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Influence of Reactive Transport on the Reduction of U(VI) in the Presence of Fe(III) and Nitrate: Implications for U(VI) Immobilization by Bioremediation / Biobarriers

FINAL REPORT

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1 Executive Summary

Subsurface contamination by metals and radionuclides represent some of the most challenging remediation problems confronting the Department of Energy (DOE) complex. In situ remediation of these contaminants by dissimilatory metal reducing bacteria (DMRB) has been proposed as a potential cost effective remediation strategy. **The primary focus of this research is to determine the mechanisms by which the fluxes of electron acceptors, electron donors, and other species can be controlled to maximize the transfer of reductive equivalents to the aqueous and solid phases.** The proposed research is unique in the NABIR portfolio in that it focuses on (i) the role of flow and transport in the initiation of biostimulation and the successful sequestration of metals and radionuclides [specifically U(VI)], (ii) the subsequent reductive capacity and stability of the reduced sediments produced by the biostimulation process, and (iii) the potential for altering the growth of biomass in the subsurface by the addition of specific metabolic uncoupling compounds. A scientifically-based understanding of these phenomena are critical to the ability to design successful bioremediation schemes.

The laboratory research will employ *Shewanella putrefaciens* (CN32), a facultative DMRB that can use Fe(III) oxides as a terminal electron acceptor. Sediment-packed columns will be inoculated with this organism, and the reduction of U(VI) by the DMRB will be stimulated by the addition of a carbon and energy source in the presence of Fe(III). Separate column experiments will be conducted to independently examine: (1) the importance of the abiotic reduction of U(VI) by biogenic Fe(II); (2) the influence of the transport process on Fe(III) reduction and U(VI) immobilization, with emphasis on methods for controlling the fluxes of aqueous species to maximize uranium reduction; (3) the reductive capacity of biologically-reduced sediments (with respect to re-oxidation by convective fluxes of O_2 and NO_3^-) and the long-term stability of immobilized uranium mineral phases after bioremediation processes are complete, and (4) the ability for metabolic uncoupling compounds to maintain microbial growth while limiting biomass production. The results of the laboratory experiments will be used to develop mathematical descriptive models for the coupled transport and reduction processes.

This research is based on three areas of scientific research focused around the theme of coupling DMRB-mediated redox process, chemical transport, and control of microbial growth. The hypotheses associated with these themes can be stated as follows.

1.1 Investigation of the Reduction of U(VI) by Biogenic Fe(II)

Hypothesis 1.1 (Abiotic U(VI) Reduction by Fe(II). Adsorbed to Crystalline Fe(III) Oxides). Biogenic Fe(II) adsorbed to crystalline Fe(III) oxides will reduce U(VI) at carbonate/bicarbonate concentrations relevant to natural groundwaters. The net rate and extent of abiotic reduction of U(VI) will be influenced primarily by: (i) the crystalline Fe(III) mineral, (ii) the concentration of adsorbed Fe(II), (iii) the pH, and (iv) the carbonate/bicarbonate concentration as it affects the formation of uranyl complexes. Fe(II) adsorbed as ion exchange complexes on phyllosilicates will not be as effective a reductant as Fe(II) adsorbed to crystalline Fe oxides. Convective-dispersive transport will affect this abiotic reduction process by altering both the Fe(II) and the carbonate/bicarbonate concentrations (through removal), and by changing the distributions of U(VI) and adsorbed Fe(II) along the flowpath.

Hypothesis 1.2 (Abiotic U(VI) Reduction by Structural Fe(II)). Microbially reduced Fe-containing clays will be effective reductants of U(VI) at carbonate/bicarbonate concentrations relevant to natural groundwaters. For a particular Fe-containing clay mineral, the rate and extent of the abiotic reduction process will be affected by: (i) the pH, and (ii) the carbonate/bicarbonate concentration as it affects the formation of uranyl complexes. Convective-dispersive transport will affect this reduction process primarily by altering the carbonate/bicarbonate concentrations (through removal), and by changing the distribution of U(VI) along the flowpath.

1.2   Influence of Transport on Fe(III) Reduction and U(VI) Immobilization

Hypothesis for Task 2.1 (Nitrate Flux Control of DMRB Growth and Distribution). In systems in which facultative DMRB are present, control of the aqueous fluxes of nitrate and electron acceptor though the system will influence: (i) the spatial distribution of microbial biomass in the sediments, and (ii) the spatial location and time at which the transition from nitrate-reducing to iron-reducing conditions is established. The growth of facultative DMRB on nitrate prior to the establishment of iron-reducing conditions will lead to a greater net rate and extent of Fe-containing mineral reduction than would be observed if no nitrate were present.

Hypothesis for Task 2.2 (Flux Effects on Coupled Abiotic and Biotic U(VI) Reduction). Once Fe-reducing conditions are established, either the biotic or abiotic reduction of U(VI) can dominate the reaction pathway depending on the aqueous and solid phase geochemical conditions. As the system evolves, two biogeochemical process will tend to decrease the net rate of U(VI) reduction: (i) the generation of Fe(II) causing the passivation of the biotic reduction of U(VI), and (ii) the generation of CO_2 promoting an increase in uranyl-carbonato complexes which are not abiotically or biotically available for reduction. The presence of convective flux will moderate these effects by exporting CO_2 and Fe(II), and increase the rate of U(VI) reduction over what would be observed under static conditions.

1.3   Reductive Capacity of Bioreduced Sediments and Stability of U(IV) Phases

Hypothesis for Task 3.1 (Reductive Capacity). Subsurface biostimulation can produce Fe(II)-containing sediments that persist in the reduced state after microbial processes have ceased. Under the convective fluxes oxidants (O_2 and NO_3^-) that are generally found in groundwaters, this reduced state can persist for hundreds of pore volumes, which may correspond to a time period of decades at typical natural groundwater flow velocities.

Hypothesis for Task 3.2 (U(IV) Stability). Under reducing conditions, U(IV) precipitates will become increasingly stable as they are incorporated into iron oxide mineral phases or as they assume more crystalline U(VI)-containing minerals. Increasing the amount of time that U(IV) precipitates are exposed to reducing conditions will lead to increased persistence of U(IV) mass in solid phases and resistance to remobilization.

This research is multidisciplinary, and involves a strong research team who have expertise in conducting biogeochemical experimental studies in flowing systems, and who have expertise in developing mechanistic models of such processes for the purposes of interpretation and prediction. Team members are familiar with DOE site problems and remediation needs, and

have experience with the experimental and modeling techniques required to successfully examine and interpret coupled biogeochemical phenomena in subsurface systems.

1.4 Promotion of Catabolic Pathways in Biostimulation

Recent evidence has suggested that the control of microbial biomass in the subsurface is one of the primary problems facing sustainable biostimulation in the subsurface. Unlike the bioreactors used in industry or for wastewater treatment, the subsurface biomass is inaccessible, and can not be controlled using the methods (e.g., cell wasting, increasing dilution rate) that are available for conventional bioreactors. There is potentially a tremendous supply of TEA in the form of iron available in the subsurface. Even a 1% weight concentration of bioreducable iron would yield on the order of 300 moles of TEA per cubic meter. Compare this with, for example, the very high nitrate concentration of 1000 mg/L, which would deliver only on the order of 6 moles of TEA per cubic meter. The problems that have been experienced at field sites such as Rifle, CO appear to arise not because of TEA limitations, but because of the uncontrolled growth of biomass (1) potentially blocking access to iron oxides, and (2) providing uncontrolled secondary sources of labile carbon.

