

Natural Gene Transfer to Develop Resistance to Metal Toxicity in Microbial Communities at the Oak Ridge FRC

Project Number EE-595-EEDA

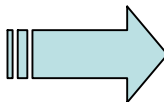
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FRC conditions present challenges to in-situ bioremediation strategies

Problematic conditions ^{JF1}

1. *High nitrate (~1000 ppm in Area 2)*
2. *Acidity*
3. *Heavy metals (Ni, Al...)*



Result

1. *Too high redox for stimulating sulfate and Fe reducers*
2. *Affects metal bioavailability, and thus, toxicity*
3. *Inhibit nitrate reducers*

Will introduction of nickel resistance into indigenous microbial community have a positive effect on nitrate reducers and stimulate iron and sulfate reducers?

JF1

WATERSHED COMMUNITY CHARACTERIZATION UPDATE - SHIFTS IN GROUNDWATER MICROBIAL COMMUNITY STRUCTURE OBSERVED ALONG CONTAMINANT GRADIENTS (YAN, GENTRY, WATSON, AND ZHOU)

Microbial communities were compared by Yan, Gentry, and Zhou in 6 samples along contaminant transport pathways in shale (FW106, FW413, GW537, and PTMW02) and gravel substrates (FW231 and FW410) in addition to an uncontaminated background sample (FW301). Genomics DNAs were isolated from groundwater, purified, amplified with primers 27F and 1492R, and cloned to produce 16S rDNA libraries. Over 1,500 clones were sequenced, and data was analyzed using DOTUR. The most highly contaminated groundwater, FW106 and FW413 (pH ~3.7; ~2,000 mg nitrate/l; ~20 mg uranium/l), had limited diversity with Shannon Index values of 0.66 to 1.33 compared to 4.89 at the background site. Diversity values increased along the contaminant gradient in the shale pathway with decreased contaminant levels. Diversity values were higher in the gravel pathway (~3) which also had higher pH and lower contaminant levels. Clones from the highly contaminated groundwater in the shale pathway were dominated by γ -Proteobacteria, primarily an unclassified Xanthomonadaceae related to *Frateuria* spp. The proportion of this organism was directly correlated with the level of contamination. Less contaminated groundwater along the shale and the gravel pathway contained communities strikingly different than the highly contaminated groundwater. Our results suggest that microbial community composition at the FRC is strongly influenced by contaminant (i.e., nitrate, uranium) and pH levels. Further data analysis is being performed to determine the primary determinant(s) of community composition at the FRC. Additional samples from each pathway along with samples from limestone, surface water, and springs are also being analyzed.

In addition, Kerkhof, Kostka, and McGuinness are assaying microbial populations using DNA-based (presence/absence) measurements by TRFLP of 16S rRNA genes. These results will be summarized in a future update but results so far are consistent with the above findings.

Slide 2 (Continued)

J Fitts, 3/22/2006

Project overview



Goal: Immobilize uranium in contaminated sediments via microbial reduction and precipitation

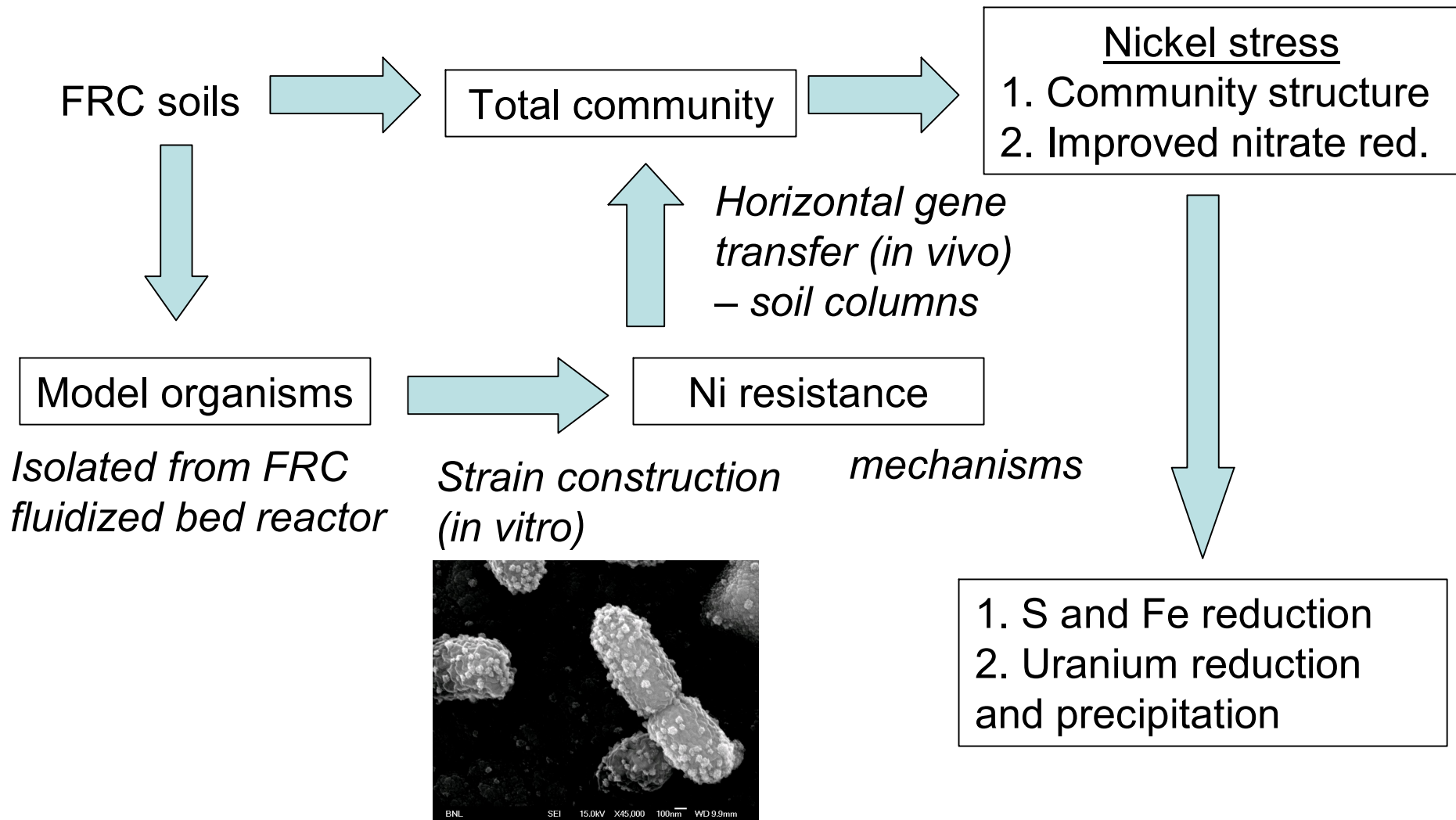
Problem: Active uranium reducers are inhibited by co-contaminants in complex waste streams (e.g., heavy metals)

