Working Group Report
Microbial Community Analysis

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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

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

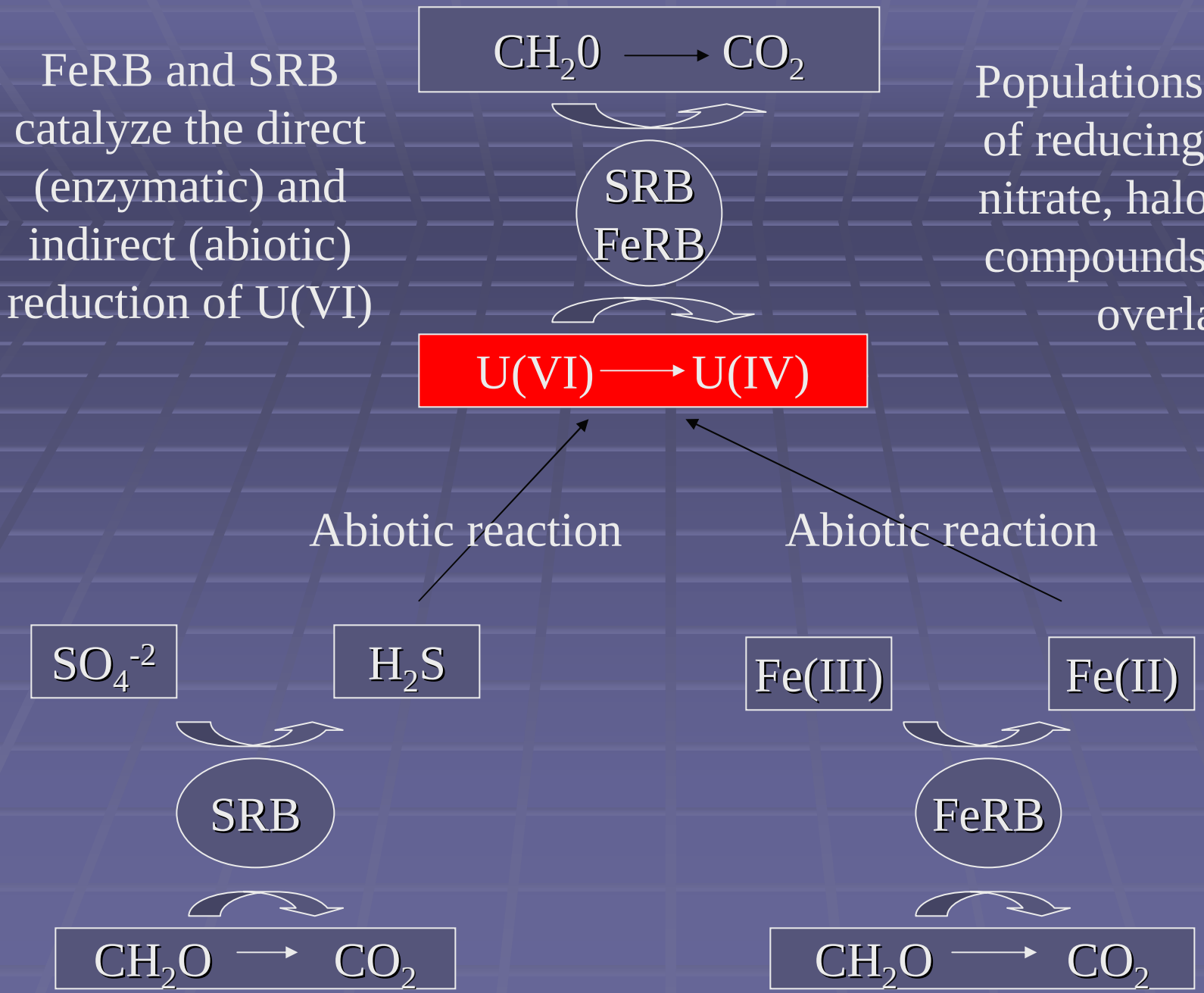
FRC - What do we know?

- 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
- Nitrate must be removed first before U(VI) will occur (Finneran et al., 2002; Senko et al., 2002)

Intro to FRC

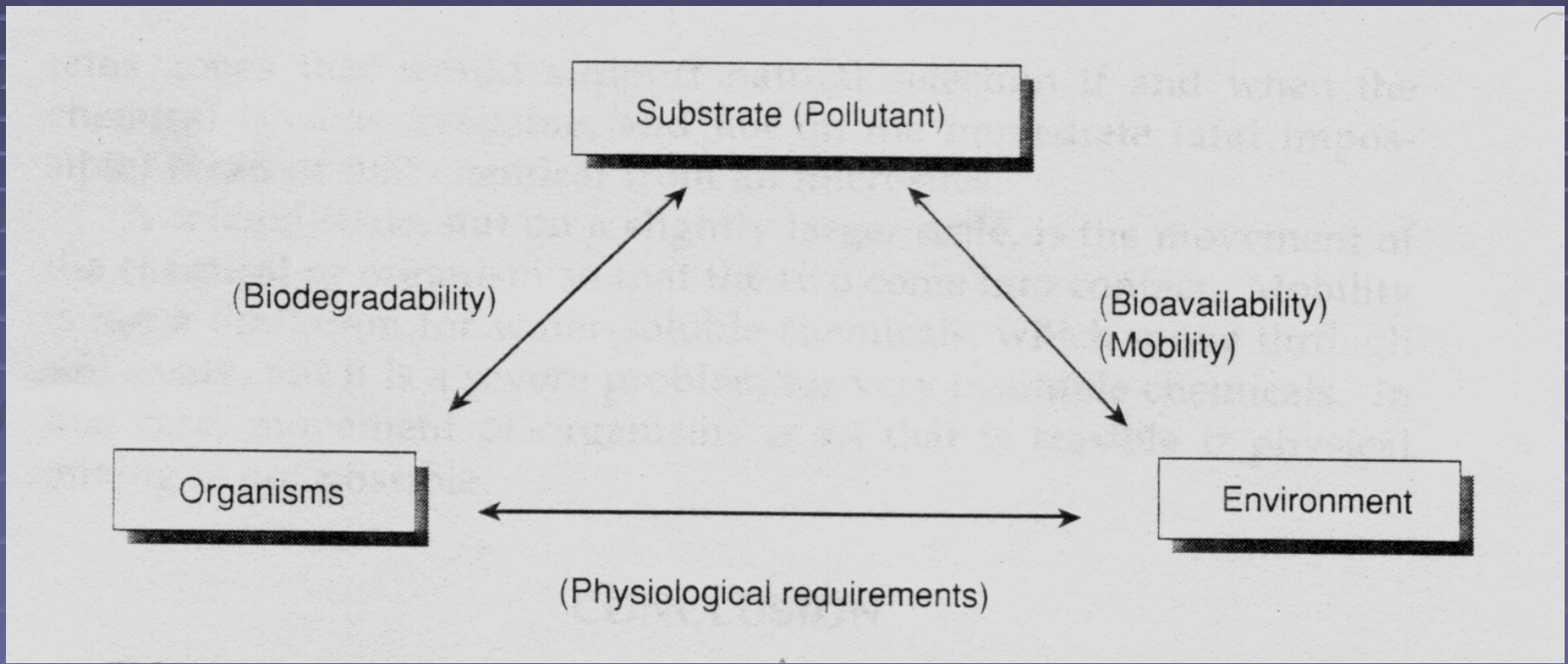
- FRC = harsh environment for microorganisms; Ex. pHs often 3-4 in contaminated areas
- Upon addition of electron donor and pH neutralization, extensive nitrate and metal reduction have been observed
- Thus, communities believed to be limited by: low C, pH and high nitrate, toxic metals
- What we don’t know… a great deal.

