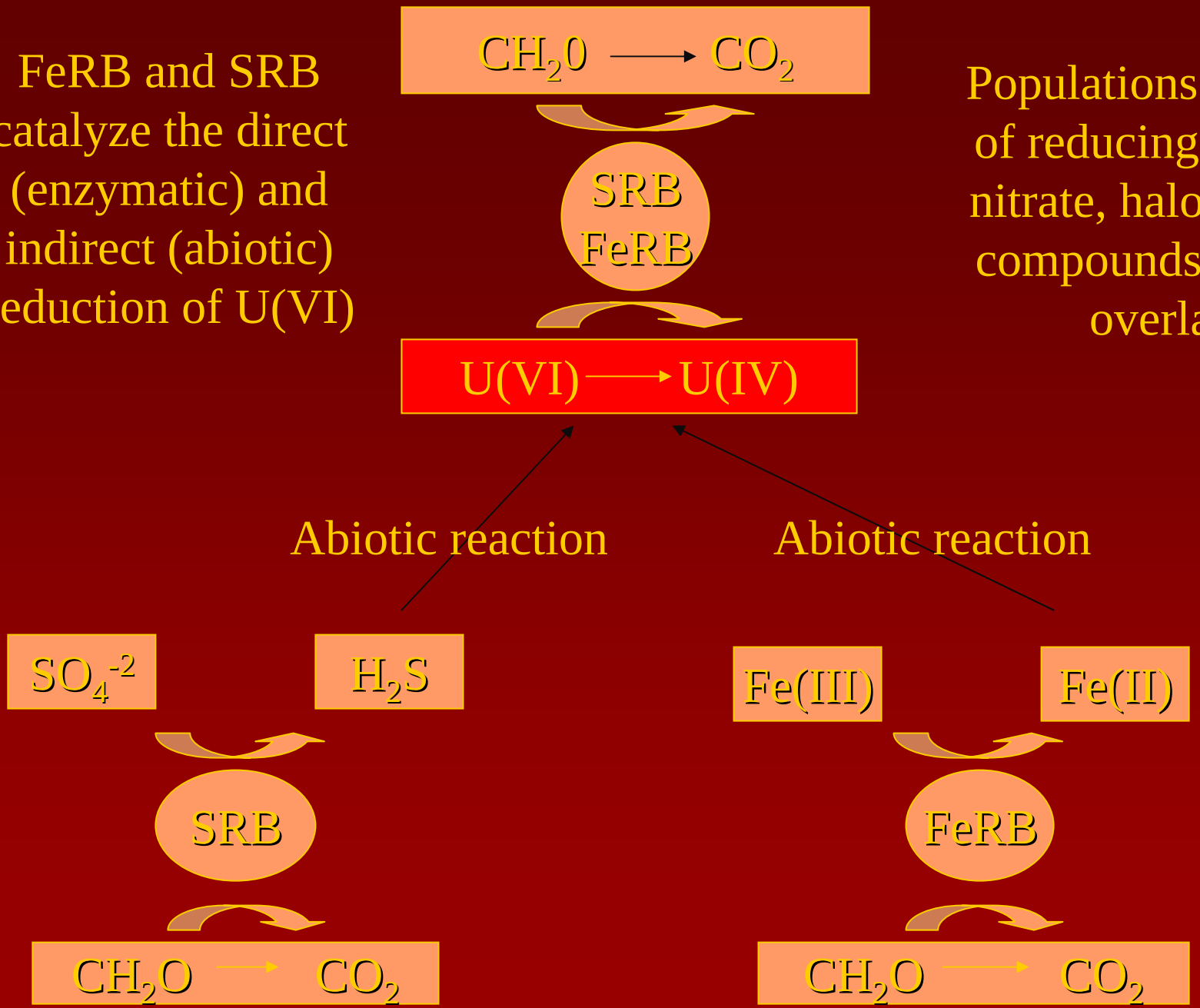
Working Group Report
Microbial Community Analysis