Major project objectives

Demonstrate application of natural gene transfer to improve community function under increased levels of toxic metal stress (van der Lelie, BNL Biology Dept.)

Demonstrate ability to enhance uranium immobilization in ORNL sediments by indigenous microorganisms that have adopted the toxic metal resistance marker (Fitts, BNL Environmental Sci. Dept.)

Project design schematic – presentation outline



Construction of model organisms

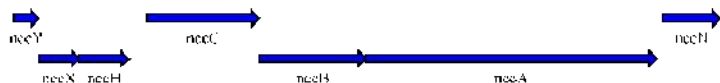
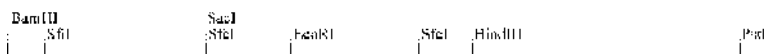
~~*Ralstonia*~~
Cupriavidus
 Metal resistant gene



ncc



nre



Minimum required for Ni resistance: *nreB*

- **Plasmid pMOL222 (IncQ): broad host replication and mobilization** JF3
- **Mini Tn5 single hopper transposons: provide stability due to loss of transposase gene**

Slide 5

JF3

Plasmid is the carrier of ncc nre genetic marker
extra chromosomal DNA (plasmid can exist on its own, separate from the genome)
can replicate in a broad range of bacterial species

transconjugation - e. coli transfers genetic material to future transconjugant

transposons - virus (isolated from a virus): recognizes specific site on bacterial genome and cuts, and inserts itself into the genome

will not hop again (provides stability) by losing transposase gene

In aqueous solution electroporation: voltage across (+mV) dense culture in a cuvette and

Chemoporation: make holes in bug, not as efficient

Shotgunporation: inject DNA coated bullet into single cell

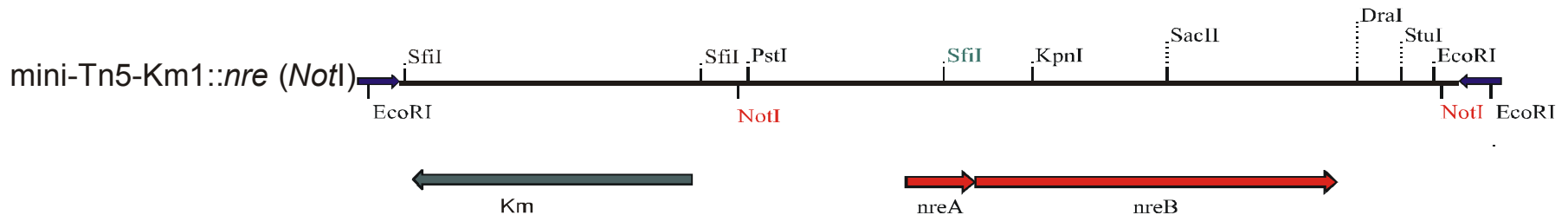
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nre gene provides Ni resistance to broad range of strains

1. *Broad expression range for nre encoded Ni resistance in both proteo and gram positive bacteria*

- *proven concept (Dong et al., 1998; Taghavi et al., 2001)*

2. *Kanamycin selection marker*



JF2

Slide 6

JF2

FIG. 1. Schematic representation of different mini-Tn5 Ni resistance transposons. The positions of the kanamycin resistance marker (Km), the Ni resistance determinants ncc and nre, the inverted repeats at the extremities of the minitransposons, and important restriction sites are indicated.

The sizes of the minitransposons are given in parentheses.