FeRB and SRB catalyze the direct (enzymatic) and indirect (abiotic) reduction of U(VI)



Populations capable of reducing metals, nitrate, halogenated compounds largely overlap

Ecological approach - contaminant remediation



from Tiedje (1993)

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

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.

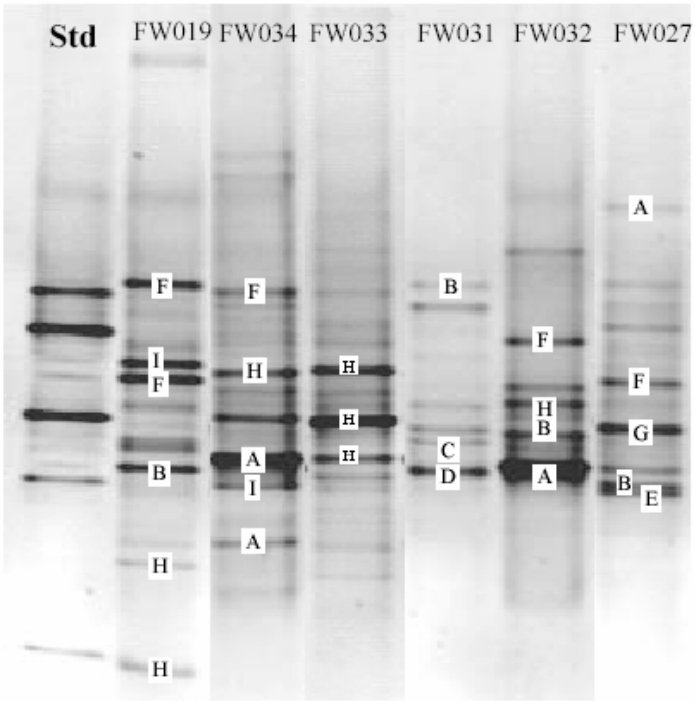
Microbial Community Composition - Stimulating ?'s

- 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., submitted to 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

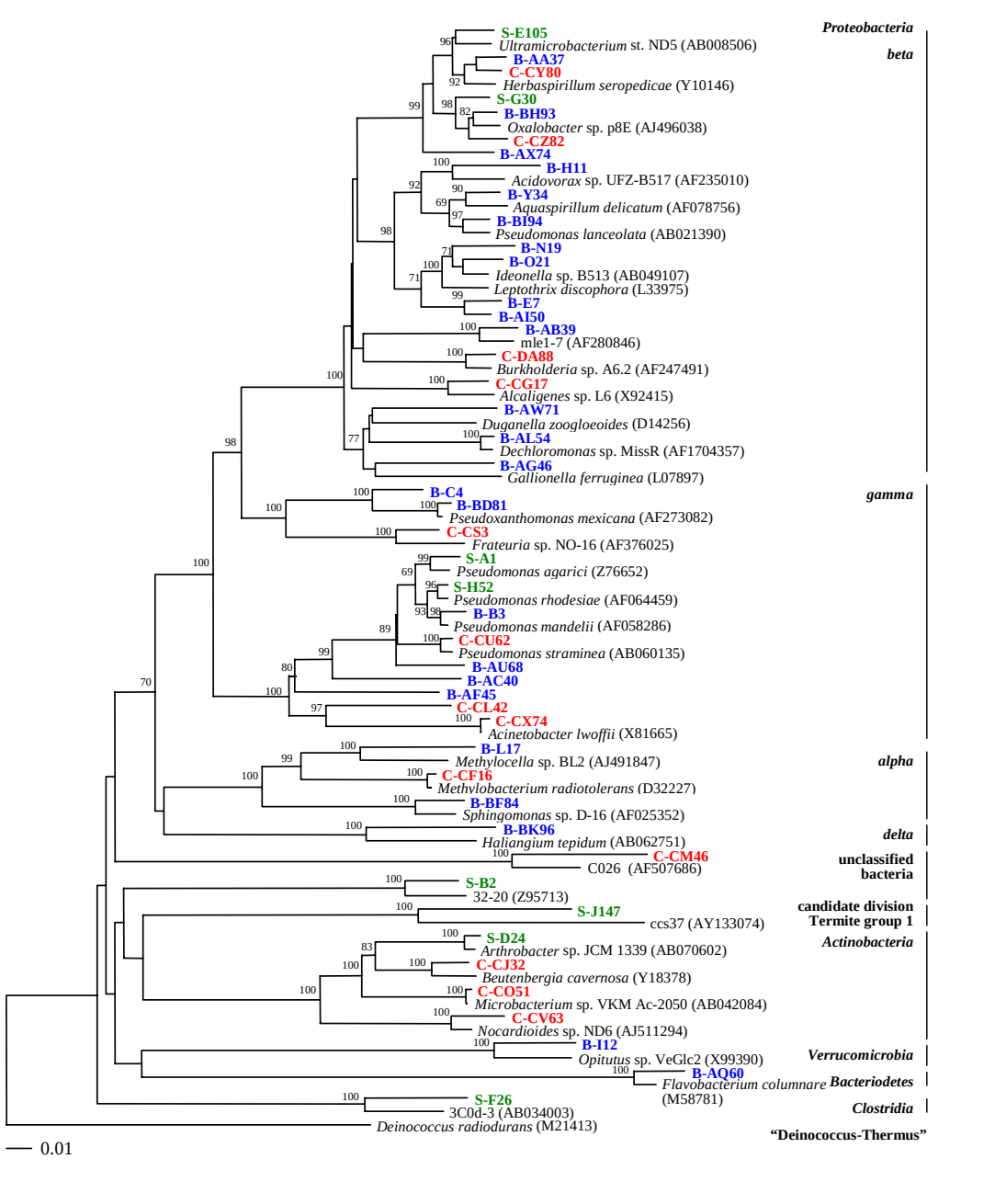
[a] Frequency of a given RFLP-type out of 85 total clones.