In Task 1, we propose methods to promote controlled microbial growth two phases are: (1) a biomass growth and distribution phase, and (2) a phase that seeks to minimize additional growth and maximize and sustain the transfer of electrons from the electron donor to the subsurface.

Hypothesis for Task 4.1. Maximizing Electron Transfer / Minimizing Biomass Production Hypothesis. The use of chemical metabolic uncouplers can be used in subsurface bioremediation schemes to shunt energy (carbon) to catabolic pathways, and maximize electron transfer while minimizing additional microbial growth.

2 Research Background and Objectives

2.1 Uranium Contamination in the Subsurface

Waste disposal from the past production of defense materials has generated a wide variety of both organic and inorganic contaminants on DOE lands. An inventory of many of the contaminant of concern has been compiled by Riley et al. [*Riley, et al.*, 1992]. Across the DOE complex, metals and radionuclides form one of the most significant classes of contaminants of concern. Metals and radionuclides such as As, Cd, Co, Cr, Ni, Pb, Pu, Tc, and U constitute a major portion of the soil, vadose zone, and groundwater contamination problems associated with many DOE sites. Of these, CrO_4^{2-}, UO_2^{2+}, and TcO_4^- are highly mobile and pose the major risks at sites such as Hanford.

Uranium in particular is a widespread contaminant, occurring in the soil and groundwater at over fifty percent of the DOE facilities examined in the report by Riley et al. [*Riley, et al.*, 1992], including the Hanford and the Oak Ridge sites. As an example of the magnitude of this problem, a recently declassified Hanford Works monthly report from 1951 makes note that "approximately 91,600 gallons of supernatant containing an estimated 22.5 tons of depleted uranium were lost to the soil…" [*Hanford_Works*, 1951] in an overflow accident at an underground storage tank. At the Oak Ridge Field Research Center (FRC), the groundwater concentrations of uranium species are typically in the 0 to 4 $mg\ L^{-1}$ range (with an average near 3 $mg\ L^{-1}$), which up to 100 times the maximum contaminant level for drinking water currently being imposed by the EPA. Uranium contamination often occurs in the presence of nitric acid, which was used in various dissolution and extraction processes. Both at the FRC and at Hanford (e.g., the Hanford 300 Area [*Hartman, et al.*, 2003]), significant quantities of nitrate appear in the groundwater with uranium. Ultimately, biologically-mediated immobilization of uranium in the subsurface must be considered in the context of potentially low pH and the presence of NO_3^-. The presence of nitrate is particularly troublesome because it may serve as a competitive TEA for DMRB, a potential oxidant for Fe(II) and U(IV) [*Senko, et al.*, 2002], and as a TEA for U(VI) oxidizing bacteria [*Finneran, et al.*, 2002].

2.2 In Situ Remediation of Uranium

It has been known for some time that some microorganisms can effectively use iron or uranium as a terminal electron acceptor (e.g., [*Gorby and Lovley*, 1992; *Lovley, et al.*, 1991b]) and may be useful in transforming mobile U(VI) into relatively immobile and insoluble U(VI) precipitates. This recognition has led to the idea that mobile U(VI) might be *immobilized* through a bioremediation process involving the active in situ stimulation of native organisms [*Abdelouas, et al.*, 1999; *Anderson, et al.*, 2003; *Lovley and Coates*, 1997; *Lovley and Phillips*, 1992]. Of the various schemes available, one of the most tractable appears to be the generation fixed zone of metal reduction, often referred to as a "biobarrier" (e.g., [*Salanitro, et al.*, 2000]).

Recently, in situ uranium reduction has been attempted at several field sites including a biobarrier demonstration in Rifle, CO [*Anderson, et al.*, 2003], a 'push-pull' tests at a landfill in Norman, OK [*Senko, et al.*, 2002] and at the Oak Ridge FRC [*Istok, et al.*, 2004]. These tests all indicated that in situ reduction of U(VI) was possible, but they also highlighted several areas that are in need of additional research. In particular, U(VI) reducing conditions were not sustainable

at the Rifle site. It has been noted by Anderson et al. [*Anderson, et al.*, 2003] that the problems that were experienced at that site appear to arise not because of TEA limitations *per se*, but because of the uncontrolled growth of biomass (1) potentially blocking access to iron oxides, and (2) providing uncontrolled secondary sources of labile carbon. The development of a thorough understanding of the interaction between microbial reductive processes and transport processes in the subsurface need to be better understood so that effective schemes for creating and sustaining biobarriers can be developed.

2.3   Uranium Geochemistry

Uranium is present in the environment primarily in the U(IV) and U(VI) oxidation states. In the predominantly oxic soils and groundwaters at DOE sites, uranium is present in almost exclusively in the mobile U(VI) state. Although the mobility of U(VI) is disadvantageous from the perspective of potential for groundwater contamination, it is actually desirable from the perspective of initiating remediation [*MacDonald and Kavanaugh*, 1994]. Uranium geochemistry in the presence of iron minerals is complicated, and there are several physiochemical interactions that dramatically influence the distribution and biological availability of uranium in the subsurface.

Aqueous Geochemistry

Uranium geochemistry is fascinatingly complex, so much so that it is nearly a discipline unto itself (e.g., ref. [*Guillaumont, et al.*, 2003]). Hydrolysis species and uranyl-carbonato complexes appear to be the most environmentally-relevant forms of complexed uranium in natural waters. At *high* total inorganic carbon concentrations (> 30 mmol L^{-1} C), atmospheric P_{CO_2}, and near-neutral pH, the predominant species are U(VI) dicarbonates [$UO_2(CO_3)_2^{2-}$] and tricarbonates [$UO_2(CO_3)_3^-$]. At *lower* total inorganic carbon values that are more typical of natural groundwaters (< 2mmol L^{-1} C), uranyl hemicarbonate [$(UO_2)_2CO_3(OH)_3^-$], dicarbonate, and hydrolysis species [UO_2OH^+ and $UO_2(OH)_2^0$] predominate; the distribution of species for an inorganic carbon concentration of 0.2 mmol L^{-1}.

Iron oxides and other Fe(III)-containing minerals have high capacities for sorption of metals from aqueous solution, and are believed to be of particular importance to metals and radionuclide remediation in natural sediments because of their common occurrence as grain coatings and precipitates. Iron oxides have been shown to significantly adsorb a large number of cations, including many metals (such as Cd, Co, Cr, Hg, and Pb), and radionuclides (such as Np, Pu, Th, and U); a summary of much the available information can be found in refs. [*Brown, et al.*, 1999; *Cornell and Schwertmann*, 1996; *Dzombak and Morel*, 1990].

