Working Group Report
Microbial Community Analysis

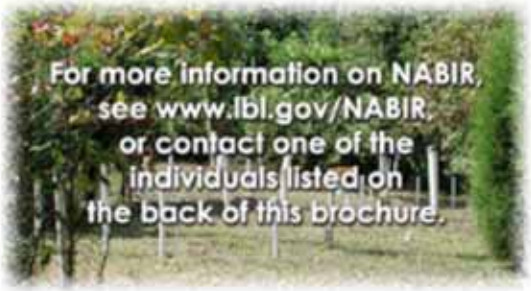
Joel E. Kostka

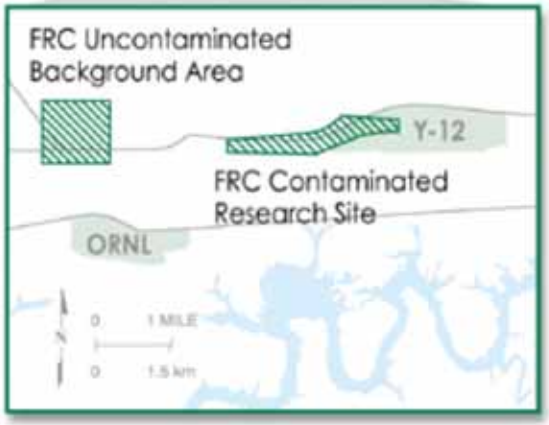
Outline

- Introduction
  - Working group objectives
  - Intro to FRC research
  - Status of working group
- Latest results from research teams
  - Abundance/ biomass
  - Microbial community composition
  - Reports from each team
- Conclusions
- Future work

U.S. DOE NABIR Field Research Center

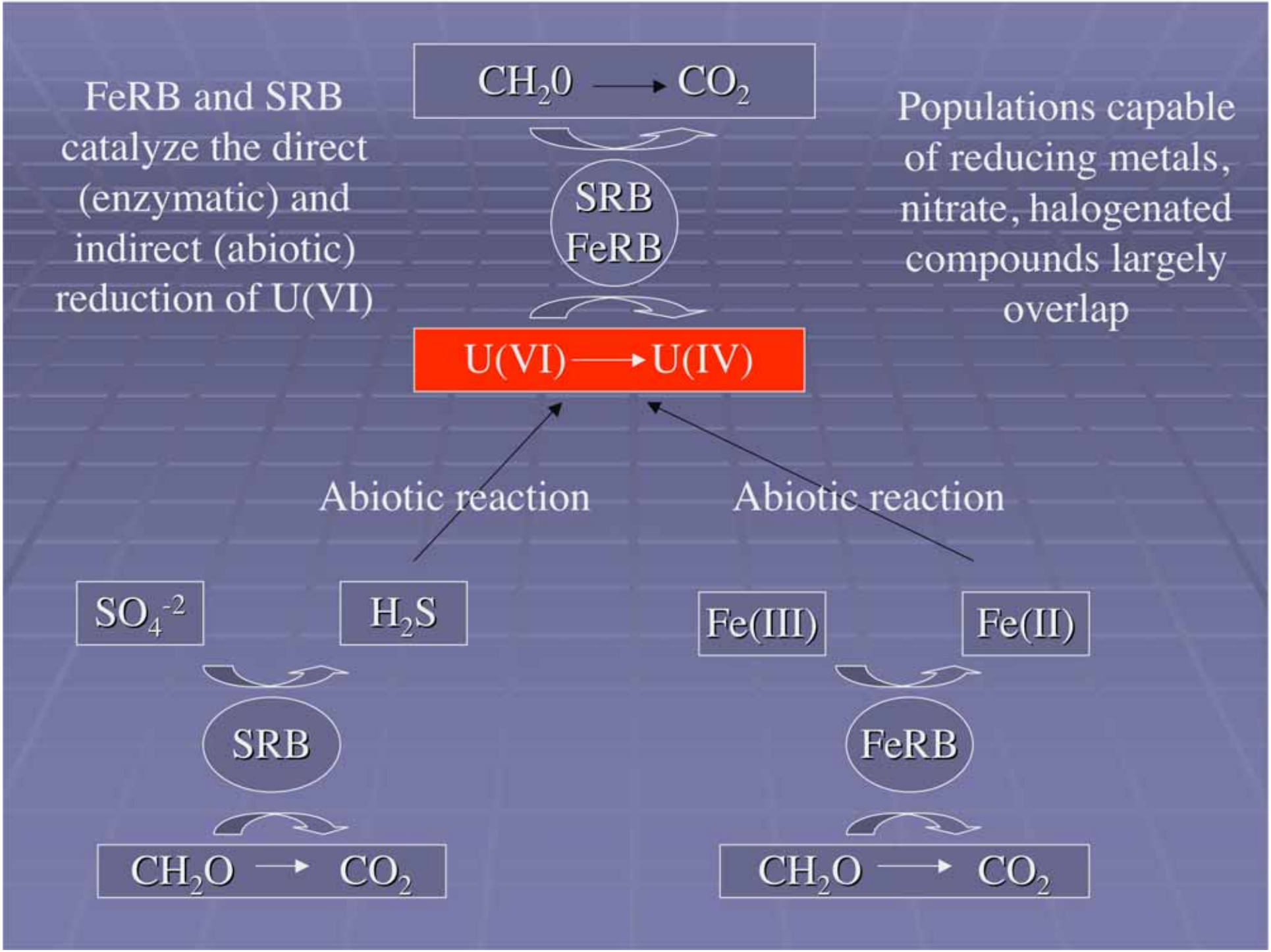
- Located at Y-12 on Oak Ridge Reservation
- Site constructed as part of the secret WWII Manhattan Project to produce nuclear weapons
- FRC (Field Research Center) is centered on groundwater plumes that originate from former waste disposal ponds