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)



Reardon et al., AEM (submitted)

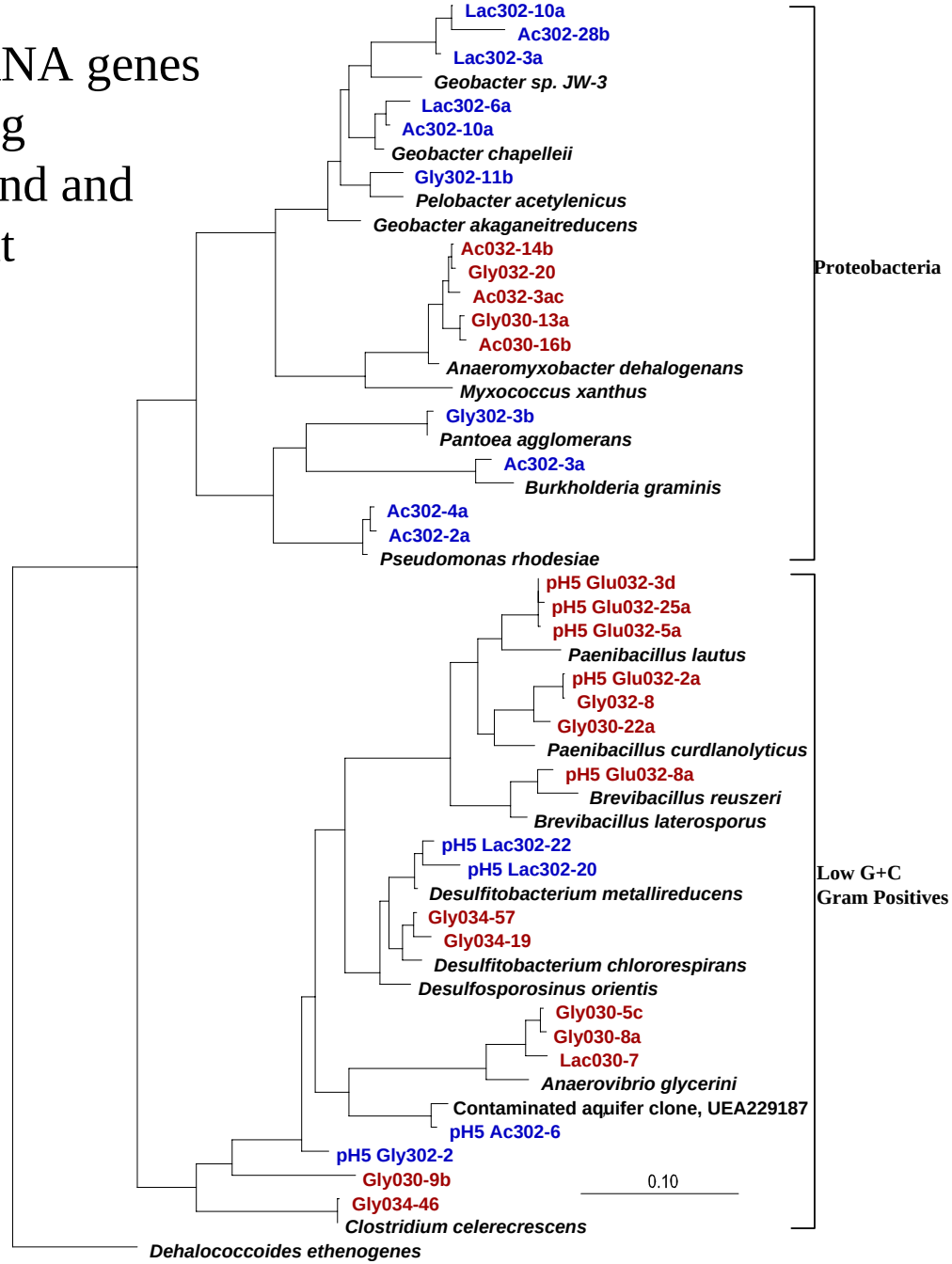
Cultivation-dependent analysis

- ✓ Up to 50% of 4-6 mM U(VI) was reduced in <48 hours

Phylogenetic tree of 16S rRNA genes cloned from Fe(III)-reducing enrichments from background and contaminated FRC sediment

Blue=background

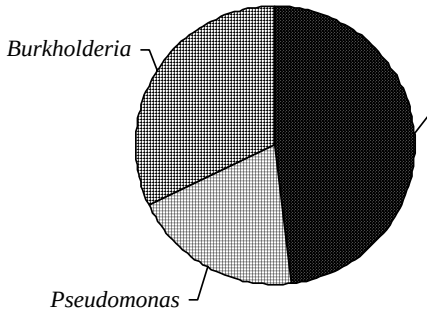
Red=contaminated



Background:

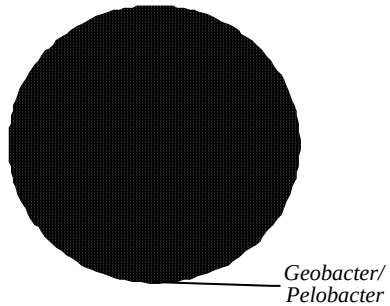
Cultured at pH 7

Acetate



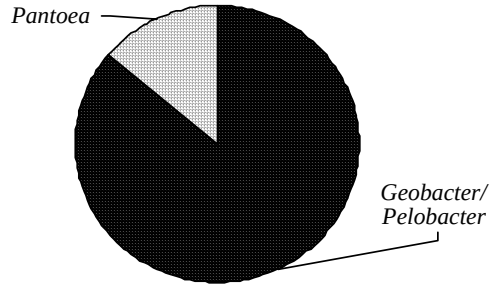
(50 clones)

Lactate



(39 clones)

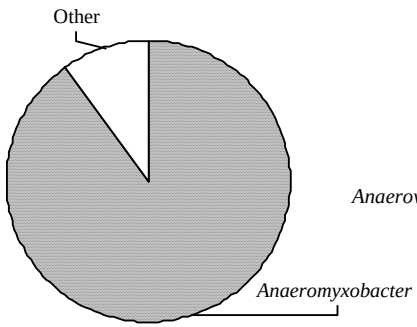
Glycerol



(49 clones)

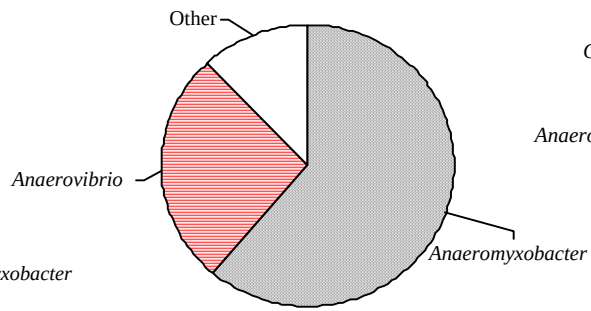
Contaminated:

