STRUCTURE AND FUNCTION OF SUBSURFACE MICROBIAL COMMUNITIES AFFECTING RADIONUCLIDE TRANSPORT AND BIOIMMOBILIZATION

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This project employed a multidisciplinary approach incorporating five research groups at the Florida State University (FSU), Rutgers University (RU), Georgia State University (GSU), the Oak Ridge National Laboratory (ORNL), and the University of Illinois (U of I). These groups were selected because of their expertise in subsurface microbiology and molecular ecology of anaerobic prokaryotes (Kostka, Kerkhof, Chin), bioinformatics and phylogenetic reconstruction (Green), high throughput cultivation (Keller, Elkins), geochemistry and mineralogy (Stucki).

Our objectives were to: 1) isolate and characterize novel anaerobic prokaryotes from subsurface environments exposed to high levels of mixed contaminants (U(VI), nitrate, sulfate), 2) elucidate the diversity and distribution of metabolically active metal- and nitrate-reducing prokaryotes in subsurface sediments, and 3) determine the biotic and abiotic mechanisms linking electron transport processes (nitrate, Fe(III), and sulfate reduction) to radionuclide reduction and immobilization. Mechanisms of electron transport and U(VI) transformation were examined under near *in situ* conditions in sediment microcosms and in field investigations. Field sampling was conducted at the Oak Ridge Field Research Center (ORFRC), in Oak Ridge, Tennessee. The ORFRC subsurface is exposed to mixed contamination predominated by uranium and nitrate.

This project was initiated in March, 2007 and completed in March, 2011 (with a one year no cost extension). Denise Akob successfully defended her Ph.D. dissertation at FSU based on ERSP/ SBR research in July, 2008. Since that time, Dr. Akob has been working as a postdoc in Germany where she continues her research on microbial communities in uranium-contaminated sediments under the direction of Professor Kirsten Kuesel at Friedrich Schiller University in Jena, Germany. Puja Jasrotia joined the Kostka lab as a Ph.D. student at FSU. She began her training during summer, 2008, and as was the case for Dr. Akob, the main focus of Puja's dissertation research has been this ERSP/ SBR project. From 2007 to 2010, Dr. Stefan Green directed the cultivation-independent molecular work as a postdoc on this project. Dr. Green recently accepted a faculty position at the University of Illinois at Chicago. Dr. Om Prakash directed the culture-based aspects as a postdoc on this project. Dr. Prakash has recently accepted a faculty position at the National Centre for Cell Science in Pune, India.

Project meetings were held each year to facilitate completion of the stated objectives, two in Tallahassee and two in conjunction with the ERSP principal investigator meetings. The first meeting, held in December of 2007, was attended by all coPIs except for Martin Keller. This meeting included one day of presentations followed by a day of breakout sessions to brainstorm on future experiments. During the second meeting, held in June of 2008, Karsten Zengler of the University of California-San Diego and Jim Elkins from Keller's group at ORNL visited FSU to design high throughput cultivation experiments to be conducted at ORNL. Dr. Zengler was a consultant on this project. The same format was used as the first project meeting with presentations made by all investigators.

I. Summary of project results

In short, we effectively addressed all 3 stated objectives of the project. In particular, we isolated and characterized a large number of novel anaerobes with a high bioremediation potential that can be used as model organisms, and we are now able to quantify the function of subsurface sedimentary microbial communities *in situ* using state-of-the-art gene expression methods (molecular proxies).

Research Highlights:

Task I-Isolation and characterization of novel anaerobes

- A wide range of pure cultures of metal-reducing bacteria, sulfate-reducing bacteria, and denitrifying bacteria (32 strains) were isolated from subsurface sediments of the Oak Ridge Field Research Center (ORFRC), where the subsurface is exposed to mixed contamination of uranium and nitrate. These isolates which are new to science all show high sequence identity to sequences retrieved from ORFRC subsurface.
- Based on physiological and phylogenetic characterization, two new species of subsurface bacteria were described: the metal-reducer *Geobacter daltonii*, and the denitrifier *Rhodanobacter denitrificans*.
- Strains isolated from the ORFRC show that *Rhodanobacter* species are well adapted to the contaminated subsurface. Strains 2APBS1 and 116-2 grow at high salt (3% NaCl), low pH (3.5) and tolerate high concentrations of nitrate (400mM) and nitrite (100mM). Strain 2APBS1 was demonstrated to grow at in situ acidic pHs down to 2.5.
- *R. denitrificans* strain 2APBS1 is the first described *Rhodanobacter* species shown to denitrify. Nitrate is almost entirely converted to N_2O, which may account for the large accumulation of N_2O in the ORFRC subsurface.
- *G. daltonii*, isolated from uranium- and hydrocarbon-contaminated subsurface sediments of the ORFRC, is the first organism from the subsurface clade of the genus *Geobacter* that is capable of growth on aromatic hydrocarbons.
- High quality draft genome sequences and a complete eco-physiological description are completed for *R. denitrificans* strain 2APBS1 and *G. daltonii* strain FRC-32.
- Given their demonstrated relevance to DOE remediation efforts and the availability of detailed genotypic/ phenotypic characterization, *Rhodanobacter denitrificans* strain 2APBS1 and *Geobacter daltonii* strain FRC-32 represent ideal model organisms to provide a predictive understanding of subsurface microbial activity through metabolic modeling.

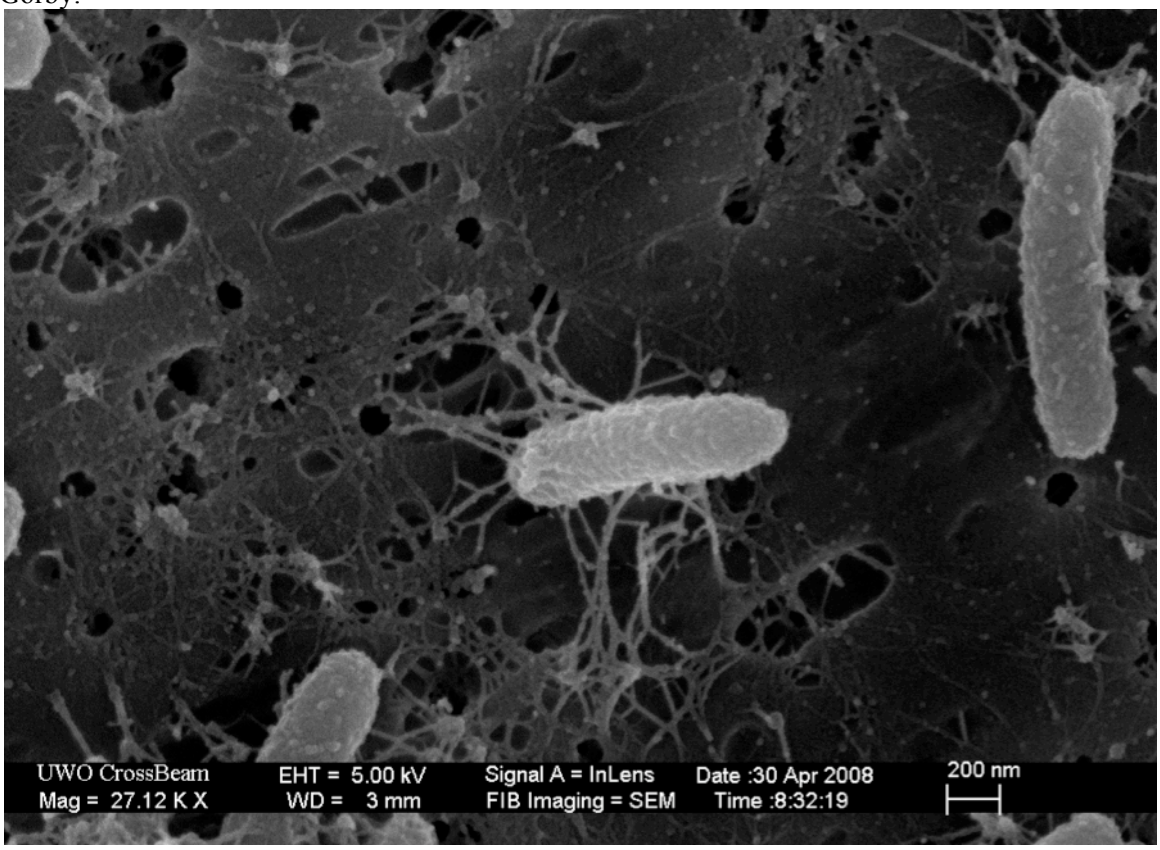
Tasks II & III-Diversity and distribution of active anaerobes and Mechanisms linking electron transport and the fate of radionuclides

- Our study showed that members of genus *Rhodanobacter* and *Geobacter* are abundant and active in the uranium and nitrate contaminated subsurface. In the contaminant source zone of the Oak Ridge site, *Rhodanobacter* spp. are the predominant, active organisms detected (comprising 50% to 100% of rRNA detected). *G. daltonii* was also detected in abundance at other U.S. DOE nuclear legacy waste sites, including the uranium-contaminated subsurface of the Rifle site.
- We demonstrated for the first time that the function of microbial communities can be quantified in subsurface sediments using messenger RNA assays (molecular proxies) under in situ conditions. The application of dsr and gltA gene expression as molecular proxies for the metabolically active sulfate-reducing and Fe(III)-reducing prokaryotes, respectively, was extended to low biomass core samples from the uranium-contaminated subsurface of the Oak Ridge and Hanford sites.

- Active *Geobacteraceae* were identified and phylogenetically characterized from the cDNA of messenger RNA extracted from ORFRC subsurface sediment cores. This is arguably the best way to identify the metabolically-active members of in situ microbial communities. Multiple clone sequences were retrieved from *G. uraniireducens*, *G. daltonii*, and *G. metallireducens*.
- Results show that *Geobacter* strain FRC-32 is capable of growth on benzoate, toluene and benzene as the electron donor, thereby providing evidence that this strain is physiologically distinct from other described members of the subsurface *Geobacter* clade.
- Fe(III)-reducing bacteria transform structural Fe in clay minerals from their layer edges rather than from their basal surfaces. This is a new finding, not previously known, and was discovered only because of our ability to obtain Mossbauer spectra at temperatures below 10 K.

A) Task I-Isolation and characterization of novel anaerobes

Figure 1. SEM image of *Geobacter* FRC-32 isolated from highly uranium-contaminated subsurface sediments of the ORFRC, Oak Ridge, Tennessee. Image was provided by Yuri Gorby.



Isolation and characterization of a novel species from the subsurface *Geobacter* clade (Prakash et al., 2010).
An Fe(III)- and uranium(VI)-reducing bacterium, designated strain FRC-32T, was isolated from a contaminated subsurface of the Oak Ridge Field Research Center (ORFRC) in Oak Ridge, Tennessee, where the sediments are exposed to mixed waste contamination of radionuclides and

hydrocarbons (see Figure 1). Analyses of both 16S rRNA gene and the Geobacteraceae-specific citrate synthase (gltA) mRNA gene sequences retrieved from ORFRC sediments indicated that this strain was abundant and active in ORFRC and Rifle subsurface sediments undergoing uranium(VI) bioremediation. The organism belonged to the subsurface clade of the genus Geobacter and shared 92–98% 16S rRNA gene and 75–81% rpoB gene sequence similarities with other recognized species of the genus. In comparison to its closest relative, Geobacter uraniireducens Rf4T, according to 16S rRNA gene sequence similarity, strain FRC- 32T showed a DNA–DNA relatedness value of 21 %. Cells of strain FRC-32T were Gram negative, non-spore-forming, curved rods, 1.0–1.5 mm long and 0.3–0.5 mm in diameter; the cells formed pink colonies in a semisolid cultivation medium, a characteristic feature of the genus Geobacter. The isolate was an obligate anaerobe, had temperature and pH optima for growth at 30 6C and pH 6.7–7.3, respectively, and could tolerate up to 0.7% NaCl although growth was better in the absence of NaCl. Similar to other members of the Geobacter group, strain FRC-32T conserved energy for growth from the respiration of Fe(III)-oxyhydroxide coupled with the oxidation of acetate. Strain FRC-32T was metabolically versatile and, unlike its closest relative, G. uraniireducens, was capable of utilizing formate, butyrate and butanol as electron donors and soluble ferric iron (as ferric citrate) and elemental sulfur as electron acceptors. Growth on aromatic compounds including benzoate and toluene was predicted from preliminary genomic analyses and was confirmed through successive transfer with fumarate as the electron acceptor. Thus, based on genotypic, phylogenetic and phenotypic differences, strain FRC-32T is considered to represent a novel species of the genus Geobacter, for which the name Geobacter daltonii sp. nov. is proposed. The type strain is FRC-32T (5DSM 22248T5JCM 15807T).

Isolation of 5 novel species of denitrifying bacteria and reassessment of PCR-based methods for detecting these organisms in the contaminated terrestrial subsurface (Green et al., 2010).