Immobilization of Uranium

- The soluble form of uranium, U(VI), can be reduced to an insoluble form, U(IV)
- U(VI) can be reduced to U(IV) through enzymatic and abiotic reactions with microorganisms
- In effect, organisms capable of uranium reduction can control the removal or release of uranium in the groundwater

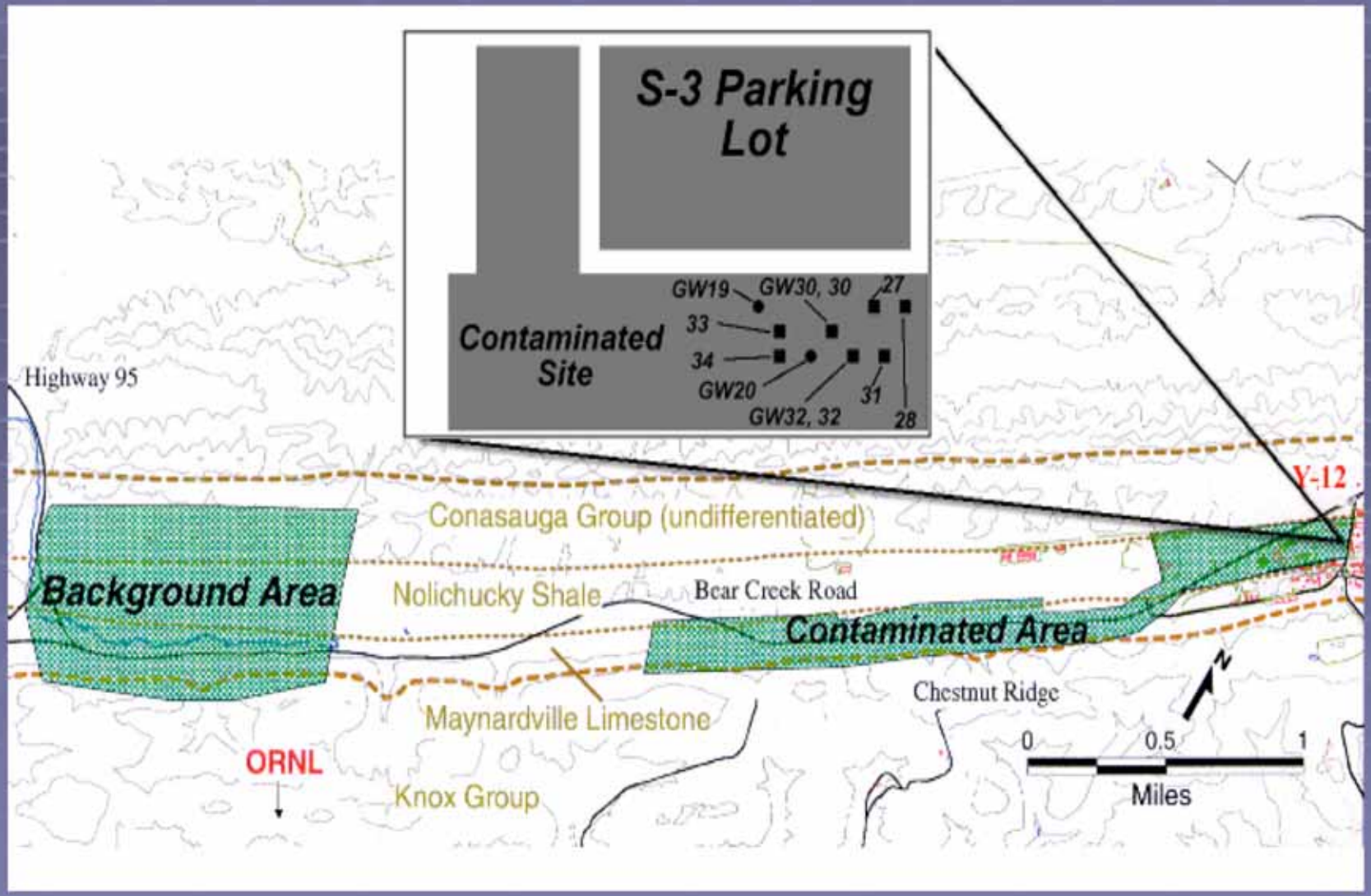

FeRB and SRB catalyze the direct (enzymatic) and indirect (abiotic) reduction of U(VI)
$CH_20 \longrightarrow CO_2$
SRB
FeRB
U(VI) → U(IV)
Populations capable of reducing metals, nitrate, halogenated compounds largely overlap
Abiotic reaction
Abiotic reaction
SO_4^{-2}
H_2S
Fe(III)
Fe(II)
SRB
FeRB
$CH_2O \longrightarrow CO_2$
$CH_2O \longrightarrow CO_2$

FRC - What do we know?

