

## **PROGRESS REPORT:**

### **Investigating In Situ Bioremediation Approaches for Sustained Uranium Immobilization Independent of Nitrate Reduction**

ERKP555

Proposal for LAB 04-06

Environmental Remediation Sciences Program

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#### **Research Objective:**

The daunting prospect of complete nitrate removal at DOE sites, such as the ERSP Oak Ridge Field Research Center (FRC), provides strong incentive to explore bioremediation strategies that will allow for uranium bioreduction and long-term stabilization in the presence of nitrate. The cost and effort required for complete nitrate removal from the FRC and similar DOE-contaminated sites may prove to be unworkable. For example, field tests of uranium bioreduction at the FRC have shown that nitrate levels rebound quickly and completely after cessation of active biostimulation.

The goal of this research is to improve our understanding of microbiological and geochemical interactions in low pH high nitrate environments. It is our hypothesis that reduced metals and radionuclides can be stable in low pH deoxygenated waters, such that if even a fraction of the microbial reduction potential is directed toward biologically mediated reduction of U(VI) and metals, there may be no need for complete and sustained nitrate/nitrite removal. In other words, so long as the water is kept anaerobic, stability of immobilized species may be sustained and additional metal and U reduction and immobilization achieved, thereby mitigating the necessity for nitrate removal. Our approach is based on optimizing biogeochemical conditions in enrichment cultures from FRC sediments. Additionally, experiments detailing the biogeochemical coexistence of U(IV) and  $\text{NO}_3^-/\text{NO}_2^-$  are crucial for predicting the long-term evolution of bioreduced uranium in groundwater and sediment. The addition of phosphate through organophosphate additives such as triethylphosphate (TEP) is anticipated to stabilize the bioreduced system against remobilization.

#### **Research Progress and Implications:**

This report summarizes work after 2 years of a 3-year project.

### *Nitrate-indifferent uranium reduction*

Sediment microcosm enrichments and subsequent transfers to growth media have demonstrated that microbial communities from FRC sediments do indeed have the capacity to reduce uranium in the presence of nitrate when stimulated with the appropriate donor and redox conditions. Those enrichments demonstrating this capacity were analyzed by various molecular biology techniques to determine the phylogenetic diversity of the microbial community. Anaerobic enrichments were prepared with MOPS/TRIS buffers at pH 4.9-7, bicarbonate, and electron donor. Methanol and glycerol (10-20 mM) were found to be successful electron donors for stimulating ~90% reduction of uranium (~10 ppm) with less than 10% loss of nitrate (~850 ppm). Higher pH enrichments demonstrated similar U reduction potential with 5-30% nitrate loss.

Molecular investigations of the microbial community by complimentary 16S rRNA (data not shown) and Terminal Restriction Length Fragment Polymorphism (T-RFLP, Fig. 1) analyses have provided a consistent picture. In uranium-reducing enrichments, clone sequences most closely related to *Desulfotomaculum guttoideum* and *Anaerosinus glycerini* were the dominant organisms in nearly all cases. Both organisms tree within the broadly defined Clostridiaceae family, although all clones related to *Anaerosinus glycerini* had similarity ranks of 0.7 or less, indicating the likely presence of unknown organisms with variable physiological traits. Differences in T-RFLP profiles were consistent between both methanol and glycerol treatments and across a range of acidic pHs (Fig. 1). Gas chromatographic analyses suggest acetate as a dominant end product, consistent with the phylogenetic identification of members of the Clostridiaceae, some of which are known to be capable of methanol fermentation through the reaction  $4 \text{ MeOH} + 2 \text{ HCO}_3^- \rightarrow 3 \text{ Acetate} + \text{ H}^+ + 4 \text{ H}_2\text{O}$ .

This research has demonstrated that the indigenous FRC microbial community contains the capacity for nitrate-indifferent uranium bioreduction and paves the way for in-situ bioimmobilization of uranium co-existing with nitrate. Considering not only the rigid dogmatic framework of terminal electron acceptor utilization, but rather the holistic view of the subsurface as a complex system with many coupled microbial and biogeochemical processes, microbial physiology can be manipulated to produce those conditions best suited to nitrate-indifferent uranium reduction.