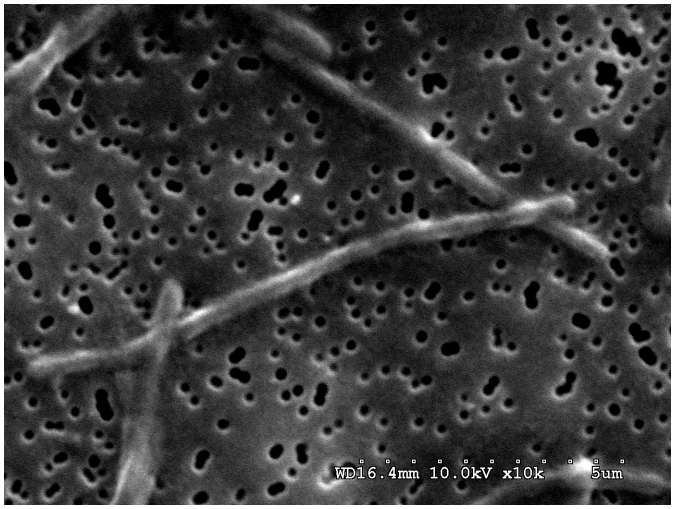
In terrestrial subsurface environments where nitrate is a critical groundwater contaminant, few cultivated representatives are available to verify the metabolism of organisms that catalyze denitrification. In this study, five species of denitrifying bacteria from three phyla were isolated from subsurface sediments exposed to metal radionuclide and nitrate contamination as part of the U.S. Department of Energy's Oak Ridge Field Research Challenge (ORFRC). Isolates belonged to the genera *Afipia* and *Hyphomicrobium* (*Alphaproteobacteria*), *Rhodanobacter* (*Gammaproteobacteria*), *Intrasporangium* (*Actinobacteria*), and *Bacillus* (*Firmicutes*). Isolates from the phylum *Proteobacteria* were complete denitrifiers, whereas the Gram-positive isolates reduced nitrate to nitrous oxide. rRNA gene analyses coupled with physiological and genomic analyses suggest that bacteria from the genus *Rhodanobacter* are a diverse population of denitrifiers that are circumneutral to moderately acidophilic, with a high relative abundance in areas of the acidic source zone at the OR-IFRC site. Based on genome analysis, *Rhodanobacter* species contain two nitrite reductase genes and have not been detected in functional-gene surveys of denitrifying bacteria at the OR-IFRC site. Nitrite and nitrous oxide reductase gene sequences were recovered from the isolates and from the terrestrial subsurface by designing primer sets mined from genomic and metagenomic data and from draft genomes of two of the isolates. We demonstrate that a combination of cultivation and genomic and metagenomic data is essential to the *in situ* characterization of denitrifiers and that current PCR-based approaches are not suitable for deep coverage of denitrifiers. Our results indicate that the diversity of denitrifiers is significantly underestimated in the terrestrial subsurface.

Description of new species and a novel clade of denitrifying bacteria from the contaminated terrestrial subsurface (Prakash et al., 2011, in review).

Bacterial strains 2APBS1^T and 116-2 were isolated from uranium and nitrate contaminated subsurface sediments from the Oak Ridge Integrated Field Research Challenge (OR-IFRC) site

located in Oak Ridge, TN (see Figure 2). A combination of physiological and genetic assays revealed that both isolates are capable of complete denitrification, and can use nitrate, nitrite and nitrous oxide as terminal electron acceptors. Phylogenetic studies using small subunit ribosomal RNA (SSU rRNA) gene sequencing placed the strains within the genus *Rhodanobacter* of the *Gammaproteobacteria*. Cells of strain 2APBS1^T and 116-2 were Gram negative, non-spore-forming, curved rods, 1.0–1.5 um long and 0.3–0.5 μm in diameter. The cells formed yellow colonies on solid cultivation medium, a characteristic feature of the family *Xanthomonadaceae*. The isolates were facultative anaerobes, and had temperature and pH optima for growth at 30ºC and pH 4 - 8, respectively, and could tolerate up to 2.0% NaCl, though growth was better in its absence. Strains 2APBS1^T and 116-2 contained high levels of 15:0 iso and 17:1 iso w9c fatty acids, consistent with previously published fatty acids profiles of other *Rhodanobacter* species. Although strains 2APBS1^T and 116-2 have high SSU rRNA gene sequence similarity to *R. thiooxydans* (>99%), DNA-DNA hybridization values were substantially below the 70% threshold used to designate novel species. In addition, this is the first described *Rhodanobacter* species capable of denitrification. Thus, based on genotypic, phylogenetic, chemotaxonomic and physiological differences, strain 2APBS1^T and 116-2 are considered to represent a novel species of the genus *Rhodanobacter*, for which the name *Rhodanobacter denitrificans* sp. nov is proposed. The type strain is 2APBS1^T (=DSM 25369^T =JCM 17641^T). Strain 116-2 (= DSM 24678 = JCM 17642) is a reference strain.

Figure 2. SEM image of *Rhodanobacter denitrificans strain 2APBS1* isolated from highly uranium-contaminated subsurface sediments of the ORFRC, Oak Ridge, Tennessee.



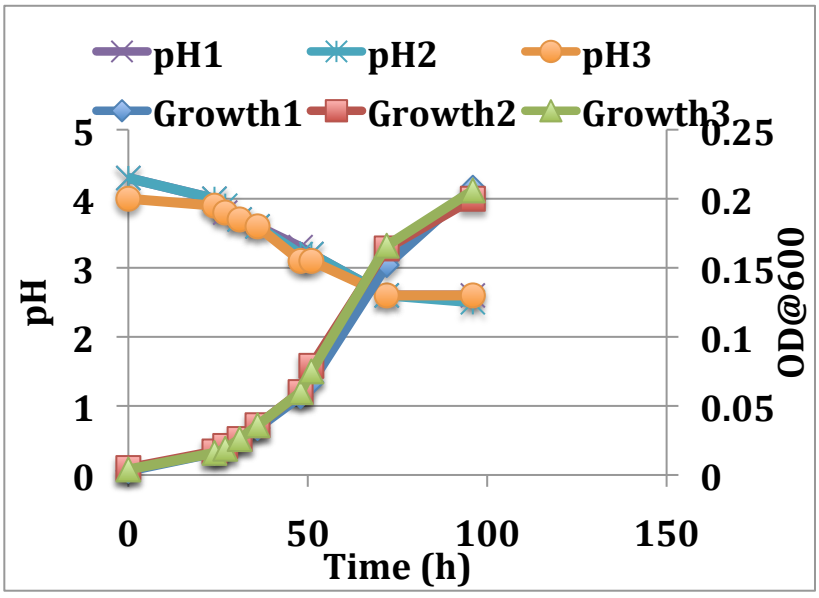
Denitrifying bacteria from the genus *Rhodanobacter* dominate microbial communities in a uranium- and nitrate-contaminated subsurface environment (Green et al., 2011, in review).

The effect of long-term mixed-waste contamination on the microbial community in the terrestrial subsurface was investigated at the field scale at the Oak Ridge Integrated Field Research Challenge (ORIFRC) site in Oak Ridge, TN. The primary contaminants of concern are uranium and nitrate, and are present in elevated concentrations in the acidic, near-source zone. Our primary hypothesis was that pH and nitrate would both exert strong selective pressure on the subsurface bacterial community. To test this hypothesis, we conducted a systematic survey of the groundwater microbial community across the site during two

seasonal sampling events. On a more limited scale, subsurface sediment was also examined from two cores, one taken from the most heavily contaminated area of the site and one from an area with low contamination. A suite of DNA- and RNA-based molecular tools were employed for site characterization, including quantitative PCR of ribosomal RNA (rRNA) and nitrite reductase genes, and community composition analysis using a fingerprinting technique and direct sequencing of rRNA genes. The results demonstrate a dramatic effect of pH on the subsurface microbial community, particularly of putative denitrifying bacteria from the genus *Rhodanobacter* (class Gammaproteobacteria). The relative abundance of bacteria from this genus was most strongly correlated with low pH, and the bacteria were highly abundant and active in many wells in the most highly contaminated area of the site. Other factors, such as concentration of nitrate, oxygen and sampling season do not appear to influence the distribution of *Rhodanobacter*. Our data are consistent with the hypothesis that these organisms are acid-tolerant denitrifiers, well suited to the acidic, nitrate-rich subsurface conditions. The data also confirm pH as the most dominant driver of bacterial community structure in the contaminated subsurface environment.

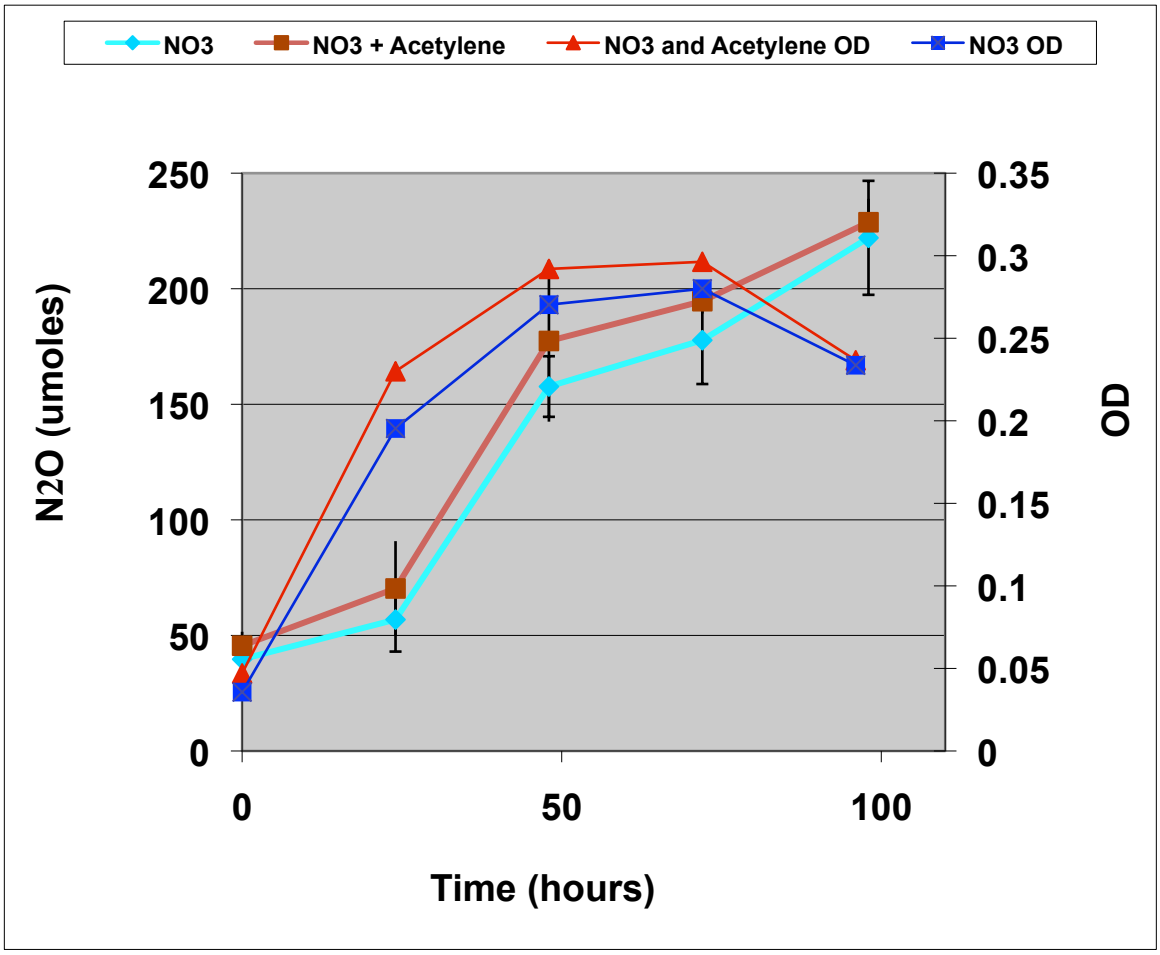
Rhodanobacter completely denitrifies and is well adapted to it's subsurface habitat, the contaminant source zone at the Oak Ridge FRC.
Rhodanobacter strains 2APBS1 and 116-2 were isolated from uranium-contaminated OR-IFRC subsurface sediments. Consistent with their source zone habitat, strains 2APBS1 and 116-2 were shown to be highly tolerant of key stressors: like pH (grow down to pH 3.0), salt (3 % NaCl), nitrite (100 mM) and nitrate (400 mM). Substantial growth of strain 2APBS1 under aerobic conditions was demonstrated at the in situ pH of 3.0 to 3.5 (see Figure 3). Whereas organisms within this genus are not known to denitrify, phenotypic and genotypic analyses confirm that strains 2APBS1 and 116-2 contain a complete denitrification pathway.

Figure 3. Growth of *Rhodanobacter* strain 2APBS1 on glucose in a synthetic groundwater medium. Growth is demonstrated at the in situ pH of 3.0 to 3.5.