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Ni resistance introduced into nitrate reducers

| Nickel-sensitive wild type | | | | |
|--------------------------------|-------------------------|--------------------|------------------------------|--------------------|
| Species | pMOL222 transconjugants | | Tn5::ncc-nre transconjugants | |
| | Aerobic MIC (mM) | Anaerobic MIC (mM) | Aerobic MIC (mM) | Anaerobic MIC (mM) |
| <i>Enterobacter</i> M-53 | 3 - 6 | 3 | 2 - 6 | 3 - ≥ 3 |
| <i>Enterobacter</i> DM-S | 3-6 | 3 | Not available | Not available |
| <i>Klebsiella</i> DM-C3 | 1-2 | 2 - ≥ 3 | 1 - 2 | 2 - ≥ 2 |
| <i>Pseudomonas</i> DM-Y2 | 1 - 2 | <1 - 1 | 1 - 2 | < 1 - 1 |
| <i>Iodobacter</i> DM-K3 | < 1 - 2 | 1 | < 1 - 2 | 1 |
| <i>Chromobacterium</i> DM-N3 | < 1 - 2 | 1 | 2 | 2 |
| <i>Janthinobacterium</i> M-A11 | < 1 - 3 | 1 | Not available | Not available |
| <i>Stenotrophomonas</i> M-A15 | Not available | Not available | Not available | Not available |
| <i>Shewanella</i> MR-1 | 1 - 2 | 1 | Not available | Not available |
| Nickel-resistant wild type | | | | |
| | Aerobic MIC (mM) | | Anaerobic MIC (mM) | |
| <i>Pseudomonas</i> M-16 | 6 | | 2 | |
| <i>Pseudomonas</i> DM-H2 | 6 | | 2 | |

- **Organisms isolated from fluidized bed reactor treating FRC groundwater**
- **MIC values independent of plasmid vs. genomic insertion**

JF6

Slide 7

JF6

all nitrate reducing bacteria

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Successful gene transfer is confirmed on the genome level

BOX PCR

1: receptor P. DM-Y2

2: transconjugant

3: Donor E. coli CM2034 (in case of ncc-nre E. coli CM 2520)

nreB PCR

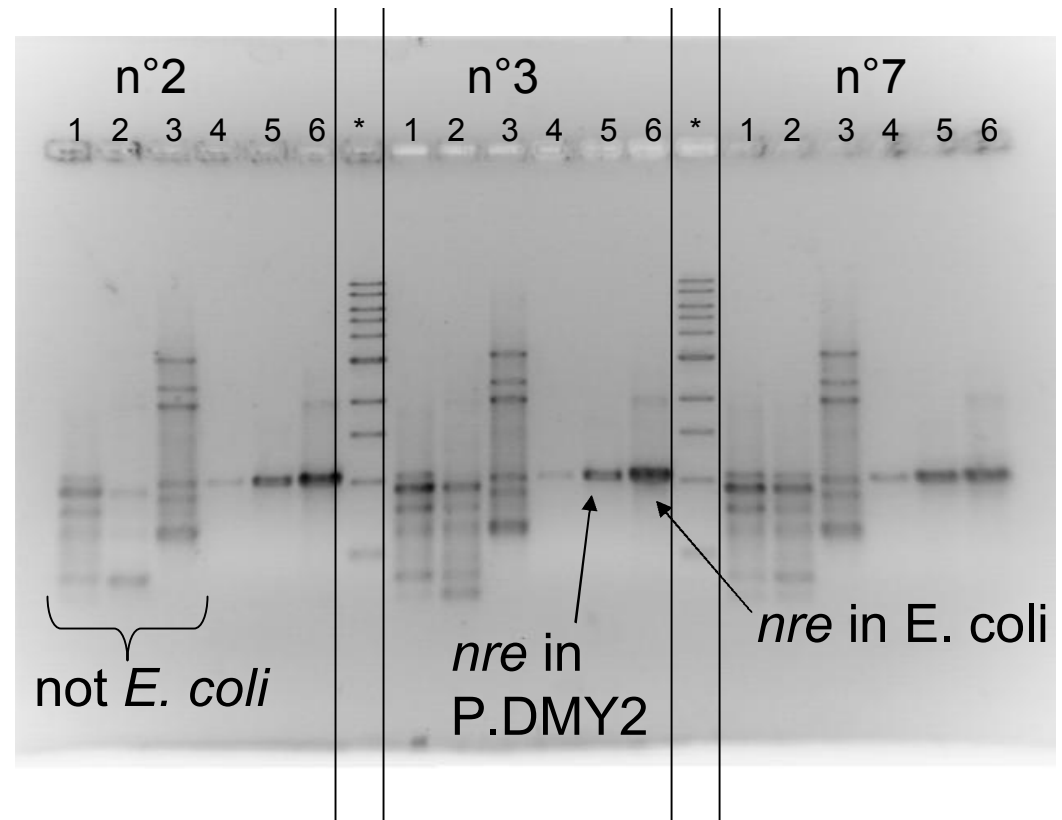
4: receptor P. DM-Y2

5: transconjugant

6: Donor E. coli CM2034 (in case of ncc-nre E. coli CM 2520)

* 1 kb ladder

Pseudomonas DM-Y2 pMOL222



Both plasmid and genomic insertion confirmed for all strains

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JF7

1-3 box pcr - amplify w/

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Plasmid transfer produces equally stable *nre* gene