Although there has been general agreement in the literature that the uranium complexes UO_2OH^+ and $(UO_2)_3(OH)_5^+$ form important surface complexes in the absence of carbonates [*Hsi and Langmuir*, 1985; *Waite, et al.*, 1994], until recently the adsorption of uranyl-carbonato complexes has been hypothesized only on the basis of theory. Spectroscopic evidence has now been collected showing that ternary uranyl-carbonato complexes are formed at the Fe(III) oxide surface at a wide range of pH values [*Bargar, et al.*, 1999; *Bargar, et al.*, 2000]; uranyl-carbonato complexes containing two or three carbonato ions are thought to be excluded from the surface due to steric or coordinative constraints [*Waite, et al.*, 1994]. Complexes capable of being adsorbed predominate at circumneutral pH and low carbonate concentrations.

Microbial reduction of U(VI) typically produced U(IV) as uraninite, $UO_{2(s)}$ [*Abdelouas, et al.*, 1999; *Liu, et al.*, 2002; *Lovley, et al.*, 1991b; *Wielinga, et al.*, 2000b]. Although the reoxidation of biogenic U(IV) has not been widely investigated, the oxidation of $UO_{2(s)}$ as spent nuclear fuel has been extensively studied [*De Pablo, et al.*, 1999; *Sharma, et al.*, 1996; *Shoesmith, et al.*, 1998]. These studies showed that $UO_{2(s)}$ could be rapidly oxidized in contact with O_2 and other oxidants. The oxidation kinetics are controlled by four steps including the diffusion of oxidants to $UO_{2(s)}$ surface, surface coordination of the oxidants, electron transfers, and detachment of U(VI) from the surfaces. The first and last steps are controlled by the mass transfer processes, while the second and third steps were controlled by surface chemistry and electrochemical processes, respectively. Limited studies of reoxidation of biogenic $UO_{2(s)}$ also indicated that $UO_{2(s)}$ could be reoxidized in presence of O_2 [*Abdelouas, et al.*, 1999], manganese oxides [*Fredrickson, et al.*, 2002; *Liu, et al.*, 2002], and nitrate reduction intermediates including NO_2^-, N_2O, and NO [*Senko, et al.*, 2002]. The oxidation rates of biogenic U(IV) followed the similar kinetic processes as observed for the spent nuclear fuels in presence of oxidants [*Liu, et al.*, 2002].

Although these studies indicate that biogenic $UO_{2(s)}$ could be readily oxidized in contact with various oxidants including O_2 and nitrate reduction intermediates, they also suggested a potential means to slow down the reoxidation rates, that is to provide a mass transfer resistance between U(IV) and oxidants. This mass transfer resistance will decrease the rates of oxidants to U(IV), and decrease the detachment rate of U(VI) after U(IV) oxidation. The decreased detachment of U(VI) will decrease the surface coordination sites for oxidants and thus further slow the kinetics of $UO_{2(s)}$ oxidation. The batch studies performed by this project demonstrated that such mass transfer resistance created by Fe(II) oxidation into iron oxide precipitates was effective in slowing down the remobilization of U and may be practically applicable and warrant further test under more field relevant flowing conditions.

A few studies have suggested that U(IV)-containing minerals maintained under reducing conditions can be incorporated into crystalline Fe(III) oxides over time [*Ménager, et al.*, 1994; *Payne, et al.*, 1994; *Szecsody, et al.*, 1998]. The re-oxidation and solubility of U(IV)-containing mineral phases produced by microbially-mediated reactions has not been studied in detail, but is a highly-relevant issue for the bioremediation of U(VI). Because all bioremediation/biobarrier applications will eventually return to oxic conditions, it is important to build further understanding of the stability of U(IV)-containing minerals formed under reducing conditions, and the potential for creating conditions that will ultimately limit the rates of reoxidation.

2.4 Influence of Dissimiltory Metal Reducing Bacteria (DMRB) on Subsurface Metal and Radionuclide Chemistry

It has been known for some time that many species of bacteria can bring about the reduction of metals, but it has been only recently that it has been shown that metal reduction can be coupled to the oxidation reactions of cellular metabolism (see refs. [*Lovley*, 1987; *Nealson and Saffarinini*, 1994] for reviews). As a group, the organisms that can directly transfer electrons to metals (and radionuclides) have been termed dissimilatory metal reducing bacteria (DMRB). These bacteria belong to several phylogenic groups, and bacteria that have been specifically shown to reduce U(VI) include *Geobacter* sp. [*Lovley*, 1987], *Shewanella* sp. [*Lovley, et al.*, 1991a], *Desulfotomaculum* sp. [*Tebo and Obraztsova*, 1998], *Desulfovibrio* sp [*Lovley, et al.*, 1993], *Desulfosporosinus* sp. [*Suzuki, et al.*, 2003], and *Clostridium* sp. [*Francis,*

et al., 1994]. Organisms of the *Geobacter* species have been shown to be widely prevalent in the subsurface, and have been shown to be active in the field [*Anderson, et al.*, 2003; *Holmes, et al.*, 2002]. Organisms of the *Shewanella* species have been found at sites with uranium contamination [*Abdelouas, et al.*, 1998], but have not yet been systematically shown to have an important role in metal reduction in the subsurface environment. Recently, a novel myxobacterium. *Anaeromyxobacter dehalogenans*, was discovered that is able to use Fe(III) as a terminal electron acceptor [*He and Sanford*, 2003; *Sanford, et al.*, 2002]. At the Oak Ridge FRC, it was determined that *Anaeromyxobacter* sp. were the dominant metal reducing organisms in the contaminated portions of the site [*Petrie, et al.*, 2003], and it was suggested that these organisms may play an important role in subsurface U(VI) reduction in acidic sediments.

Iron and Uranium Reduction Mechanisms by DMRB in the subsurface

Because Fe(III) oxides and other Fe(III)-containing minerals are highly insoluble, it has generally been thought that DMRB require direct contact with the mineral surface to use Fe(III) as a terminal electron acceptor (TEA) [*Lovley*, 1991]. Experimental results, however, have suggested that humic substances or even U(VI) may act as electron shuttles for the reduction of Fe(III) or Mn(IV) in natural systems [*Liu, et al.*, 2002; *Nevin and Lovley*, 2000]. Quinones appear to be one of the primary electron accepting moieties in humic substances, and the experimental use of anthraquinone-2,6-disulfonate (AQDS) has verified that such quinones can operate as electron shuttles [*Fredrickson, et al.*, 2000; *Lovley and Anderson*, 2000]. It has been recently shown that *Geobacter metallireducens* specifically expresses flagella and chemotactic motility only under conditions where insoluble Fe(III) or Mn(IV) oxides are used as the TEA [*Childers, et al.*, 2002]. The implication is that there are at least two microbial schemes for improving contact with solid-phase TEAs: the use of quinines (in the case of *Shewanella* sp.), and the development of protein structures that allow chemotactic motility (in the case of *Geobacter* sp.).

