1. Close out technical report for Grant DE-FG02-97ER62469

1997-2000

Noncompetitive microbial diversity patterns in soils: their causes and implications for bioremediation

2000-2003

Understanding the roles of spatial isolation and carbon in microbial community structure dynamics and activity for bioremediation

2003-2006

Towards understanding population dynamics of metal and radionuclide reducers at field remediation sites

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2. There are no patents pending or protected data reported on herein.

3. Executive summary

This funding provided support for over nine years of research on the structure and function of microbial communities in subsurface environments. The overarching goal during these years was to understand the impact of mixed contaminants, particularly heavy metals like uranium, on the structure and function of microbial communities. In addition we sought to identify microbial populations that were actively involved in the reduction of metals because these species of bacteria hold the potential for immobilizing soluble metals moving in subsurface water. Bacterial mediated biochemical reduction of metals like uranium, technetium and chromium, greatly reduces their mobility through complexation and precipitation. Hence, by taking advantage of natural metabolic capabilities of subsurface microbial populations it is possible to bioremediate contaminated subsurface environments with a cost-effective *in situ* approach. Towards this end we have i.) identified bacterial populations that have thrived under the adverse conditions at the contaminated FRC site, ii.) phylogenetically identified populations that respond to imposed remediation conditions at the FRC, iii.) used metagenomics to begin a reconstruction of the metabolic web in a contaminated subsurface zone, iv.) investigated the metal reducing attributes of a Gram-positive spore forming rod also capable of dechlorination.

4. Comparison of original goals with accomplishments.

1997-2000

The objectives were to determine the structure and composition of microbial communities as influenced by mixed wastes, and to determine the mechanisms controlling microbial community diversity. To the extent possible at the time, we interrogated contaminant impacted microbial communities to test our hypotheses regarding response to toxicity. Twenty nine soil samples were taken from contaminated and uncontaminated sites at Dover Air Force Base, Deleware; the heavy metal contaminated site at Cannelton, Michigan; and site in MI, Abbott's Pit in Virginia; and the noncontaminated longterm experimental research site at the Kellogg Biological Station in Michigan. Over six hundred 16S rRNA clones were partially sequenced. This approach provided insight into the impact of contaminant on soil communities as well as the causes of diversity and identified the populations that were able to adapt to the new conditions. This work identified spatial isolation as a major controller of microbial diversity.

2000-2003

Two objectives were cited in this proposal: (1) To determine the key mechanisms controlling soil microbial community structure, and (2) to determine the impacts of radioactive and mixed waste contaminants on the structure and composition of microbial communities and the effects of spatial isolation on the responses of microbial communities to such contaminants. These objectives were met by examining spatial isolation, carbon source heterogeneity and contaminant levels in field samples and laboratory microcosms.

2003-2006

Our objectives and associated hypotheses were stated in the original proposal as:
(1.) Deleted in revised workplan, as requested.
(2.) To determine the microbial populations important for the reduction of U, Cr and Tc at the NABIR-FRC using metagenomics-based approaches.
(3.) Apply the developed molecular technologies for quantifying which microbial populations grew during field implementation of remediation.

These goals were met though the construction of metagenomic libraries and 16S rRNA clone libraries from FRC sediment. In addition, microcosms were cultivated with ^{13}C-ethanol to mimic the subsurface conditions established by the Criddle-Jardine reduction zone at the FRC. Nucleic acid was extracted, separated based on buoyant density and fractionated to isolate the ^{13}C-labeled fraction. The ^{13}C-labeled DNA was used for the construction of 16S rRNA clone libraries to identify the metabolically active members of a metal reducing community.

5. Summary of Project Activities.

a. **1997-2000: Noncompetitive microbial diversity patterns in soils: their causes and implications for bioremediation.** Twenty nine soil samples were taken from four geographically distinct locations with a range of contaminant concentrations as well as soil carbon levels. Using diversity indices applied to 16S rRNA clone libraries it was shown that uniform diversity characteristic of the surface communities had diversity index values that were 2 to 3 orders of magnitude greater than those for the

high-dominance, saturated, subsurface communities. A uniform or evenly structured community suggests that competition between species does not shape the structure of the community, rather spatial isolation may be the driving pressure for these communities. In addition, high carbon concentrations may support more uniform distributions, even in saturated soils. Figs. 1 & 2 are from publication #23 (listed below).