Acetate



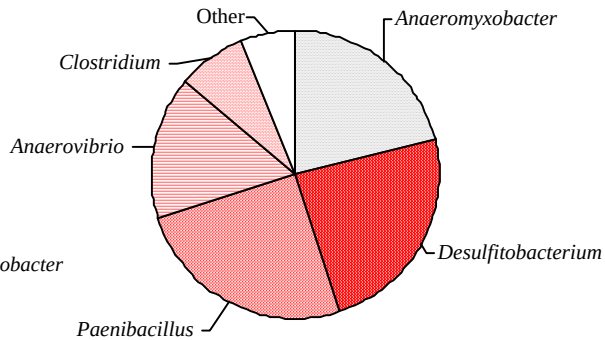
(152 clones)

Lactate



(41 clones)

Glycerol



(238 clones)

Conclusions: Cultivation-dependent Investigation

- The diversity of culturable FeRB appears to depend on pH
- Bioremediation studies of uranium-contaminated sediment should be widened to include some Gram positive organisms and *Anaeromyxobacter (*organisms that may be better adapted to environmental extremes)
- The ability of neutrophilic organisms cultured from contaminated sediment to reduce U(VI) could be of great importance

Cultivation-independent Analysis

Organisms Chosen for MPN-PCR

Geobacter

- ✓Fe(III)-reducing organism
- ✓Dominant in many subsurface environments (including contaminated)
- ✓Can become motile
- ✓Some species can reduce nitrate
- ✓Detected in background Fe(III)-reducing enrichments

Anaeromyxobacter

- ✓ Fe(III)-reducing organism
- ✓Detected in several sedimentary environments (including contaminated)
- ✓Can dechlorinate chlorinated compounds
- ✓Has gliding motility
- ✓Can reduce nitrate
- ✓Microaerophilic
- ✓Can form spore-like structures
- ✓Detected in contaminated Fe(III)-reducing enrichments

Paenibacillus/Brevibacillus

- ✓ Detected in low pH contaminated Fe(III)-reducing enrichments

Primer Design for MPN-PCR

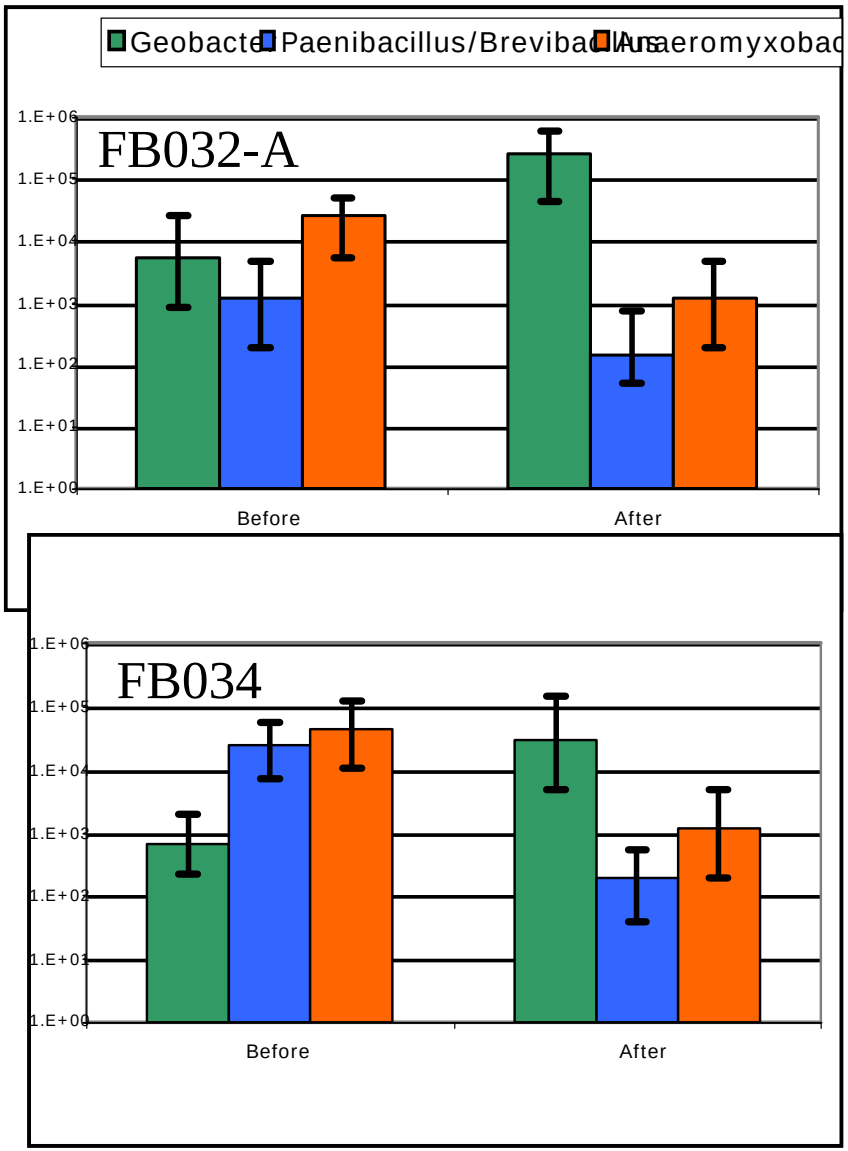
Target Organism(s)	Primer Name	Primer Sequence (5'-3')	Amplicon Length (bp)	Reference
Anaeromyxobacter	60F	CGA GAA AGC CCG CAA GGG T	401	this study
	461R	ATT CGT CCC TCG CGA CAG T		
Paenibacillus / Brevibacillus	160F	TGA GTA ACA CGT AGG CAA CCT	174	this study
	334R	TAA TGC GCC GCA GGC CCA T		
Geobacter	494F	AGG AAG CAC CGG CTA ACT CC	331	(Holmes et al., 200
	825R	TAC CCG CRA CAC CTA GTC T		

