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DETACHMENT OF ESCHERICIA COLI FROM SATURATED POROUS MEDIA  
IN LABORATORY COLUMNS

BY  
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DETACHMENT OF *ESCHERICIA COLI* FROM SATURATED POROUS MEDIA  
IN LABORATORY COLUMNS

BY

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## ABSTRACT

### DETACHMENT OF *ESCHERICHIA COLI* FROM SATURATED POROUS MEDIA IN LABORATORY COLUMNS

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Recent studies suggest that bacterial contamination of groundwater is a national health concern in the United States. The movement of bacteria-laden water through soil and aquifer sediments often results in significant removal of bacteria from the aqueous phase, however, elevated concentrations of indicator organisms in drinking water aquifers are often detected and may be due, in part, to the slow release of sediment-associated bacteria. This condition, referred to as tailing, is caused by the detachment of previously attached cells over time, with aqueous concentrations often orders of magnitude below the peak concentration. Extended tailing has often been observed in laboratory and field transport experiments. The factors controlling bacterial attachment to aquifer sediments have been well investigated, however the processes controlling bacterial detachment from sediment surfaces at steady state are not well understood.

To address this research gap in understanding bacterial detachment in the subsurface, laboratory column experiments were performed to investigate the attachment and detachment of a nonmotile strain of *Escherichia coli* cells at resting state through uncoated or Fe-coated quartz sand (350-500  $\mu\text{m}$  diameter) in KCl solution at low or high ionic strength (0.001 M or 0.01 M). For each experimental run, a pulse of  $^3\text{H}$ -labeled *E. coli* cells was injected into flow-through columns, and column effluent was sampled for ~17-23 pore volumes (8.5-11.5 h). To account for biological effects on detachment,

experiments were also run using *E. coli* cells treated with 0.5% formaldehyde rendering them “dead”. To calculate bacterial attachment and detachment rates, the one dimensional advection-dispersion equation modified to account for deposition and detachment was fit to the bacterial breakthrough curves (BTCs) for each treatment combination. To compare protein composition within cells, protein analysis was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Tailing of “live” *E. coli* cells was higher than the cells that were rendered “dead” and therefore biologically inactive, which was opposite than expected. This difference was attributed to cell surface characteristics and to the low carbon content within the columns. In addition, protein analysis indicated that there was a difference between the protein profiles of “live” and “dead” cells. As expected, bacterial BTC peaks for coated sand experiments were significantly lower than peaks for experiments using uncoated sands, indicating that attachment was more prevalent in coated sands. Tailing was observed for experiments using Fe-coated sands, however, which is contradictory to the reporting of others. Bacteria have been found to irreversibly attach to Fe-coated sands due to the positive charge of the coating and negative charge of bacteria. This irreversible attraction is thought to permanently remove bacteria from the aqueous phase, yet this research indicates that *E. coli* cells can reversibly attach to coated sand. In addition, detachment rates did not significantly change for the different treatments under the steady state conditions, indicating detachment may be more of a concern in perturbed systems. More research is necessary to elucidate the factors controlling detachment for the future protection of groundwater supplies from contamination by pathogenic microorganisms.

## I. INTRODUCTION

Bacterial contamination of groundwater is a national health concern in the United States. The movement of bacteria through groundwater has therefore been a focus of research. Although water quality is improving and water supplies in the US are among the safest in the world, more information on detection and elimination is still needed (US EPA 1999a).

In addition to concerns about bacterial contamination, recent research has focused on the use of bacteria for *in situ* groundwater bioremediation (Harvey 1991; Scheibe and Wood 2003). *In situ* bioremediation involves the injection of contaminant-degrading bacteria to the subsurface, and successful remediation depends on bacterial transport to an area of contamination. Understanding the processes controlling bacterial movement through the subsurface, including the mechanisms controlling both attachment to and detachment from sediment surfaces, will assist in the prevention of pathogen transport and the remediation of aquifers (Bengtsson and Ekere 2001; Scheibe and Wood 2003).

The effects of biological and physicochemical factors on cell attachment have been well investigated in field and laboratory experiments and by numerical modeling. The factors influencing detachment, however, have not been thoroughly investigated. The goal of this study was to determine if bacteria have any biological effect on attachment/detachment and to elucidate the biological and physicochemical characteristics that could affect detachment for the development of future models.

## **Bacterial Contamination of Groundwater**

Drinking water contamination by pathogenic (disease causing) microorganisms was recognized by scientists beginning in the early 20<sup>th</sup> century (USEPA 1999a). Contamination of drinking water sources occurs when wells are poorly constructed or when contamination sources are too close to wells. Public disease outbreaks occur when these waters are not treated properly prior to use. Approximately 80% of the 54,000 community water systems, defined as drinking water systems that serve a resident population year-round, use groundwater as the main source of drinking water in the United States (USEPA 1999a). Furthermore, 53% of the United States population relies to varying degrees on groundwater supplies (USEPA 1994). In New Hampshire alone, over 500,000 people are served by more than 2,000 groundwater systems (USEPA 2000).

Fecal contamination has been detected in almost half of all drinking water wells in the United States (Macler and Merkle 2002). Fecal contamination includes both pathogenic and non-pathogenic microorganisms including bacteria, viruses and protozoa (Mawdsley et al. 1995). Microorganisms naturally inhabit the intestines of warm-blooded mammals and are consequently found in animal excreta (Craun et al. 1997; USDA and SCS 1992). Surface infiltration and runoff are two ways in which these microorganisms can enter groundwater. If pathogenic, microorganisms have the potential to cause disease outbreaks when the contaminated groundwater is improperly treated and used for drinking water. Contaminated groundwater can also be transferred to surface waters in areas of groundwater discharge (USEPA 1993). Surface waters carrying pathogens can cause recreational beach closures and shellfishery restrictions (USEPA 1994).

Outbreaks caused by the ingestion of pathogens in drinking water have been recorded throughout North America. The largest outbreak caused by waterborne pathogens reported to date in the United States occurred when over 400,000 people were infected by *Cryptosporidium* in Milwaukee, Wisconsin (Craun et al. 1997). Many outbreaks in North America have been traced to wells carrying the pathogen *E. coli* O157:H7. *E. coli*, members of the coliform family, are Gram-negative bacteria found in the digestive system of warm-blooded mammals and are commonly used as indicators of fecal contamination. Although many are nonpathogenic, strains of *E. coli* are pathogenic, including *E. coli* O157:H7. *E. coli* O157:H7 is a member of a pathogenic group of *E. coli* serotypes, enterohemorrhagic *E. coli* (EHEC). EHEC can cause hemorrhagic colitis, which is characterized by abdominal complications (Griffin and Tauxe 1991). Such conditions can worsen and cause kidney failure, known as hemolytic uremic syndrome (HUS), which can lead to death in children, the elderly and other immunocompromised individuals (Griffin and Tauxe 1991).

The following cases are some examples of recent outbreaks that were caused by the ingestion of groundwater contaminated with *E. coli* O157:H7 in North America. In August 1999, approximately 1,000 people attending the Washington County Fair in Albany, New York were infected from ingesting well water carrying *E. coli* O157:H7 (CDC 1999). This ingestion resulted in two deaths. The source of contamination was traced to water from a shallow groundwater well that was not treated prior to use at the fair. The cause of the well contamination was suspected to be from infiltration of cow manure (Gagliardi and Karns 2002; Olsen et al. 2002).

In a similar case in Canada, more than 3,000 people were infected and five died from the ingestion of groundwater drawn from the town supply and contaminated with *E. coli* O157:H7 in Walkerton, Ontario in May 2000. The source was not positively identified but may have been the result of runoff contaminated with manure (Gagliardi and Karns 2002). Olsen et al. (2002) reported the results of an investigation of a 1998 outbreak of *E. coli* O157:H7 in Alpine, Wyoming. An unconfined aquifer that served as the unchlorinated municipal groundwater supply in Alpine caused an outbreak of *E. coli* O157:H7 in 1998. Elk and deer feces were found to be the cause (Olsen et al. 2002).

