Linking the structure and function of subsurface microbial communities for the assessment of bioremediation potential at the Oak Ridge Field Research Center

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Biostimulation of Iron Reduction and Uranium Immobilization: Microbial and Mineralogical Controls

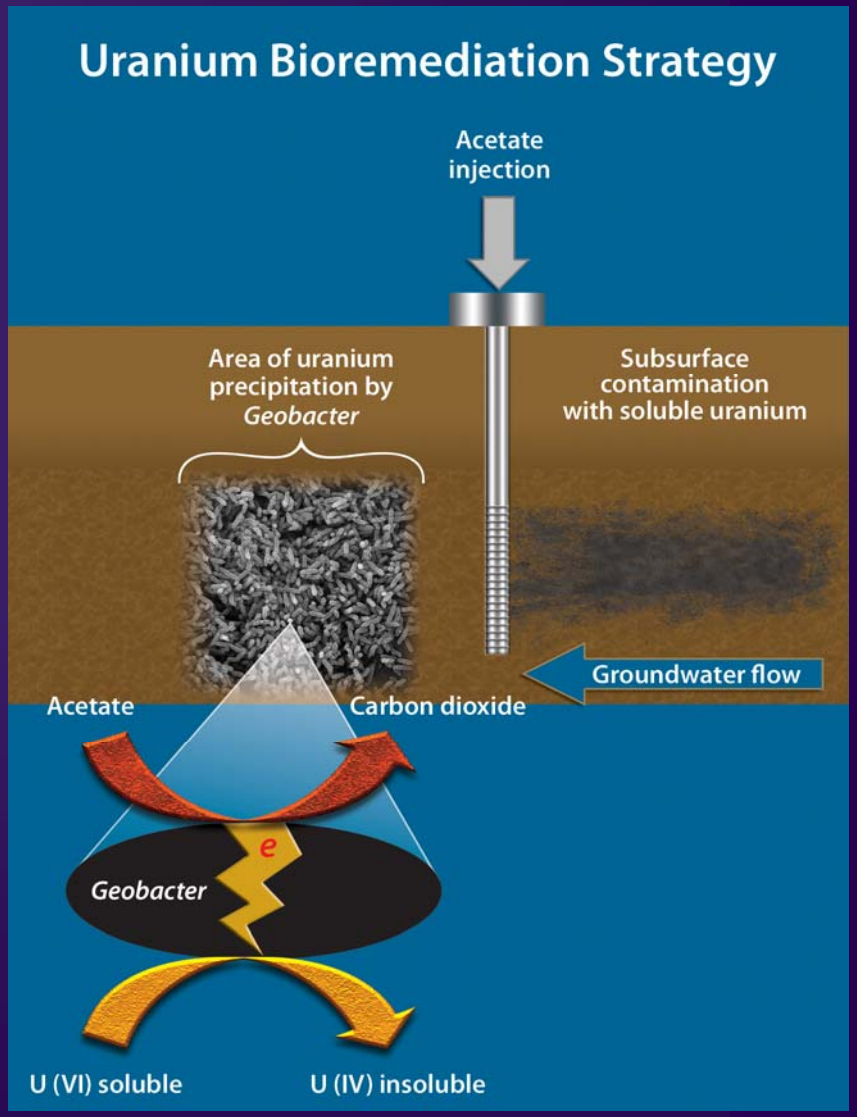
- NABIR/ ERSP Integrative Studies
- Investigators
  - Joel Kostka- Florida State University
  - Lee Kerkhof- Rutgers University
  - Joe Stucki- University of Illinois

Overall Objectives

1. Determine the microbial and geochemical mechanisms controlling the reduction and immobilization of U(VI) during biostimulation
2. Focus on metal- and nitrate-reducing prokaryotes that mediate electron flow as well as on iron minerals that are likely to make strong contributions to the transport/ transformation of U(VI)

Remediation of metal radionuclides at DOE sites

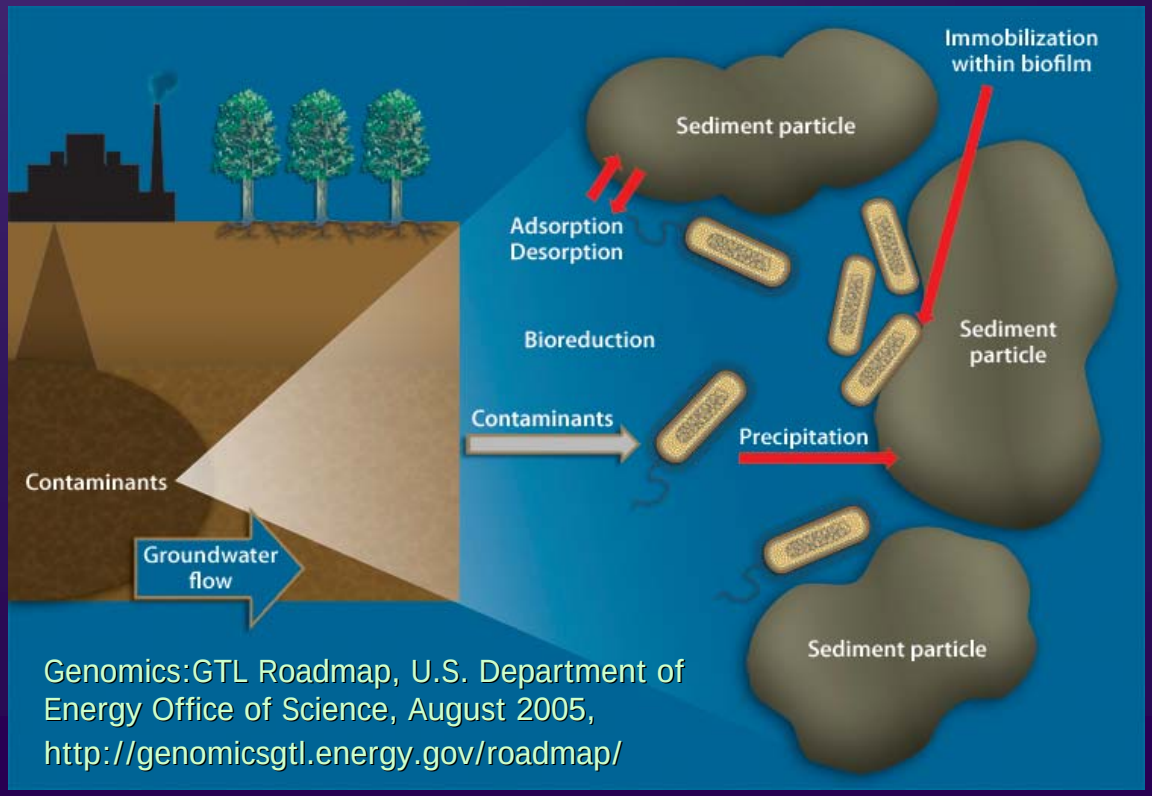
- Field experiments indicate that indigenous microorganisms could provide more cost-effective solutions for remediating mobile metal contaminants near source zones than current practices (Anderson et al., 2003; Istok et al., 2004; Wu et al., 2006)



Genomics:GTL Roadmap, U.S. Department of Energy Office of Science, August 2005, http://genomicsgtl.energy.gov/roadmap/

Remediation of metal radionuclides at DOE sites

- Bioremediation potential dictated by the physiological requirements for the growth and metabolism of microorganisms
- Bioremediation approaches have yet to be implemented partly because large gaps remain in our knowledge of the *in situ* diversity, distribution and metabolic capability of microorganisms in contaminated subsurface environments



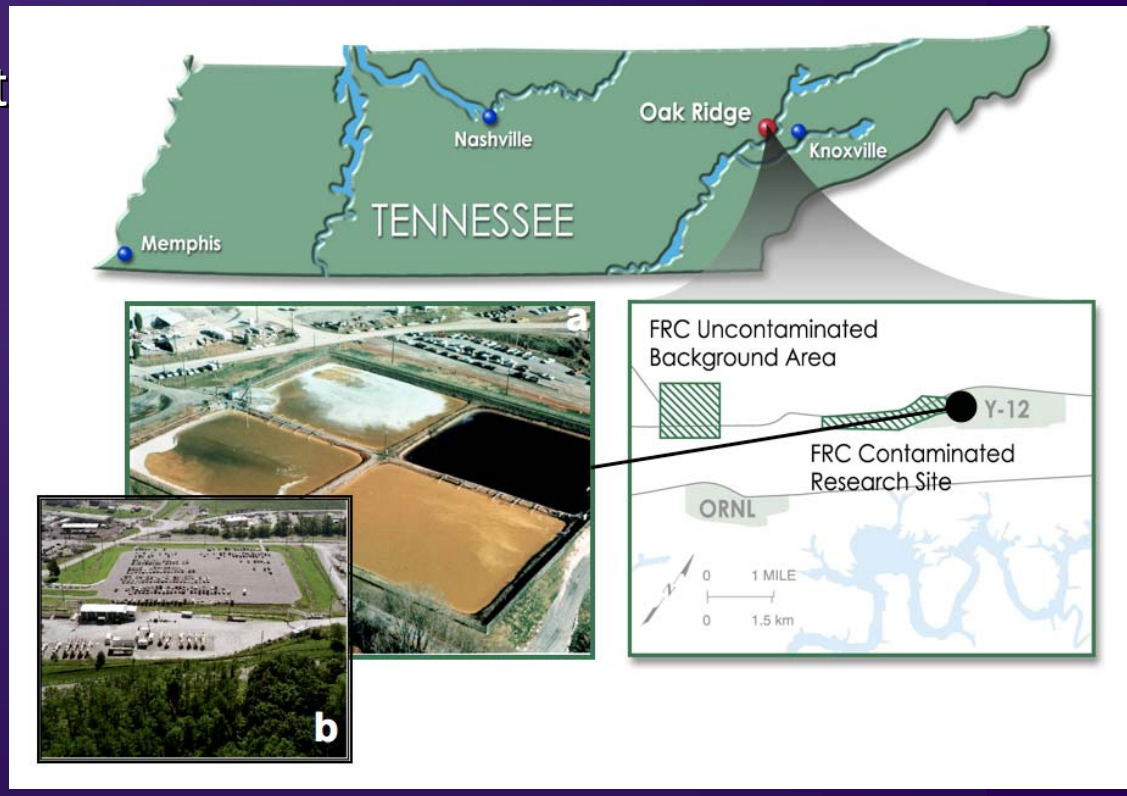
Genomics:GTL Roadmap, U.S. Department of Energy Office of Science, August 2005, http://genomicsgtl.energy.gov/roadmap/

