Transport and activity of dissimilatory metal-reducing bacteria in porous media for the remediation of heavy metals and chlorinated hydrocarbons
by Robin Gerlach

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Engineering
Montana State University
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Abstract:
Dissimilatory metal-reducing bacteria (DMRB) are capable of reducing a wide range of metals and the injection of DMRB is considered for the cleanup of contaminated soil and groundwater. The success of such an approach, among other factors, relies upon the bacterial transport through porous media and the contaminant transformation by DMRB.

Long term starvation of Shewanella algae BrY, the model DMRB used in this research, allows for significantly improved transport through quartz sand porous media columns. However, the reasons for the improved transport remain unclear. Changes in cell size, net electrostatic charge, hydrophobicity, buoyant density, and effective diffusion coefficient do not provide a sufficient explanation and this question will have to remain the focus of future research. In addition, it became evident that the employed mathematical model based on the colloid filtration theory is not capable of appropriately describing scale and physiology dependent effects on bacterial transport through porous media. A complete understanding of the processes and factors influencing the porous media transport of starved bacteria is still lacking and will remain as the focus of future research.

The reduction of chromium [Cr(VI)], a compound of significant environmental concern at many Department of Energy, Department of Defense, and Environmental Protection Agency Superfund sites, can be facilitated by DMRB. Starved S. algae BrY cells can be resuscitated into an actively metabolizing state in the presence of ferric iron [Fe(III)], which is abundant in the environment. Active S. algae BrY cells can either directly reduce Cr(VI) or produce surface reactive ferrous iron [Fe(II)]. Fe(II) chemically reduces and precipitates Cr(VI) eliminating it from contaminated water. S. algae BrY cells can also potentially contribute towards the long term reactivity of zero valent iron subsurface barriers. The microbial reduction of Fe(III) allows for the removal or activation of surface associated corrosion products and results in increased transformation rates of carbon tetrachloride, another widespread environmental contaminant.

The results summarized in this dissertation indicate that the injection of starved DMRB is a promising technology for the remediation of subsurface environments contaminated with heavy metals and chlorinated hydrocarbons.
TRANSPORT AND ACTIVITY OF DISSIMILATORY METAL-REDUCING BACTERIA IN POROUS MEDIA FOR THE REMEDIATION OF HEAVY METALS AND CHLORINATED HYDROCARBONS

by

Robin Gerlach

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Engineering

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APPROVAL

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This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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The results summarized in this dissertation indicate that the injection of starved DMRB is a promising technology for the remediation of subsurface environments contaminated with heavy metals and chlorinated hydrocarbons.
CHAPTER 1

INTRODUCTION

Every year, large quantities of organic and inorganic compounds are released into the environment as the result of human activities. These releases can be deliberate or accidental. Due to their toxicity and recalcitrance in the environment, many of these compounds can accumulate to unacceptable levels and pose a severe risk to humans and the environment. Davis (1972) was one of the first to suggest the application of bacteria in the remediation of contaminated groundwater. The use of indigenous or exogenous bacteria for the cleanup of contaminated groundwater has since become a widely applied approach.

In situ bioremediation has become one of the preferred technologies for the cleanup of contaminated sites since it avoids the need for excavation and subsequent disposal of hazardous waste. While the bacterial degradation of fuel hydrocarbons and polyaromatic hydrocarbons is well documented (Alexander, 1999; Crawford and Crawford, 1996), the use of bacteria for the cleanup of soils and groundwater contaminated with chlorinated hydrocarbons (U.S.EPA, 2000) and metals (Evanko and Dzombak, 1997; McCullough et al., 1999) remains the focus of much research.

The increased need and interest for biological metal and radionuclide remediation is partially based on more than 50 years of using nuclear energy for both peaceful and military purposes. Many Department of Energy (DOE) and Department of Defense (DOD) sites are contaminated with hazardous and radioactive waste. These sites create a legacy that is estimated to cost the DOE alone more than US $ 60 billion over the next 10 years (McCullough et al., 1999). The contamination at many DOE facilities is spread over large areas and deep below the surface (McCullough et al., 1999). Conventional treatment strategies, such as pump and treat or excavation with subsequent treatment, might not be practical, economical, or advisable due to the long term liability that might be created. Thus, the development of alternative strategies for the treatment of DOE sites can potentially save billions of dollars.
Two subsurface remediation strategies have received much attention over the recent years and have demonstrated potential for the development of economically viable, long term, low maintenance cleanup strategies.

1. The use of permeable reactive barriers (PRBs). PRBs established downstream of contaminated sites have proven to effectively prevent off-site migration of chlorinated hydrocarbons, metals, and radionuclides (Scherer et al., 2000; U.S.EPA, 1998; Yuyun and Allen, 1999).

2. The metabolism of dissimilatory metal-reducing bacteria (DMRB). DMRB have demonstrated potential for the remediation of groundwater contaminated with chlorinated hydrocarbons, heavy metals, and radionuclides (Amonette et al., 2000; McCullough et al., 1999; Workman et al., 1997).

Dissimilatory metal-reducing bacteria (DMRB) gain energy for growth by coupling the oxidation of organic compounds or hydrogen to the dissimilatory reduction of ferric iron [Fe(III)] and other oxidized metals. DMRB can enzymatically reduce large amounts of a wide range of metal ions, including Fe(III), Cr(VI) and U(VI) (Harding, 1997; Lovley, 1993; Lovley, 1995b). Although the direct enzymatic reduction of heavy metals by DMRB potentially allows for the development of remediation strategies, the indirect reduction of soluble contaminants such as Cr(VI), U(VI), and carbon tetrachloride might be more important and feasible in subsurface environments (Fredrickson and Gorby, 1996a; Harding, 1997; McCullough et al., 1999). DMRB can reduce a wide range of ferric iron minerals to produce redox reactive ferrous iron [Fe(II)] (Caccavo, Jr. et al., 1992). Ferrous iron can chemically react with soluble contaminants including Cr(VI), U(VI), and carbon tetrachloride (Amonette et al., 2000; Eary and Rai, 1988; McCullough et al., 1999).

Over the last decade, it has become apparent that the biological and geochemical processes described above can govern the fate of metals and radionuclides in the subsurface. Consequently, a number of governmental agencies have begun to support research addressing the fate of heavy metals and radionuclides in the subsurface. The DOE, for instance, established the Natural and Accelerated
Bioremediation Research (NABIR) program in 1997. The NABIR program focuses on the development of a strong theoretical foundation for the assessment and remediation of DOE sites contaminated with mixtures of metals and radionuclides. The high interest in the biogeochemical processes involved also recently led to a five day symposium on “Chemical-Biological Interactions in Contaminant Fate”, organized by the Division of Environmental Chemistry at the 220th American Chemical Society National Meeting in Washington D.C. (August 20-24, 2000). The majority of the presentations focused on the influence of iron minerals and dissimilatory metal-reducing bacteria on the fate of chlorinated hydrocarbons, heavy metals, and radionuclides. The presentations clearly demonstrated the importance of metal-reducing bacteria in contaminated subsurface environments.

The idea of utilizing DMRB to establish and maintain permeable reactive barriers (PRB) in the subsurface is intriguing. DMRB, present in the subsurface or injected into the subsurface, could be stimulated to produce redox reactive ferrous iron from indigenous or injected ferric iron. The produced ferrous iron would chemically react with chlorinated hydrocarbons, Cr(VI), U(VI), and other contaminants. However, reports of field trials evaluating the use of DMRB for the establishment of redox reactive subsurface barriers are lacking. Thus, this research project is designed to improve the basis for future field demonstrations involving the use of DMRB for environmental cleanup.

The major research goals of this dissertation are:

1. The development of a strategy for the enhancement of bacterial transport through porous media, and

2. The assessment of using dissimilatory metal-reducing bacteria for the \textit{in situ} bioremediation of heavy metals and chlorinated hydrocarbons.

These two main research goals are reflected in the structure of this dissertation. The first part of the introduction reviews the processes and parameters governing bacterial transport through porous media. This review is the basis for Chapters 2 and 3, which evaluate bacterial starvation as a promising strategy for the bacterial transport enhancement (Chapter 2), and document the changes in transport-related bacterial cell properties during long term starvation (Chapter 3). The second part of the introduction provides an
overview of the current literature on permeable reactive subsurface barriers and microbial metal-reduction. This overview provides the background for the Chapters 4 and 5, which describe the potential use of DMRB to establish and maintain redox reactive zones in the subsurface capable of the continuous precipitation of Cr(VI) (Chapter 4) and the potential of DMRB to influence the performance of zero valent iron redox reactive subsurface barriers (Chapter 5).

Bacterial Transport Through Porous Media for Bioaugmentation (Research Goal I)

The transformation rates achievable by stimulation of the indigenous microflora at contaminated subsurface sites are often insufficient to develop economically viable remediation technologies. An insufficient number of indigenous microorganisms capable of degrading the compounds of interest or the inability to effectively stimulate the indigenous microflora are commonly encountered reasons for low transformation rates (Vogel, 1996; Walter, 1997). Therefore, bioaugmentation, the injection of microorganisms which can biotransform environmental contaminants, has been proposed and implemented (Dybas et al., 1998; Ellis et al., 2000; McCullough et al., 1999; Salanitro et al., 2000; Steffan et al., 1999). Unfortunately, the injection of previously cultivated bacteria into subsurface formations can cause excessive biofilm growth in injection wells. The resulting clogging of well screens poses a major problem in the development of subsurface bioaugmentation strategies (Molz et al., 1986; Nelson et al., 1985, Malusis et al., 1997; Maxwell and Baqai, 1995). Thus, for the successful development of subsurface bioaugmentation strategies, the ability to avoid excessive adhesion, limit biofilm growth, and to facilitate bacterial transport through porous media needs to be improved. Much research has focused on the development of strategies for the enhancement of bacterial transport through porous media. However, a promising technology applicable to a wide range of bacterial strains and to different hydrogeological conditions has yet to be developed.

A bacterial strain or consortium chosen for subsurface bioaugmentation should have the following characteristics. The microorganisms should easily obtain regulatory approval for injection. The microorganisms should be easily transported to avoid the plugging of injection wells and readily distributed
over large areas. The cells should yet be adhesive enough to distribute themselves along the flowpath. They should be compatible with the indigenous microflora, i.e. they should not negatively influence the existing microflora, but should be able to survive and actively metabolize the compounds of interest. Bacterial strains or consortia for bioaugmentation should be inexpensive to obtain and easy to culture.

The following literature review on bacterial transport through porous media will justify the choice of bacterial starvation as a means for enhancing bacterial transport through porous media.

**Parameters Influencing Bacterial Transport Through Porous Media**

Bacterial transport is typically governed by aqueous phase advective movement coupled with retardation by adhesion onto surfaces and straining or trapping in interstitial pores. Bouwer et al. (2000), Mills (1997), Lawrence and Hendry (1996), and Harvey (1991) provide excellent reviews of the factors influencing bacterial transport through porous media. The numerous parameters influencing bacterial transport and adhesion in porous media can be grouped into three major categories, 1) solution chemistry, 2) porous media characteristics and hydraulic conditions, and 3) properties of the bacterial cells. These categories are discussed in the following paragraphs.

**Influence of Solution Chemistry on Bacterial Transport Through Porous Media.** A wide range of solute characteristics has been reported to influence bacterial adhesion to surfaces and transport through porous media. An increase in ionic strength has been correlated with increasing attachment (Fontes et al., 1991; Gannon et al., 1991b; Jewett et al., 1995; Mills et al., 1994; Scholl et al., 1990; Tan et al., 1994) and is usually attributed to the compression of the electrostatic double layer in the presence of high ion concentrations (Das and Caccavo Jr., 2000; Deshpande and Shonnard, 1999; Mills et al., 1994). Scholl and Harvey (1992), Kinoshita et al. (1993), and Jewett et al. (1995) report the effect of changes in pH values on bacterial transport and attachment, however, no uniform results are found. Increased attachment with decreasing pH is reported by Scholl and Harvey (1995) and Kinoshita et al. (1993), while Jewett et al. (1995) state that changes in pH from 5.5 to 7 do not affect bacterial transport through porous media.
columns. Temperature has also been shown to influence bacterial transport, however the effect seems to be case dependent. While Bellamy et al. (1985) describe higher cell removal at higher temperatures, Sarkar et al. (1994) describe lower removal at higher temperatures. Johnson and Logan (1996) investigated the influence of dissolved and sediment organic matter (DOM, SOM) on bacterial transport through porous media columns and describe an increase in travel distance in the presence of SOM. This increase in travel distance is attributed to an increase in negative surface charge of the iron-coated quartz sand through the addition of SOM. Like SOM, surfactants or dispersants can also result in decreased attachment and therefore facilitate the transport of bacteria through porous media. This is most likely due to decreased cell porous media interactions in the presence of surface active compounds. However, the activity or viability of the bacteria may be influenced negatively by the addition of such compounds (Goldberg et al., 1990; Gross and Logan, 1995b; Jackson et al., 1994; Paul and Jeffrey, 1984; Sarkar et al., 1994).

Influence of Porous Media Properties and Hydraulic Conditions on Bacterial Transport Through Porous Media. A number of porous media characteristics and hydraulic conditions have been reported to influence bacterial adhesion and transport through porous media. For instance, the pore size, grain size, and their distributions can influence bacterial transport through porous media (Fontes et al., 1991; Marlow et al., 1991; Sharma and McInerney, 1994; Smith et al., 1985; Wood and Ehrlich, 1978). Sharma and McInerney (1994) report that penetration rates of non-motile strains increase linearly with the size of the glass beads used, however, penetration rates of motile strains become independent with bead diameters of 398 μm or larger. Presumably, the lower specific surface area provided by larger grains and the higher hydraulic conductivity of the coarse grained porous medium allow for increased transport (Fontes et al., 1991; Marlow et al., 1991). Wood and Ehrlich (1978) and Smith et al. (1985) attribute enhanced bacterial transport to preferred flowpaths, which are provided by fractures or macropores present in the native material. The soil mineralogy (e.g. presence of Fe-minerals) and the organic matter content can influence the porous media surface charge and surface hydrophobicity, respectively. Both, the surface charge and the surface hydrophobicity, can influence bacterial adhesion to surfaces and transport through porous media.
The presence of positively charged surfaces like Fe-minerals in soils is reported to lead to increased attachment of bacterial cells, which carry a net negative charge at neutral pH values (Johnson and Logan, 1996; McCaulou et al., 1994; Mills et al., 1994; Scholl and Harvey, 1992; Scholl et al., 1990). Hydrophobic surfaces have been described to increase the number of cells adhering in batch and column systems (Absolom et al., 1983; Fletcher and Loeb, 1979; McCaulou et al., 1994) however, hydrophilic cells might show decreased attachment to hydrophobic surfaces (Paul and Jeffrey, 1985).

In addition to the surface chemistry, the physical characteristics, such as surface roughness, can significantly influence attachment regardless of the prevailing chemistry (Geesey and Costerton, 1979; Mueller, 1996). Thus, it is not surprising that hydrodynamic conditions have also been found to play a very important role in bacterial adhesion to surfaces. Rijnaarts et al. (1993) and Scheuerman et al. (1998) suggest that the hydrodynamics can become much more important in view of bacterial attachment than the physicochemical conditions.

The interstitial fluid velocity can vary widely when cells are actively injected into the subsurface. The interstitial fluid velocity depends on the pumping rates during injection and the radial distance from the point of injection. Most authors report an increase in breakthrough and dispersal of bacteria at higher interstitial fluid velocities (Gannon et al., 1991b; Marlow et al., 1991; Sarkar et al., 1994; Tan et al., 1994), although Camesano and Logan (1998) report higher dispersal at low pumping velocities and Gross et al. (1995) observe no influence of fluid velocity on bacterial transport through porous media.

**Influence of Cell Properties on Bacterial Transport through Porous Media.** A number of bacterial cell properties have an influence on bacterial adhesion and bacterial transport through porous media. The cell surface charge, often measured as zeta potential, has been shown to influence bacterial adhesion to surfaces. Cell adhesion has been correlated mostly inversely but also directly with surface net electrostatic charge (Gilbert et al., 1991; Sharma et al., 1985; VanLoosdrecht et al., 1987a). Cell hydrophobicity has been reported to influence bacterial adhesion to surfaces and transport through porous media (Caccavo, Jr. et al., 1997; DeFlaun et al., 1999; Fletcher and Marshall, 1982; VanLoosdrecht et al., 1987b) and separate
mechanisms have been found for the bacterial adhesion to hydrophilic and hydrophobic surfaces (Paul and Jeffrey, 1985). Both, the hydrophobicity and net electrostatic charge, are influenced by the type of surface molecules present on the outside of the cells. Proteins and (lipo)polysaccharides are believed to influence bacterial adhesion to surfaces and transport through porous media but it cannot yet be predicted how certain surface molecules influence bacterial transport and adhesion (Abbott et al., 1983; Caccavo, Jr., 1999; Caccavo, Jr. et al., 1997; Costerton et al., 1978; Fletcher, 1976; Fletcher and Floodgate, 1973; Rijnaarts et al., 1996; Williams and Fletcher, 1996).

Cell motility and chemotaxis can have an influence on transport through porous media. While the influence of bacterial motility on bacterial transport through porous media seems to be mostly negligible at flow velocities typically encountered in aquifers (Barton and Ford, 1995; Barton and Ford, 1997; Camper et al., 1993), bacterial motility is suggested to result in increased dispersal of cells (Camesano and Logan, 1998) and in increased penetration under static conditions (Jenneman et al., 1985; Reynolds et al., 1989). Cell appendages, such as flagella and pili, have also been shown to become important during initial bacterial attachment to surfaces. This influence is attributed to an increase in collisions due to the greater effective diameter, an increase in effective diffusivity, and to the ability of the bacterial appendages to reach across the boundary layer between the liquid and the solid phase (Busscher et al., 1990a; Busscher et al., 1990b; Busscher et al., 1992; Mueller, 1996; O'Toole and Kolter, 1998a; O'Toole and Kolter, 1998b; Piette and Idziak, 1991). Das and Caccavo suggest that the flagellum acts as a motility-independent adhesin (Das and Caccavo, unpublished results).

Other bacterial cell properties correlated with transport through porous media include the cell size and cell shape. Smaller and more spherical cells are commonly thought to be transported better through porous media than larger and elongated cells (Fontes et al., 1991; Gannon et al., 1991a; Weiss et al., 1995). Harvey et al. (1997) show that the buoyant density of bacterial cells can influence the sedimentation rates of bacteria in porous media environments, but it is considered negligible at ambient groundwater flow velocities (Corapcioglu and Haridas, 1984).
Many of the cell properties listed above are influenced by the physiological state of the bacteria and can be significantly different for the same bacterium depending on the environmental conditions (Grasso et al., 1996; VanLoosdrecht et al., 1987a). Thus, controlling the physiology of a bacterial strain before injection offers great potential for the development of effective bacterial transport strategies.

**Implications for Bioaugmentation Strategies**

The review of the current literature implies that bacterial transport through porous media is influenced by a combination of numerous parameters. The importance of any single parameter is specific to any given situation and cannot be predicted easily. Camper et al. (1993) and Harvey (1997) state that bacterial transport experiments should be performed with the aquifer material of interest and as close as possible to the expected conditions in the field since the determination of individual characteristics of cells or the porous medium will not allow an accurate prediction. A good overview on how to design and standardize bacterial transport experiments is given by Harvey (1997).

The examination of the parameters influencing bacterial adhesion to surfaces and transport through porous media makes evident that only a very limited number of parameters can be effectively manipulated in field scale applications. Attempts to change the solution chemistry or the physicochemical properties of the porous medium are likely to become an economical and technological challenge. Thus, manipulating parameters falling into these two categories might be extremely difficult or not advisable. In addition to the economical and technological challenges, lowering the ionic strength or changing the pH of groundwater could lead to undesirable changes in the groundwater chemistry potentially resulting in increased contaminant mobility. Manipulating the physicochemical properties of the aquifer by for instance hydraulically or pneumatically fracturing the aquifer is difficult to control over large areas and might result in increased contaminant transport through zones with high hydraulic conductivity. *Manipulating and controlling the bacterial inoculum and the interstitial fluid velocity appear to have the greatest potential of economical and technological success in field scale subsurface bioaugmentation strategies.*
Bacteria are commonly cultured in large amounts before injection in bioaugmentation projects. The cultivation step allows for the manipulation of the inoculum in many different ways. Strains with a decreased tendency to adhere to surfaces can be selected or bacteria can be harvested at a specific growth rate. Procedures on how to select for such strains have been described in Caccavo et al. (1997) and DeFlaun et al. (1990, 1999). Unfortunately, these procedures can be time consuming and might not be successful for every bacterial strain or situation. Transport enhancement strategies which are potentially applicable to any bacterial strain and consortium are more desirable. The following paragraph will indicate that a potentially applicable strategy involves the use of starved bacteria.

**Bacterial Starvation as a Transport Enhancement Strategy**

Starvation is believed to be a common survival mechanism for bacteria which cannot form spores or cysts (Hood and MacDonell, 1987; Kjelleberg et al., 1993b; Tabor et al., 1981). Starvation of bacteria can result in radical size reduction and a rapid decrease in metabolic activity until the bacteria approach complete dormancy (Kjelleberg, 1993a). Starved bacteria can survive for years in the absence of nutrients (Amy and Haldeman, 1997; Kjelleberg, 1993a) and the improved transport of metabolically dormant cells is believed to contribute towards the presence of bacteria in the deep subsurface (Fredrickson and Onstott, 1996b; Lappin-Scott and Costerton, 1990). Starved bacteria can be resuscitated relatively rapidly by the addition of suitable nutrients (Amy and Morita, 1983; Cunningham et al., 1997; Kjelleberg, 1993a; Novitsky and Morita, 1978), making bacterial starvation a potentially effective means of facilitating transport of through porous media for a number of engineering applications (Bouwer et al., 2000; Cunningham et al., 1997; Cusack et al., 1992; Gerlach et al., 1998; Lappin-Scott and Costerton, 1992; Lappin-Scott et al., 1988a; Lappin-Scott et al., 1988b; MacLeod et al., 1988; Sharp et al., 1999).