The actual extent of pathogen outbreaks is underestimated due to underreporting (Olsen et al. 2002). Often only novel cases are reported, making it difficult to track occurrences for the prevention of future incidences (O'Brien and Adak 2002). The best way to prevent outbreaks from occurring is to eliminate the possibility of pollutants entering a source of drinking water (USEPA 1999a). Nationally, the Wellhead Protection Program, part of the Safe Drinking Water Act, requires the individual states to create pollution prevention plans for every public drinking water source (USEPA 1999a).

Some of the most cited groundwater pollutant sources of fecal contamination are agricultural activities and septic systems (USEPA 1994). Other sources include land application of sludge byproducts from wastewater treatment, sewer line breaks and sanitary landfill leachate. The EPA/State Feedlot Workgroup states that agricultural groundwater contamination sources include leaky manure storage lagoons and excess manure application to cropland (USEPA 1993). Utilizing manure for crop fertilization can cause contamination when manure is not treated for bacteria prior to spreading (Gagliardi and Karns 2002), when it is applied in excess (Bicudo and Goyal 2003), or

when manure is spread on porous soils (Unc and Goss 2003). Other sources include the pasturing of livestock and wash water from animal housing (Mawdsley et al. 1995).

States have taken individual measures to control and prevent surface and groundwater contamination from agricultural runoff, such as the Nonpoint Source Management Plan created by the New Hampshire Department of Environmental Services (NHDES 1999). NHDES, in cooperation with the Natural Resources Conservation Service (NRCS), has been successful in installing Best Management Practices (BMPs) such as constructed wetlands and concrete structures for manure storage and containment. These BMPs have effectively reduced bacterial loading from farms around the state (Landry, 2003). The USDA and SCS (1992) list several methods for reducing bacterial contamination, including reducing the number of animals from areas draining to a drinking water source, reducing manure application by dividing applications throughout the growing season, increasing the amount of vegetative cover and reducing the amount of irrigation to lessen the extent of manure leaching.

Septic systems, the other commonly sited source of groundwater contamination, were first used in the United States in 1860 at the community scale. Today, approximately 25% of United States households use septic systems to treat their sanitary waste (USEPA 2001). This number is increasing in rural and suburban areas (Stevik et al. 2004). Septic systems can be an effective way to fully treat the waste of a household if the system is properly installed and maintained (USEPA 1999b). If soils become saturated, however, microorganisms can become mobilized. Septic systems can fail (i.e. release incompletely treated wastewater into a groundwater system) when soils become clogged, are too permeable or are not permeable enough (Schueler 1999). Septic systems

can contain pathogenic bacteria and viruses. System failures can cause outbreaks of infectious disease such as gastrointestinal illness, hepatitis A, cholera and typhoid (USEPA 2001). To prevent failures, systems should be pumped out every 2 to 5 years (USEPA 2001; USEPA 1999b), inspected biannually and replaced after the recommended 15 to 30 years. This preventive maintenance is often done at the discretion of the homeowner (Schueler 1999).

### **Bioremediation**

Another compelling reason for subsurface bacterial transport research is *in situ* bioremediation, defined as the addition of contaminant degrading non-pathogenic bacteria to the subsurface for pollutant elimination. One method of bioremediation involves the injection of contaminant degrading bacteria into the subsurface (Crawford 1991; McClaine and Ford 2002). In order for this process to be effective, bacteria must travel to a source of contamination without attaching to surfaces. At this point, bacteria can then remove contamination from the aqueous phase (Bolster et al. 2001; Gannon et al. 1991; Johnson and Logan 1996). The success of this technique relies on a number of factors, including microorganism biology (Crawford 1991), groundwater chemistry (Johnson and Logan 1996) and characteristics of the porous media (Johnson and Logan 1996; Vance 1995). For example, bacterial transport is less hampered in coarse-grained versus fine-grained groundwater systems (Vance 1995).

In addition, the use of contaminant degrading bio-curtains, regions of sediment-associated bacteria, is a focus of current research (Bolster et al. 2001). Their formation requires that injected bacteria attach to surfaces, multiply, produce extracellular

polysaccharides and eventually form a biofilm. Biofilms, defined as a community of cells permanently attached to a surface and bound together in several layers, can effectively degrade contaminants. The development of a biofilm requires cells to attach and remain attached. Bacterial detachment from areas of contaminant remediation could potentially reintroduce pollutants into groundwater systems.

Methods for effective *in situ* bioremediation are still under development (Crawford 1991). Understanding the factors controlling attachment and detachment of bacteria from porous media are therefore necessary for predicting aquifer filtration efficiency and for successful bioremediation (Bengtsson and Ekere 2001).

### **Bacterial Transport Through Saturated Porous Media**

Before the 1970s, it was assumed that groundwater was free of fecal-borne microorganisms due to the natural filtering affects of the soil (USEPA 1999a). Although the movement of bacteria-laden water through soil and aquifer sediments often results in significant removal of bacteria from the aqueous phase, the efficiency of filtration depends on many factors, such as soil type and geology (USEPA 2001). Fine-textured topsoil can almost completely filter bacteria from the water column, but more coarse or disturbed soils are not as efficient (USDA and SCS 1992). More permeable soils may facilitate bacterial transport through soils to groundwater (Mawdsley et al. 1995) whereas saturated soils are less able to effectively filter bacteria (USDA and SCS 1992).

One way to gain a better understanding of the potential for aquifer filtration and bioremediation is to investigate how bacteria are transported through the subsurface. Bacteria can move unimpeded through saturated porous media or can interact with the

substrate. Planktonic, or free-floating cells, are transported by processes such as advection, dispersion and Brownian motion. Advection is transport caused by the flow of water, while dispersion occurs when a zone of mixing spreads mass beyond the region of advection. This process is termed mechanical dispersion (mixing caused by the spatial variability of a porous medium) or molecular diffusion (mixing caused by molecular motions). Cells are also affected by Brownian motion, which is random but constant movement caused by the bombardment of surrounding molecules when cells are suspended in a fluid or gas. This is important on a small scale when bacteria are close enough to surfaces to possibly become attached.

As bacteria move through aquifer materials, cell-substrate interactions affect bacteria movement. Research has investigated bacteria/surface associations since the 1930s (Mills 2003). Bacteria interact with aquifer substrates through two processes, mechanical and sorptive (Stevik et al. 2004; Vance 1995). Mechanical processes include gravitational settling and straining. Straining traps bacteria that are too large to move through pore spaces and consequently removes bacteria from the water column (Harvey 1991). Straining occurs if cell diameters are greater than 5% of the diameter of the porous media (Harvey 1991). Sizes of most bacteria range from 0.2 (starved cells) to 5  $\mu\text{m}$  (Vance 1995).

### **Bacterial Attachment**

If bacteria are much smaller than pore spaces, sorption is the dominant process affecting bacteria/surface interactions (Stevik et al. 2004). Bacteria can attach to surfaces either reversibly or irreversibly. Cells that are reversibly attached are held by temporary

cellular bonds which can be broken by changes in flow, solution chemistry, or cell motility (Marshall 1980). Reversibly sorbed cells do not come in direct contact with surfaces and are held at separation distances from surfaces by repulsive forces in an area termed the “secondary minimum” (Marshall 1980) (Figure 1). Bonds between cells and surfaces are much more difficult to break when cells are irreversibly attached (Mills 2003). Irreversible attachment occurs when repulsive forces are weak and cells can directly connect to surfaces in an area termed the “primary minimum” (van Loosdrecht et al. 1990). Cells can also become irreversibly attached because of cell surface structures or because of the excretion of extracellular polysaccharides. Irreversible attachment followed by attachment of cells to other cells is required for the formation of biofilms. Attachment of cells is also referred to as adhesion, deposition or retention.

