Final Report:
**An *In Situ* Method for Establishing the Presence and Predicting the
 Activity of Heavy Metal-Reducing Microbes in the Subsurface**

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Introduction:

The primary objective of this research was to establish an *in situ* tracer method for detecting the presence, distribution, and activity of subsurface heavy metal-reducing microorganisms. Research focused on microbial systems responsible for the reduction of chromium and a suite of biotracers coupled to the reduction process. The tracer method developed may be used to characterize sites contaminated with chromium or expedite bioremediation; and although research focused on chromium, the method can be easily extended to other metals, organics, and radionuclides.

This brief final report contains three major sections. The first identifies specific products of the research effort such as students supported and publications. The second section briefly presents major research findings, while the last section summarizes the overall research effort.

Research Products:

Funding for this research supported two PhD students and graduated one Masters (see Table 1). One of the students, Dr. Kanika Sharma, was a runner-up for the Young Scientist Award in the Agrochemical Division of ACS.

From the two dissertations and one thesis, one book chapter was published, and a total of and 11 journal articles are expected (see Table 2). Six of the journal articles are in various stages of peer-review and draft copies can be provided. Finally, 7 oral presentations of research results were made at national meetings (See table 3).

Significant Findings:

The first four papers under Table 2 present results of laboratory research investigating the effects of various biotracers (electron donors and acceptors) on chromate reduction by indigenous Cr(VI)-reducing bacteria isolated from Cr(VI) contaminated soils. Enrichment cultures were established to maximize the diversity of chromium reducing bacteria recovered from the highly contaminated soil. Anaerobic enrichments utilizing a variety of electron donating tracers, including acetate, lactate, citrate, glucose, and

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benzoate were coupled with electron accepting tracer systems that included combinations of Fe(III), Cr(VI), and the humic acid analog anthraquinone disulfonate (AQDS).

Table 1: Students Supported Under the Grant

Student	Degree and Date	Department	Title of Dissertation/Thesis
Mohamed M.A. Mohamed	PhD. 2001	Civil and Coastal Engineering	METABIOTRANS: A 3-D finite element model for metals transport and biotransformation in saturated groundwater
Kanika Sharma	PhD. 2002	Soil and Water Science	Microbial Cr(VI) reduction: Role of electron donors, acceptors, and mechanisms, with special emphasis on <i>Clostridium</i> Sp
Khansith Boupah	MS. 2000	Civil and Coastal Engineering	Applying laplace Transforms and the De Hoog algorithm to solve contaminant transport equations.

Bacterial isolates from these cultures were screened for growth as obligate anaerobes and as facultative anaerobes, and characterized by 16S rDNA sequence analysis. The glucose-Fe(III)-AQDS –Cr(VI) enrichment [now on referred as GCAF] yielded the highest diversity of strains, most of which belonged to the low GC division. The facultative strains included *Bacillus* and *Cellulomonas*. The obligate anaerobes included *Clostridium*.

Among the various electron donating biotracers tested, enrichment cultures supplemented with glucose showed the fastest microbial Cr(VI) reduction, suggesting; (i) many rapidly growing Cr-reducing bacteria preferred glucose as an electron donor; and (ii) organisms enriched by glucose were more efficient at reducing Cr(VI) than those enriched by other biotracers.

Enrichments with Cr(VI) as sole electron acceptor showed insignificant microbial growth and Cr(VI) reduction. The effect of additional electron acceptors was apparent when a higher rate of Cr(VI) reduction was observed in the presences of biotracers Fe(III) or AQDS, a humic acid analog. In the presences of both AQDS and Fe(III), the rate of