The injection, resuscitation, and subsequent plugging of high permeability zones in oil bearing formations using starved bacteria is demonstrated in the literature (Cusack et al., 1992; Lappin-Scott and Costerton, 1990; Lappin-Scott et al., 1988a; Shaw et al., 1985). The use of starved bacteria results in significant improvements in secondary oil recovery since the bacteria travel deeper into the high
permeability zones and form bacterial plugs upon resuscitation, which force the injected water through the low permeability zones to recover residual oil more efficiently.

Starved bacteria have also been used in a series of laboratory and field scale experiments demonstrating the possibility to form hydraulic groundwater barriers. Starved cells derived from bacteria known to produce large amounts of extracellular polysaccharides (EPS) were injected into porous media and resuscitated. The bacteria are reported to form thick biofilms upon resuscitation and to decrease the porous media permeability dramatically (Cunningham, 2000; Cunningham et al., 1997).

It is well known that a number of changes occur during long term starvation of non spore-forming bacteria. A large amount of RNA and protein appear to be degraded rapidly at the onset of starvation (Kaplan and Apirion, 1975b; Pine, 1965), however synthesis of new proteins appears to be required for long term survival of starving cells (Groat et al., 1986; Hengge-Aronis, 1993; Kaplan and Apirion, 1975a; Kjelleberg et al., 1993b; Reeve et al., 1984). These proteins are believed to be part of a general stress response, often involving RpoS, and allow bacteria to become more stress resistant and more efficient scavengers (Cappelier et al., 2000; Fischer et al., 1998; Foster and Spector, 1995; Matin, 1991; Matin et al., 1989; Spector, 1998; Spector et al., 1999; Svensater et al., 2000; Teich et al., 1999; Zinser and Kolter, 1999; Zinser and Kolter, 2000). Increased stress resistance is verified by the observation that starved cells survived better under a range of stress conditions than their vegetative counterparts (Albertson et al., 1990; Hartke et al., 1994; Nystrom et al., 1988; Nystrom et al., 1990; Nystrom et al., 1992) and that slow growing cells are better adapted for starvation stress survival than their fast growing counterparts (Muller and Babel, 1996).

In the section “Influence of Cell Properties on Bacterial Transport through Porous Media” cell surface properties were discussed, which are known to influence bacterial attachment to surfaces and transport through porous media. It was discussed that the growth state of bacteria and the presence of nutrients can influence attachment. Thus, it is not surprising that bacterial starvation can significantly influence the attachment of bacteria to surfaces. Short term starvation of bacteria can result in an increased tendency to attach to surfaces (Dawson et al., 1981; Kjelleberg, 1984) whereas long term starvation (weeks
to months) may decrease bacterial attachment and enhance bacterial transport through porous media (Bouwer et al., 2000; Cusack et al., 1992; Gerlach et al., 1998; Lappin-Scott and Costerton, 1992; Lappin-Scott et al., 1988a; Lappin-Scott et al., 1988b; MacLeod et al., 1988; Sharp et al., 1999).

It is one goal of this research to develop and evaluate strategies for the enhancement of bacterial transport through porous media, which are potentially applicable to field relevant scales, a wide range of bacterial strains, and different hydrogeological conditions. Ideally, such a strategy should allow to culture a large number of bacteria inexpensively, which are mobile enough to transport over large distances and yet adhesive enough to attach to soil particles. This would allow establishing a sessile population capable of transforming dissolved and sorbed contaminants. Long term nutrient starvation appears to provide such a widely applicable strategy for the enhancement of bacterial transport through porous media.

The motivation for using the DMRB, *Shewanella algae* BrY, for these studies is explained in the following section.

**Permeable Reactive Subsurface Barriers and Microbial Metal-Reduction (Research Goal 2)**

The use of permeable subsurface treatment zones (or barriers) established downstream of contaminated areas provides potential for preventing the off-site migration of contaminants without severely influencing the natural groundwater flow. Permeable reactive barriers (PRBs) can provide long term control of a contamination problem, if their reactivity is maintained over extended periods of time. PRBs are commonly constructed by excavating trenches perpendicular to the groundwater flow direction and refilling these trenches with the reactive material of choice. These systems represent semi-passive approaches, which minimize the exposure of operating personnel to potentially hazardous compounds.

Permeable reactive barriers can be made in a number of different designs and consist of many different materials (Figure 1.1 and Figure 1.2, Gavaskar et al., 1998; Sacre, 1997; Scherer et al., 2000; U.S.EPA, 1997; U.S.EPA, 1998; Yuyun and Allen, 1999).
The most commonly used material is zero valent iron (Fe(0)). Fe(0) has been employed in permeable reactive subsurface barriers which are capable of remediating a wide range of contaminants including chlorinated organics (R-Cl, Eykholt and Davenport, 1998, Gatpagar et al., 1997, Gillham and O'Hannesin, 1994, Helland et al., 1995, Johnson et al., 1996, Johnson et al., 1998, Roberts et al., 1996, Sayles et al., 1997, Siantar et al., 1996), heavy metal and radionuclide oxyanions, such as chromate (CrO$_4^{2-}$, Gould, 1982, Cantrell et al., 1995) and oxycations, such as uranyl (UO$_2^{2+}$) (Fiedor et al., 1998), nitroaromatics (R-NO$_2$, Cao et al., 1999, Agrawal and Tratnyek, 1996), and nitrate (NO$_3^-$, Huang et al., 1998, Till et al., 1998, Siantar et al., 1996). The corrosion of Fe(0) provides the electrons necessary for the reduction of these contaminants according to the following half reactions.

\[
\text{Fe}(0) \rightarrow \text{Fe}^{2+} + 2 \, e^- \quad \text{(Eq. 1.1)}
\]
\[
\text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + e^- \quad \text{(Eq. 1.2)}
\]

The electrons provided react with the different contaminants according to following half reactions.

\[
\text{R-Cl} + 2 \, e^- + \text{H}^+ \rightarrow \text{R-H} + \text{Cl}^- \quad \text{(Eq. 1.3)}
\]
\[
\text{CrO}_4^{2-} + 3 \, e^- + 8 \, \text{H}^+ \rightarrow \text{Cr}^{3+} + 4 \, \text{H}_2\text{O} \quad \text{(Eq. 1.4)}
\]
\[
\text{UO}_2^{2+} + 2 \, e^- \rightarrow \text{UO}_2 \quad \text{(Eq. 1.5)}
\]
R-NO$_2$ + 6 e$^-$ + 6 H$^+$ → R-NH$_2$ + 2 H$_2$O \hspace{1cm} (Eq. 1.6)$^1$

NO$_3^-$ + 8 e$^-$ + 9 H$^+$ → NH$_3$ + 3 H$_2$O \hspace{1cm} (Eq. 1.7)$^2$

Figure 1.2. Reactive Media employed in permeable reactive barriers, adapted from Sacre (1997).

The simplicity, low maintenance requirements, and robustness of the approach have made Fe(0) PRBs one of the preferred technologies for the remediation of groundwater contaminated with chlorinated hydrocarbons and heavy metals.

However, no reliable data on the long term performance of Fe(0) PRBs are available yet. Laboratory studies simulating long term performance of Fe(0) indicate that contaminant degradation rates can either decrease (Devlin et al., 1998, Gatpagar et al., 1997) or more rarely increase (Helland et al., 1995)

$^1$ Other potential reaction products include nitroso and hydroxylamino compounds. (Klausen et al., 1995)

$^2$ Other potential reaction products are nitrogen gas and nitrite (Till et al., 1998)
as a function of time. This temporal effect on contaminant degradation rates is usually attributed to the development of corrosion products that passivate the metal surface and influence electron transport to the contaminant (Helland et al., 1995; Johnson and Tratnyek, 1994; Odziemkowski et al., 1998).

A wide range of iron corrosion products has been found in laboratory and field studies. The corrosion products commonly found in the presence of contaminated groundwater include iron (hydr)oxides such as goethite (Pratt et al., 1997), magnetite (Bonin et al., 1998; Odziemkowski and Gillham, 1997; Odziemkowski et al., 1998), maghemite (Peterson et al., 1997), hematite (Pratt et al., 1997), mixed chromium-iron-hydroxides (Powell et al., 1995a, Blowes et al., 1997b), green rust (Odziemkowski and Gillham, 1997; Odziemkowski and Gillham, 1997), and siderite (Mackenzie et al., 1995). The corrosion product distribution appears to be dependent on the type of iron used, contaminant present, geochemical conditions, and time of exposure to contaminated groundwater.

Fe(0) PRBs are usually established using trench and fill methods to place the iron into the subsurface. However, the installation can become uneconomical at depths of more than 12 to 15 meters and alternative methods for establishing permeable reactive zones in the subsurface have been proposed (Yuyun and Allen, 1999). Since injection wells can be installed much deeper than trenches the injection of colloidal Fe(0) particles to establish redox reactive zones has been proposed (Cantrell and Kaplan, 1997a; Cantrell et al., 1997b; Cantrell et al., 1995). A group at Pacific Northwest National Laboratory (PNNL) has demonstrated and patented a method using dithionite (S$_2$O$_4^{2-}$) as a chemical reductant (Amonette et al., 1998; Amonette et al., 1994; Istok et al., 1999; Scott et al., 1998). The dithionite is injected into the subsurface in a pH 11 bicarbonate buffer and reduces indigenous ferric iron to ferrous iron, which chemically reacts with heavy metals, radionuclides, and chlorinated hydrocarbons. This technology has been demonstrated at DOE’s Hanford site in Richland, WA and at an army base in Fort Lewis, WA (Beets, 1998; Fruchter et al., 1997).

Alternatively, indigenous ferric iron could be reduced by microorganisms. Dissimilatory metal-reducing bacteria (DMRB) are widely distributed in both pristine and contaminated terrestrial, aquatic and subsurface environments and can reduce many iron minerals found in the environment and on passivated
Fe(0) (Caccavo, Jr. et al., 1992; Fredrickson and Gorby, 1996a; Heijman et al., 1995; Heijman et al., 1993; Kostka and Nealson, 1995; Lovley, 1995b; Nealson and Saffarini, 1994; Roden and Zachara, 1996). DMRB can also reduce a large number of heavy metals and radionuclides enzymatically and therefore offer a potentially useful mechanism for the remediation of contaminated subsurface environments (Beets, 1998; Fredrickson and Gorby, 1996a; Fruchter et al., 1997; Lovley, 1995a; Lovley, 1995b; Lovley, 1997). Alternatively, DMRB can produce ferrous iron from indigenous ferric iron minerals in the subsurface, which in turn chemically reduces carbon tetrachloride (CT) to chloroform (CF) and Cr(VI) to Cr(III) (Amonette et al., 2000; Caccavo, Jr. et al., 1996; Gorby et al., 1994; Gorby et al., 1995).

In summary, the potential use of DMRB in subsurface remediation has gained considerable interest from engineers, microbiologists, and geochemists. DMRB are known to influence the geochemistry of subsurface environments and have the potential to be part of successful strategies for the bioremediation of heavy metal and radionuclide contaminated subsurface environments. DMRB are also likely to contact existing subsurface remediation installations such as Fe(0) PRBs and to influence their performance.

**Research Goals**

This research is designed to evaluate the use of dissimilatory metal reducing bacteria (DMRB) for the bioaugmentation of contaminated subsurface environments. Two major research needs are identified based on the literature review.

1. The transport of DMRB through porous media has to be enhanced and injection well clogging avoided.

2. The applicability of DMRB to mediate the precipitation of heavy metals and the dechlorination of chlorinated hydrocarbons has to be evaluated.

This dissertation addresses these needs by utilizing the dissimilatory metal-reducing bacterium, *Shewanella algae* BrY, as the model organism. The following four objectives are addressed in the four main chapters of this dissertation.
Objectives

1. Starvation of *Shewanella algae* BrY is evaluated as a strategy for the enhancement of bacterial transport through porous media.

2. The reasons for the improved transport of starved *S. algae* BrY bacteria are elucidated by monitoring changes in transport related cell properties during nutrient starvation.

3. The possibility of using *S. algae* BrY to establish and maintain redox reactive zones for the elimination of chromium(VI) from contaminated groundwater is tested.

4. The potential influence of *S. algae* BrY on existing remediation systems based on zero valent iron is evaluated.
CHAPTER 2

ENHANCING TRANSPORT OF SHEWANELLA ALGAE BrY THROUHG POROUS MEDIA BY STARVATION

Abstract

The successful bioaugmentation of contaminated subsurface environments relies on the effective transport and distribution of bacteria through porous media. This chapter provides evidence that starvation of the dissimilatory metal-reducing bacterium Shewanella algae BrY results in a significant enhancement of bacterial transport. Intermediate scale column studies (30 cm and 3 m length) were conducted to compare the transport of starved and freshly cultivated (vegetative) cells of S. algae BrY through quartz sand columns. Plate counts were utilized to estimate bacterial numbers and activity. The fractional recovery (number of cells recovered in the effluent compared to number of cells injected) and the normalized breakthrough concentration were statistically significantly greater for starved cells in both 30 cm and 3 m long columns. The distribution of starved cells sorbed to the sand along the flowpath was in addition more homogeneous for starved cells. It was concluded that bacterial starvation facilitates the transport and distribution of S. algae BrY through porous media.

Introduction

The literature review in Chapter 1 suggests that nutrient starvation is potentially a reliable way to control the physiological state of bacteria and thus many transport-related parameters, including the cell size, shape, surface charge, hydrophobicity, buoyant density, type and amount of cell surface molecules, the presence of pili and flagella, and thus the motility and chemotaxis of cells. Starvation of bacteria results in radical size reduction and a rapid decrease in metabolic activity until the bacteria approach complete
dormancy (Kjelleberg, 1993a). Starved bacteria can survive for years in the absence of nutrients but can be resuscitated relatively rapidly by the addition of suitable nutrients (Amy and Haldeman, 1997; Kjelleberg, 1993a). Short term starvation (hours to days) of bacteria can result in increased attachment to surfaces (Dawson et al., 1981; Kjelleberg, 1984) while long term starvation (weeks to months) can decrease attachment and enhance bacterial transport through porous media significantly (Bouwer et al., 2000; Gerlach et al., 1998; Lappin-Scott and Costerton, 1992; Sharp et al., 1999). The injection of starved bacteria has been suggested to promote plugging of subsurface formations for enhanced secondary oil recovery (Cusack et al., 1992; Lappin-Scott et al., 1988a; Lappin-Scott et al., 1988b; MacLeod et al., 1988) and for groundwater containment (Cunningham et al., 1997). This chapter describes the results of transport experiments and the derivation of parameters which quantitatively describe the transport of starved and vegetative cells of *Shewanella algae* BrY through porous media.

**Quantification of Bacteria in Porous Media**

The quantification of bacteria in groundwater and soil poses one of the greatest challenges in microbiology. There is a lack of established methods for the reliable quantification of bacterial cell numbers and activity in porous media, which are applicable to field scale investigations. The methods employed in laboratory and field scale investigations include traditional plate counts, most probable number (MPN) methods, direct microscopic counts, the use of genetically engineered bacteria containing fluorescent markers (e.g. green fluorescent protein (gfp), Burlage et al., 1996) or an antibiotic resistance marker (Berry and Hagedorn, 1991), radiolabeled cells (DeFlaun et al., 1999; Gross et al., 1995a), ferromagnetic antibody aided concentration (Johnson et al., 2001; Zhang et al., 1999), and $^{13}$C-labeled cells together with isotope selective mass spectrometric measurements (DeFlaun et al., 1997). Some of these methods provide powerful tools for the quantification of bacteria in soil columns in the laboratory but are not practical for field scale applications due to regulatory concerns (i.e. radiolabeled cells or antibiotic markers). The use of ferromagnetic antibodies and $^{13}$C labeling allow for the selective enumeration of cells in groundwater but cannot effectively assess the number or activity of sorbed cells. Recent developments in
molecular biology have significantly improved our ability to selectively detect and quantify bacteria in environmental samples. However, affordable methods to reliably quantify bacterial activity in porous media are still under development.

Bacterial plate counts have been used in a number of research projects evaluating the bacterial transport through porous media (Alexander et al., 1991). Plate counts of attached bacteria require the desorption of bacteria before culturing, which is commonly achieved by shaking, blending, or sonicating a soil sample in the presence of a buffered solution. The desorption of bacteria from soils is an important step since it represents a trade-off between complete desorption of bacteria and bacterial injury or decay due to stress factors during the desorption procedure. Stress due to buffer constituents, such as surfactants, or agitation procedures can significantly influence the outcome of the subsequent culturing step. Stevenson (1958) already reported that increasing the sonication time during an ultrasound aided desorption procedure increases the number of bacteria recovered from soils. However, extensive sonication can lead to a sharp decrease in viable cell numbers most likely due to heating and disruption of the cell integrity by ultrasound.

It is well known that bacterial plate counts underestimate the number of bacterial cells especially when attempting to quantify the number or activity of bacteria sorbed to surfaces (Wagner et al., 1993; Zuberer, 1994). However, since plate counts provide a lower estimate of bacterial numbers and activity, they were utilized in this research. It can be assumed that the reported numbers commonly underestimate the number of active bacteria since plate counts do not account for viable but not culturable (VBNC) cells.

Experiments comparing the transport of starved and vegetative cells of *Shewanella algae* BrY were performed on two different scales. First, short columns (30 cm length) were utilized to develop a test system for the relatively rapid assessment of bacterial transport in porous media. Larger scale experiments involving columns of 3 m length were utilized to test the applicability of the parameters derived from the short column experiments and to test the starvation transport enhancement strategy on a more field relevant scale.
Materials and Methods

Strain and Culturing Methods

Cultures of *Shewanella algae* BrY (formerly *Shewanella alga* BrY, Caccavo, Jr. et al., 1992) were maintained on tryptic soy agar (40 g L\(^{-1}\); Difco, Detroit, MI) at room temperature. Cells were aerobically grown to the late exponential, early stationary phase in tryptic soy broth (30 g L\(^{-1}\); Difco, Detroit, MI) at room temperature for 15 hours on a rotary shaker at 150 rpm. The cells were harvested by centrifugation (5860 \(\times\) g, 20 minutes, 4 °C), washed twice in phosphate buffered saline solution (PBS; 8.5 g L\(^{-1}\) NaCl, 0.96 g L\(^{-1}\) K\(_2\)HPO\(_4\), 0.61 g L\(^{-1}\) KH\(_2\)PO\(_4\), pH 7.0), and resuspended in PBS. Washed *S. algae* BrY cells were either used directly (vegetative state) or starved by aseptically stirring on a magnetic stir plate at room temperature as described by Caccavo et al. (1996). Starved cells were harvested after 7 weeks of starvation by centrifugation and resuspended in PBS before incorporation into the transport experiments.

Transport studies

Cells of *S. algae* BrY, starved or vegetative, were injected into porous media columns filled with 40 mesh quartz sand (Unimin Corp., Emmet, ID). The columns were established using commercially available polyvinylchloride (PVC) pipe. Columns of two different dimensions were used, 3 m long and 2.54 cm in diameter, and 30 cm long and 1.5 cm in diameter. The column inlets and outlets were layered with nylon mesh, and pea gravel to prevent settling of sand into the inlet tubing and washout of sand through the outlet tubing. All columns were equipped with sample ports at the influent and effluent points and a number of columns were equipped with additional sample ports evenly distributed along the length of the column. The columns were packed wet to avoid the inclusion of air pockets and facilitate reproducible packing. The water level was maintained just below the top of the column during packing by periodically draining part of the water by gravity. The columns were always packed by slowly pouring the porous
medium into the water filled column to the desired settled bed height. The columns were operated upflow using a constant head tank or a peristaltic pump for 30 cm and 3 m long columns, respectively.

The pore volume and hydrodynamic dispersion coefficient of each column were determined utilizing Na-fluorescein (Aldrich, Milwaukee, WI) as a tracer and by fitting the experimental data to an analytical solution of the advection dispersion equation described below (Van Genuchten and Alves, 1982). The interstitial fluid velocity \((v)\) in the bacterial transport experiments was calculated using the measured flowrate, the cross sectional area of the column, and the porosity, which was estimated from the tracer studies.

The bacterial concentrations were expressed as fractions of their influent concentrations in each experiment and were plotted as a function of the number of pore volumes eluted from the columns. In addition, the fractional recovery was calculated from the influent and effluent data. The fractional recovery was defined as the number of bacteria in the effluent of the column (integral of the effluent concentration over time) divided by the total number of bacteria injected into the column (integral of the influent concentration over time).

The columns were prepared for inoculation by flushing with 2 pore volumes 0.5 % NaOCl (dilute commercial bleach), 1 pore volume sterile PBS, 2 pore volumes 0.01 M sodium thiosulfate to eliminate residual chlorine and 2 pore volumes sterile PBS. A commercial kit (HACH, Loveland, CO) was used to ensure residual chlorine concentrations below the detection limit (0.05 mg L\(^{-1}\)) in the effluent of the columns. Plate counts routinely performed on effluent samples after this treatment did not show any growth. During the transport experiments, the columns were inoculated with approximately two pore volumes of starved or vegetative cells of \(S.\ algae\) BrY suspended in PBS. The transport of bacteria was monitored by sampling the influent, effluent, and the pore water along the flowpath of the columns for culturable cells. Aliquots (0.1 mL and 1 mL for 30 cm and 3 m columns, respectively) were taken using sterile, disposable 1 mL syringes equipped with 22 gauge needles. Dilutions in PBS were performed immediately and colony forming units were determined on tryptic soy agar (40 g L\(^{-1}\); Difco, Detroit, MI;
incubation for 24 hours at 30°C). The reported values are the arithmetic means of the three appropriate plate counts.

**Porous Media Sampling**

The distribution of sorbed cells throughout the columns was estimated at the end of the experiments by sectioning the columns and analyzing the sand cores for culturable cells after desorption from the sand. The 30 cm columns were divided into six sections of each five centimeter length while the 3 m long columns were sectioned into ten 30 cm long sections. The desorption of cells was accomplished by diluting a desorption solution, described by Camper et al. (1985), 1:5 in PBS. Higher concentrations of the desorption solution appeared to inhibit growth of *S. algae* BrY cells on TSA plates. An aliquot (5 ml) of this solution was added to 1 g of sand in a test tube, which was vortexed for 1 second. The test tube was placed on a horizontal shaker (150 rpm) for 30 min and then vortexed again for 3 seconds. The supernatant was sampled immediately after coarse particles had settled and plate counts on tryptic soy agar were performed after dilution in PBS.