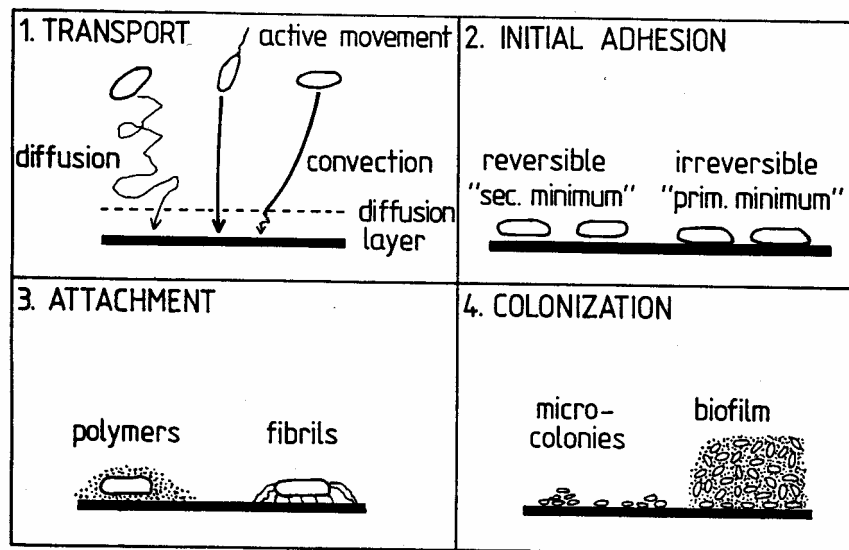


Figure 1. Diagram of the bacterial transport process including diffusion, reversible and irreversible attachment and ultimately the formation of biofilms (from van Loosdrecht et al. 1990).

Bacteria can adhere to a variety of surfaces, including other cells, mucous, teeth, plant roots, pipelines, soil and gravel (Marshall 1991). Benefits for cells to attach to surfaces may include the organic and inorganic nutrients found on surfaces, the ability to remain stationary until nutrient conditions become more favorable and for protection from predation. Pathogens usually must adhere to host surfaces prior to dividing, after which time they can colonize a site in order to infect hosts (Doyle and Sonnenfeld 1989). The factors that affect bacterial attachment to aquifer substrates can be divided into biological and physicochemical processes (Murphy and Ginn 2000).

#### Effects of Biological Factors on Attachment

Research has shown that biological factors have significant affects on cell transport (Becker et al. 2004) and can assist in irreversible attachment of cells to surfaces. Biological factors involved in cell transport include growth and survival (DeFlaun et al. 1990), cell concentrations (Camesano and Logan 1998), cell size (Fontes et al. 1991), bacterial surface characteristics (McCaulou et al. 1994; Williams and Fletcher 1996) and motility (Camesano and Logan 1998).

Growth can affect attachment rates. DeFlaun et al. (1990) reported that higher percentages of bacteria attached during log-phase of growth versus stationary phase. Although growth rates in the subsurface are much lower than in surface sediments, growth within aquifers is a factor over long time periods (Harvey 1991). Survival of bacteria in the subsurface is affected by the length of time cells remain attached to surfaces and also by predation, starvation, competition and environmental conditions including groundwater chemistry and temperature (Harvey 1991).

Lower cell concentrations, whether due to starvation or predation, may reduce the amount of bacteria transported through porous media (Gannon et al. 1991). Johnson et al. (1996) found that as bacterial concentrations increased and cell coverage on substrates increased, attachment rates decreased because attached cells blocked the attachment of unattached cells. The process of attached cells preventing the attachment of other cells, termed the blocking effect, was stronger at low ionic strengths. Tan et al. (1994) observed greater bacterial breakthrough when cell concentrations were increased. At concentrations  $10^8$  cells  $\text{ml}^{-1}$  or greater they observed that cell concentration no longer affected bacterial breakthrough and speculated that this was caused by a blocking effect.

Small cells usually move through pore spaces more easily than large cells (Gannon et al. 1991) because of fewer interactions between small cells and substrates (Hendry et al. 1999). Smaller particles move more easily through pore spaces because there are more potential flow paths for small cells (Sirivithayapakorn and Keller 2003). Gannon et al. (1991) found that higher percentages of smaller cells than larger cells were transported through soil columns. In column experiments, Weiss et al. (1995) found that the average length of cells was greater in column influent than effluent. Fontes et al. (1991) performed column experiments using two differently shaped bacteria strains (a coccus 0.75- $\mu\text{m}$  diameter and rod that was 0.75- by 1.8- $\mu\text{m}$ ) and observed lower recovery of larger rod-shaped cells in column effluent.

Although research has examined the involvement of cells in attachment, the role cell surface properties play in bacterial attachment is not completely understood (Williams and Fletcher 1996). Cell surface characteristics previously investigated include hydrophobicity, cell surface charge, cell membrane proteins and the excretion of

extracellular polysaccharides. Less than 0.1% of cell surfaces actually come into direct contact with a surface, therefore cell/surface interactions are fairly indirect (van Loosdrecht et al. 1990). Surfaces of cells can also change with environmental conditions (van Loosdrecht et al. 1989).

Hydrophobicity is defined as the distortion of polar water molecules around nonpolar molecules due to an inability to form hydrogen bonds. A hydrophobic surface is repellant to water and a hydrophilic surface can bond with water molecules. Surfaces of bacteria are generally hydrophilic with hydrophobic sites. Hydrophobic groups on cell and substrate surfaces can decrease separation distances by removing water films between a cell and the surface (Marshall 1991). Hydrophobicity varies for every bacterial strain. Hydrophobic bacteria have higher surface potentials than hydrophilic bacteria and attach to a greater extent than hydrophilic cells (van Loosdrecht et al. 1987a). Bacteria also become more hydrophobic during exponential growth phase (van Loosdrecht et al. 1987b).

Rijnaarts et al. (1999) found that bacteria surface polymers containing hydrophobic groups adsorbed to hydrophobic Teflon. In column experiments, McCaulou et al. (1994) observed slower attachment rates of hydrophilic bacteria than hydrophobic bacteria. They speculated that this was the result of the hydrophilic bacteria traveling farther prior to attaching. Once attached, however, they observed that the bonds of hydrophilic cells were irreversible. They also found that hydrophobic bacteria were more attracted to hydrophobic surfaces (sand coated with polymer to simulate organic carbon) than hydrophilic surfaces (quartz sand). Hydrophilic bacteria were also attracted to the hydrophobic surfaces but to a lesser extent than hydrophobic bacteria, and attachment

rates of hydrophilic cells to hydrophilic surfaces were slightly higher than to hydrophobic surfaces.

Additionally, electrokinetic potential affects attachment and plays a bigger role as hydrophobicity decreases (van Loosdrecht et al. 1987b). In order to determine information about bacterial electrostatic attraction and repulsion, the electrokinetic potential (or zeta potential) of a cell is usually calculated from electrophoretic mobility and conductance (van Loosdrecht et al. 1987b). Van Loosdrecht et al. (1987b) reported that hydrophobic cells have high electrokinetic potentials, yet they speculate that charged groups cover less than 8% of cell surfaces. McCaulou et al. (1994) reported that higher electrophoretic mobility of bacteria (higher negative charge) can increase affinity for positively charged surfaces in column experiments. Gannon et al. (1991), however, found that bacteria surface charge was not related to transport.