Joel E. Kostka

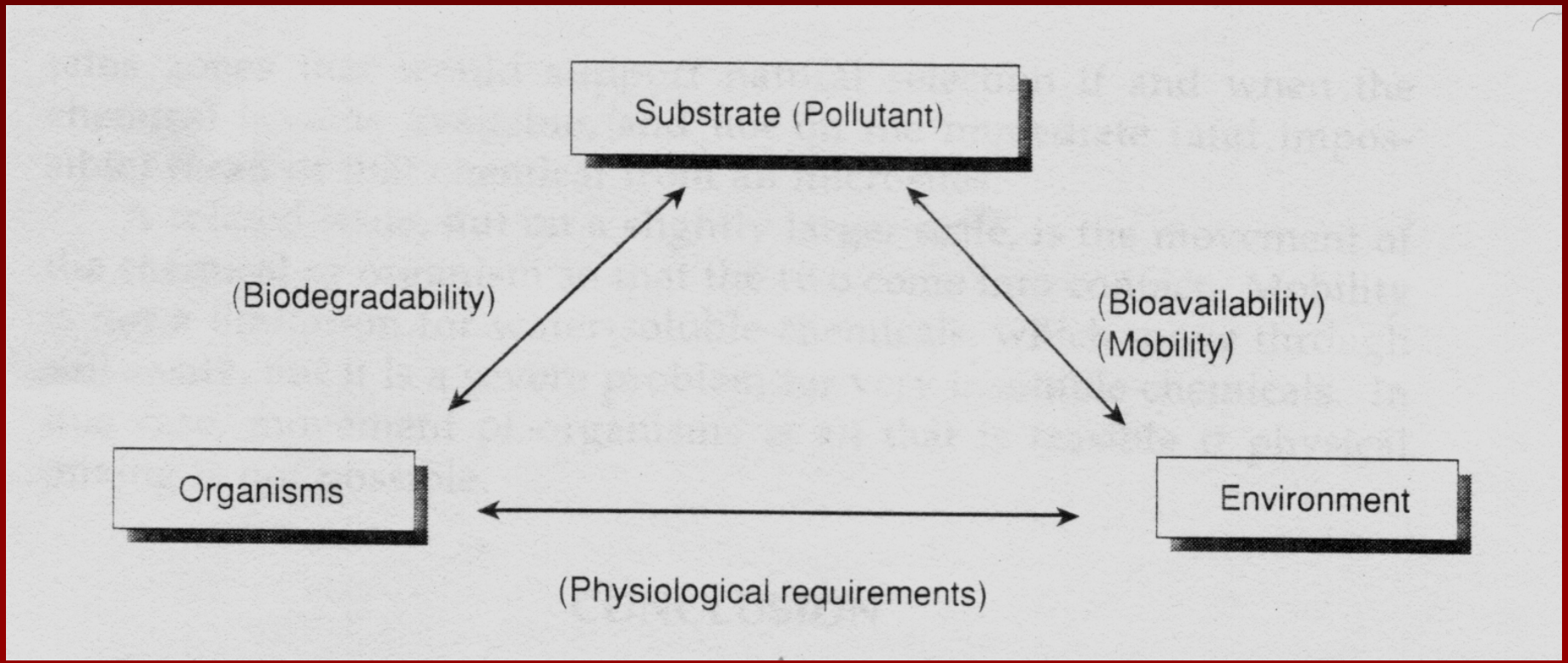
NABIR

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 - bioremediation potential (Tiedje, 1993)



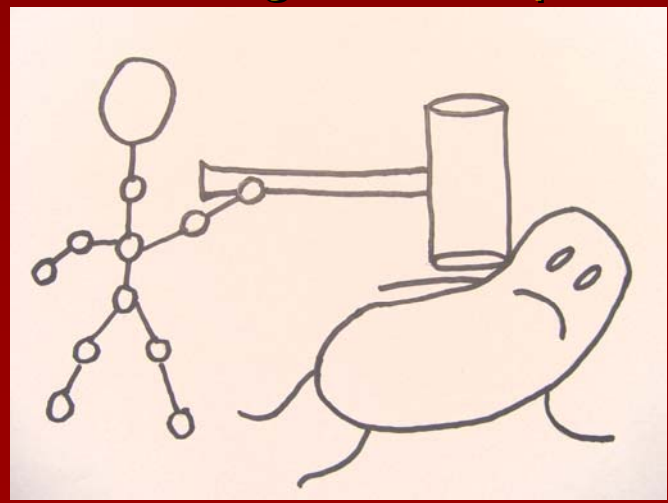
- ✓ Remediation potential dictated by physiological requirements for growth and metabolism

Ecological approach- what do we need to know?