**Modeling**

Bacterial transport through porous media has often been described using clean bed filtration models. Yao et al. (1971) suggested a set of equations describing the removal of suspended particles in a porous media bed. Improved and alternative clean bed filtration models were developed subsequently (Logan et al., 1993; Logan et al., 1995; Rajagopalan and Tien, 1976; Tien and Payatakes, 1976). Logan et al. (1995) summarized, clarified, and compared clean bed filtration model equations and proposed the following steady state solution.

\[
\frac{c}{c_0} = \exp \left[ -3 \frac{(1 - \Theta)}{2} \frac{\alpha \eta x}{d_c \Theta} \right]
\]

(Eq. 2.1)
In Equation 2.1, \( c \) is the effluent cell concentration (cells length\(^{-3}\)), \( c_0 \) the influent cell concentration (cells length\(^{-3}\)), \( d_c \) is the diameter of the spherical collectors (length), \( \Theta \) the porosity, \( \alpha \) the collision efficiency factor (i.e. the number of collisions of suspended bacteria with collectors which result in adhesion of a bacterium compared to the number of total collisions), \( \eta \) is the single collector efficiency (i.e. the rate at which particles strike a single collector divided by the rate at which particles flow towards the collector), and \( x \) is the distance into the porous medium (length). Assuming spherical collectors, the overall single collector efficiency (\( \eta \)) can be calculated as follows (Logan et al., 1995).

\[
\eta = 4N_{pe}^{-\frac{1}{3}} + \frac{3}{2}N_R^2 + N_G
\]  
(Eq. 2.2)

The three terms involving \( N_{pe}, N_R, \) and \( N_G \) represent the removal processes based on diffusion, interception, and gravitational settling, respectively (Logan et al., 1995). They can be calculated from

\[
N_{pe} = \frac{\nu d_c}{D_p}
\]  
(Eq 2.3)

\[
N_R = \frac{d_p}{d_c}
\]  
(Eq 2.4)

\[
N_G = \frac{(\rho'_p - \rho)g d_p^2}{18 \mu \nu}
\]  
(Eq 2.5)

where \( d_p \) is the diameter of the bacteria (length), \( D_p \) the particle diffusion coefficient (length\(^2\) time\(^{-1}\)), \( \rho'_p \) and \( \rho \) are the densities of the bacteria and the fluid (mass length\(^{-3}\)), respectively, \( g \) is the gravitational constant (length time\(^{-2}\)), \( \mu \) the fluid viscosity (mass length\(^{-1}\) time\(^{-1}\)), and \( v \) the interstitial fluid velocity (length time\(^{-1}\)). \( D_p \) can be calculated from

\[
D_p = 0.1051 \frac{kT}{\mu d_p}
\]  
(2.6)

with \( k \) the Boltzman constant, \( T \) the absolute temperature (Logan et al., 1995).

The steady state solution (Equation 2.1) is based on the application of the advection dispersion equation (Equation 2.7) assuming first order removal of particles from the suspension.
\[
\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} - v \frac{\partial c}{\partial x} - k_{\text{att}} c \tag{Eq. 2.7}
\]

\(D\) in Equation 2.7 is the hydrodynamic dispersion coefficient (length\(^2\) time\(^{-1}\)).

Equation 2.7 describes the filtration process using a simple first order reaction term with \(k_{\text{att}}\) (time\(^{-1}\)) as rate coefficient. Additional terms for cell death or lysis can be included. However, cell death and lysis were considered negligible in this study since plate counts in the influent remained relatively constant and commonly varied less than 10% during the time of the transport experiment.

Dispersion can commonly be neglected when calculating particle collision efficiencies in porous media beds at steady state (Unice and Logan, 2000). Thus, Equation 2.7 becomes at steady state.

\[
\frac{\partial c}{\partial x} = -\frac{k_{\text{att}}}{v} c \tag{Eq. 2.8}
\]

Integration yields an equation containing a first order attachment coefficient in distance \((k_{\text{att}}/v, \text{length}^{-1})\), which can be estimated from the suspended cell concentration profile over the length of the column.

\[
\frac{c}{c_0} = \exp\left(-\frac{k_{\text{att}}}{v} x\right) \tag{Eq. 2.9}
\]

The comparison of Equations 2.1 and 2.9 finally reveals that

\[
k_{\text{att}} = \frac{3}{2} \frac{(1 - \Theta)}{d_e \Theta} v \alpha \eta \tag{Eq. 2.10}
\]

\(k_{\text{att}}\) was estimated independently from two different sets of data; from suspended cell concentration profiles over the length of the column after 2 pore volumes and by fitting an analytical solution of Equation 2.7 (Equation 2.11, Van Genuchten and Alves, 1982, p. 60) to the effluent concentration profiles over time observed in the transport experiments. The analytical solution (Equation 2.11) was also utilized to model the experimental results.

\[
c(x,t) = \frac{c_0}{2} \left[ \exp\left(\frac{(v-u)x}{2D}\right) \text{erfc}\left(\frac{x-ut}{2\sqrt{Dt}}\right) + \exp\left(\frac{(v+u)x}{2D}\right) \text{erfc}\left(\frac{x+ut}{2\sqrt{Dt}}\right) \right] \tag{Eq. 2.11}
\]
where
\[ u = v \left( 1 + \frac{4k_{\text{att}}D}{v^2} \right)^{0.5} \]  
(Eq. 2.12)

Results and Discussion

Short (30 cm) Column Experiments

Triplicate experiments utilizing 30 cm long columns were performed for both starved and vegetative cells. The dispersion coefficient and porosity which were estimated from tracer studies varied between 0.63 cm² min⁻¹ and 2.83 cm² min⁻¹, and 46 % and 50 %, respectively. The interstitial fluid velocity varied between 1.84 cm min⁻¹ and 3.09 cm min⁻¹ and the average influent concentration between 6.86 x 10⁷ CFU mL⁻¹ and 2.65 x 10⁸ CFU mL⁻¹ (Table 2.1).

Table 2.1. Summary of parameters for bacterial transport experiments in 30 cm porous media columns filled with 40 mesh quartz sand

<table>
<thead>
<tr>
<th></th>
<th>(D^1)</th>
<th>(\Theta^2)</th>
<th>(v^3)</th>
<th>(C_0^4)</th>
<th>(k_{\text{att}}^5)</th>
<th>(k_{\text{att}}^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>starved</td>
<td>0.63</td>
<td>0.50</td>
<td>3.09</td>
<td>7.79 x 10⁷</td>
<td>0.0018</td>
<td>n.d.³</td>
</tr>
<tr>
<td>cells</td>
<td>1.21</td>
<td>0.50</td>
<td>1.86</td>
<td>2.65 x 10⁸</td>
<td>0.0008</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>1.67</td>
<td>0.46</td>
<td>2.41</td>
<td>1.43 x 10⁸</td>
<td>0.0014</td>
<td>0.0011</td>
</tr>
<tr>
<td>vegetative</td>
<td>2.83</td>
<td>0.48</td>
<td>2.51</td>
<td>8.75 x 10⁷</td>
<td>0.0029</td>
<td>n.d.</td>
</tr>
<tr>
<td>cells</td>
<td>1.98</td>
<td>0.50</td>
<td>2.27</td>
<td>6.86 x 10⁷</td>
<td>0.0023</td>
<td>0.0020</td>
</tr>
<tr>
<td></td>
<td>2.32</td>
<td>0.50</td>
<td>1.84</td>
<td>9.84 x 10⁷</td>
<td>0.0022</td>
<td>0.0017</td>
</tr>
</tbody>
</table>

1 Dispersion coefficient [cm² min⁻¹], estimated from tracer studies
2 porosity, estimated from tracer studies
3 interstitial fluid velocity [cm min⁻¹]
4 influent cell concentration [CFU mL⁻¹]
5 first order attachment coefficient [s⁻¹] estimated using Equation 2.7
6 first order attachment coefficient [s⁻¹] estimated using Equation 2.9
7 n.d.: not determined
Breakthrough of Bacteria. Starved cells of *Shewanella algae* BrY were detected in the effluent of the columns at higher normalized concentrations than vegetative cells (Figure 2.1). The average normalized breakthrough concentration for starved cells was 38 ± 6% compared to 13 ± 1% for vegetative cells. The higher breakthrough of starved cells is also reflected in greater fractional recoveries (20 ± 3%) compared to vegetative cells (6 ± 1%). A multiple regression analysis was performed to evaluate the effects of bacterial physiology (i.e. starved vs. vegetative cells), influent cell concentration, and interstitial fluid velocity on the fractional recovery and normalized breakthrough concentration of *Shewanella algae* BrY after transport through 30 cm quartz sand columns. The regression analysis revealed that, even after correcting for the potential influence of influent cell concentration and interstitial fluid velocity, the physiological state of the cells significantly influenced the transport behavior of cells in the column experiments. The resulting P-values of \( P = 0.006 \) and \( P = 0.005 \) for fractional recovery and normalized breakthrough concentration, respectively, clearly indicated the statistically significant difference between starved and vegetative cells of *S. algae* BrY.

![Figure 2.1](image)

Figure 2.1. Normalized breakthrough concentration (c/c₀) of starved (♦, ■, △) and vegetative (◇, □, △) cells of *Shewanella algae* BrY in the effluent of 30 cm long quartz sand columns and corresponding model fits (Equation 2.7, solid lines).
The corresponding model fits provided additional information about the transport behavior of starved and vegetative *Shewanella algae* BrY cells (Figure 2.1). Vegetative cells appeared to break through slightly earlier (i.e. at 0.95 and 0.91 pore volumes) than predicted by the model in two columns. The third column, however, showed a delayed breakthrough (at 1.14 pore volumes) for vegetative cells in comparison to the model predictions. Starved cells were detected in the effluent approximately at (one column) or after the predicted times (two columns, at 1.08 and 1.14 pore volumes). Since the experiments were not designed to determine the exact time of breakthrough, it was difficult to derive definitive statements about the time of breakthrough. An early breakthrough of cells was maybe due to size dependent exclusion since the effective porosity for particles can be significantly lower than for solutes (Bales et al., 1989; Bitton et al., 1974; Morley et al., 1998). Since an early breakthrough was observed more frequently for the larger vegetative cells, pore size exclusion may have influenced the transport of *S. algae* BrY in the quartz sand columns.

**Distribution of Bacteria in the Pore Water.** The enhanced transport of starved *S. algae* BrY cells was also reflected in the profiles of suspended cell concentrations for starved and vegetative cells after the injection of 2 pore volumes of cell suspension (Figure 2.2). A total of only four columns was considered for this analysis because plate counts for the third set of columns were unsuccessful. Using a least square method to fit Equation 2.9 to the experimental data, first order sorption coefficients (*k*<sub>sat</sub>) of 5.3 x 10<sup>-4</sup> s<sup>-1</sup> and 1.1 x 10<sup>3</sup> s<sup>-1</sup> were obtained for starved cells (R<sup>2</sup>-values: 0.580 and 0.870) and 2.0 x 10<sup>-3</sup> s<sup>-1</sup> and 1.7 x 10<sup>3</sup> s<sup>-1</sup> for vegetative cells (R<sup>2</sup>-values: 0.930 and 0.932). Despite the low regression coefficients for the starved cell concentration profiles, the smaller *k*<sub>sat</sub> values provided supporting evidence that starved cells of *S. algae* BrY transport better through the quartz sand columns than their vegetative counterparts. A steady state model allowing for a bimodal distribution of first order sorption coefficients (Bolster et al., 2000) was applied to fit the experimental data but did not result in obvious improvements (data not shown). The *k*<sub>sat</sub> values estimated using Equation 2.9 were in the same range, but consistently lower than the *k*<sub>sat</sub> values derived from fitting the analytical solution of Equation 2.7 to the experimental data (see Table 2.1). These
differences might be due to the uncertainties in estimating the steady state breakthrough concentration from the experimental data (Figure 2.1), which was necessary for the calculation of $k_{att}$ using Equation 2.7. In addition, Equation 2.9 assumes steady state conditions, which might not have been achieved completely after two pore volumes in the 30 cm long columns. Since steady state could not be assumed, the $k_{att}$ values derived from fitting Equation 2.7 were used for subsequent calculations. The $k_{att}$ values were used to predict the breakthrough of starved and vegetative cells through the 3 m long columns.

![Image of graph showing normalized concentrations of starved and vegetative cells along the flowpath.](image)

Figure 2.2. Normalized concentrations of starved (♦, ▲) and vegetative (□, ○) cells of *S. algae* BrY in the pore water along the flowpath of 30 cm long quartz sand columns after injection of approximately 2 pore volumes of cell suspension. Model fits (Equation 2.9) are represented by the solid lines.

Sorption of Bacteria Along the Flowpath. The distribution of sorbed cells throughout the 30 cm columns at the end of each experiment further supported the hypothesis that starved cells were better transported, less retarded, and more homogeneously distributed throughout the columns (Figure 2.3). The
number of cells desorbed per g of sand in each 5 cm section was normalized to the total number of cells injected to allow comparison between the different columns. It was evident that vegetative cells sorb more strongly than starved cells within the first few cm of the columns. The relative number of vegetative cells (CFU) detected per g of sand decreased from $7.17 \times 10^3 \pm 2.41 \times 10^3$ g$^{-1}$ in the first 5 cm section to $3.63 \times 10^3 \pm 4.82 \times 10^4$ g$^{-1}$ (51% of sorbed bacteria detected in first 5 cm of column) at 15 to 20 cm into the column and then remained constant. The quantification limit of this method was approximately $1.5 \times 10^4$ CFU g$^{-1}$ sand or approximately $10^6$ g$^{-1}$. Starved cells appeared to be more homogeneously distributed along the flowpath. The normalized concentration of starved cells decreased only slightly from $3.22 \times 10^3 \pm 2.24 \times 10^4$ g$^{-1}$ within the first 5 cm of the column to $2.49 \times 10^3 \pm 3.97 \times 10^4$ g$^{-1}$ (77%) between 15 to 20 cm into the column and remained constant through the remainder the column. The large error bars obtained for sorbed vegetative cells in the influent region of the columns suggested a relatively high variability in the behavior of the three different vegetative cell cultures. Differences in the transport behavior of freshly cultivated cells were also noted by Johnson and Logan (1996) who reported significant day to day variations in transport behavior of equivalently treated cell cultures. The surface coverage of the quartz sand with cells was less than 0.5%. This estimate was established under the assumption that all cells, which were not detected in the effluent of the columns, adhered to the first 5 cm of the porous media column. An equivalent surface area for vegetative cells was calculated using the cell dimensions (2.2 mm x 0.63 mm) given by Caccavo et al. (1996). The total surface area covered by vegetative cells was then compared to the total surface area of the quartz sand in the first five centimeter of the column (15 g x 0.168 m$^2$ g$^{-1}$, as determined by surface area analysis using nitrogen gas).
Figure 2.3. Sorption of starved (♦) and vegetative (■) cells of *S. algae* BrY to quartz sand along the flowpath of 30 cm long columns. The sorbed cell concentrations were normalized to the total number of cells injected into each column. The averages of each three different columns and their corresponding standard errors of the mean are plotted.

**Recovery of Bacteria from Columns.** The normalized effluent concentrations, fractional recoveries, total recoveries of cells from the experimental systems, and the distribution of the recovered cells in the effluent, sorbed to sand, and present in the pore water are listed in Table 2.2.

As mentioned above, the normalized breakthrough concentration and fractional recoveries were significantly higher for starved cells than for vegetative cells. Total recoveries of cells from the experimental systems were limited using the desorption and plate count methods described. Between 37 % and 69 % of the total number of cells injected into each column were recovered by analyzing the effluent, the sand, and the pore water separately. Total recoveries were around 40 % with the exception of one case in which 69 % of the vegetative cells added were accounted for. No clear difference was observed between
the ability to recover starved or vegetative cells (2-sample t-test $P = 0.482$). Such low and inconsistent recoveries were reported by other researchers who used plate counts for the enumeration of bacteria in porous media transport experiments (Alexander et al., 1991; Camper et al., 1993; Fontes et al., 1991; Tan et al., 1994). Since a decay of cells in the feed reservoir was not observed (the commonly observed variations remained within 10% of the average influent concentration), the inability to recover all bacteria from the porous media columns might have been due to incomplete recovery of bacterial cells sorbed to the quartz sand. Cells may have been injured during the desorption procedure and were thus not recoverable using the plate count method employed. Bacterial decay or a change of bacterial cells into the viable but not culturable state during the passage through the porous media column might have also caused incomplete recoveries. The relative distribution of the recovered cells to the three phases (cells in the effluent, sorbed to sand, and in the pore water) appeared to be significantly different for starved and vegetative cells. While 36 ± 6% of the recovered starved cells were detected in the effluent, only 15 ± 3% of the recovered vegetative cells were detected there (2-sample t-test $P = 0.031$). Relative recoveries of sorbed bacteria were higher for vegetative cells (72 ± 3% compared to 47 ± 8% for starved cells; $P = 0.039$). No significant difference was observed for the relative number of bacteria recovered in the pore water (17 ± 4% and 13 ± 1% for starved and vegetative cells, respectively; $P = 0.261$). These distributions further supported the hypothesis that starved cells of *S. algae* BrY were better transported, less retarded, and more homogeneously distributed through porous media.
Table 2.2. Normalized breakthrough concentrations, absolute, and relative recoveries of cells from 30 cm columns.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Normalized Fractional Breakthrough Recovery</th>
<th>Total Recovered Concentration in Effluent</th>
<th>Fraction of Recovered Cells Detected on Sand</th>
<th>Fraction of Recovered Cells Detected in Effluent</th>
<th>Fraction of Recovered Cells Detected in Pore Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starved</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>34%</td>
<td>17%</td>
<td>37%</td>
<td>50%</td>
<td>31%</td>
</tr>
<tr>
<td>2</td>
<td>45%</td>
<td>22%</td>
<td>42%</td>
<td>37%</td>
<td>43%</td>
</tr>
<tr>
<td>3</td>
<td>35%</td>
<td>20%</td>
<td>48%</td>
<td>53%</td>
<td>36%</td>
</tr>
<tr>
<td>vegetative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14%</td>
<td>6%</td>
<td>43%</td>
<td>75%</td>
<td>12%</td>
</tr>
<tr>
<td>2</td>
<td>12%</td>
<td>5%</td>
<td>69%</td>
<td>70%</td>
<td>18%</td>
</tr>
<tr>
<td>3</td>
<td>13%</td>
<td>7%</td>
<td>40%</td>
<td>71%</td>
<td>15%</td>
</tr>
</tbody>
</table>

1 fractions calculated based on the total recovery of cells from the system. I.e., distribution of recovered cells to the three phases: Sorbed (detected on sand), transported through column (detected in effluent), and present in pore water at the end of the experiment (detected in pore water).

Large (3 m) Column Experiments

In order to develop economically viable bioaugmentation strategies, it is necessary to predict and control the transport of bacteria through porous media at field relevant scales. Intermediate scale experiments, utilizing 3 m long (2.54 cm diameter) columns were performed in duplicate to evaluate scale dependent effects on bacterial transport and to test the ability of the filtration model to predict bacterial transport at larger scales. A summary of the column parameters is given in Table 2.3.
Table 2.3. Summary of parameters for bacterial transport experiments in 3 m porous media columns filled with 40 mesh quartz sand

<table>
<thead>
<tr>
<th></th>
<th>( D_1 )</th>
<th>( \Theta )</th>
<th>( \nu )</th>
<th>( c_0 )</th>
<th>( k_{at}^5 )</th>
<th>( k_{at}^6 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>starved cells</td>
<td>3.85</td>
<td>0.49</td>
<td>3.98</td>
<td>( 2.57 \times 10^7 )</td>
<td>0.000552</td>
<td>n.d. 7</td>
</tr>
<tr>
<td>vegetative cells</td>
<td>3.64</td>
<td>0.50</td>
<td>3.98</td>
<td>( 3.86 \times 10^7 )</td>
<td>0.0029</td>
<td>n.d. 7</td>
</tr>
<tr>
<td></td>
<td>3.02</td>
<td>0.48</td>
<td>3.43</td>
<td>( 7.60 \times 10^7 )</td>
<td>0.00091</td>
<td>0.0027</td>
</tr>
</tbody>
</table>

1. Dispersion coefficient [cm² min⁻¹], estimated from tracer studies
2. Porosity, estimated from tracer studies
3. Interstitial fluid velocity [cm min⁻¹]
4. Influent cell concentration [CFU mL⁻¹]
5. First order attachment coefficient [s⁻¹] estimated using Equation 2.7
6. First order attachment coefficient [s⁻¹] estimated using Equation 2.9
7. n.d.: not determined

Breakthrough of Bacteria. The trends in concentrations of culturable cells in the effluent of the 3 m long columns were in agreement with the 30 cm scale results (Figure 2.4). However, the breakthrough occurred at lower concentrations since the bacteria had to pass through a longer column causing greater removal. The breakthrough of starved *S. algae* BrY cells occurred at higher concentrations (10 ± 3 %) than for their vegetative counterparts (0.5 ± 0.7 %) and the fractional recoveries were 4 ± 1 % for starved and 0.2 ± 0.3 % for vegetative cells. Despite the obvious visual difference between the breakthrough curves of starved and vegetative cells, two sample t-tests indicated only a low statistical significance for the observed differences in the normalized breakthrough concentration (\( P = 0.136 \)) and fractional recovery (\( P = 0.079 \)) of starved and vegetative cells. The low statistical significance was mainly due to the limited number of samples available (n = 4) and the high variability in normalized breakthrough concentrations and fractional recovery for vegetative cells. Multiple regression analyses could not be performed due to the limited number of samples available. It should also be noted that a steady state was possibly not completely achieved after two pore volumes in the 3 m long columns. The effluent cell concentrations appeared to still
increase when the experiments were terminated after two pore volumes of bacterial cell suspension were injected.