- Contaminants present: uranium, nitrate, technetium, chlorinated compounds (TCE, PCE), fuel hydrocarbons (toluene, benzene)
- Uranium and nitrate are primary contaminants driving remediation; therefore focus has been on metal- and nitrate-reducers
- "Biostimulation" or substrate addition is a promising strategy for U(VI) immobilization by indigenous microorganisms

FRC (continued)

- Harsh subsurface environment for microorganisms; pHs 3-4, [nitrate] mM to M
- Low viable counts and little activity observed in microcosms of unaltered sediments
- Upon addition of electron donor and pH neutralization, extensive nitrate and metal reduction have been observed
- Thus, communities believed to be limited by: low C, pH and high nitrate, toxic metals


S-3 Parking Lot
Contaminated Site
GW19
GW30, 30
27
33
34
GW20
GW32, 32
31
28
Highway 95
Y-12
Conasauga Group (undifferentiated)
Background Area
Nolichucky Shale
Bear Creek Road
Contaminated Area
Chestnut Ridge
Maynardville Limestone
ORNL
Knox Group
N
0
0.5
1
Miles

Contaminated Area - Waste Ponds During Operation

Background Area

•Pristine site with a similar parent rock mineralogy and sediment characteristics to contaminated areas of the FRC

Objectives of Working Groups

- Identify how FRC can best be used
- Determine level of site characterization and post-experimental monitoring to be conducted by FRC vs. research teams
- Stimulate cross disciplinary collaboration
- Expand involvement to new and more NABIR researchers

Microbial Community Analysis Working Group

- List of potential participants drafted after NABIR PI mtg
- 15-20 PIs contacted; 5 responded with detailed summaries of FRC-related research; more have responded in past few weeks
- Information was taken from submitted publications
- Barkay/ Sobecky, Fields/ Zhou/ Tiedje et al., Geesey/ Cummings et al., Kostka, Krumholz, Lovley, Marsh, Roden, Wan/Firestone/Hazen/Brodie, White/ Peacock
- See written report for details; next draft will be available after workshop
- Please let me know if you want to be included with this list!!

Abundance/ Biomass

- Comprehensive study across a range of FRC environments lacking
- Direct counts have not revealed any dramatic differences between contaminated and pristine sites
- PLFA biomass measurements?
- Viable counts have shown decreased abundance in contaminated environments, but results vary, especially for anaerobes

NABIR

Viable counts of aerobic heterotrophs (Balkwill lab)

- No growth observed in majority of plates from contaminated FRC samples
- When growth observed, counts were 10^2 to 10^3 CFU g^{-1}
- UMTRA sediments: 10^3 to 10^7 CFU g^{-1}

Microbial Community Composition - Approaches

- Focus on metal- and nitrate-reducers
- Overall community composition must be understood in order to understand competition for substrates
- Majority of researchers have studied 16S rRNA gene sequences thus far
- Several groups have investigated functional genes (nirS, nirK)
- Most approaches have been qualitative to semi-quantitative (clone libraries)

Microbial Community Composition - Stimulating ?'s

- How does community composition vary between groundwater, sediments, microbial samplers? Does it matter for remediation strategies?
- In other words, where should we focus our efforts in order to refine bioremediation strategies?
- What are common microbial groups detected by multiple research teams?
- Does diversity of contaminated environments differ from that of pristine? It appears so.

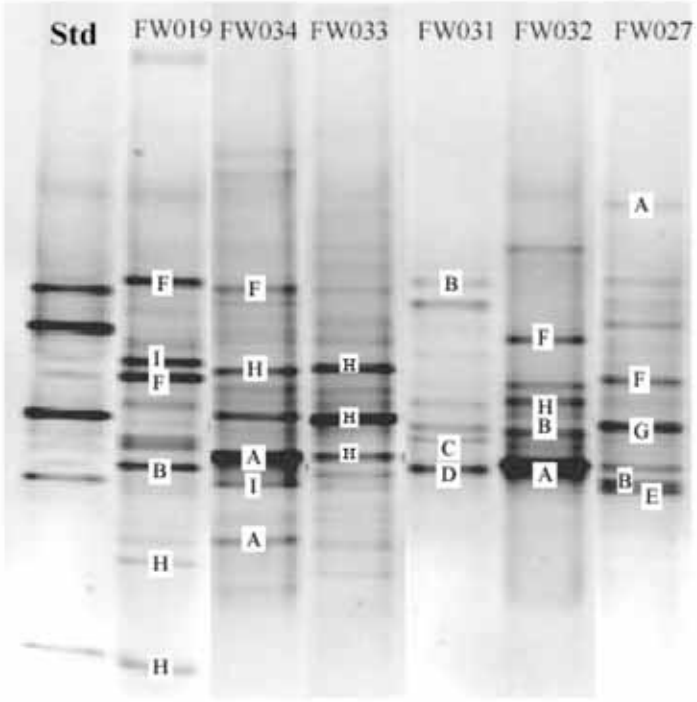
Microbial Community Composition - Stimulating ?'s

- How does diversity relate to desired metabolism for remediation?
- Are desired contaminant transformations (metal, nitrate reduction) catalyzed by competing or largely overlapping functional groups of organisms

Isolates

- Barkay/ Sobecky: Gram positive, aerobic heterotrophs (Bacillus, Arthrobacter)
- Fields: nitrate-reducers, 200 isolates (beta and gamma Proteobacteria, Gram positives)
- Kostka: metal-reducers (Geobacter, Anaeromyxobacter?)
- Krumholz: nitrate-reducers (Agrobacterium, Pseudomonas, Klebsiella)
- Lovley: nitrate and uranium-reducer (Salmonella)

DGGE profiling of eubacterial 16S rRNA gene sequences - microbial samplers
D.C. White, A. Peacock - Istok et al., EST



Bands	Phylogenetic Affiliation
A	*Dechloromonas*
B	*Alcaligenes*
C	*Ralstonia*
D	*Frateuria*
E	*α-proteobacteria* (*Rhodopseudomonas*)
F	*ß-proteobacteria* (*Aquaspirillum*-like)
G	*Sphingomonas*
H	*Geobacter* and *Geobacter*-like
I	*Unclassified*

Fig. 12 (left) DGGE eubacterial community profile of the microbial samplers deployed during field tests. The portion of the gel shows the range of 30-52% denaturant, in which all visible bands were found. Labeled bands were excised and sequenced and correspond to the grouping shown on the right. (right) Phylogenetic affiliation obtained from neighbor-joining analysis of 16S V3 fragments retrieved from DGGE band excisions.