- Identification and distribution of organisms driving desired metabolism
- Quantification of important metabolic groups
- Determination of physiological potential
- Significance of diversity

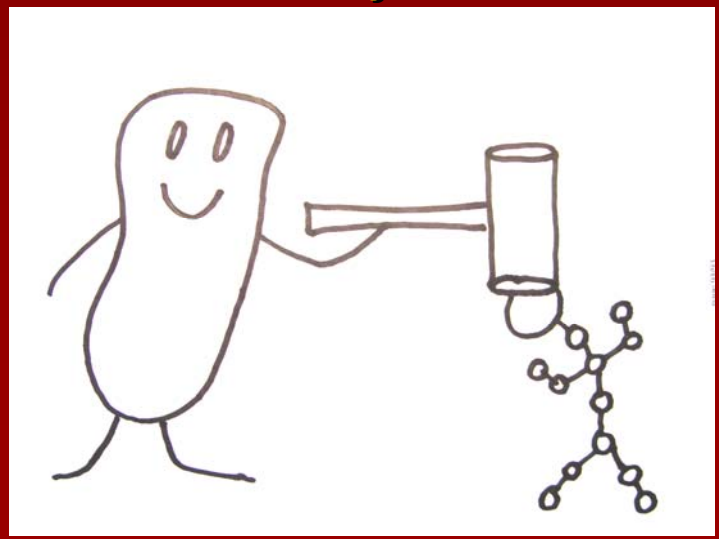
FRC - What do we know?

- 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
- Harsh subsurface environment for microorganisms; pHs 3-4, [nitrate] mM to M



FRC (continued)

- Microbial metabolism believed to be limited by: low C, acidic pH, and high nitrate, toxic metals
- Upon addition of electron donor and pH neutralization, extensive nitrate and metal reduction have been observed
- Thus, “Biostimulation” or substrate addition is a promising strategy for U(VI) immobilization by indigenous microorganisms



Objectives/ Activities of Working Group

- Overall Objectives
  - Optimize use of FRC
  - Determine level of site characterization and post-experimental monitoring to be conducted by FRC vs. research teams
  - Stimulate collaboration
- Specific to Microbial Communities Group
  - Breakout session on “Biodiversity and Bioremediation” at last PI meeting
  - 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

Microbial Community Analysis Working Group

- Barkay/ Sobecky
- Geesey/ Cummings et al.
- Fields et al.
- Hazen/Brodie
- Kerkhof
- Kostka
- Krumholz
- Kuske
- Loeffler
- Lovley
- Roden
- Tiedje/ Marsh et al.
- White/ Peacock
- Zhou

✓ Please let me know if you want to be included with this list!!

Current Questions

- How does community composition vary between groundwater, sediments, microbial samplers?
- Origin/ distribution of organisms driving remediation?
- 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.

Current Questions

- 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

Abundance/ Biomass

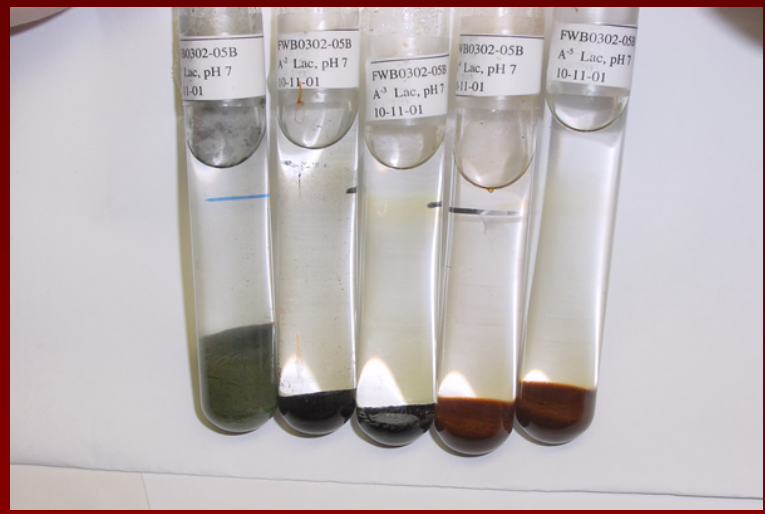
- Comprehensive study across a range of FRC environments lacking
- Direct counts have not revealed any dramatic differences between contaminated and pristine sites
- PLFA biomass measurements
- Viable counts have shown decreased abundance in contaminated environments, but results vary, especially for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