after 100 generations

| Nickel-sensitive wild type | | | | | | |
|--------------------------------|-------------------------|--------------------|---------------|--------------------------------------|--------------------|---------------|
| Species | pMOL222 transconjugants | | | Tn5:: <i>ncc-nre</i> transconjugants | | |
| | Aerobic MIC (mM) | Anaerobic MIC (mM) | Stability (%) | Aerobic MIC (mM) | Anaerobic MIC (mM) | Stability (%) |
| <i>Enterobacter</i> M-53 | 3 - 6 | 3 | 62 - 94 | 2 - 6 | 3 - \geq 3 | 14 - 100 |
| <i>Enterobacter</i> DM-S | 3 - 6 | 3 | 82 - 100 | Not available | Not available | Not available |
| <i>Klebsiella</i> DM-C3 | 1 - 2 | 2 - \geq 3 | 0 - 96 | 1 - 2 | 2 - \geq 2 | 0 - 92 |
| <i>Pseudomonas</i> DM-Y2 | 1 - 2 | <1 - 1 | 100 | 1 - 2 | < 1 - 1 | 99 - 100 |
| <i>Iodobacter</i> DM-K3 | < 1 - 2 | 1 | 0 - 83 | < 1 - 2 | 1 | 100 |
| <i>Chromobacterium</i> DM-N3 | < 1 - 2 | 1 | 99 - 100 | 2 | 2 | 82 |
| <i>Janthinobacterium</i> M-A11 | < 1 - 3 | 1 | 0 | Not available | Not available | Not available |
| <i>Stenotrophomonas</i> M-A15 | Not available | Not available | Not available | Not available | Not available | Not available |
| <i>Shewanella</i> MR-1 | 1 - 2 | 1 | 0 - 3 | Not available | Not available | Not available |

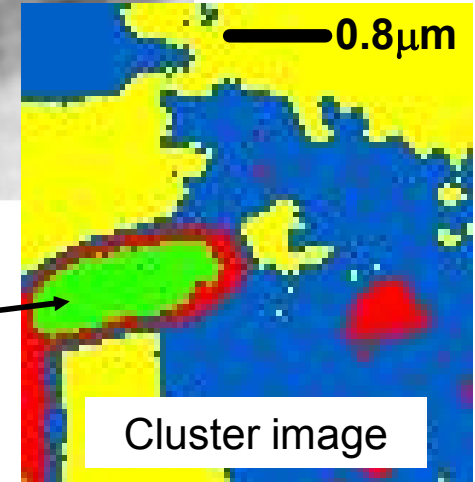
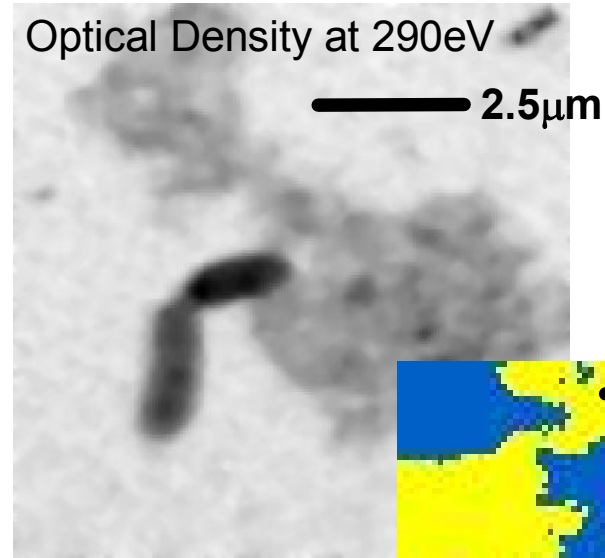
Both plasmid and genomic insertion exhibit equal stability in culture

Ni resistance mechanisms

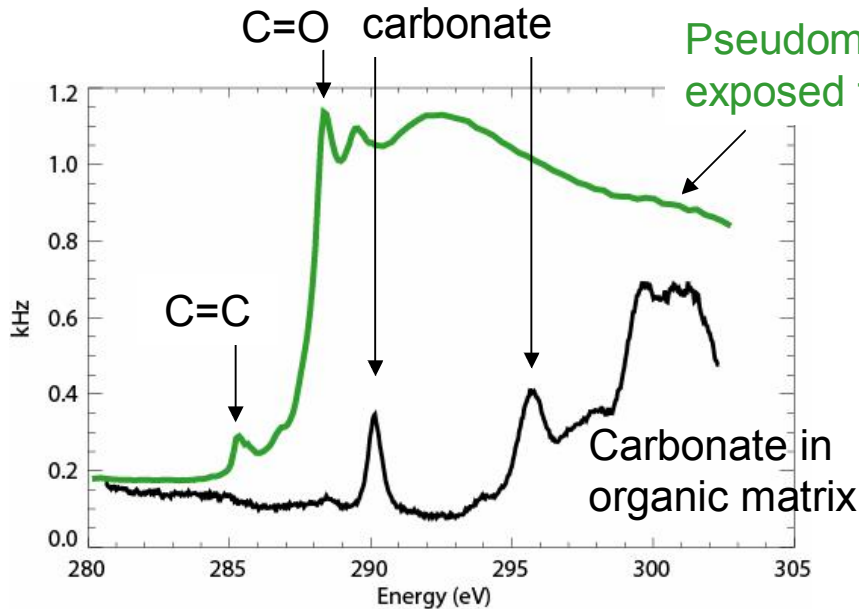
Scanning Transmission X-ray Microscope
imaging at the carbon K-edge

Washed cells exposed to Ni for 2 hrs

Rinsed cells are dried on microscope window



Pseudomonas DMY2::ncc-nre
exposed to 2 mM NiCl₂



Carbonates not observed
O K-edge will be sensitive to
NiO formation



Column experiments

Area 2 sediments (high nitrate low uranium)

Homogenization under atmospheric conditions

Inoculated columns with extracted indigenous community

Anaerobic mineral growth media w/ C:N:P of 100:10:1 (ethanol carbon source)