Specific Objectives

1. Elucidate key microbial groups that catalyze electron transport processes leading to radionuclide immobilization
2. Differentiate from unwanted groups (take up carbon but not involved in contaminant transformation)
3. Provide more quantitative information on metabolically active microbial groups that can serve as an inputs for modeling

Study Site: ERSP Oak Ridge Field Research Center

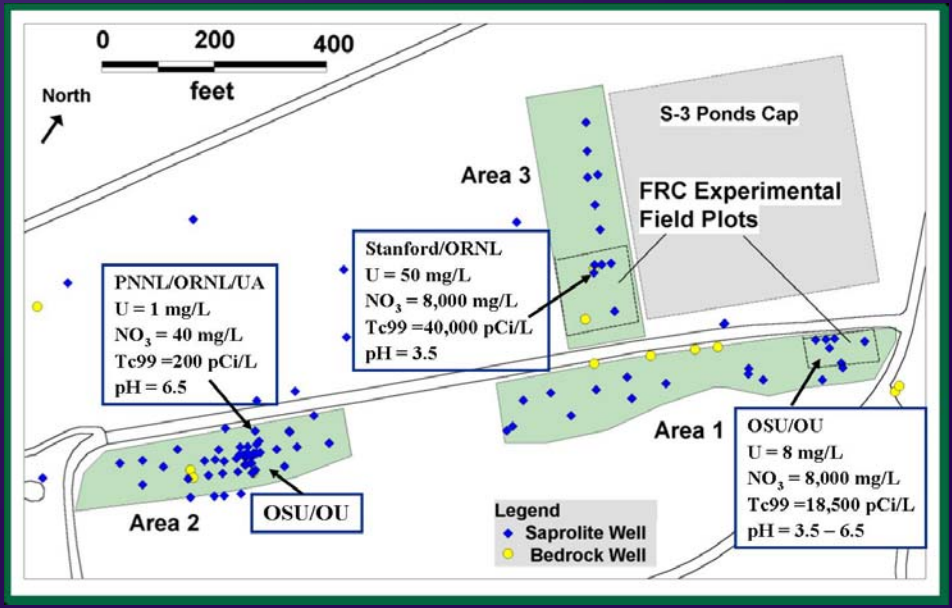
- Waste disposal: the S-3 ponds
  - 7 km contaminant plume
  - Mixed wastes
    - Radionuclides
    - Nitric acid
    - Toxic metals



- Because of their widespread significance as groundwater contaminants at DOE sites, our research has centered on the cleanup of uranium(VI) and nitrate.

ORFRC Experimental Plots

- Contaminated Plots
  - Area 1
    - High uranium and nitrate concentrations
    - Acidic to neutral pH
  - Area 2
    - Moderate to high uranium and nitrate concentrations
    - Sulfate present
    - Neutral pH



Results/ Discussion

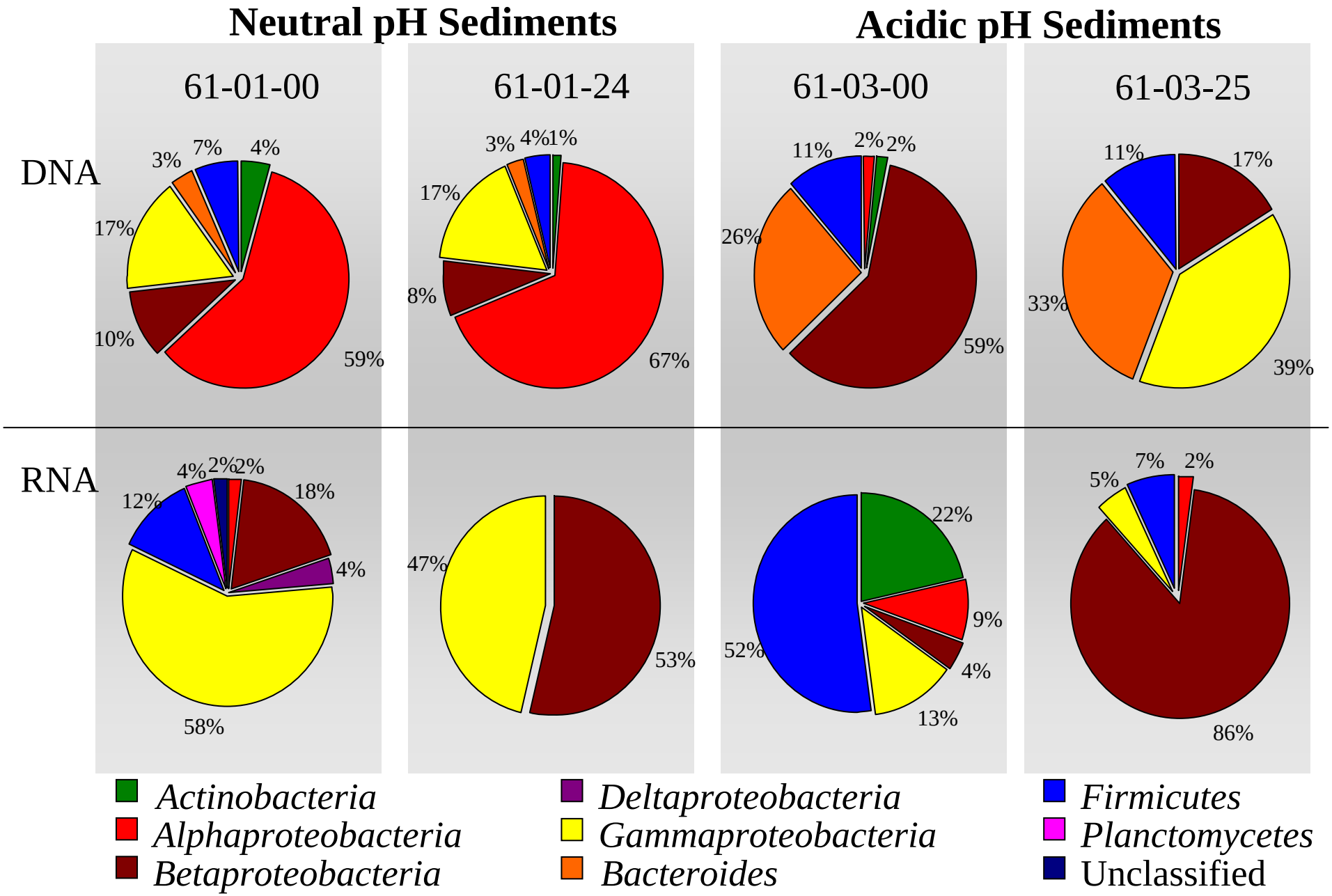
- *In situ* characterization of metabolically active microbial groups using rRNA targets
- Direct linkage of microbial function with phylogenetic structure in subsurface sediments
  - Stable isotope probing (SIP)
  - Reverse transcription realtime PCR
- *In situ* characterization of iron minerals as agents of electron transfer or sorption of radionuclides

SUBSURFACE SEDIMENTS

Sediment Characteristics of Area 1 Borehole FB61

Sample	Depth (m)	pH	Nitrate	Fe(III)
61-01-00	2.4-3.1	6.7	0.6	31.5
61-01-24	3.1-3.7	6.1	0.1	17.0
61-03-00	4.9-5.5	3.9	17.8	17.3
61-03-25	5.5-6.1	3.7	40.1	18.6