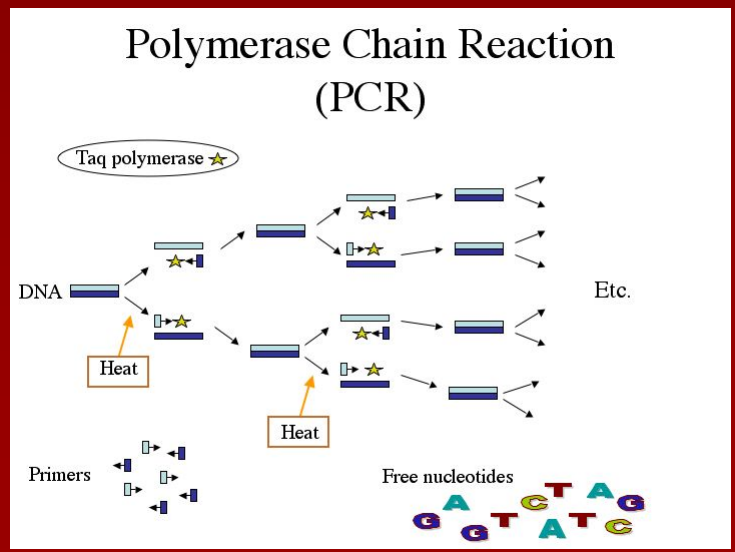
Microbial Community Composition - Approaches

- 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)

Methods

- Cultivation
- Cloning/ sequencing- DNA, RNA targets
- Quantitative PCR
- Stable isotope probing (SIP)
- High density oligonucleotide arrays





Target Organisms- Metal-reducers

- Dissimilatory metal-reducers
  - Deltaproteobacteria: Geobacter (I), Anaeromyxobacter
  - Betaproteobacteria: Rhodoferax
  - Gammaproteobacteria: Salmonella (I)
  - Gram positives: Desulfitobacterium, Desulfosporosinus
  - Acidobacteria: Geothrix
- Fermentative metal-reducers
  - Gram positives: Clostridium, Anaerovibrio, Bacillus, Paenibacillus
  - Gammaproteobacteria: Pseudomonas, Serratia

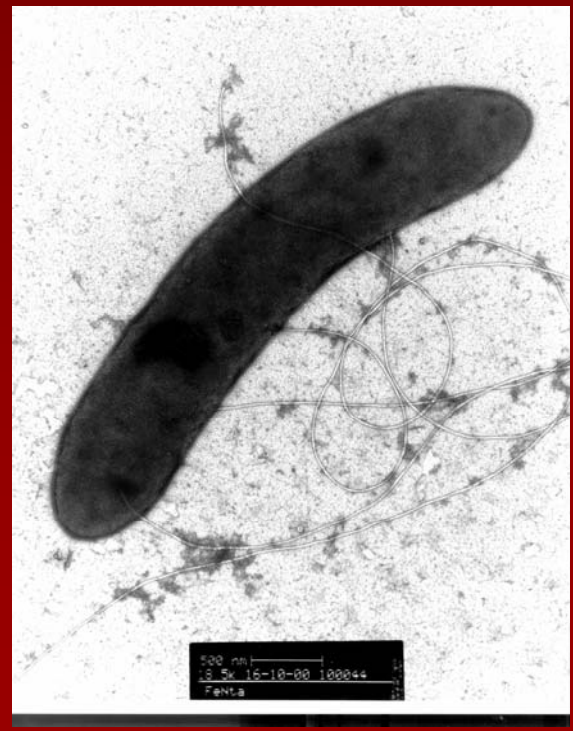
✓I = Isolated

✓Published evidence: Petrie et al., 2003; Istok et al., 2003; Peacock et al., 2003; Shelobolina et al., 2003; North et al., 2004

