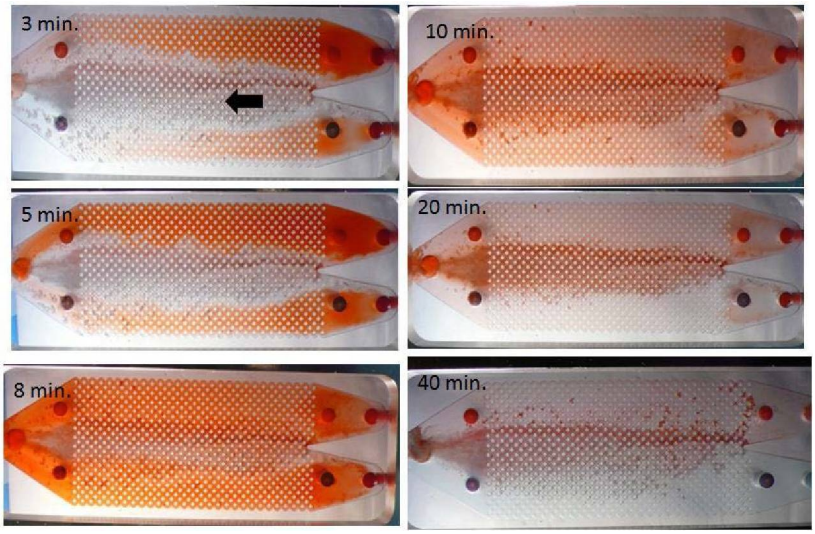

Figure 5. Time-lapse of tracer injection in flow cell on day 3 of Ca carbonate precipitation by MJK2. Parallel flows of complementary media (CMM+urea [below], CMM+Ca [top]) run right to left (direction of arrow). Red dye was injected to both ports simultaneously.

The increased area of mixing/reaction was seen in the results of dye tracer studies (Figure 5). A tracer study in the uninoculated flow cell demonstrated low flow in the center region (data not shown); however, that area is narrower than in the experimental flow cell containing the MJK2 by about 0.7 cm. These results imply that in our flow cell, the decrease in porosity by day three was accompanied by decreasing surface area that is contacted by the migrating fluid. We also noted progressive precipitation downstream over the 8 cm length of the flow cell. The effective pore volume of the flow cell apparently decreased throughout the study, but this could not be calculated exactly due to an unknown flow rate on day three when the tracer study was performed.

Because MJK2 is both ureolytic and has GFP we could visualize the interactions between microorganisms, flow paths and precipitation. GFP allowed us to locate the ureolytic organisms in the flow cell during calcium carbonate precipitation (in Figure 6, the cells are the small white objects). On day two, direct microscopic evidence of GFP fluorescing-*E. coli* MJK2 was noted in all areas of the flow cell but especially in the mixing zone at the front of the flow cell. By day six after more precipitation had occurred within the flow cell the organism could not be seen microscopically. The microscopy also supported the macroscale observation that MJK2 was precipitating calcium carbonate preferentially in the mixing zone as evidenced by the blurred image due to the mineral.

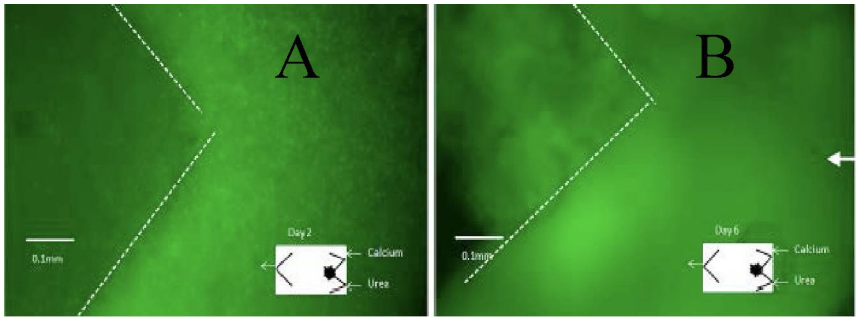

Figure 6. Photomicrograph (200x; GFP filter) of MJK2 and calcium carbonate precipitates in flow cell at the first post (see inset) where two media merge on days 2 (A) and 6 (B). Flow is right to left. Dashes outline the edge of the first post.

While the GFP may not be able to tell us about the overall activity of cells in a heterogeneous environment like the flow cell, future studies are likely to find the constructed ureolytic GFP organism helpful. For example, the constructed ureolytic GFP organisms can be used within a more controlled, uniform environment such as a 96 well assay plate to define the activity of the cells under controlled conditions. In this environment one would be able to measure cellular activity in CMM base medium with urea, perhaps varying conditions to optimize activity. Alternatively, future studies interested in cell location and changes to their distributions over time may use the constructed ureolytic GFP strains in smaller flow cells such as micro flow cells or capillary tubes as these can be used with a confocal microscope. In these smaller flow regimes more detailed imaging including 3D information may be ideal for linking the cells' activities and locations in the context of overall distributions of calcium carbonate precipitation.