Table 2. Reviewed Publications

Paper Number	Title	Authors	Type	Status
1	Analysis of microbial community structures	Ogram, A.V. and K. Sharma	Book Chapter. <i>Manual of Environmental Microbiology, ASM</i>	Published. 2002
2	<i>Clostridium chromoreductans</i> sp. nov., a novel anaerobic Cr(VI)-reducing bacterium isolated from soil	Sharma, K., H. Alrich, K. Hatfield, and A.V. Ogram	Journal article. <i>Int. J. System. Evol. Microbiol.</i>	In Review (draft copies available)
3	Metal reduction by <i>Clostridium chromoreductans</i>	Sharma, K., K. Hatfield, and A.V. Ogram	Journal article	In preparation
4	Role of electron donors and acceptors in Cr(VI) reduction in soil microcosms	Sharma, K., K. Hatfield, and A.V. Ogram	Journal article	In preparation
5	Modeling microbial-mediated reduction in batch reactors	Mohamed, M.M. and K. Hatfield	Journal article <i>ASCE J. Envir. Eng.</i>	In Review (draft copies available)
6	MDL groundwater software: Laplace transforms and the De Hoog algorithm to solve contaminant transport equations	K. Boupha, M. Jacobs, and K. Hatfield	Journal article <i>Computers and Geosciences</i>	In Review (draft copies available)
7	Sensitivity Analysis of metals biotransformation in two-dimensional heterogeneous aquifers	Mohamed, M.M., and K. Hatfield	Journal article <i>Journal of Contaminant Hydrology</i>	In Preparation

Table 2. Reviewed Publications Continued

Paper Number	Title	Authors	Type	Status
8	Monte Carlo Evaluation of heavy metal biotransformations in two-dimensional heterogeneous aquifers	Mohamed. M.M., K. Hatfield, and A. Hassan	Journal article <i>Journal of Hydrology</i>	In Review (draft copies available)
9	Bioremediation of heavy metal contaminated groundwater under natural gradient conditions	Mohamed. M.M., and K. Hatfield	Journal article <i>Journal of Contaminant Hydrology</i>	In Preparation
10	Application of moment analysis for the inverse estimation of biological parameters associated with heavy metal biotransformations in the subsurface	Mohamed. M.M., and K. Hatfield	Journal article <i>Journal of Contaminant Hydrology</i>	In Preparation
11	Enhancing gradient-based parameter estimation with an evolutionary approach (In Review)	Agyei. E. and K. Hatfield	Journal article <i>Journal of Hydrology</i>	In Review (draft copies available)
12	Simultaneous in situ measurements of specific discharge and Cr(VI) mass flux in porous media	T.J. Campbell, K. Hatfield, M. Annable, and P.S.C. Rao	Journl article <i>ES&T</i>	In Review (draft copies available)

Table 3. Presentations			
Presentation Number	Title	Authors	Reference
1	Numerical solutions for biological reactions in groundwater	Mohamed M.M.A., and K. Hatfield	<i>EOS</i> .1999 Fall meeting of the AGU. San Francisco. CA. pp F308. 1999
2	Quinone mediated reduction of Cr(VI)	Sharma, K., K. Hatfield, and A.V. Ogram	100 th American Society of Microbiology Meeting, Los Angeles, CA. 2000.
3	Biological treatment of metals in the subsurface	Mohamed M.M.A., and K. Hatfield	<i>EOS</i> .2001 Fall meeting of the AGU. San Francisco. CA. pp S99. 2001
4	Isolation and characterization of Cr(VI) reducing bacteria	Sharma, K., K. Hatfield, and A.V. Ogram	101st American Society of Microbiology Meeting, Orlando, FL. 2001.
5	An iterative solution for modeling microbial-mediated metal reduction	Mohamed M.M.A., and K. Hatfield	<i>EOS</i> .2001 Fall meeting of the AGU. San Francisco. CA. pp S147. 2001
6	Humic mediated reduction of Cr(VI) by a <i>Clostridium</i> sp GCAF-1	Sharma, K., K. Hatfield, and A.V. Ogram	102nd American Society of Microbiology Meeting, Salt Lake City UT. 2002.
7	Cr(VI) reduction by <i>Clostridium chromreductens</i>	Sharma, K	National meeting of the American Chemical Society, New Orleans, LA

Cr(VI) reduction was further augmented. These results indicated the following: (i) microbially-mediated reduction of Cr(VI) occurred as result of cooperative activity between Fe(III)-reducing, AQDS-reducing, and Cr(VI)-reducing bacteria, and (ii) a reduction in Cr(VI) toxicity occurred in the presence of Fe(III) or AQDS.