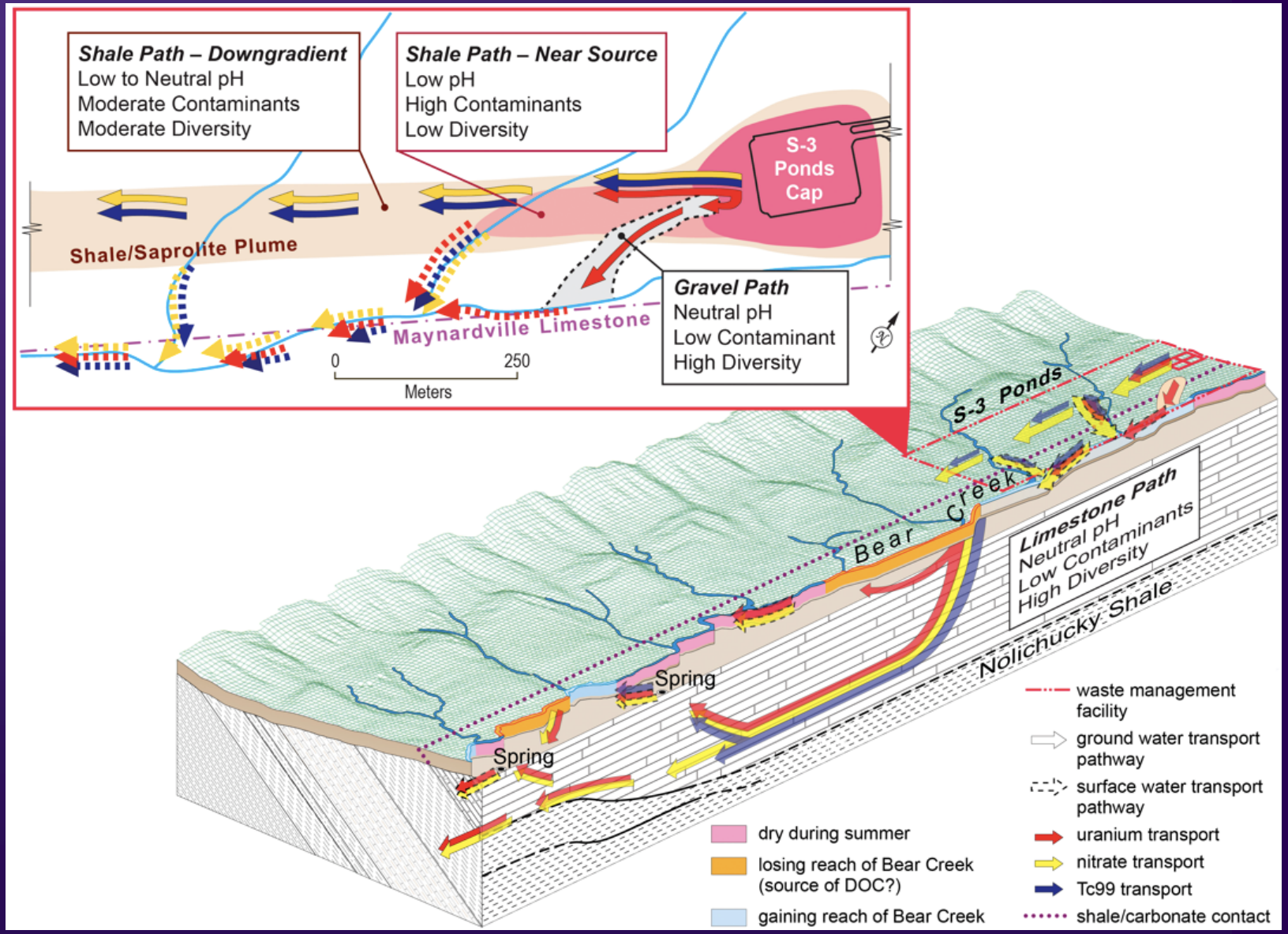
SSU rRNA gene (DNA-derived) and SSU rRNA (RNA-derived) clone libraries constructed


Neutral pH Sediments
Acidic pH Sediments
61-01-00
61-01-24
61-03-00
61-03-25
DNA
RNA
3%
7%
4%
17%
10%
59%
3%
4%
1%
17%
8%
67%
11%
2%
2%
26%
59%
11%
17%
33%
39%
4%
2%
2%
12%
18%
4%
58%
47%
53%
22%
9%
52%
4%
13%
7%
2%
5%
86%
Actinobacteria
Alphaproteobacteria
Betaproteobacteria
Deltaproteobacteria
Gammaproteobacteria
Bacteroides
Firmicutes
Planctomycetes
Unclassified

Conclusions - Sediments

- Successfully extracted rRNA from low biomass subsurface sediment
- RNA-derived phylotypes were predominantly subsets of the DNA-derived library from the same sample
- Comparison of DNA- and RNA-derived libraries indicated that nitrate-reducing taxa are available for bioremediation
- Sequences closely related to nitrate-reducing bacteria comprised 28% and 43% of clones from the total and metabolically active microbial community, respectively
- Akob, D.M., H.J. Mills, and J.E. Kostka. 2006. Metabolically-active microbial communities in uranium-contaminated subsurface sediments. FEMS Microbiology Ecology doi: 10.1111/j.1574-6941.2006.00203.x

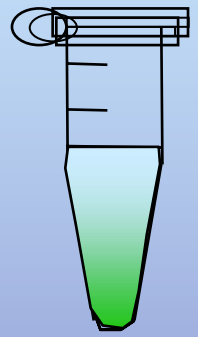
Groundwater



Kostka, Watson, Kerkhof, Gentry, Zhou. 2006. Submitted.

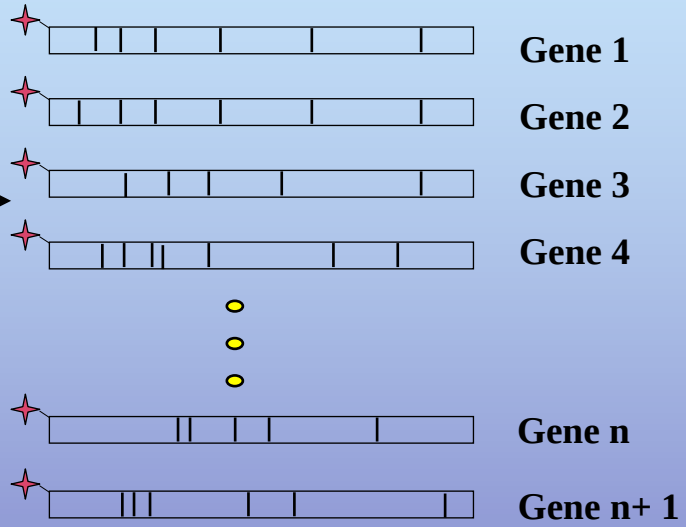
How does Terminal Restriction Fragment Length Polymorphism work?