NO_3 utilization and N_2O production with concomitant growth of strain 2APBS1 indicated that bacterium has a complete pathway for denitrification (see Figure 4). Over 80 % of N is converted to N2O. This could explain the accumulation of N2O in groundwaters of the Oak Ridge IFRC. High quality draft genome sequence and complete description of *Rhodanobacter* sp. strain 2APBS1 are available in our laboratory.

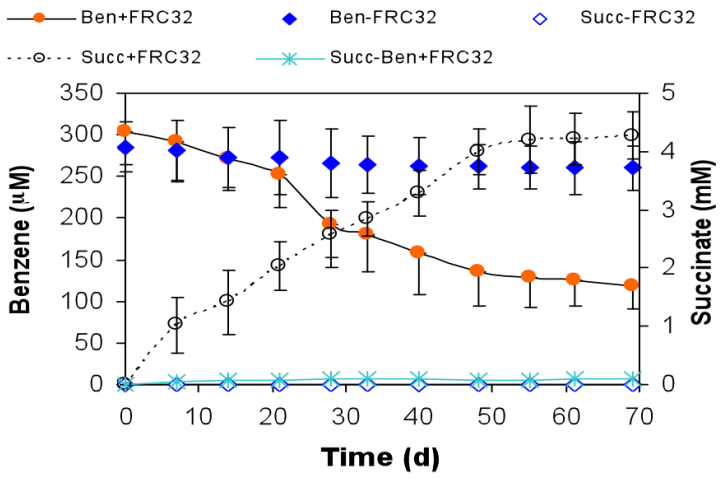
Figure 4. NO_3 utilization and N_2O production with concomitant growth of strain 2APBS1 indicated that bacterium has a complete pathway for denitrification.



***Geobacter daltonii* strain FRC-32 is the first organism from the subsurface clade of the genus *Geobacter* that is capable of growth on aromatic hydrocarbons.**
Groundwaters at over half of DOE sites contain organic in addition to radionuclide contaminants, and compounds such as monoaromatic hydrocarbons could provide an important source of carbon and electrons for biotransformation processes in the oligotrophic subsurface, thereby limiting the fate and transport of inorganic contaminants under natural attenuation conditions. *Geobacter daltonii* was isolated from uranium- and hydrocarbon-contaminated subsurface sediments of the ORFRC, and is the first organism from the subsurface clade of the genus *Geobacter* that is capable of growth on aromatic hydrocarbons (Figure 5). BLAST analysis was performed on the *G. daltonii* genome and compared to the *G. metallireducens* genome, the only other *Geobacter* species capable of aromatic contaminant degradation. As in *G. metallireducens*, genes predicted to play a role in aromatics degradation were identified and shown to cluster into large and small genomic islands. Of note, *G. daltonii* has uniquely two copies of the benzylsuccinate synthase

(*bssA*) gene, which is involved in the first step of toluene oxidation. The α subunits have 76% identity to one another. Also interesting, these copies lie within completely independent operons, each with its own transcriptional regulator and accessory genes downstream. RT-PCR analysis revealed that the expression level of *bssAs* vary when *G. daltonii* is grown on toluene vs. benzene, however, expression analysis suggests that benzene oxidation proceeds through use of the toluene oxidation pathway in *G. daltonii* (Figure 6). Iterative cultivation and genomic approaches need to be integrated to understand the growth and physiological controls of microbially catalyzed contaminant transformation as well as to develop metabolic models for key organisms in the subsurface.

Figure 5. Growth of G. daltonii strain FRC-32 on benzene and fumarate.



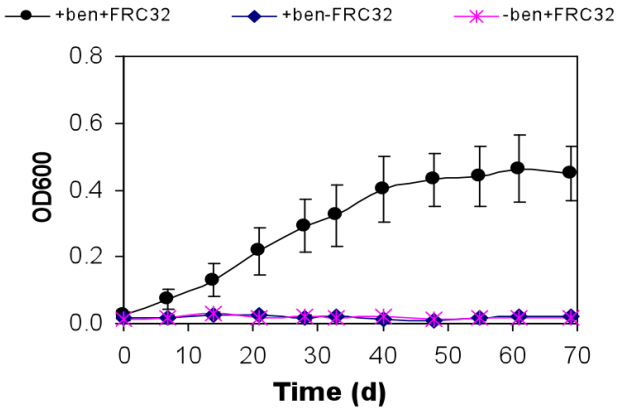
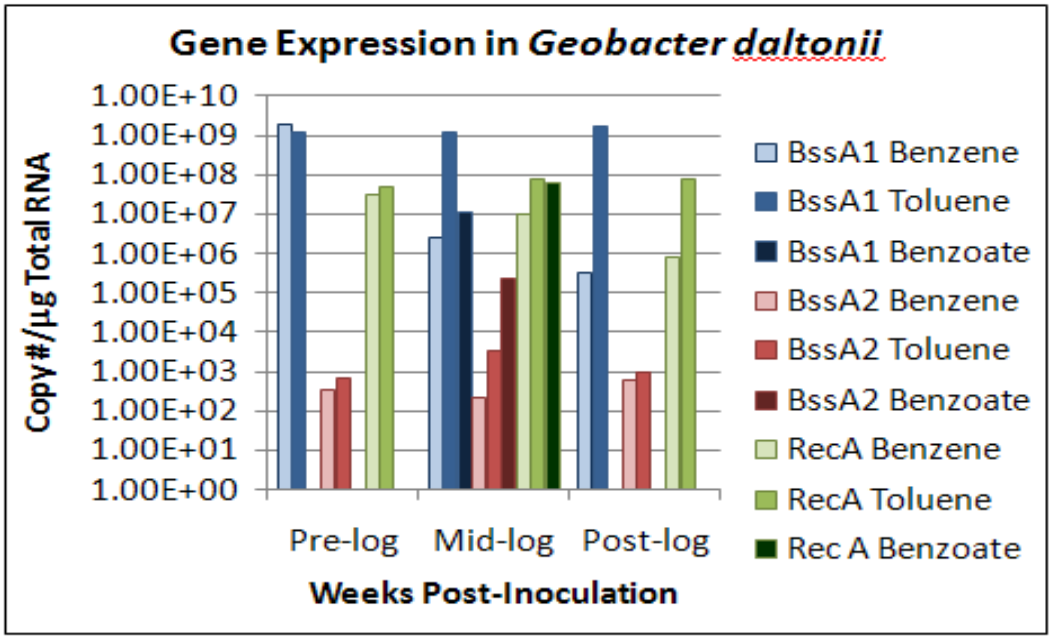


Figure 6. Differential Expression of *bssA1* & *bssA2* genes in *G. daltonii* grown on different aromatic substrates & fumarate. Transcript levels of *bssA* were determined with quantitative real-time RT-PCR. The housekeeping gene *recA* was used as the internal standard.



B) Tasks II & III-Diversity and distribution of active anaerobes and Mechanisms linking electron transport and the fate of radionuclides

Linking specific heterotrophic bacterial populations to bioreduction of uranium and nitrate using stable isotope probing in contaminated subsurface sediments (Akob et al., 2011, in review).

Shifts in terminal electron accepting processes during biostimulation of uranium-contaminated sediments were linked to the composition of stimulated microbial populations using DNA-based stable isotope probing. Nitrate reduction preceded U(VI) and Fe(III) reduction in ^{13}C-ethanol-amended microcosms. The predominant, active denitrifying microbial groups were identified as members of the *Betaproteobacteria*, whereas *Actinobacteria* dominated under metal-reducing conditions.

At radionuclide-contaminated sites managed by the U.S. Department of Energy (DOE) microbially mediated uranium reduction is limited under *in situ* conditions often due to low pH and electron donor limitation and the presence of competing and more energetically favorable terminal electron acceptors, *e.g.,* nitrate. Field manipulation experiments have shown that ethanol is an effective electron donor for the *in situ* biostimulation of terminal electron accepting processes (TEAPs) resulting in the complete reduction of U(VI) after nitrate reduction.

Although biostimulation is known to promote the removal of U(IV) and nitrate from solution, the identity of bacterial populations catalyzing these processes in contaminated sediments remains uncertain. Previous work was largely based on limited DNA sequence information and the activity of bacterial populations that mediate the fate of U(VI) in response to electron donor addition was not often confirmed with RNA or stable isotopic tracer approaches. Thus, a microcosm-based stable isotope probing (SIP) experiment was employed to assess

whether distinct microbial populations are catalyzing the removal of nitrate and immobilization of U(VI) in subsurface radionuclide-contaminated sediments.

Microbial activity during biostimulation of uranium-contaminated sediments. Sediments from borehole FB097 were sampled from the Area 2 experimental plot at the U.S. DOE's Oak Ridge Field Research Center (ORFRC), on 01 May 2006, 5 to 7 m below the surface within the saturated zone, as described previously. Microcosms were prepared and sampled as previously described except that the sediment was diluted 1:5 (w/v) with sterile, anoxic artificial groundwater (composition per liter: 1.0 g NaCl, 0.4 g $MgCl_2*6H_2O$, 0.1 g $CaCl_2*2H_2O$, 0.5 g KCl, 1 ml selenite-tungstate solution, 1 ml trace elements solution, and 5.0 mM $NaHCO_3$). Duplicate microcosms were prepared for treatments N1 (^{13}C- labeled ethanol amended, (> 99 atom % ^{13}C; Cambridge Isotopes, USA) and N2 (unlabeled ethanol amended).

Addition of ^{13}C-labeled or unlabeled ethanol stimulated microbial activity. No difference in rates of carbon or electron acceptor utilization was observed for treatments N1 and N2 indicating that there was no negative effect of adding a ^{13}C-enriched substrate (data not shown). Nitrate was rapidly consumed in the microcosms from day 2 to 6 (Figure 7A) corresponding to the initial consumption of supplemental ethanol (Figure 7B). The concentration of uranium was observed to increase on day 6, whereas, U(VI) removal from solution was observed from days 8 to 16 and began prior to the onset of Fe(III) reduction, which occurred from days 11 to 16 (Figure 7A). Supplemental ethanol was completely consumed at the end of the incubation (day 22) and was incompletely oxidized to acetate (Figure 7B). Acetate accumulation was observed on day 8 and the accumulated acetate was not utilized as a secondary carbon substrate during the course of the incubation.

Shifts in terminal electron-accepting processes (TEAPs) were consistent with thermodynamic predictions and previous studies in which the addition of ethanol stimulated microbial populations to rapidly reduce nitrate allowing for a shift to metal reduction once nitrate was depleted. The concentration of soluble U(VI) was observed to increase during nitrate reduction, likely due to abiotic oxidation of U(IV) by nitrate reduction intermediates. The onset of U(VI) reduction preceded Fe(III) reduction in the experiment and was hypothesized to occur via direct enzymatic reduction. However, rapid U(VI) reduction can occur via abiotic reduction with Fe(II) in the presence of Fe(III) (oxyhydr)oxides and U(IV) can rapidly reduce Fe(III). This rapid redox cycling of Fe(III)-Fe(II) could mask the onset of metal reduction and be mistakenly be attributed to direct enzymatic processes. At the end of the experiment, dissolved U(VI) concentrations decreased to < 3 μM. Although, U(VI) was not completely removed, the final concentration was below the EPA guidelines for drinking water indicating successful bioremediation. The incomplete removal of U(VI) may have been influenced by carbon availability, however, previous work indicated that incomplete U(VI) reduction in Area 2 sediments was likely due to geochemical and not microbiological factors.

Characterization of metabolically active microbial populations. For microbial community analyses, samples from each replicate microcosm were collected at days 3, 6 and 22. DNA was extracted from 0.25 g of solid-phase microcosm sample using a modified phenol-chloroform procedure. Density gradient centrifugation and separation of ^{13}C- and ^{12}C-DNA bands was performed according to the method of Gallagher et al. 2005, with ^{13}C-labeled carrier DNA added to assist visualization of the ^{13}C-DNA band in the density gradient. SSU rRNA genes from ^{13}C- and ^{12}C-DNA bands were PCR amplified, purified, screened using terminal restriction fragment length polymorphism (TRFLP) analysis.

Microbial communities clearly assimilated supplemental ^{13}C-labeled ethanol and shifts in phylogenetic structure were observed to coincide with changes in electron-accepting processes. ^{13}C-labeled substrate incorporation into microbial biomass was detected by day 3, corresponding to the onset of nitrate reduction and ethanol depletion. DNA extracts from day 0 of the experiments were below the minimum concentration required for PCR amplification. ^{13}C-DNA was not present in the unlabeled-ethanol treatment (N2) and profiles generated from this

treatment are representative of the total microbial community present in the microcosms. Peaks detected in the ^{12}C fractions from the ^{13}C-treatments represent those groups not utilizing supplemental carbon substrates, such as dead, senescent, autotrophic or sporeforming bacteria. Similar TRFLP profiles were obtained from the ^{13}C- and ^{12}C-DNA bands from the two treatments indicating that the majority of the microbial community assimilated ^{13}C from the addition of ^{13}C-ethanol. Clustering analysis, based on Bray-Curtis distance of the percent peak area of TRF peaks, revealed significant changes in community structure with incubation time, treatment and incorporation of the ^{13}C-label (Figure 8). TRFLP profiles obtained from treatments N1 and N2 during nitrate reduction were most similar and clustered separately from the active community profiles obtained from days 6 and 22 (Figure 8). The ^{13}C-enriched community incorporating ^{13}C during metal reduction was significantly different from the inactive (treatment N1, ^{12}C-enriched DNA) and total microbial community (N2) analyzed on the same day.

