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Natural Gene Transfer to Develop Resistance to Metal Toxicity in Microbial Communities at the Oak Ridge Reservation Field Research Center

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Office of Science U.S. DEPARTMENT OF ENERGY

BACKGROUND & OBJECTIVES

BACKGROUND

Uranium (VI) is the most common radionuclide contaminant found within the U.S. nuclear weapon complex at the Oak Ridge Reservation Field Research Center (ORR FRC) which is managed by the U.S. Department of Energy. Nitrate is often a cocontaminant with U(VI) in subsurface environments because of the use of nitric acid in the processing of U(VI) waste, which also is responsible for the often low pH found at these sites. Previous studies of microbial U(VI) reduction in sediments indicate that no net U(VI) reduction occurs until nitrate is reduced. Many Fe(III)-reducing and sulfate-reducing bacteria such as members of the *Geobacteraceae* and *Shewanellae* families possess the ability to reduce nitrate[1]. However these organisms are known to be inhibited by the presence of heavy metals such as nickel, which frequently contaminate these sites.

OBJECTIVES

To demonstrate the feasibility of applying natural horizonral gene transfer (HGT) to improve the performance of natural microbial communities under conditions imposed by metal stress, using Ni toxicity and resistance as a model system and as such improve the onset of nitrate reduction followed by sulphate reduction under lab conditions.

METHODS

This study focuses on contaminated subsurface sediments from the U.S. Department of Energy FRC, located at Area 2 from the Oak Ridge National Laboratory in Oak Ridge, Tennessee. Three replicate microcosms were constructed in an anaerobic chamber under a N_2 atmosphere for each set of condition in 125 ml glass serum vials (figure 1): 70 g of sediment and 60 ml of modified and N_2-flushed Postgate C medium [2] with 2.02 g/l KNO_3; leaving 50% of headspace. $NiCl_2$ was added at a concentration of 2 mM. In the bioaugmentation conditions 3-7 (table 1), a culture washed in 10mM $MgSO_4$ was injected at a cell density of 10^7 ml/cells. The cultures were grown overnight on 869 at 29.5 °C and 200 rpm. *Pseudomonas* DM-Y2 pMOL222 and *Pseudomonas* DM-Y2::ncc-nre were genetically engineered *Pseudomonas* DM-Y2 containing either a plasmid bearing the nickel resistance genes or constructed with a genomic insertion of the nickel resistance genes [3]. The microcosms were stopped with butyl rubber stoppers. All microcosms were incubated at room temperature, statically. An overview of the different applied conditions is given in table 1. Following start-up, samples were periodically taken with syringes and the same amount of modified Postgate C medium was added. The samples were filtered with 0.45 µm pore size syringe filters and stored at 4°C in the dark in 5ml amber bottles. Each sample was analyzed for nitrate and sulfate using a colorimetric method based on [4, 5] and for pH. U oxidation state and spatial distribution within the sediments was determined using synchrotron-based x-ray microspectroscopy at beamline X27A at BNL's National Synchrotron Light Source (figure 2). As a function of batch incubation time a dilution series (10^0-10^4) of unfiltered microcosm samples was made in 10mM $MgSO_4$, and spread-plated on rich media 869 [6], 284 [6] + carbon mix (0.5 g/l of glucose, gluconate, acetate, succinate and lactate) and 284 + carbon mix and either 1, 2 or 3 mM $NiCl_2$. These plates were incubated at 30°C.

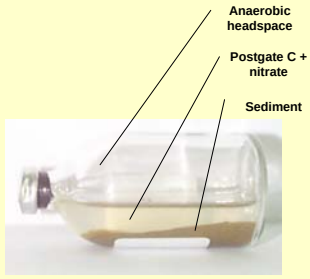

Figure 1. Microcosm, 125 ml glass serum vial

Figure 2. NSLS Beamline X26A Experimental Table

Table 1. Overview of the implemented conditions

Condition	Soil	Postgate C + nitrate	$NiCl_2$	*Pseudomonas* GN33 #1	*Pseudomonas* DM-Y2 pMOL222 #7	*Pseudomonas* DM-Y2::*ncc-nre* #7	*Ralstonia metallidurans* CH34	*Ralstonia metallidurans* AE104
1	+	+	+	-	-	-	-	-
2	+	+	-	-	-	-	-	-
3	+	+	+	+	-	-	-	-
4	+	+	+	-	+	-	-	-
5	+	+	+	-	-	-	+	-
6	+	+	+	-	-	+	-	-
7	+	+	+	-	-	-	-	+