The model fits (solid lines in Figure 2.4) indicated that both starved and vegetative cells tended to break through slightly later than the model predicted. It is possible that retardation of cells occurred in the columns, which would explain the delayed breakthrough (Gannon et al., 1991b; Harvey et al., 1989; McCaulou et al., 1994). Retardation can occur independently from the attenuation of cells (Harvey et al., 1989) and it might be that retardation, although it might have occurred in the smaller scale columns, was only observable consistently in the larger scale columns.

![Normalized breakthrough concentration of starved (♦, ■) and vegetative (◇, □) cells of *Shewanella algae* BrY in the effluent of 3 m long quartz sand columns and corresponding model fits (Equation 2.7, solid lines).](image)

**Figure 2.4.** Normalized breakthrough concentration of starved (♦, ■) and vegetative (◇, □) cells of *Shewanella algae* BrY in the effluent of 3 m long quartz sand columns and corresponding model fits (Equation 2.7, solid lines).

**Distribution of Bacteria in the Pore Water.** Concentration profiles of suspended cells after two pore volumes were acquired for two columns, one for starved cells, one for vegetative cells (Figure 2.5). The $k_{att}$ value obtained for starved cells using Equation 2.9 was $6.2 \times 10^4\ s^{-1}$ ($R^2 = 0.964$) and $2.7 \times 10^3\ s^{-1}$.
($R^2 = 0.998$) for vegetative cells. In contrast to the 30 cm long columns, the $k_{att}$ values estimated using Equation 2.9 were higher than the corresponding $k_{att}$ values derived from fitting the analytical solution of Equation 2.7 to the experimental data (see Table 2.3). The difference between the first order sorption coefficients estimated with Equation 2.9 and Equation 2.7 was already discussed for the 30 cm long columns and might have been due to incomplete recovery of bacteria in the pore water or the effluent of the columns.

Although the $k_{att}$ values derived for 30 cm and 3 m long columns appeared to fall in a similar range, the $k_{att}$ values derived from the short columns could not predict the breakthrough concentrations in the 3 m long columns. Using the average $k_{att}$ derived from the 30 cm long columns, the predicted normalized breakthrough concentration for 3 m long columns was 0.01 % and 0.0000009 % for starved and vegetative cells, respectively. These predicted concentrations did not agree with the normalized breakthrough concentrations of starved (10 %) and vegetative cells (0.5 %) observed in 3 m column experiments. Due to the exponential relationship of Equation 2.9 slight changes in $k_{att}$ resulted in significant changes in the effluent concentration. Scale dependent effects on bacterial transport through porous media were described by Harvey et al. (1993) and Martin et al. (1996). Harvey et al. (1993) stated the importance of small and large scale variability of heterogeneities on the results of bacterial transport, Martin et al. (1996) observed decreases in the collision efficiency factor ($\alpha$, also called sticking efficiency), and thus decreasing $k_{att}$ with increasing distance into the porous media columns. These observations indicated that the estimation of $k_{att}$ and $\alpha$ from laboratory scale experiments do not necessarily allow the accurate prediction of field scale results. In addition, a number of researchers recently suggested the existence of subpopulations with different transport behaviors in monoclonal cultures (Albinger et al., 1994). The presence of a subpopulation of sticky bacteria resulted in a higher than predicted number of bacteria adhering to the porous media grains close to the injection point while the less sticky subpopulation traveled extremely well through the porous medium, resulting in higher than expected effluent concentrations.
Figure 2.5. Normalized concentrations of starved (♦) and vegetative (■) cells of *S. algae* BrY in the pore water along the flowpath of 3 m long quartz sand columns after injection of approximately 2 pore volumes of cell suspension. Model fits (Equation 2.9) are represented by the solid lines.

**Sorption of Bacteria Along the Flowpath.** The distribution of sorbed cells throughout the columns at the end of each experiment (Figure 2.6) showed the same trend as for the 30 cm column experiments. Vegetative cells appeared to attach more strongly to the quartz sand close to the injection point than starved cells. The normalized cell concentrations of vegetative cells decreased rapidly with distance into the column. Between 120 cm and 150 cm into the column, the relative concentration of vegetative cells sorbed to sand had dropped to approximately 0.5 % (9.31 x 10^{-6} g^{-1}) of the concentration detected in the first 30 cm of the column (1.71 x 10^{-3} g^{-1}). The relative concentration of starved cells sorbed to sand between 120 cm and 150 cm into the column (6.35 x 10^{-5} g^{-1}) was at 9 % of the concentration detected in the first 30 cm (7.28 x 10^{-4} g^{-1}). The relative sorbed cell concentrations in the last 30 cm of the column (270 cm to 300 cm section) was 2.75 x 10^{-6} g^{-1} (0.2 % of sorbed cell concentration in first 30 cm of column) and 1.54 x 10^{-5} g^{-1}
(2 %) for vegetative and starved cells, respectively. As in the short column experiments, large standard errors were observed for sorbed vegetative cells, indicating higher culture to culture variability for vegetative cells. As in the 30 cm columns, the surface coverage of the quartz sand with vegetative cells was less than 0.5 %.

Figure 2.6. Sorption of starved (♦) and vegetative (■) cells of *S. algae* BrY to quartz sand along the flowpath of 3 m long columns. The sorbed cell concentrations were normalized to the total number of cells injected into each column. The averages of each two different columns and their corresponding standard errors of the mean are plotted.

Recovery of Bacteria from Columns. Table 2.4 lists the normalized effluent concentrations, fractional recoveries, total recoveries of cells from the experimental systems, and the distribution of the recovered cells in the effluent, sorbed to sand, and present in the pore water.
Table 2.4. Normalized breakthrough concentrations, absolute, and relative recoveries of cells from 3 m columns

<table>
<thead>
<tr>
<th>replicate</th>
<th>normalized breakthrough concentration</th>
<th>fractional recovery in effluent</th>
<th>total recovered</th>
<th>fraction of recovered cells detected on sand</th>
<th>Fraction of recovered cells detected in effluent</th>
<th>fraction of recovered cells detected in pore water</th>
</tr>
</thead>
<tbody>
<tr>
<td>starved</td>
<td>1</td>
<td>8%</td>
<td>4%</td>
<td>51%</td>
<td>91.5%</td>
<td>7%</td>
</tr>
<tr>
<td>cells</td>
<td>2</td>
<td>12%</td>
<td>5%</td>
<td>38%</td>
<td>86.3%</td>
<td>13%</td>
</tr>
<tr>
<td>vegetative</td>
<td>1</td>
<td>0.004%</td>
<td>0.0006%</td>
<td>37%</td>
<td>99.9%</td>
<td>0.002%</td>
</tr>
<tr>
<td>cells</td>
<td>2</td>
<td>1.0%</td>
<td>0.4%</td>
<td>55%</td>
<td>98.7%</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

1 fractions calculated based on the total recovery of cells from the system. I.e. distribution of recovered cells to the three phases: Sorbed (detected on sand), transported through column (detected in effluent), and present in pore water at the end of the experiment (detected in pore water)

The normalized breakthrough concentration and fractional recoveries were compared above ("Breakthrough of Bacteria"). The total recoveries of cells from the 3 m long columns fall in the same range as for the 30 cm columns; between 37 % and 55 % of the total number of cells injected into each column were recovered. The relative distribution of recovered cells to the three phases (effluent, sorbed to the sand, and pore water) also followed the same pattern that was described for the 30 cm long columns. Due to the limited number of total samples (n = 4) and the high variability in the behavior of the vegetative cell cultures, significance levels of two sample t-tests remained low. The fraction of recovered vegetative cells sorbed to the quartz sand (99 ± 1 %) was higher than for starved cells (89 ± 4 %; P = 0.160). This was balanced by a higher fraction of recovered starved cells in the effluent (10 ± 4 %) compared to vegetative cells (0.4 ± 0.5 %; P = 0.163). The difference between the fraction of recovered starved and vegetative cells detected in the pore water was less significant than for the other two phases (P = 0.222).

Modeling and Scale Up Issues

In order to apply the starvation transport enhancement strategy in the field, predictions about the transport behavior of starved cells must be made on field relevant scales. The first order attachment
coefficients \((k_{att})\) estimated in this work and their observed decrease at larger scales agreed with the observations made by others (McCaulou et al., 1995; Schijven et al., 1999). The scale dependency of \(k_{att}\) however, did not allow to predict the breakthrough of starved or vegetative cells through 3 m long columns based on 30 cm column results. A scale dependency of \(k_{att}\) was described and is known to limit the applicability of the simple first order attachment terms to predict results on larger scales (Martin et al., 1996). In addition, the transport model used was derived for spherical collectors. The quartz sand used in this study was irregularly shaped and this might have contributed to the observed discrepancies between the experimental data and the modeling predictions at both, the 30 cm and the 3 m scale. Another potential explanation for the inability to model the experimental results using the filtration theory with first order attachment is population variability within the bacterial cultures. The presence of subpopulations with different transport behaviors and the observed scale dependent effects would require the application of transport models, which could potentially account for spatial and intrapopulation heterogeneities of transport related parameters. Detailed studies recently revealed the presence of subpopulations within monoclonal cultures (Albinger et al., 1994; Baygents et al., 1998; Bolster et al., 2000; Simoni et al., 1998) and thus multi-parameter models might be more appropriate to describe bacterial transport through porous media. However, for the development of a conceptual model describing population- and scale-dependent effects, very specific knowledge of the parameters influencing bacterial transport is required. This research, however, did not allow to reveal specific mechanisms that might be responsible for the inability to use short columns in order to predict bacterial transport at larger scales. Thus, the choice of a more appropriate transport model was not obvious and was not pursued in this research.

Influence of Influent Cell Concentration and Fluid Velocity

Additional improvements in bacterial transport through porous media could be achieved by optimizing other parameters which are known to influence bacterial transport and which can be controlled in field applications. Preliminary experiments evaluating the influence of influent cell concentration and interstitial fluid velocity on the transport of starved \(S. algae\) BrY were performed in 30 cm long columns. A
change in influent cell concentration from $2.28 \times 10^6$ CFU ml$^{-1}$ to $1.27 \times 10^7$ CFU ml$^{-1}$ resulted in a change in fractional recovery from 21% to 17% for the low and high cell concentration, respectively. A change in interstitial fluid velocity from approximately 3 cm min$^{-1}$ to approximately 13 cm min$^{-1}$ only led to an increase in fractional recovery from 17% to 19% for low and high fluid velocity, respectively. These data suggest that the control of influent cell concentration and fluid velocity can potentially influence the bacterial transport through porous media but their importance appears to be low compared to the influence of starvation.

Conclusions

A major problem that is preventing the widespread implementation of bioaugmentation strategies is the tendency of bacteria to strongly adhere to the surfaces in the immediate vicinity of the injection point. Biofouling of these areas can lead to a failure of the injection system and limited dispersion of the bacterial inoculum in the subsurface. The use of starved bacteria might overcome this problem since starved bacteria typically adhere less, transport farther, and distribute more homogeneously through porous media than vegetative cells. The promising potential of starved cells for bioaugmentation strategies is indicated by higher fractional recoveries (3.2 fold higher for 30 cm columns, 22.6 fold higher for 3 m columns), higher normalized breakthrough concentrations (2.9 and 19.9 fold), and lower first order attachment coefficients ($k_{att}$ 1.8 fold lower for 30 cm columns, and 3.2 fold lower for 3 m columns). In addition to their improved transport behavior, starved cells are metabolically dormant. Their metabolic dormancy and lag phase before regaining full metabolic activity would allow for the simultaneous or subsequent injection of nutrients without the danger of immediate biofouling of the employed injection system.

There is sufficient evidence to assume that transport enhancement by bacterial long term starvation is not limited to \textit{Shewanella algae} BrY but is a rather general starvation response across many bacterial strains (Amy and Haldeman, 1997; Kjelleberg, 1993a; Lappin-Scott and Costerton, 1990). Thus, the starvation transport enhancement strategy could be applicable to a large number of bacterial strains with
a variety of metabolic capabilities. If successfully developed, the starvation transport enhancement strategy would provide the practitioner with a versatile tool for the development of bioaugmentation technologies.

Additional Research Needs

In order to better understand the transport behavior of *S. algae* BrY cells, the parameters governing the transport of starved bacteria have to be identified. According to the filtration theory (Equation 2.10) \( k_{\text{att}} \) is influenced by \( \eta \) and \( \alpha \), \( v \), and \( \Theta \). While the fluid velocity (\( v \)) and the porosity (\( \Theta \)) are independent from the inoculum used, \( \eta \) and \( \alpha \) account for properties of the bacteria. The single collector efficiency (\( \eta \)) can be calculated using Equations 2.2 through 2.6. The cell diameter (\( d_p \)), diffusion coefficient (\( D_p \)), and buoyant density (\( \rho_p \)) are the cell properties which can account for differences in \( \eta \) between starved and vegetative cells. The collision efficiency (\( \alpha \)) incorporates all physicochemical interactions between the bacteria and the porous medium, and in addition any inaccuracies in the calculation of \( \eta \) (Martin et al., 1996). Equations for the calculation of \( \alpha \) however are not available for bacteria. This indicates that the currently existing filtration models do not yet account for these physicochemical interactions and inaccuracies.

Changes in cell shape, surface charge, hydrophobicity, and the presence of pili and flagella are likely to influence \( \alpha \) and therefore influence bacterial attachment to surfaces and transport through porous media. It was considered important to monitor the change of several of these bacterial cell properties in an attempt to identify parameters responsible for the starvation transport enhancement. The results of these investigations and their implications are summarized in Chapter 3.

Once a better understanding of the factors governing the transport of starved *S. algae* BrY cells has been gained, a model accounting for these factors must be developed. However, until the processes governing bacterial transport through porous media are sufficiently understood and appropriate models developed, transport experiments should be conducted at field relevant scales, with the bacteria and subsurface material of interest, and by mimicking field conditions as closely as possible.
Since there is not necessarily a clear relationship between attenuation and retardation of bacteria in porous media it is extremely difficult to extrapolate from column breakthrough data to the distribution of sorbed cells (Harvey et al., 1989). Therefore, the development of more accurate methods for the quantification of sorbed bacteria and their activity is essential to develop a thorough understanding of bacterial transport through porous media. A more complete recovery of cells from porous media would allow to better estimate the distribution of cells along the flowpath and therefore allow to describe the adhesion processes better. Using plate counts, between 30 % and 60 % of the injected cells remained unaccounted for in this study. Such a low recovery allows for much speculation and can result in the development of mathematical models which, although they accurately describe the experimental data, might not be based on a thorough understanding of the processes involved.

Since the activity of sorbed bacteria is the most important factor determining the success of bioaugmentation strategies, it would be desirable to develop procedures which allow for the enumeration and activity assessment of sorbed bacteria simultaneously. The recent development of molecular techniques has rapidly improved our ability to selectively detect and quantify bacteria in environmental samples. The most commonly used suite of molecular methods for the identification and quantification of bacterial numbers and activity consists of selective ribosomal RNA (rRNA) and DNA extraction, quantitative polymerase chain reaction (qPCR) and reverse transcriptase PCR (RT-PCR), denaturing gradient gel electrophoresis (DGGE) fingerprinting, and nucleic acid sequencing. The detection and quantification of rRNA and rDNA (DNA encoding for the rRNA) could be utilized for quantification and activity assessments. The rRNA content of cells can be correlated with bacterial activity while the amount of rDNA estimates bacterial numbers (Schafer et al., 2001). Consequently, the rRNA to rDNA-ratio can be used as an estimate for the specific activity of bacterial cells (Duineveld et al., 2001). Unfortunately, the techniques currently available are 1) mostly designed for aqueous samples, 2) still fairly work intensive, and 3) require the use of expensive equipment and supplies (qPCR equipment and fluorescent labels). The current development of nucleic acid microarrays might provide an opportunity to develop these methods more
inexpensively. Successfully developed microarray technologies might allow for the inexpensive and selective enumeration and activity assessment of bacterial cells in environmental samples.
CHAPTER 3

CHANGE OF TRANSPORT-RELATED CELL PROPERTIES DURING STARVATION OF SHEWANELLA ALGAE BrY

Abstract

The enhanced transport of long term nutrient starved bacteria has often been attributed to the decrease in average cell size during starvation. However, according to the filtration theory, cell size alone is not sufficient to explain the difference in transportability between starved and vegetative cells of Shewanella algae BrY. This chapter documents the changes of a number of transport-related cell properties of Shewanella algae BrY during carbon and nitrogen starvation. The cell buoyant density decreased during starvation, the apparent diffusion coefficient and the cell surface charge (measured as zeta potential) increased but no change in hydrophobicity was observed. According to the filtration model applied, the cell size, buoyant density, and diffusion coefficient of starved cells could partly explain the improved transport behavior of starved cells through porous media while the transport of vegetative cells was drastically overpredicted. The observed slight changes in cell surface charge and hydrophobicity were not sufficient to explain the difference between starved and vegetative cells. It was concluded that parameters beyond the ones investigated appear to be responsible for the differences in porous media transport of starved and vegetative S. algae BrY cells and that a better understanding of these parameters must be obtained before an appropriate mathematical model can be developed.

Introduction

Chapter 2 clearly demonstrated that starved Shewanella algae BrY cells are transported better through porous media than their vegetative counterparts. However, explanations for the improved
transportability of starved cells are lacking. Changes in cell size and cell shape have been correlated with transport through porous media (Bitton et al., 1974; Fontes et al., 1991; Gannon et al., 1991a; Weiss et al., 1995) and were suggested to be at least partly responsible for the improved transport of starved bacteria (Lappin-Scott and Costerton, 1992; Lappin-Scott et al., 1988b).

However, based on the filtration theory, the differences in cell size between starved and vegetative cells of *Shewanella algae* BrY were not sufficient to explain the observed differences in bacterial transport.

Caccavo et al. (1996) reported a change in cell size from approximately 2.2 μm x 0.63 μm (length x width) to more spherical cells of approximately 1.04 μm x 0.63 μm during starvation. Based on the one-dimensional filtration theory (Equation 2.1), this change would lead to an only slightly increased breakthrough \((c/c_0)\) of starved cells. Figure 3.1 shows the steady state concentration in the effluent of 30 cm long columns for particles in the range from 0.1 μm to 5 μm. A list of the parameters used in these calculations is given in Table 3.1. According to these calculations, starved cells of *S. algae* BrY break through the 30 cm long porous media columns at approximately 47.2 % of the influent cell concentration while vegetative cells are predicted to break through at 46.6 %. The experimental data summarized in Chapter 2 show slightly lower breakthrough concentrations for starved cells (38 %) and significantly lower concentrations for vegetative cells (13 %). Thus, parameters other than the cell size are likely to govern the transport of starved and vegetative *S. algae* BrY cells through porous media columns.

Equation 2.1 suggests that, if the filtration theory and the parameters applied here are appropriate for the description of bacterial transport through porous media, changes in the single collector efficiency \((\eta)\) or the collision efficiency factor \((\alpha)\) must be responsible for the observed changes in normalized breakthrough concentrations \((c/c_0)\). The single collector efficiency \((\eta)\) is a measure for the rate at which particles strike porous media grains and is influenced by the size, diffusion coefficient, and buoyant density of the bacterial cells (see Equations 2.2 through 2.6). The collision efficiency factor \((\alpha)\) is a measure for the number of collisions, which result in the adhesion of particles to the porous media grains. The collision efficiency factor is also referred to as sticking efficiency in bacterial transport experiments. In contrast to \(\eta\) there is no theoretical basis available for the calculation of \(\alpha\); it allows to account for physicochemical
interactions between the bacteria and the porous media grains (Martin et al., 1996) and should remain
between zero and one (Logan et al., 1995).

Figure 3.1. Predicted, normalized, steady-state breakthrough concentrations through 30 cm quartz
sand columns of particles with diameters between 0.1 μm and 5 μm according to the
filtration theory (Equation 2.1, Logan et al., 1995). The arrows indicate the calculated
breakthrough concentrations for starved and vegetative cells of *S. algae* BrY, whose sizes
were reported to be 1.04 μm and 2.2 μm, respectively (Caccavo, Jr. et al., 1996). The
curve was generated with the values listed in Table 3.1.

The observed differences in porous media transport between starved and vegetative cells and the
inaccurate model predictions discussed in Chapter 2 make it necessary to investigate the parameters which
potentially influence \( \eta \) and \( \alpha \), and thus the transport and adhesion of bacteria in porous media. The
diameter \( (d_p) \), buoyant density \( (\rho_p) \) and diffusion coefficient of the cells \( (D_p) \) are known to influence \( \eta \) (see
Equations 2.2 through 2.6). Changes in cell hydrophobicity, net electrostatic charge, character and
properties of surface molecules, and the presence of flagella and pili are likely to influence the ability of
cells to adhere to surfaces after a collision has occurred, i.e. influence the collision efficiency or stickiness
of cells \( (\alpha) \).
Table 3.1: Parameters used for the calculation of normalized breakthrough concentrations in 30 cm columns as shown in Figure 3.1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\rho$</td>
<td>998.2 kg m$^{-3}$</td>
<td>$v$</td>
<td>2 cm min$^{-1}$</td>
</tr>
<tr>
<td>$\rho_p$</td>
<td>1044 kg m$^{-3}$</td>
<td>$g$</td>
<td>9.80565 m s$^{-2}$</td>
</tr>
<tr>
<td>$d_c$</td>
<td>0.5 mm$^{-1}$</td>
<td>$\mu$</td>
<td>$1.002 \times 10^{-3}$ kg m$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>$T$</td>
<td>293.16 K</td>
<td>$\theta$</td>
<td>0.5</td>
</tr>
<tr>
<td>$k$</td>
<td>$1.3048 \times 10^{23}$ J K$^{-1}$</td>
<td>$\alpha$</td>
<td>1$^2$</td>
</tr>
<tr>
<td>$d_p$</td>
<td>0.1 $\mu$m to 5 $\mu$m</td>
<td>$x$</td>
<td>0.30 m</td>
</tr>
</tbody>
</table>

1 effective filtration size (manufacturer information, Unimin Corp., Emmet, ID)

2 $\alpha$ = 1 indicates complete destabilization of the bacterial suspension, i.e. every collision of a bacterial cell with a porous media grain results in the removal of the bacterium from the cell suspension.