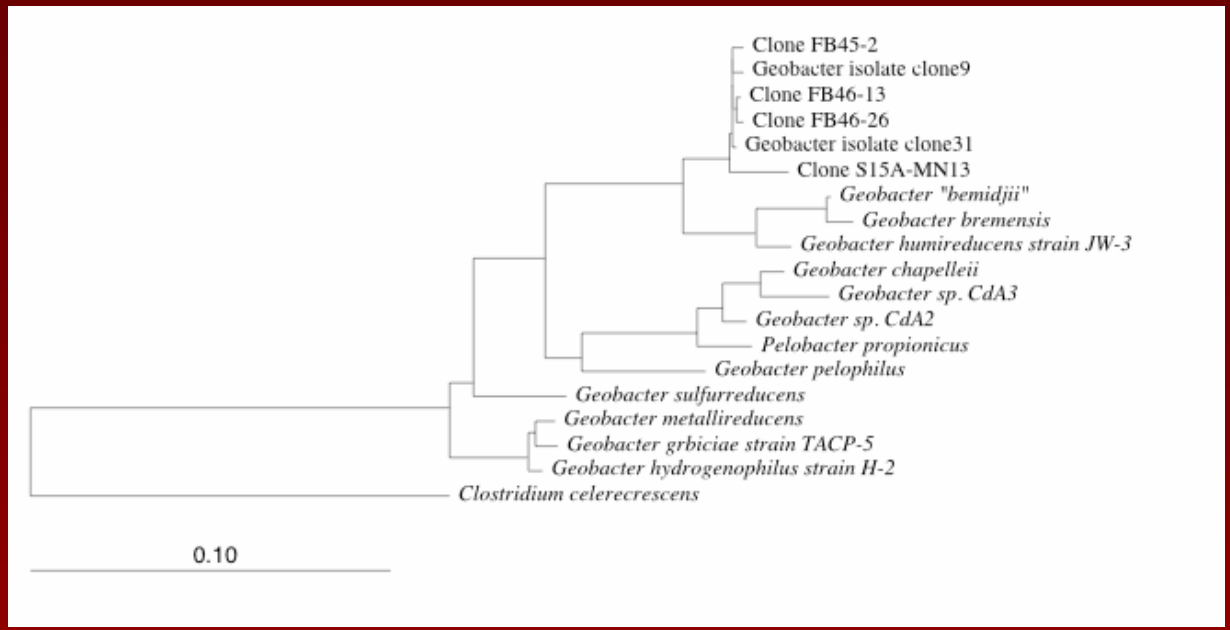
Target Organisms- Nitrate-reducers

- Dissimilatory reduction of nitrate to ammonium
  - Deltaproteobacteria: Geobacter (I), Anaeromyxobacter
  - Gram positives: Desulfitobacterium
- Denitrification
  - Betaproteobacteria: Alcaligenes (I), Ralstonia, Azospirillum, Acidovorax (I), Dechloromonas
  - Gammaproteobacteria: Pseudomonas (I), Klebsiella (I)
  - Alphaproteobacteria: Hyphomicrobium, Bradyrhizobium, Rhizobium, Blastobacter, Agrobacterium (I)

✓**Published evidence: Yan et al., 2003**



Geobacter strain FRC 32



- ✓Isolate shares high sequence identity with phylotypes from acidic FRC subsurface (North et al., AEM, 2004)
- ✓Growth with FeOOH as sole electron acceptor
- ✓Limited substrate utilization
- ✓Approved for draft genome sequencing

Key observations

- Diversity and biomass appear to be lower in contaminated environments
- In situ GW and sediment communities dominated by proteobacteria (alpha, beta, gamma)
- Nitrate and metal reduction stimulated by C2 to C6 electron donors
- Uranium reduction concurrent with Fe(III) reduction
- Low pH (≤ 5) toxic to nitrate-reducers

Key observations

- Ammonium does not accumulate under nitrate-reducing conditions in field or microcosms
- Both community composition and biomass change substantially during biostimulation
- Geobacteraceae make up small portion of in situ communities (GW, sediment) but predominate after electron donor addition
- Many OTUs detected by microarray but not in clone library

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

Publications to date

- Fields, M.W., 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).
- Istok, J.D., 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. 38: 468-475.
- Kostka, J.E., 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. Change in bacterial community structure during in situ biostimulation of subsurface sediment cocontaminated with uranium and nitrate. Appl. Environ. Microbiol. 70: 4911-4920.
- Peacock, A.D., Y.-J. Chang, J.D. Istok, L. Krumholz, R. Geyer, B. Kinsall, D. Watson, K.L. Sublette and D.C. White. 2003. Utilization of microbial biofilms as monitors of bioremediation. Microbial Ecology (in press).

Publications to date

- Petrie, L., 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.
- Reardon, C.L., D.E. Cummings, L.M. Petzke, B.L. Kinsall, D.B. Watson, B.M. Peyton, G.G. Geesey. 2003. Composition and diversity of microbial communities recovered from surrogate minerals incubated in an acidic uranium-contaminated aquifer. Appl. Environ. Microbiol. (in press)
- 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.
- Yan,T., 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.

Challenges for the future

- Develop effective sampling strategies for extreme heterogeneity in sediment characteristics (mineralogy, pore geometry)
- Use PI coordination to increase replicability of approaches within the same field experiment (to combat sample heterogeneity)
- QUANTIFICATION of distribution of important functional groups (GW, sediments)
- Develop methods to elucidate “active” members of populations during biostimulation
- Compare microbial communities in groundwater, sediments, microbial samplers

Challenges for the future

- Add comprehensive study of biomass in sediments and groundwater
- Develop and deploy quantitative, cultivation-independent approaches in conjunction with field experiments and geochemical analysis
- Free ourselves from bonds of PCR

Outline

- Introduction
  - Intro to FRC research
  - Working group objectives
  - Status of working group
- Summary of group results
  - Abundance/ biomass
  - Microbial community composition
- Conclusions
- Future challenges