Table 3. Bacterial 16S rDNA clones from biofilms formed on hematite in FRC Background Area well FW303.

Clone ID	GenBank no.	Frequency[a]	Affiliation[b] (% similarity) (Accession)	Putative division
B-Y34		38	*Aquaspirillum delicatum* (97%) (AF078756)	β-Proteobacteria
B-B3*		6	*Pseudomonas mandelii* (98%) (Z76652)	γ-Proteobacteria
B-BH93		5	*Oxalobacter* sp. p8E (97%) (AJ496038)	β-Proteobacteria
B-BD81		5	*Pseudoxanthomonas mexicana* (98%) (AF273082)	γ-Proteobacteria
B-C4		4	*Pseudoxanthomonas mexicana* sp. UR374_02 (95%) (AF273082)	γ-Proteobacteria
B-AA37*		4	*Herbaspirillum seropedicae* (97%) (Y10146)	β-Proteobacteria
B-E7		3	*Variovorax* sp. HAB-30 (94%) (AB051691)	β-Proteobacteria
B-BF84*		2	*Sphingomonas* sp. D-16 (96%) (AF025352)	α-Proteobacteria
B-AQ60		2	*Flavobacterium columnare* (96%) (M58781)	Bacteroidetes
B-L17		1	*Methylocella* sp. BL2 (92%) (AJ491847)	α-Proteobacteria
B-BI94		1	[*Pseudomonas*] *lanceolata* (97%) (AB021390)	β-Proteobacteria
B-AI50		1	*Leptothrix discophora* (95%) (L33975)	β-Proteobacteria
B-AL54		1	*Dechloromonas* sp. MissR (98%) (AF170357)	β-Proteobacteria
B-AG46*		1	*Gallionella ferruginea* (91%) (L07897)	β-Proteobacteria
B-AX74		1	*Aquaspirillum arcticum* (95%) (AB074523)	β-Proteobacteria
B-AB39		1	Clone mlel (98%) (AF280846)	β-Proteobacteria
B-H11		1	*Acidovorax* sp. UFZ-B517 (98%) (AF235010)	β-Proteobacteria
B-AW71*		1	*Zoogloea* sp. strain DhA-35 (91%) (AJ011506)	β-Proteobacteria
B-N19		1	*Ideonella* sp. B513 (97%) (AB049107)	β-Proteobacteria
B-O21		1	*Ideonella* sp. B513 (96%) (AB049107)	β-Proteobacteria
B-AU68		1	*Pseudomonas rhodesiae* (96%) (AF064459)	γ-Proteobacteria
B-AF45		1	*Pseudomonas putida* (90%) (AF094737)	γ-Proteobacteria
B-AC40		1	*Pseudomonas* sp. NZ111 (92%) (AY014825)	γ-Proteobacteria
B-BK96		1	*Haliangium tepidum* (92%) (AB062751)	δ-Proteobacteria
B-I12		1	*Opitutus* sp. VeGlc2 (93%) (X99390)	Verrucomicrobia

[a] Frequency of a given RFLP-type out of 85 total clones.

C. L. Reardon, D. E. Cummings, L. M. Petzke, D. B. Watson, B. L. Kinsall, B. M. Peyton, and G. G. Geesey. Comparison of attached communities in pristine and uranium-contaminated regions of a Department of Energy subsurface site using molecular analysis of colonized hematite. (submitted)

Table 4. Bacterial 16S rDNA clones from biofilms formed on hematite in FRC Area 3 well FW026.

Clone ID	GenBank no.	Frequency[a]	Affiliation[b] (% similarity) (Accession)	Putative division
C-CG17		59	*Alcaligenes* sp. strain L6 (95%) (X92415)	β-Proteobacteria
C-CS3*		24	*Frateuria* sp. NO-16 (96%) (AF376025)	γ-Proteobacteria
C-CF16		4	*Methylobacterium radiotolerans* (99%) (D32227)	α-Proteobacteria
C-CU62*		3	*Pseudomonas straminea* (99%) (AB060135)	γ-Proteobacteria
C-CJ32		2	*Beutenbergia cavernosa* (96%) (Y18378)	Actinobacteria
C-CY80*		1	*Herbaspirillum seropedicae* (96%) (Y10146)	β-Proteobacteria
C-DA88		1	*Burkholderia* sp. A6.2 (98%) (AF247491)	β-Proteobacteria
C-CZ82*		1	*Duganella zoogloeoides* (98%) (D14256)	β-Proteobacteria
C-CL42		1	*Pseudomonas syringae* (89%) (AB001450)	γ-Proteobacteria
C-CX74*		1	*Acinetobacter lwoffii* (99%) (X81665)	γ-Proteobacteria
C-CO51		1	*Microbacterium* sp. VKM Ac-2050 (99%) (AB042084)	Actinobacteria
C-CV63		1	*Nocardioides* sp. ND6 (96%) (AJ511294)	Actinobacteria
C-CM46		1	Clone CO26 (93%) (AF507686)	Unknown

Reardon et al., AEM (submitted)

Research Questions

- Via cultivation-dependent methods:
  - Identify and characterize the Fe(III)-reducing bacteria in the FRC subsurface in contrasting geochemical environments
- Via cultivation-independent methods:
  - Determine structure/ function relationships of metal-reducing bacteria and competing heterotrophs during *in situ* bioremediation

Conclusions: cultivation-dependent Investigation

- The abundance and community composition of culturable FeRB is dependent upon geochemical parameters (pH, nitrate)
- Microorganisms capable of producing spores or spore-like bodies were representative of acidic sediments
- Neutrophilic organisms cultured from contaminated acidic sediment likely to be important since pH neutralization used for bioremediation