Detailed kinetic studies were conducted with an enriched Cr(VI)-reducing consortium that was dominated by organisms belonging to *Clostridium* sp. Reduction of Cr(VI) was observed only in the presence of an additional electron accepting biotracer [Fe(III) or AQDS]. The rate of Cr(VI) reduction observed was highest when both Fe(III) and AQDS were present in the medium compared to Fe(III) alone. It was speculated that microbially generated Fe(II) acted as an electron shuttle in the reduction of Cr(VI). In the presence of AQDS, the rate of Cr(VI) reduction increased due to the stimulatory effect of AQDS on Fe(III) reduction. Research results supported the role of AQDS as an electron shuttle to enhance Fe(III) reduction.

Similar studies were conducted with GCAF-1, a pure isolate from GCAF consortium. The focus of the studies was to elucidate the mechanism of Cr(VI) reduction by the *Clostridium* sp. and to compare Cr(VI) reduction rates of consortium and the pure isolate. These studies showed comparable rates of Cr(VI) reduction in the presence of Fe(III) and AQDS. Results suggested that the Cr(VI) reducing ability of GCAF-1 was maintained at the same level when in isolation or when in a consortium with other organisms. Studies further demonstrated the requirement of Fe(III) by *Clostridium* sp. GCAF-1 to reduce Cr(VI) concentrations as high as 20 ppm. Studies also showed that the amount of Cr(VI) reduced per mole of glucose in Fe(III) systems was lower than when AQDS was present along with Fe(III) in the medium.

Research showed GCAF-1 was capable of reducing 5 ppm Cr(VI) directly and in the absence of Fe(III) and AQDS. It was not determined if *Clostridium* sp. GCAF-1 was capable of growing when Cr(VI) was the only available electron acceptor. Reduction of Cr(VI) by GCAF-1 was found to be dependent on the presence of glucose as no reduction occurred in the absence of glucose.

An analysis of metabolic products of the consortium and GCAF-1 revealed that acetate, lactate, and butyrate were the major biotracers or fermentation products formed during glucose oxidation. No difference was observed in the ratios of metabolic products of cultures grown in the absence of an external electron acceptor and those grown in the presences of Fe(III) and AQDS. However, cultures grown with Fe(III), AQDS, and Cr(VI) showed an increase in the acetate/butyrate and acetate/lactate ratios. These changes in cell metabolism may serve two purposes; (i) energy generation by adenosine triphosphate (ATP) formation; and/or (ii) reduction of Cr(VI) to detoxify the immediate environment. Formation of acetate in the glycolytic pathway is coupled with ATP production, and an increase in acetate concentration is perhaps a strategy adopted by strain GCAF-1 to generate more energy to survive in toxic Cr(VI) environments. A decrease in butyrate and lactate concentrations was accomplished by reduction of Cr(VI), suggesting that NADH, the electron donor for the reduction of pyruvate to butyrate and

lactate was channeling its electrons toward the reduction of Cr(VI) via the electron transport chain.

The primary focus of **Paper 2**, was the identification and phylogenetic characterization of isolate GCAF-1. Efforts revealed GCAF-1 strain belonged to the genus *Clostridium*. DNA-RNA hybridization with all type strains showing 98% similarity in 16s rDNA indicated the strain to be a novel *Clostridium* species. GCAF-1 also exhibited the presence of at least two 16s rDNA copies. Other detailed biochemical analyses of GCAF-1 were documented in this study. In view of the ability of this strain to reduce Cr(VI), the species name *Clostridium chromoreductans* sp. nov. was proposed.

From the above described research findings, that characterize of the effects and biochemical roles of various biotracers (electron donors and acceptors) in the reduction of chromate by indigenous Cr(VI)-reducing bacteria, several mathematical models were developed to describe the fate and transport of Cr(VI) and biotracers in batch systems and in Cr(VI) contaminated soils. These models are fully described in **Papers 5, 6, 7, 8, and 9**. Models were needed in subsequent research efforts to define relationships between measurable quantities such as biotracer and Cr(VI) concentrations and mass fluxes and the presence, distribution, and activity of subsurface heavy metal-reducing microorganisms.