Operate under anaerobic conditions

Pseudomonas DMY2 tested in column studies

2,6 - FRC community

3,7 - FRC community + Pseudomonas wild type

4,8 - FRC community + Pseudomonas pMol222

5,9 - FRC community + Pseudomonas::ncc-nre

Kill 1

2

3

4

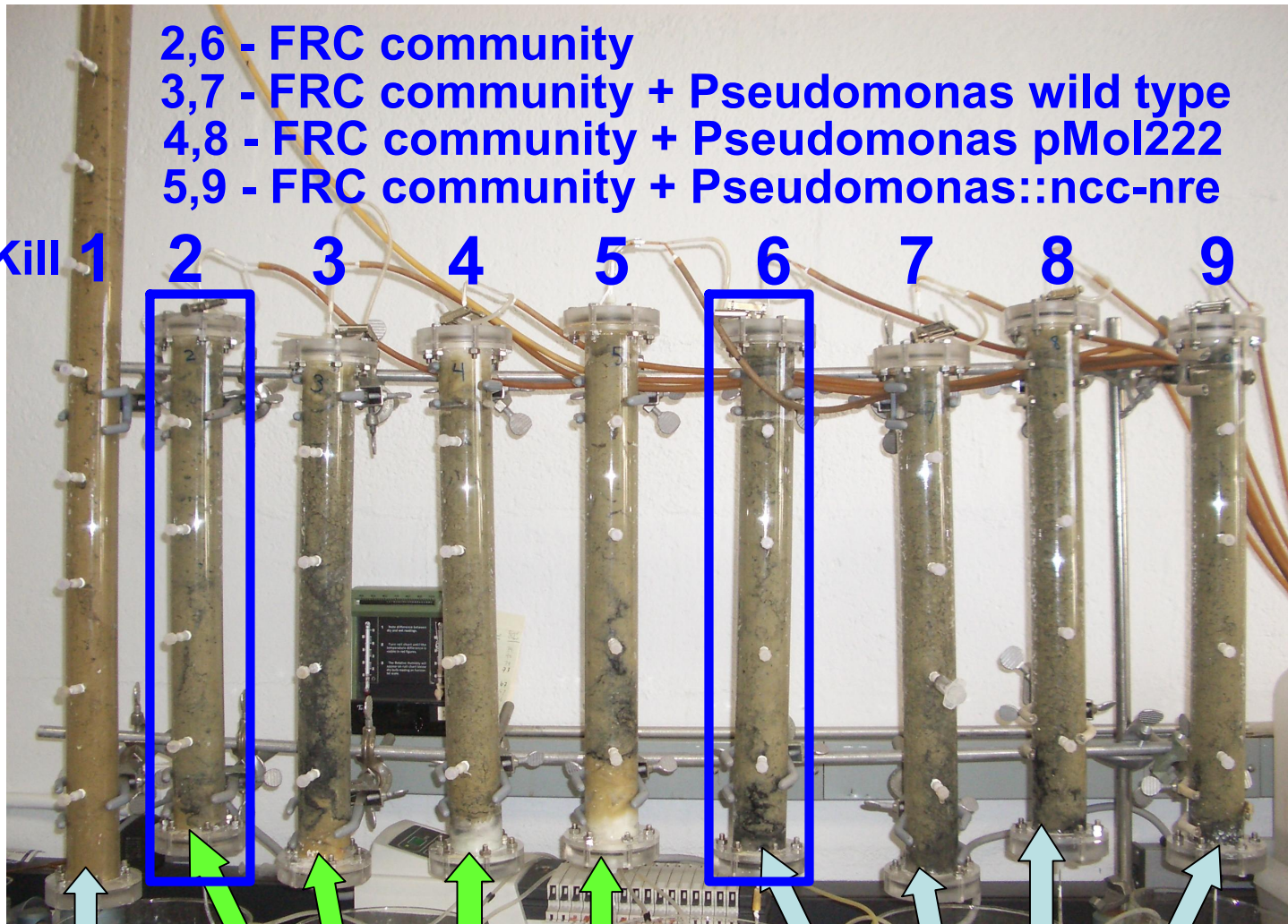
5

6

7

8

9