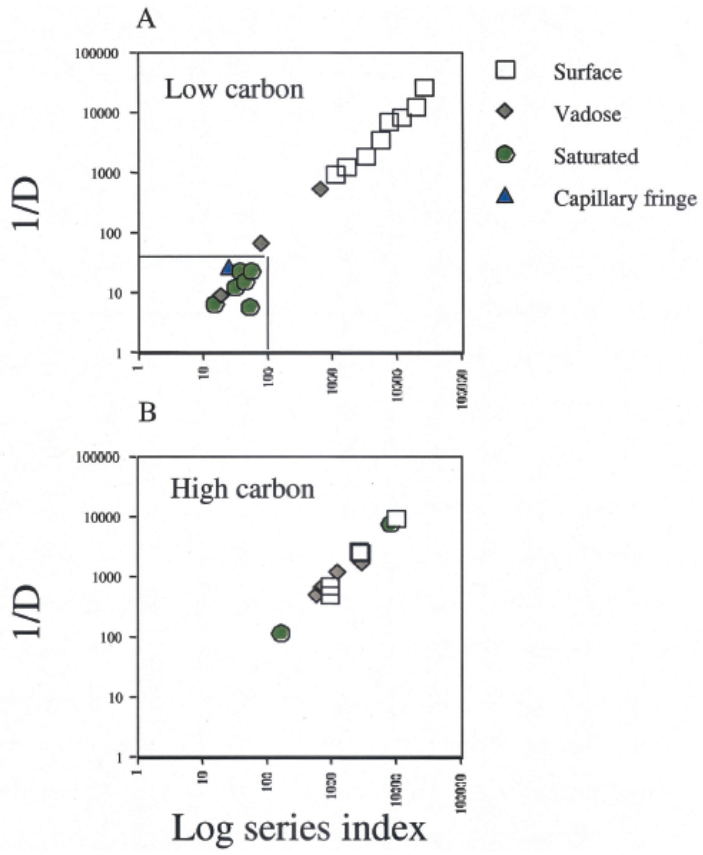

Figure 1. Plots of reciprocal of Simpson's index (1/D) versus the log series index for the microbial communities at different soil depths. Note the difference between low-carbon (A) and high-carbon (B) sites. Boxed values indicate dominance.

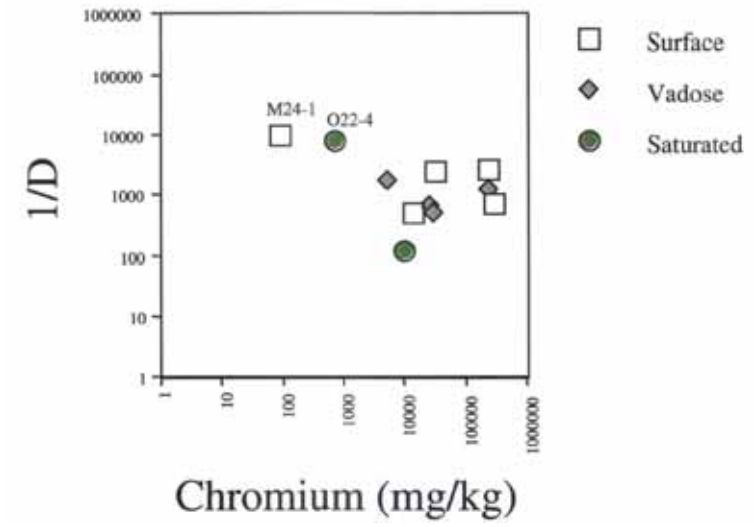

Figure 2. Comparison of microbial diversity and chromium levels at the high-carbon Cannelton site. Note that there appears to be little dominance and relatively high diversity, even at high concentrations of chromium.

b. **2000-2003: Understanding the roles of spatial isolation and carbon in microbial community structure dynamics and activity for bioremediation.** Microbial community diversity and heterogeneity in saturated and unsaturated subsurface soils from Abbott's Pit in Virginia (1.57, 3.25, and 4.05 m below surface) and Dover Air Force Base in Delaware (6.00 and 7.50 m below surface) were analyzed using a culture-independent small-subunit (SSU) rRNA gene (rDNA)- based cloning approach. We detected 4-6 dominant OTUs in clone libraries from 33 to 100 unique sequences. Most of the bacterial clones were affiliated with members of the *Proteobacteria* family (90%), gram-positive bacteria (3%), and members of the *Acidobacteria* family (3%). No OTUs were shared by the two sites. In addition, samples from nonsaturated regions grouped together and apart from samples derived from saturated soils. The diversity diminished with depth. Table 1 below is from publication 16 (listed below).