The focus of **Paper 5** was to summarize an investigation of numerical solutions to a system of coupled non-linear ordinary differential equations (ODE) that describe the reduction of chromate by indigenous Cr(VI)-reducing bacteria in batch reactors. Four methods were evaluated including (1) the Quasi-steady-state-approximation (QSSA), (2) the Finite difference method (FDM), (3) the Taylor expansion series approximation, and (4) the Fourth-order Runge-Kutta method (RKM). Solutions from each method were compared to those of analytical solutions and another numerical model (BIOKEMOD). Both the QSSA and the FDM produced good solutions compared to those of the analytical solution and the RKM; however, the QSSA showed slightly better agreement with the RKM than the FDM. The Taylor series expansion, on the other hand, delivered inaccurate results.

The QSSA was easily implemented to solve the coupled biological ODE's, and it proved to be a robust solution method. For these reasons, it was selected to model experimental data of Cr(VI) reduction in batch experiments. The QSSA method delivered excellent simulations of the Cr(VI) reduction compared to the laboratory observations. The strong agreement between QSSA results and those of RKM, BIOKEMOD, analytical solution, and laboratory data suggests that QSSA can be used with more complicated cases when more than one substrate is being utilized in batch culture experiments. Furthermore, QSSA can be easily implemented to solve systems of nonlinear biological reaction ODEs derived from coupled subsurface transport equations.

Paper 6 presented the MDL Groundwater Modeling system which was specifically developed to provide a suite of models suitable for simulating subsurface reactive transport of contaminants (i.e., chromium and organics), biotracers, and microorganisms.

Several models within the program were used to investigate the fate and transport of biotracers and microorganism associated with the reduction of chromium: while others were created to expand an understanding of various physical, biological, and chemical processes affecting the fate and transport of Cr(VI) and reducing microorganisms in one-, two- and three-dimensional systems. Simulated transport processes were advection and dispersion in mobile pore water and rate-limited contaminant mass transfer between mobile and immobile pore water regions. Constituent reactions incorporated in various models included first-order production and decay and linear reversible sorption that was either instantaneous or rate-limited. Between models, a choice of contaminant source-loading functions were incorporated including as constant contaminant fluxes, pulse loads, and exponential decaying mass fluxes. Each particular model was represented in MDL as a closed-form Laplace-space or frequency domain solution to a specific set of transport equation(s), boundary conditions and initial conditions. Concentrations of tracer, contaminant, or microbial transport in the time-space domain were simulated using the De Hoog algorithm to numerically invert each analytical Laplace-space model.

Papers 7, 8, and 9 describe and apply a three-dimensional finite element model, METABIOTRANS that was developed for simulating the transport and biotransformation of metals in saturated groundwater. The model simulates steady-state groundwater flow in saturated porous media, transient transport of multiple solutes (i.e., metals, hydrocarbons) in heterogeneous/anisotropic porous media, sorption of dissolved constituents, microbially-mediated redox reactions between multiple electron donors and acceptors, and growth of multiple bacterial species coupled to redox reactions. METABIOTRANS uses Michaelis-Menten kinetics relationships to describe the reduction of available electron acceptors, the consumption of electron donors, and the growth of microbial biomass. These relationships were modified to incorporate two different mechanisms of microbial growth inhibition. The first mechanism evolves from a competition for the most desired electron acceptor in the aquifer: while the other inhibition mechanism reflects the toxicity of the metal itself on bacterial species.

METABIOTRANS was verified using analytical solutions to eleven complex problems. These problems were employed to evaluate the model performance under different groundwater flow, transport, and boundary conditions for one, two, and three-dimensional problems. Results of the validation study suggest METABIOTRANS is capable of simulating the transport of multi-solutes in heterogeneous/anisotropic saturated aquifers as influenced by advection, dispersion, sorption and biodegradation/biotransformation under different modeling conditions. Once the model was verified, it was used to study three important aspects of remediating sites contaminated with Cr(VI). The first aspect was to determine the relative importance of various biological parameters on the natural attenuation of Cr(VI) in the subsurface (**Paper 7**). The second aspect was to investigate effects of both physical and biological heterogeneities on Cr(VI) fate and transport [**Paper 8**]. And finally, **Paper 9** examined the effects of nutrients and electron donor availability on the in situ active remediation of Cr(VI) contaminated groundwater.