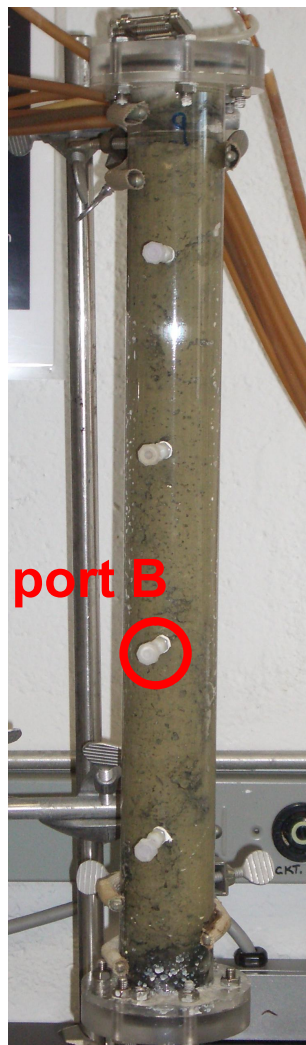


Media +
Formaldehyde

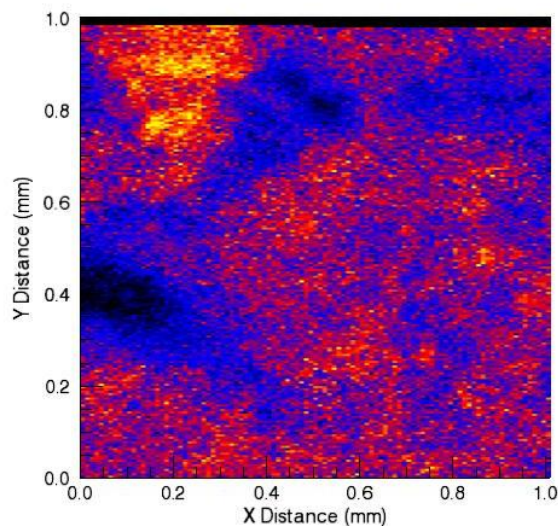
Media +
1 mM NiCl₂

Media
no Nickel added

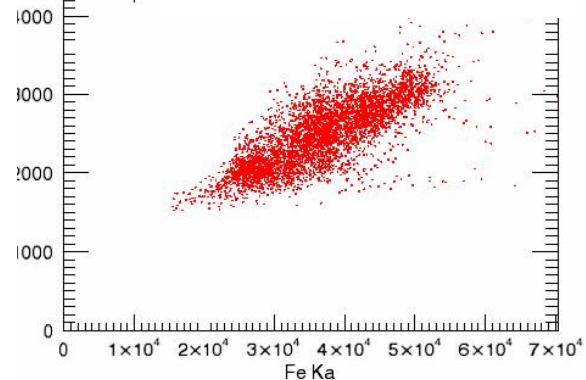
Geochemical interrogation: S, Fe & U at time zero



U distribution

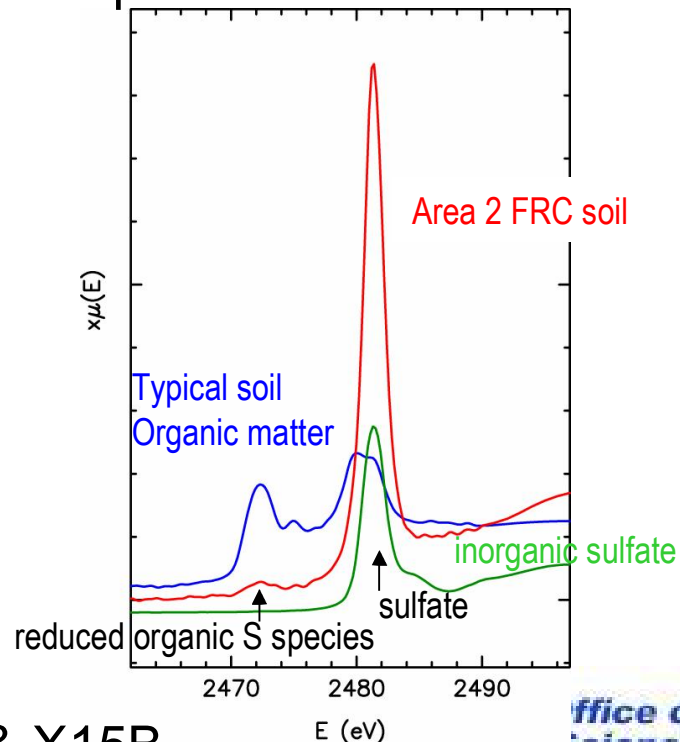
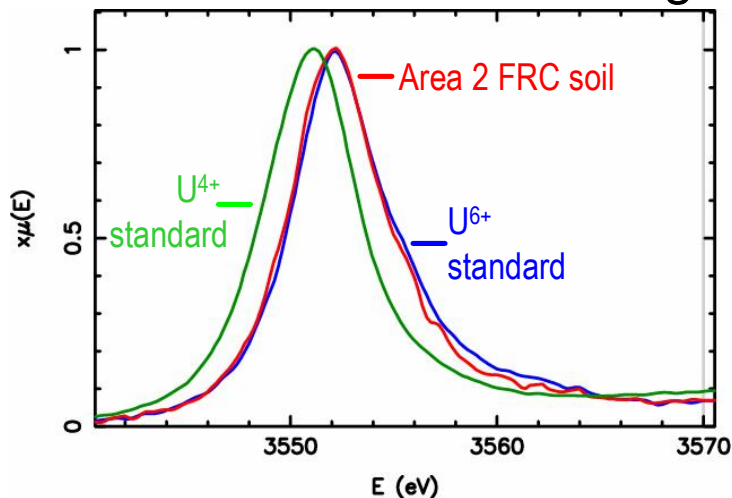