Active members of the microbial community were identified by matching peak sizes to predicted terminal restriction fragment (TRF) sizes of sequences in the ORFRC Sequence Database. Peaks in ^{13}C-DNA TRFLP profiles matched to members of the *Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Bacteroidetes,* and *Chloroflexi* (Figure 9). The *Chloroflexi* phylum represented 16.2% of the total peak area at day 3 of the experiment, when active nitrate reduction was observed, and decreased in abundance during the metal reduction phase (Figure 9). This suggests that these organisms mediated nitrate reduction and were not actively involved in metal-reduction in the microcosms. Members of the Chloroflexi contain genes in the denitrification pathway (nirK, nosZ; Green et al., 2010), the group is often detected in sediments under nitrate reducing conditions, and recent evidence from bacterial populations in wastewater treatment plants indicates that the Chloroflexi are capable of denitrification. Further, the Chloroflexi were shown to assimilate ^{13}C-acetate under nitrate-reducing conditions in activated sludge.

Once nitrate was removed as a competing electron acceptor, U(VI) and Fe(III) reduction could proceed. During the metal reduction phase of the incubation, we observed an increase in peaks matching to the TRF size of the *Actinobacteria* phyla*, e.g.,* a peak at 245 bp matching to *Arthrobacter* (Figure 9). Members of the *Actinobacteria* such as *Cellulomonas* were shown to reduce U(VI) and Fe(III) in pure culture. Although *Arthrobacter* is not a confirmed metal-reducer, it has been implicated in the bioprecipitation of U(IV). We show a direct link between the *Actinobacteria* and U removal during biostimulation, and further work is needed to identify the mechanism behind this activity.

Members of the *Betaproteobacteria*, *e.g., Dechloromonas, Diaphorobacter, Azoarcus*, *Ralstonia*, and *Alcaligenes*, dominated microcosm communities under all treatment conditions, in corroboration with previous studies of nitrate-rich uranium-contaminated subsurface sediments. As the genera to which these organisms belong all contain known denitrifiers, it is likely that they are actively catalyzing nitrate removal in this experiment. Surprisingly, in addition to being linked to nitrate reduction, *Betaproteobacteria* taxa predominated the active communities under metal-reducing conditions when nitrate was depleted. Thus, we hypothesize that members of the *Betaproteobacteria* were coupling oxidation of supplemental carbon to Fe(III) and/or U(VI) reduction or were detected in high abundance due to cross-feeding of supplemental ^{13}C. The prolonged dominance of *Betaproteobacteria* members during the entire incubation and the short incubation time suggests that these organisms are active at later time points and not an artifact of the SIP method. In corroboration, previous studies by our group and others indicate that organisms belonging to the *Betaproteobacteria* catalyze metal reduction in sediments and in uranium-contaminated soils without nitrate as a co-contaminant. In addition, our findings confirm the importance of this group to the removal of nitrate and maintenance of reducing conditions during bioremediation in contaminated ORFRC subsurface sediments.

Addition of carbon substrates is an effective strategy for biostimulating the reductive immobilization of U(VI) in the subsurface at U.S. DOE sites contaminated with nuclear legacy

waste. Using a SIP approach, we were able to show that ethanol is readily utilized and incorporated into microbial biomass by subsurface microbial communities in uranium-contaminated sediments. In addition, carbon utilization by specific microbial populations was observed to coincide with the reduction of nitrate and Fe(III) and the removal of U(VI). Availability of electron acceptors in subsurface sediments affects the composition and activity of microbial populations actively involved in U(VI) reduction. By directly linking specific electron-accepting processes with microbial community characterization, we provide direct evidence for denitrifying and metal reducing capability of taxa (*Betaproteobacteria, Chloroflexi, Actinobacteria*, *Bacteroidetes,* and *Firmicutes*) previously detected in the subsurface during U(VI) bioremediation.

Figure 7. (A) Electron acceptor and (B) electron donor utilization in ethanol-amended microcosms. Arrows indicate sampling points for TRFLP community fingerprinting. Values are averages ± standard deviations of the results for the four microcosms established for treatments N1 (^{13}C-ethanol) and N2 (unlabeled ethanol).

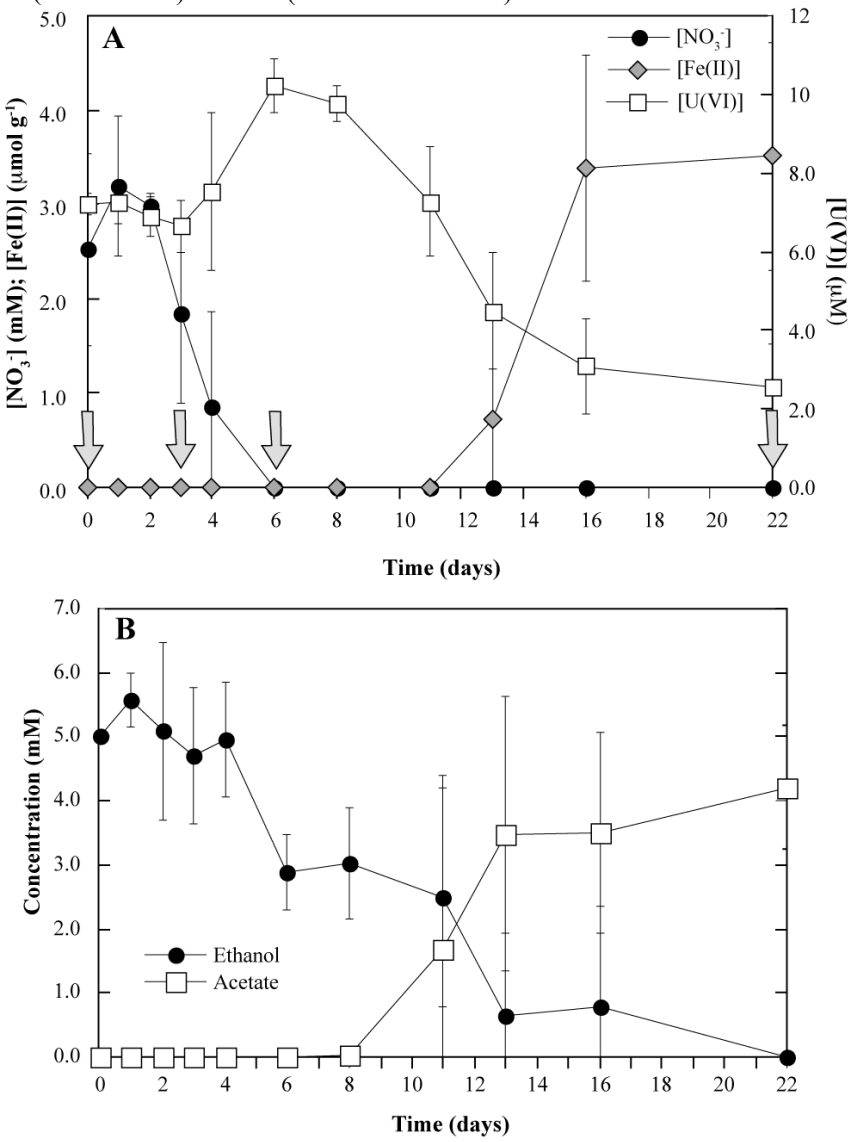


Figure 8. Dendrogram showing the similarity of *MnlI* TRFLP profiles for ^{13}C and ^{12}C fractions obtained from microcosm samples of treatments N1 (^{13}C-ethanol) and N2 (unlabeled ethanol). Clustering analysis based on Bray-Curtis distance, incorporating the relative abundance for each peak, and UPGMA methods. One thousand bootstrap analyses were conducted and circles represent bootstrap values greater than 50%.

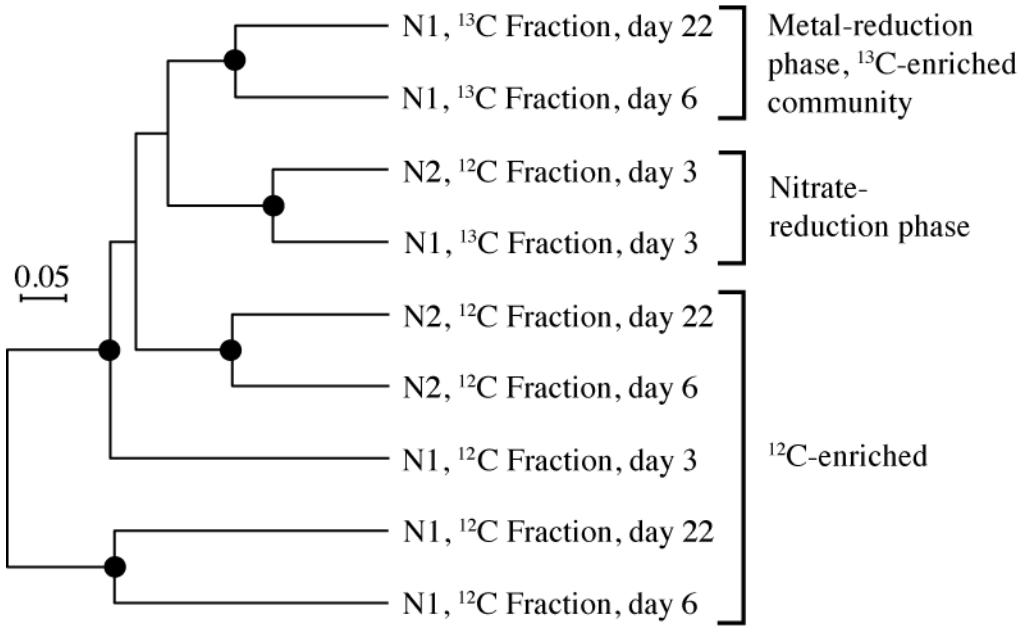
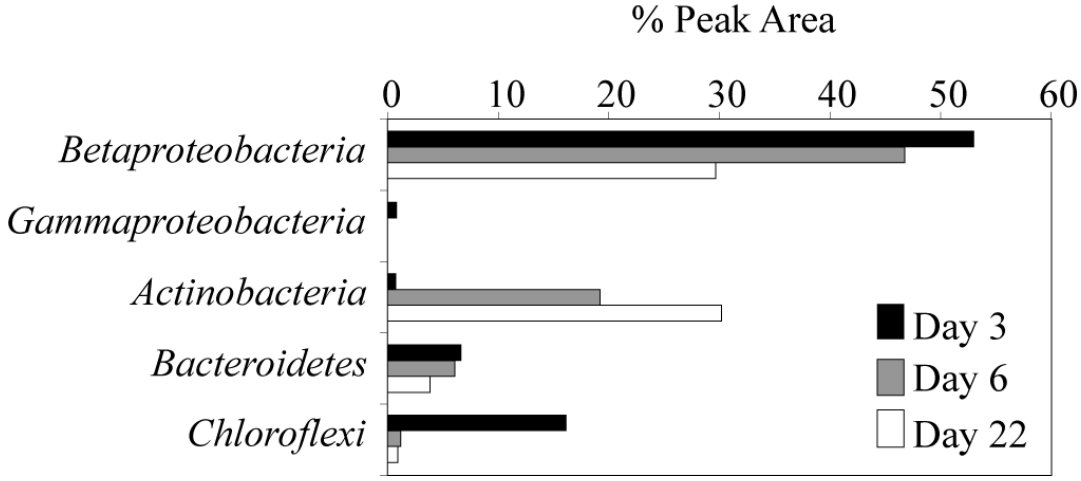


Figure 9. Relative abundance of phylogenetic groups detected in ^{13}C-DNA fractions of treatment N1 (^{13}C-ethanol amended microcosms). Phylogenetic affiliation of TRFLP peaks was determined by matching peak sizes from *MnlI* profiles against the ORFRC Sequence Database.



Metabolic activity of sulfate- and metal-reducing bacteria in U(VI)-contaminated sediments (Akob et al., 2011, in review).

Uranium bioremediation in the subsurface at DOE sites is currently focused towards stimulation of indigenous subsurface microbial communities to immobilize U(VI) by reducing it to U(IV). The aim of this study was to assess the potential for functional groups of organisms to be involved in U immobilization.

Site and sample description. The study was conducted at the Oak Ridge Field Research Center (ORFRC) in Oak Ridge, Tennessee. Sediments were sampled from borehole FB107, within the Area 2 experimental plot, 5 to 7 m below the surface, on 12 September 2007 and from borehole FB124, Area 3 experimental plot, 1.23 to 15.08 m below the surface, on 7 February 2008 within the saturated zone, using a Geoprobe equipped with polyurethane sleeves lining the corer. Cores were aseptically sectioned under strictly anoxic conditions in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, Michigan) and stored anaerobically in gas-tight containers at 4°C prior to overnight shipment to Florida State University.