Paper 7 explored the nature of various microbial reaction parameters incorporated in METABIOTRANS. Sensitivity analyses were performed with the maximum microbial growth rate, $\mu_{\max}$; the half saturation coefficient for the electron donor, K_a ; the half saturation coefficient for Cr(VI), K_c ; the cell death rate, B ; and the cell growth inhibition factor, K_i . The objective was to assess parameter effects on the attenuation of Cr(VI) and the growth of the metal-reducing bacteria. Two and Three dimensional transport examples were presented. Ten different model runs were used in each example to elucidate the significance of each microbial parameter.

Increasing $\mu_{\max}$, increased the rate of Cr(VI) reduction and, therefore, reduced the time required for natural attenuation. Changing this parameter did not alter the transient behavior of first and second spatial moments of the Cr(VI) plume normalized with respect to the current plume mass. Bacterial biomass distribution was quite sensitive to a change in $\mu_{\max}$. For example, an increase in $\mu_{\max}$ by a factor of four caused a 300% increase in the biomass. Increasing the microbial growth rate produced a shift in the biomass centroid towards the location of the initial Cr(VI) release and, as a result, increased the skewness of the microbial distribution. The variance and the peakness of the biomass distributions were not sensitive to perturbation in the microbial growth rate. Perturbing the Cr(VI) half saturation coefficient (K_c) produced the opposite effect of the microbial growth rate when K_c was much greater than the local Cr(VI) concentration. As K_c approached the magnitude of local Cr(VI) concentrations the effect of the half saturation coefficient was largely dependent on metal concentrations and the inhibition factor, K_i .

In general, both metal and bacterial concentrations were not extremely sensitive to variations in the inhibition factor (K_i) as compared to the other parameters. However, an order of magnitude variation in its value produced a significant change in the biomass distribution. The inhibition factor forced bacteria to grow more uniformly in the aquifer. Therefore, minor changes were observed in the centroid and skewness of microbial biomass distribution. In addition, unlike other parameters, reducing the inhibition factor slightly changed the contaminant variance in both longitudinal and transverse directions.

Increasing the microbial death coefficient (B) decreased the rate of biomass accumulation in the aquifer and, therefore, less Cr(VI) was reduced. Thus, B affected the size of the Cr(VI) plume and also the second, third, and fourth moments [normalized by the initial Cr(VI) mass] of the Cr(VI) plume. Unlike the maximum growth rate coefficient, the microbial death coefficient equally affects all bacteria in the aquifer independent of the Cr(VI) concentration. Because of this, no effect was observed on the biomass distribution variance, skewness, or kurtosis.

Finally, the effects of all these biological parameters on the contaminant mass flux were investigated. Mass flux was most sensitive to variations in $\mu_{\max}$ and K_c and least sensitive to K_i and B . Hence, measuring fluxes could be one way of estimating the growth and half saturation coefficients.

Paper 8 presents results of METABIOTRANS used in a Monte Carlo scheme to evaluate the effects of both physical (hydraulic conductivity) and biological (initial biomass concentrations) heterogeneities on metal biotransformations in the subsurface. In addition, effects of a correlation between both heterogeneities were also addressed. Four general cases were investigated. In the first case, only physical heterogeneity was considered. Biological heterogeneity was examined next; and then for the third case, the effects of both heterogeneities were studied as spatially correlated processes. Finally, in case four, the effects of variable biotracer concentrations were investigated. Within each case, a sensitivity analysis was performed involving other parameters such as the microbial maximum growth rate and the initial biomass concentration variance in the aquifer.

Physical heterogeneity alone did not affect the estimation of the mass of Cr(VI) reduced (compared to the deterministic case) as long as uniform initial biomass and constant biological parameters were assumed. As expected, physical heterogeneity increased dispersion in both longitudinal and transverse directions and the skewness and the peakness of metal concentration distributions normalized with the current Cr(VI) mass; nonetheless, these changes were indistinguishable between reactive metal and non-reactive constituents. For moments normalized by the initial Cr(VI) mass, effects of biological activity on contaminant distributions were not obscured. For example, it was evident from the second moments that Cr(VI) concentrations variances decreased with respect to a non-reactive contaminant; and that increasing the microbial maximum growth rate magnified such differences. Finally, the increase in the hydrodynamic dispersion (created by physical heterogeneity) enhanced Cr(VI) transport which in turn encouraged greater than expected microbial growth such that higher ensemble mean biomass concentrations appeared further down-gradient than simulated with a homogeneous aquifer.