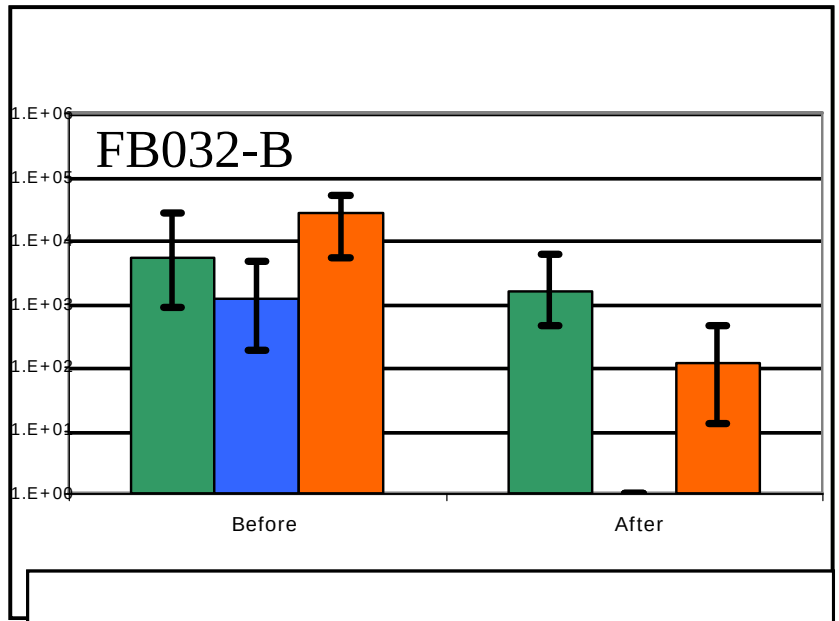
- ✓Clone libraries were produced from each primer set to ensure primer specificity
- ✓Primer sets were also compared to 16S rRNA gene sequences in two databases (RDP and GenBank)

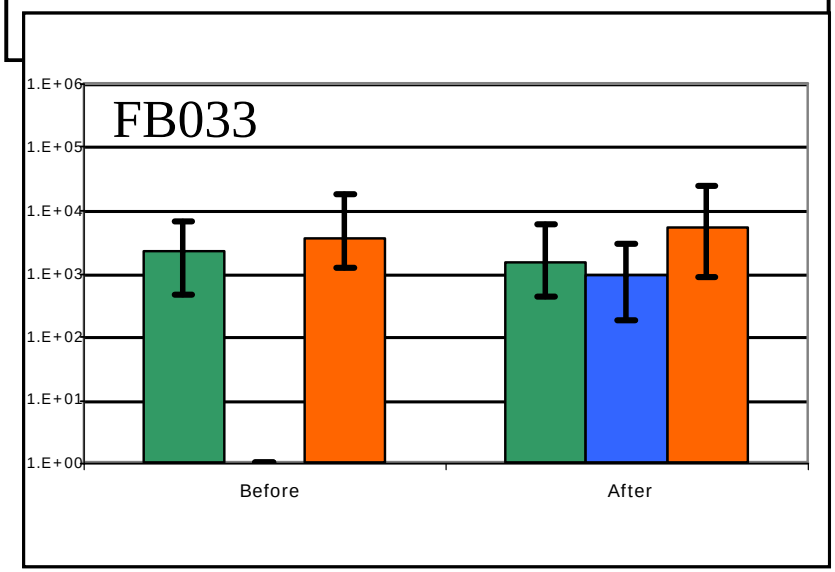
Sediment Chemistry Before and After Carbon Source Addition

		pH		Nitrate (mM)	
Core (Carbon sourc added)	Corresponding unstimulated core	Before biostimulatic	After biostimulatic	Before biostimulatic	After biostimulatic
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

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

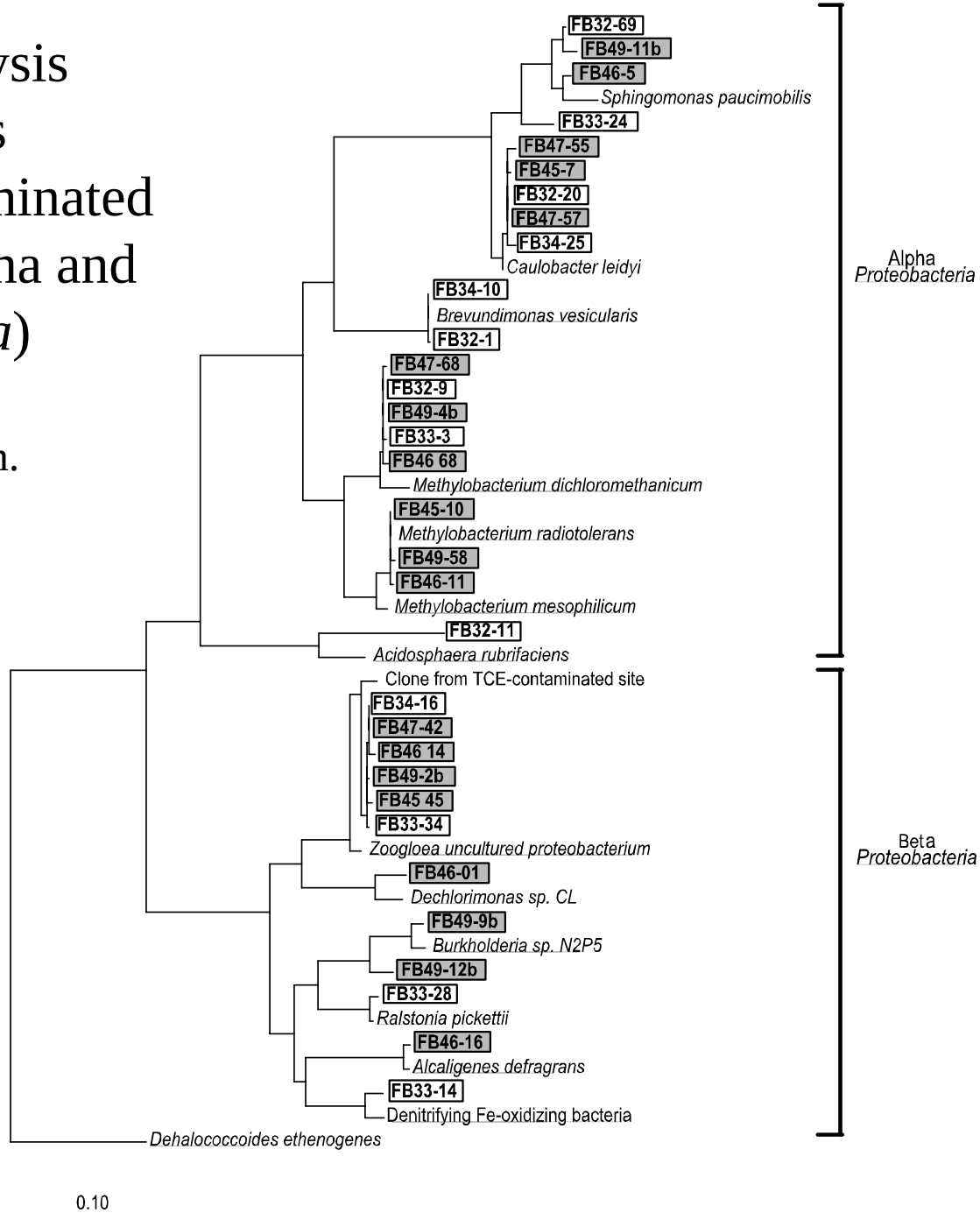






Phylogenetic Analysis of 16S rRNA genes cloned from contaminated FRC sediment (alpha and beta *Proteobacteria*)

White box: before biostim.
Gray box: after biostim.