### *The influences of oxygen and nitrate on uranium (IV) stability*

Uranium (IV) was produced by electrochemical reduction of U(VI) acetate solutions. The precipitate was injected into stoppered 28-mL pressure tubes with varying oxygen content, including tap, degassed, or deoxygenated water giving a total U concentration of ~1.5 ppm. Nitrate (1000 ppm) or nitrite (100 ppm) and were added selectively. While degassing involves cooling the water under ultra-high purity N<sub>2</sub> gas, deoxygenation involves an additional vacuum flushing procedure. Replicate tubes were prepared using Fe(II) instead of uranium. For more than 100 days, the uranium remained at initial levels (~0.1 ppm, equivalent to controls) before rising slightly after 215 days (Fig. 2). The minimal increase in U(VI) was not related to the oxygen removal or nitrogen species content, suggesting it was due to infiltration of oxygen during sampling or through needle-holes in the butyl rubber septa. Fe(II)-bearing tubes after 100 days demonstrated marked differences (Fig. 3a), suggesting that while Fe(II) is rapidly oxidized by nitrite and not nitrate, U(IV) is not.

Due to the importance of nitrite-dependent iron oxidation, additional experiments with similar redox treatments are being performed in mixed U-Fe-N systems. Hydroxy-sulfate green rust (GR) was reacted with U(VI)-acetate to produce a U(IV)-GR suspension. Synchrotron x-ray absorption spectroscopy confirmed that the uranium was completely reduced and that the U(IV)-GR suspension was robust against U(IV) reoxidation (Fig. 4). Ongoing experiments will elucidate the relative importance of oxygen removal, nitrate, nitrite, and iron oxidation on U(IV) reoxidation processes (Fig 3b, 3c).

These experiments are relevant to the long-term stewardship of uranium and nitrate co-contaminated sites; many remediation strategies involve uranium immobilization through bioreduction. Evidence from field experiments at the FRC demonstrate that nitrate will persist in the absence of active biostimulation. Thus, the long-term fate of uranium will be determined in the presence of redox-active iron and nitrate.

### **Planned Activities:**

Ongoing research includes: characterization of nitrate reducers within the enrichments in order to identify conditions more partial to nitrate reducers (completed early '07), isolation physiological/ molecular characterization of potential FRC Clostridaceae which may be contributing to the uranium reduction (continuing until end of funding), verification of the sustainability of U reduction through repeated transfers, enrichments, and microcosms, some including triethylphosphate (continuing until end of funding), and reoxidation experiments involving varying levels of oxygen stress in the presence of iron and (continuing until end of funding).

### **Institution-specific tasks:**

U(IV) oxidation experiments, enrichment set-up and sampling, isolation, and geochemical analyses were performed at ORNL. Molecular biological analyses such as 16S rRNA, T-RFLP, and amplification of nitrate reducing genes were performed at FSU. Synchrotron XAS was accomplished by ORNL researchers at the Advanced Photon Source (ANL) with the assistance of Bruce Ravel through the General User Program.

### **Information Access:**

- Smith AC, Balkwill DL, Madden AS, Phelps TJ (2006) Microbial community responses to nitrate-indifferent uranium bioreduction. *American Geophysical Union, San Francisco*.
- Madden AS, Smith AC, Balkwill DL, Fagan LA, and Phelps TJ (2006) Nitrate-independent uranium bioreduction. *ERSP Fall Meeting, Oak Ridge*.
- Smith AC, Madden AS, Balkwill DL, Fagan LA, Phelps TJ (2006) Bioremediation approaches for sustained uranium immobilization independent of nitrate reduction. *American Society for Microbiology, Orlando*.
- Madden AS, Smith AC, Balkwill DL, Fagan LA, Phelps TJ (2006) Bioremediation approaches for sustained uranium immobilization independent of nitrate reduction. *DOE Environmental Remediation Sciences Program Principal Investigators April 2006 meeting, Airlie, VA*.