The changes in cell diameter during starvation of *S. algae* BrY were previously described by Caccavo et al. (1996). These data were used for the development of Figure 3.1. The research summarized in this chapter describes the changes of cell numbers (colony forming units, direct cell counts, and absorbance at 600 nm), hydrophobicity, apparent diffusion coefficient, electrostatic charge, apparent buoyant density, adhesivity to quartz sand, and transportability through 30 cm quartz sand columns of *Shewanella algae* BrY over a starvation period of 7 weeks. The findings are expected to improve the understanding of bacterial transport enhancement by starvation and contribute towards the development of a model appropriately describing the transport of starved and vegetative bacteria through porous media.

Materials and Methods

Strains and Culturing Methods

Cultures of *Shewanella algae* BrY were maintained and grown as described in Chapter 2. Starved cells were harvested weekly by centrifugation and resuspended in PBS for all assays, except for microscopic direct counts, viable cell counts, and absorption measurements, which were performed with
samples taken directly from the starvation cultures. *Shewanella algaee* BrY-gfp (gfp = green fluorescent protein) was graciously provided by Yuri Gorby (Pacific Northwest National Laboratory, Richland, WA). *S. algaee* BrY-gfp was cultured and maintained in the presence of 20 mg L\(^{-1}\) kanamycin.

**Cell Quantification and Cell Imaging**

Cells were quantified using absorbance measurements at 600 nm, microscopic direct counts, and dilution plate counts using tryptic soy agar (TSA, Difco). Absorbance measurements were performed using a Shimadzu spectrophotometer (UV-2101 PC). Microscopic direct counts were performed after fixing cells in 4 % formaldehyde solution and staining cells on dark polycarbonate filters (Whatman, Clifton, NJ) with 50 mg L\(^{-1}\) acridine orange or 10 mg L\(^{-1}\) 4',6-diamidino-2-phenylindole (DAPI) solution for one to five minutes. The filters were examined using a Nikon Eclipse E800 epifluorescence microscope with a mercury lamp UV source. The filter blocks used had an excitation/emission bandwidth of 450-490 nm/\(>515\) nm and 340-380 nm/435-495 nm for acridine orange and DAPI, respectively. Electronic images of *S. algaee* BrY-gfp cells were acquired using the same microscope with mercury lamp, the 450-490 nm/\(>515\) nm filter block and a charge coupled device (CCD) camera (Princeton Instruments, Trenton, NJ). Cell sizes were estimated manually from the acquired images by measuring cell width and length and comparing it to the appropriate scale bar. Plate counts were performed after dilution in PBS by spreading 0.1 mL of bacterial cell suspension onto tryptic soy agar. Plates were incubated at 30 °C for 24 hours and colonies were counted using a 5x magnification glass.

**Hydrophobicity**

Changes in cell hydrophobicity were estimated using the bacterial adhesion to hydrocarbons (BATH) assay (Rosenberg, 1984; Rosenberg et al., 1980). In brief, cell suspensions (5 mL) with an absorbance (600 nm) between 0.120 and 0.180 were added to acid washed screw cap vials. Hexadecane (2 mL, Fisher, Pittsburgh, PA, previously filtered through a 0.2 \(\mu\)m membrane) was added, the vials were
closed, and vortexed for 30 seconds. The hexadecane and aqueous phases were allowed to separate for 15 minutes and an aliquot of the aqueous phase was transferred carefully to a cuvette using a glass pasteur pipette. The absorbances (600 nm) of the treatments were compared to control treatments lacking hexadecane. All measurements were conducted in triplicate. The hydrophobicity index (HI) was calculated according to

$$HI = \frac{A_{600\text{nm, initial}} - A_{600\text{nm, final}}}{A_{600\text{nm, initial}}}$$

(Eq. 3.1)

Zeta Potential and Diffusion Coefficient

The zeta potential and the apparent diffusion coefficient of the cells was determined from cell suspensions in PBS at pH 7.0 and 25 °C using a Zetasizer 2c (Malvern Instruments). Zeta potentials and apparent diffusion coefficients were determined from the average of 10 measurements.

Buoyant Density

The apparent buoyant density was calculated using the density gradient centrifugation procedure described by Harvey et al. (1997). In brief, 2.5 mL of a bacterial suspension was carefully layered on top of 40 mL of a Percoll/PBS solution at pH 7 (Sigma, St. Louis, MO) and the tubes were centrifuged at 15,000 x g for 60 minutes. Immediately after centrifugation, the position of the bacterial bands in the tubes was compared to bands formed by brightly colored beads of known density (DMB-10, Sigma, St. Louis, MO).

Adhesion Assay

Adhesion studies were performed as described previously (Caccavo, Jr. et al., 1997). In brief, cell suspensions in PBS were added to 2 mL microcentrifuge vials containing 0.1 g of quartz sand (approximate surface area 0.0168 m²). The vials were placed on a horizontal shaker at 150 rpm for 30 minutes, centrifuged at 300 x g for 5 minutes, and the supernatant was sampled for total microscopic cell counts.
using acridine orange. Cell concentrations between $1.17 \times 10^7$ and $1.93 \times 10^8$ cells per ml were used and the adhesion assays were performed in triplicate. The number of adhered cells was calculated by subtracting the number of unadhered cells from the number of total cells. Adhesion of cells to the tubes and cell lysis were accounted for with controls of adhesion assay buffer without quartz sand.

**Transportability Through Porous Media**

Transport experiments were performed with vegetative cells, and cells starved for one, four, and seven weeks in the same manner as described in Chapter 2.

**Results and Discussion**

Figure 3.2 shows images of *Shewanella algae* BrY-gfp cells during long term starvation. Cells immediately after culturing and washing were between 1.8 μm and 3.0 μm in length and approximately 0.6 μm in width. Cells fluoresced brightly when observed under ultraviolet (UV) light with the appropriate filter set. In the upper left corner of image (a) ongoing cell divisions were captured. Two cells were separating from each other at the end of a cell division cycle at the time the image was acquired. *S. algae* BrY-gfp cells shrunk over time and were approximately 1.5 μm in length and 0.6 μm in width after 7 weeks but still fluoresced due to the presence of gfp (image b). The cells appeared to be perfectly spherical with an approximate diameter of 0.6 μm after 6 months of starvation (image c). *S. algae* BrY-gfp cells still fluoresced after 6 months and could be resuscitated on tryptic soy agar (data not shown). Visual observation indicated that *S. algae* BrY-gfp cells were motile until the third week but mostly non motile at longer starvation times.
Cell Quantification

Viable cells from *Shewanella algae* BrY starvation cultures were estimated as colony forming units on tryptic soy agar. The concentration of viable cells slightly increased during the first week of starvation from initially $3.83 \times 10^9 \pm 1.33 \times 10^8$ CFU mL$^{-1}$ to $4.93 \times 10^9 \pm 4.84 \times 10^8$ CFU mL$^{-1}$ (Figure 3.3). The concentration of cells decreased to $2.10 \times 10^8 \pm 1.04 \times 10^7$ CFU mL$^{-1}$ after seven weeks of starvation. Initially total cell counts were approximately ten times higher ($3.15 \times 10^{10} \pm 2.05 \times 10^9$ cells mL$^{-1}$) than the concentration of colony forming units. The total concentration of cells decreased slightly during the first week to $2.77 \times 10^{10} \pm 2.02 \times 10^9$ cells mL$^{-1}$ and was approximately 2.5 times higher than the concentration of viable cells. The numbers further decreased over time and reached $4.98 \times 10^9 \pm 1.05 \times 10^8$ cells mL$^{-1}$ after 7 weeks, which was approximately 25 times higher than the concentration of colony forming units at that time. The absorbance (600 nm) of the cell suspensions decreased almost linearly from $1.22 \pm 0.06$ to $0.172 \pm 0.01$ over seven weeks of starvation. These results concur with the observations by Caccavo et al. (1996) who reported a decrease in viable cell numbers during starvation. The change in the
ratio of culturable to total cells from initially ten to approximately 25 after seven weeks of starvation indicated an increase in the fraction of cells that were physically intact but not culturable. Resuscitation methods other than plate counts on full strength tryptic soy agar might have allowed for a better recovery of *S. algae* BrY cells from long term starvation, however, the development of improved recovery methods was not focus of this research.

![Figure 3.3](image)

**Figure 3.3.** Concentration of total cells (♦), culturable cells (■), and absorbance at 600 nm (▲) during starvation of *Shewanella algae* BrY. Error bars represent the standard error of the means (n = 3).

**Hydrophobicity and Zeta Potential**

The change in hydrophobicity and zeta potential during starvation is shown in Figure 3.4. The hydrophobicity index appeared to slightly increase during the first 3 weeks of starvation but decreased again starting in week 4. It remained between 27 % and 42 % until week 5 and dropped slightly to 18 ± 13 % after 7 weeks of starvation. The standard errors obtained using the BATH assay, however, were too
large to determine definite trends. An analysis of variance was performed to obtain pooled variances for a two sample t-test. The t-test failed to indicate a statistically significant difference between the initial and final hydrophobicity indices ($P = 0.66$).

The zeta potential slightly increased over the 7 week period. Initially $-39.5 \pm 1.6$ mV were measured, which agrees with the value of $-37.7 \pm 0.7$ mV reported by Caccavo et al. (1997). The zeta potential remained at approximately $-40$ mV until week 4 but then increased to reach $-33.3 \pm 2.8$ mV after 7 weeks. However, this change in zeta potential could not explain the observed transport improvement reported in Chapter 2. A change of net electrostatic charge to more positive values should have resulted in increased electrostatic interaction between the negatively charged quartz sand and the bacterial cells (Sharma et al., 1985). However, as shown in Chapter 2, the transport of $S. \text{algae}$ BrY cells was improved and not inhibited by starvation. Thus, electrostatic interactions were not likely to be responsible for the improved transport of starved $S. \text{algae}$ BrY cells through quartz sand at circumneutral pH.

![Figure 3.4](image-url)

Figure 3.4. Change in hydrophobicity index (♦) and zeta potential (■) during starvation of *Shewanella algae* BrY. Error bars represent the standard error of the means (n = 3 and n=10 for hydrophobicity index and zeta potential, respectively).
Diffusion Coefficient and Buoyant Density

The apparent diffusion coefficient and the apparent buoyant density both changed slightly during starvation (Figure 3.5). The diffusion coefficient increased from $2.7 \times 10^{-9} \pm 1.11 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ to $4.79 \times 10^{-9} \pm 1.43 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ after seven weeks and the apparent buoyant density decreased from $1.044 \text{ g cm}^{-3}$ to $1.020 \text{ g cm}^{-3}$.

![Figure 3.5. Change of apparent buoyant density (♦) and apparent diffusion coefficient (■) during starvation of *Shewanella algae* BrY. Error bars represent the standard error of the means (n = 3 and n=10 for buoyant density and diffusion coefficient, respectively).](image)

Modeling Results

**Influence of Diffusion Coefficient and Buoyant Density, Calculation of \( \eta \).** The measured values for the cell diameter (1.04 \( \mu \text{m} \) and 2.2 \( \mu \text{m} \), Caccavo et al. 1996), buoyant density (1.020 g cm\(^{-3}\) and 1.044 g cm\(^{-3}\)), and diffusion coefficient (2.7 \( \times \) 10\(^{-9}\) cm\(^2\) s\(^{-1}\) and 4.79 \( \times \) 10\(^{-9}\) cm\(^2\) s\(^{-1}\)) of starved and vegetative cells
(respectively) were used to calculate the theoretical breakthrough concentrations according to Equations 2.1 through 2.5. All other values were used as listed in Table 3.1.

The observed increase of the apparent diffusion coefficient and the decrease in buoyant density alone were not sufficient to explain the differences in breakthrough concentrations of starved and vegetative cells. The breakthrough concentrations calculated for starved cells (45.4 %) were slightly closer to the experimentally observed breakthrough (38 %) after accounting for buoyant density ($\rho_p$) and diffusion coefficient ($D_p$) indicating that $\rho_p$ and $D_p$ have a slight influence on the transport behavior of bacterial cells. However, the model failed to predict the breakthrough of vegetative cells. A normalized breakthrough concentration of 42.0 % was predicted and only 13 % were observed in the 30 cm column experiments summarized in Chapter 2.

The determination of buoyant density ($\rho_p$) and diffusion coefficient ($D_p$) also allowed the calculation of the single collector efficiencies ($\eta$) for starved and vegetative cells. However, the differences in $\eta$ between starved ($7.62 \times 10^{-4} \pm 1.30 \times 10^{-4}$, see Table 3.2 for listing) and vegetative cells ($9.37 \times 10^{-4} \pm 1.24 \times 10^{-4}$) could only account for part of the differences in breakthrough concentrations and were not statistically significantly different (two sample t-test, $P = 0.190$). Thus, if all other parameters of the model were correct, changes in the collision efficiency factor ($\alpha$) must have been responsible for the differences between starved and vegetative cells.

Table 3.2. Estimated single collector efficiencies ($\eta$) and collision efficiency factors ($\alpha$) for starved and vegetative S. algae BrY cells, 30 cm columns.

<table>
<thead>
<tr>
<th>starved</th>
<th></th>
<th>vegetative</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$\eta$</td>
<td>$\alpha$</td>
<td>$\eta$</td>
<td>$\alpha$</td>
</tr>
<tr>
<td>0.000637</td>
<td>1.846</td>
<td>0.000794</td>
<td>2.660</td>
</tr>
<tr>
<td>0.000896</td>
<td>1.006</td>
<td>0.001008</td>
<td>2.494</td>
</tr>
<tr>
<td>0.000752</td>
<td>1.284</td>
<td>0.001008</td>
<td>2.418</td>
</tr>
</tbody>
</table>
Calculation of Collision Efficiencies $\alpha$. The estimation of $\eta$ for each column allowed the calculation of $\alpha$ values for starved and vegetative cells using the first order attachment coefficients estimated in Chapter 2 (Table 2.1) and Equation 2.10. The $\alpha$ values obtained for starved cells ($1.38 \pm 0.43$) were statistically significantly lower than for vegetative cells ($2.55 \pm 0.12$, $P = 0.049$). This difference in $\alpha$ values indicated that differences in the sticking efficiencies ($\alpha$) were partly responsible for the observed differences in porous media transport of starved and vegetative cells, assuming that all other values used for the calculations, such as cell size were correct (see Table 3.2 for list of $\alpha$ values).

The sticking efficiency ($\alpha$) was introduced into the filtration theory to account for physicochemical interactions between the particles (bacteria) and the porous media grains (Martin et al., 1996). However, since there is no theoretical basis available for the calculation of $\alpha$, it is commonly estimated from filtration experiments. It, thus, also indirectly serves to account for any inaccuracies in the filtration theory. Since the filtration model was derived for spherical collectors, such inaccuracies can derive from the application of the filtration model to porous media grains that are irregularly shaped like the quartz sand used in this study. According to the filtration theory (Logan et al., 1995), values for $\alpha$ should remain between zero and one. However, values of $\alpha$ greater than one have been reported to be necessary to accommodate for differences in model predictions and experimental data (Baygents et al., 1998; Logan et al., 1995; Martin et al., 1996). It was concluded that the filtration theory, as applied here, was not completely appropriate for describing the experimental data from bacterial transport experiments (Chapter 2) unless values of greater than one were assigned to the sticking efficiency ($\alpha$).

The cell surface hydrophobicity and the net electrostatic charge could have potentially influenced the physicochemical interaction between bacteria and porous media grains, and therefore $\alpha$. However, as discussed above, no significant changes in cell surface hydrophobicity were observed using the BATH assay. However, since the BATH assay measurements were associated with high standard errors, future research should evaluate other methods that allow to estimate cell surface hydrophobicity more accurately. Hydrophobic interaction chromatography utilizing hydrophobic resins such as phenyl- or octyl-sepharose is
potentially suitable for this purpose. The observed changes in zeta potential to less negative values during starvation cannot explain the increased transport of starved bacteria either. A change of cell surface charge to less negative values should have resulted in increased cell quartz sand interactions and thus decreased transportability.

**Applicability of Filtration Model to 3 m Column Results**

The measurement of the buoyant density \( \rho_p \) and diffusion coefficient \( D_p \) were used to predict the transport through the 3 m long columns. However, as discussed in Chapter 2, the model drastically underpredicted the breakthrough concentrations for both starved and vegetative cells. While breakthrough concentrations of 0.04 % were predicted for starved cells, 10 % ± 3 % were observed in the experiments described in Chapter 2. The breakthrough of vegetative cells was also underpredicted; a normalized effluent concentration of 0.017 % was predicted but 0.4 % ± 0.5 % were observed.

While \( \eta \) was not significantly different for starved and vegetative cells (starved: \( 5.37 \times 10^{-4} \pm 0 \); vegetative \( 5.85 \times 10^{-4} \pm 2.88 \times 10^{-5}, \) see Table 3.3), \( \alpha \) values were lower for starved cells (0.46 ± 0.06) than for vegetative cells (1.38 ± 0.87). However, a two sample t-test failed to indicate a statistically significant difference between the \( \alpha \) values of starved and vegetative cells \( (P = 0.374) \) due to the high variability observed in the transportability of vegetative cells. The \( \alpha \) values calculated for the 3 m columns were significantly smaller than the ones calculated for 30 cm long columns (see Table 3.2). A decrease of collision efficiency factors \( (\alpha) \) with increasing distance into a porous medium was also observed by Martin et al. (1996) and is a further indication for scale dependent effects on bacterial transport.

<table>
<thead>
<tr>
<th>starved</th>
<th>vegetative</th>
</tr>
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<tbody>
<tr>
<td>( \eta )</td>
<td>( \alpha )</td>
</tr>
<tr>
<td>0.000537</td>
<td>0.502</td>
</tr>
<tr>
<td>0.000537</td>
<td>0.412</td>
</tr>
</tbody>
</table>

Table 3.3. Estimated single collector efficiencies \( (\eta) \) and collision efficiency factors \( (\alpha) \) for starved and vegetative *S. algae* BrY cells, 3 m columns.
Batch Adhesion Assays as a Predictive Tool

The tendency of *S. algae* BrY cells to adhere to quartz sand in batch systems was monitored in order to provide a tool that would potentially allow to screen bacterial strains for their applicability to the starvation transport enhancement strategy. The fraction of cells adhered to quartz sand in batch experiments and the fractional recovery of cells after passage through 30 cm quartz sand columns are shown in Figure 3.6. The adhesion assay showed a significant decrease in the fraction of cells adhered to quartz sand over the first two weeks of starvation (t-test with pooled standard errors, \( P = 0.05 \)) and the fraction attached remained below 10% in average during the seven weeks of starvation. It should be noted that the adhesion assay yielded high standard errors and negative adhesion values after two and five weeks, i.e. a higher cell number was observed in the treatments containing quartz sand than in the controls. It is obvious, though, that there was an inverse relationship between the number of cells adhered to quartz sand and the fractional recovery of cells (Figure 3.6).

![Graph showing change in adhesivity and transportability](image-url)

**Figure 3.6.** Change in adhesivity (fraction of cells adhered to quartz sand, ◆), and transportability (fractional recovery of viable cells after passage through 30 cm of quartz sand, □) during starvation of *Shewanella algae* BrY. Error bars represent the standard error of the means.
A linear relationship was suggested after the average fractional recovery from 30 cm porous media columns was plotted against the average fraction of cells adhered to quartz sand in the batch adhesion studies (Figure 3.7). The regression analysis resulted in a correlation coefficient of $R^2 = 0.9421$ and a slope of $(\text{fractional recovery}) \times (\text{fraction adhered})^{-1} = -0.2365$. Thus, a decrease in the fraction of cells adhered to quartz sand in batch experiments resulted in an increase in fractional recovery during transport through 30 cm quartz sand columns. These results imply that it might be possible to use less time- and cost-intensive batch experiments to qualitatively predict the transport behavior of cells through porous media. However, to obtain quantitative results, the reproducibility of adhesion assays will have to be improved. The acquisition of sorption isotherms would potentially allow for better quantitative assessments. However, the acquisition of sorption isotherms for bacterial cultures is expected to be approximately as work intensive as column studies and therefore would have only little potential as a screening tool. In addition, batch adhesion experiments are not likely to emulate the potential influence of hydrodynamics and kinetics on bacterial adhesion in porous media columns.

$$\text{fractional recovery} = -0.2365 \times (\text{fraction adhered in batch}) + 0.2413$$

$R^2 = 0.9421$

Figure 3.7. Plot of fractional recovery from 30 cm quartz sand columns over the fraction of cells adhered in batch experiments.
Conclusions

The research summarized in this chapter demonstrated that, according to the filtration theory, changes in cell size, buoyant density, and diffusion coefficient could partly explain the enhanced transport of starved cells. The remaining differences between starved and vegetative cells had to be accounted for by modifying the collision efficiency factor ($\alpha$), which had to take on values of greater than one. Although values of $\alpha$ greater than one have been reported in the literature, they are conceptually not appropriate to use in the filtration model. Thus, the applicability of the filtration theory for bacterial transport through porous media in its currently applied form has to be questioned and the development of a mathematical model capable of appropriately describing bacterial transport through porous media is necessary. Such a model should be capable of appropriately describing the observed scale- and physiology-dependent changes influencing the transport and attachment behavior of bacterial cells, which were described in Chapter 2 and this chapter.