One of the primary issues associated with the sustained microbial reduction of Fe(III)-containing minerals is the passivating effect of aqueous Fe(II) generated during the bioreduction process. In batch experimental systems where DMRB reduce Fe(III) oxides in the presence of excess organic substrates, it has been observed that the reduction process initially proceeds rapidly but eventually slows and stops before Fe(III) becomes limiting [*Roden and Zachara*, 1996]. Adsorption or precipitation may physically block or otherwise interfere with the electron transfer from DMRB to Fe(III) [*Liu, et al.*, 2001a; *Liu, et al.*, 2001b; *Zachara, et al.*, 1998]. This hypothesis has been supported by experimental work in which the removal of Fe(II) from the aqueous phase of either semi-continuous batch or column experiments allowed Fe(III) oxides to be reduced nearly to completion [*Roden and Urrutia*, 1999; *Roden, et al.*, 2000]. In any event, the presence of passivating Fe(II) appears to be reversible, and some experiments have shown complete recovery of DMRB from such effects [*Liu, et al.*, 2001b].

Experimental observations have also suggested that the form of Fe(III) oxide can influence the rate and extent of the reductive process [*Fredrickson, et al.*, 1998; *Fredrickson, et al.*, 2000; *Wielinga, et al.*, 2000a]. Recent results have favored the idea that surface-area is the primary controlling variable involved [*Roden*, 2003]. It has also been reported that structural Fe(III) in clays can be reduced by DMRB [*Dong, et al.*, 2003; *Kostka, et al.*, 1999a; *Kostka, et al.*, 1999b]. Fe-containing phyllosilicates may prove to be a significant reservoir of reductive potential in the subsurface. For example, it has been shown [*Zachara, et al.*, 1995a; *Zachara, et al.*, 2000] that and Fe-containing smectite is abundant in the Hanford Ringold formation, and these materials may be the primary electron acceptor for DMRB in these sediments.

Past experimental evidence [*Liger, et al.*, 1999; *Marcos, et al.*, 2000] has suggested that surface-associated Fe(II) may be an effective reductant for U(VI). In the experiments of Fredrickson et al. [*Fredrickson, et al.*, 2000], however, no reduction of U(VI) was observed in the presence of high carbonate concentrations; however, the aqueous species may have been dominated by the di- or tri-carbonato complexes which do not associate strongly with the oxide surface. Recent work completed on our current NABIR project (see section 3, 'Preliminary Studies' below) have shown that this mechanism may not be an important at field-relevant carbonate concentrations.

DMRB promote the formation of a variety of Fe(II) mineral phases (e.g., magnetite, siderite, green rust, goethite) depending upon the source of Fe(III) [HFO or more crystalline Fe(III) oxides], pH, reduction rate, and other geochemical conditions [*Fredrickson, et al.*, 1998; *Fredrickson, et al.*, 2000]. Recently, it has been shown that several of these structural Fe(II) phases have the ability to reduce U(VI) [*O'Loughlin, et al.*, 2003; *Wersin, et al.*, 1994]. This is currently an area of funded NABIR research (O'Loughlin et al., "Investigation of the Transformation of Uranium under Iron-Reducing Conditions: Reduction of U(VI) by Biogenic FeII/FeIII Hydroxide [Green Rust]"). Recent research [*Roden, et al.*, 2000], suggests that the presence of convective fluxes substantially diminishes the precipitation of these secondary minerals. On the basis of this observation, and because the ability for Fe(II)-containing minerals to act as reductants of radionuclides is part of the currently-funded NABIR projects mentioned above, these minerals will not be a primary focus of our proposed research. If other secondary Fe(II) containing minerals are found to be a significant pool of reductive capacity in our experimental systems, the effects of these minerals will be incorporated into our experimental design and analysis.

2.5 Microbial growth Kinetics of DMRB: Promoting Catabolic Over Anabolic Pathways

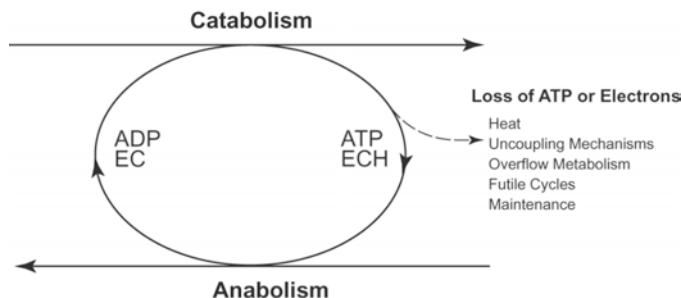It has been recognized that understanding and controlling the dynamics and growth of the microbial community during subsurface remediation by biostimulation is paramount to the ultimate success of this remediation technique (cf., ref. [*Anderson, et al.*, 2003]). One of the primary obstacles currently is a thorough understanding of how to promote the biological transfer of electrons to uranium in the solid phase in a sustainable manner. Although biological reduction of U(VI) has been achieved in the field [*Istok, et al.*, 2004], in the most extensive field test yet conducted [*Anderson, et al.*, 2003], it was not possible to maintain U(VI) reduction for long periods of time. The problem was hypothesized to have been caused by overgrowth or microorganisms near the well and subsequent formation of sulfate reducing conditions. Although the exact cause of the treatment failure can not be determined, it is likely that enough biomass was formed in situ such that (1)

Fig. 1. A highly simplified model of the balance between catabolic and anabolic processes in a cell. These two processes are tightly coupled when energy is limited, but in situations with excess energy, it is possible to decouple the pathways. In this figure, ATP/ADP is the conventional phosphorous containing energy couple, and EC/ECH represents a generic electron transport couple.

microbial access to Fe(III) was limited, (2) sulfate was present in the aqueous phase in significant enough quantities to allow sulfate reducers to be competitive. It is possible that the subsurface biomass that was formed also provided secondary substrate for sulfate reducing organisms, effectively removing the ability to control the flux of electron donor into the subsurface. Hence, for bioremediation in the field, it may be important to understand how one might promote catabolic (with associated electron transfer to the extracellular environment) over anabolic pathways for the purposes of controlling the amount of biomass in the subsurface and maximizing electron transfer.

The importance of promoting catabolic over anabolic pathways in microbial treatment systems is not new. It has been recognized for some time that it is possible to uncouple anabolic and catabolic pathways in microbial systems [*Cook and Russell*, 1994; *Russell and Cook*, 1995; *Teixeira de Mattos and Neijssel*, 1997; *Westerhoff, et al.*, 1982] to produce metabolic shifts of carbon and electron flow to less efficient pathways. This idea has been applied in biological wastewater treatment to maximize the oxidation of organic carbon loads (i.e., maximize electron transfer from organic C to O_2) while minimizing microbial growth [*Chudoba, et al.*, 1992; *Liu*, 1998; *Liu and Tay*, 2000b; *Low and Chase*, 1999; *Low, et al.*, 2000; *Mayhew and Stephenson*, 1998; *Van Loosdrecht and Henze*, 1999; *Wei, et al.*, 2003]. There are several mechanisms that have been proposed for explaining how catabolic and anabolic processes can be decoupled. These include: (1) simple maintenance requirements [*Pirt*, 1965], (2) overflow metabolism [*Tempest and Neijssel*, 1978], (3) metabolic uncoupling [*Senz*, 1962], and (4) futile enzymatic cycles [*Katz and Rognstad*, 1978]. A review of these mechanisms for shifting the catabolic and anabolic pathways in organisms is presented by Russell and Cook [*Russell and Cook*, 1995]. Although these ideas have not yet been applied to subsurface remediation, they do provide a framework in which one can logically designing a scheme that maximizes electron transfer to the extracellular environment while minimizing microbial growth. In this proposal, our focus will be on methods to increase electron flow by inducing metabolic uncoupling by the use of chemical metabolic uncouplers.