Microcosm design, sampling, and geochemistry. A microcosm experiment was performed with Area 2 FB107 sediments to assess the activity and composition of microbial communities under simulated bioremediation conditions. Microcosms were prepared as previously described except that the sediment was diluted 1:5 (w/v) with sterile, anaerobic artificial groundwater (composition per liter: 1.0 g NaCl, 0.4 g $MgCl_2*6H_2O$, 0.1 g $CaCl_2*2H_2O$, 0.5 g KCl, 1 ml selenite-tungstate solution, 1 ml trace elements solution, and 5.0 mM $NaHCO_3$). Three replicate microcosms were established for each of the following treatments: amendment with ethanol, amendment with ethanol + molybdate, amendment with acetate, and amendment with acetate + molybdate; with molybdate added to inhibit sulfate reduction. Sulfate (2.5 mM final concentration), molybdate (5 mM final concentration), and electron donors (5 mM ethanol or acetate) were added from sterile anaerobic stocks.

Microcosms were sampled by removing ~5 ml every 1-3 days using a sterile syringe equipped with an 18G needle under a H_2/N_2 atmosphere in a Coy anaerobic chamber. HCl extractable Fe(II) content was measured as previously described. Porewaters were extracted for determination of uranium and sulfate concentrations and carbon substrate utilization as previously described. At select time points, samples from each replicate microcosm were collected and pooled for cultivation-independent community characterization by centrifuging samples at 7,000

× g for 7 minutes, discarding the supernatant, and freezing the solid phase at -80°C until nucleic acid extraction.

Geochemistry of borehole sediment. Soluble geochemistry was determined on sediment samples from each depth interval by extracting sediment in a 1:1 (w/v) ratio in deionized water followed by a 1 hr incubation at 200 rpm on a shaker. Samples were centrifuged at 5,000 x g for 5 min, followed by filtration through a 0.22 µm nylon syringe filter. The liquid phase was then analyzed for SO_4^{2-} by the $BaSO_4$ turbidimetric method, for nitrate using the colorimetric method described by Cataldo et al., and the pH was measured with a calibrated digital pH meter. Samples for uranium determination were acidified with nitric acid and stored at -20°C prior to kinetic phosphorescence analysis using a KPA-11 analyzer (Chem-Chek Instruments, Richland, Washington).

Total iron and Fe(II) in each depth interval were determined by oxalate extraction and colorimetric quantification using ferrozine, as previously described. In brief, sediment samples for Fe(II) determination were kept under strictly anaerobic conditions and were extracted in anaerobic oxalate (0.2 M ammonium oxalate, 0.2 M oxalic acid, pH 2.5) for 4 hours in the dark, shaking at 200 rpm. After extraction, samples were centrifuged for 5 min at 5,000 rpm and the extract was added to ferrozine reagent (50 mM HEPES, 0.1% ferrozine, pH 7.0). The extract and ferrozine were incubated for 10 min in the dark then measured spectrophotometrically at 562 nm. Sediment samples for total Fe determinations were dried aerobically then extracted in oxalate in the dark at room temperature by shaking at 200 rpm for 4 hr. After extraction, samples were centrifuged for 5 min at 5,000 rpm and the extract was reacted with total Fe reagent (1% hydroxylamine hydrochloride in ferrozine) for 4 hr in the dark prior to measurement on a spectrophotometer at 562 nm.

Geochemical data of borehole sediments was published previously as supplemental material in Green et al. 2010 (Green *et al.* 2010).

Real-time PCR quantification of mRNA transcripts. Total RNA and mRNA were extracted from a total of 3.9 ml of microcosm sample according to previously described methods from our labs. The *gltA*-specific primers used for mRNA real-time PCR quantification were CS375nF (5'–AAC AAG ATG RCM GCC TGG G– 3') and CS598nR (5'–TCR TGG TCG GAR TGG AGA AT– 3'). Primers for *dsr*A-specific mRNA real-time PCR quantification were DSR1F (5'–AC[C/G] CAC TGG AAG CAC G–3') and DSRQP3R (5'–CGC ATG GTR TGR AAR TG–3' (this study). cDNA synthesis was performed using the method described by Chin et al. with 0.5 µg template mRNA and 2 µM reverse primer incubated at 52°C for 60 min, followed by enzyme inactivation at 70°C for 15 min. cDNA was purified, quantified and prepared for real-time PCR quantification using SYBR Green and the primer sets described above. Quantitative analysis of the cDNA was carried out with the Applied Biosystems 7500 Real-Time PCR system using 7500 Real-Time PCR System Sequence Detection Software (Version 1.3.1). Thermocycling for *glt*A was performed with an initial activation step at 50°C for 2 min and denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 1 min, and elongation at 65°C for 30 s, with a final extension step at 65°C for 6 min. The detection limits of PCR assays were determined and had a minimum sensitivity of 10^0 to 10^1 target molecules per reaction. The precision and reproducibility of quantification were carefully optimized, and correct lengths of PCR products were verified.

Phylogenetic analysis of partial *dsr*A and *glt*A cDNA clone sequences was performed. A clone library was generated with RT-PCR amplicons obtained from mRNA isolated from microcosm sediment samples according the methods described above. Resulting nucleotide *gltA* sequences were compared to the GenBank protein databases according to the BLASTx algorithm and the amino acid sequences were aligned with reference sequences using Clustal W. The resulting 75 amino acid alignment was used to infer phylogenetic trees using neighbor-joining methods.

Expression of *dsr*A and *glt*A during biostimulation. The addition of ethanol or acetate stimulated microbial activity with sulfate reduction observed only in treatments without molybdate, whereas Fe(III) reduction (accumulation of Fe(II)) was observed in all treatments. *Dsr*A transcripts were detected at nearly all time points of the incubation but correlated with sulfate reduction in the ethanol and acetate only amended treatments (Figures 10A and 11A). In the ethanol-amended treatment, *dsr*A transcripts increased linearly to a maximum of 1.52×10^4 copies per µg RNA at day 9 then decreased on day 14 when sulfate reduction ceased (Figure 10A). Approximately 3.2 mM of sulfate was reduced in the ethanol treatment in contrast to only 1.3 mM in the acetate treatment. The maximum abundance of *dsr*A transcripts was also lower with 7.29×10^3 copies per µg RNA detected on day 22 when sulfate reduction ceased (Figure 2A). In treatments where molybdate inhibited sulfate reduction (Figures 10A and 11A), *dsr*A transcripts were detected but at a very low abundance ($> 2.00 \times 10^3$ copies per µg RNA).

Abundance of *glt*A transcripts and Fe(II) increased proportionally in all treatments with the highest amount of Fe(II) and *glt*A transcripts observed on the same day (Figures 10B and 11B). The majority of Fe(II) accumulated between days 5 and 8 and the addition of molybdate did not impact the amount of Fe(II) produced or the quantity of transcripts (Figures 10B and 11B). In the ethanol only treatment at day 22, 8.4×10^4 *glt*A copies per µg RNA were observed, whereas, 1.2×10^5 *glt*A copies per µg RNA were observed on the same day in the ethanol with molybdate treatment (Figure 10B). Approximately 9.8 and 9.6 mM Fe(II) accumulated and the maximum copies of *glt*A transcripts was between 8.4×10^4 and 1.2×10^5 (copies per µg RNA) in the acetate and acetate with molybdate treatments, respectively (Figure 11B).

During the initial 5 days of incubation, U(VI) concentrations decreased in all treatments, however a more substantial decrease was observed in ethanol treatments (data not shown). U(VI) concentrations were observed to increase in conjunction with Fe(III) reduction and subsequently decreased by day 16 in the all treatments. Approximately 12 and 5 µM U(VI) was removed from

solution in the ethanol and acetate treatments, respectively. Ethanol was consumed from days 0 to 6 in ethanol-amended treatments and was incompletely oxidized to acetate. Acetate accumulated until day 6 and then was slowly consumed for the remainder of the incubation. In acetate-amended treatments, acetate was consumed slowly starting on day 4 and ceased on day 22 of the incubation.

In situ activity of SRB and FeRB in contaminated sediments. We quantified *dsr*A and *glt*A expression along with detailed geochemical analysis of sediments over a depth profile in order to determine whether the *in situ* geochemistry correlated with microbial activity. The expectation was that the abundance of *dsr*A transcripts would be low in regions with high sulfate due to low on going *in situ* sulfate reduction. As an indicator of on going iron reduction, we expected high copy numbers of *glt*A were in regions with high concentrations of Fe(II). In borehole FB124, from the highly contaminated Area 3 of the ORFRC, expression of *dsr*A and *glt*A transcripts and the geochemistry indicated on going microbial activity in a depth of 9.75 to 9.88 m below surface (Figure 12). The highest abundance of *dsr*A and *glt*A were observed in this depth interval along with low sulfate and high Fe(II) concentrations. The other depth intervals of FB124 had a low level of gene expression and little geochemical evidence of sulfate or iron reduction. The two deepest sediment samples from FB124 were also the most contaminated, as seen in the high uranium concentrations (Figure 12B) and expression of *glt*A and *dsr*A was low (Figure 12C). In contrast, depth profiling of gene expression and geochemistry in FB107 did not provide clear evidence for *in situ* microbial activity (Figure 13). In this core, the abundance of transcripts was much lower than FB124 (Figures 12C and 13C, note different scales) and the depth interval (6.30-6.34 m below surface) with the highest abundance did not have high Fe(II) or low sulfate (Figure 13). The region with the highest Fe(II) (5.84-5.89 m below surface) had moderate expression of *glt*A.

We demonstrated for the first time that the function of microbial communities can be quantified in subsurface sediments using messenger RNA assays (molecular proxies) under in situ conditions. The application of dsr and gltA gene expression as molecular proxies for the metabolically active sulfate-reducing and Fe(III)-reducing prokaryotes, respectively, was extended to low biomass core samples from the uranium-contaminated subsurface of the Oak Ridge and Hanford sites. Active *Geobacteraceae* were identified and phylogenetically characterized from the cDNA of messenger RNA extracted from ORFRC subsurface sediment cores. This is arguably the best way to identify the metabolically-active members of in situ microbial communities. Multiple clone sequences were retrieved from *G. uraniireducens*, *G. daltonii*, and *G. metallireducens*.

Figure 10: Expression of *dsr*A (A) and *glt*A (B) genes related to electron acceptor usage in ethanol-biostimulated microcosms. The lower panels show treatments amended with molybdate as an inhibitor of sulfate reduction. Values are averages ± standard deviations of the results for triplicate microcosms.

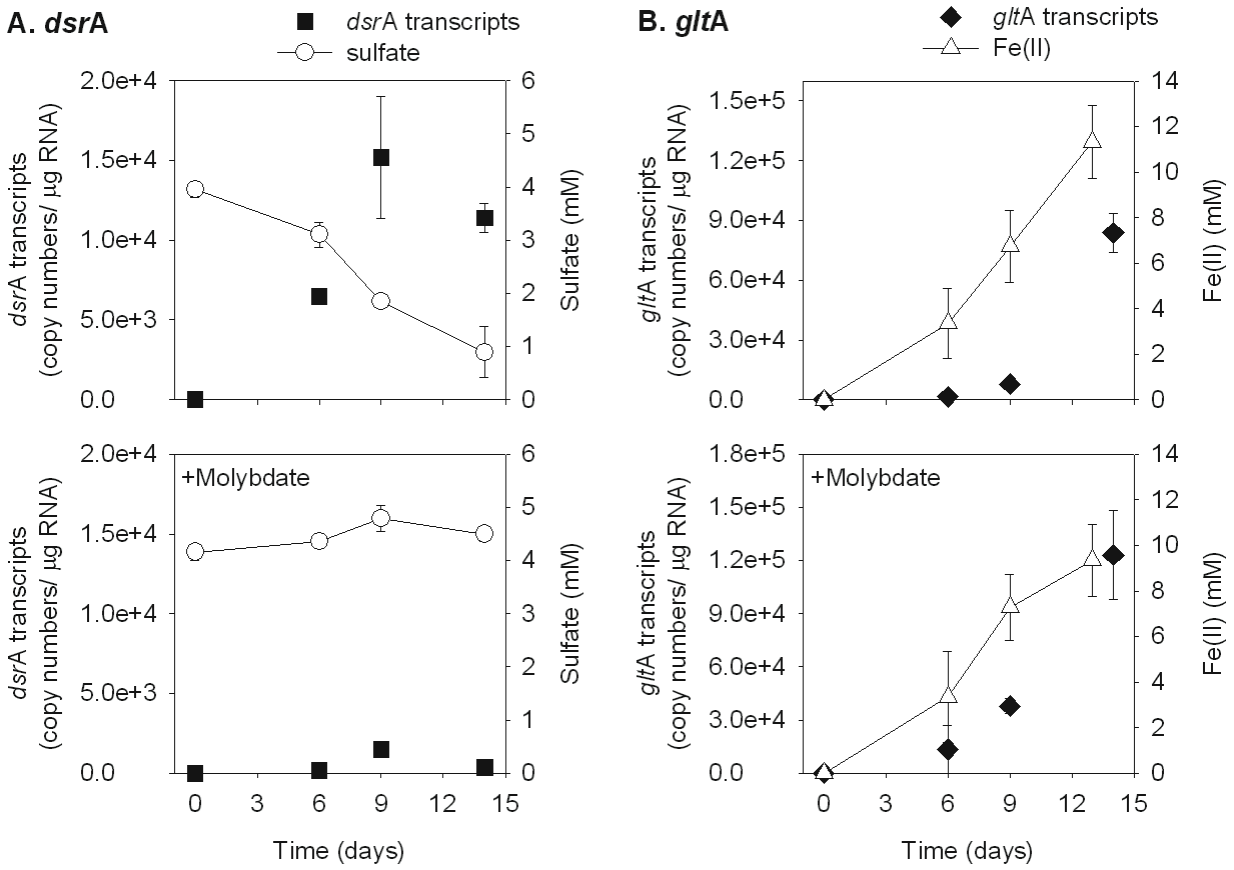


Figure 11: Expression of *dsr*A (A) and *glt*A (B) genes related to electron acceptor usage in acetate-biostimulated microcosms. The lower panels show treatments amended with molybdate as an inhibitor of sulfate reduction. Values are averages ± standard deviations of the results for triplicate microcosms.