The involvement of cell proteins in attachment has also been investigated. The cell envelope is comprised of proteins that may be involved in attachment and detachment of Gram-negative bacteria. Gram-negative bacteria are distinguished from Gram-positive bacteria because of their inability to retain the Gram stain, which indicates whether a lipopolysaccharide outer membrane is present (Gram-negative) or not (Gram-positive). The cell envelope is comprised of an inner and outer membrane and of peptidoglycan, which provides cell wall structure (Özkanca and Flint 2002). The permeable outer membrane covers the peptidoglycan layer and is made up of proteins (including lipoproteins), phospholipids and lipopolysaccharide. These surface components contribute to cell surface hydrophobicity (Hancock 1991) and are involved in attachment. Lipopolysaccharides, which are nonpolar, may dominate in binding to

hydrophobic surfaces, whereas polysaccharides, capable of polar or electrostatic interactions, may be involved in attachment of cells to hydrophilic surfaces (Williams and Fletcher 1996). Bidle et al. (1993) investigated the role of S-layer proteins (proteins that form lattices on the outside of cells and can contribute to cell hydrophobicity) in attachment and found that S-layers are involved in attachment but that the degree of S-layer attachment varies with environmental conditions. Fimbriae, proteinaceous rod-shaped cell surface structures 0.2 to 2  $\mu\text{m}$  long, are thought to be hydrophobic (Ward 1980) and also play roles in attachment (Doyle and Sonnenfeld 1989). One method commonly used to examine cell protein profiles is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which involves separating proteins by isoelectric charge or molecular weight.

Extracellular polysaccharides, excreted by bacteria during the breakdown of organic material, are also involved in attachment (Doyle and Sonnenfeld 1989). These excretions form a “cement” that holds cells to surfaces. Cells attached by extracellular polysaccharides can become irreversibly attached and therefore have the potential to form biofilms.

Cell motility can also be involved in cell attachment. Motility is the means for some species of bacteria to move, in a process termed taxis. For example, movement may occur towards or away from areas which are either rich or deficient in nutrients, light or oxygen (chemotaxis, phototaxis, or aerotaxis, respectively). Motility may increase the chances of a cell arriving at a substrate, overcoming electrostatic repulsive barriers, and attaching to surfaces (Stevik et al. 2004; van Loosdrecht et al. 1989).

One mode of motility is the flagella, which can be up to 20- $\mu$ m long. In some cases, flagella have been found to be involved in actual cell attachment to substrates (McClaine and Ford 2002) and to and from other cells. Flagella also facilitate bacterial adhesiveness as they protrude through the hydrophilic outer membranes of cells exposing hydrophobic sites on bacteria (Hancock 1991). Fibrils are other structures on cell surfaces involved in motility that could act as bridging structures binding cells irreversibly to substrates.

Camesano and Logan (1998) observed an increase in attachment of nonmotile bacteria but found a decrease in cell retention of motile bacteria when fluid velocities were decreased. They also reported that decreasing ionic strength by two orders of magnitude decreased retention of motile bacteria strains more than nonmotile strains. Van Schie and Fletcher (1999) reported that flagellated cells attached to surfaces while still motile at log phase. At stationary phase, however, cells became nonmotile and attachment to surfaces was no longer observed. They also stated that the relationship between motility and attachment remained unclear.

#### Effects of Physicochemical Factors on Attachment

As cells move close to substrate surfaces they are subject to attractive London-van der Waals and repulsive electrostatic forces. The combination of these two forces form the weak attraction described previously as reversible attachment (Stevik et al. 2004). Van der Waals forces are the only adsorptive forces effective at cell-substrate separation distances greater than 50-nm (Marshall 1991) and occur when electron clouds form

temporary dipoles. The fluctuating dipoles induce dipoles in surrounding molecules by distorting adjacent electron clouds, resulting in temporary attractions.

As bacteria approach substrate surfaces, electrostatic forces increase. Gram-negative bacteria are generally negatively charged with some positively charged sites (Doyle and Sonnenfeld 1989) in natural groundwater conditions. The negative charge is due to phosphate, carboxylate and sulfate groups found in cell walls (Hancock 1991). The  $pH_{iep}$ , which is the isoelectric point at which charges are balanced, ranges from approximately 2 to 3.65 in Gram-negative bacteria (Mills 2003). At higher solution pH, Gram-negative bacteria are therefore negatively charged.

The negative layer of charge around bacteria and solids is surrounded by a layer of counterions that thins further from surfaces, creating a double layer of charge. The thickness of this double layer is determined by ionic strength, decreasing with increasing ionic strength (Mills 2003). This phenomena is described by the commonly used DLVO theory, named for Derjaguin and Landau (1941) and Verwey and Overbeek (1948), which calculates total interaction potential ( $G_{total}$ ) as the sum of attractive van der Waals ( $G_A$ ) and repulsive electrostatic ( $G_E$ ) forces,  $G_{total} = G_A + G_E$  (Figure 2) (Harvey 1991; Mills 2003; van Loosdrecht et al. 1990). DLVO theory predicts that in groundwater at high ionic strength, the double layer is compressed and van der Waals forces dominate. Electrostatic repulsion is the dominant force in groundwater at low ionic strength (Rijnaarts et al. 1999).

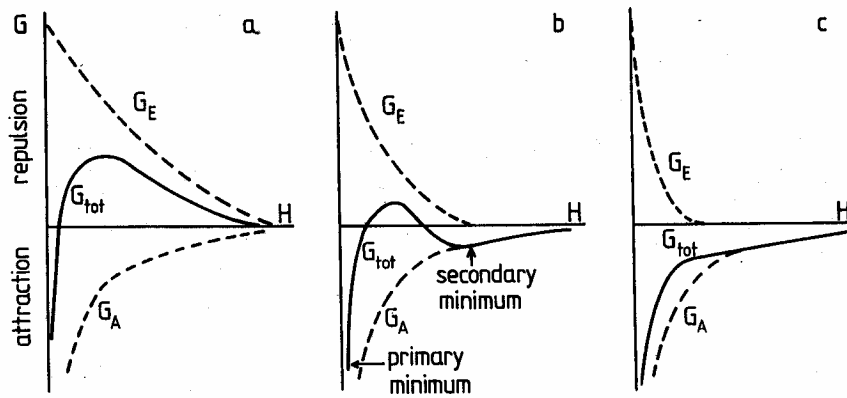


Figure 2. Total Gibbs energy interaction potential ( $G_{tot}$ ), van der Waals forces ( $G_A$ ) + electrostatic forces ( $G_E$ ) versus distance of particle H from a like-charged flat surface at (a) low ionic strength, (b) intermediate ionic strength and (c) high ionic strength (from van Loosdrecht et al. 1990). The particle is repelled at low ionic strength as a result of electrostatic forces, lies reversibly attached within the secondary minimum at intermediate ionic strength, and rests in an irreversibly attached state within the primary minimum at high ionic strength.

Van der Waals and electrostatic forces both interact at separation distances of about 10 to 20-nm until cells reach within 1 to 1.5-nm of a surface (Marshall 1991) in groundwater of intermediate ionic strength ( $<0.01$  M). This area, called the secondary energy minimum, is where cells do not directly contact surfaces but can remain in a state of reversible attachment (van Loosdrecht et al. 1990). Energy is required to overcome the 1.5-nm barrier. At ranges below 1.5-nm (the deep primary minimum), van der Waals forces attract bacteria because of a lack of repulsive kinetic energy (Mills 2003). Bacteria can then become irreversibly attached barring changes in solution chemistry (Harvey 1991). There is skepticism, however, that DLVO theory is applicable to microorganisms because exact separation distances are unknown because of the presence of cell wall structures such as fimbriae and because of variable cell surface chemistry (Meinders et al. 1995).