Genus	Group	% of units of bacteria in sample					Mean %	No. of diff[b]	PC[c]	Loading[d]
		DBW2	DBW1	AAW	AVZ	ABW				
Pseudomonas	Gamma	19.8	50.5	76.8	48.2	44.5	47.96	20	2	−0.234
Agrobacterium	Alpha	16.6	10.8	1.9	3.7	31.4	12.88	1	1	0.206
Ralstonia	Beta	33.4	15.3	0	0	0	9.74	1	2	0.268
Halomonas	Gamma	1	0.5	13.6	16.8	4.3	7.24	2	2	−0.326
Staphylococcus	Low G+C	0	0	4.1	3.7	0	1.56	1	2	−0.317
Stenotrophomonas	Gamma	3.4	0.5	0	0	0	0.78	1	3	−0.260
Burkholderia	Beta	0.5	1	0	0	1.2	0.54	4	2	0.243

[a] Data are presented as percentage of each sample.
[b] diff, the number of different strains represented.
[c] The PC on which the unit loads most heavily.
[d] Loading values represent the results of PCA of the 33 units.

Table 1. Summary of the units of bacteria found that were present at the five sites.

c. **2003-2006: Towards understanding population dynamics of metal and radionuclide reducers at field remediation sites.** A microbial community indigenous to a highly contaminated area at the FRC was sequenced by JGI using metagenomic techniques. Biomass was collected from approximately 1,700 L of FRC well FW106 groundwater. This groundwater had a pH of 3.4 and contained approximately 50 mg of uranium, 4,000 mg of nitrate, and 2,000 mg of sulfate/L. Direct bacterial counts were between 10^4 and 10^5 cells/ml. Groundwater was collected using peristaltic pumps and passed through filters to collect the biomass and the DNA was extracted via a freeze-thaw grinding method. A 16S rRNA gene clone library was constructed and approximately 200 clones were obtained and sequenced. Extracted DNA was also sent to JGI for generation of 3-, 8-, and 40-kb BAC/fosmid libraries and whole genome shotgun sequencing. The 16S rRNA gene library indicated that the community contained ~14 different bacterial populations. The community was dominated (>90 % of clones) by a Gamma-Proteobacterium population whose closest isolated relatives (~96 % similarity) were *Rhodanobacter* and *Frateuria* spp. Smaller populations (<10 % of clones) of beta-Proteobacteria were identified that were similar (approx. 98 %) to *Acaligenes* and *Herbaspirillum* spp. Approximately 60 Mb have been sequenced by JGI. These sequences are currently being run through the ORNL pipeline for sequence annotation. Preliminary analysis of the metagenomic sequence data using phylogenetic markers including 16S rRNA genes, *gyrB* and *recA* suggested that the community was dominated by the gamma-Proteobacterial population similar to *Rhodanobacter* and *Frateuria* spp., but also indicated that the sample may contain a higher proportion of Beta-Proteobacteria than was detected by the 16S rRNA gene clone library. Several putative metal resistance and denitrification genes have been identified. These results reveal the genetic capabilities of microorganisms indigenous to highly contaminated FRC groundwater and will potentially allow researchers to more effectively utilize the microorganisms' genetic potential to remediate the FRC site.

We have also further evaluated the application of our newly developed Random Primer mRNA Amplification (RPRA) strategy for the investigation of environmental samples. The method uses a modified T7 RNA polymerase-based approach to amplify prokaryotic mRNAs for microarray analysis. We have previously tested this method with respect to representative amplification, sensitivity, and quantification by microarray hybridization using whole genome microarrays and *Shewanella oneidensis* MR-1. In addition, we have recently tested mixed cultures of bacteria and two different environmental samples. For the mixed culture, aliquots of 100 ng of each total RNA sample from four bacteria, *S. oneidensis, D. radiodurans, R. palustris,* and *N. europaea* were mixed and subjected to RPRA. A mixture of 2.5 ug of unamplified RNAs was used as the control. Amplified cDNAs or the control cDNAs were co-hybridized with unamplified cDNA from each of four bacteria to the corresponding whole genome microarray slides. Our results suggested that some amplification bias was introduced as compared to self-hybridization results from pure *S. oneidensis* unamplified RNAs. The number of hybridized spots increased (from 3365 to 3704), indicating that cross hybridization by cDNAs from other bacteria might have occurred. It was also observed that the percentage of spots with a 1.5-fold