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

Conclusions: cultivation-dependent Investigation

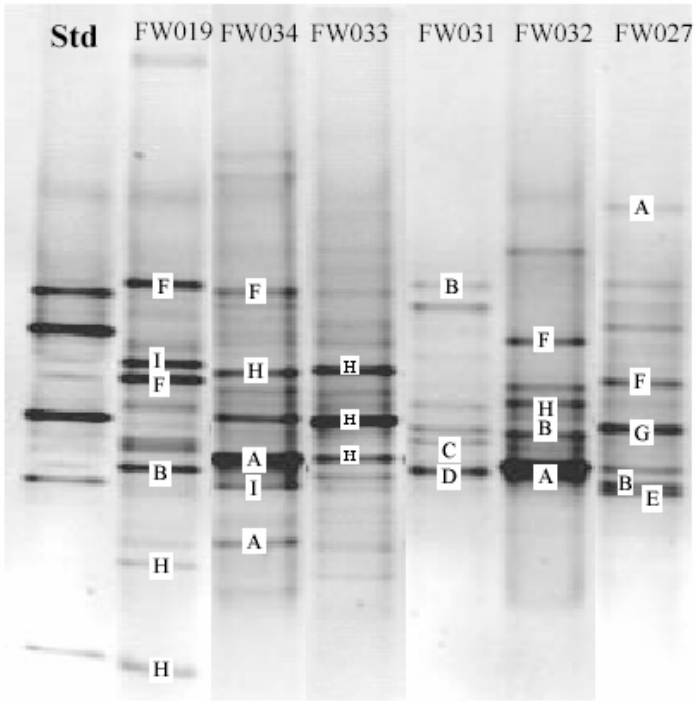
- The abundance and community composition of culturable FeRB is dependent upon geochemical parameters (pH, nitrate)
- Microorganisms capable of producing spores or spore-like bodies were representative of acidic sediments
- Neutrophilic organisms cultured from contaminated acidic sediment likely to be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

Petrie et al., 2003, AEM

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%

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.

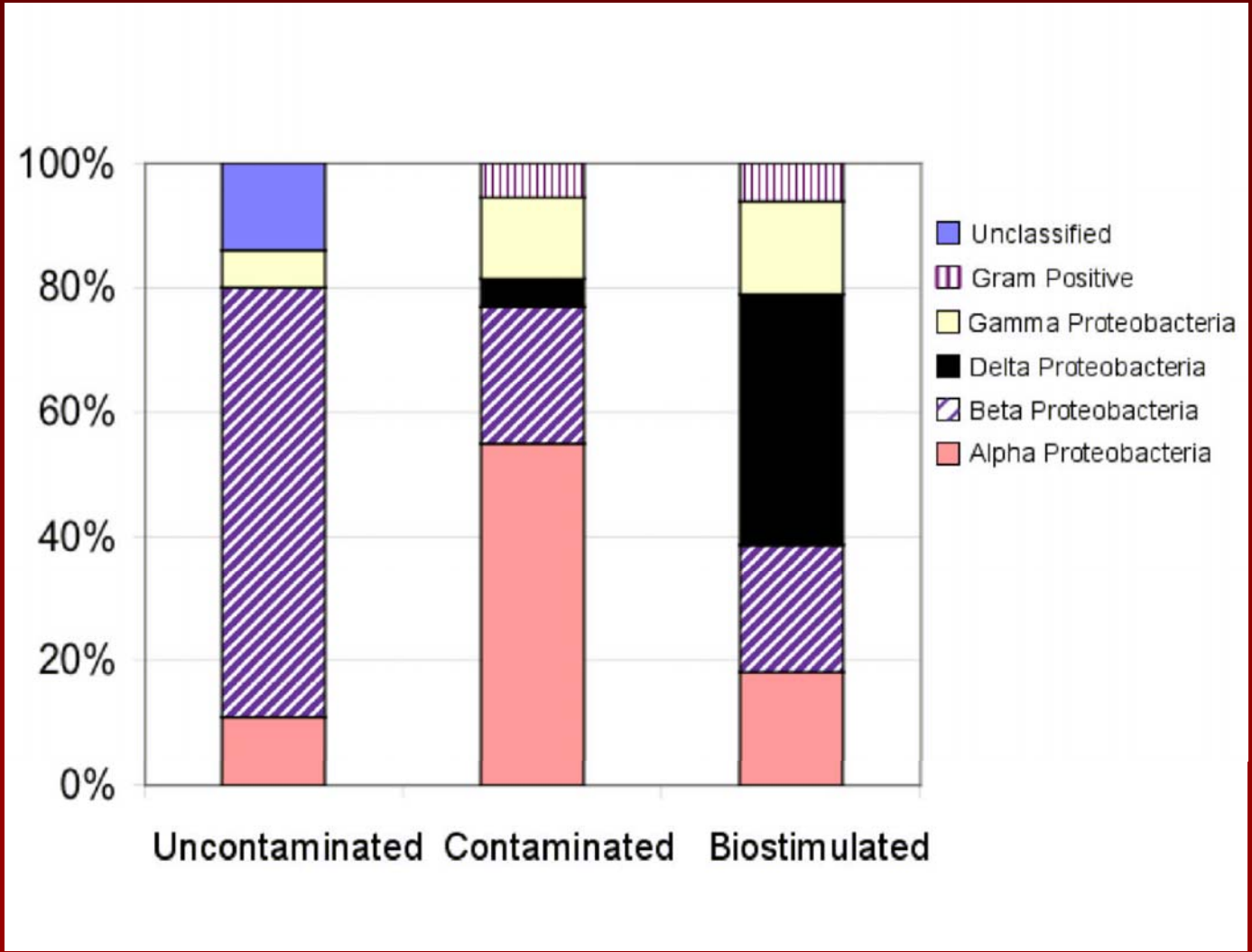
Clone ID	GenBank no.	Frequency[a]	Affiliation[b] (% similarity) (Accession)	Putative division
C-CG17		59	*Alcaligenes* sp. strain L6 (95%) (X92415)	β-Proteobacteria
C-CS3*		24	*Frateuria* sp. NO-16 (96%) (AF376025)	γ-Proteobacteria
C-CF16		4	*Methylobacterium radiotolerans* (99%) (D32227)	α-Proteobacteria
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)