Fontes et al. (1991) and Mills et al. (1994) found that decreasing ionic strength by an order of magnitude decreased bacterial attachment rates to quartz sands. Deshpande and Shonnard (1999) also observed that increasing ionic strength increased attachment rates of bacteria to silica sand. Similarly, Bolster et al. (2001) reported that decreasing ionic strength decreased attachment rates of bacteria to uncoated quartz sand. In contrast, DeFlaun et al. (1990) reported that increases in ionic strength did not increase attachment of bacteria to sand. At the field scale, Mailloux et al. (2003) found that high ionic strength did not induce higher attachment rates for hydrophilic *Comamonas* sp. strain DA001.

Attachment rates have been shown to increase if positively charged sediments are present. A common aquifer substrate, quartz ( $\text{SiO}_2$ ), is negatively charged at circumneutral pH, and therefore repels negatively charged bacteria (Mills 2003). Aquifer sands, however, are often coated with an iron oxyhydroxide coating, which has a  $\text{pH}_{\text{iep}}$  greater than 8 (Johnson and Logan 1996) thus carrying a positive charge at neutral and acidic pH values. An electrostatic attraction between iron coated sands and negatively charged bacteria therefore becomes possible. Bolster et al. (2001) and Johnson and Logan (1996) both reported that in miniature sand columns, the presence of coated sands increased attachment rates. In sorption isotherm experiments, Mills et al. (1994) reported almost complete removal of bacteria from the aqueous phase at bacterial concentrations up to  $10^8$  cells  $\text{ml}^{-1}$  in coated quartz sands and only 28 to 75% of the bacteria were adsorbed when uncoated sands were used. McCaulou et al. (1994) found that more bacteria remained attached to quartz sand coated with positively charged hematite than to other surfaces. Bolster et al. (2001) also examined the combination of ionic strength and

grain coatings and reported that a decrease in ionic strength by an order of magnitude decreased sticking efficiency in uncoated but not coated sands.

Other physicochemical factors known to affect bacterial attachment include fluid velocity (Becker et al. 2004; Camesano and Logan 1998), grain size (Bolster et al. 2001; Fontes et al. 1991) and media heterogeneity (Fontes et al. 1991). As flow velocity is increased, bacterial breakthrough may increase (Wollum and Cassel 1978) because opportunities for bacterial attachment decrease. McClaine and Ford (2002) also found that attachment rates decreased as flow increased. Hendry et al. (1999) observed higher percent recovery of bacteria in column experiments as linear groundwater velocity increased.

Fontes et al. (1991) found that bacterial attachment was greater for smaller grain sizes ( $\sim 330 \mu\text{m}$ ) than for coarse grained sand ( $\sim 1000 \mu\text{m}$ ) and that grain size was the most important factor in controlling transport. Bolster et al. (2001) reported that grain size did not have a significant effect on bacterial retention capacity of sand in column experiments.

Heterogeneity of porous media, including differences in media sizes or surface coatings, can affect transport. Johnson et al. (1996) and Loveland et al. (2003) observed a direct relationship between colloidal attachment rates and the fraction of quartz sand coated with iron oxyhydroxide to uncoated sand, with attachment rates increasing as the percentage of fully or partially coated sands increased. Bolster et al. (1999) observed significant spatial variability in attached bacteria after dissecting laboratory columns. They speculated this was a result of media heterogeneity. In addition, they hypothesized that the variability was a result of different attachment capabilities within influent

bacteria populations. At the field scale, Mailloux et al. (2003) found that aquifer heterogeneity had much less effect than microbial population heterogeneity in an aquifer in Oyster, Virginia.

### **Bacterial Detachment**

Once bacteria have become attached to sediment surfaces, they can become detached from the solid phase and re-enter the aqueous phase by a process called detachment, also referred to as entrainment or desorption. This transfer of bacteria from the solid phase to the aqueous phase can result in the long-term release of bacteria back into drinking water supplies, posing a potential human health risk if the bacteria are pathogenic (Zhang et al. 2001). Trace amounts of bacteria can be eluted for long periods of time following a source pulse (Bales et al. 1991) and may have adverse effects if conditions in distant locations foster growth (Hornberger et al. 1992). McCaulou (1994) observed that time scales for detachment were 0.5 to 75 d versus 0.2 to 2 h attachment time scales, indicating that if attachment rates remained the same, bacteria with higher detachment rates would move greater distances at higher concentrations. Quantifying detachment in various subsurface conditions is therefore necessary to determine the potential for bacterial transport.

This detachment process over time, referred to as tailing, has been observed as extended tailing in breakthrough curves (BTCs, Figure 3) in both laboratory and field experiments (Zhang et al. 2001) including studies by Camesano and Logan (1998), Fontes et al. (1991), McCaulou et al. (1994), Johnson et al. (1995), Wollum and Cassel (1978) and Zhang et al. (2001). Aqueous concentrations in BTC tails are often orders of

magnitude below peak concentrations, yet are still measurable. Wollum and Cassel (1978) observed BTC tailing of streptomycete conidia in sand column experiments and reported very low concentrations in column effluent after 36 pore volumes of sterile water had been flushed through. Zhang et al. (2001) reported low concentrations of bacterial tailing that lasted for months in their field experiment. Fontes et al. (1991) attributed the bacterial tailing observed in their column experiments to the flushing of previously attached cells. Figure 3 illustrates the typical sections of BTCs.

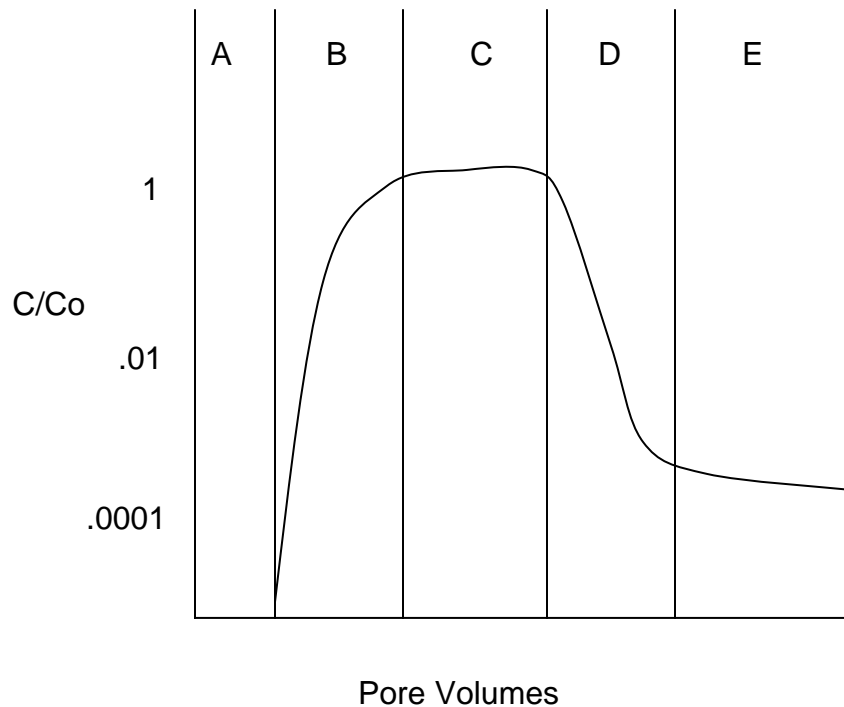


Figure 3. A typical breakthrough curve with five distinctive regions dictating the stages of a pulse of bacteria transported through porous media (from Johnson et al. 1995). Section A shows the delay before any concentrations are detected in the effluent, B displays the breakthrough, C represents steady state conditions, D is the effluent portion of the curve demonstrating the effect of dispersion and a high rate of reversible attachment, and E represents detachment over time. Breakthrough must be plotted on a semi-log scale in order to observe tailing.

Detachment has been observed in breakthrough curves for decades, yet the factors controlling detachment from sediment surfaces at steady state have not been as well investigated as the factors determining attachment (Bergendahl and Grasso, 2003). Recent studies have recognized the importance of detachment and have begun to examine the biological and physical factors controlling bacterial detachment in addition to attachment.