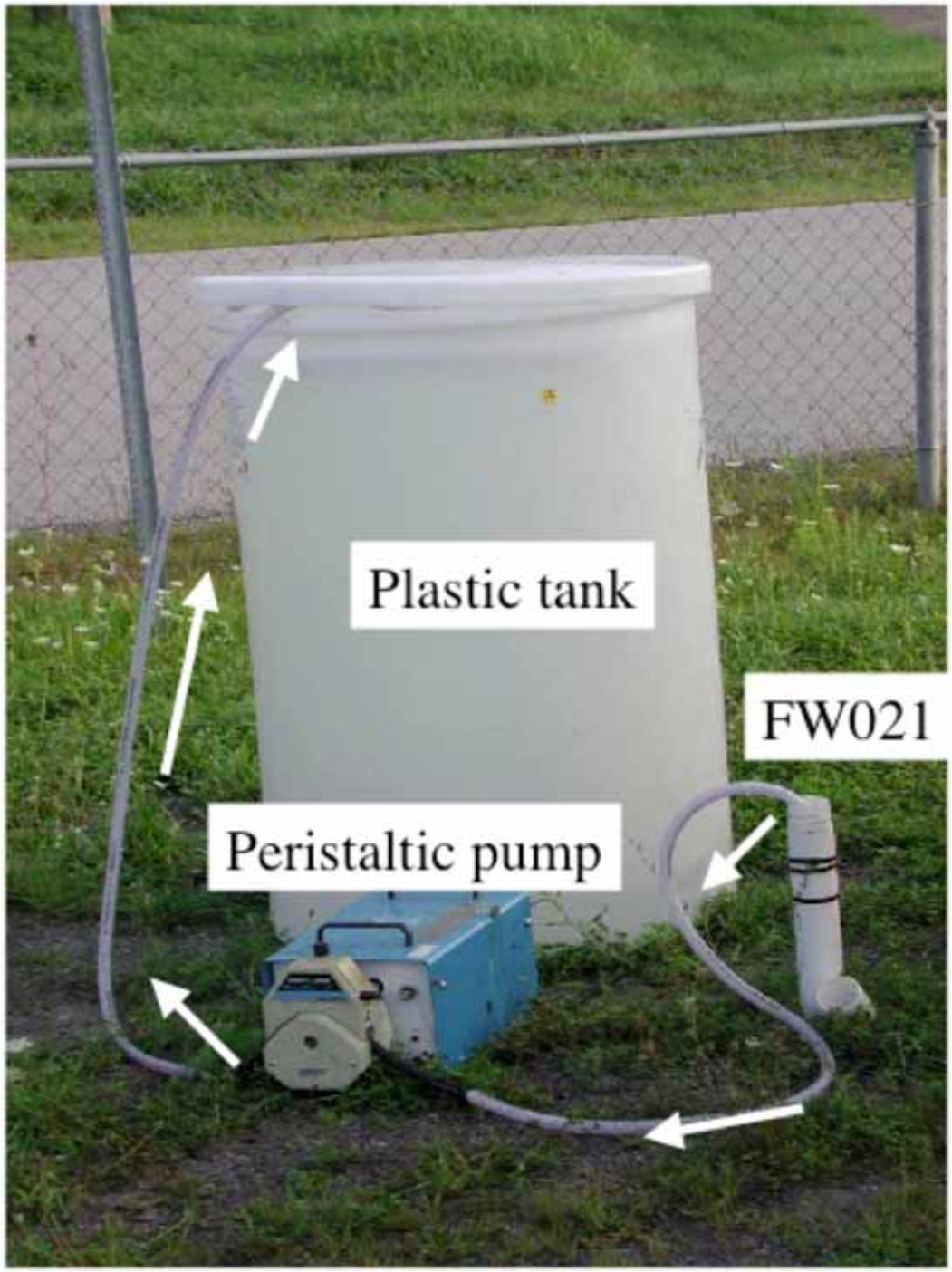
Petrie et al., 2003, AEM

Objectives of *In situ* Biostimulation Experiment

- To determine structure/ function relationships of metal-reducing bacteria and competing heterotrophs during *in situ* bioremediation in acidic subsurface environments
- Quantify microbial activity using geochemical analysis of groundwater/ sediments (push-pull activity tests)
- In parallel, quantify the change in the abundance/ diversity of sedimentary microbial communities using cultivation-independent methods
  - Quantitative MPN (most probable number)-PCR
  - Cloning and sequencing of 16S rRNA genes

In Situ Biostimulation using Push-Pull Activity Tests
Jack Istok - OSU

- Biostimulation: addition of electron donors to increase microbial activity
- Push-pull activity tests: wells were injected with site groundwater, bicarbonate, an inert tracer, and an electron donor (glucose or ethanol)
- Groundwater chemistry was monitored over time to determine the kinetics of electron donor and acceptor utilization
- Sediment cores collected in the zone of influence surrounding wells, before and after electron donor addition