change was increased in both unamplified and amplified samples. However, the differences between them were consistent with those from the *S. oneidensis* pure culture in most cases. Therefore, these results may simply indicate that cross-hybridization among genomes occurred, which has no relation to the fidelity of RPRA. To further test the representative and quantitative nature of RPRA, experiments were carried out with our ~2,000 probe functional gene array (FGA) using total RNA obtained from two environmental samples, a fluidized bed reactor sample from the Criddle-Jardine site and a biostimulated groundwater sample from the FRC. For the fluidized bed reactor analysis, RPRA was used to amplify 100 ng of environmental RNA. A portion of the amplified RNA (5 ug) was hybridized to the FGA slides along with unamplified RNA (5 ug) as a control. Eight genes showed significant hybridization signals from unamplified RNA. However, 15 genes were detected from the amplified RNA including all 8 genes found with the control RNA. The detected genes included various denitrification and sulfate-reduction genes and their identity and diversity agreed with results from other analytical methods. As these results indicate, a major potential advantage of this method is the ability to detect the less dominant community members that cannot be detected without amplification. The RPRA method was then used to analyze groundwater samples from the biostimulated FRC well FW029. Single dye hybridization with 5 ug of amplified RNA (from 100 ng of starting RNA) detected hybridization to 40 probes. The detected genes included genes relevant to bioremediation including the *boxA* and *boxB* of *Azoarcus evansii*. Interestingly, only 12 of the detected genes overlapped with those detected from DNA hybridizations from the same sample suggesting the potential of the approach to distinguish only active populations. Figure 3 below shows the results of a bioinformatics analysis of our metagenomic results. In this figure we have attempted to reconstruct the metabolic web of the FRC site.