The observed changes in cell hydrophobicity and zeta potential were not sufficient to explain the differences in sticking efficiency between starved and vegetative cells. Research elucidating the change of other potentially transport related cell properties is ongoing. Currently scanning and transmission electron microscopy (SEM, TEM) are utilized to observe the fate of flagella during long term starvation of *Shewanella algae* BrY. The SEM and TEM work is performed by Alice Dohnalkova at the Environmental and Molecular Science Laboratory (EMSL) located at Pacific Northwest National Laboratory (PNNL, Richland, WA). Caccayo et al. (1992) reported that vegetative cells of *Shewanella algae* BrY have a single polar flagellum. The presence of flagella could significantly increase the effective diameter of vegetative cells and therefore result in a greater removal of cells according to the filtration theory. Long term starvation might result in the loss of the flagellum and could therefore provide an explanation for the transport differences observed for starved and vegetative cells. Preliminary results utilizing a flagella deficient mutant (*S. algae* BrY-NF, which was kindly provided by Amitabha Das from PNNL), however indicate that the absence of the flagellum, though it potentially increases the transportability of vegetative cells, does not result in the same
increase in transportability as for starved cells (data not shown). Mueller (1996) discussed the influence of flagella and cell motility on the attachment of *Pseudomonas fluorescens* to the surface of microscopic flow cells. The higher adsorption rate coefficient of the flagellated, motile strain was attributed to superior transport properties. The non-motile strain however showed higher sticking efficiencies suggesting that the flagellum was not necessary for attachment but increased the specific adsorption rate coefficient. Das and Caccavo in contrast suggest that the flagellum acts as a motility-independent adhesin for *Shewanella algae* BrY (Das and Caccavo, unpublished results). Mueller (1996) however states that the hydrodynamic conditions can significantly influence the attachment of bacteria and it is likely that the hydrodynamic conditions in porous media are different from microscopic flow cells. Thus, the change of other parameters might be responsible for the improved transport behavior of starved cells.

A more thorough characterization of the cell surface of *S. algae* BrY during starvation might provide additional information about the change of transport related cell surface properties. Information about protein and carbohydrate patterns on the cell surface could potentially be obtained using X-ray Photoelectron Spectroscopy (XPS) and Time of Flight Secondary Ion Mass Spectroscopy (ToF SIMS) as described by Caccavo et al. (1997). In addition, an alternative method should be applied to verify the hydrophobicity results obtained with the BATH assay (e.g. hydrophobic interaction chromatography).

In view of potential field applications of the starvation transport enhancement strategy, the starvation time should be optimized to produce cells with optimal transport properties in the least amount of time. The fractional recoveries observed for *S. algae* BrY (Figure 3.6) suggest that similar transport efficiencies can be achieved after four and seven weeks of starvation. Thus, four weeks of starvation might be sufficient to achieve a significant improvement in bacterial transport. Seven weeks of starvation time were initially chosen because previous work by Caccavo et al. (1996) indicated an almost constant cell size after seven weeks of starvation.

A thorough observation of the results by Caccavo et al. (1996) indicate that after five weeks of starvation the iron reductase activity reached levels that were below the detection limit of the assay utilized. One can therefore speculate that, if the iron reductase activity is an indicator for the overall metabolic
activity and if metabolic activity is necessary for irreversible attachment, the decrease in enzyme activity might also be responsible for the decreased attachment of starved *S. algae* BrY cells to the quartz sand in porous media columns. Additional research is necessary to test this hypothesis.
Abstract

Ferrous iron [Fe(II)] reductively transforms heavy metals in contaminated groundwater and the bacterial reduction of indigenous ferric iron [Fe(III)] to Fe(II) has been proposed as a means of establishing redox reactive barriers in the subsurface. The reduction of Fe(III) to Fe(II) can be accomplished by stimulation of indigenous dissimilatory metal-reducing bacteria (DMRB) or injection of DMRB into the subsurface. The microbially produced Fe(II) can chemically react with contaminants such as Cr(VI) to form insoluble Cr(III)-precipitates.

In batch and column experiments, the DMRB *Shewanella algae* BrY reduced surface-associated ferric iron to ferrous iron, which chemically reduced highly soluble Cr(VI) to insoluble Cr(III). Once the chemical Cr(VI)-reduction capacity of the Fe(II)/Fe(III) couple in the experimental systems was exhausted, the addition of *S. algae* BrY allowed for the repeated reduction of Fe(III) to Fe(II), which again reduced Cr(VI) to Cr(III).

The research presented herein indicates that a biological process using DMRB allows the establishment of a biogeochemical cycle that facilitates chromium precipitation. Such a system could provide a means for establishing and maintaining remedial redox reactive zones in Fe(III)-bearing subsurface environments.

Introduction

Chromium(VI) compounds have been used extensively in the manufacture of alloys, the electroplating industry, the manufacture of dyes and pigments, the preservation of wood, in the leather
tanning industry, and as corrosion inhibitors in conventional and nuclear power plants (Hayes, 1997; Langard, 1980). Due to leakage, poor storage, and improper disposal practices, Cr(VI) has become one of the most frequently detected contaminants at hazardous waste sites (National Research Council, 1994; Watts, 1997). The spills are often extensive and the frequent presence of co-contaminants such as chlorinated aliphatics, radionuclides, metals, and organic solvents makes Cr(VI) remediation an environmental challenge (James, 1994; Watts, 1997; Pagilla and Canter, 1999).

Cr(VI) is highly soluble and therefore easily transported with the ground water (Bartlett and James, 1988). It is toxic and mutagenic to humans and other organisms (Committee on Biologic Effects of Atmospheric Pollutants, 1974; Gibb, 1999; Keith and Telliard, 1979; Watts, 1997). In its reduced trivalent state [Cr(III)], chromium forms insoluble hydroxides (Hug and Laubscher, 1997; Richard and Bourg, 1991) and remains immobile under most environmentally-relevant pH and redox conditions (Blowes et al., 1997a; James, 1996; Lantz, 1992; Pohland et al., 1993; Richard and Bourg, 1991). Cr(III) is less toxic than Cr(VI) and is neither readily taken up by organisms or plants nor transferred through the food chain (Hayes, 1997; National Research Council, 1994).

Traditional remediation technologies such as excavation with subsequent treatment or Pump and Treat have often proven to be expensive and can create a long term liability for the responsible party (Mackay and Cherry, 1989; Seaman et al., 1999). Technologies to treat large volumes of soils and groundwater inexpensively are lacking. Passive treatment systems, such as subsurface barriers, offer an alternative to traditional cleanup technologies. Subsurface barriers minimize the need for expensive operating equipment and minimize human exposure to contaminants. Treatment systems relying on geochemical processes have attracted increased attention in recent years.

Permeable reactive subsurface barriers made from iron metal have been successfully used for the elimination of a variety of contaminants including chlorinated solvents (O'Hannesin and Gillham, 1998), heavy metals (Cantrell et al., 1995; Gould, 1982), and radionuclides (Fiedor et al., 1998). The iron metal is usually introduced into the aquifer using trench and fill methods. However, the installation of trenches can become uneconomical at depths of more than approximately 12 m (Scott et al., 1998; Fortner, 1995). Thus,
alternative technologies allowing for the establishment of redox reactive zones in the subsurface have been proposed and implemented in the field. These technologies involve the injection of small Fe(0) particles under high pressure or after hydraulic or pneumatic fracturing of the aquifer (Landis and Vidumsy, 1999; Cantrell et al., 1997b).

In situ redox manipulation has also been suggested for sites where trench and fill methods are impractical (Seaman et al., 1999). Cr(VI) can be reduced by ferrous iron, sulfides, and natural organic compounds (Wittbrodt and Palmer, 1996) and the reduction of Cr(VI) in several field studies has been attributed to the presence of ferrous iron (Anderson et al., 1994; Henderson, 1994; Pagilla and Canter, 1999; Patterson et al., 1997). Industrially, the addition of ferrous iron as a reductant represents a common treatment scheme for Cr(VI) contaminated water (Eary and Rai, 1988) and the use of ferrous iron in subsurface barriers for the remediation of chromium has been proposed (Blowes et al., 1997a; Powell et al., 1995b). During a field demonstration, at the Department of Energy (DOE) site in Hanford (WA), an inorganic reductant (sodium dithionite, Na2S2O5) was injected into the aquifer and allowed to reduce indigenous Fe(III)-minerals to Fe(II) (Scott et al., 1998). The dithionite-containing carbonate-bicarbonate buffer (pH 11) was injected into the subsurface and reduced between 60% and 100% of the Fe(III) present in the Hanford sediments. The resulting reductive capacity of the reduced aquifer material was calculated to be sufficient for the treatment of 51 to 85 pore volumes (or 7 to 12 years) of groundwater at 1 ppm Cr(VI) and 9 ppm dissolved oxygen (Scott et al., 1998).

Dissimilatory Metal-Reducing Bacteria (DMRB) have been found to use a wide range of iron minerals as electron acceptors at ambient groundwater pH (Caccavo, Jr. et al., 1992; Fredrickson and Gorby, 1996a; Roden and Zachara, 1996). DMRB are widely distributed in pristine and contaminated terrestrial, aquatic, and subsurface environments (Lovley, 1995b). They gain energy for growth by coupling the oxidation of organics or H2 to the dissimilatory reduction of Fe(III) minerals and other metals.

Gorby et al. (1994) and Amonette et al. (2000) demonstrated that DMRB can reduce Fe(III) oxides, which can in turn reduce carbon tetrachloride (CT) to chloroform (CF) and Cr(VI) to Cr(III) according to the following reactions.
\[ 2 \text{Fe}^{2+} + \text{CCl}_4 + \text{H}^+ \rightarrow 2 \text{Fe}^{3+} + \text{CHCl}_3 + \text{Cl}^- \]  
(Eq 4.1)

\[ 3 \text{Fe}^{2+} + \text{CrO}_4^{2-} + 5 \text{H}^+ \rightarrow 3 \text{Fe}^{3+} + \text{Cr(OH)}_3^{\delta^+} + \text{H}_2\text{O} \]  
(Eq 4.2)

Depending on the Fe(II)/Cr(VI) ratio and reaction time, reaction (2) will result in the precipitation of pure chromium-hydroxides or mixed iron-chromium-hydroxides with iron and chromium in different ratios (i.e. Fe\(_{x}\)Cr\(_{3-x}\)(OH)\(_3\), x=0,1,2,3) (Cary et al., 1977; Powell et al., 1995b).

If DMRB can repeatedly generate surface reactive ferrous iron from indigenous iron minerals in the subsurface, they could potentially be used to establish \textit{in situ} bioreactors or permeable reactive barriers. A viable remediation strategy based on this approach must provide continuous reduction and precipitation of chromium from contaminated groundwater. The continuous reduction of Cr(VI) relies upon the renewal of Fe(II) through microbial reduction of the Fe(III) present. To the author's knowledge the ability of DMRB to repeatedly generate surface reactive Fe(II) for the chemical reduction of Cr(VI) to Cr(III) in the presence of iron-chromium precipitates has not yet been demonstrated. Laboratory-scale model systems were used to evaluate the potential for such a process.

Materials and Methods

Experimental

Test Organism and Culture Methods. \textit{Shewanella algae} BrY (Caccavo, Jr. et al., 1992) was used as the model DMRB in these studies. \textit{S. algae} BrY was maintained on tryptic soy agar (40 g L\(^{-1}\), Difco Laboratories) at ambient temperature and grown to the late log, early stationary phase in tryptic soy broth (30 g L\(^{-1}\), Difco Laboratories) at 150 rpm for 15 hours at ambient temperature. Cultures were centrifuged at 5,860 x g for 20 minutes at 4 °C, washed twice in phosphate buffered saline solution (PBS) to remove nutrients, and resuspended in the same buffer (Caccavo, Jr. et al., 1996). \textit{S. algae} BrY cells immediately after this treatment were defined as vegetative cells and were used after resuspension and dilution in
oxygen-free minimal medium. Starved cells were obtained by aseptically stirring cell suspensions on magnetic stir plates at room temperature. Cells were harvested after 7 weeks. Centrifugation and resuspension in oxygen-free minimal medium served to remove cell debris from the starvation cultures (Caccavo, Jr. et al., 1996). Starved cells were used in parts of this study because it was previously demonstrated that starved cells are transported more effectively through porous media than vegetative cells and would therefore have an advantage in subsurface bioaugmentation with DMRB (Chapter 2; Bouwer et al., 2000).

**Amorphous Iron Coating.** Artificially iron-coated sand was obtained by soaking 200 g of quartz sand (40 mesh, Unimin Corp., Emmet, ID) for several days in twice its volume of 1 N HNO₃, subsequent washing with distilled water until the pH of the rinsewater exceeded 5.0, and mixing with 400 mL of a suspension of amorphous iron(III)oxyhydroxide (Lovley and Phillips, 1986). This suspension was prepared by dissolving 13.8 g FeCl₃ in 400 mL of deionized water and slowly adjusting the pH to 7.0 using NaOH. A layer of clear liquid formed after the mixture of sand and iron(III)oxyhydroxide was briefly shaken and allowed to settle. The clear liquid was decanted after approximately 12 hours of settling time and replaced with an equal volume of 0.1 mM NaCl at pH 7.0. The entire overlying suspension was decanted after an additional 12 hours, leaving only the sand. The above procedure was repeated four additional times over the course of 96 hours. The coated sand was washed to remove loosely attached iron, allowed to air-dry, and passed through a 2 mm sieve to break up larger aggregates. The coating procedure yielded a coating of amorphous iron(III)oxyhydroxide of about 1.3 ± 0.12 mg Fe(III) per gram of sand. No Fe(II) was detected on the sand using the analytical method described below.

**Batch Experiments.** Iron Reduction. Oxygen free minimal medium containing NaHCO₃, 2.5; NH₄Cl, 1.5; KH₂PO₄, 0.6; KCl, 0.1 (in grams per L of deionized water); vitamins 10 mL per L, and trace minerals 10 mL per L (Balch et al., 1979; Lovley and Phillips, 1988) amended with 10 mM lactate was prepared under an N₂:CO₂ (80:20) gas atmosphere (Balch and Wolfe, 1976). Oxygen free minimal medium (9 mL) and 1 mL of bacterial culture, either starved or vegetative, were added to test tubes containing 1 g
of iron-coated sand and the vials were crimp sealed using thick butyl rubber stoppers (Bellco Glass, Vineland, NJ). All manipulations were performed under an oxygen-free N₂/CO₂ purge. The resulting bacterial concentrations were approximately 1.5 x 10⁷ colony forming units (CFU) per mL. Control vials were established lacking either bacteria or lactate and all vials were incubated statically in the dark. Triplicate sets of vials were sacrificed periodically to determine the amount of ferrous iron in the supernatant and on the sand separately.

**Batch Experiments. Chromium Reduction.** A subset of vials containing biologically generated Fe(II) was autoclaved to eliminate microbial activity and thus to distinguish between chemical and biological Cr(VI)-reduction. Cr(VI) (0.15 mg) was injected from a K₂CrO₄ stock solution to a final concentration of 15 mg L⁻¹. Cr(VI) concentrations in the aqueous phase were monitored over time as described below. Vials were sacrificed at the end of each experiment and analyzed for Cr(VI) and total Cr in the aqueous and solid phase separately. Controls containing oxidized iron oxyhydroxide coated sand were established to account for abiotic losses of Cr(VI).

Experiments evaluating the ability of *S. algae* BrY to reduce Cr(VI) directly were also performed. Cr(VI) was injected into vials containing vegetative cells of *S. algae* BrY cells in oxygen free minimal medium supplemented with 10 mM lactate and Cr(VI) concentrations were monitored over time.

**Regeneration of Ferrous Iron in the Presence of Chromium Precipitates and Repeated Chromium Reduction.** A set of vials containing microbially reduced iron was sterilized by autoclaving and then repeatedly injected with a Cr(VI) solution until their reduction capacity for Cr(VI) was exhausted. The capacity of these vials to reduce Cr(VI) was regarded as exhausted if 48 hours after the last injection of 0.05 mg Cr(VI), more than 0.05 mg/vial (=5 mg L⁻¹) Cr(VI) were still detected. Vials were sacrificed for analysis of Fe(II), total Fe, Cr(VI), and total Cr after exhaustion of the Cr(VI) reduction capacity. A subset of the remaining vials was reinoculated with *S. algae* BrY to evaluate the potential for regeneration of reduced iron through the addition of DMRB. Reinoculated vials were sacrificed over time for iron analysis.
Vials that showed evidence of Fe(II) production were autoclaved and their ability to repeatedly reduce Cr(VI) was tested as described above.

**Column Experiments.** Porous media columns were established to evaluate the microbially mediated geochemical elimination of Cr(VI) from contaminated waters in flow through systems (Figure 4.1). Polytetrafluoroethylene (PTFE) columns, 5 cm in length, fitted with stainless steel nuts and ferrules, and PTFE and Tygon® tubing (Cole Palmer) were established. Each column was filled with seven grams of iron-coated sand. A PTFE grid was placed at the inlet and outlet of each column to prevent washout of sand into the outlet or settling of sand into the inlet tubing. The columns were operated upflow with a peristaltic pump at flowrates of 4 mL min⁻¹ and up to 15 columns were operated simultaneously via a manifold.

Each column was inoculated with approximately 6 x 10⁸ CFU of starved *S. algae* BrY and the columns were operated in semi-batch mode. Approximately 20 mL of oxygen-free minimal medium supplemented with 10 mM lactate was pumped through each column every two days. Columns were sacrificed over time, and the sand was analyzed for Fe(II) and total Fe. After evidence of Fe(II) production, a Cr(VI) solution was injected concurrently with a tracer (fluorescein) at a flowrate of 1.16 mL min⁻¹, resulting in a residence time of approximately 6 min in the columns. Inlet and outlet concentrations of Cr(VI) and the tracer concentrations were monitored over time. The columns were sacrificed at the conclusion of the experiment and analyzed for both total Cr and Cr(VI).

A subset of columns was reinoculated with starved *S. algae* BrY cells after depletion of the Cr(VI) reduction capacity and the ability of the columns to reduce Cr(VI) was assessed as described above after allowing adequate time for iron reduction.
Analytical

Iron Quantification. Amorphous iron-coated sand was analyzed for both total iron and ferrous iron [Fe(II)]. The method described by Lovley and Phillips (Lovley and Phillips, 1987; Lovley and Phillips, 1988) was modified as follows. Instead of 0.5 M HCl, 2.5 M HCl was used, which proved to be sufficient for the solubilization of all Fe from the sand after incubation on a horizontal shaker for 30 minutes at 150 rpm. Fe(II) was determined by adding 5 mL of 2.5 N hydrochloric acid to 1 g of iron coated sand. Total iron was quantified by adding 5 mL of 0.25 N hydroxylamine solution in 2.25 N hydrochloric acid to 1 g of iron coated sand. Aliquots of the acid extracts were added to 5 mL of a solution of 1 g ferrozine (Sigma, St.
Louis, MO) in 1 L 750 mM HEPES buffer at pH 7 and the absorbance was determined spectrophotometrically at 562 nm.

**Chromium Quantification.** Aqueous Cr(VI) was quantified using diphenylcarbazide (Urone, 1955). A 500 µL sample was added to 10 mL 0.2 N sulfuric acid and vortexed. A diphenylcarbazide (DPC) solution (0.5 mL, 0.25% of DPC in 100% acetone) was added and the absorbance was read at 540 nm.

Cr(VI) was extracted from the sand using a modified procedure described by Vitale et al. (1994). A 0.28 M Na$_2$CO$_3$/0.5 M NaOH solution (5 mL) was added to 1 g of sand, mixed, and heated to 90-95 ºC for 60 minutes. The vials were swirled repeatedly during the heating process and deionized water was added, if needed, to replace losses due to evaporation. The vials were allowed to cool to room temperature and the total volume was filtered through a 0.2 µm nylon membrane (Fisher Scientific, Pittsburgh, PA). The pH was adjusted to 7.5 using concentrated HNO$_3$ and 0.5 mL of the DPC solution was added. The pH was adjusted to 1 with concentrated H$_2$SO$_4$ and the total volume of the sample was adjusted to 10.5 mL with 0.2 N H$_2$SO$_4$ before the absorbance was read at 540 nm.

Aqueous phase total Cr was determined after filtration using a modified standard method (Anonymous 1992). One drop of methyl orange indicator solution was added to 1 mL of sample. Concentrated NH$_4$OH was added until the solution turned yellow, diluted H$_2$SO$_4$ (1:2 from concentrated H$_2$SO$_4$) was added until the indicator turned red, and the total volume was adjusted to approximately 5 mL. Boiling chips were added, the vial was closed with a rubber stopper, and a 20 gauge needle was inserted as exhaust; the solution was heated to a boil. One drop of a KMnO$_4$ solution (40 g L$^{-1}$) was added and boiling was continued. If fading occurred, another drop of KMnO$_4$ was added and boiling was continued for at least 2 minutes after addition of the last drop of KMnO$_4$. Sodium azide (NaN$_3$, 5 g L$^{-1}$) was added in 0.1 mL increments and boiling continued until the color disappeared. The vials were allowed to cool after addition of 1 drop of concentrated H$_3$PO$_4$. Concentrated H$_2$SO$_4$ was used to adjust the pH to 1.0 and the whole content of each vial was filtered through a 0.2 µm nylon filter. The volume was adjusted to 10 mL using 0.2 N H$_2$SO$_4$ and 0.5 mL of DPC solution were added before measuring the absorbance at 540 nm.
Total Cr from the sand was determined analogously substituting the 1 mL liquid sample with 1 g of Fe-coated sand.

Results and Discussion

Microbial Fe(III) Reduction

*S. algae* BrY can use Fe(III) as electron acceptor to grow in the absence of oxygen with lactate as the electron donor. Starved and vegetative cells of *S. algae* BrY reduced surface associated ferric iron [Fe(III)] to ferrous iron [Fe(II)] over time (Figure 4.2). The reduction was faster in vials inoculated with vegetative bacteria than in those inoculated with starved bacteria. Vegetative cells reduced approximately 90% (1.19 ± 0.09 mg) of the ferric iron available on the sand (1.3 ± 0.12 mg) within 24 hours. Starved cells exhibited a slower rate of Fe(III) reduction and reduced approximately 80% (1.04 ± 0.02 mg) of the ferric iron within 10 days. Control vials lacking either lactate or *S. algae* BrY cells showed no evidence of Fe(II) production, indicating that the observed reduction was due to electron donor-dependent microbial activity.

It has been shown that starved cells of *S. algae* BrY reduce soluble ferric pyrophosphate slower than vegetative cells (Caccavo, Jr. et al., 1996). The data presented here are consistent with these results. Although starved cells of *S. algae* BrY reduce surface-associated Fe(III) at a slower rate than vegetative cells, the amount of Fe(II) produced by starved cells after 10 days is only slightly lower than the amount produced by vegetative cells.