#### Effects of Biological Factors on Detachment

Cell size and surface characteristics can affect bacterial detachment. Meinders et al. (1995) observed the transport of three bacteria types and reported that the smallest cells had the lowest detachment rates. They speculated that this could have been related to collisions between attached and planktonic cells.

Becker et al. (2004) compared Gram-positive, Gram-negative, motile and nonmotile bacteria transport through uncoated and iron-oxyhydroxide coated latex beads in column experiments and observed greater breakthrough tailing in Gram-positive bacteria than Gram-negative in the presence of coated sands. They suggested that the coating may have an effect on cell wall/substrate interactions, which may be related to the lipopolysaccharide outer membrane which is only present in Gram-negative bacteria. Williams and Fletcher (1996) previously found that lipopolysaccharide was involved in bacterial transport.

Motility has varied affects on bacterial detachment. McCaulou et al. (1995) found that motile bacteria detached three times as fast as nonmotile cells in laboratory column experiments. Conversely, Becker et al. (2004) reported that at low flow rates, motile

bacteria had lower detachment rates than nonmotile bacteria. They also suggested that flagella aid in detachment. McClaine and Ford (2002) observed that fewer motile bacteria remained attached at low flows than high flows in experiments examining the attachment of *E. coli* cells to glass. They speculated that flagella aided in detachment at low flow rates but not at high flows.

#### Effects of Physicochemical Factors on Detachment

Effects of chemical perturbations on colloid and bacteriophage (viruses that attack bacteria) detachment, such as decreases in ionic strength and increases in pH, have been reported in the literature (Bergendahl and Grasso 2003). Grolimund and Borkovec (1999) observed that a decrease in ionic strength increased colloid detachment. They suggested that as ionic strength increases, the activation energy for particle release required to overcome energy barriers increases. The effects of ionic strength on bacterial detachment under steady state conditions are not well understood.

Bales et al. (1991) and Kinoshita et al. (1993) reported that increasing pH during laboratory column experiments increased bacteriophage detachment rates. Kinoshita et al. (1993) found that detachment rates were slow at steady state conditions but speculated that drastic increases in pH would increase virus detachment.

The effects of grain coatings on bacterial detachment are also not well known. Scholl et al. (1990) found that bacterial detachment from Fe-hydroxide coated sand did not occur in column experiments. When solution chemistry was changed to pH 8, however, detachment from Fe-coated sands did occur in field and laboratory experiments

(Scholl and Harvey 1992). In column experiments, Becker et al. (2004) also observed bacterial tailing in the presence of iron-oxyhydroxide coatings.

Brownian motion can cause bacterial detachment (Meinders et al. 1995) as can flow perturbations. Detachment can occur when flow velocities exceed the strength of interaction between cells and surfaces and when the velocity is high enough to exert shear (McClaine and Ford 2002). Zhang et al. (2001) reported that detachment rates of the bacterial strain *Comamonas* DA001 decreased by an order of magnitude as velocity decreased at the field scale. Becker et al. (2004) concluded that detachment was more sensitive to flow rates than sorption rates were. In laboratory column experiments, however, Tan et al. (1994) examined bacteria transport at three flow velocities and found that detachment did not correlate with flow velocity.

### **Mathematical Modeling**

Attachment and detachment rates are important controls on bacterial transport (Harvey 1991) and numerical modeling is used to determine these rates (Deshpande and Shonnard 1999). Bacteria are the size of colloids. Colloid-filtration theory (Rajagopalan and Tien 1976; Yao et al. 1971), therefore, is typically incorporated into a one-dimensional advection-dispersion equation in order to model bacterial transport (Harvey 1991; Johnson et al. 1995). Colloid-filtration theory is used to describe colloid removal during filtration. It is based on bacterial transport processes that transport cells to surfaces including Brownian motion, gravitational settling (Harvey 1991) and collector efficiency, defined as the ratio of the rate of particles that strike a collector to the rate of those that flow towards it.

Flow-through columns have often been used in laboratory experiments to investigate bacterial transport and are modeled by fitting multiple parameter models to BTCs (Becker et al. 2004). The parameters are derived by fitting a modified one-dimensional advection-dispersion equation that accounts for forward (attachment) and reverse (detachment) rates (similar to kinetic sorption) to the BTCs. No model to date, however, has been successful in accurately predicting the entire shape of BTCs (Johnson et al. 1995).

Numerous modeling techniques have been developed to describe the attachment process. Johnson et al. (1996) used a second-order model modified to account for the effect of previously attached bacteria on attachment rates. Deshpande and Shonnard (1999) called this a “blocking” model since the sorbed bacteria prevent further attachment. Bolster et al. (1999) used a similar approach and also accounted for an influent concentration of bacteria composed of two populations with different sticking efficiencies. Deshpande and Shonnard (1999) included intermediate-order kinetics into first- and second-order models to account for filter ripening, which is when attachment rates increase over time due to cell-to-cell attachment. They found that this model provided a better fit to observed data and was more flexible than first- and second- order models, although at low ionic strength first- and second- order models provided better agreement with data.

Models that account for detachment have progressed from first-order filtration to residence time-dependent models (Becker et al. 2004). Hornberger et al. (1992) treated detachment as a first-order kinetic process but found this model overpredicted tailing and emphasized BTC peaks at the expense of the tails. The model did, however, provide a

better fit than models neglecting detachment. In addition to modeling bacterial transport as a kinetic process, Becker et al. (2004) and Johnson et al. (1995) used residence time-dependent models that described detachment as a function of residence time and accounted for both the peak and tail of BTCs equally. Johnson et al. (1995) found that by accounting for quick detachment followed by slow detachment in a two-rate model, they were able to simulate either breakthrough or elution portions of observed BTCs but not both simultaneously. They found that by utilizing a two-site model that included irreversible attachment in addition to accounting for residence times, they were able to model both breakthrough and elution simultaneously. Harvey and Garabedian (1991) and McCaulou et al. (1994) also utilized two-site models assuming two potential attachment sites. Despite these differences from the model used by Hornberger et al. (1992), modeling bacterial transport as a first-order process is still one of the most common approaches to describing bacteria detachment in aquifers.

### **Objectives**

As cells move through the subsurface, the likelihood of cell attachment to or detachment from surfaces depends on many factors, including the biology of the cells, solution chemistry, and subsurface types. It is necessary to understand the processes that control bacterial movement through aquifers in order to develop models that accurately describe and predict the attachment and detachment of bacteria from aquifer sediments (Harvey 1991). Studies that have investigated the relationship between factors affecting attachment and detachment are not always in agreement, possibly because of the many variables involved, including the wide variety of bacterial strains that have been

investigated. In addition, the factors controlling detachment have not been as well investigated as the factors controlling attachment. The goal of this current study, therefore, was to elucidate some of the biological and physicochemical factors affecting the detachment of bacteria by examining the movement of *E. coli*, a commonly used indicator of fecal contamination, through saturated porous media in laboratory columns and by determining the protein profiles of these cells. The objectives of this study were (1) to determine to what extent, if any, *E. coli* cells are actively involved in the detachment process, and (2) to determine the extent of bacterial detachment in the presence or absence of surface coatings at varied ionic strengths.

### **Hypotheses**

To address these objectives, the following hypotheses were tested:

H1: “Live” bacterial cells will have lower detachment rates because “live” cells will be actively involved in the attachment/detachment process.

H2: Iron coatings will generate lower detachment rates than uncoated sand due to the attraction of negatively charged bacteria to positively charged iron coated sand grains.

H3: Higher ionic strength will reduce detachment rates because of stronger attractive van der Waals forces.