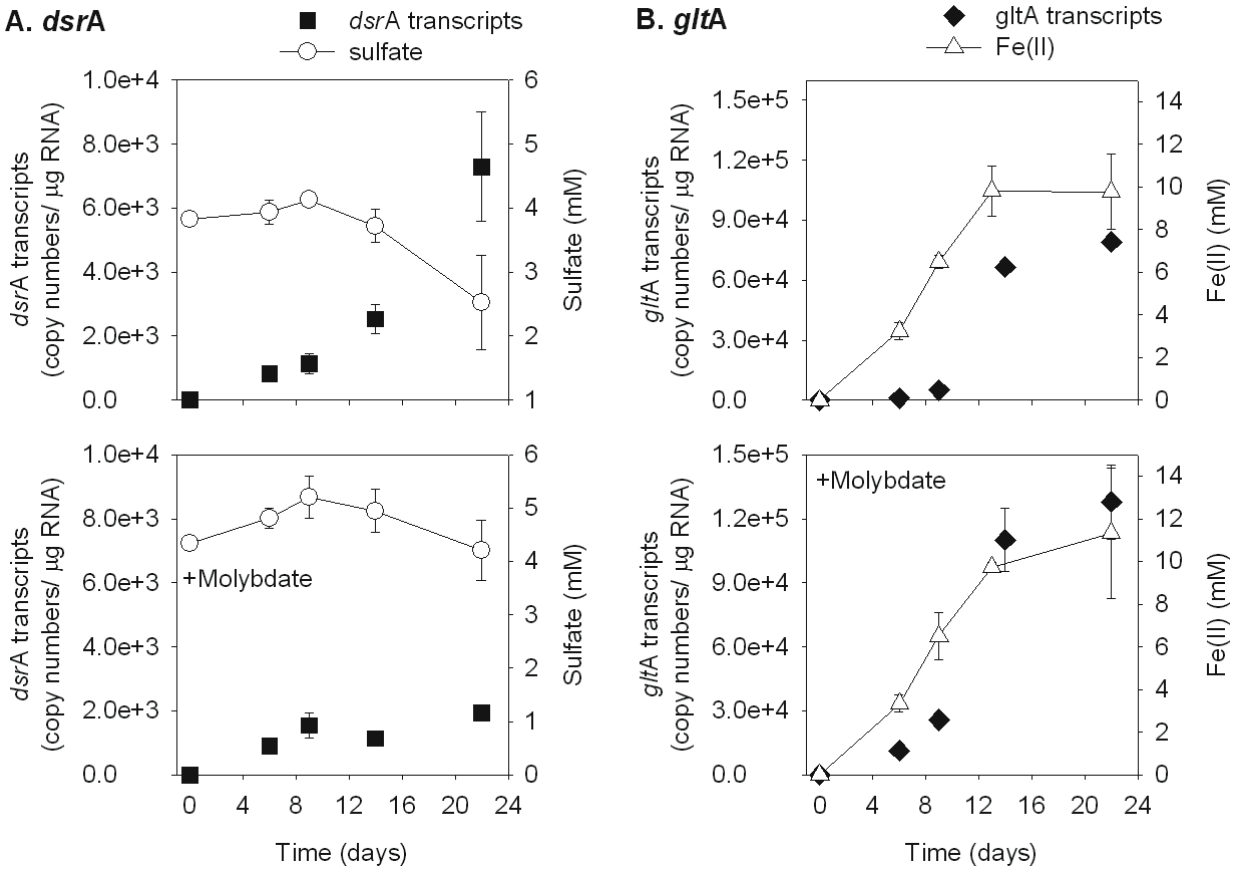


Figure 12: Geochemistry (A, B) and abundance of *dsr*A and *glt*A transcripts (C) in depth intervals of Area 3 borehole FB124. Values for transcript abundance are averages ± standard deviations of the results for triplicate samples.

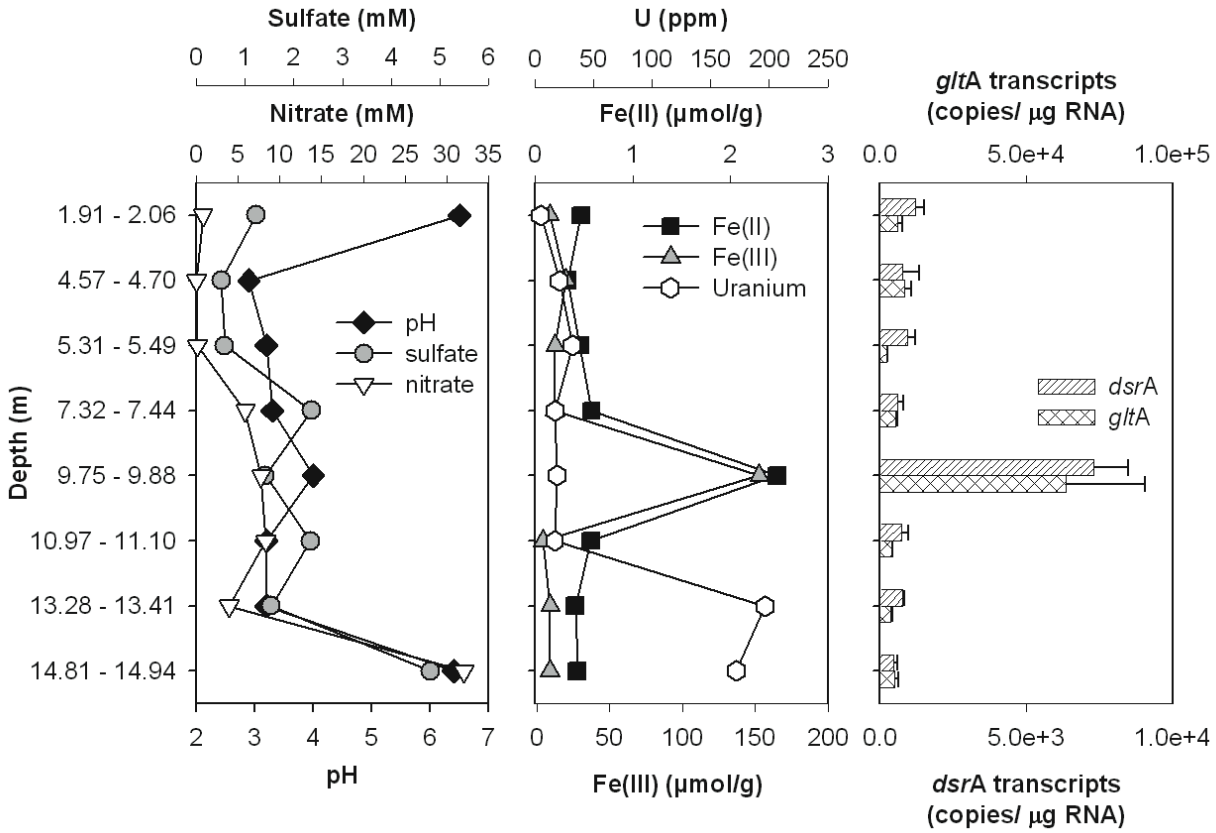
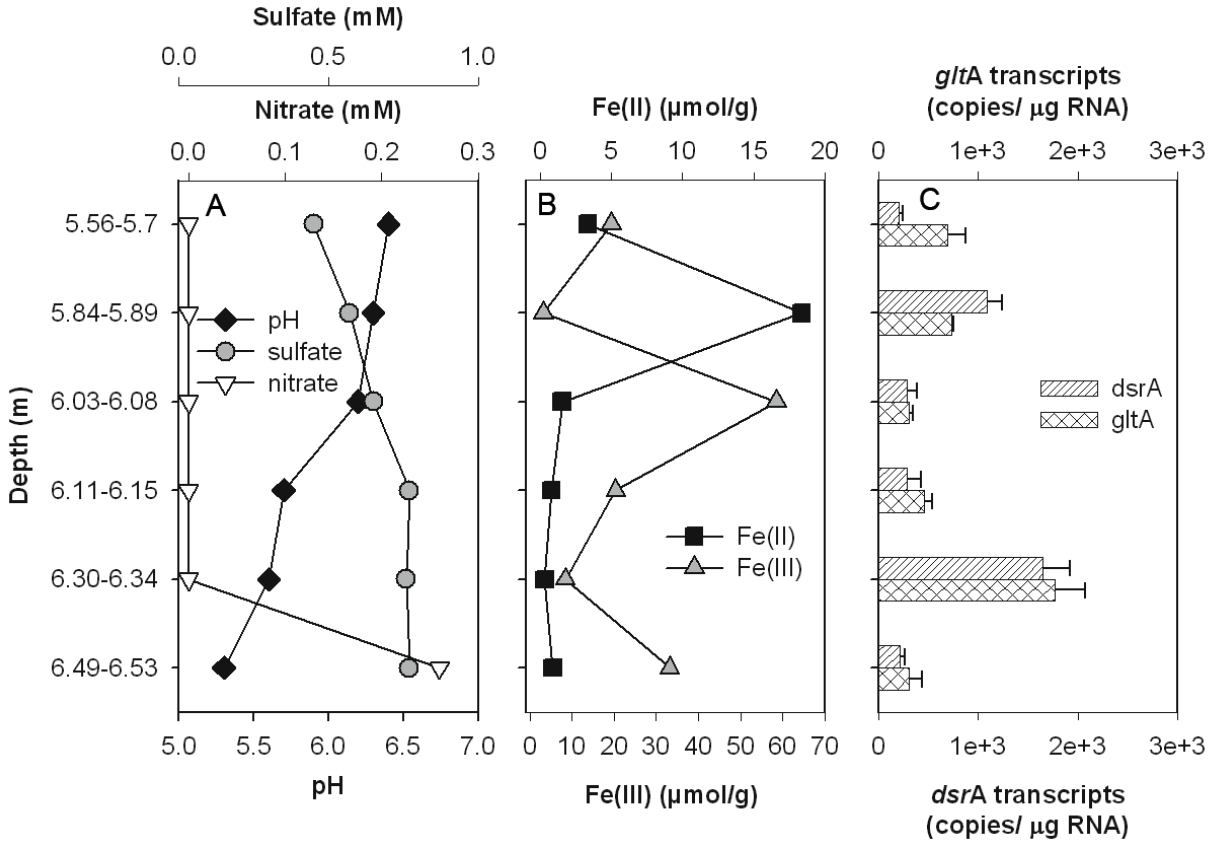


Figure 13: Geochemistry (A, B) and abundance of *dsr*A and *glt*A transcripts (C) in depth intervals of Area 2 borehole FB107. Values for transcript abundance are averages ± standard deviations of the results for triplicate samples. Uranium was not analyzed.



Iron Mineralogy Phase (Ribeiro et al., 2009)

The surface and colloidal behavior of phyllosilicate clay minerals significantly influences many important processes occurring in soils, sediments, and industrial formulations, including the fate of redox-active radionuclides in ORFRC sub-soils. Bacterial activity is now well known to change the oxidation state of Fe in phyllosilicates, but also alters the abundance and nature of Fe (oxyhydr)oxides external to the phyllosilicates. The purpose of this aspect of the study was to focus on the Fe in the phyllosilicates. The oxidation state of iron (Fe) in the crystal structures of smectite clay minerals has been found to be a critically important factor underlying the behavior of the clay. Changes in Fe oxidation state may occur either biotically due to the presence of Fe-reducing bacteria or abiotically by reaction with inorganic reductants such as dithionite. The link between clay behavior and Fe oxidation state was originally made from laboratory studies using chemical reductants. In natural systems, however, biotic pathways are the more likely method for altering oxidation state in the clay, but only a few studies have investigated whether similar effects are achieved by these two pathways. Because the largest body of evidence derives from the chemical pathway, but the biotic pathway is more important for most practical or natural processes, an understanding of the differences, if any, between the effects of these two pathways on clay mineral properties is important.