Sample



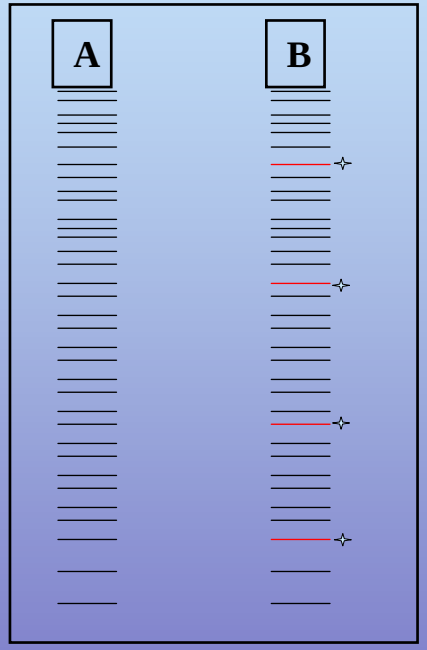
Purify DNA

PCR with fluorescent primers



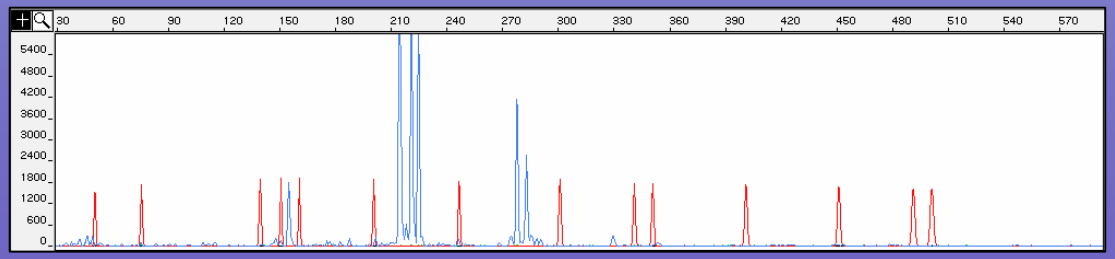
Restriction Enzyme Digest

Sizes separate on automated sequencer

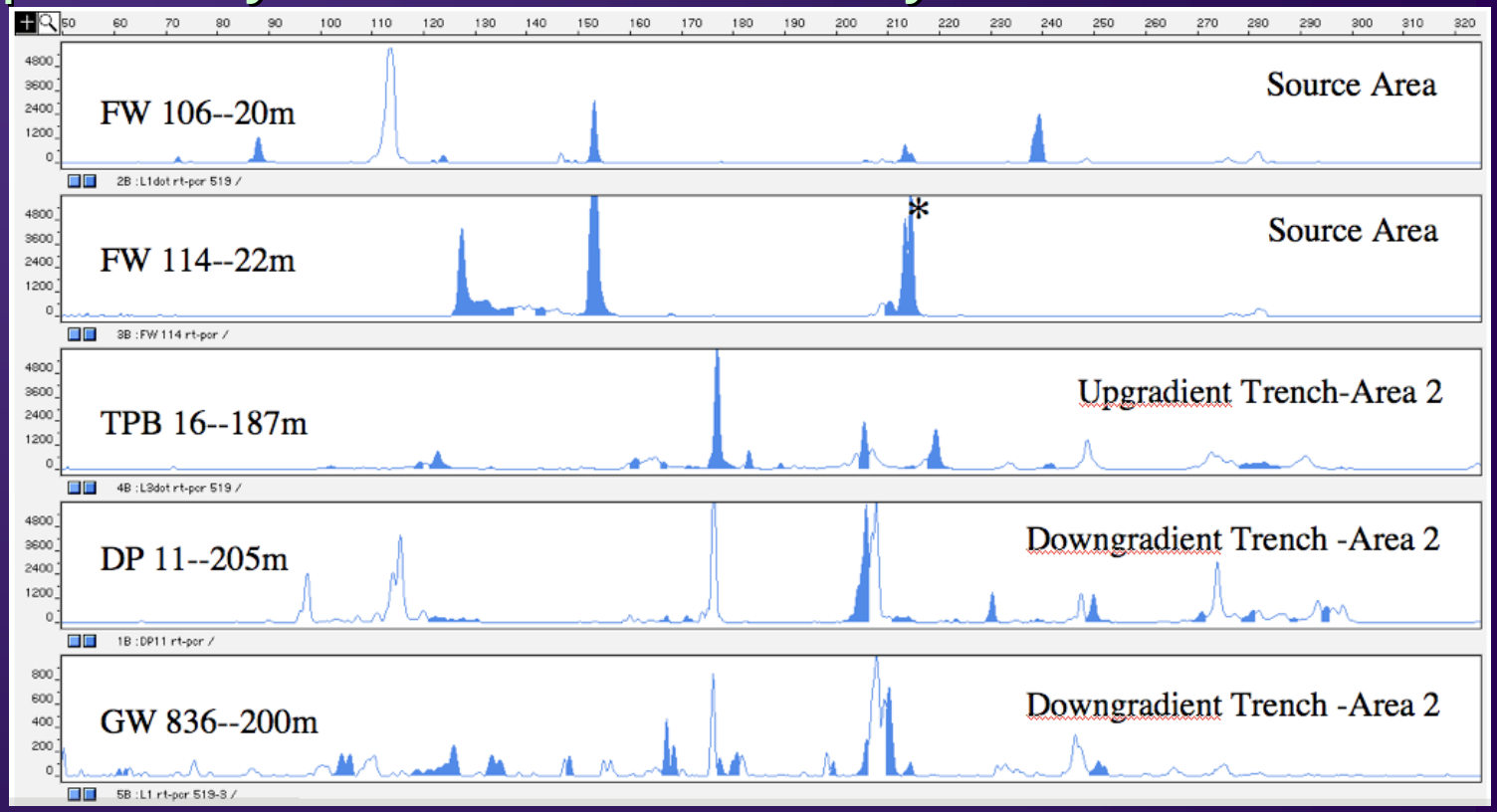


ABI Software Analysis

Only labeled fragments appear as peaks



GROUNDWATER: Phylogenetic composition largely impacted by contaminant chemistry



- T-RFLP profiles of RT-PCR reactions of 16S rRNA extracted along the contaminant plume
- Metal-reducing *Deltaproteobacteria* (*Anaeromyxobacter* and *Geobacter*) detected often at DNA level

Lee Kerkhof, Rutgers University

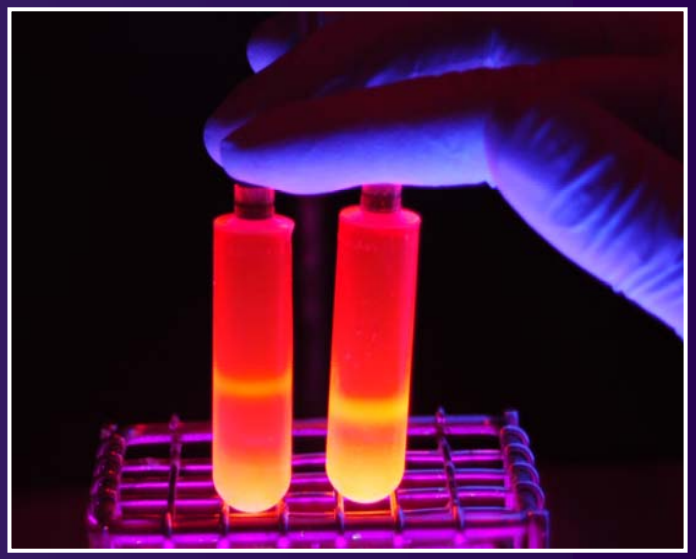
Results/ Discussion

- *In situ* characterization of metabolically active microbial groups using RNA targets
- Direct linkage of microbial function with phylogenetic structure in subsurface sediments
  - Stable isotope probing (SIP)
  - Reverse transcription realtime PCR
- *In situ* characterization of iron minerals as agents of electron transfer or sorption of radionuclides

Microcosms

- Area 2 sediment combined with groundwater
  - pH neutralized
  - Flushed with N_2
- Treatments (3 replicates each):
  - 20 mM Ethanol
  - 10 mM Glucose
  - No carbon control
- Incubated at 30°C
- Monitored activity using geochemical analysis
- 6 experiments