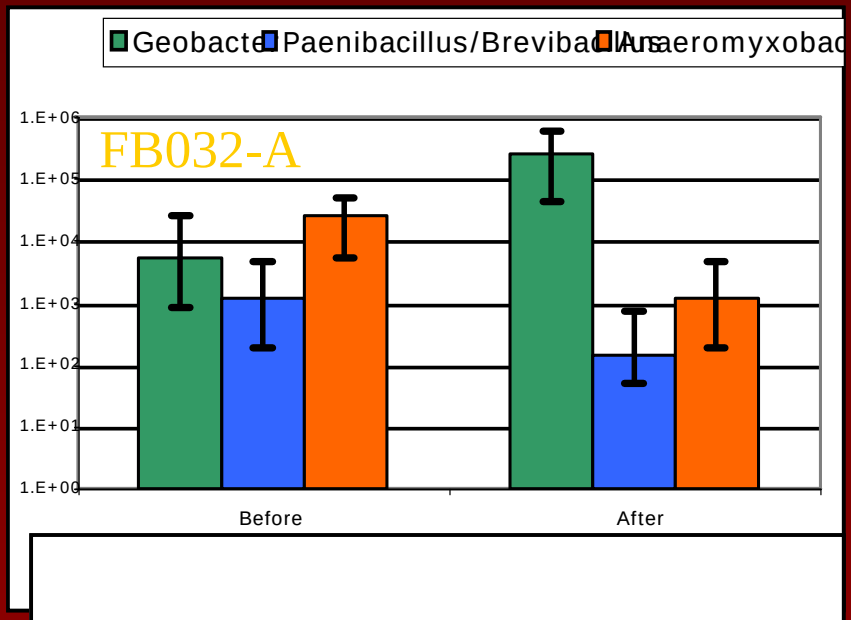
Viable counts of aerobic heterotrophs (Balkwill lab)