In a companion study funded by the ERSP program, Lee et al. (2006) used infrared spectroscopy to compare the effects of biotic and chemical reduction pathways on the clay

structure. Results revealed that: (1) the amount of structural Fe(II) that can be produced by the biotic pathway appears to be limited to less than approximately 1 mmole Fe(II)/g clay; (2) within this range of reduction, structural changes in the smectite were small compared to the dramatic changes observed by Fialips et al. (2002a,b) for reduction levels exceeding 3 mmole Fe(II)/g clay; (3) the effects of biotic and abiotic reduction were similar; and (4) reoxidation generally restored the infrared spectrum of the smectite to its original appearance. These conclusions contradict assertions that bacterial reduction of structural Fe in smectites evokes extensive and irreversible changes in the clay structure (Kim et al., 2003, 2004; Dong et al., 2004). Because the clay crystal is complex, consisting of many different types of chemical interactions, and infrared spectroscopy probes only the vibrational energies of interatomic bonds, further comparisons using complementary tools for structural analyses were warranted.

The significance of observation (2) above is that the relative fraction or percent of reduction in any given smectite will vary depending on the total Fe content. For Upton montmorillonite, with ~0.4 mmole/g, the fraction of reduction is virtually 100%; whereas, for Garfield nontronite, with ~4.5 mmole/g, the extent of reduction is only about 20%.

Because of its specificity for Fe, Mossbauer spectroscopy is uniquely suited to provide further structural information from redox-modified smectites, especially if employed over a wide range of temperatures (4 to 298 K). The purpose of this study was to provide further comparative information regarding the changes in clay structure that occur due to biotic or abiotic reduction, as probed by variable-temperature Mossbauer spectroscopy.

In this study we found that bacteria reduce structural Fe in clay minerals from their layer edges rather than from their basal surfaces. This is a new finding, not previously known, and was discovered only because of our ability to obtain Mossbauer spectra at temperatures below 10 K. We also found important differences in the extent and distribution of reduced Fe in the layers of clay minerals, depending on their total Fe content and Mg and Al composition.

II. Papers and other products delivered

A total of 20 publications (16 published or "in press" and 4 in review), 10 invited talks, and 43 contributed seminars/ meeting presentations were completed during the past four years of the project. Two symposia were chaired and one plenary talk was given by the PI at national/ international meetings. Please see a listing of publications and presentations below.

PI Kostka served on one proposal review panel per year for the U.S. DOE Office of Science during the four year project period. The PI leveraged funds from the state of Florida to purchase new instrumentation that aided the project. The PI initiated an international collaboration on uranium-contaminated subsurface environments in the former East Germany with scientists from the Friedrich Schiller University, Jena, Germany. This collaboration led to several publications and many meeting presentations (see below).

Honors/ Awards/ Service:

PI Kostka served on the following proposal review panels for the U.S. DOE Office of Science and other federal agencies:

U.S. Department of Energy, Environmental Remediation Sciences Program (2007)

U.S. Department of Energy, National Synchrotron Light Source II Imaging and Spectroscopy Workshop (2008)

National Institutes of Health, Development and Application of Nanotechnology-based Tools to Understand Mechanisms of Bioremediation (R01) (2008)

U.S. Department of Energy, Early Career Scientist Competition, Genome Science & Environmental Remediation Sciences Programs (2009)

PI Kostka was elected **Chair** (2009-present) for Division N, Microbial Ecology, American Society of Microbiology.
PI Kostka was elected as **Editor** (2011-2016) of the journal of Applied and Environmental Microbiology.

PI Kostka served as an instructor for the week-long Graduate Research School "Alteration and element mobility at the microbe-mineral interface" financially supported by the German Research Foundation DFG (1257), Friedrich Schiller University, Jena, Germany, November, 2009.

The following grants were leveraged from state and local sources in support of this DOE project:
FSU Research Foundation, Equipment and Infrastructure Enhancement Grant (EIEG), "NimbleGen Microarray Facility for Functional Genomics Analyses," 3/15/08-3/14/11, **$52,965** (coPI).
FSU Research Foundation, Equipment and Infrastructure Enhancement Grant (EIEG), "Real-Time PCR Detection System for Quantitative Analysis of Gene-Expression and Gene Copy Numbe**r**," 6/04/09-6/04/12, **$32,221** (coPI).

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2) Akob, D.M. 2008. Structure and function of microbial communities controlling the fate and transformation of U(VI) in radionuclide contaminated subsurface sediments. Ph.D. Dissertation. Earth, Ocean, and Atmospheric Science Dept., Florida State University, Tallahassee, FL.

3) Akob, D.M., H.J. Mills, T.M. Gihring, L. Kerkhof, J.W. Stucki, A.S. Anastacio, K.J. Chin, K. Kusel, A.V. Palumbo, D.B. Watson, and J.E. Kostka. 2008. Functional diversity and electron donor dependence of microbial populations capable of U(VI) reduction in radionuclide-contaminated subsurface sediments. Applied and Environmental Microbiology, 74, 3159-3170.

4) Akob, D.M., H.J. Mills, and J.E. Kostka. 2007. Metabolically active microbial communities in uranium-contaminated subsurface sediments. FEMS Microbiology Ecology, 59, 95-107.

5) Burkhardt, E.-M., D.M. Akob, S. Bischoff, J. Sitte, J.E. Kostka, D. Banerjee, A.C. Scheinost, and Kirsten Kuesel. 2010. Impact of biostimulated redox processes on metal dynamics in an iron-rich creek soil of a former uranium mining area. Environmental Science & Technology 44: 177-183.

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14) Winch, S., H. J. Mills, J. E. Kostka, D. Fortin, D. R.S. Lean. 2009. Identification of sulfate-reducing bacteria in methylmercury contaminated mine tailings by analysis of SSU ribosomal RNA genes. FEMS Microbiol. Ecol. 68: 94-107.

15) Wu, W., J. Carley, S.J. Green, J. Luo, S. D. Kelly, K. Lowe, T. Mehlhorn, S. Carroll, B. Boonchayanant, D. Watson, K. M. Kemner, J. Zhou, P. K. Kitanidis, J.E. Kostka, P. M. Jardine, and C. S. Criddle. 2010. Effects of nitrate on the stability of uranium in a bioreduced region of the subsurface. Environmental Science & Technology 44: 5104-5111.

16) W. Wu, J. Carley, D. Watson , B. Gu, S. Brooks, S. D. Kelly, K. Kemner, J. D. van Nostrand , L. Wu, M. Xu, J. Zhou, J. Luo, E. Cardenas, C. Hwang, M. W. Fields, T. L. Marsh, J. M. Tiedje, S. J. Green, J. E. Kostka, P. K. Kitanidis, P. M. Jardine, C. S. Criddle. 2011. Bioreduction and immobilization of uranium in situ: a case study at a USA Department of Energy radioactive waste site, Oak Ridge, Tennessee. Acta Scientiae Circumstaniae (Chinese journal; in press).

17) Akob, D.M., L. Kerkhof, K. Küsel, D. B. Watson, A. V. Palumbo, and J. E. Kostka. 2011. Linking specific heterotrophic bacterial populations to bioreduction of uranium and nitrate using stable isotope probing in contaminated subsurface sediments. Applied and Environmental Microbiology (in review).

18) S.J. Green, O. Prakash, P. Jasrotia, W.A. Overholt, E. Cardenas, D. Hubbard, J.M. Tiedje, D.B. Watson, P.M. Jardine, S.C. Brooks, and J.E. Kostka. 2011. Denitrifying bacteria from the

genus Rhodanobacter dominate microbial communities in a uranium- and nitrate-contaminated subsurface environment. Applied and Environmental Microbiology (in review).

19) Akob, D.M., S. H. Lee, M. Sheth, K-J. Chin, K. Küsel, D. B. Watson, A. V. Palumbo, and J.E. Kostka. 2011. Metabolic activity of sulfate- and metal-reducing bacteria in U(VI)-contaminated sediments. Applied and Environmental Microbiology (in review).

20) O. Prakash, S. J. Green, P. Jasrotia, W. A. Overholt, A. Canion, D. B. Watson, S. C. Brooks, and J. E. Kostka. Description of *Rhodanobacter denitrificans* sp. nov., isolated from uranium and nitrate contaminated subsurface sediment. International Journal of Systematic and Evolutionary Microbiology (in review).

Conferences, Symposia, and Invited Seminars:

The PI Kostka chaired the following symposia which included several ERSP/ SBR investigators. He also presented a talk in each of the symposia (cited below):

"The Shallow Subsurface Biosphere: Critical Geobiological Interface and Window into the Deep Biosphere," Chair of symposium, 108th American Society for Microbiology General Meeting, Boston, MA, June, 2008

"Iron Geomicrobiology," Co-chair of session, Goldschmidt Conference, Knoxville, TN, June, 2010

Invited Seminars:

1) Kostka, J.E. 2010. Extreme Denitrification: Sedimentary Microorganisms that Breathe Nitrogen from the Acidic Subsurface to the Permanently Cold Arctic. Georgia Institute of Technology, Atlanta, Georgia, January.
2) Kostka, J.E. 2010. Extreme Denitrification: Acid Tolerant Denitrifying Microbes that Drive Nitrate Attenuation in the Terrestrial Subsurface. Argonne National Laboratory, Argonne, IL, February.
3) Kostka, J.E. 2010. Ecosystem functional genomics: inferring environmental forcing of community structure and function, Institute for Computing in Science Workshop: Computational Methods and Terabase Genomics, Sponsored by Argonne National Lab and U.S. DOE, July, 2010.
4) S.J. Green. 2010. Current molecular methods that target denitrification genes do not detect key denitrifying taxa in soils. Second Annual Argonne Soils Workshop, Argonne, IL, October.
5) Kostka, J.E. 2009. Extreme Geomicrobiology: Ecology of Prokaryotes that Catalyze Critical Geobiological Processes in the Subsurface, Oak Ridge National Laboratory, Oak Ridge, TN, November.
6) Green, S.J. Microbial Community Analyses of a Nitrate- and Uranium-Contaminated Subsurface Environment: Prospects for Bioremediation. Montclair State University, Department of Biology and Molecular Biology Seminar (Montclair, NJ). November 2009.
7) Kostka, J.E. 2009. Keynote presentation: Ecology of Fe(III)-reducing bacteria that catalyze critical geobiological processes in sedimentary ecosystems. Goldschmidt Conference, Davos Switzerland, June.
8) Kostka, J.E. 2009. Ecology of Iron Microbes that Catalyze Critical Geobiological Processes in Sedimentary Ecosystems, Graduate Research School, "Alteration and element mobility at the microbe-mineral interface" financially supported by the German Research Foundation DFG (1257), November.

9) Kostka, J.E. 2009. Digging in the Dirt: Field Methods in Geomicrobiology, Graduate research school "Alteration and element mobility at the microbe-mineral interface" financially supported by the German Research Foundation DFG (1257), November.
10) Kostka, J.E. 2009. Extreme Geomicrobiology: Ecology of Prokaryotes that Catalyze Critical Geobiological Processes in the Subsurface, Helmholtz Institute for Environmental Research, Leipzig, Germany, November.