In Fig. 1 we present a highly simplified model of the balance between anabolic and catabolic pathways. Under energy-limited growth conditions, catabolic and anabolic pathways are tightly coupled [*Russell and Cook*, 1995]. However, when energy is in excess, bacteria can spill ATP and electrons by one ore more of the mechanisms identified above that consume energy but do not yield cell mass. If one is attempting to maximize the number of reductive equivalents transferred to the subsurface media, as is the case for bioremediation schemes for the in situ immobilization of U(VI), then promoting such reactions may yield better and more sustainable treatment.

As an example, it is well known that there are chemical metabolic uncouplers that can decouple catabolic energy production and electron transfer from anabolism [*Weigel and Englund*, 1977]. Many of these compounds are antibiotics (e.g, vancomycin), but some are relatively benign and inexpensive compounds (e.g., 2,4 dinitrophenol [*Mayhew and Stephenson*, 1998]; 3,3',4',5-tetrachlorosalicylanilide (TCSA) [*Chen, et al.*, 2002]). As a class, these chemicals work by impeding the cell's ability to generate a proton gradient across cell membranes; this, in turn, prevents the production of ATP. The conduction of electrons remains uninhibited, however, so the cells are able to continue oxidizing electron donors, and continue the transfer electrons to TEAs in the extracellular environment (often at increased rates). The net result is that cells are able to oxidize energy sources and transfer electrons to the extracellular environment, but the yield (biomass fixed per unit electron donor) is reduced.

There have been full scale tests on the use of such chemical uncouplers in wastewater treatment plants in Phoenix, AZ [*Okey and Stensel*, 1993], and it was shown in those studies that the net transfer of electrons to the extracellular environment (O_2 as the TEA in this case) was *increased* by the uncouplers, whereas *synthesis of new cell mass was essentially zero*. Such chemical uncouplers may have a role in subsurface bioremediation efforts aimed at metals and radionuclide immobilization because they present another option for promoting the release of electrons to the environment without the production of excessive biomass.

The description of microbial growth and substrate conversion under conditions of metabolic uncoupling have been studied extensively [*Russell and Cook*, 1995; *Zeng and Decker*, 1995]. Substrate consumption can be considered to consist of three parts, a growth-independent maintenance requirement, grow-rate dependent uptake, and a "spilling" term indicating that electron donor is being used in a way that is uncoupled from anabolism. Mathematically, this can be represented by [*Zeng and Decker*, 1995]

$$q_s = m_s + \frac{\mu}{Y_s^{max}} + \Delta q_{max} \frac{C_s - C_s^*}{C_s - C_s^* + K_s^*} \tag{1}$$

Here, q_s is the total substrate uptake rate, and the quantity $m_s + \mu / Y_s^{max}$ represents the conventional Monod-type kinetic term for substrate uptake by cell growth, C_s is the electron donor concentration, C_s^* is the critical substrate concentration, and K_s^* is a new semi-empirical saturation constant. The third term on the right-hand side of Eq. (1) represents the substrate uptake that is shunted to non-growth (non-maintenance) pathways.

Bioremediation of subsurface materials is inherently less controllable than more highly engineered systems such as bioreactors. However, the use of chemical metabolic uncouplers can add an element of control that is currently missing (and desperately needed).

2.6 Putting the Pieces Together: Coupling of Biological, Geochemical, and Transport Processes

Although in principal the biogeochemical processes that occur when convective-dispersive transport is important are not fundamentally changed by the transport processes, their *manifestation* can be dramatically different. The presence of convective dispersive fluxes in the formation and longevity of biologically reduced subsurface sediments is important for many reasons. As an example, consider the generation of Fe(II) by microbial reduction of Fe(III)-containing minerals. In a batch system, one typically starts out with a fixed initial concentration of electron donors and acceptors. If one were to initiate microbial reduction of iron by adding a fixed amounts of Fe(III) and an electron donor such as lactate, the reduction of Fe(III) would lead to a continual increase in the Fe(II) concentration, leading to passivation of the microbial reduction process. In a column system under the same conditions, the process of convective-dispersive transport would continually remove Fe(II) [*Roden, et al.*, 2000]. Although the Fe(II) concentration would initially increase in the column, eventually a near steady-state condition would arise where the generation of Fe(II) was just balanced by the convective-dispersive removal of Fe(II). Under these conditions, the aqueous Fe(II) concentration would be fixed at some maximum value that was determined by the ratio of the rate of generation of Fe(II) to the rate of convective-dispersive removal of Fe(II).

Our conceptualization of the subsurface U(VI) reduction process is illustrated in Fig. 2, where we have focused on representing important processes rather than a specific accounting of the various chemical species that may be involved.

The primary features of the conceptual model illustrated in Fig. 2 is that *convective dispersive fluxes of aqueous species can have a major impact on the dynamics and evolution of the biogeochemical processes involved in the reduction of U(VI) and in the re-oxidation of bioreduced sediments*. By understanding how the transport and reaction processes are coupled, we can determine how the fluxes of electron acceptors, electron donors, and other species can be controlled to *maximize* the transfer of reductive equivalents to the aqueous and solid phases. The coupling of the biogeochemical and transport processes can lead to effects that are observed in flowing systems that are not generally seen in batch. Specific examples include:

Fig 2. Conceptual process model for U(VI) transport and reactions in the presence of Fe(III) oxides under nominally iron-reducing conditions. Note that this conceptual picture implies that U(VI) and Fe(II) may be *simultaneously* present because of mixing and transport; however, this does not necessarily imply that U(VI) and F(III) are simultaneously reduced.

- **The *form* of the reactions can be influenced when characteristic time of reactions is greater than characteristic time for transport.** Under such conditions, a reaction that appears to be in equilibrium at low flow rates may become kinetic as the flow rate increases (e.g., ref. [*Gabriel, et al.*, 1998]). We have observed these effects for uranium transport in natural sediments in our currently supported research.

- **The transport process may affect the *extent* of the reactions.** For example, if biogenic Fe(II) is continually removed by convective-dispersive transport, the passivating effects that it has on biogeochemical reactions will be reduced. This may allow more U(VI) to be reduced under these conditions than would be possible in batch [*Roden and Urrutia*, 1999].