Both the construction of ureolytic GFP organisms and the investigation of parallel flow mixing zones are first steps towards understanding and predicting outcomes of larger, field-scale remediation efforts.

Modeling: Integration of data from a 3-dimensional column into a model was performed by adapting COMSOL to consider phenomena such as pressure drops in the column studies. Gabe Iltis (OSU) worked closely with James Connolly (MSU) to troubleshoot the adaptation of this software for imaging applications.

Project Outcomes:

Summary

- Two microbes were designed with urease functionality and green fluorescent protein (GFP).
- Urease functionality in the microbes was enhanced by addition of Ni.
- One of the microbes, MJK2, was observed microscopically, along with Ca carbonate precipitates, during flow cell experiments aimed at demonstrating strategies for amendment addition.
- Presence of MJK2 enhanced the mixing of the two media and promoted a broader pattern of calcium carbonate distribution.
- Distinguishing between fluorescing cells and Ca carbonate was difficult.

In the final stages of the project, Colwell helped to organize a breakout group session at the 2012 SBR PI meeting that aimed to consider new directions for subsurface biogeochemical research. The presentation and ensuing discussion focused on how SBR research contributions would be valuable in addressing new fundamental research areas in energy production, usage and storage. Also, the findings from experiments aimed at design and testing of the GFP-constructs and their first use in flow cells was published (Connolly et al., 2013). This research also contributed to a manuscript describing the breadth of subsurface biogeochemical processes and future directions of the field which was published in a 2013 issue of *Reviews in Mineralogy and Geochemistry*.

Following is your previous report of products delivered:
None

Please briefly (7000 chars or less) describe papers and other products delivered:
Publications:
Colwell, F. and S. D'Hondt. 2013. Nature and Extent of the Deep Biosphere. In, *Reviews in Mineralogy and Geochemistry, Volume 75, Carbon in Earth (*R. M. Hazen, A. P. Jones, J. A. Baross, eds.). Mineralogical Society of America, Geochemical Society, pp. 547-574.

Connolly, J., M. Kaufman, A. Rothman, R. Gupta, R. Gerlach, M. Schuster, and F. Colwell. 2013. Construction of two ureolytic model organisms for the study of microbially induced calcium carbonate precipitation. J. Microbiol. Meth. 94: 290-299.

Kaufman, M. J. 2011. Construction of a model organism for performing calcium carbonate precipitation in a porous media reactor. Oregon State University, Corvallis. MS Thesis. 114 pages.

Wildenschild, D., M.L. Rivers, M.L. Porter, G.C. Iltis, R.T. Armstrong, and Y. Davit, 2012. Using Synchrotron-based X-ray Microtomography and Functional Contrast Agents in Environmental Applications, Invited book chapter for 2nd edition of *Tomography and Imaging of Soil-Water-Root Processes* edited by S.H. Anderson and J.W. Hopmans. CSA Books.

Presentations:
Colwell, F. 2012. Presentation in Breakout Session 3: Subsurface Biogeochemical Processes Associated with Energy Production, Usage and Storage. Subsurface Biogeochemical Research Annual Meeting, April 30-May 2, 2012.

Iltis, G., Y. Davit, R.T. Armstrong, B,D, Wood, and D. Wildenschild, 2011. Imaging Microbial Biofilm in Three-dimensional Opaque Porous Media with X-ray Tomography (Invited). *Interpore 2011, 3rd International Conference on Porous Media & Annual Meeting of the International Society for Porous Media*, Bordeaux, France, March 28-31, 2011.

Iltis, G., Y. Davit, J. Connelly, B. D. Wood, D. Wildenschild, 2010, In situ imaging of biofilm within opaque porous media (Invited). Eos Trans. AGU, Fall Meet. Suppl., Abstract NS33A-03.

Iltis, G., R.T. Armstrong, and D. Wildenschild, 2010. Visualization and quantification of biofilm architecture within porous media using synchrotron based x-ray computed microtomography. Geox2010: 3rd International Workshop on X-Ray CT for Geomaterials, New Orleans, LA, March 1-3. 2010.

Following is your previous report of other notes concerning the project:
None

Please provide any new notes (7000 chars or less) concerning the project:

A new OSU graduate course was developed as a result of this research. Applied Imaging and Image Processing (ENVE 599 Special Topics; 3 credits; spring term 2012) involves a combination of lectures and hands-on exercises using primarily freeware and university-licensed software (ImageJ and Matlab). The course focus is on 3D x-ray based methods, however, many of the processing steps port easily to other imaging modalities.

Other Project Information Sources:
Project URL: None

Related URL at institution:
None