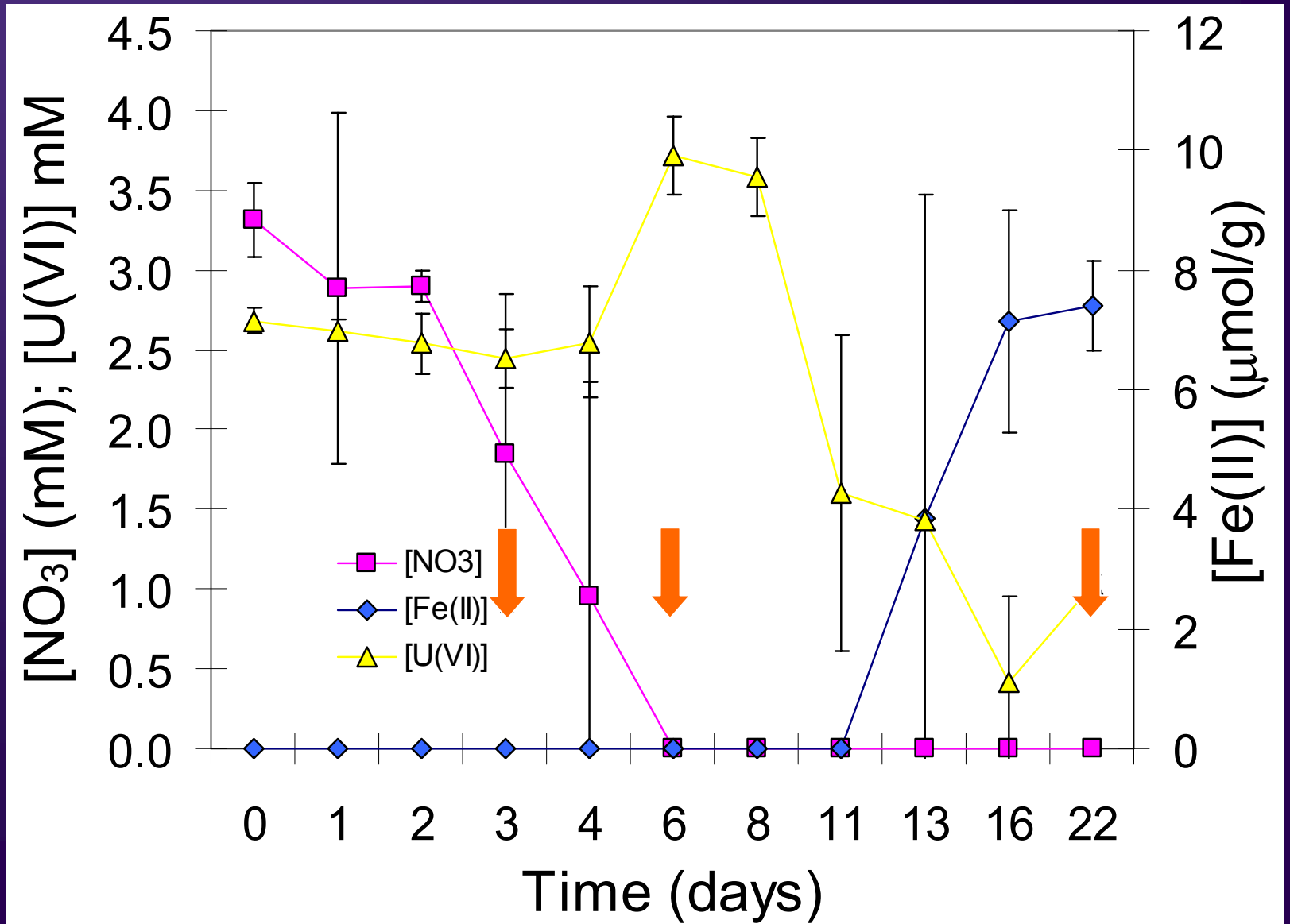
Stable Isotope Probing (SIP) Experiment



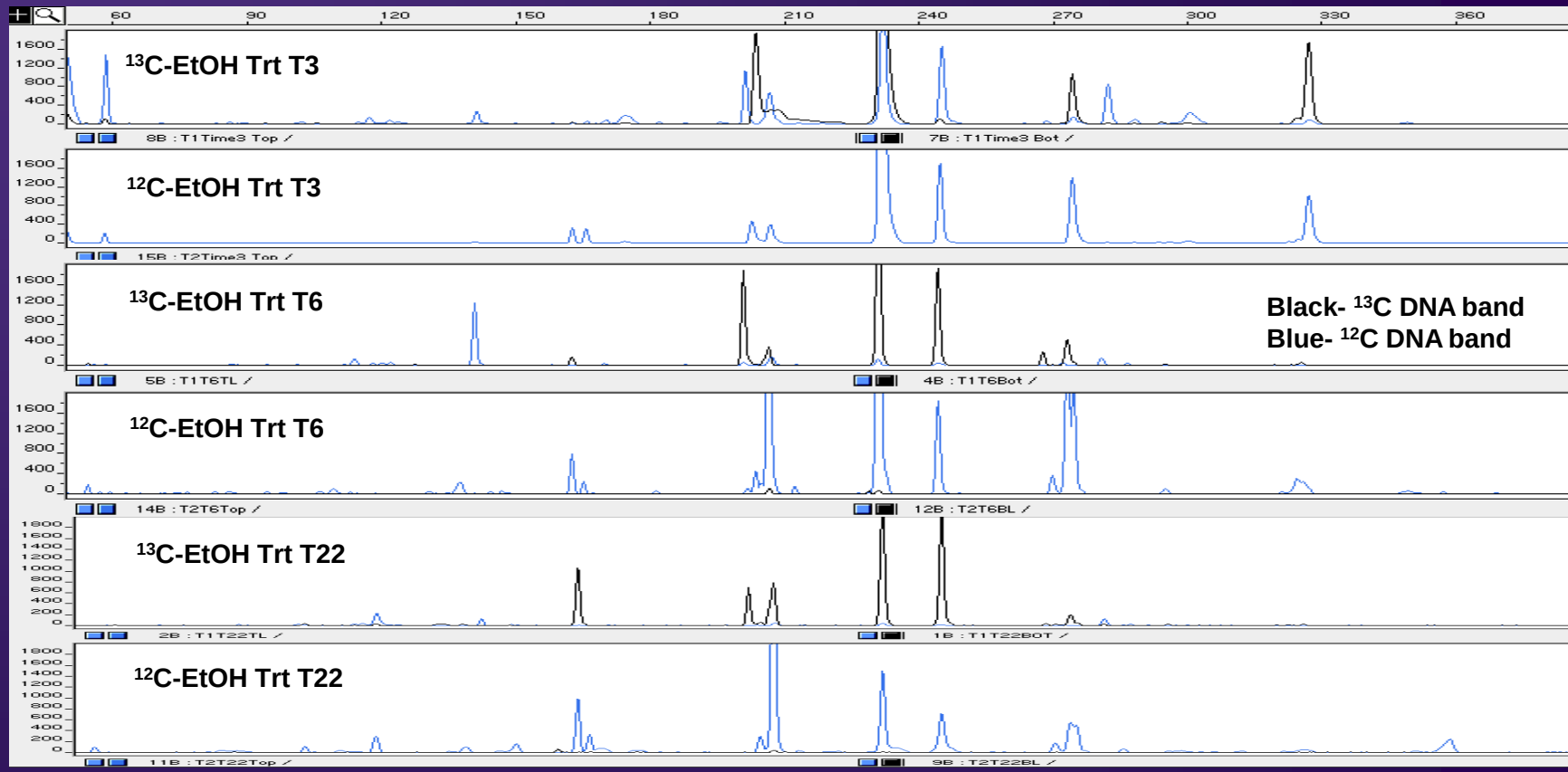
- Microcosm Treatments:
  - ^{13}C-Ethanol (heavy)
  - ^{12}C-Ethanol (light)
- Targeting metabolically active bacteria:
  - Bacteria utilizing ^{13}C-Ethanol incorporate isotopically heavy C molecules into their DNA
  - $^{12}C/^{13}C$-DNA is separated by density gradient centrifugation
  - Community profiling by TRFLP

SIP: Electron Acceptor Utilization

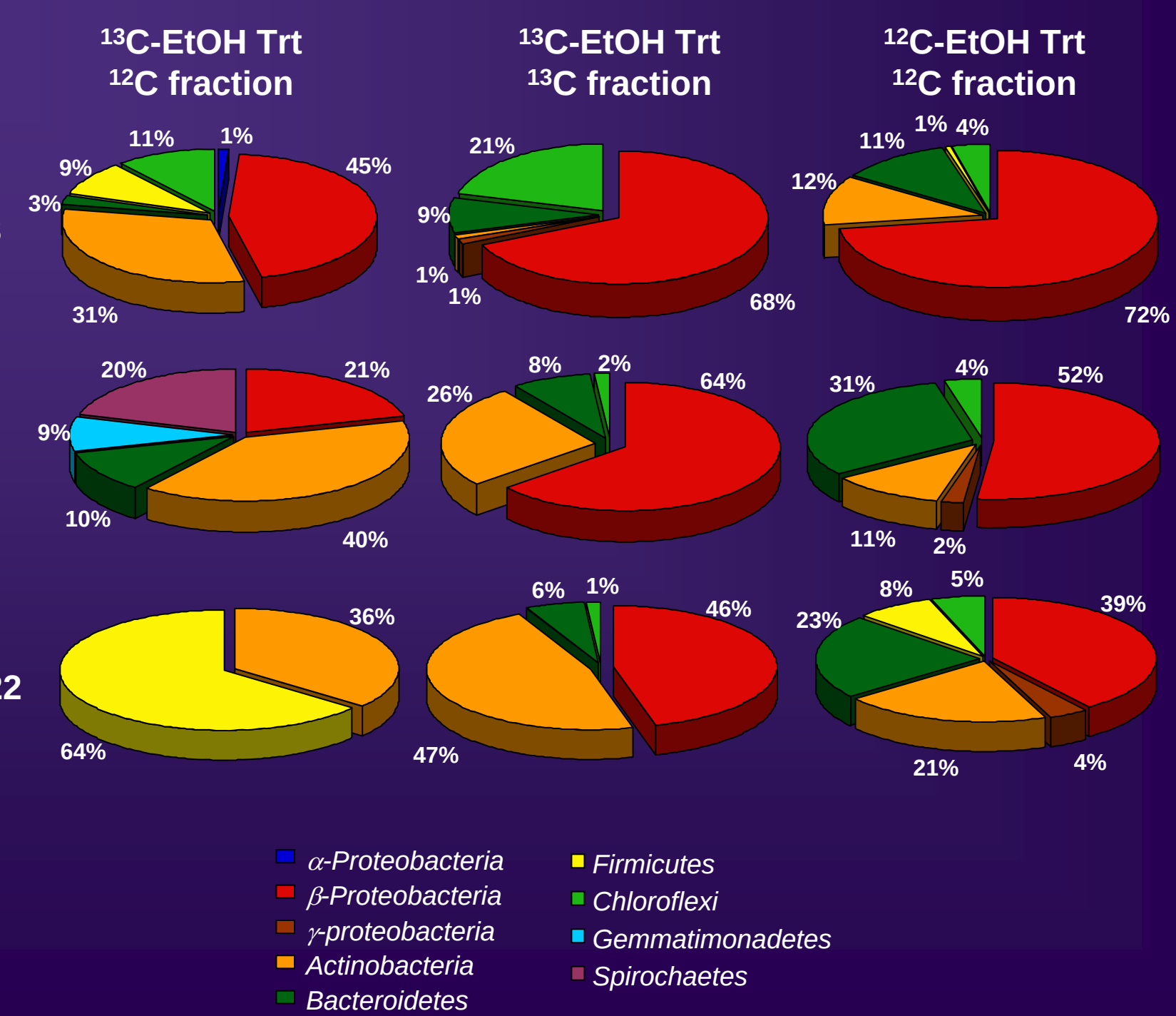
- *Shifts in TEAPs in both C treatments were temporally equivalent*



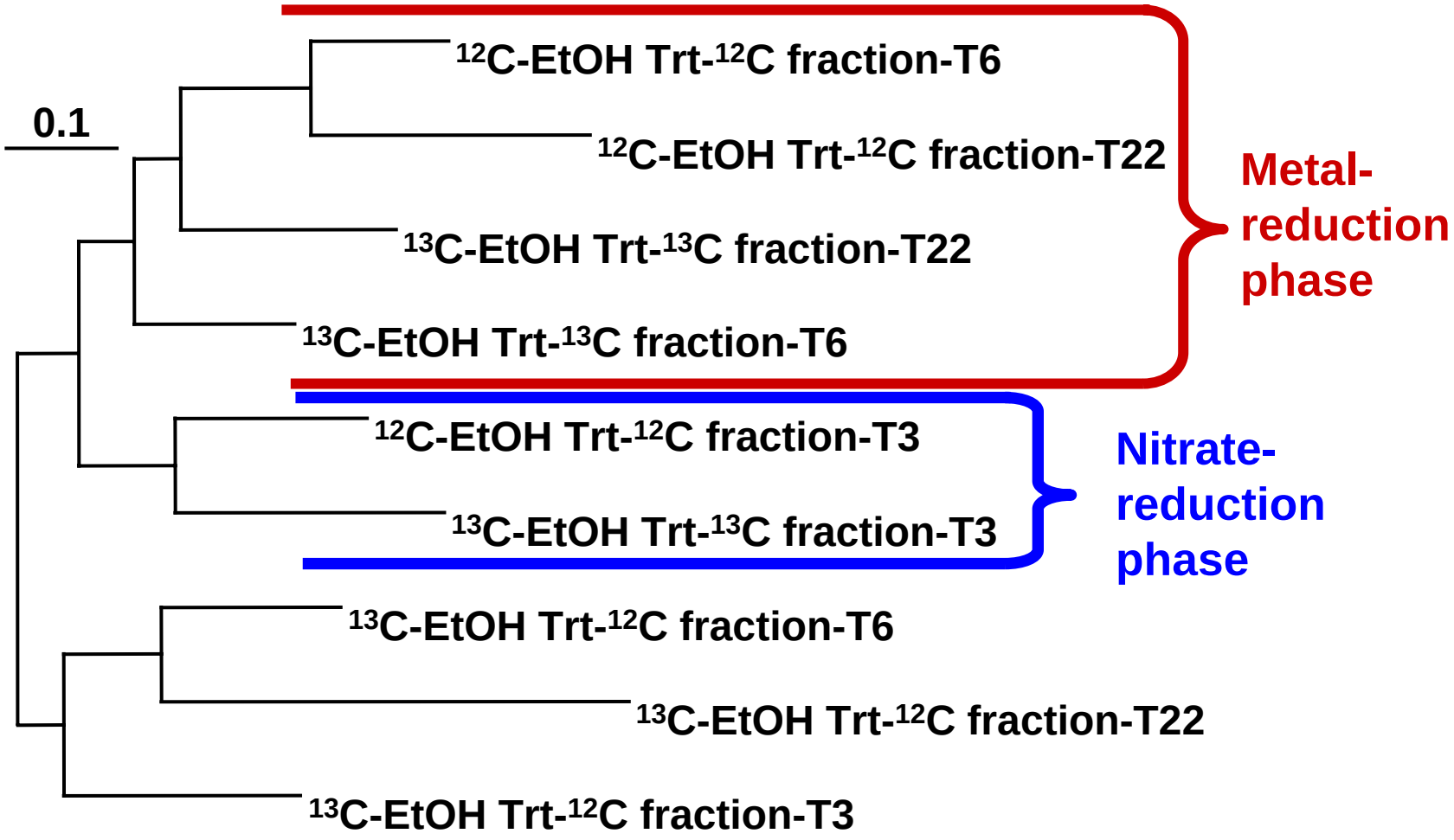
SIP: Community Profiles (TRFLP)