Physical heterogeneity had no effect on the cumulative mass flux, although, peak mass fluxes were attenuated due to increased dispersion. Two periods of high uncertainty in mass flux were observed: the first occurred as the leading edge of the plume approached the section, while the other occurred as the trailing edge traversed the same section. Biotransformation decreased Cr(VI) concentrations between sections, which in turn decreased the cumulate mass flux and the flux variance. For the same reason, a greater decrease in the flux variance was observed at the trailing edge of the plume than at the leading edge.

Biological variability alone did not change the reduction in Cr(VI) mass compared to that of the homogeneous case when a low growth rate was used. However, when higher microbial maximum growth rate was used, the homogeneous case overestimates the biotransformed mass of Cr(VI) and therefore, underestimates the time required to remediate an aquifer. Thus, for remediation scenarios characterized with low microbial growth rates, a homogeneous assumption could be used to obtain an acceptable approximation of the required remediation time. The higher the microbial growth rate the more noticeable the influence of the biological heterogeneity on changes in Cr(VI) plume size, moments, and mass fluxes.

Increasing the spatial variance of the initial biomass distribution resulted in a decrease in estimated bioremediation efficiency. An increased biomass variance produces regions of extremely high and low biomass concentrations: as a result, opportunities increase for less efficient interception with the Cr(VI) plume and microbial reduction of Cr(VI). Increasing biological heterogeneity increases all spatial moments normalized by the initial Cr(VI) mass as compared to the homogeneous case. Little if any effect was observed with moments normalized by the current Cr(VI) mass.

When negative correlationw between physical and biological heterogeneities were considered, higher biological activity was expected in zones of lower groundwater velocity and vise versa. Thus, a negative correlation lead to expected Cr(VI) concentrations at the leading edge that were higher than those for the case of zero correlation; and the opposite was simulated at the trailing edge of the plume. Finally, increasing the microbial growth rate augmented differences simulated between the two cases.

When a positive correlations were considered, enhanced Cr(VI) reduction was predicted over the high velocity regions. Effects on the ensemble mean concentrations of Cr(VI) were opposite to those observed with a negative correlation. Increasing the variance of initial biomass concentration from 1.0 to 3.0 (assuming positive correlation), decreased effectiveness of the natural attenuation of Cr(VI). In addition, uncertainties in the mean Cr(VI) concentration increased.

When the correlation between the physical and biological heterogeneities was changed from negative to positive, the initial position of the bacterial center of mass changed accordingly from 0.07 to -0.07 meters (referenced to the center of the aquifer). This occurred because random generations of biomass distributions were derived from a common spatial distribution of hydraulic conductivities, which exhibited slightly higher conductivities in the upgradient half of the flow domain. Because all other parameters were preserved between simulations, the trajectory of the first moment followed parallel paths. This means that the correlation between physical and biological heterogeneities did not affect the trajectory of the biomass centroid. Similar effects were also seen in other spatial moments (i.e. curves of the different spatial moments of negative and positive correlations were on opposite sides of that of the simulation where biological heterogeneity was ignored). The effect of changing the variance of the initial biomass concentration is more crucial than that of the type of the correlation itself (positive or negative) on the spatial moments of both Cr(VI) plume and biomass distribution. Using a unit spatial variance of the initial biomass was not enough to visualize the importance of the correlation to predicting the mean mass fluxes. For example, when the variance was increased to three there was a distinctive increase in the expected mass flux and mass flux variance.

Variability in the initial concentrations of electron donor reduced microbial growth; and it increased the uncertainty in estimated Cr(VI) concentrations. For the conducted simulations, there was at least a 30% reduction in the biotransformation efficiency (total

biotransformed Cr(VI) mass in the aquifer). Like biological variability, electron donor variability does not affect the dispersion of the ensemble mean concentration of Cr(VI). However, this was not the case when Cr(VI) initial mass was used to normalize moments. Clear increases were seen with second, third and fourth moments. On the other hand, a decrease in spatial moments of the biomass mean concentration is observed. Finally, ignoring variability in the initial concentration of an electron donating biotracer can lead to an underestimation of the mean mass flux and the uncertainty associated with that flux.