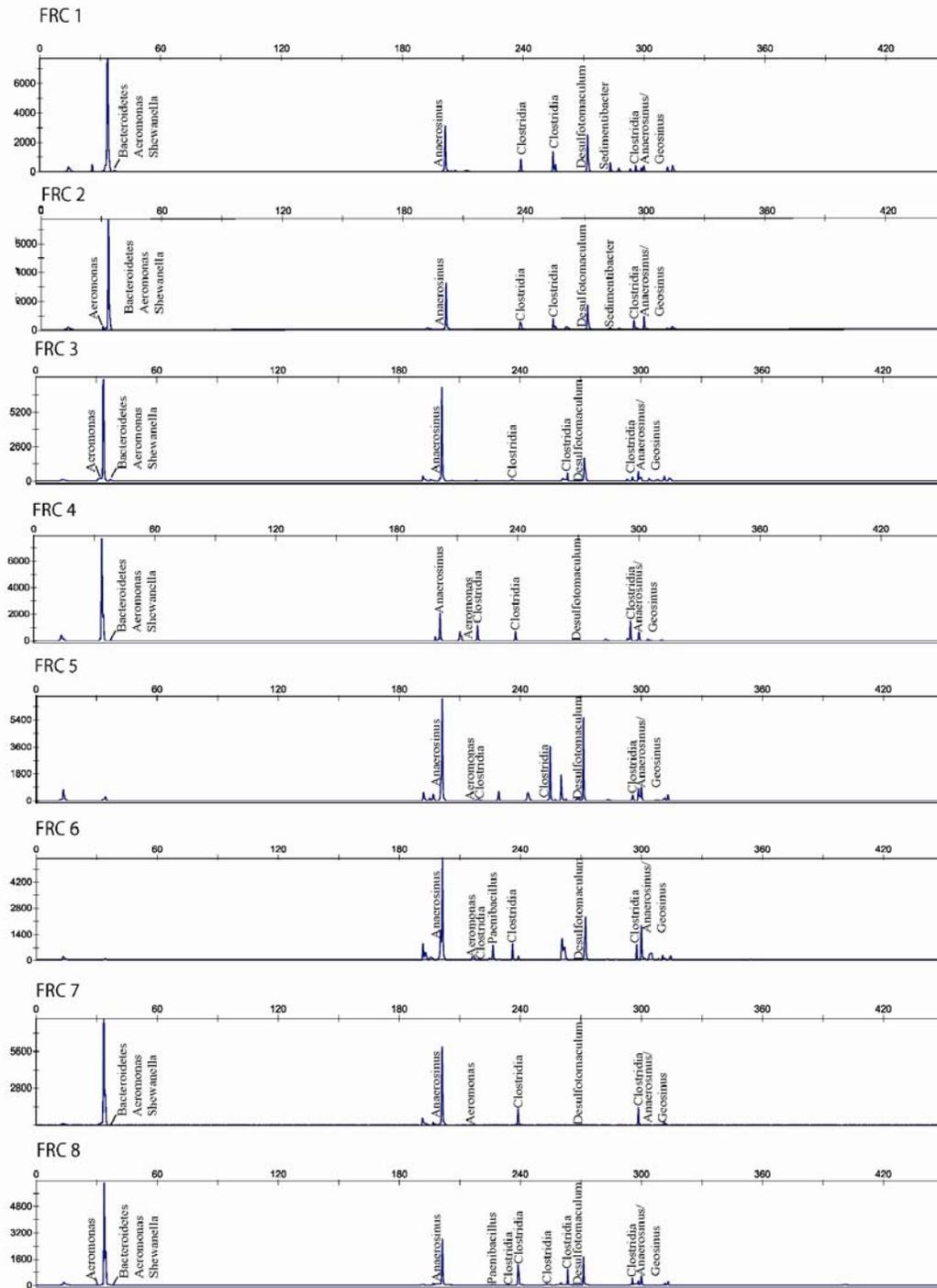


Figure 1. T-RFLP profiles of uranium-reducing enrichment cultures.