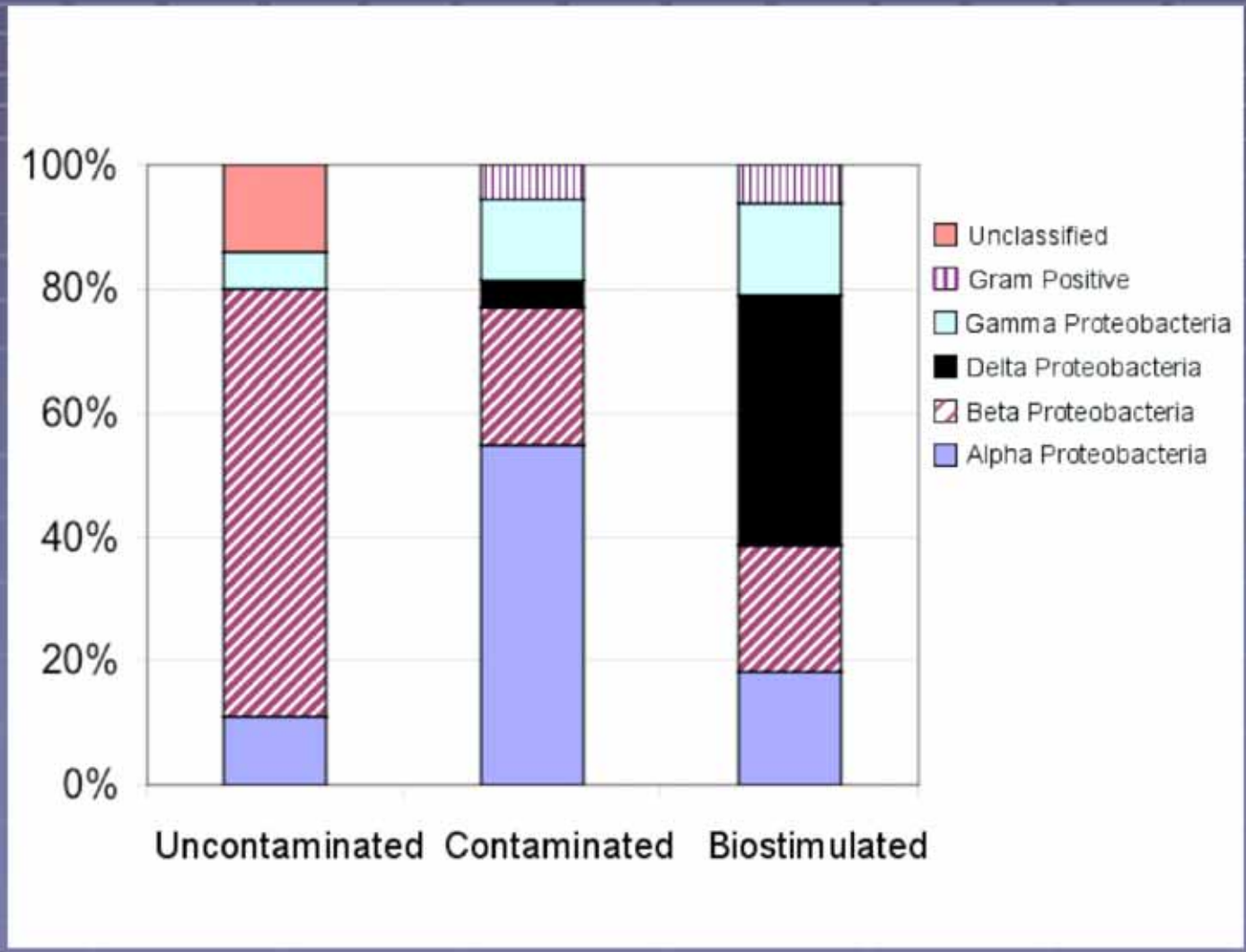
Push-Pull Activity Tests

<u>Step 1.</u>
Collect ~200 L groundwater from FW021

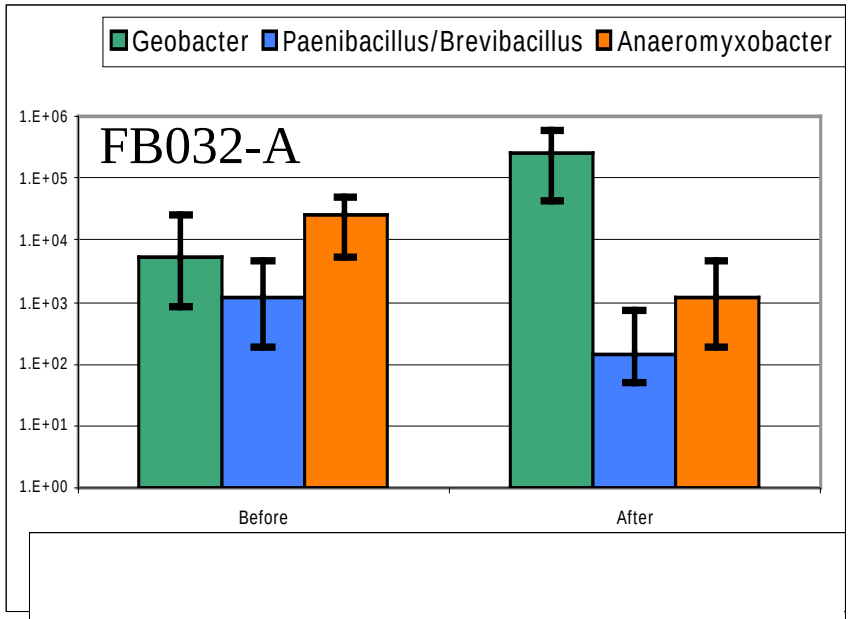
Sediment Chemistry Before and After Carbon Source Addition