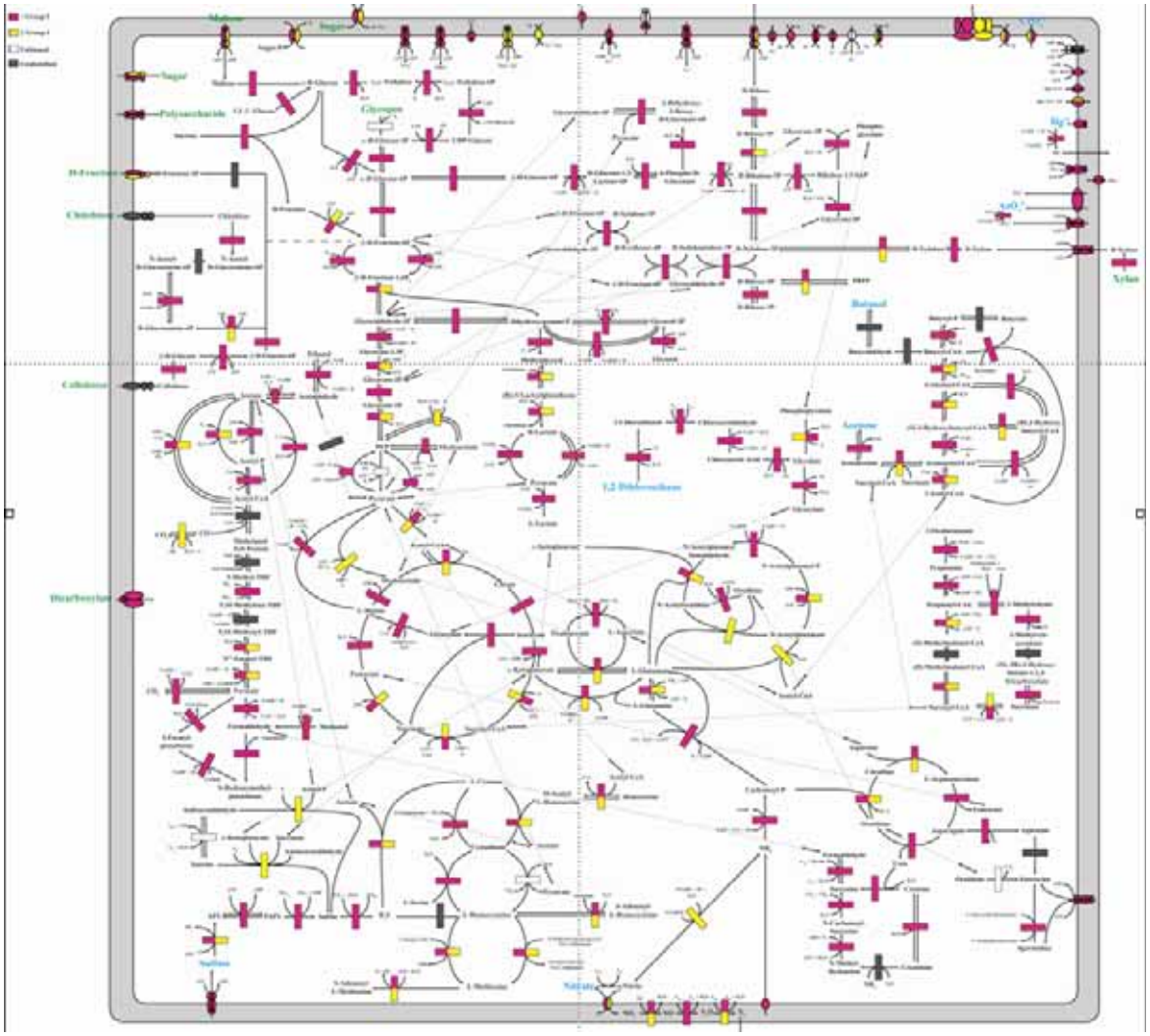

Figure 3. Metabolic reconstruction of FW106 community. Important stressors present at FW106 are shown in blue, likely carbon sources in green. The presence of specific enzymes indicated by color: magenta, γI; yellow, βI; white, unassigned; grey, not identified. The community shows specific adaptations for degradation of 1,2-dichloroethene and a diverse number metal resistance mechanisms. Fermentation does not appear to occur with the community instead based on respiration of nitrate (denitrification) or oxygen. Assimilatory nitrate reductase genes were only found in βI, suggesting that this bacterial species may serve as a keystone species producing biological nitrogen for the rest of the community. The most likely mechanism of acid resistance is a combination of A) proton and cation transporters acting to maintain the chemiosmotic gradient and B) multiple organic acid metabolism pathways designed to degrade protonated acids that permeate the cell under acidic conditions.

Stable isotope probing (SIP) methods, 16S rRNA sequencing and functional gene array (FGA) analyses have identified the organisms and functional genes that were biostimulated in both the denitrifying Criddle-Jardine fluidized bed reactor (FBR) and in the FRC site where uranium reduction is occurring. Advances in SIP methodology have also been achieved to detect functional genes important to FRC processes possessed specifically by the ethanol-biostimulated populations.

FBR samples were collected in . 2004 and clone libraries of 16S rRNA genes representing the reactor community were constructed. The reactor community was dominated by the Beta-Proteobacteria (62% of clones sequenced), with the most significant genera being *Thauera* (19%) and *Hydrogenophaga* (18%). Other protobacteria present were *Dechloromonas, Zoogloea, Azoarcus, Cystobacter, Rhodobacter* and *Chitinophaga*. The remainder of the community was comprised of Bacteroides (32%), with *Flavobacterium* (24%) being the dominant bacteroides genus. The majority of these organisms are previously reported denitrifiers, with the exception of *Cystobacte*r. Similar analyses were performed on sediment samples taken directly from the FRC site. Figure 3 below presents a phylogenetic tree of 16S clones and their closest cultivated relatives along with metabolic capabilities associated with each in sediment samples taken from the FRC.

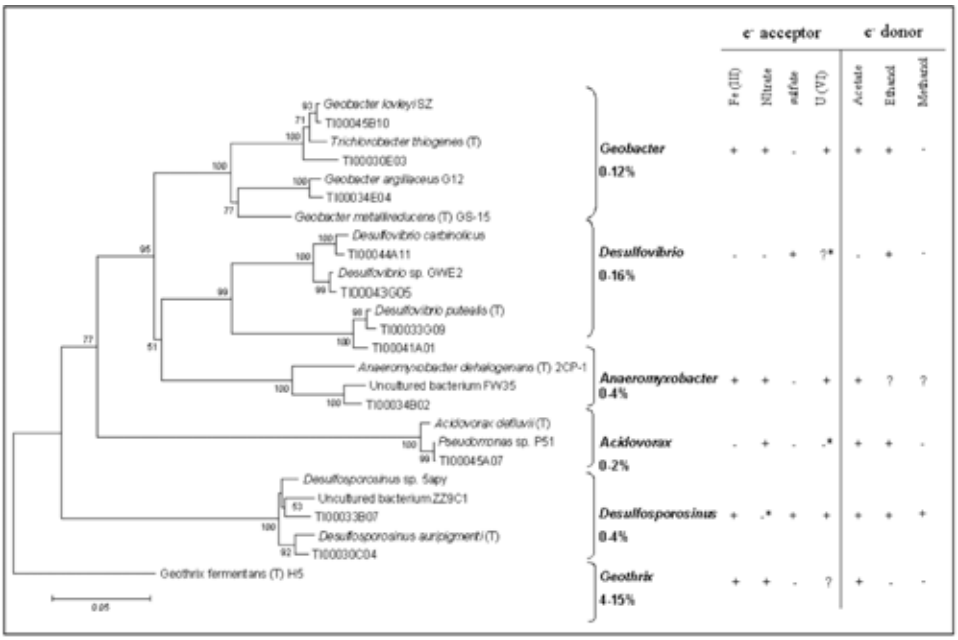

Figure 4. Metabolic abilities of the closest relatives of the sequences detected in sediment samples of the active area and their phylogenetic relation with sequences from the libraries (shown as TI000XXXXX). The activities are based on published literature of the closest relatives where available.