Contributed Seminars and Meeting Presentations:

1) Green, S.J., Jasrotia, P., Overholt, W.A., Prakash, O., Gihring, T.M., Jardine, P.M., Watson, D.B., Brown, S.D., Palumbo, A.V., Schadt, C., Brooks, S., Sul, W.-J., Tiedje, J., Reed, J. and Kostka, J.E. 2011. Why are Rhodanobacter spp. so dominant in a uranium-contaminated subsurface environment? American Society for Microbiology Annual Meeting, 2011. New Orleans, LA.
2) Hu, P., Wu, C.H., DeSantis, T., Jasrotia, P., Woo, H., Kearcher, K., Meiss, S., Torok, T., Taylor, L.D., Overholt, W.A., Green, S.J., Andersen, G.L., Kostka, J.E., and Hazen, T.C. 2011. Validation of MycoChip – A Microarray for Fungal Community Studies. American Society for Microbiology Annual Meeting, 2011. New Orleans, LA.
3) Kanak, A., M. Patel & K-J. Chin. 2011. Functional Analysis of *Geobacter daltonii* to Determine Anaerobic Benzene Degradation Processes. American Society for Microbiology Annual Meeting, 2011. New Orleans, LA.
4) Jasrotia, P., Overholt, W.A., Green, S.J., Schadt, C.W., Watson, D.B., Brooks, S., and Kostka, J.E. 2011. Watershed scale fungal community characterization along a pH gradient in an aquifer co-contaminated with uranium and nitrate. American Society for Microbiology Annual Meeting, 2011. New Orleans, LA.
5) Kostka, J.E., Prakash, O., Green, S.J., Jasrotia, P., Kerkhof, L., Chin, K.-J., Keller, M., Venkateswaran, A., Elkins, J.G., Stucki, J.W., Brown, S.D., Palumbo, A.V. 2011. Structure and Function of Subsurface Microbial Communities Affecting Radionuclide Transport and Bioimmobilization. Department of Energy Subsurface Biogeochemical Research Program Annual PI Meeting. Washington, D.C., April.
6) Gihring, T., Schadt, C.W., Zhang, G., Yang, Z., Carroll, S., Lowe, K., Mehlhorn, T.L., Jardine, P.M., Watson, D.B., Brooks, S.C., Wu, W., Kostka, J.E., Green, S.J. 2010. Changes in microbial community structure during amendment with long-term electron donor sources for bioreduction of groundwater contaminants. Department of Energy Environmental Remediation Sciences Program 5th Annual PI Meeting. Washington, D.C, April.
7) Gihring, T., Schadt, C.W., Zhang, G., Yang, Z., Carroll, S., Lowe, K., Mehlhorn, T.L., Jardine, P.M., Watson, D.B., Brooks, S.C., Wu, W., Kostka, J.E., Overholt, W.A., Green, S.J., Zhou, J., Zhang, P., Von Nostrand, J. 2010. Changes in microbial community structure and activity during amendment with long-term electron donor sources for bioreduction of groundwater contaminants. Goldschmidt 2010. Knoxville, TN, June.
8) Green, S.J., Jasrotia, P., Hubbard, D., Overholt, W.A., Prakash, O., Gihring, T.M., Akob, D.M., Jardine, P.M., Watson, D.B., Brown, S.D., Palumbo, A.V., Sul, W.J., Tiedje, J., Kostka, J.E. 2010. Current molecular methods that target denitrification genes do not detect key denitrifying taxa. International Society for Microbial Ecology Symposium 2010. Seattle, WA, August.
9) Green, S.J., Jasrotia, P., Hubbard, D., Prakash, O., Kostka, J.E., Gihring, T., Schadt, C.W., Watson, D.B., Jardine, P.M., Brooks, S. 2010. Characterization of denitrifying microbial communities in the subsurface co-contaminated with uranium- and nitrate from the molecular to the watershed scales. Department of Energy Environmental Remediation Sciences Program 5th Annual PI Meeting. Washington, D.C., April.
10) Jasrotia, P., Prakash, O., Canion, A.K., Green, S.J., Kostka, J.E. 2010. Isolation and characterization of acid tolerant denitrifying bacteria and fungi from the terrestrial

subsurface. Department of Energy Environmental Remediation Sciences Program 5th Annual PI Meeting. Washington, D.C., April.

11) Sheth, M., D. Akob, J. E. Kostka, K-J. Chin. 2010. Metabolically Active Microbial Communities in Uranium(VI)-Contaminated Hanford 300 Area Subsurface Sediments. American Society for Microbiology Annual Meeting. San Diego, CA May.
12) Jasrotia, P., Prakash, O., Canion, A.K., Green, S.J., Kostka, J.E. 2010. Isolation and characterization of acid tolerant denitrifying fungi and bacteria from the terrestrial subsurface. American Society for Microbiology Annual Meeting. San Diego, CA May.
13) Jasrotia, P., S. J. Green, W. Overholt, D. Hubbard, and J. E. Kostka. 2010. Probing the denitrifying microbial community in uranium-contaminated subsurface environments with multi-faceted molecular- and cultivation-based approaches. Graduate Student Symposium, Earth Ocean & Atmospheric Science Department, Florida State University, November.
14) Kostka, J.E., Prakash, O., Green, S.J., Jasrotia, P., Kerkhof, L., Chin, K.-J., Keller, M., Venkateswaran, A., Elkins, J.G., Stucki, J.W., Brown, S.D., Palumbo, A.V. 2010. Structure and Function of Subsurface Microbial Communities Affecting Radionuclide Transport and Bioimmobilization. Department of Energy Environmental Remediation Sciences Program 5th Annual PI Meeting. Washington, D.C., April.
15) Overholt, W.A., Green, S.J., Prakash, O., Gihring, T., Akob, D.M., Jasrotia, P., Jardine, P.M., Watson, D.B., Brown, S.D., Palumbo, A.V., Sul, W.J., Tiedje, J., Kostka, J.E. 2010. Denitrifying bacteria from the genus Rhodanobacter are key members of acidic and uranium-contaminated subsurface environments from Oak Ridge, TN. American Society for Microbiology Annual Meeting, 2010. San Diego, CA., May.
16) Salome, K.R., Beazley, M.J., Green, S.J., Martinez, R.J., Kostka, J.E., Sobecky, P.A., Taillefert, M. 2010. Competition Between U(VI) Bioreduction and Biomineralization in a Contaminated Sediment. Department of Energy Environmental Remediation Sciences Program 5th Annual PI Meeting. Washington, D.C., April.
17) Burkhardt, E.-M., S. Bischoff, D. M. Akob, J. E. Kostka, and K. Küsel. 2009. Metal mobility changes during microbial redox processes in contaminated creek soils. Goldschmidt Conference, Davos Switzerland, June.
18) Akob, D.M., E.-M. Burkhardt, D.M. Akob, J. Sitte, L. Kerkhof, K. Kuesel, D.B. Watson, A.V. Palumbo, and J.E. Kostka. 2009. Identification of active microbial communities linked to bioremediation and natural attenuation of radionuclides and heavy metals in contaminated aquifers. Goldschmidt Conference, Davos Switzerland, June.
19) Burkhardt, E.-M., D.M. Akob, S. Bischoff, J. E. Kostka, and K. Küsel. 2009. Metal Mobilization by Fe(III)-reducing Microbial Communities in Contaminated Creek Soils. VAAM/GBM Annual Conference, Bochum, Germany, March.
20) Sitte, J., D. Akob, C. Kaufmann, K. Pollok, K. Finster, J. Kostka, F. Langenhorst, and K. Küsel. 2009. Activity and Identification of Sulfate Reducing Prokaryotes in Heavy Metal and Radionuclide Contaminated Creek Soil. VAAM/GBM Annual Conference, Bochum, Germany, March.
21) Overholt, W.A., S. J. Green, W. Wu, D. Watson, P. Jardine, T. Mehlhorn, K. Lowe, J. Carley, S. Carroll, G. Zhang, C. Schadt, C. Criddle and J. E. Kostka. 2009. Tracking long-term shifts in microbial community structure in an experimentally-manipulated subsurface environment exposed to high levels of radionuclides and nitrate. 109th American Society for Microbiology General Meeting, Philadelphia, PA, May.
22) Prakash, P., S. J. Green, T. M. Gihring, P. Jardine, D. Watson and J. E. Kostka. 2009. Denitrification activity and novel denitrifying bacteria isolated from a terrestrial aquifer exposed to mixed contamination. 109th American Society for Microbiology General Meeting, Philadelphia, PA, May.

23) Green, S.J., D. M. Akob, W. J. Sul, J. Tiedje, and J. E. Kostka. 2009. Combined Geochemical and Pyrosequencing Community Analysis of a Uranium-contaminated Subsurface Sediment Profile. 109th American Society for Microbiology General Meeting, Philadelphia, PA, May.
24) Lee, S.H., D. M. Akob, J. E. Kostka, K-J. Chin. 2009. Metabolically Active Prokaryotes in Uranium(VI)-Contaminated *in situ* Subsurface Sediments. 109th American Society for Microbiology General Meeting, Philadelphia, PA, May.
25) Wu, W.-M., G. Zhang, S. D. Kelly, F. Zhang, T. Mehlhorn, S. Green, K. M. Kemner, S. Broocks, J. Kostka, C. S. Criddle, C. Schadt, D. Watson, P. M. Jardine. 2009. Reduction of Uranium (VI) in Sediments with Complex Organic Electron Donors. 109th American Society for Microbiology General Meeting, Philadelphia, PA, May.
26) P. Jasrotia, S. J. Green, D. Akob, W. J. Sul, J. M. Tiedje, P. M. Jardine, D. B. Watson, and J. E. Kostka. 2009. Profiling of microbial community structure across physico-chemical gradients using deep sequencing in the uranium-contaminated subsurface. Fourth Annual DOE-ERSP Principal Investigator Meeting, Landsdowne, VA, April.
27) S. J. Green, W. A. Overholt, W. Wu, D. Watson, P. Jardine, T. Mehlhorn, K. Lowe, J. Carley, S. Carroll, G. Zhang, C. Schadt, C. Criddle, J. D. Van Nostrand, J. Zhou, and J. E. Kostka. 2009. Distinctive shifts in subsurface microbial community structure correlate with uranium redox phases during *in situ* field manipulation at the Oak Ridge Integrated Field Research Challenge (OR-IFRC). Fourth Annual DOE-ERSP Principal Investigator Meeting, Landsdowne, VA, April.
28) J.E. Kostka. 2009. Microbial Community Response Parallels Uranium Immobilization and Remobilization during *In Situ* Field Manipulation. Fourth Annual DOE-ERSP Principal Investigator Meeting, Landsdowne, VA, April.
29) Chin, K.-J. 2008. Quantifying functional gene expression of sulfate- and iron-reducing bacteria in anaerobic environments. International Symposium on Environmental Microbiology (Invited Talk), Tokyo Metropolitan University, Tokyo, Japan, February.
30) Kostka, J.E. 2008. "Cold War Biogeochemistry: Nuclear Legacy Waste in the Subsurface of U.S. DOE Sites," FAMU-FSU College of Chemical & Biomedical Engineering, Tallahassee, February.
31) Kostka, J.E. 2008. "Cold War Microbiology: Bioremediation of Nuclear Legacy Waste in the Subsurface of U.S. DOE Sites," Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, April.
32) Kostka, J.E. 2008. "Identification and quantification of metal-reducing bacteria that catalyze redox reactions in radionuclide-contaminated subsurface soils." Workshop: Clay Surface Redox Processes: Fundamentals and Characterization Techniques, 45th Annual Meeting of the Clay Minerals Society, April.
33) Kostka, J.E. 2008. Structure and Function of Subsurface Microbial Communities Affecting Radionuclide Transport and Bioimmobilization. ERSP Principal Investigator Meeting, Landsdowne, VA, April.
34) D.M. Akob, K. Chin, L. Kerkhof, K. Kuesel, D.B. Watson, A.V. Palumbo, and J.E. Kostka. 2008. Targeting the metabolically active iron(III)- and sulfate-reducing bacteria with a high potential for U(VI) bioimmobilization in contaminated subsurface sediments. Third Annual DOE-ERSP PI Meeting, Landsdowne, VA, April.
35) D. M. Akob, L. Kerkhof, K.-J. Chin and J. E. Kostka. 2008. Characterization of the Metabolically Active Iron(III)- and Sulfate-Reducing Bacteria in Uranium(VI) Contaminated Subsurface Sediments Using DNA-SIP Analysis. 108th American Society for Microbiology General Meeting, Boston, MA, June.
36) E. Ewald, D. Akob, J. Kostka, J. Sitte, and Kirsten Küsel. 2008. Effect of Iron Reduction on Heavy Metal Mobilisation. 108th American Society for Microbiology General Meeting, Boston, MA, June.

37) Kostka, J.E. 2008. "Quantification of metabolically active prokaryotes in the shallow subsurface," 108th American Society for Microbiology General Meeting, Boston, MA, June.
38) Kostka, J.E., 2008. "Microbial Ecology of Fe(III)-reducing Bacteria," Telluride Workshop: Biogeochemical Processes of the Iron Cycle: From Microbes to Mineral Surfaces, Telluride, CO, July.
39) Lee, S., D. Akob, J. E. Kostka, K.-J. Chin. 2008. Quantification of functional gene expression of sulfate- and iron(III)-reducing bacteria and phylogenetic analysis of metabolically active bacteria in uranium(VI)-contaminated subsurface sediments. American Society for Microbiology (ASM) General Meeting, Boston, MA, June.
40) Stucki, J. W., A. S. Anastacio, and P. Sellin. 2008. Interaction of Bentonite and Iron(0) in Aqueous Suspension. Annual Meeting of The Clay Minerals Society, Abstracts.
41) Pereira, Marcio C., Jose Domingos Fabris, and Joseph W. Stucki. 2008. Characterization of structural changes in redox-modified smectites using near-infrared reflectance spectroscopy. Fourth Middle European Clay Conference, Abstracts, p. 125.
42) Ribeiro, Fabiana R., Joel E. Kostka, Peter Komadel, and Joseph W. Stucki. 2008. Comparison of structural iron reduction in smectites by bacteria and dithionite: A variable-temperature Mossbauer spectroscopic study. Fourth Middle European Clay Conference, Abstracts, p. 139.
43) Stucki, Joseph W. 2008. Common histories between the Soil Science Society of America and The Clay Minerals Society. Invited presentation to a special symposium on the history of soil science. Houston, Texas, October 6, 2008.