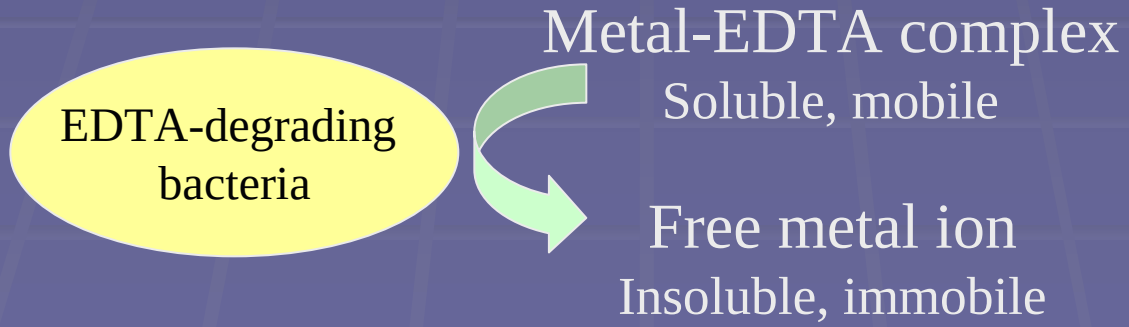


Methylobacterium

- *Methylobacterium*-type sequences were the most abundant sequences detected through cloning and sequencing both before and after biostimulation
- *M. dichloromethicanum:* Dechlorinating species
- *M. radiotolerans:* Tolerates radiation
- *M. mesophilicum:* Involved in the degradation of metal-EDTA complexes

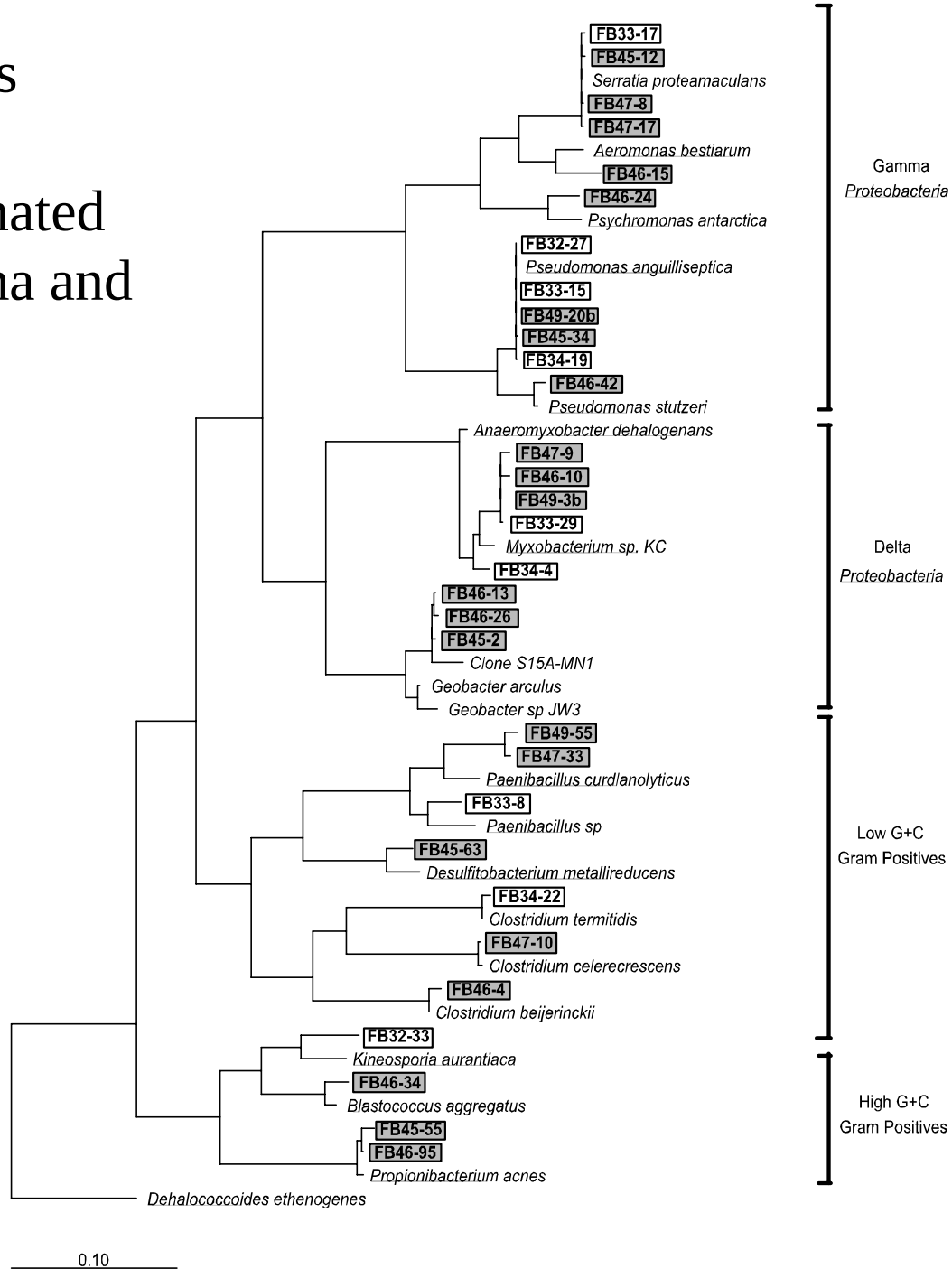
Metal-EDTA Complexes

- EDTA (ethylenediamine tetraacetic acid) was used as a cleaning agent, and was co-disposed with radioactive materials at the FRC
- EDTA forms stable, soluble complexes with metals (including uranium) hindering their adsorption to sediment surfaces
- To limit the migration of uranium through the groundwater, biodegrade the co-occurring organic ligand