U-Fe correlation



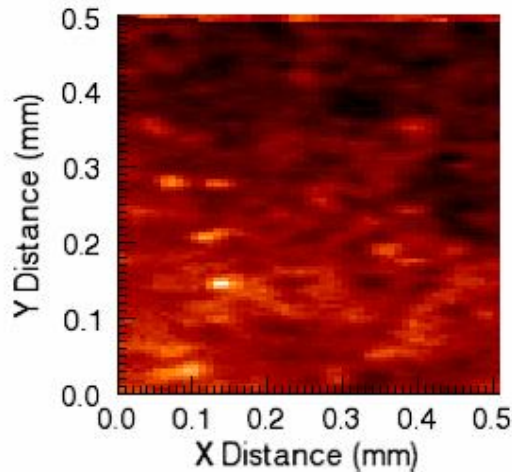
S speciation and redox state

U oxidation state at M5 edge



Geochemistry of columns after 65 days

Ni distribution in Column 2



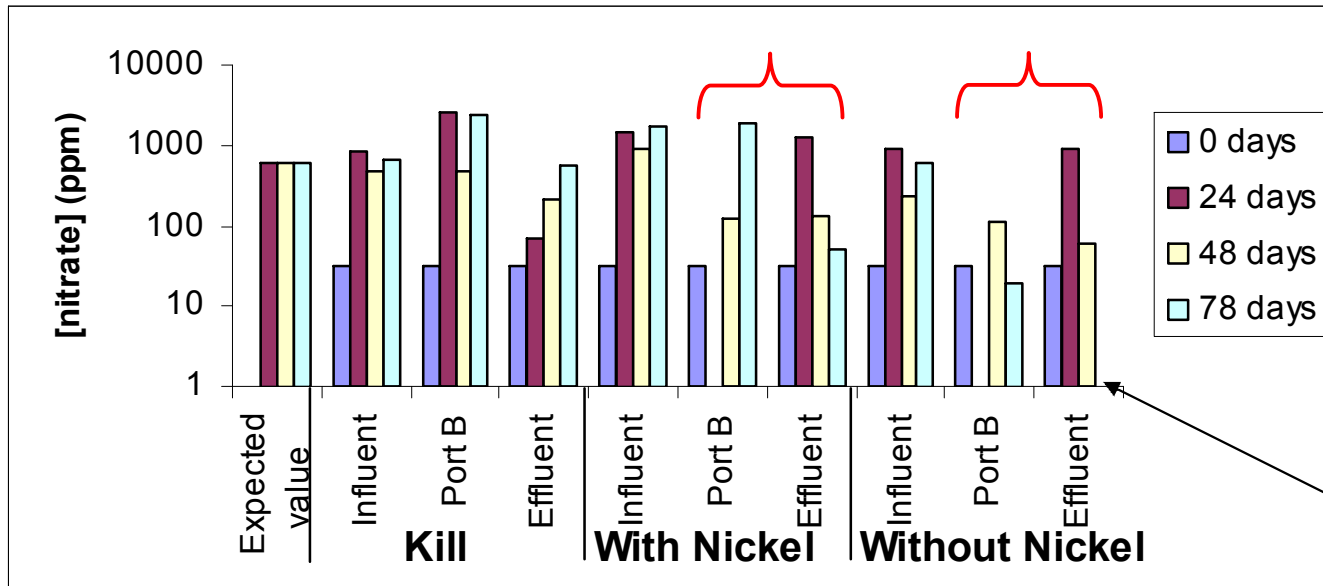
Column effluent indicators

- *Initial mobilization of Uranium*
- *Nickel breakthrough observed but significant adsorption occurs*

Soil indicators by x-ray absorption spectroscopy

- *Small increase in sulfide relative to kill (oxidation during transfer may be problem)*
- *Fe(III) oxides still dominate*
- *No reduction of Uranium observed*

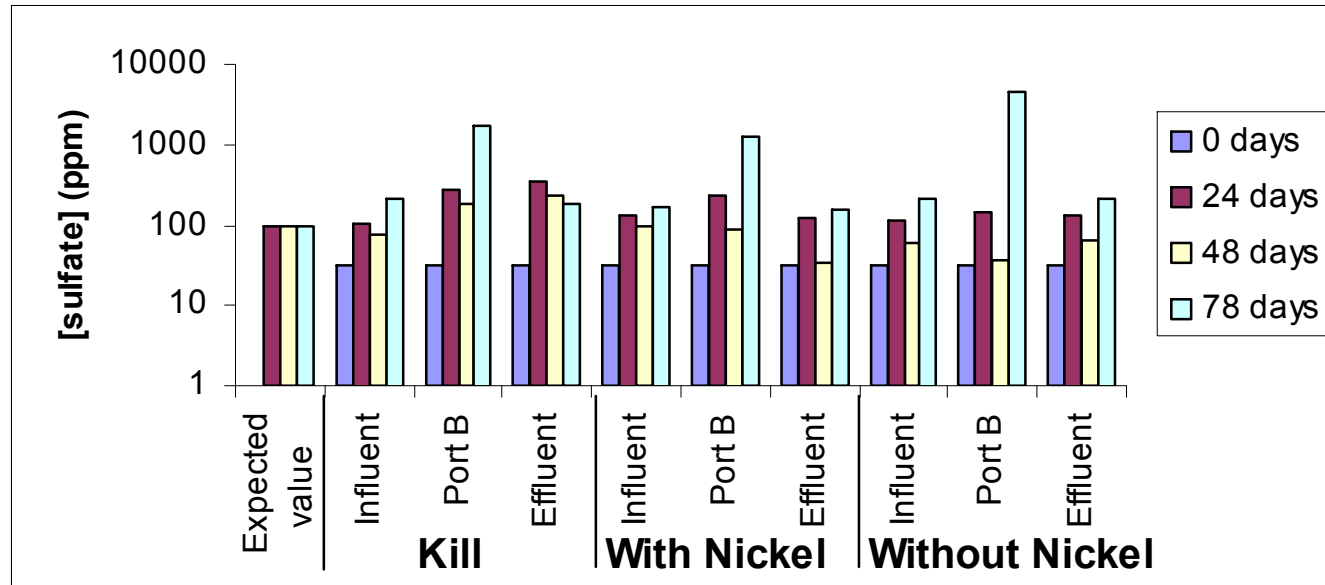
Column experiment after 78 days – nitrate analyses



Below detection

- After 78 days significant nitrate reduction in the viable columns
- Negative effect of nickel on nitrate reduction