- **Transport may *distribute reactions spatially*.** This may influence reactions rates by accessing a larger volume of the subsurface. As and example, physical/chemical/microbial heterogeneity may interact with the transport process to allow spatial distribution of the redox state of the subsurface. This would be critical information for understanding the immobilization of metals and radionuclides in the subsurface, because it may allow the reduction of metals in a spatial pattern that is substantially different than would be predicted otherwise.
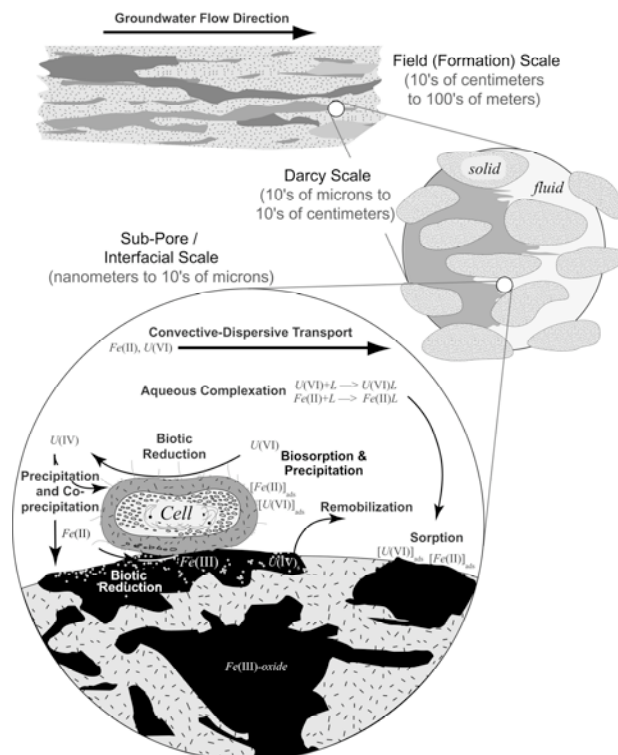
3 Results

In this section we have summarized the results of this cycle of NABIR supported research. As a result of this NABIR funding, we have published eight papers in peer reviewed journals; these papers are are included in the Appendix. Below, we summarize some of the primary results from this work.

3.1 Kinetics of U(VI) Sorption on Natural Sediments Under Conditions of Transport

Sorption experiments were conducted under flowing conditions to determine the U(VI) sorption rate to Fe-containing minerals under a two carbonate concentrations (1 and 10 mmol/L) and a range of pH values (4-9) (Fig. 3). Carbonates can form complexes with U(VI), and these complexes appear to adsorb most strongly at circumneutral pH. Previous work by other NABIR researchers (S. Brooks) has reported the equilibrium sorption isotherms for U(VI) in batch systems. We have extended this work to examine the importance of the kinetics of U(VI) sorption under conditions of transport. Our experiments were conducted in two DOE site sediments (Oak Ridge FRC and Hanford) at groundwater velocities relevant to field remediation. The systems have been constructed at intermediate scale (50 cm long) and using artificial groundwaters that approximate geochemical conditions found at Hanford and at the FRC. Our results indicate that the sorption of U(VI) in natural sediments cannot generally be treated as an equilibrium process This is an important observation, because it indicates that under conditions that apply to bioremediation, it may not be possible to assume equilibrium partitioning of U(VI) species to the solid phase. We are currently assessing whether the sorption is a diffusion- or rate-limited process, and this work will be completed in FY04. One manuscript is currently in process on this work.
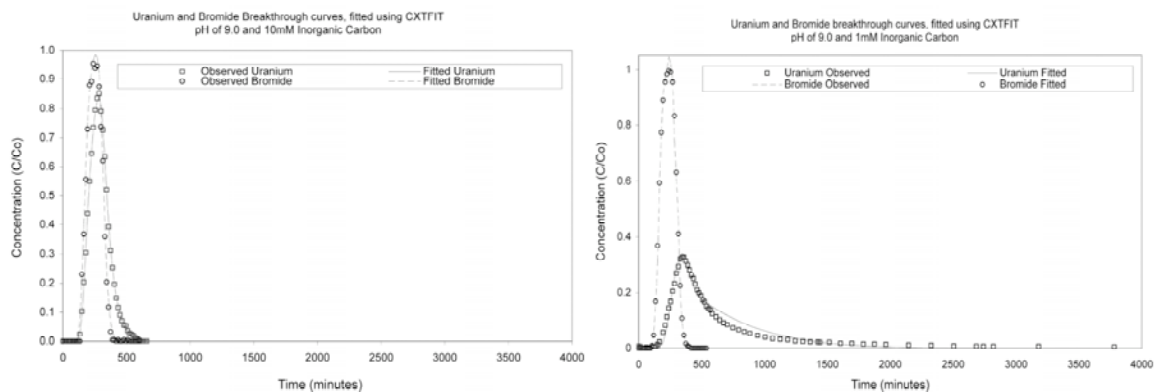

Fig 3. Effect of inorganic carbon concentration on uranium sorption and transport. For the figure on the left, the inorganic carbon concentration was 1 0mM; on the right 1 mM. Uranium concentration was 1 mg/L.

3.2 Biogenic U(IV) Remobilization In Presence of Fe(II)

The kinetics of $UO_{2(s)}$ oxidation (see background section) suggests that increasing the mass transfer resistance between oxidants and U(IV) precipitates may decrease the rate of biogenic U(IV) reoxidation and remobilization. This concept was tested by adjusting the Fe(II) content in the bioreduced FRC saprolite sediments mixed with biogenic U(IV). It was hypothesized that the iron oxides produced during the reoxidation process were could cover the existing U(IV) solid phases, and thus provide a mass transfer resistance that might help limit U(IV) reoxidation. Because iron oxides adsorb U(VI) under most geochemical conditions, this

adsorption process was also hypothesized to retard the rates of U(VI) release to aqueous phase when U(IV) oxidation did occur.

We initiated such testes in batch systems with the goal of understanding whether protective iron oxides could prevent or slow down the remobilization of biogenic U(IV). The FRC sediments and U(VI) were reduced by *S. putrefaciens* CN32 in separate reactors using lactate as electron donor and PIPES as pH buffer. After 60 days of incubation, the bioreduced sediments contained 0.14 mmol/g of acid (0.5 M HCl) extractable Fe(II), which was about half of the iron oxide content (0.27 mmol/g) in the sediment; about a third (0.05 mmol/g) of the acid extractable Fe(II) was associated with the sorbed phase with the remainder in precipitated phases. The samples of the bioreduced sediments were then mixed with biogenic U(IV) in 0.1 M $NaNO_3$ electrolyte, and spiked with different Fe(II) concentrations in an anaerobic box. The suspensions were mixed on a linear mixer (20 rpm) and maintained at pH 7. The suspensions were exposed to the atmosphere O_2, the U(VI) in the aqueous phase was monitored as a function of time using Kinetic Phosphorescence Analysis (KPA).

The increase of U(VI) in the aqueous phase is indicative of the reoxidation of U(IV) (Fig. 4a). The addition of Fe(II) in the suspensions significantly decreased the rates of U(VI) release. At spiked Fe(II) = 0.2 mmol/g, the mobilized U(VI) was stabilized below 44 ppb for the entire duration of the experiments. The U(VI) was initially released fast in the suspensions with 0 and 0.02 mmol/g of Fe(II) spikes (Fig. 4a) and then decreased and stabilized after 10 days. This fast initial remobilization disappeared in the suspension with 0.20 mmol/g of Fe(II) spike. These results indicated that iron oxides could play a protective role to slow the remobilization rate of biogenic U(IV). The results also suggested that the iron oxides produced from spiked Fe(II) oxidation provided such protection. This observation is consistent with the effect of the mass transfer limitation because the oxidative precipitation of the spiked Fe(II) could cover on U(IV).