In **Paper 9**, remediation of a hypothetical Cr(VI) plume was studied through 36 different scenarios. These scenarios were designed to investigate the effects on remediation produced by modifying the number and position of injection wells and the injection rates of nutrient and electron donor. This paper also examined the sensitivity of remediation scenarios to the inhibition induced by elevated nutrient and electron donor concentration. Simulations showed placing the nutrient and electron donor injection wells near the location of the initial contaminant release did not produce efficient remediation. Furthermore, treatment wells should not be situated an excessive distance downgradient the point of Cr(VI) release, as treatment of a much larger plume could become problematic. Locating injection well(s) a moderate longitudinal distances downstream of the initial contaminant release (5 m in the simulations presented in this study) produced the highest efficiencies of Cr(VI) reduction.

Increasing the number of wells and injection rates clearly had a direct effect of increasing Cr(VI) reduction in the aquifer. In general, line sources produced the highest levels of Cr(VI) transformation. This is not to say that this injection pattern made the most efficient use of electron donors and nutrients. Hence, there appears exist a need for coupling METABIOTRANS to an optimization algorithm for the purpose of identifying an optimal remediation design that considers not just the physical/biological processes currently presented in the model but also the effective delivery of required nutrients and electron donors.

Finally, it was shown in **Paper 9** that it was important to accurately characterize the microbial species (or consortium) responsible for metal reduction. Inaccurate determination of constituent inhibition factors for Cr(VI), nutrients, or electron donors could undermine efforts to bring about effective remediation. For example, if added electron donors or nutrients induced growth inhibition at low levels, use of multiple-properly-spaced wells with lower injection rates was the preferred alternative to a single well where maintaining constituent concentrations below toxic levels would be more difficult.

Papers 10 and 11 respectively introduce direct and indirect inverse methods for estimating important microbial and physical parameters of a Cr(VI) reducing system using spatial data on microbial biomass and concentrations and fluxes of Cr(VI) and biotracers. To complement the inverse methods, a novel method for direct *in situ* measurement of Cr(VI) and biotracer fluxes was proposed and demonstrated in **Paper 12**.

Quantifying the presence, distribution, and activity of subsurface heavy metal-reducing microorganisms through biotracers and models requires estimation of flow, transport, and microbiological parameters. Using laboratory-based parameters in models to simulate field scale processes could result in false predictions of contaminant attenuation in the subsurface; which could in turn result in inaccurate assessments of remediation. Scaling relationships were developed that use the spatial moments of microbial biomass, Cr(VI), and biotracers (see **Paper 10**). These relationships used spatially distributed biomass concentrations and biotracer concentrations and fluxes to estimate the microbial parameters including the maximum growth rate coefficient ($\mu_{\max}$), the substrate half saturation coefficient (K_S), and the yield coefficient (Y_S). Two different cases were examined. In the first case, one microbial population was assumed to consume two solutes: one serves as electron donor, while the other acts as an electron acceptor. Two microbial populations are simulated in case two and assumed to compete for a common electron donor. The finite element model METABIOTRANS was used to test the scaling relationships. Results from several test cases validated the scaling relationships. In addition, it was shown that microbial parameter estimates were more robust if the scaling relationships used concentration and flux data rather than concentration data alone.

Traditional gradient-based inverse modeling methods have been used extensively in the past to estimate the parameters of an aquifer formation but not microbial distributions and biological parameters typically associated with highly nonlinear systems. The major drawback of gradient-based techniques is their inability to yield accurate results for highly nonlinear problems. Some of the inherent difficulties have been attributed to (1) the ill-posedness of the inverse problem, (2) the insufficiency of the quality and quantity of observation data, (3) the existence of several local minima that in turn preclude the finding of a global minimum, and (4) the domination of other errors associated with the model structure. **Paper 11** introduces a hybrid inverse modeling approach whereby a gradient-based parameter estimation algorithm was coupled to an evolutionary technique for purposes of estimating modeled biological and aquifer parameters for a nonlinear, coupled flow and solute transport. Results indicated the combined gradient-evolutionary method greatly improves parameter estimates but at a cost of increased computation time. The tool developed can be used in conjunction with an appropriate fate and transport model such as METABIOTRANS to estimate the presence, distribution, and activity of subsurface heavy metal-reducing microorganisms through biotracers.