To investigate the presence and diversity of denitrification genes, PCR targeting *nirS* was amplified and a clone library was constructed. Exploratory sequencing of 35 clones revealed five different groups of *nirS* sequences in the FBR. GenBank

BLASTn searches of the approximately 900-b.p. sequences indicated that the sequences may be novel, with percent similarities to nearest matches (all uncultured *nirS* clones) ranging from 90.2 - 99.4%. The nearest named matches all were *nirS* from either *Thauera chlorobenzoica* or *Thauera aromatica*, the latter of which was also detected in the 16S rRNA gene clone library, although homologies were low (80.3 to 92.5%) suggesting that the predominant *nirS* genes in the FBR may be previously uncharacterized *nirS* genes from *Thauera* species.

SIP experiments were conducted on FBR material from all three sampling dates. Microcosms were provided with ^{13}C-ethanol, and timecourse detection of ^{13}CO2 in the microcosm headspace and culturable cell counts indicated that growth and ^{13}C-ethanol metabolism began rapidly (detected within 2 hrs) and continued over the course of the incubations (up to 24 hrs). Both the initial cell numbers and growth rates differed in the three samples, with the largest population increase occurring in the May sample, which became the primary focus of in-depth SIP analyses. ^{13}C-DNA was isolated by density gradient centrifugation and then 16S rRNA genes were PCR amplified and analyzed by T-RFLP. T-RF peaks representing biostimulated organisms were tentatively identified based on *in silico* digestions of total community 16S rRNA gene clone library data as *Thauera, Acidovorax, Dechloromonas, Hydrogenophaga* and *Sterolibacterium.*

Significant progress was made toward integrating SIP with functional gene analyses to determine what relevant capabilities were possessed by the active biostimulated community. Using *nirS* primers, denitrification genes were successfully amplified from FBR ^{13}C-DNA indicating that the denitrifying population is clearly deriving carbon from the biostimulatory substrate, ethanol. Recently, preliminary tests have been conducted to simultaneously probe for thousands of functional genes in biostimulated populations by integrating SIP with functional gene array (FGA) analysis. Comprehensive FGAs were developed at ORNL that include probes for C, N, S and P cycling, metal resistance and contaminant degradation genes, and were produced in both 8000-probe and ~23000-probe formats. One challenge to integrating SIP with FGA analysis is the small amount of ^{13}C-DNA recoverable (10 - 50 ng), which is too little for direct FGA analysis (~3 ug per array). This limitation was overcome using a modified rolling circle amplification method known as whole community genome amplification (WCGA), developed and verified for use in FGA analyses at ORNL. As proof-of-concept, ^{13}C-DNA obtained from a SIP experiment probing for biphenyl-utilizers in soil was amplified with WCGA and hybridized to 8000-probe FGAs. Several genes involved in degradation of aromatic compounds, and genes belonging to *Arthrobacter, Pseudomonas* and *Rhodococcus* strains were detected in SIP samples and confirmed absent in negative controls, which is promising since PCB-degrading strains belonging to these three genera were previously isolated from this model soil. This novel SIP-FGA approach will be applied to FBR and FRC sediment samples in the near future.

SIP studies have been initiated with sediment samples collected from Area 3 in the FRC where uranium reduction is successfully being biostimulated to identify active microorganisms and their functional capabilities. Sediment slurries were collected from three wells (injection well FW-104, sampling well FW101-2 and extraction well FW-026) using the technique of well surging. Most probable number

(MPN) analyses revealed that nitrate, sulfate and iron reducing bacteria were present in the samples, and numbers decreased with distance from the injection well. One liter anaerobic microcosms were constructed with each slurry and were incubated with ^{13}C-ethanol plus nitrate and sulfate provided in realistic field concentrations. An array of processes were monitored over a time course, including the reduction of nitrate, sulfate, and uranium (VI), ethanol disappearance, acetate concentrations, pH and COD. SIP analyses were completed for several time points that represent periods of active nitrate, sulfate and uranium (VI) reduction.

To investigate microbial community composition in sediments throughout the FRC biostimulation zone and to characterize the three-dimensional distribution of microorganisms we processed 17 sediment slurry samples from wells throughout Area 3, including at different depths, during active biostimulation. Clone libraries of 16S rRNA genes were constructed and sequenced, and data analyzed statistically in relation to spatial location and geochemical parameters including nitrate, sulfate, sulfide, U(VI), metals, COD, ethanol and acetate concentrations. The results showing the phylogenetic distribution of clones across the sampling area is shown in Fig. 5 below.