Most of the Fe(II) produced (> 95%) remained associated with the sand surfaces as observed by separate analyses of the supernatant and the sand (data not shown). The fact that most of the reduced iron remains surface-associated is important in view of a possible field application since surface-associated Fe(II) is more reactive with dissolved contaminants than dissolved Fe(II) (Kriegman-King and Reinhard, 1994). Furthermore, in aquifers dissolved Fe(II) would be transported out of the system over time with the flowing groundwater minimizing the efficacy of this technology.
Figure 4.2. Production of Fe(II) from amorphous Fe(III) oxyhydroxide-coated sand by starved (●) and vegetative cells (■) of *S. algae* BrY. Every system contained 1 g of sand coated with 1.3 ± 0.12 mg of Fe(III) initially. Controls lacking either lactate (▲) or *S. algae* BrY (□) did not produce significant amounts of Fe(II). Error bars represent the standard error of the means (n=3).

The injection of DMRB into the subsurface and the stimulation of DMRB in the subsurface to enhance contaminant transformation has been proposed (Caccavo, Jr. et al., 1996, Gorby et al., 1994). Here it was demonstrated that starved *S. algae* BrY cells, which can be transported through porous media better than vegetative cells (Chapter 2; Bouwer et al., 2000), can reduce surface-associated Fe(III) and produce surface-associated, reactive Fe(II). Thus zones of surface reactive Fe(II) could be established in contaminated aquifers by stimulating naturally-occurring DMRB or by bioaugmenting the contaminated subsurface with starved DMRB.
Cr(VI) Reduction by Microbially Produced Fe(II)

The reduction of Cr(VI) by Fe(II) has been documented using soluble Fe(II) (Seaman et al., 1999) and surface associated Fe(II) in natural aquifer material (Caccavo, Jr. et al., 1996; Gorby et al., 1994; Kriegman-King and Reinhard, 1994).

Cr(VI) concentrations decreased immediately after injection in the experimental vials containing biologically produced, surface-associated Fe(II) (Figure 4.3). The reaction of Fe(II) with Cr(VI) occurred so fast in these vials that at the first sampling event after 30 seconds, the concentration of Cr(VI) was already significantly lower than the control concentration of 15 mg L\(^{-1}\). Vials that were sterilized immediately before the injection to eliminate microbial activity showed the same initial decrease, providing evidence that chemical processes are governing the reduction of Cr(VI) by Fe(II) initially. Control vials containing oxidized iron oxyhydroxide-coated sand did not show a significant decrease in Cr(VI) over the duration of the experiment, indicating that sorption of Cr(VI) to the iron-coated sand was negligible. The initial increase in Cr(VI) concentrations in the control vials (see Figure 4.3) can be attributed to incomplete mixing before the first sampling event. Since the reaction of Cr(VI) with Fe(II) was expected to occur rapidly, the systems were sampled as soon as possible after addition of Cr(VI), i.e. after 30 seconds. Thus, there was only a limited time available for mixing and the resulting incomplete mixing might account for initially lower Cr(VI) concentrations.

Based on the stoichiometry of reaction (2), the vials contained sufficient Fe(II) to completely reduce the Cr(VI) added (0.15 mg, resulting in an initial concentration of 15 mg L\(^{-1}\)). However, 11 minutes after the injection of Cr(VI), the reduction of 15 mg L\(^{-1}\) Cr(VI) remained incomplete. This incomplete conversion of Cr(VI) could be due to mass transport limitations within the experimental systems or the presence of Fe(II) sites which were not reactive with Cr(VI). Powell et al. (1995) stated that the reactivity of surface-associated Fe(II) can vary depending on the mineralogy. Although no mineralogical analyses were performed during this research, the white color of the precipitate during biological iron reduction and the high carbonate content of the buffer suggest that siderite (FeCO\(_3\)) formed at least partly on the sand grains. Siderite has been described as only moderately reactive with dissolved phase contaminants relative
to other iron minerals (Agrawal and Tratnyek, 1996; Blowes et al., 1997a). This may explain the fraction of moderately and non reactive Fe(II) in the batch vials. Diffusion limitations (Agrawal and Tratnyek, 1996) and the accumulation of chromium precipitates can also limit Cr(VI) reduction by surface reactive Fe(II).

![Graph showing Cr(VI) transformation](image)

Figure 4.3. Cr(VI) transformation in vials containing (■) microbially reduced Fe-coated sand and active *S. algae* BrY, (○) microbially reduced Fe-coated sand (autoclaved to eliminate microbial activity), and (▲) oxidized Fe-coated sand. The reaction of Fe(II) with Cr(VI) occurred so fast that at the first sampling event, the concentration of Cr(VI) was already significantly lower in vials containing Fe(II) than in control vials. The target concentration of 15 mg L\(^{-1}\) is indicated by (○). Error bars represent the standard error of the means (n = 3).

The vials were analyzed for total chromium and Cr(VI) at the conclusion of each experiment. The majority (> 95%) of the chromium recovered from the sand was recovered as Cr(III) (data not shown) demonstrating that Cr(VI) was transformed to Cr(III) in the presence of biologically produced surface associated Fe(II).
Complete conversion of Cr(VI) within 24 hours was only observed in the vials containing active *S. algae* BrY cells. This decrease suggests either direct reduction of Cr(VI) by *S. algae* BrY or an indirect mechanism in which *S. algae* BrY re-reduced ferric iron resulting from reaction (2) to ferrous iron, which then again reduced Cr(VI) to Cr(III). Direct microbial reduction of Cr(VI) by metal-reducing bacteria and soil indigenous microbial populations has been reported (Bader et al., 1999; Bopp and Ehrlich, 1988; Turick et al., 1998). It was demonstrated here that direct microbial reduction of Cr(VI) by active *S. algae* BrY is possible, although significantly slower than the chemical reduction with surface reactive Fe(II).

Regardless of whether surfaces were available (quartz sand or iron-coated sand) or not, active *S. algae* BrY cells did not significantly reduce 5 mg L\(^{-1}\) Cr(VI) within 11 minutes (Figure 4.4). However, the concentration of Cr(VI) dropped to below 1 mg L\(^{-1}\) in all vials containing *S. algae* BrY after 24 hours, indicating slow direct microbial reduction of Cr(VI) by *S. algae* BrY. As mentioned previously, at the first sampling event after 30 seconds, the target concentration of 5 mg L\(^{-1}\) Cr(VI) was not observed consistently in the vials, presumably due to incomplete mixing. The concentrations stabilized to approximately 5 mg L\(^{-1}\) Cr(VI) after 1-2 minutes into the experiment and repeated mixing. Compared to the systems containing microbially produced Fe(II) (Figure 4.3), the direct microbial reduction of Cr(VI) was much slower. These observations concur with Caccavo et al. (1996), Eary and Ray (1988), and Fendorf and Li (1996) who reported that the reduction of Cr(VI) by dissolved and surface associated ferrous iron is rapid compared to the direct microbial reduction.

Reactions with surface associated Fe(II) usually occur within minutes to hours at environmentally relevant pH values and temperatures. These reactions can require stoichiometric or excess amounts of ferrous iron (Davis and Olsen, 1995; Eary and Rai, 1988; Seaman et al., 1999). Fe(II) has been found to reduce Cr(VI) at pH values of less than 10 (Palmer and Wittbrodt, 1991), and the rate of reduction increases with decreasing pH (Powell et al., 1995b; Eary and Rai, 1991). Because the pH of ground water rarely exceeds 10, the reaction should be feasible in field situations and has been proposed for the treatment of industrial waste water and groundwater containing Cr(VI) (Eary and Rai, 1988; Seaman et al., 1999).
Figure 4.4. Direct Cr(VI) transformation by active *S. algae* BrY (▲) in the absence of sand, (□) in the presence of quartz sand, and (●) in the presence of iron-coated sand. The time between injection of bacteria and Cr(VI) (minutes) was not sufficient for significant Fe(II) production in the vials. The target concentration of 5 mg L⁻¹ is indicated by (○). Error bars represent the standard error of the means (n = 3).

Regeneration of Surface Reactive Ferrous Iron in the Presence of Chromium Precipitates

These experiments were designed to determine whether surface reactive Fe(II) can be regenerated in the presence of Fe(III)Cr(III)precipitates formed during the reduction of Cr(VI) by Fe(II). Surface reactive Fe(II) was produced initially by inoculating vials containing amorphous iron(oxy)hydroxide coated sand with starved *S. algae* BrY. Figure 4.5 shows the total mass of Fe(II) detected in the vials over the duration of the experiment. More than 90% (1.19 ± 0.10 mg) of the ferric iron available in the vials (1.3 ± 0.12 mg) was reduced to Fe(II) in the absence of Cr(VI), 20 days after the inoculation with starved *S. algae* BrY. The Fe(II) containing vials were autoclaved after 20 days to eliminate microbial activity and repeatedly injected with a Cr(VI) solution until their reduction capacity for Cr(VI) was exhausted. Only
approximately 40% (0.15 mg Cr(VI) per vial) of the theoretically reducible Cr(VI) was reduced over a period of twelve days (day 20 to day 32). Destructive analysis of triplicate vials revealed that the majority of the chromium detected on the sand (> 95%) was present as Cr(III) suggesting that Cr(VI) was reduced to Cr(III) which precipitated (data not shown). The incomplete reduction of Cr(VI) indicates the presence of slowly or non reactive fractions of Fe(II).

However, the conversion of Cr(VI) to Cr(III) could only account for approximately 80% of the decrease in ferrous iron concentration (from 1.19 ± 0.10 mg to 0.78 ± 0.10 mg) indicating that ferrous iron was possibly oxidized by compounds other than Cr(VI). Despite the fact that syringes were flushed with oxygen-free gas before sampling, trace amounts of oxygen could have been injected into the vials and lead to the oxidation of Fe(II) to Fe(III).

After the Cr(VI) reduction capacity of the vials was exhausted (day 32), a subset of the remaining vials was reinoculated with *S. algae* BrY to evaluate the possible regeneration of surface reactive ferrous iron through the addition of DMRB. Fe(II) increased from 0.78 ± 0.10 mg per vial to 1.10 ± 0.07 mg per vial in reinoculated vials only between day 32 and day 55 (Figure 4.5). Vials, which were not reinoculated, did not show an increase but rather a slight decrease to 0.73 ± 0.15 mg Fe(II), which might be due to slow reactions of Cr(VI) with Fe(II) in these vials. Cr(VI) was still detectable in these vials at day 55 while no Cr(VI) was detected in reinoculated vials. Although the amount of total Fe(II) in the reinoculated vials (1.10 ± 0.07 mg) was lower than the amount of Fe(II) produced during the initial incubation in the absence of Cr-precipitates (1.19 ± 0.10 mg) it is clear that *S. algae* BrY was able to re-reduce a significant amount of the Fe(III) which was generated during the prior injection of Cr(VI).
Reinoculated vials were examined for their ability to reduce Cr(VI) a second time after production of Fe(II). Cr(VI) was injected to a concentration of 15 mg L$^{-1}$ and Cr(VI) concentrations were monitored over time. Cr(VI) concentrations in reinoculated vials decreased immediately after injection (Figure 4.6) and only approximately 1 mg L$^{-1}$ Cr(VI) was detected after 30 seconds. The rate of Cr(VI) reduction in these vials was even faster than during the first cycle (compare to Figure 4.3) where approximately 5 mg L$^{-1}$ remained after 11 minutes. No decrease in Cr(VI) was observed in vials which were not reinoculated. The final concentration of approximately 18 mg L$^{-1}$ in these vials is the result of residual Cr(VI) in the vials before the injection of Cr(VI) from the stock solution.

These results suggest that *S. algae* BrY can regenerate the reduction capacity of iron minerals by producing surface reactive Fe(II) from Fe(III) even in the presence of Cr(III)-precipitates. It is likely that
the rate and extent of Fe(II) production in natural systems, before and after precipitation of Cr(III), will depend on the type of iron mineral present, the concentration and ability of DMRB to reduce the Fe-Cr-mineral phases after precipitation of Cr, and on the time allowed for Fe-reduction.

![Cr(VI) transformation in vials](image)

**Figure 4.6.** Cr(VI) transformation in vials with initially exhausted Cr(VI)-reduction capacity (●) after reinoculation and incubation with *S. algae* BrY, (■) without reinoculation. The Cr(VI) spike increased the initial Cr(VI) concentration in the vials by 15 mg L⁻¹ (indicated by ○). Error bars represent the standard error of the means (n = 3).

**Iron and Chromium Reduction in Columns**

In order to develop the basis for a viable restoration technology, Cr(VI) must be reductively precipitated by surface reactive Fe(II) in flow through systems at short contact times. Column studies were designed to more closely approximate field situations, in which the contact time of Cr(VI) with biologically produced surface-associated Fe(II) is limited. Columns inoculated with starved *S. algae* BrY were fed in semi-batch mode and were sacrificed periodically to analyze for Fe(II).
Fe(II) was produced over time (data not shown), albeit at a significantly slower rate than in the batch experiments described above. Starved *S. algae* BrY reduced more than 95% of the Fe(III) present in the columns after 2 months as compared to 80% after 7 to 10 days in batch experiments. The production of ferrous iron in the columns indicated that starved *S. algae* BrY colonized the Fe-coated sand and utilized the surface-associated Fe(III) as electron acceptor.

After evidence of Fe(II) production, a Cr(VI) solution (6.6 ± 0.1 mg L⁻¹) was injected into the columns concurrently with a tracer (Fluorescein, Na-salt). Influent and effluent concentrations of the tracer and Cr(VI) were monitored over time. The data were expressed as fractions of their influent concentration and were plotted as a function of the number of pore volumes eluted from each column. Figure 4.7 shows the tracer breakthrough curves of three different columns with their corresponding model fits (Fetter 1993) and their corresponding Cr(VI) breakthrough data. It is evident that one of the columns had a higher dispersivity likely due to differences in packing of the sand. However, despite this difference in tracer breakthrough it is evident that the breakthrough of Cr(VI) in all three columns was clearly retarded as compared to fluorescein. Statistical examination of the breakthrough curves using a two sample ψ-test (Gibbons, 1971, pp. 131-137) clearly indicates that in all three columns containing microbially produced Fe(II), the breakthrough of Cr(VI) was retarded (ψ-test, *P value* = 0.05).

The mass of Cr(VI) retarded in the columns was calculated on the basis of the breakthrough curves for each column, approximating complete breakthrough of Cr(VI) after four pore volumes (see Figure 4.7). The mass of Cr(VI) in the effluent of the column (integral of the effluent concentration over time) was compared with the mass of Cr(VI) injected into the column (integral of the influent concentration over time). As in the batch experiments only a fraction of the stoichiometrically possible mass of Cr(VI) was retarded in each column, however, the fraction of chromium retarded in the columns was even smaller (1 ± 0.7% (n=3) of the total reduction capacity for Cr(VI)) than for the batch studies (approximately 40% of the total reduction capacity for Cr(VI)).
It is possible that the short contact time in the columns (approximately 6 minutes) is not sufficient for more extensive Cr(VI) reduction to occur. Mass transport limitations, the presence a slowly or non reactive Fe(II)-fraction (such as siderite), and the accumulation of chromium precipitates on surface reactive sites could be responsible for the slow and incomplete reaction. The sand was analyzed for total chromium and Cr(VI) by sacrificing triplicate columns after the injection of Cr(VI). More than 95% of the chromium was recovered as Cr(III) (data not shown), indicating that the retardation of chromium was due to reductive precipitation within the columns.

Three different processes are likely to be responsible for the reductive precipitation of chromium. Cr(VI) may react chemically with surface reactive Fe(II) or active *S. algae* BrY cells may directly reduce Cr(VI) to precipitate Cr(III). These processes in addition to DMRB mediated biogeochemical cycling of Fe(III) back to Fe(II) (process 2 in Figure 4.8) may explain the attenuated increase of Cr(VI) towards the
end of the experiment. All three processes together with the limited reduction capacity of the columns would cause the slow but continued reductive precipitation of chromium within the columns and would result in Cr(VI) effluent concentrations that approach the influent concentration asymptotically. It is likely that all three processes occur within the columns and therefore it is surprising that of the total reduction capacity of surface associated Fe(II) for Cr(VI), only approximately 1% is utilized after approximately 4 pore volumes and that Cr(VI) concentrations in the effluent are almost equal to the influent concentration.

Figure 4.8. Processes governing the fate of Cr(VI) in environments containing surface associated iron and dissimilatory metal-reducing bacteria (DMRB). (1) Direct Cr(VI)-reduction by DMRB; (2) chemical reduction of Cr(VI) by surface associated ferrous iron (Fe(II)_{sur}) and biological re-reduction (regeneration) of surface associated ferric iron (Fe(III)_{sur}) to Fe(II)_{sur} by DMRB.

The remaining columns were reinoculated after depletion of the Cr(VI) reduction capacity to evaluate the possibility of regenerating the reactivity of the columns by injection of S. algae BrY. Approximately two months after reinoculation, Fe(II) production in these columns was evident and Cr(VI) was injected a second time concurrently with fluorescein. The normalized breakthrough curves were very
similar to those plotted in Figure 4.7 (data not shown) indicating that the reduction capacity of the columns was regenerated by the reinoculation of *S. algae* BrY.

**Conclusions**

The results presented here suggest the possibility of artificially establishing a biogeochemical cycle (Figure 4.8) in Cr(VI) contaminated subsurface environments. DMRB reduce ferric iron [Fe(III)] to ferrous iron [Fe(II)], which in turn reduces Cr(VI) to Cr(III) while Fe(II) is oxidized (back) to Fe(III). DMRB can then re-reduce the Fe(III) to Fe(II) to close the biogeochemical cycle.

The use of DMRB potentially allows the establishment and maintenance of redox reactive zones (permeable subsurface barriers) in Fe(III)-bearing subsurface environments. The applicability of this technology to any given field site will depend on our ability to produce surface reactive Fe(II) from the iron minerals present in the aquifer. This can be achieved by stimulating indigenous microorganisms or, if microbial iron reduction cannot be sufficiently supported, by our ability to augment the existing populations with metal-reducing bacteria. The use of starved metal-reducing bacteria has potential for such an approach. Starved bacteria are transported better through porous media (Chapter 2; Bouwer et al., 2000) and, as shown here, can produce surface reactive Fe(II).

However, like in most bioremediation strategies, there are limitations and concerns that have to be considered. High initial Cr(VI) concentrations or the presence of other toxic compounds might slow down or prevent biological iron and chromium reduction. High or low pH values as well as a lack of electron donor can also hinder biological iron reduction and, subsequently, chromium reduction.

The process described herein does not remove chromium from the aquifer but immobilizes it by precipitation. Although Fe(III)Cr(III)hydroxides and pure Cr(III)hydroxide have very low solubilities and are unlikely to result in chromium concentrations above the drinking water limit for chromium, the reoxidation of Cr(III) compounds is a concern. Although microbially mediated reoxidation of Cr(III) is not a proven concern in systems containing natural soil material (James, 1994, Bader et al., 1999), manganese oxides have been found to quickly oxidize Cr(III) in soils (Rai and Zachara, 1988, Bartlett and James,
Therefore, the application of oxidants such as potassium permanganate or hydrogen peroxide, commonly used in certain remediation schemes, can potentially lead to a remobilization of immobilized chromium.

Future research is needed to address the identification of mineral phases developing during the microbial reduction of iron (hydr)oxides and iron chromium (hydr)oxides. The stability of these precipitates will be assessed and the recycling frequency of the ferric/ferrous iron redox couple in the presence of Cr(VI) will be evaluated in batch and flow through systems.
CHAPTER 5

DISSIMILATORY IRON-REDUCING BACTERIA CAN INFLUENCE THE REDUCTION OF CARBON TETRACHLORIDE BY IRON METAL


Abstract

Little is known about the long term performance of zero valent iron (Fe(0)) subsurface barriers. Groundwater exposure induces corrosion processes that can passivate the Fe(0) surface and decrease barrier reaction rates. This chapter presents evidence that dissimilatory iron-reducing bacteria (DIRB) can stimulate the rate of carbon tetrachloride (CT) transformation in the presence of corroded iron. The DIRB, *Shewanella algae* BrY, adhered to the corroded Fe surfaces that showed little or no capacity to transform CT. The addition of BrY to these systems with decreased CT transformation rates resulted in increased ferrous iron concentrations and increased CT transformation to chloroform (CF). The results suggest that DIRB can have an influence on the long term performance of Fe(0) barriers.

Introduction

Permeable, reactive, subsurface barriers of zero valent iron (Fe(0)) have been successfully employed for the remediation of contaminated groundwater. Fe(0) is effective in degrading or transforming a wide range of contaminants including chlorinated organic (Eykholt and Davenport, 1998; Gatpagar et al., 1997; Gillham and O'Hannesin, 1994; Helland et al., 1995; Johnson et al., 1998; Johnson et al., 1996; Roberts et al., 1996; Sayles et al., 1997; Siantar et al., 1996), heavy metal oxyanions (Cantrell et al., 1995; Gould, 1982) and oxycations (Fiedor et al., 1998), nitroaromatics (Agrawal and Tratnyek, 1996; Cao et al.,
and nitrate (Huang et al., 1998; Siantar et al., 1996; Till et al., 1998). The corrosion of Fe(0) provides the electrons necessary for the reduction of these contaminants.

Laboratory studies simulating long term performance of Fe(0) have indicated that contaminant degradation rates can either decrease (Devlin et al., 1998; Gatpagar et al., 1997) or more rarely increase (Helland et al., 1995) as a function of time. This temporal effect on contaminant degradation rates is usually attributed to the development of corrosion products that develop on the metal surface (Helland et al., 1995; Johnson and Tratnyek, 1994; Scherer et al., 1998). Johnson and Tratnyek (1994), Scherer et al. (1998), and Odziemkowski et al. (1998) have proposed that the accumulation of corrosion products on Fe(0) surfaces either inhibits contaminant access to the metal surface or forms new sites for contaminant adsorption, reaction, and catalysis. A decrease in reaction rates can be due to the accumulation of thick layers of amorphous corrosion products on Fe(0) surfaces, which may function as a physical barrier between dissolved contaminants and the underlying reactive sites. The accumulation of highly redox reactive corrosion products such as green rust, however, can also significantly contribute to the reduction of contaminants in Fe(0) systems (Erbs et al., 1999).