Measuring Cr(VI) and biotracer fluxes can lead to a direct assessment of microbial activity and robust estimates of microbial parameters. In addition, a measured Cr(VI) fluxes can be used directly to assess the effectiveness of remedial activities. In **Paper 12**, a novel method is introduced for the simultaneous measurement of local groundwater specific discharge (q) and Cr(VI) mass flux (J_{Cr}) using a permeable adsorbent device. The proposed method allows for determination of both q and J_{Cr} as two-dimensional vectors, with each vector composed of an angular and a radial component. The device consists of a cylindrical container whose outer shell is constructed of highly permeable mesh material with impermeable end caps. The interior of the device is divided into a center well and three outer sectors, each packed with a granular anion exchange resin having high adsorption capacity for the Cr(VI) oxyanions CrO_4^{2-} and $HCrO_4^-$. The

adsorbent in the center well of the device is loaded with benzoate as resident tracer. Experiments were conducted in which these devices were placed in porous packed bed columns through which was passed a measured quantity of simulated groundwater containing Cr(VI). The resin was then removed from the four sectors separately and extracted to determine the masses of Cr(VI) and resident tracer bound in each. A nonlinear solute transport model was used to relate the observed rate of resident tracer displacement to vector components of discharge, and a simple mass balance equation was used to calculate J_{Cr} .

Experimental results showed q was measured to an accuracy of $\pm 15^\circ$. Accuracy of the radial specific discharge vector component depended on the degree of displacement of the resident tracer. Cr(VI) mass fluxes were measured to an accuracy of $\pm 17\%$. These results demonstrate the potential utility of permeable adsorbent devices for simultaneous measurement of local groundwater specific discharge and Cr(VI) mass flux in contaminated aquifers, and more generally for monitoring transport and attenuation rates of various groundwater contaminants. Further study is needed to test the accuracy and practicality of these devices in actual field conditions.

Summary:

The primary objective of this research was to establish an *in situ* tracer method for detecting the presence, distribution, and activity of subsurface heavy metal-reducing microorganisms. Research focused on microbial systems responsible for the reduction of chromium and a suite of biotracers coupled to the reduction process. Extensive laboratory experiments were conducted to establish relevant constitutive relationships between Cr(VI), microbial activity, and several biotracers including Fe(III), AQDS, and Glucose. Investigative effort lead to the identification of a novel *Clostridium* species capable of direct Cr(VI) reduction.

Batch reactor models and subsurface Cr(VI) transport models were developed based on the findings of laboratory experiments and the conceptual model of the Cr(VI)-glucose-AQDS-Fe(III) system. These models were used in subsequent research efforts to define relationships between measurable concentrations and fluxes of biotracers and Cr(VI) and the presence, distribution, and activity of subsurface heavy metal-reducing microorganisms. Sensitivity studies were performed to assess the importance of various microbial parameters on Cr(VI) reduction in groundwater. In addition, an exhaustive study was conducted to examine the effects of both physical (hydraulic conductivity) and biological (initial biomass concentrations) heterogeneities on metal biotransformations. Inverse modeling methods were developed to facilitate the estimation of field-scale biological parameters. One of the methods was direct while the other must be coupled to a transport model such as METABIOTRANS. Once essential microbial parameters have been estimated, predictions can be made that describe the spatial and temporal distribution of microbial mass, the potential efficacy of remediation, and the expected flux of chromate leaving the zone of active remediation.

Model Predicted Cr(VI) fluxes can be compared to measured fluxes using a new in situ approach introduced from this work. The data generated can be used to assess the performance of remedial activities.

Finally, funding from DoE supported two PhD students and one Masters. A total of 12 peer-reviewed papers are expected from this research effort; one has already been published and 6 are currently in review.