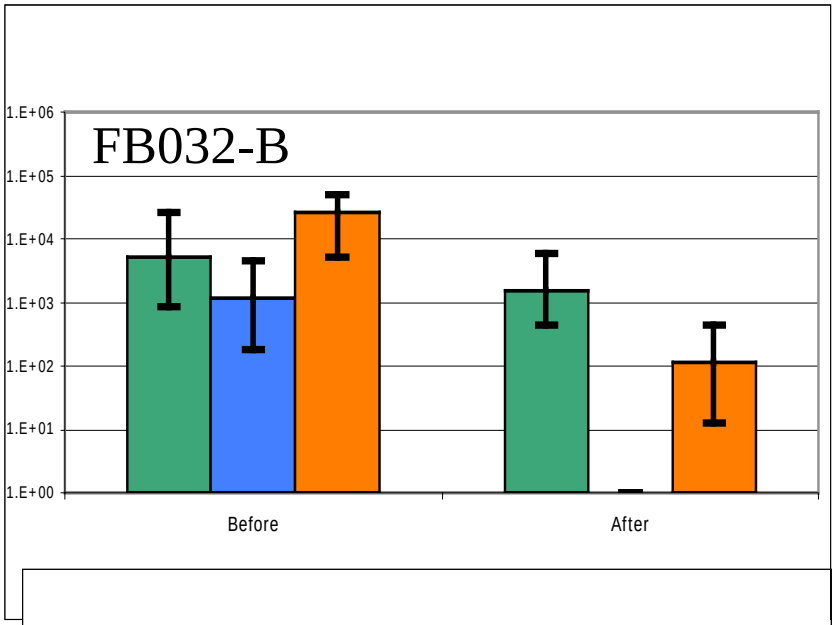
		pH		Nitrate (mM)	
Core (Carbon source added)	Corresponding unstimulated core	Before biostimulation	After biostimulation	Before biostimulation	After biostimulation
FB045 (Glucose)	FB032	4.4	4.1	8.6	1.5
FB046 (Glucose)	FB032	4.4	6.6	8.6	2.2
FB047 (Glucose)	FB033	3.6	4.5	154.3	6.8
FB049 (Ethanol)	FB034	3.8	4.9	36.9	0.1

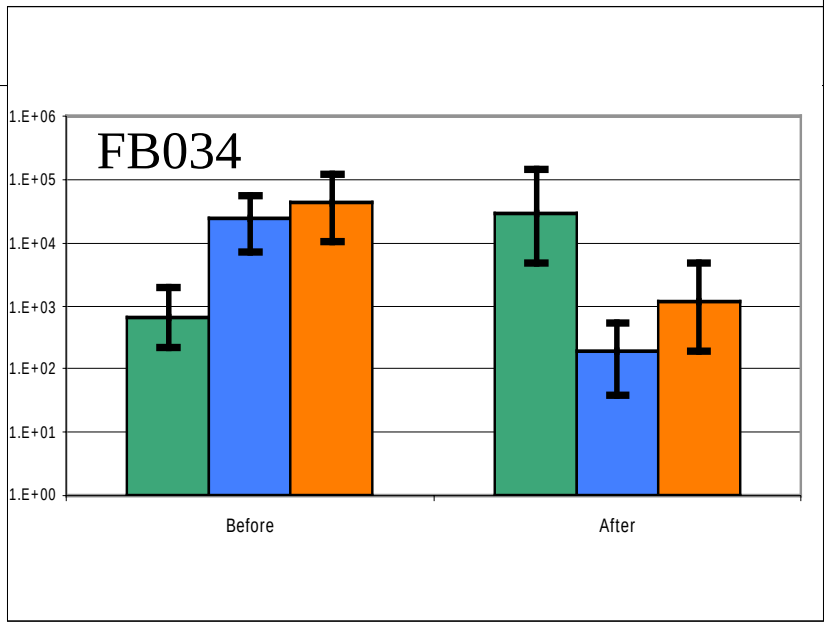
Bacterial Communities Before and After Biostimulation

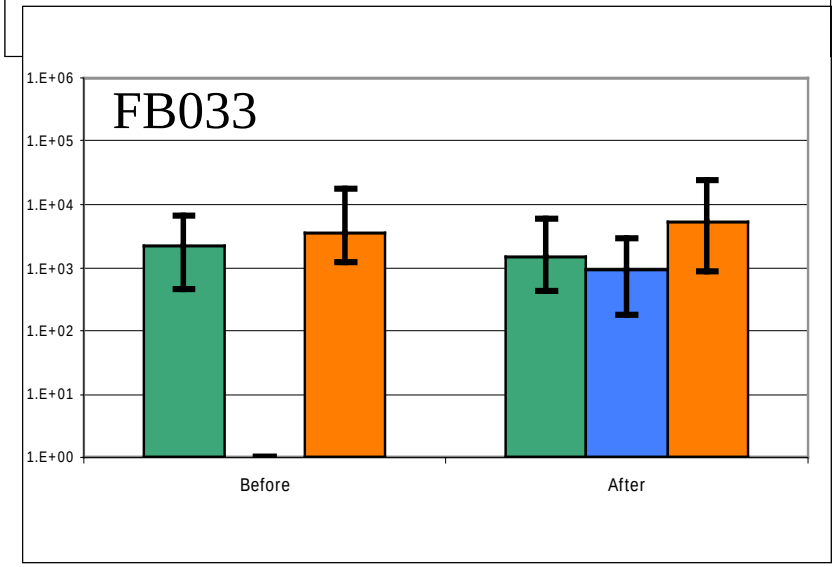


MPN-PCR Results (16S rRNA gene copies/gram sediment)









Change in Inferrred Physiology from Phylogeny

			Clone library	
FRC Contaminant	**Physiological potential**	**Potential bioremediating organisms**	% Before	% After
Uranium	Reduction and immobilization by FeRB	*Geobacter sp.* (58) *Anaeromyxobacter dehalogenans* (31) *Desulfitobacterium metallireducens* (23)	4.5%	37.0%
	Reduction and immobilization by fermentative FeRB	*Clostridium beijerinckii* (96) *Serratia proteamaculans* (58)	5.7%	10.5%
Nitrate	Reduction	*Pseudomonas stutzeri* (71) *Alcaligenes defragans* (heyen) *Ralstonia pickettii* (park) *Anaeromyxobacter dehalogenans* (84) *denitrifying Fe-oxidizing clone* (straub) *Paenibacillus sp.* (Shida)	22.0%	27.1%
Chlorinated hydrocarbons	Dechlorination	*Methylobacterium dichloromethanicum* (39) *Anaeromyxobacter dehalogenans* (84) Clone from TCE-contaminated site (13) *Dechloromonas sp.* (Prok)	42.5%	34.4%
Polychlorinated biphenyls	Dechlorination	*Acidosphaera rubrifaciens* (Nogales) *Caulobacter leidyi* (Nogales)	14.9%	2.2%
Fuel hydrocarbons	Degradation	*Burkholderia sp. N2P5* (70) *Sphingomonas paucimobilis* (70)	5.7%	14.9%