A wide range of Fe(0) corrosion products has been found in laboratory and field studies, and corrosion product distribution appears to be dependent on the type of Fe(0) used, the contaminant present, geochemical conditions, and the time of exposure to contaminated groundwater. The corrosion products commonly found in the presence of contaminated groundwater include goethite (α-FeOOH; Pratt et al., 1997), magnetite (Fe₃O₄; Bonin et al., 1998; Odziemkowski and Gillham, 1997; Odziemkowski et al.; 1998; Peterson et al., 1997), maghemite (γ-Fe₂O₃; Peterson et al., 1997), hematite (α-Fe₂O₃; Pratt et al., 1997), mixed chromium-iron-hydroxides (Blowes et al., 1997b; Powell et al., 1995a), green rust (mixed Fe(II)-Fe(III)-hydroxy salts; Odziemkowski and Gillham, 1997), and siderite (FeCO₃; Mackenzie et al., 1997).

Many of these iron minerals are used by dissimilatory iron-reducing bacteria (DIRB) as electron acceptors (Caccavo, Jr. et al., 1992; Fredrickson and Gorby, 1996a; Heijman et al., 1995; Heijman et al., 1993; Kostka and Nealson, 1995; Roden and Zachara, 1996). DIRB are widely distributed in both pristine
and contaminated terrestrial, aquatic, and subsurface environments (Lovley, 1995b) and are thus likely to contact Fe(0) barriers in situ. DIRB gain energy for growth by coupling the oxidation of organic matter to the dissimilatory reduction of Fe(III) minerals.

Gorby et al. (1994) demonstrated that DIRB can reduce structural Fe(III) oxides, which can in turn reductively dechlorinate carbon tetrachloride (CT) to chloroform (CF). The bacterial reduction of Fe(III) to Fe(II) allowed for the following reaction to occur:

\[
2 \text{Fe}^{2+} + \text{CCl}_4 + \text{H}^+ \rightarrow 2 \text{Fe}^{3+} + \text{CHCl}_3 + \text{Cl}^- \quad (\text{Eq. 5.1})
\]

DIRB have also been shown to influence the corrosion of mild steel by solubilizing the protective ferric iron film on the passivated metal surface (Obuekwe et al., 1981a; Obuekwe et al., 1981b). A decrease in corrosion layer thickness due to solubilization of corrosion products in Fe(0) systems could also lead to increased reaction rates of iron metal with chlorinated aliphatics (Devlin et al., 1998; Gatpagar et al., 1997; Scherer et al., 1998).

It was hypothesized that DIRB can influence the performance of Fe(0) subsurface barriers by adhering to corroded iron and reducing Fe(III) corrosion products to Fe(II)-compounds. The reduction of Fe(III) to Fe(II) can lead to the formation of surface reactive Fe(II) species or result in the removal of passivating ferric precipitates on the Fe(0) surface. Either one of these processes will potentially increase the reductive transformation of carbon tetrachloride (CT).

Materials and Methods

Organism and Culture Conditions

*Shewanella algae* BrY (Caccavo, Jr. et al., 1992) was used as a model DIRB in these studies. *S. algae* BrY was maintained on tryptic soy agar (40 g L⁻¹, Difco Laboratories) at ambient temperature. *S. algae* BrY cultures were grown in tryptic soy broth (30 g L⁻¹, Difco Laboratories) at 28 °C and 150 rpm for 15 hr. Cultures were centrifuged at 5,520 x g for 20 minutes at 4 °C, washed once in anaerobic 10 mM
HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffer (pH 7.0) amended with 2.5 g liter\(^{-1}\) NaCl and suspended in the same buffer. All buffers were made anaerobic by boiling and cooling under a constant stream of O\(_2\)-free N\(_2\) (Balch and Wolfe, 1976). All manipulations were performed under an O\(_2\)-free N\(_2\) purge.

CT Reduction

The ability of \textit{S. algae} Br\(\gamma\) to influence CT reduction by passivated Fe(0) was examined. Fe(0) powder (20 mg, 100 mesh, Fisher Scientific) was added to 10 ml of aerobic HEPES buffer (pH 7.0), sealed in culture tubes under an aerobic headspace and vigorously shaken on a wrist action shaker for approximately 40 hours. This method was found to be efficient for pre-exhausting the CT-reducing capacity of the Fe(0) powder. Following pretreatment, the tubes were purged with O\(_2\)-free N\(_2\) for 15 minutes by lowering a stainless-steel canula into the bottom of each tube and letting the anaerobic gas percolate through the aqueous phase. Anaerobic sodium lactate (final concentration 10 mM) and either heat-killed or active cells of \textit{S. algae} Br\(\gamma\) (final concentration of \(10^{10}\) cells ml\(^{-1}\)) were added to the tubes using degassed syringes and needles. The tubes were then filled to their maximum capacity (27.5 ml) with anaerobic HEPES buffer to minimize loss of CT into the headspace. The gas-sparging canula was slowly removed from the tube as a PTFE-lined silicon septum was inserted. Control tubes containing either heat-killed or active \textit{S. algae} Br\(\gamma\) cells without iron powder were also established. The experiments were initiated by injecting 0.5 ml of a CT (Sigma) stock solution into the tubes. The stock solution was made by adding 1 ml of pure phase CT to 40 ml of deionized water. Excess aqueous phase was allowed to escape from the tube by allowing it to equilibrate with a N\(_2\) filled syringe. Aqueous phase samples (0.1 ml) were taken with a gas-tight syringe through the PTFE lined septum immediately after CT addition and periodically over the duration of the experiment. At each time-point, the sampling-syringe was flushed with N\(_2\) and approximately the same volume of N\(_2\) was injected into each tube. The sample was injected into 1 ml of hexane and allowed to equilibrate for at least 10 minutes on a horizontal shaker (150 rpm). The hexane phase was then used for gas chromatographic analysis. The total headspace volume at the end of the
experiment was less than 5% of the vessel volume, ensuring that less than 4% of CT remaining and less than 0.5% of CF produced in the tubes were in the headspace (using the Henry constants given by Gossett, 1987). All CT reduction treatments were conducted in triplicate.

Iron Reduction

The ability of *S. algae* BrY to use Fe(0) corrosion products as a terminal electron acceptor was examined in an experiment analogous to that described above, omitting the CT and replacing the PTFE-lined septa with blue butyl rubber stoppers. The initial cell concentration was $1.60 \times 10^{10}$ cells ml$^{-1}$. Sampling was performed by removing aqueous phase-aliquots, with a disposable syringe and 22-gauge needle. Samples were injected directly into 0.5 N HCl for ferrous iron analysis. All iron reduction treatments were conducted in triplicate.

Adhesion of BrY to Iron

Adhesion studies were performed as described previously (Caccavo, Jr. et al., 1997) with the exception that the Fe(III) oxide-bicarbonate buffer was replaced with Fe(0)-HEPES buffer. Anaerobic tubes of HEPES-pretreated iron were prepared as described above. A final concentration of $4.2 \times 10^8$ cells ml$^{-1}$ was used and adhesion assays were performed in triplicate.

Analytical Methods

CT, CF, and dichloromethane (DCM) were analyzed using a HP 6890 gas chromatograph (GC) equipped with an electron capture detector. An aliquot (10 µl) of hexane phase of the liquid-liquid extracts was injected onto a 60/80 Carbopack B/1% SP-1000 column (8 ft x 1/8 in). The GC was operated under the following conditions: Injector temperature of 275 °C, initial temperature of 100 °C, increased at 15 °C min$^{-1}$ up to 200 °C, detector temperature of 250 °C, and N$_2$ flow of 40 ml min$^{-1}$. The GC was controlled by HP Chem Software, Vers. A.04.01. This method permitted for the detection of concentrations of less than
10 μg l⁻¹ of CT, CF, or DCM. Ferrous iron analysis was performed with Ferrozine as described by Lovley and Phillips (1987).

**Results and Discussion**

A number of studies have suggested that contact between DIRB cells and insoluble Fe(III) minerals is a necessary prerequisite for Fe(III) mineral reduction (Arnold et al., 1988; Caccavo, Jr. et al., 1992; Caccavo, Jr. et al., 1996; Lovley and Phillips, 1988; Tugel et al., 1986). The ability of *S. algae* BrY to catalyze reactions on the surface of Fe(0) particles would thus be predicated on the ability of *S. algae* BrY cells to adhere to those particles. The results in Table 4.1 demonstrate that 82% of the *S. algae* BrY cells adhered to 2 mg ml⁻¹ of HEPES-pretreated iron, while cells were unable to adhere to untreated Fe(0). When 20 mg ml⁻¹ of HEPES-pretreated iron were provided, more than 99% of the cells adhered while only 10% adhered to 20 mg ml⁻¹ of untreated Fe(0). The difference in the ability of *S. algae* BrY to adhere to each of these forms of Fe(0) is most likely due to the presence of Fe(III) oxides on the surface of the corroded iron. Previous studies have demonstrated that DIRB preferentially adhere to Fe(III) coated surfaces (Caccavo, Jr. et al., 1997; Grantham and Dove, 1996). The results of the adhesion experiments suggest that DIRB would be more likely to catalyze reactions on the surface of corroded iron than on the surface of untreated Fe(0).

**Table 4.1. Adhesion of *S. algae* BrY cells to HEPES-pretreated iron and Fe(0)**

<table>
<thead>
<tr>
<th>treatment</th>
<th>% adhered cells ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mg HEPES-pretreated iron</td>
<td>82.2 ± 6.2</td>
</tr>
<tr>
<td>2 mg Fe(0)</td>
<td>-5.4 ± 8.2</td>
</tr>
<tr>
<td>20 mg HEPES-pretreated iron</td>
<td>99.3 ± 5.8</td>
</tr>
<tr>
<td>20 mg Fe(0)</td>
<td>10.6 ± 6.3</td>
</tr>
</tbody>
</table>

¹ numbers represent the means of three replicate assays ± the standard errors of those means
Following adhesion, active *S. algae* BrY cells were capable of restoring the capacity of HEPES-pretreated iron to transform CT to CF. After 20 hours of incubation CT concentrations were statistically significantly lower (t-test, *P*-value ≤ 0.025) in treatments with active *S. algae* BrY than in controls containing HEPES-pretreated iron without cells or cells without iron (Figure 5.1A). In fact, neither *S. algae* BrY nor HEPES-pretreated iron caused significant transformation of CT. No direct CT transformation by BrY was observed, which agrees with previous work by Gorby et al. (1994) and Workman et al. (1997). Workman et al. (1997) were able to stimulate CT transformation in the presence of BrY through the addition of Vitamin B12. However, the levels of Vitamin B12 used by Workman et al. (1997) were much higher than normally observed in natural systems and are unlikely to have existed in these experiments.

![Figure 5.1](image)

Figure 5.1. (A) Carbon tetrachloride degradation and (B) chloroform production by (■) passivated Fe(0), (●) passivated Fe(0) with active *S. algae BrY* cells and (▲) active *S. algae BrY* cells alone. Error bars represent the standard error of the means (n = 3).

Treatments containing both HEPES-pretreated iron and active cells also accumulated more CF than the controls (Figure 5.1B) (*P* ≤ 0.0003). Approximately 60 % of the added CT were converted to CF in tubes containing both HEPES-pretreated iron and *S. algae* BrY. No dichloromethane (DCM) or other transformation products were detected in any of the setups.
Active cells of *S. algae* BrY were capable of generating ferrous iron with lactate as an electron donor and HEPES-pretreated iron as a sole terminal electron acceptor (Figure 5.2). These experiments were conducted in the absence of CT since the presence of CT could have resulted in instantaneous re-oxidation of microbially produced Fe(II) to Fe(III), thus masking the production of Fe(II) by DIRB. The sum of dissolved and colloidal Fe(II) was measured in these systems. Dissolved Fe(II) is reported to be a weak reductant for CT transformation to CF (Doong and Wu, 1992) while surface associated Fe(II) is thought to be a stronger reductant (Kriegman-King and Reinhard, 1992; Kriegman-King and Reinhard, 1994; Matheson and Tratnyek, 1994). Using conventional extraction methods, however, it is difficult to measure the quantity of adsorbed Fe(II) selectively, because the grains of Fe(0) provide a large reservoir of extractable metal ions (Johnson et al., 1998). Figure 5.2 clearly shows that, over a period of ca. 6 days, the treatments with active *S. algae* BrY cells resulted in significantly higher Fe(II) concentrations than either control ($P \leq 0.027$).
Conclusions

The influence of microorganisms on the performance of Fe(0) subsurface barriers has only recently been recognized. Methanogenic and denitrifying bacteria have been shown to have a positive effect on the degradation of CT and CF by Fe(0) (Novak et al., 1998; Till et al., 1998; Weathers et al., 1997).

It is reported here for the first time that DIRB can influence the transformation of a chlorinated aliphatic in the presence of passivated iron. The results indicate that DIRB can have a positive influence on the performance of zero valent iron by restoring the reactivity of passivated iron to transform CT to CF.

The work described herein did not allow for the identification of the exact reaction mechanism. It is possible that DIRB restore the reactivity of corroded iron by producing surface-bound reactive Fe(II) sites through microbial reduction of Fe(III) precipitates or by reductive dissolution of Fe(III) corrosion products. Reductive dissolution of ferric iron precipitates would lead to a decrease in corrosion layer thickness, which could increase the mass transport of contaminants to surface reactive sites or facilitate the electron transport from the Fe(0) to reaction sites on the corrosion product-covered surface of the iron grains (Scherer et al., 1998). Thin passive films composed of magnetite, maghemite, and other mixed valent (hydr)oxides have been modeled as semiconducting Fe$_2$O$_3$ doped with Fe(II) (Schmuki et al., 1995). Thicker corrosion layers tend to be amorphous with fewer semiconductor characteristics (Scherer et al., 1998). The reductive dissolution of amorphous iron (oxy)hydroxides from the grain surface would lead to a decrease in corrosion layer thickness and could result in enhanced electron transport from the underlying iron metal to surface reactive sites.

More research is needed to identify the mechanisms by which DIRB influence the performance of Fe(0). Specifically designed experiments involving surface analytical equipment should allow for the identification of the reaction mechanisms. Future research should also address the role of iron-reducing bacteria in pilot and field scale barriers. DIRB could be isolated from core samples and their effects could be evaluated under more field realistic conditions (alkaline pH, field-weathered iron material).
A thriving DIRB population in Fe(0) barriers could also have a negative effect on the reactivity of Fe(0). The development of thick biofilms could inhibit mass transport of dissolved contaminant to the iron surface (Grady and Lim, 1980; Revsbech and Jørgensen, 1986), thus lowering the overall reactivity of Fe(0) barriers. Although currently not observed in field applications, excessive microbial growth within subsurface barriers could potentially lead to a decrease in porosity, which could result in hydraulic failure of the barrier. DIRB activity in Fe(0) barriers could also result in enhanced dissolution of reactive corrosion products thus decreasing the overall reactivity of Fe(0) subsurface barriers.
The research summarized in this dissertation was designed to evaluate the use of dissimilatory metal-reducing bacteria (DMRB) for the bioaugmentation of subsurface environments contaminated with heavy metals and chlorinated hydrocarbons.

Two important aspects determining the success or failure of bioaugmentation strategies were evaluated including the effective delivery of DMRB into the subsurface and the biogeochemical influence of DMRB on the fate of contaminants. The four objectives listed in the Introduction were addressed in four separate but interrelated research projects, which are summarized in the four main chapters of this dissertation. This chapter summarizes the findings, discusses their implications, and identifies future research needs.

Chapter 2 compared the porous media transport of starved and vegetative cells of *Shewanella algae* BrY, the model DMRB used in this dissertation. Bacterial long term starvation had been suggested as a transport enhancement strategy previously to this work, but a quantitative comparison had not been attempted. Based on the fractional recovery of cells (i.e. the total number of bacteria transported through a porous media column divided by the total number of cells injected) starved cells were transported better through porous media than vegetative cells. A 3.2 fold increase in fractional recovery was observed in 30 cm long columns for *S. algae* BrY cells, which were previously starved for 7 weeks. The longer (3 m) columns showed an approximately 23 fold increase in fractional recovery for starved cells. In addition, starved cells were more homogeneously sorbed to the quartz sand along the flowpath while vegetative cells tended to preferentially sorb to sand close to the influent side of the columns. The preferential sorption of vegetative cells to the vicinity of the injection point could potentially cause the clogging of injection wells in field situations, a well known problem in the application of bioaugmentation technologies.
The research described in Chapter 3 was designed to evaluate potential reasons for the transport enhancement by starvation. A number of cell properties known to influence bacterial transport through porous media was monitored over seven weeks of starvation and an existing mathematical model, based on the colloid filtration theory, was used to evaluate their importance. Changes in cell size, buoyant density, diffusion coefficient, hydrophobicity, and net electrostatic charge could only partly explain the transport enhancement by starvation. However, a comparison of the results of batch adhesion assays with the fractional recoveries from 30 cm long columns suggested that less labor intensive batch experiments might provide an effective screening tool. Such a tool would allow to screen bacterial strains for their starvation transport enhancement potential without the need to perform labor intensive column studies.

Chapters 2 and 3 suggest additional experimental work, which will improve the understanding of physiological changes and mechanisms responsible for the transport enhancement by bacterial starvation. The influence of flagella, the change in surface characteristics of the cells, and the relationship between metabolic activity and bacterial transport through porous media should be evaluated. Once the transport mechanisms are understood, efforts should be focused on the development of an appropriate mathematical model. The employed model based on the filtration theory was not easily adapted to accurately describe scale- and physiology-dependent effects on bacterial transport through porous media. Methods for the reliable, selective, and quantitative assessment of bacterial numbers and activity in porous media should be developed in order to facilitate the model development process. Chapter 2 suggests a method based on the use of molecular techniques, which, if successfully developed, could provide the necessary information in the future.

In view of potential field applications, the starvation time should be optimized. The results summarized in this dissertation suggest that for *Shewanella algae* BrY four weeks of starvation result in bacteria that transport approximately as well as bacteria starved for seven weeks. However, the concentration of bacteria in the starvation cultures were approximately five fold greater after four weeks than after seven weeks, which would allow to produce larger numbers of bacteria from the same initial volume of cells.
Chapters 4 and 5 focused on the ability of *Shewanella algae* BrY cells to directly and indirectly influence the fate of heavy metals and chlorinated hydrocarbons in the subsurface.

Chapter 4 described the resuscitation of starved *S. algae* BrY into actively metabolizing cells in the presence of surface associated iron minerals. Active *S. algae* BrY cells reduced chromium(VI) directly and in addition allowed the establishment of a biogeochemical cycle involving surface reactive iron which facilitated the precipitation of chromium(VI) from contaminated water. A research project, based on these findings is currently addressing the potential feasibility of this technology by characterizing the mineral phases developing during iron and chromium reduction and by evaluating the stability of the chromium precipitates under different redox conditions.

Chapter 5 described the potential influence of DMRB on the performance of zero valent iron (Fe(0)) permeable reactive barriers (PRBs). DMRB are ubiquitous in the environment and therefore likely to come in contact with existing PRBs. However, the potential effects of DMRB on Fe(0) PRBs had not been evaluated previously to this work. The microbial reduction of corrosion products, which accumulated on the Fe(0) surface by *S. algae* BrY cells was presumably responsible for the increase in reaction rates of Fe(0) with carbon tetrachloride. Chapter 5 was published in “Environmental Science and Technology” and is reprinted in this thesis with permission of the American Chemical Society. This publication has stimulated widespread interest and a number of funding requests and research projects in the scientific community. Drs. Michelle Scherer and Pedro Alvarez at the University of Iowa are currently investigating the influence of DMRB on the mineralogy and reactivity of Fe(0) in the presence of chlorinated aliphatics. Research groups at the Oakridge National Laboratory and MSE Technology Applications (Butte, MT) are interested in the effect of DMRB on the performance of Fe(0) PRBs for uranium elimination from groundwater.

**Implications for Field Applications**

The author of this dissertation is convinced that the research summarized herein contributes towards an improved understanding of bacterial transport through porous media. Continued research in this
area will contribute towards the development of a widely applicable technology for subsurface bioaugmentation.

There is ample evidence that long term nutrient starvation of bacteria enhances the transport of a number of different bacterial strains and the knowledge about starvation responses suggests that starvation could be a generally applicable strategy for transport enhancement. The development of a generally applicable transport enhancement strategy would allow for the use of any bacterium of interest. Indigenous and exogenous bacterial strains and potentially whole consortia could be used. The bacteria could be starved after isolation and enrichment, and injected into contaminated subsurface environments to accelerate bioremediation processes.

The ultimate success of bioaugmentation strategies will be based on the survival and activity of the injected microorganisms. The survival and activity of these organisms is influenced by many factors including nutrient availability, moisture content, physical and chemical characteristics of the environment (pH, toxicity, electron acceptors, etc.), predation, parasitism, and competition with the indigenous microbial population. Future research should, therefore, focus on further improvement of bacterial transport strategies and on the development of environmentally benign, however competitive and metabolically active inocula.

Research focusing on the optimization of fluid velocity and inoculum concentration for bacterial transport and the use of site groundwater or artificial groundwater for starvation might lead to further improvements of the starvation transport enhancement strategy.

Future research should evaluate the bacterial transport, bacterial activity, and long term stability of chromium precipitates on bench- and meso-scale. The long term stability of chromium precipitates will be addressed in bench scale experiments in a collaborative research project between the Idaho National Environmental and Engineering Laboratory (INEEL) and the Center for Biofilm Engineering at Montana State University. In view of a potential field scale application alternative carbon sources should be evaluated. An excess of lactate was used in the studies described. The type and amount of carbon source added to any subsurface environment should be optimized to minimize costs and the danger of excessive microbial growth close to the injection well. The transport of starved *Shewanella algae* BrY through porous
media, their resuscitation in the presence of iron minerals, and the reaction of ferrous iron with Cr(VI) should be evaluated on a larger scale. A meso-scale subsurface testing facility such as the VEGAS in Stuttgart (Germany) would be ideal for this purpose.
REFERENCES CITED