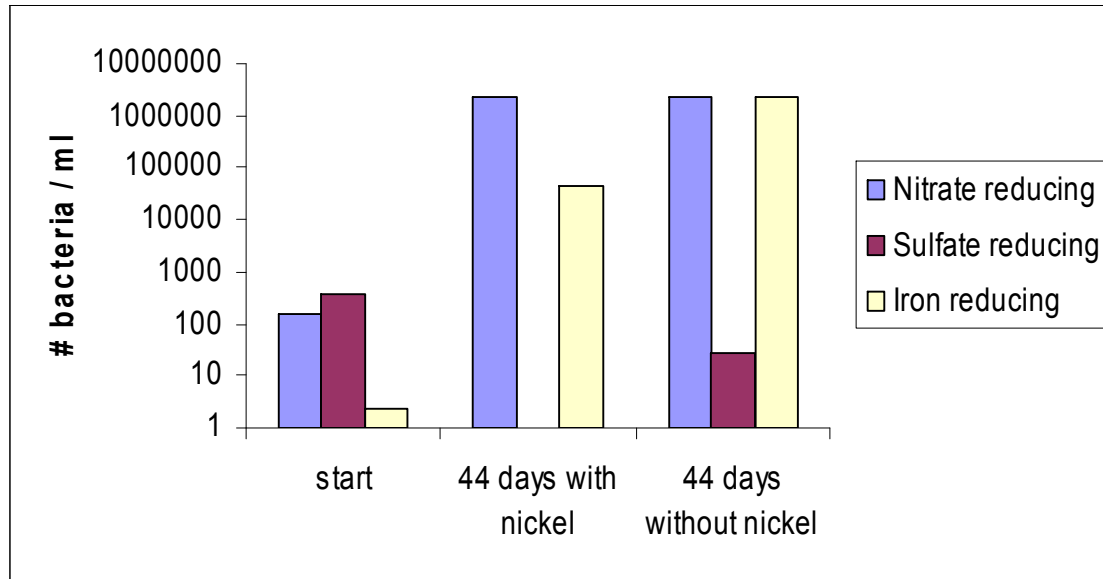
Column experiment after 78 days – sulfate analyses



- No significant reduction in sulfate is observed

Community analysis – Most Probable Number Counts

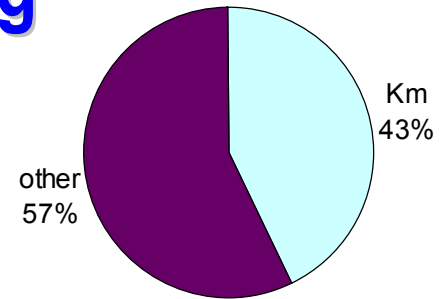
MPN Counts for nitrate, sulfate and iron reducers



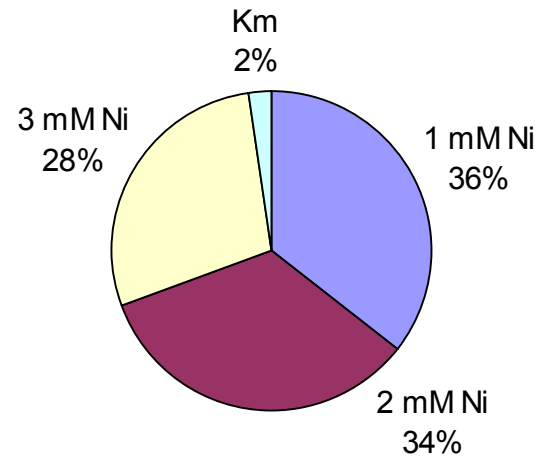
- MPN for nitrate, sulfate and iron reducers
 - Increase of nitrate reducing & iron reducing organisms
 - Decrease of sulfate reducing organisms
 - Nickel has a negative effect on sulfate and iron reducing organisms

Community analysis - ongoing

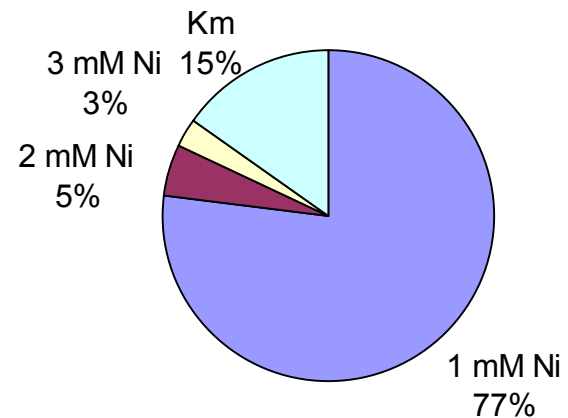
- Relative number counts of nickel and kanamycin resistant bacteria
 - Enrichment of nickel resistant organisms
 - More nickel resistant organisms under pressure of nickel



Start



44 days with nickel



44 days without nickel

Community analysis - ongoing

- 1. Constructed library with DNA extracted from community in the homogenized soil*
 - TOPO TA cloning: 100 clones*
- 2. Presently being sequenced; community composition will be determined at least on genus level*

Findings and Future directions

1. *Determine community composition of columns bioaugmented with engineered Pseudomonas DMY2*
2. *Quantitative PCR with nre targets to look for evidence of horizontal gene transfer in FRC column communities* JF4
3. *Ni bioavailability measured with Ni biosensor, based on lux fusion (light production) with cnr (nickel resistance operon) in strain CH34* JF5
4. *Explore Cupriavidus metallidurans strain CH34 which has natural resistance to a variety of metals*
5. *Extend findings to other systems, e.g. Hg (II) and Cr(VI) resistance*

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JF4 increasing level of nre beyond introduced species

J Fitts, 3/30/2006

JF5 naturally resistant to a range of heavy metals and high concentrations;
- apply approach to other metals
- overcome problem of introducing engineered organism b/c this is natural

J Fitts, 3/30/2006

Acknowledgements

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