RESULTS & DISCUSSION

Figure 3a indicates that nitrate reduction is occurring in all batch conditions except condition 7. Comparing data for conditions 1 and 2 suggests that the presence of nickel slowed down the onset of nitrate reduction in condition 1. Although condition 7 is bioaugmented with *Pseudomonas* DM-Y2::*ncc-nre* #7, previous stability tests conducted by growing the culture for 100 generations on non-selective medium indicated that the nickel resistant phenotype was not stable, which might explain the lower nitrate reduction rate. pH measurements of filtered samples indicate (results not shown) for all soil amended microcosms an increasingly acidic environment with pH values between 4 and 6 during the first two time points and decreasing to values between 4 and 4.5 by the third time point. It has been noted that uranium contaminated sites are complex waste sites with cocontamination by nitrate, heavy metals and inorganic acids used in uranium milling, extraction and enrichment [1]. Denitrification becomes negligible at pH 4, and therefore, the increasing acidity may become a problem at longer batch incubation times. Spread plating of the microcosms, however, indicates the presence of a viable population at numbers of greater than 10^5 /ml on 869, 284 + carbon mix. The populations in the microcosms tolerated a nickel concentration up to 2 mM, in which case a value of 10^5 /ml was observed (results not shown) and no significant differences were found between the different batch conditions. Although the initial number of nickel resistant bacteria has declined, according to [6] this is not uncommon for bioaugmentation experiments as the alien species must overcome different hurdles to establish itself as a member of the community. In order to circumvent the need for the inoculum to survive and remain functional for prolonged periods, horizontal gene transfer of resistance genes to the endogenous population might be a valuable alternative strategy. Whether this has taken place in these batch experiments remains to be seen. Sulfate concentrations dropped significantly between 28 and 42 days of incubation (figure 3b) and have since remained constant. Longer incubation times and additional data points are required to descern the behavior of the sulphate-reducing population.

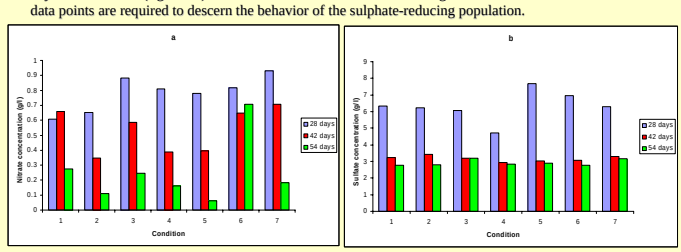

Figure 3. Results of spectrophotometric analyses of the Postgate C medium for nitrate and sulfate concentrations as a function of time with (a) nitrate concentration and (b) sulfate concentration for the conditions 1-7 as explained in table 1.

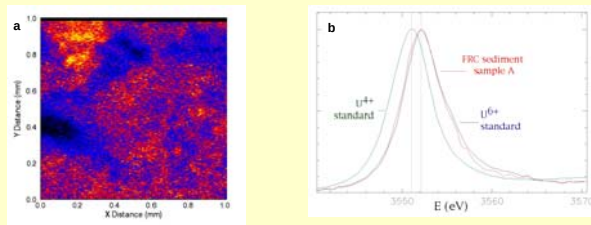

Figure 4. (a) MicroXAS two-dimensional image of U distribution in the as-delivered fine-grained fraction of sediments from FRC Area 2 shows a relatively even U distribution and (b) U M-5 edge x-ray absorption spectra indicate U is predominantly in the 6+ oxidation state at the start of the batch experiments. Data collected following addition of the high ionic strength growth medium show a decrease in sediment-bound U.

PLANNED WORK

Based on previous work done by [7], Single Point Genomic Sequence Tagging will be modified to our needs in order to determine the evolution in microbial population in the batch experiments as a function of nitrate and sulfate reduction, and couple these results to the extent of uranium immobilization and the selection for nickel resistance. Using *nre-ncc* primers quantitative PCR will be used to determine the amount of nickel resistance genes in the population as a function of time. Based on the results of the batch experiment, *in situ* simulated percolation systems will be designed which will allow us to study horizontal gene transfer of nickel resistance genes within a microbial population under *in situ* simulated nitrate and sulfate reducing conditions and couple this to the immobilization kinetics of uranium. *In situ* observations of HGT can be performed by a non-disruptive technique using fluorescent markers, such as the green fluorescent gene (GFP) [8]. Experiments are under way to label the organisms which were used in the batch experiment.

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This work is being supported by the U.S. Department of Energy, Natural and Accelerated Bioremediation Research (NABIR) Program, under Contract DE-AC02-98CH10886.