- *Betaproteobacteria* (*Dechloromonas, Azoarcus, Alcaligenes, Ralstonia*, and *Diaphorobacter)*
  - Many families metal-resistant and capable of nitrate-reduction
- *Arthrobacter* spp. member of the *Actinobacteria*
  - nitrate- and chromate-reducer
- *Firmicutes*
  - *Clostridium* –fermentative metal-reducer

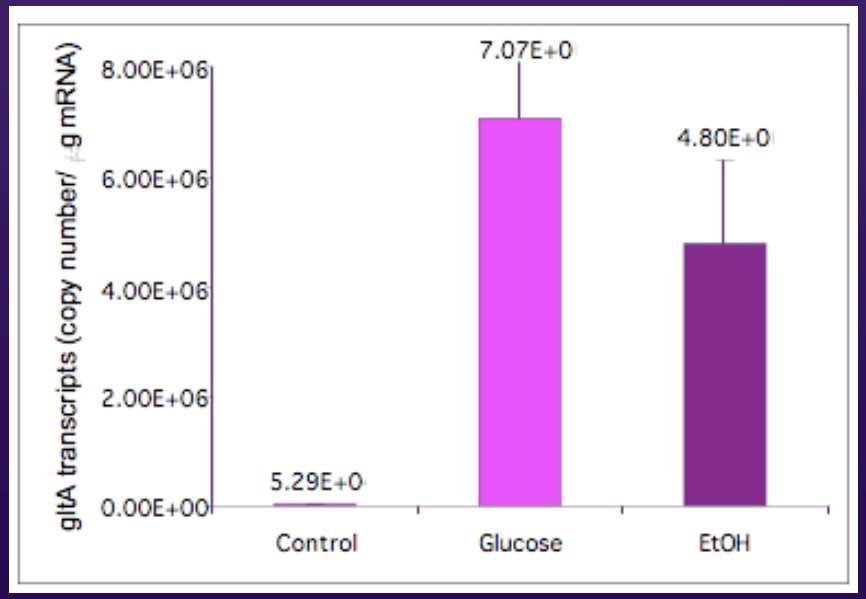

^{13}C-EtOH Trt
^{12}C fraction
^{13}C-EtOH Trt
^{13}C fraction
^{12}C-EtOH Trt
^{12}C fraction
T3
T6
T22
11%
1%
9%
45%
3%
31%
21%
9%
1%
1%
68%
11%
1%
4%
12%
72%
20%
21%
9%
10%
40%
8%
2%
26%
64%
31%
4%
52%
11%
2%
6%
1%
36%
46%
64%
47%
8%
5%
23%
39%
21%
4%
α-Proteobacteria
β-Proteobacteria
γ-proteobacteria
Actinobacteria
Bacteroidetes
Firmicutes
Chloroflexi
Gemmatimonadetes
Spirochaetes

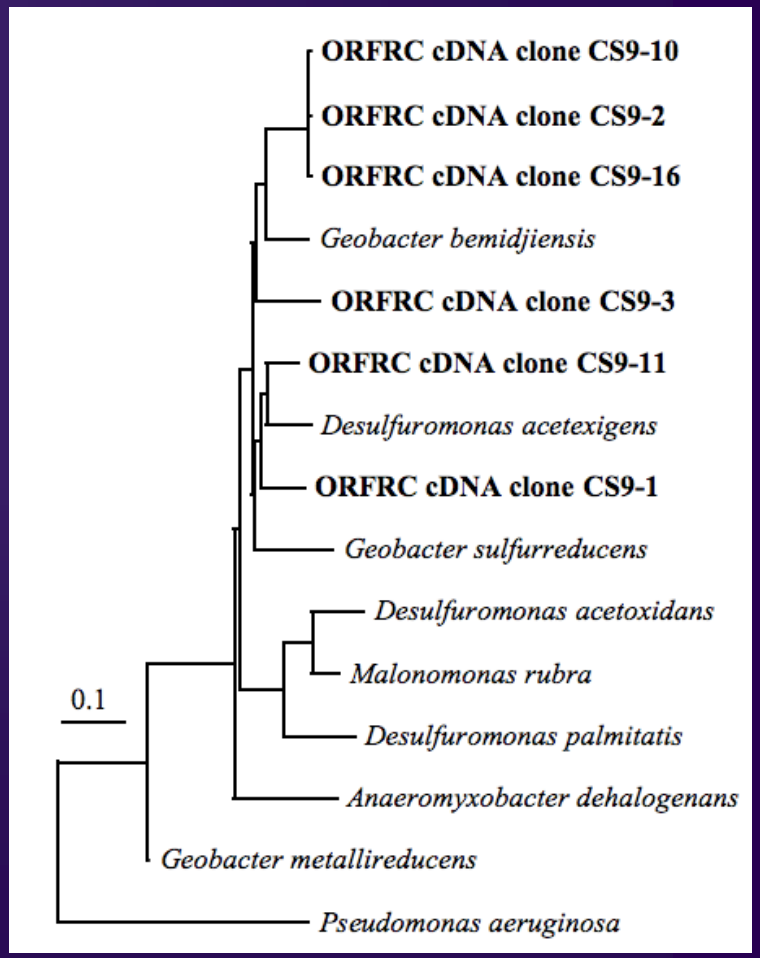
Sorensen's index derived from distance matrices of TRFLP profiles.



Quantification of gene expression in ORFRC subsurface sediments

- mRNA extracts
- Quantification by reverse transcription realtime PCR during metal reduction phase
- *Geobacteraceae* citrate synthase (gltA) gene
- Phylogenetic analysis

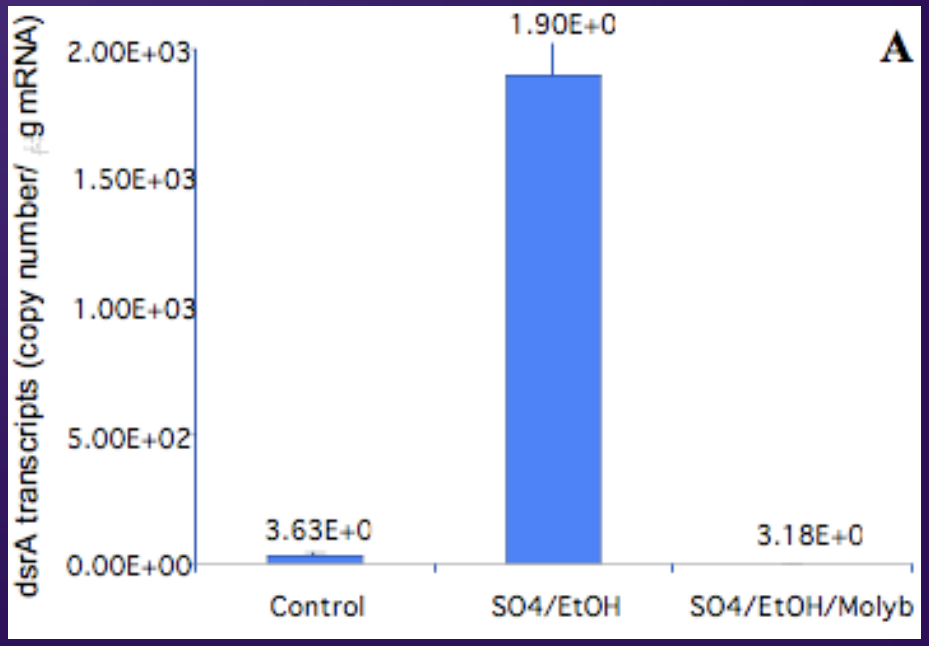




Collaboration with Dr. Kuki Chin, Georgia State University

Quantification of gene expression in ORFRC subsurface sediments

- *dsrAB* gene expression



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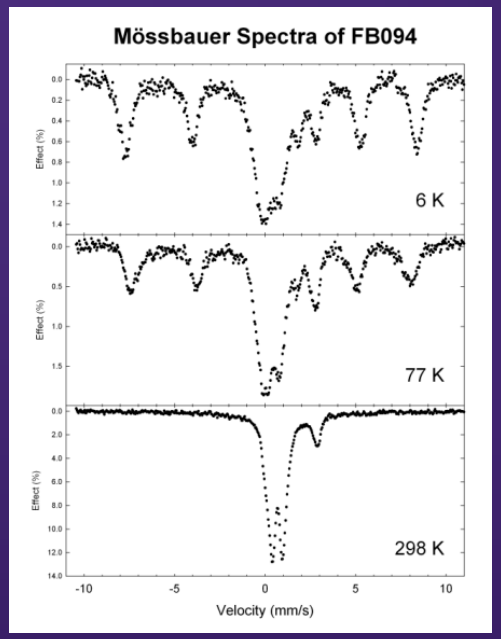
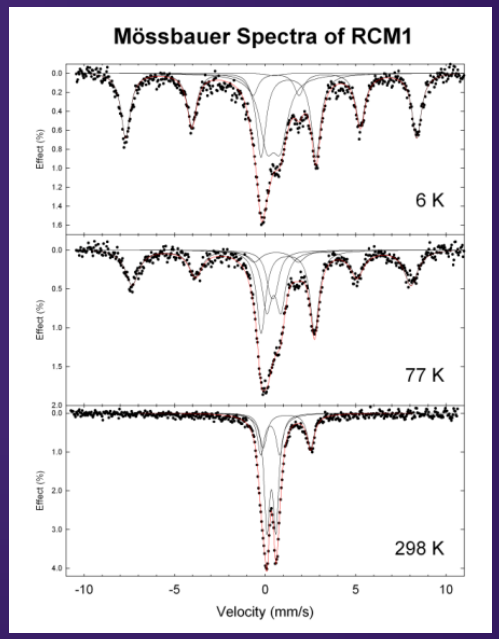
Fe Mineralogy