## II. MATERIALS AND METHODS

Laboratory column experiments were performed to investigate the detachment of a nonmotile strain of *E. coli* at resting state through saturated uncoated and Fe-coated uniform-sized quartz sand at two ionic strengths (0.001 M and 0.01 M). For each experimental run, a 0.5 pore volume (PV) pulse of  $^3\text{H}$ -labeled *E. coli* cells were injected into flow-through columns, and column effluent was sampled for ~17-23 PVs (8.5-11.5 h). To assess the importance of biological activity on the attachment/detachment process, comparisons were made between *E. coli* cells harvested and washed at stationary phase (“live” cells) with *E. coli* cells fixed with 0.5% final concentration formaldehyde (“dead” cells). To calculate bacterial detachment rates, a one-dimensional advection-dispersion equation (ADE) modified to account for both reversible and irreversible attachment was fit to the observed bacterial breakthrough curves (BTCs). To determine if there were differences in “live” and “dead” cell proteins, the protein profiles of “live” cells were compared to “dead” cells using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### **Porous Media Preparation**

Porous media was prepared in advance to ensure that sand grain sizes were uniform, grain surfaces were free of organic matter and that Fe-coated sands were thoroughly covered. Portions of commercially available quartz sand obtained from Unimin<sup>TM</sup> in New Caanan, CT were consecutively sifted through 500  $\mu\text{m}$  and 350  $\mu\text{m}$

U.S.A. Standard Testing Sieves. The fraction between 350 and 500  $\mu\text{m}$  was collected and utilized for the experiments.

In order to remove organic matter that could be utilized as a carbon source for the bacteria, the sand was acid washed and then rinsed thoroughly. First, sand was washed thoroughly with deionized water (DIW). It was then boiled in a 2 L flask containing 1 M hydrochloric acid for 2 h and finally rinsed with DIW until the rinse water pH was equal to the DIW pH. The sand was then dried in a THELCO<sup>®</sup> Lab Oven (Precision Scientific, Winchester, VA) overnight at 105°C, re-rinsed the following day, and finally dried again overnight.

Portions of the acid washed sand were coated with Fe-oxyhydroxide in a method similar to that employed by Mills et al. (1994). To coat grains, 40 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  were dissolved in 400 ml DIW. The solution pH was adjusted to >9 by slowly adding approximately 110 ml of 4 M NaOH. Two hundred grams of sand was added to the solution when the pH was >9 and placed on a Lab Rotator benchtop shaker table for 36-48 h. According to Mills et al. (1994), the overnight shaking uniformly coats the sand grains. One molar NaOH was added to the solution when the pH dropped below 9. The sand was then rinsed in DIW until the water was clear and baked again at 105°C for a 24 h period. The final step of rinsing and drying was repeated. Prior to experiments, sand was autoclaved at 121°C and 15 psi for 20 min to sterilize.

### **Column Preparation**

For every experimental run, two 30- x 4.8-cm Chromaflex<sup>™</sup> Chromatography Columns (Kontes Glass Co., Vineland, NJ) were acid washed, autoclaved for sterilization

and attached to Masterflex<sup>®</sup> Precision Tygon Food Tubing. Bed supports within the end caps were removed and replaced with stainless steel wire mesh (Stainless Steel Type 304, #60; Small Parts, Inc., Miami Lakes, FL) (Harvey et al. 1995). The mesh was then sterilized with 70% ethanol.

KCl was dissolved in DIW to yield a low or high ionic strength buffer solution (0.001 M or 0.01 M respectively). These two concentrations were chosen because they have been used in the experiments of others (Mills et al. 1994) and additionally were both “intermediate” ionic strengths, as described in Figure 2, where the effect of repulsive electrostatic forces or attractive van der Waals forces on particle separation distances from a surface is not dominant. At higher ionic strengths, electrostatic forces are known to be weak, and at lower ionic strength, effects of ionic strength will be minimal and electrostatic repulsion dominates. The pH was ~5.5 for each experiment. The buffer solution was filter sterilized through 0.2- $\mu$ m pore size, 47-mm diameter, membrane filters using a vacuum pump. This solution was then added to the columns using a Masterflex<sup>®</sup> Laboratory/Standard tubing pump in reverse upward flow to prevent the entrapment of air bubbles within the wire mesh.

Columns were wet packed with sand (uncoated or Fe-coated) by slowly pouring the sand into the buffer solution to a height of approximately 10 cm within the columns. Stirring and tapping the columns was necessary during the addition of sand in order to prevent stratification (Wollum and Cassel 1978) and air bubbles within pore spaces. Pore volumes (PV), defined as the volume of water retained in the sand columns (Wollum and Cassel 1978), ranged from 64 to 70 ml. PVs for each column were calculated as the volume of water pooled above the sand columns subtracted from the volume of water in

the column prior to the addition of sand. After packing was completed, columns were operated in the downflow direction, and approximately 10 PVs of KCl buffer solution were passed through each column for pH equilibration.

### **Bacteria Preparation**

A nonmotile (paralyzed flagella) strain of *E. coli*, K-12 HCB136, was obtained from the laboratory of Howard C. Berg at Harvard University. In nutrient deficient conditions, as aquifers typically are, starving cells have been found to lose motility (Wei and Bauer 1998). Nonmotile cells with paralyzed flagella were therefore used in these experiments in order to investigate the transport of cells in an environment similar to natural groundwater conditions. In addition, motility may affect attachment/detachment processes. In order to eliminate the possible affects of motility on detachment in these experiments, therefore, nonmotile cells were used.

Antibiotic selection was used for *E. coli* verification (Williams and Fletcher 1996); the culture was first streaked on tryptic soy agar (TSA) (Difco™) with 20 µg ml<sup>-1</sup> streptomycin (HCB136 is resistant to streptomycin) and grown for 22 h at 35°C in an air incubator. For storage and preservation, cultures were grown and then treated with Dimethyl Sulfoxide (DMSO). First, single colonies were removed from the confirmed culture following incubation and were then used to inoculate autoclave sterilized 300g ml<sup>-1</sup> tryptic soy broth (TSB) (Sigma®) in 25 ml DIW. The culture was then grown aerobically with shaking at 120 rpm at 22°C. One ml of this culture was used to inoculate a second flask of 50 ml autoclaved sterilized TSB solution which was then grown aerobically for 6 h at 22°C. Following the final incubation, DMSO (Sigma®) was

added to the culture to yield a final concentration of 7% DMSO (Greaves and Davies 1965). One ml aliquots of the treated culture were then pipetted into Cryo-Stor vials, mixed using a Mini Vortexer, and cryogenically frozen (-80°C) until use.

For each experiment, one frozen isolate was thawed and aseptically used to inoculate autoclave sterilized 300g ml<sup>-1</sup> TSB in 50 ml DIW. The culture was grown aerobically with shaking at 120 rpm for approximately 16 h at approximately 22°C. Following incubation, 0.5 ml from the culture was aseptically removed and inoculated into a second flask containing 50 ml sterilized TSB and DIW. The new culture was grown aerobically with shaking at 120 rpm for 7.5 h at 25°C in a controlled temperature room. Following the second incubation, cells were washed and harvested via centrifugation for the removal of growth media to ensure that growth was not a factor in the experiments. The cells were centrifuged at 6,037 x g for 10 min at 18°C. The supernatant was then decanted, the bacterial pellet resuspended in 35 ml of 0.0001 M KCl, and the solution centrifuged again as described above. This was repeated a third time to assure that the growth media had been completely removed. Following the third centrifugation, the bacterial pellet was suspended in 50 ml of low or high ionic strength KCl buffer solution, depending on experimental treatments. Care was taken to incubate, inoculate and centrifuge the bacteria in the same manner for each experimental run in order to reduce experimental variability.