We are now moving to study the effects of aging of the bioreduced sediments to examine whether U(IV) could be incorporated into iron oxides and Fe(II)-containing minerals under reducing conditions and whether such incorporation could slow down both U(IV) reoxidation and U(VI) release. Later we want to study the Hanford sediments to see whether the similar effects of Fe(II) and aging will exist and extend to column systems with flowing oxidants including O_2, NO_3^-, and NO_2^- and their combination. These studies will help us to understand the effects of mass transfer resistance on the remobilization of biogenic U(IV) in field relevant systems.
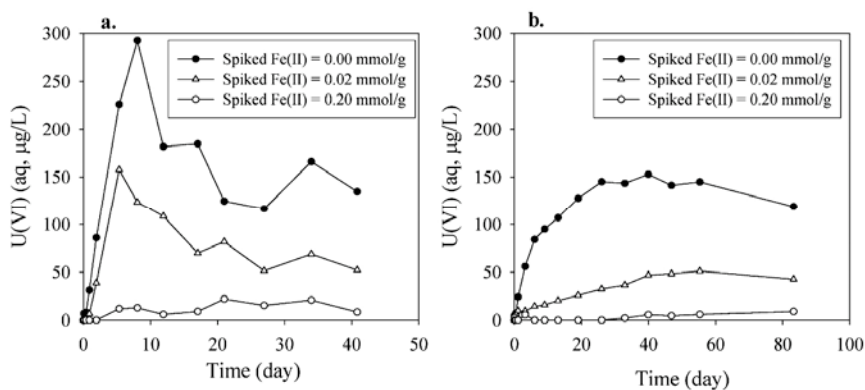
Fig. 4. Evolution of aqueous U(VI) released from the remobilization of biogenic U(IV) in presence of O_2 (atm) and NO_3^- (0.1 M). a.) Bioreduced sediments; and b.) Sediments treated by DCB to remove iron oxides. Both sediment suspensions contained 3800 ug/L of U(IV). The spiked Fe(II) concentration was provided in the Figure legends.

3.3 Metabolic Uncoupling

Microbial processes form the crux of some of the most common environmental engineering applications; examples include water treatment and subsurface remediation. Microbial applications are based on the bacterial ability to consume organics, metals and other waste products as electron-donors or accepters. This is accompanied by generation of biomass, which might be desirable in many processes as an end-product. However, the efficiency and operational cost of some of these processes is adversely affected by excessive biomass-production, resulting from lack of control over microbial growth. An example could be the disposal of highly organic sludge waste from Activated Sludge treatment scheme, which poses significant problems [Liu and Tay 2001; Liu 2003; Mayhew and Stephenson 1997; Wei et al. 2003]. Similarly, excessive biomass generation during in-situ subsurface bioremediation can lead to loss of process control or complete process failure (Anderson et al. 2003).
It has been observed by a number of researchers that, under certain growth conditions, there can be a drastic drop in cellular yield. Senez [Senez 1962] was the first one to point out this anomaly, and observed roughly 50% reduction in the yield of *Desulfovibrio desulfuricans* (strain Berre S). It is now known that the presence of excess substrate, addition of organic protonophores, toxins such as heavy metals & antibiotics, and other thermal or physico-chemical processes can lead to a partial dissociation between catabolism and anabolism [*Chen, et al.*, 2002; *Cook and Russell*, 1994; *Liu and Chen*, 1997; *Liu, et al.*, 1998; *Liu and Tay*, 2001; *Mayhew and Stephenson*, 1997; *Wei, et al.*, 2003; *Ye, et al.*, 2003]. This process is generically termed as metabolic uncoupling, and is characterized by a decrease in the amount of cell mass produced per unit growth substrate processed (i.e., the observed cell yield).

Most metabolic uncoupling studies have been restricted to mixed cultures. These include the development of mathematical models for quantification of cell yield and degree of uncoupling [*Liu*, 1998; *Liu and Chen*, 1997; *Zhang, et al.*, 1992]. There is a lack of literature on metabolic uncoupling of pure cultures, although many of them have been shown to be very useful for in-situ remediation activities. A large number of Department of Energy (DOE) sites are contaminated with heavy metals and chlorinated solvents like uranium, cadmium, TCE, PCE etc. It has been proposed that some metals and radionuclides might be bioremediated at these sites by promoting the growth of microorganisms that can use the contaminant metals and radionuclides as terminal electron acceptors, which leads to a reduction in their oxidation state. This is usually accompanied by a decrease in contaminant solubility, which renders them immobile. *Shewanella oneidensis* MR-1 is a facultative dissimilatory metal reducing bacteria (DMRB) that has been widely used as a model organism for inducing metal reduction. In application, generation of excess biomass in the subsurface during in-situ remediation activities poses problems. It can lead to changes in the composition of subsurface microbial population and possible failure of the bioremediation scheme [*Anderson, et al.*, 2003]. Controlling cellular metabolism by promoting metabolic uncoupling could provide a means for controlling biomass growth, leading to successful application of microbial processes in engineered systems.
Any microbial process is difficult to employ in the real world unless it is accompanied by a quantitative process model. Existing models for metabolic uncoupling describe either (1) the effect of excess-substrate [*Liu*, 1996], or (2) the effect of a chemical uncoupler [*Liu and Tay*, 2000a]. However, no model is currently available to explain the behavior of microbes under the influence of both the excess-substrate and the presence of a chemical uncoupler. The current

study aims to present and verify a comprehensive model to explain the simultaneous effect of these two conditions.

Excess-substrate conditions, as defined in this study, refer to growth conditions (measured in terms of S0/X0) that lead to an observed cell yield which is less than the theoretical maximum value. Batch experiments were conducted using a pure culture of wild type *S. oneidensis* MR-1 grown with pyruvate as the sole energy/carbon substrate, under aerobic conditions. The cellular yield is modeled as a function of relative initial substrate concentration (S0/X0) and relative uncoupler concentration (Cu/X). The degree of uncoupling is quantified by using an uncoupling coefficient, defined as the ratio of fractional reduction in the cell yield to the theoretical maximum observed yield ($(Y_{obs})_{max}$). The compound 3, 3', 4', 5-tetrachlorosalicylanilide (TCS), a component in the formulation of soaps, rinses and, shampoos, was used as the model uncoupler in the current work. A number of previous studies have shown it to be an efficient chemical uncoupler for activated sludge cultures. TCS is capable of reducing the cellular yield effectively at lower concentrations compared to other uncouplers [*Chen, et al.*, 2002; *Chen, et al.*, 2000; *Ye, et al.*, 2003].

Initial experimentation was focused on quantifying and modeling the effect of excess-substrate conditions on cell yield, which is defined as the ratio of cell mass to the amount of substrate utilized. Later, experiments were conducted to assess the uncoupling effect of the chemical uncoupler on the cell yield of *S. oneidensis* MR-1 cells grown under excess-substrate conditions. The cell growth was measured both in terms of number of cells as well as cellular protein content. The comprehensive uncoupling model was verified using the data obtained from these uncoupling studies.