Conclusions: *In situ* Subsurface Biostimulation

- Using qualitative and quantitative molecular techniques, a large change in the microbial communities was observed in parallel with activity
- Both the abundance and diversity of organisms changed
- *Geobacter* and *Anaeromyxobacter* are important organismal groups involved in bioremediation activity (nitrate reduction, metal reduction, dehalogenation)

Conclusions (cont.)

- Sediment heterogeniety may explain why *Anaeromyxobacter* sequences were found in abundance in cloning experiments, but not in MPN-PCR after biostimulation
- Attached organisms are participating in bioremediation, but to what extent?
- See poster in Integrative Studies session

Challenges of the FRC subsurface

- Low pH and high nitrate/ toxic metal concentrations
- Extreme heterogeneity in sediment characteristics (mineralogy, pore geometry)
- QUANTIFICATION of types and activity of metal- and nitrate-reducing members of subsurface microbial communities

- Wide heterogeneity of sediment (reflected in uranium, nitrate, iron concentrations)

Publications to date

- M.W. Fields, T. Yan, S.-K. Rhee, S.L. Carroll, J. Zhou. 2003. Microbial community structure and composition from subsurface groundwater contaminated with high levels of nitrate, heavy metals, and uranium. (Submitted).
- J.D. Istok, J.M. Senko, L.R. Krumholz, D. Watson, M.A. Bogle, A. Peacock, Y.-J. Chang, D.C. White. 2003. In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. Environ. Sci. Technol. (In press).
- J.E. Kostka, D. Dalton, H. Skelton, S. Dollhopf, and J.W. Stucki. 2002. Growth of iron(III)-reducing bacteria on clay minerals as the sole electron acceptor and a growth yield comparison on a variety of oxidized iron forms. Applied and Environmental Microbiology 68: 6256-6262.
- North, N.N., S.L. Dollhopf, L. Petrie, J.D. Istok, D.L. Balkwill, and J.E. Kostka. 2004. A cultivation-independent investigation of microbial communities during in situ biostimulation of subsurface sediment co-contaminated with uranium and nitrate (Submitted).
- L. Petrie, N.N. North, S.L. Dollhopf, D.L. Balkwill, J.E. Kostka. 2003. Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). Appl. Environ. Microbiol. 69: 7467-7479.

Publications to date

- C.L. Reardon, D.E. Cummings, L.M. Petzke, B.L. Kinsall, D.B. Watson, B.M. Peyton, G.G. Geesey. 2003. Comparison of attached communities in pristine and uranium-contaminated regions of a Department of Energy subsurface site using molecular analysis of colonized hematite. Appl. Environ. Microbiol. (Submitted)
- Shelobolina, E.S., Sullivan, S., O'Neill, K., Nevin, K.P., and Lovley, D.R. 2004. Isolation, Characterization, and U(VI)-Reducing Potential of Facultatively Anaerobic Acid Resistant Bacterium from Low pH Nitrate- and U(VI)- Contaminated Subsurface Sediment and Description of Salmonella subterranea sp. nov. Appl. Environ. Microbiol. Accepted for publication, 02/05/04
- Shelobolina, E.S., O'Neill, K., Finneran, K.T., Hayes, L.A., and Lovley, D.R. 2003. Potential for In Situ Bioremediation of a Low-pH, High-Nitrate Uranium-Contaminated Groundwater. Soil and Sediment Contamination. 12: 865-884.
- T. Yan, M.W. Fields, L. Wu, Y. Zu, J.M. Tiedje, J. Zhou. 2003. Molecular diversity and characterization of nitrite reductase gene fragments (nirK and nirS) from nitrate- and uranium-contaminated groundwater. Environ. Microbiol. 5:

Conclusions

- Revise list of isolates obtained for each functional group of organisms by all research teams
- Identify common threads between results of all groups with regard to community composition in FRC subsurface (groundwater, sediments, microbial samplers)
- List objectives for future working group activities

Suggestions for future work

- Identify specific research objectives related to sampling groundwater, sediments, microbial samplers
- Develop effective sampling strategies for each
- Improve coordination during field experiments with expanded, better replicated sampling design
- Use PI coordination to increase replicability of approaches within the same field experiment (to combat sample heterogeneity)
- Compare microbial communities in groundwater, sediments, microbial samplers

Suggestions for Future Work

- Add comprehensive study of biomass in sediments and groundwater
- Develop and deploy quantitative, cultivation-independent approaches in conjunction with field experiments and geochemical analysis
- Develop methods to elucidate “active” members of populations during biostimulation

Timetable

- April '04- revise group report to include current and future research activities; display report on FRC website for all PIs to view
- March '04 to ?- develop a review of FRC microbial communities for publication in a refereed journal (after more research has been published)
- September '04- meet again at FRC workshop

Acknowledgements

- PIs and collaborators who contributed to group report
- Jack Istok, Lee Krumholz, Dave Watson, Barry Kinsall
- FRC staff
- Nadia North, Lainie Petrie, Sherry Dollhopf
- The NABIR program, Biological and Environmental Research (BER), U.S. DOE (grant DE-FG02-DOER62986)