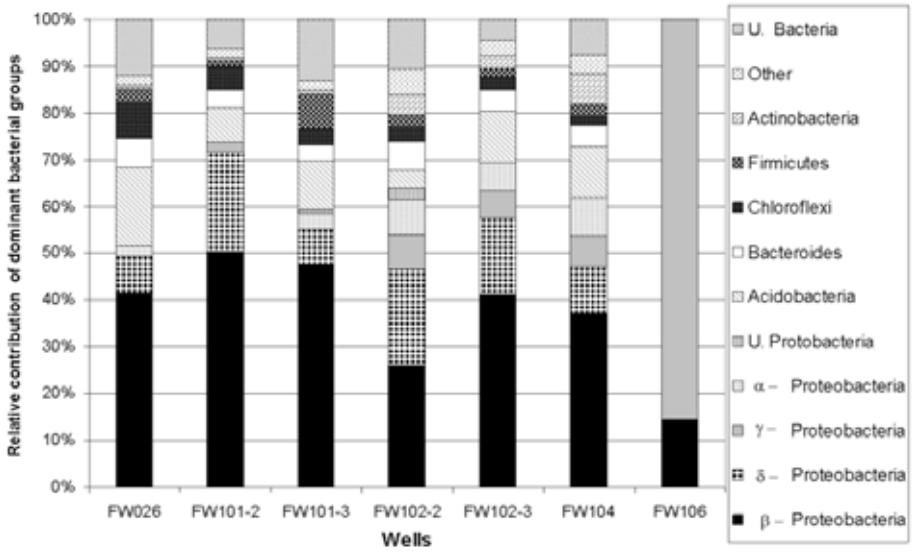

FIG. 5. Microbial composition of the clone libraries based on the RDP Classifier. The "other" group category includes the phyla Spirocheta, Gemmatimonadetes,Verrucomicrobia, Clamydia, Plantomycetes, Nitrospira, Cyanobacteria and the proposed phyla, OP11, OP10, BRC1 and TM7. Bacteria that could not be assigned with the 80% confidence bootstrap value were included in the artificial "Unclassified Bacteria" (U.Bacteria) taxon

We have investigated the metabolic capabilities of *Desulfitobacterium hafniense* (DCB-2), an anaerobic dehalogenator, for heavy metal reduction. We screened separately for Fe(III) to Fe(II), Cu(II) to Cu(I), U(VI) to U(IV), and Se(VI) to Se(IV) as sole electron acceptors in a defined minimal freshwater media. Bacterial growth under these metallorespiratory conditions was observed for Fe(III), Cu(II), and U(VI),

but not Se(VI). The reduction of uranium was recently confirmed at Argonne National Labs in collaboration with Shelly Kelly & Ken Kemner.

When grown fermentatively in DCB-1 media, *D. hafniense* was able to reduce Se(VI). Scanning electron microscopic (SEM) images of *D. hafniense* under these conditions revealed distinct polyps attached to the cell. The polyps were unique to growth in the presence of selenium. Moreover, atomic analysis using energy dispersive spectrometry (EDS) and backscatter imaging confirmed the polyps contained selenium and appeared attached to the cell via a thin filament. Cross-sectional imaging via transmission electron microscopy (TEM) coupled with EDS mapping and line scans further validated the high density of selenium contained in the polyps. A surprising decrease in carbon was also found in polyps detached from cells relative to background and *D. hafniense* cells. Additional studies on the effects of metals on cell morphology have revealed that when grown in uranium, *D. hafniense* increase in cell length by a factor of two. Respiration on Fe(III) produces a streptobacilli-type morphology, and EDS localized iron to the extracellular matrix of the cell. Similar studies have been initiated for cobalt and nickel and preliminary results suggest that cobalt can be reductively respired. Figure 6 below shows the reduction of selenium and uranium by *Desulfitobacterium hafniense*. This model Gram-positive metal reducer has potential for dealing with mixed waste including chlorinated hydrocarbons as well as heavy metals.

We have also tested *D. hafniense* for biofilm formation under conditions of fermentation (DCB-1 media) and respiration (ferric citrate media) using two different surfaces (Dupont and Siran™ beads). *Desulfitobacterium* growth and biofilm formation were observed under all conditions, although the biofilm consistency and abundance varied. Similarly, cell morphology varied depending on media and substrata. The Dupont beads may not have provided as much physical support as the Siran™ beads. Furthermore, ferric citrate media grown *D. hafniense* were significantly thinner than normal fermenting DCB-1 media grown *D. hafniense* despite pelagic or sessile growth. SEM has confirmed the streptobacilli shape of *D. hafniense* cells grown in ferric citrate and noted their smaller size. EDS has shown that iron appears to be sequestered in the EPS and not the *D. hafniense* cell during its reduction. However, further testing to validate these results is required via the more sensitive TEM EDS.