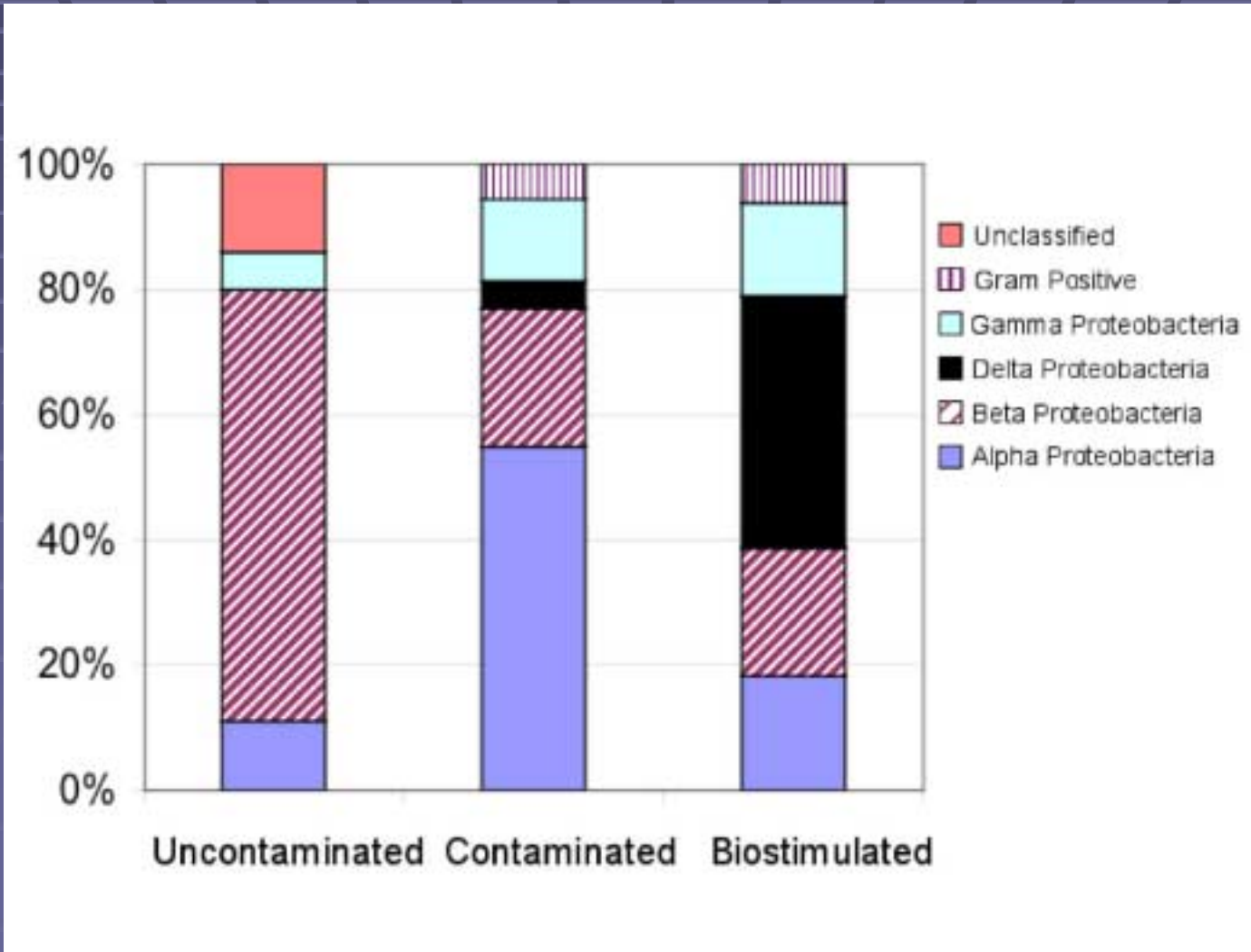


Phylogenetic Analysis of 16S rRNA genes cloned from contaminated FRC sediment (gamma and delta *Proteobacteria*, Gram-positives)

White box: before biostim.
Gray box: after biostim.



Bacterial Communities Before and After Biostimulation



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: Cultivation-independent Investigation

- Although *Geobacteraceae* sequences dramatically increased after biostimulation in half of the cores tested, other microbial groups must also be playing a role in the FRC sediment
- Heterogeneity in sediment may explain why *Anaeromyxobacter* sequences were found in abundance in cloning experiments, but not in MPN-PCR after biostimulation (this could also be due to a cloning bias)

Cultivation-independent Conclusions (cont.)

- Many sequences within the clone libraries were related to organisms with the ability to reduce nitrate, reduce iron, and/or to dechlorinate
- Organisms with the ability to utilize multiple contaminants as electron donors or acceptors may out-compete other organisms in the FRC sediment