Mössbauer Parameters at 6 K

Sample	Phyllosilicate Peak Areas		Goethite Field & Peak Areas		Chemical Analysis*
	$A_{Fe(III)}$ %	$A_{Fe(II)}$ %	B_{hf} T	A %	Fe(II) % of tot Fe
FB094	31.6	18.5	49.67	49.9	13.1
RCM7	37.8	12.9	49.72	49.3	12.5
RCM4	24.0	20.3	49.63	55.7	24.0
RCM1	30.3	25.7	49.83	43.9	31.2

*1,10-phenanthroline method of Komadel and Stucki, Clays & Clay Minerals 36:379-381.

Conclusions

- Fe was distributed in about equal quantities between aluminosilicates and Al-substituted goethite
- Silicate bound Fe(III) appeared to predominate over the Fe minerals bioreduced

Findings

- **Isolates:**
- Pure cultures have been obtained from ORFRC subsurface sediments for at least four groups of metal-reducers including (*Geobacter*, *Desulfotomaculum*, *Anaeromyxobacter, Clostridium*).
- The genome sequence of *Geobacter* strain FRC-32 is now available from JGI, and we have employed it's sequence in functional genomics as described below.

Findings

- **Microcosms / Community Analysis:**
- pH and redox (electron acceptor/donor availability) are master variables controlling not only the rates but also the pathways of microbial metabolism in the ORFRC subsurface.
  - EtOH stimulated denitrification while DNRA activity detected in glucose amended sediments
- Electron donors were determined to stimulate microbial metabolism leading to metal reduction in the following order: glucose > ethanol > lactate > hydrogen.
- Biostimulation with ethanol or glucose led to mostly incomplete carbon oxidation
- Active metal-reducers (*Anaeromyxobacter, Geobacter*, *Clostridium, Pantoea)* and nitrate-reducers *(Alcaligenes, Diaphorobacter, Dechloromonas, Arthrobacter,* and *Pseudomonas)* were detected

Findings

- **Microcosms / Community Analysis:**
- SIP revealed a distinct shift in the metabolically active communities of ethanol amended microcosms that correlated with TEAPs
- The majority of the community that incorporated ^{13}C labeled ethanol was identified to the *Betaproteobacteria* (please see Denise Akob's poster for details)
- A database of > 2600 16S rRNA gene sequences retrieved from ORFRC materials is now available; approx. 1/3 have been examined with *in silico* digestion for community profiling
- Expression of key genes of metal- and sulfate-reducing prokaryote groups was quantified and correlated with TEAPs
- Clone sequences for *gltA* were most closely related to *Geobacter bemidjiensis* and *Desulfuromonas acetexigens*.

Findings

- **Iron Mineralogy:**
- Mössbauer spectroscopy supports wet chemical analyses and is reliable for identifying Fe oxide minerals only if measurements include temperatures as low as 4.2 K.
- Mössbauer analysis shows that phyllosilicates and goethite predominate in the ORFRC subsurface and equal or greater amounts of phyllosilicates are reduced during biostimulation in comparison to goethite

Acknowledgements

FSU

- Heath Mills
- Tom Gihring
- Denise Akob
- Jon Delgardio

Rutgers

- Lee kerkhof
- Lora mcguinness

GSU

- Kuki Chin

Experimental Approach

Microbial Community Characterization

- Cultivation-dependent methods
- Cultivation-independent methods

1. DNA Extraction
2. PCR Amplification
   - SSU rRNA genes (DNA)
   - SSU rRNA (RNA)
   - Functional genes (nosZ, gltA, dsrAB)
3. Fingerprinting Methods:
   - Cloning & sequencing
   - Terminal restriction fragment length polymorphism (TRFLP)
4. Analyze:
   - Phylogenetic composition
   - Species diversity

SIP: Results

- A majority of the microbial community assimilated ^{13}C
- Peaks detected in the ^{12}C fraction of ^{13}C-EtOH treatment
  - Groups not assimilating ^{13}C from labeled ethanol
  - Dead, senescent, or sporeforming cells
- Microbial community structure changed with time and geochemistry
  - Communities at T6 and T22 were most closely related
    - Both sampled during metal-reduction
    - Communities are likely composed active in metal-reducers
  - Profiles at T3 are likely composed of active nitrate reducers

SIP: Conclusions

- Microbial groups present in Area 2 sediments have a high potential for bioremediation
  - The majority of the peak area identified to the *Betaproteobacteria*
    - Present throughout the incubation
    - *Dechloromonas, Azoarcus, Alcaligenes, Ralstonia*, and *Diaphorobacter*
    - Many families metal-resistant and capable of nitrate-reduction
  - Detected members of the *Actinobacteria* and *Firmicutes*
    - *Arthrobacter* –nitrate- and chromate-reducer
    - *Clostridium* –fermentative metal-reducer

RT-PCR of rRNA

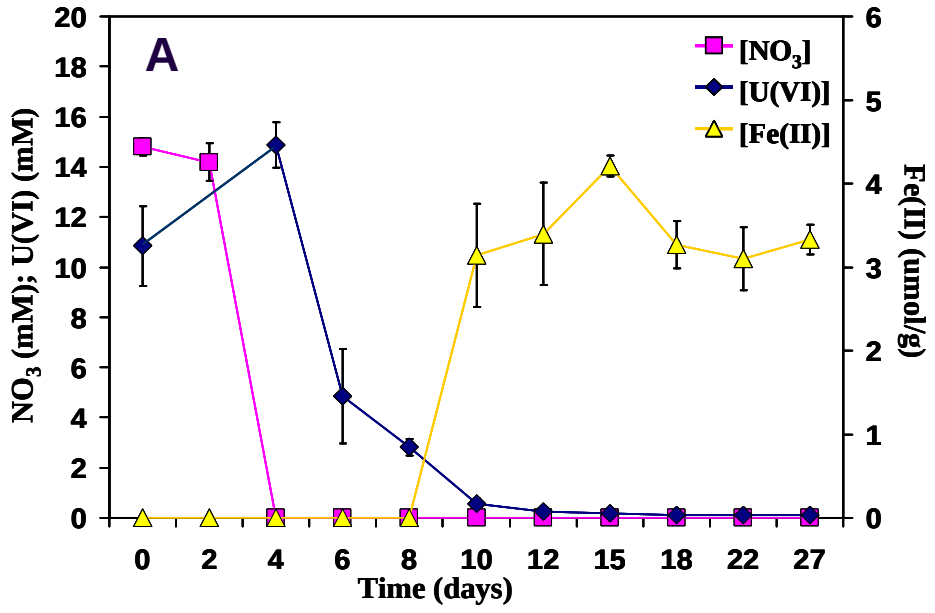
- rRNA has a higher copy number, higher turnover rate, and is produced independently of cellular respiration compared to 16S rRNA genes
- Valid approach to determine “metabolically active” community members
- Clonal analysis of same pooled samples as for DNA
  - Same phyla detected in RNA and DNA libraries
  - High sequence identity between many clone sequences retrieved from each target
- *In silico* digestion of sequences matched with digestions from DNA libraries
- cDNA can be fingerprinted for rapid screening

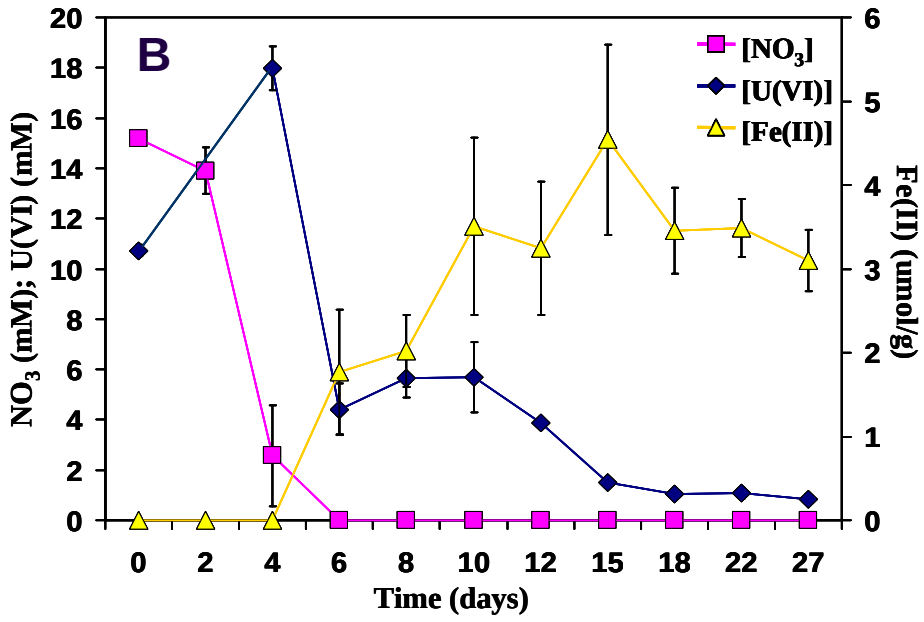
Methods:
Microbial Community Characterization