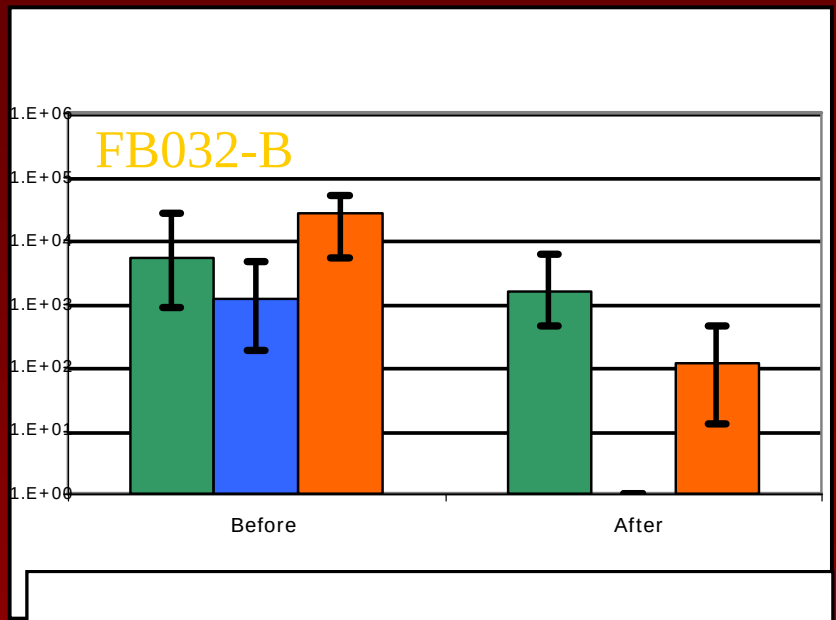
- No growth observed in majority of plates from contaminated FRC samples
- When growth observed, counts were 10^2 to 10^3 CFU g^{-1}
- UMTRA sediments: 10^3 to 10^7 CFU g^{-1}

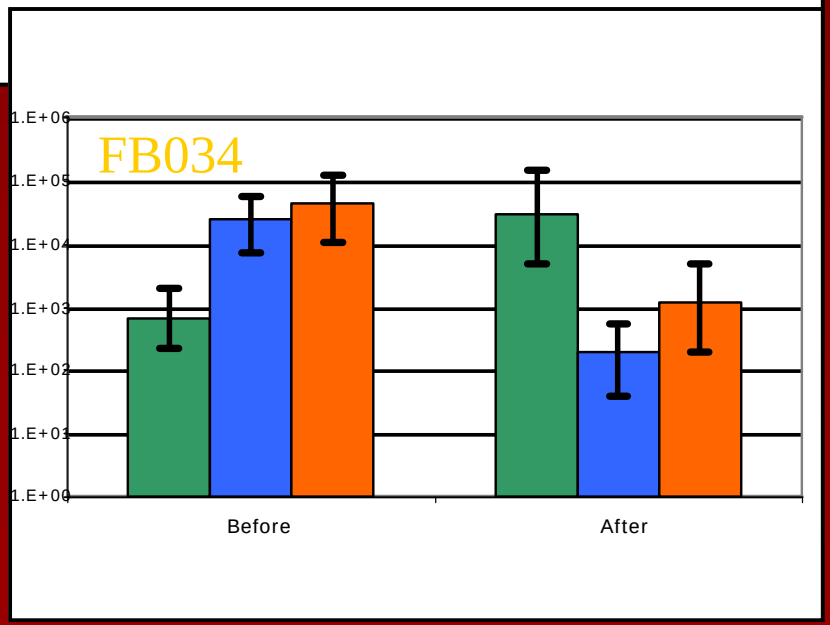
Bacterial Communities Before and After Biostimulation

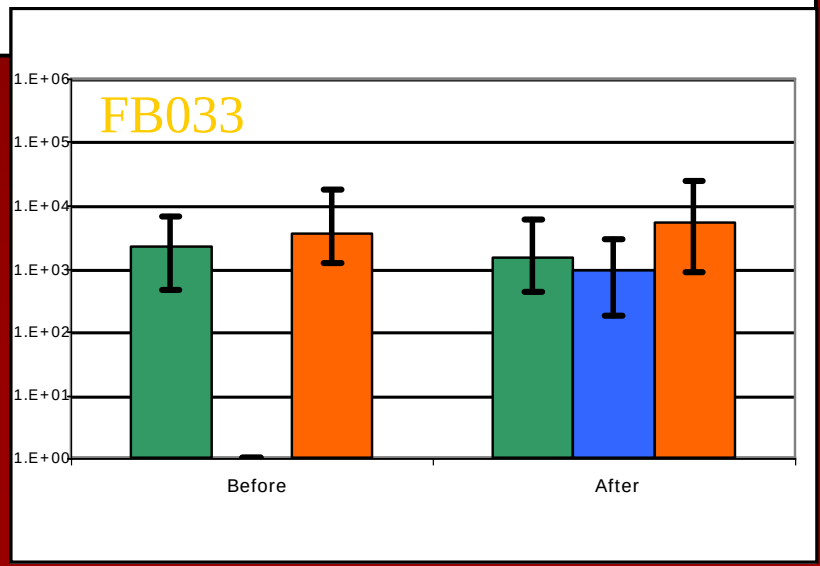


MPN-PCR Results (16S rRNA gene copies/gram sediment)









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