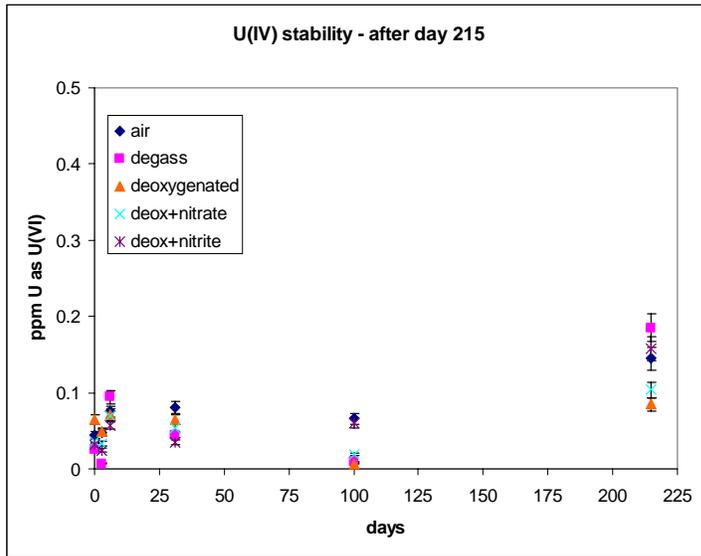


Figure 2. U(VI) concentrations measured from U(IV) stability experiments. Approximately 1.5 ppm U(IV) was injected into replicate tubes with each treatment.

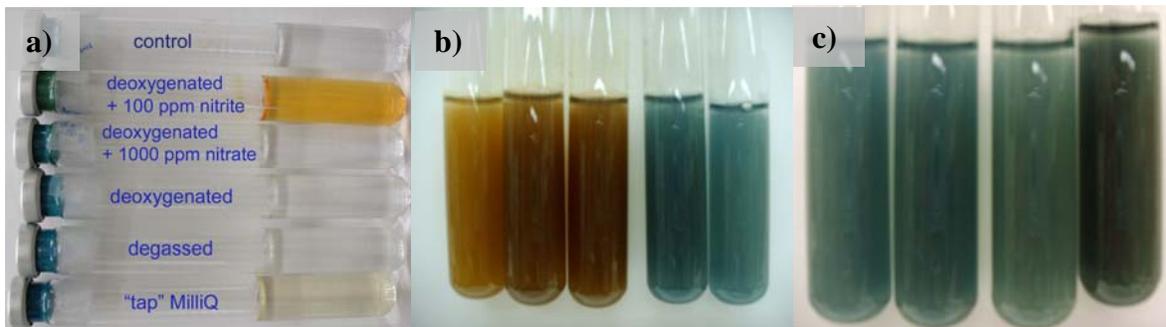


Figure 3a) 0.9 mM FeCl<sub>2</sub> after 100 days. b). L to R: tap water + GR, stagnant MilliQ water +GR, stirred MQ+GR, degassed MQ+GR, deoxygenated MQ+GR. c) All in deoxygenated MQ, L to R: as-is, +nitrate, +nitrite, +cysteine-HCl.

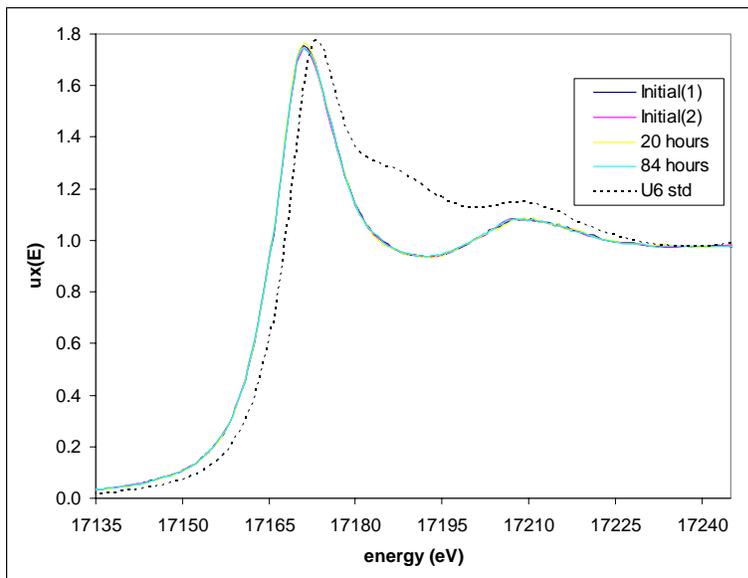


Figure 4. Synchrotron U L-3 x-ray absorption spectra of U(IV)-green rust contained in kapton tape after various exposure times to air compared with a U(VI) standard. All of the GR analyses are coincident and equivalent to the expected edge position/shape for U(IV).