Although the phenomenon of metabolic uncoupling is well known, little work has been done to define the mechanism of uncoupler action. Futile cycles, dissipation of membrane potential, ATP hydrolysis, overflow metabolism, and other processes have been discussed as the possible mechanisms of energy loss by cells [*Liu and Tay*, 2001; *Russell and Cook*, 1995]. Perhaps the best understood mechanism is the loss of proton motive force (PMF) due to a reduction in cell membrane resistance caused by some uncoupling chemicals. A class of these chemicals, also known as the protonophores (e.g., TCS, DNP etc.), is capable of short-circuiting the proton flow through the cellular membrane. This loss of proton gradient across the cellular membrane would lead to a decrease in ATP generation and subsequent energy wastage [*Cook and Russell*, 1994]. The exact mechanisms by which metabolic uncoupling is induced in other cases (e.g., heat stress, S0/X0 value etc.) still remain to be explained comprehensively. The current study makes an attempt to explain a possible pathway for metabolic uncoupling in terms of changes in cellular protein levels. The results of this study are expected to make a contribution towards a better understanding of metabolic uncoupling and bacterial growth.

An example of the influence of a chemical decoupling compound on the batch culture growth of *S. oneidensis* MR-1 cells appears in Fig. 5. In this figure, the effects of the chemical uncoupler 3, 3', 4', 5-tetrachlorosalicylanilide are shown on batch growth in 10% TSB media.
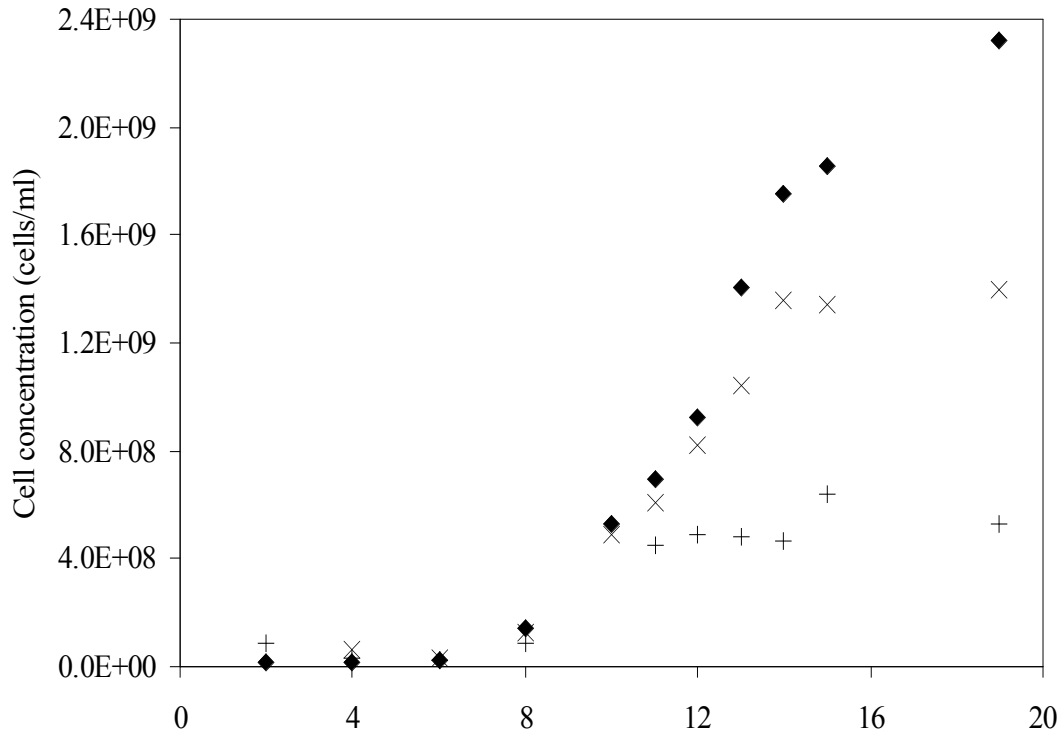
Fig. 5. Effect of uncoupler addition on cells grown in 10% TSB media. The symbol ( ) represents control culture, (x) represents culture with 2, 4-DNP addition and (+) represents culture with TCS addition.

4 Project Productivity

The following (eight) peer-reviewed papers were produced (supported in whole or part) by this project. In addition, two masters theses (both currently in preparation for publication) were prepared on the contract. The papers and theses themselves are attached in Appendix A.

Overall, we had reasonable success in developing the hypotheses represented in Sections 1.1, 1.3, and 1.4. Time did not allow the hypotheses represented in Section 1.3 to be explored in any significant detail, and similar hypotheses have since been adopted by other NABIR researchers. The papers containing the scientific information from this research are listed as follows.

(1) Wood, B.D., F. Cherblanc, M. Quintard, and S. Whitaker, 2003. Volume averaging for determining the effective dispersion tensor: Closure using periodic unit cells and comparison with ensemble averaging, Water Resour. Res., 39(8), 1210, doi:10.1029/2002WR001723.

(2) Wood, B. D., M. Quintard, and S. Whitaker (2002), Calculation of effective diffusivities for biofilms and tissues, *Biotechnology and Bioengineering*, *77*, 495-516.

(3) Wood, B. D., M. Quintard, and S. Whitaker (2004), Estimation of adsorption rate coefficients based on the Smoluchowski equation, *Chemical Engineering Science*, *59*, 1905-1921.

(4) Wood, B.D., Quintard, M., Golfier, F. and Whitaker, S., 2002, Biofilms in Porous Media: Development of Macroscopic Transport Equations via Volume Averaging with Closure, in Computational Methods in Water Resources, vol. 2, 1195-1202, edited by S.M. Hassanizadeh, R.J. Schotting, W.G. Gray and G.F. Pinder, Elsevier, Amsterdam.

(5) Ginn, T.R., B.D. Wood, K.E. Nelson, T.D. Scheibe, E.M. Murphy, and T.P. Clement, 2002. "Processes in Microbial Transport in the Natural Subsurface," Advances in Water Resources. 25:1017-1042.

(6) Scheibe, T.D., and B.D. Wood, A particle-based model of size or anion exclusion with application to microbial transport in porous media, Water Resour. Res., 39(4), 1080, doi:10.1029/2001WR001223, 2003.

(7) Zhong, L., C. Liu, J. M. Zachara, D. W. Kennedy, J. E. Szecsody, and B. D. Wood (2005), Oxidative remobilization of biogenic uranium(IV) precipitates: Effects of Iron(II) and pH, *Journal of Environmental Quality*, *34*, 1763-1771.

(8) Szecsody, J.E., Frichter, J.S., Willimams, M.D., Vermeul, V.R., and Sklarew, D. (2004), In Situ chemical reduction of aquifer sediments: Enhancement of reactive iron phases and TCE dechlorination, Environmental Science and Technology, 38: 4656-4663.

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6 Appendix A. Papers published, supported in part or in whole by NAIBIR-funded research (this project).

(Appendix A not available -- copyrighted material.)