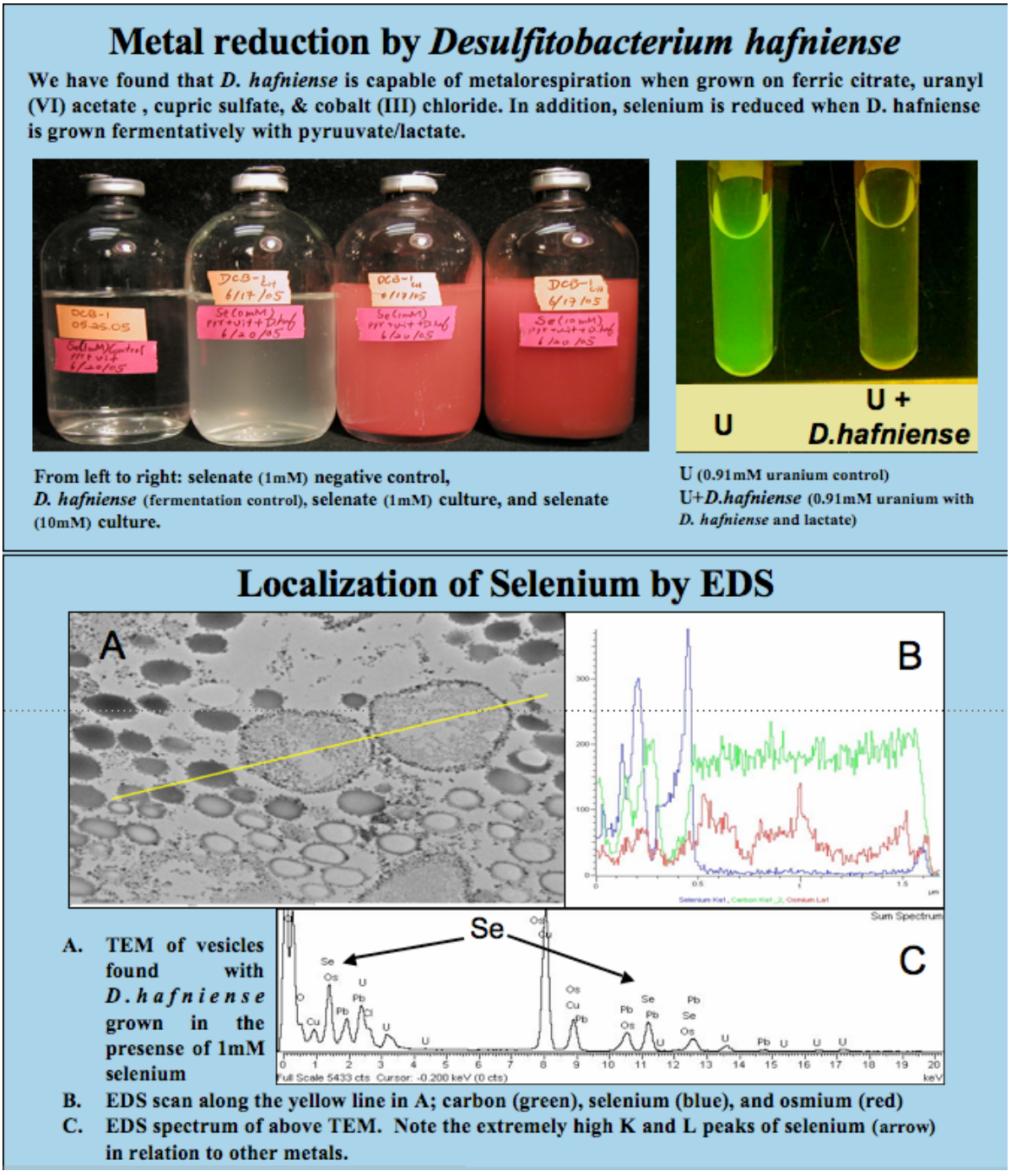

Figure 6. Upper panel shows the reduction of selenium (left) and uranium (right) be actively growing D. hafniense. The bottom panel shows an EDS scan (panel B) of D. hafniense cells and selenium loaded vesicles (large and small circles respectively, in panel A). The energy profile (panel C) clearly identifies selenium in the vesicles.

6. Publications:

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