- Enumeration of microbial functional groups
  - Nitrate- and iron-reducing bacteria
  - Most probable number (MPN) dilution series
  - Microcosm samples and Area 2 FB094 sediment

- DNA extracted from microcosm and MPN cultures
  - PCR amplified using *Bacteria* domain specific primers
  - Community fingerprinting techniques:
    - Cloning and sequencing
    - Terminal restriction fragment length polymorphism (TRFLP)
  - Quantification of functional gene expression

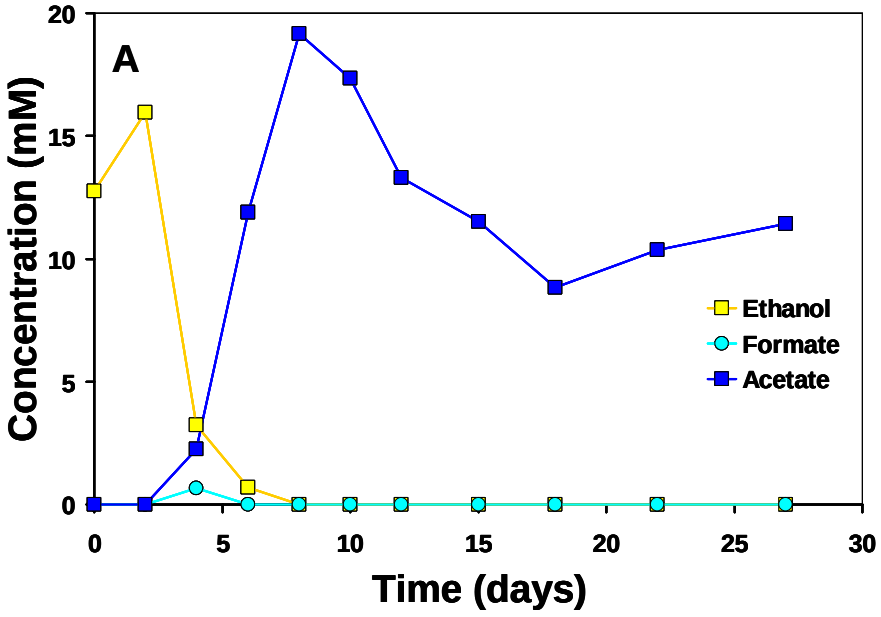
Results: Electron acceptor usage in (A) ethanol and (B) glucose amended microcosms.

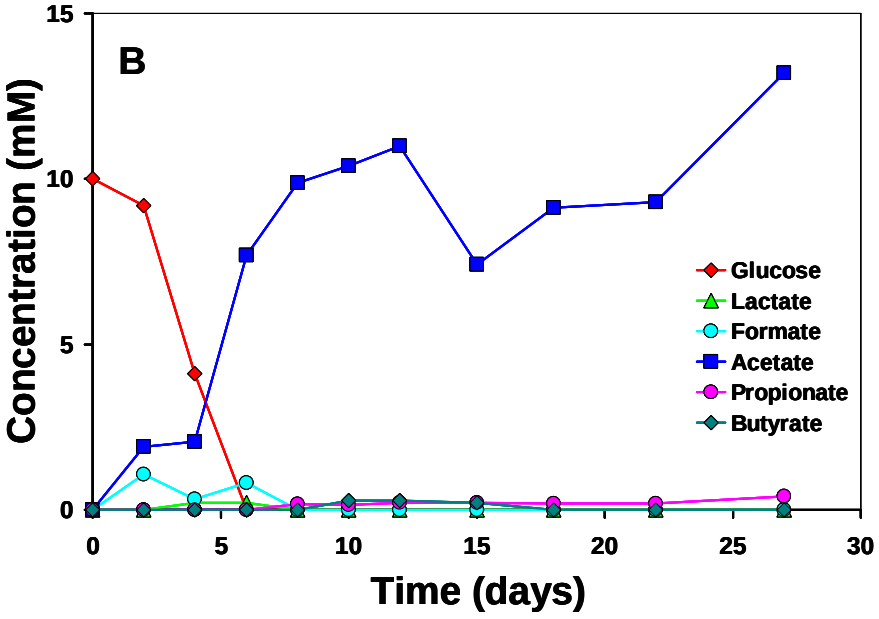




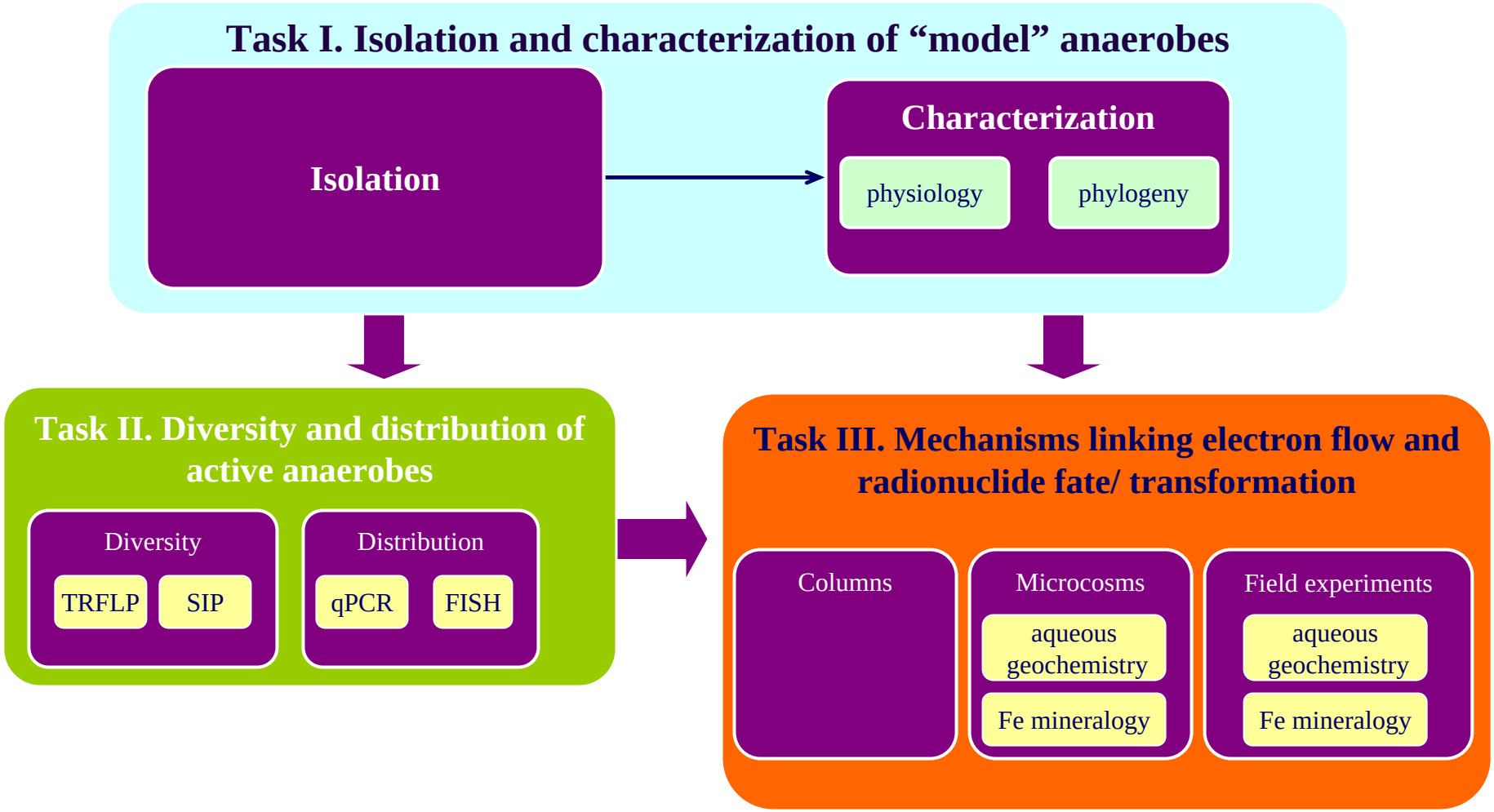
•No activity was detected in the unamended microcosms.
Values are averages of triplicate microcosms

Results: Electron donor utilization in (A) ethanol and (B) glucose amended microcosms determined using high performance liquid chromatography (HPLC).





- Incomplete oxidation of ethanol
- Fermentative metabolism


Task I. Isolation and characterization of “model” anaerobes
Isolation
Characterization
physiology
phylogeny
Task II. Diversity and distribution of active anaerobes
Diversity
TRFLP
SIP
Distribution
qPCR
FISH
Task III. Mechanisms linking electron flow and radionuclide fate/ transformation
Columns
Microcosms
aqueous geochemistry
Fe mineralogy
Field experiments
aqueous geochemistry
Fe mineralogy