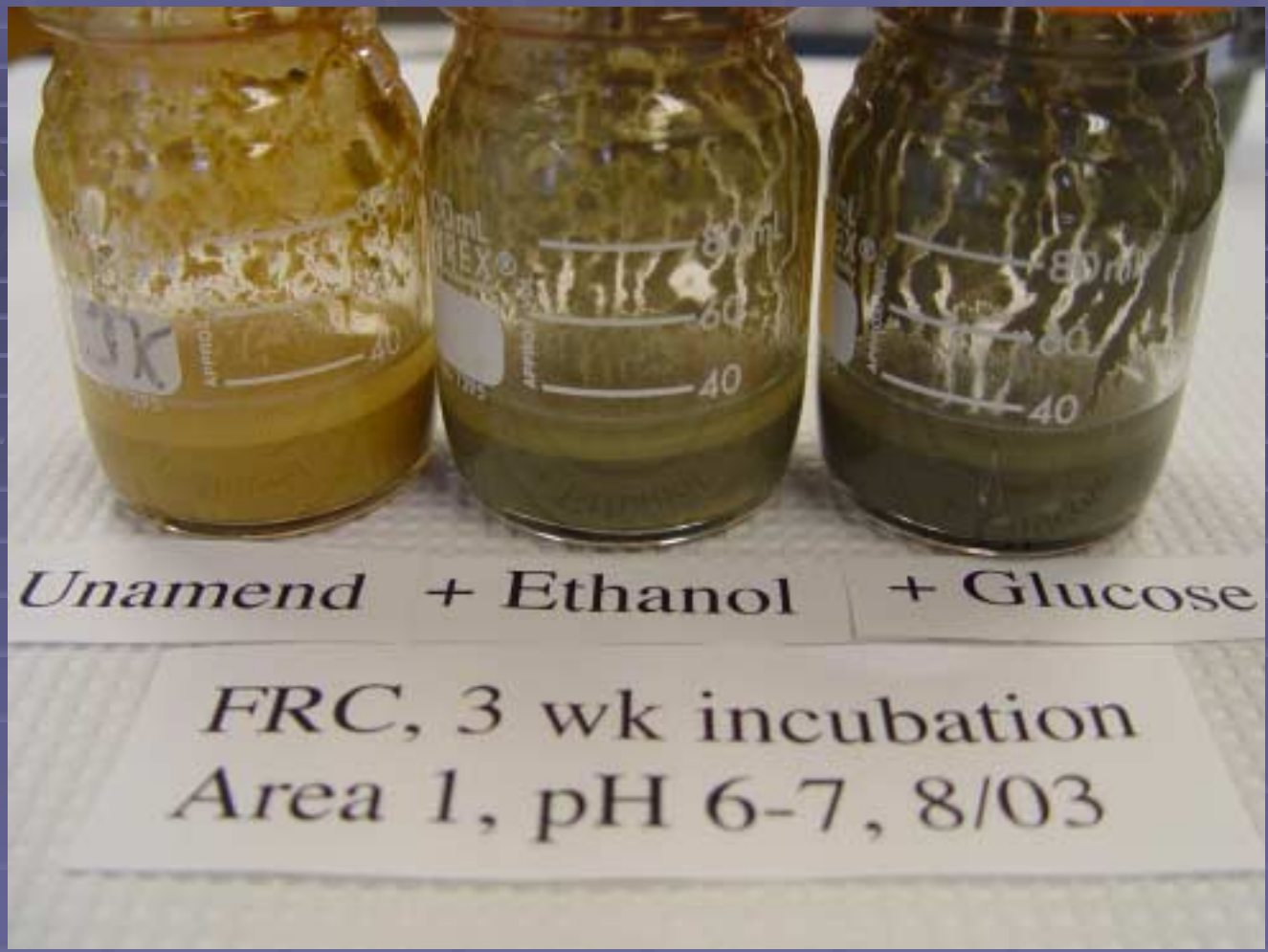
Challenges of the FRC subsurface

- 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)

Area 1 Slurries

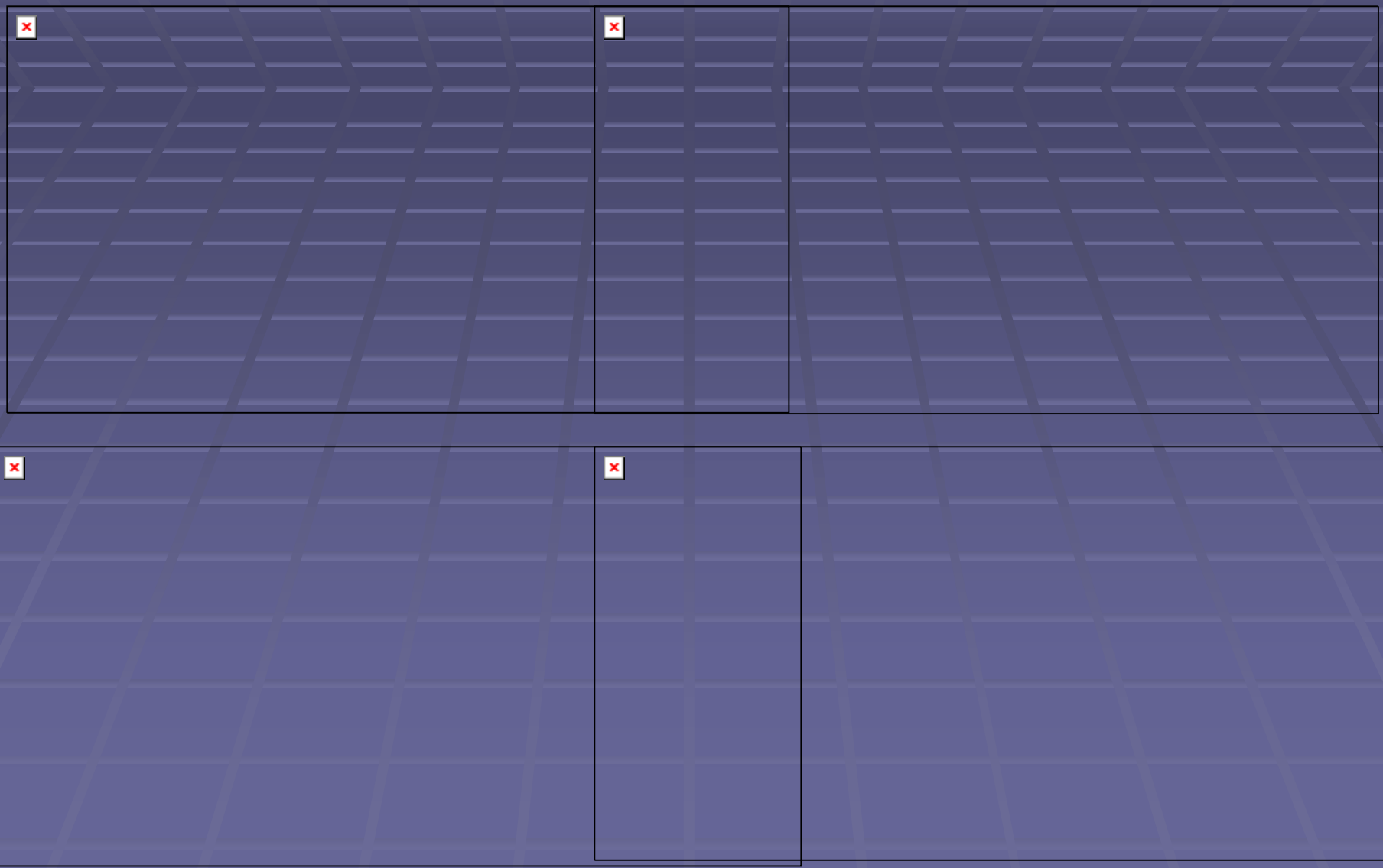
- Biostimulation clearly demonstrated
- Activity and geochemistry varies even within core
- Microbial groups display similar heterogeniety?


Unamend
+ Ethanol
+ Glucose
FRC, 3 wk incubation
Area 1, pH 6-7, 8/03


61-01-00
1
61-01-00
5
61-01-24
1
61-01-24
5
Unamend
+ Glucose
Unamend
+ Glucose
FRC, 3 wk incubation
Area 1, pH 6-7, 8/03


Unamend + Glucose
Unamend
+ Glucose
Area 1, pH 6-7
Area 1, pH 3-4
FRC, 3 wk incubation

Nitrate reduction



Publications to date

- 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: 13-24.
- 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. (In press).
- E.S. Shelobolina, K. O'Neill, K.T. Finneran, L.A. Hayes, D.R. Lovley. 2003. Potential for in situ bioremediation of a low pH, high-nitrate uranium-contaminated groundwater. Soil and Sediment Contamination (In press).
- 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. (Submitted).
- 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)
- 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).

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

- October ‘03- revise group report to include current and future research activities; display report on FRC website for all PIs to view
- November ‘03 to ?- develop a review of FRC microbial communities for publication in a refereed journal (after more research has been published)
- March ‘04- meet again at PI meeting

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