For the enumeration of bacterial concentrations in the column effluent, the diluted culture was amended with 1 mCi [4,5-<sup>3</sup>H]-leucine (DeFlaun et al. 2001). When cells are amended with leucine, an amino acid, the leucine will be incorporated into the cell proteins. The amended culture was then incubated for approximately 18 h at 22°C to

allow for  $^3\text{[H]}$  incorporation into cell biomass. Preliminary tests revealed this to be the most appropriate cell labeling method (see Appendix A). After the incubation, on the day of the experiment, the solution was split into two flasks of 25 ml each. Approximately 30 min prior to the experiment, formaldehyde (0.5% final concentration; v:v) was added to one of the two cultures (Camesano and Logan 1998; Hanein et al. 1995) to render them “dead” and therefore eliminate biological activity for comparison with the biologically active “live” cells.

Unincorporated radiolabel was removed from the bacterial solutions via backwashing filtration prior to the column experiments in a method similar to that described by Camesano and Logan (1998). This method was chosen after preliminary testing (see Appendix A). First, approximately 9 ml from each culture were filtered through 0.2- $\mu\text{m}$  pore size, 47-mm diameter, membrane filters and rinsed with DIW. The filters were then backwashed by flipping them over and filtering approximately 20 ml of low or high ionic strength KCl solution into a second filter flask. The resulting bacterial cultures were then filtered through 5.0- $\mu\text{m}$  syringe filters to prevent clumping (Bolster et al. 2001).

To ensure that these bacterial cultures were the same concentrations for each experiment, the cultures were diluted until ~75% transmittance was reached. Transmittance was measured using a Spectronic<sup>®</sup> 20D+ spectrophotometer at a wavelength of 590 nm. This transmittance was previously determined to yield  $\sim 1\text{--}2 \times 10^7$  colonies  $\text{ml}^{-1}$  in preliminary experiments. To verify that this transmittance represented  $1\text{--}2 \times 10^7$  colonies  $\text{ml}^{-1}$  for every experiment, the diluted bacterial influent cultures were also plated on TSA prior to each experiment and were enumerated the following day.

Experiments were run prior to obtaining plate counts, however, as it was assumed that the transmittance represented the cell concentrations. Plate counts for each experiment did confirm that cell concentrations were within the range of  $\sim 1-2 \times 10^7$  colonies  $\text{ml}^{-1}$ . The final diluted cultures were used as the influent bacterial pulses for the column experiments.

### **Column Experiments**

For each experimental run, a conservative tracer was first run through two adjacent columns, columns were then flushed to remove the tracer, the pulse of  $1-2 \times 10^7$  colonies  $\text{ml}^{-1}$  *E. coli* cell tracer was then injected into the columns and the bacterial concentrations in the effluent were measured over time. The experimental design included three treatments with two levels for each treatment, including bacterial state (“live” or “dead” cells), grain coatings (uncoated or Fe-coated sand), and ionic strength of the KCl buffer solution (low at 0.001 M or high at 0.01 M) (Figure 4). Each experiment was duplicated on different experimental days. On each experiment day, “live” cells were injected into one column and “dead” into the second column, while the other experimental treatments remained the same for both columns. “Live” and “dead” experiments were run on each individual experiment day instead of running duplicate experiments simultaneously because although cultures were prepared using the same procedure for each experiment, variations in growth conditions may affect results from day to day. In order to best compare “live” cell experiments to experiments using “dead” cells, it was therefore important to make comparisons using the same prepared culture. A

total of eight experiments were completed and duplicated. Experiments were run at ambient room temperature (approximately 21-23°C).

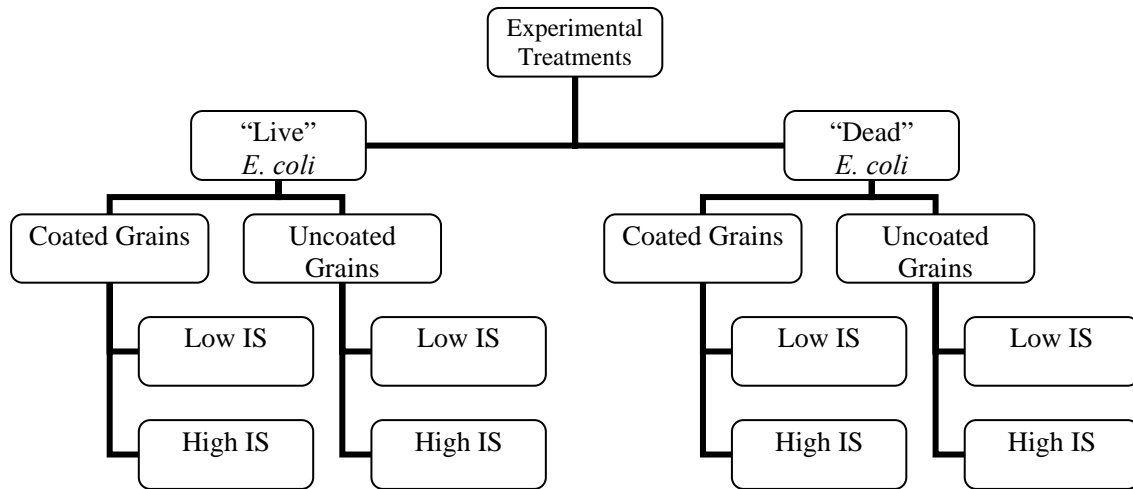


Figure 4. Flow diagram illustrating the three experimental treatments with two levels for each treatment. On any given experiment day, both levels of the first treatment, bacterial state, would be used, including “live” cells which were injected into one column and “dead” cells that were fixed with 0.5% formaldehyde to render them “dead” and were injected into the adjacent column. Both columns were packed with one of the two levels, either uncoated or Fe-coated, of the second treatment, grain coatings, and were filled with one of the two levels, either low ionic strength (IS) or high ionic strength (IS), of the third treatment, the ionic strength of the KCl buffer solution.

### **Conservative Tracer**

It was necessary to run a conservative tracer, tritiated water ( $^3\text{H}_2\text{O}$ ), through the columns prior to each experiment in order to calculate the interstitial pore water velocity and the dispersion coefficient in the columns for incorporation into equations used to estimate attachment and detachment parameters, a method often used in bacterial modeling (Becker et al. 2004; Deflaun et al. 2001). Velocity and dispersion changed each time columns were repacked, therefore it was necessary to run the tracer through

prior to each experiment. First, a stock solution of conservative tracer was prepared by mixing 0.1 mCi of 5.0 mCi ml<sup>-1</sup> tritiated water into 1L KCl buffer solution at low ionic strength. One hundred ml of the stock solution was filter sterilized through 0.2-µm pore size, 47-mm diameter, membrane filters using a vacuum pump and divided into two 250 ml flasks for each experiment. Additional KCl was added to the flasks for high ionic strength experiments.

In order to determine the initial concentrations ( $C_o$ ), 1 ml from each flask was pipetted in quadruplicate into miniature scintillation vials. The vials were filled with Ready Safe™ Liquid Scintillation Cocktail (Beckman Coulter™), loaded into vial racks, and the beta activity was counted in disintegrations per minute (dpm) mode for 10 min in a calibrated LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter™).

Prior to the introduction of the conservative tracer, columns were drained of the KCl buffer solution to the top of sand grains, and 0.5 PV (32-35 ml) of the conservative tracer was pipetted carefully into the top of the columns, followed by approximately 4 PV of radiolabel-free KCl buffer. Average linear velocity in the columns ranged from 4.0 to 4.5 m d<sup>-1</sup> as calculated from porosity, which was measured during column packing (PV divided by volume of saturated sand) and ranged from 0.36 to 0.4, and flow rate which was set at 2 ml min<sup>-1</sup>. One PV was equal to ~64-70 ml, therefore based on the flow rate, the time for one PV to pass through the columns was ~30-35 min. Conservative tracer experiments ran for approximately 2.5 h.

Effluent from each column was collected every 0.1 PV in sterilized 13 x 100 mm Pyrex® test tubes with a Spectra/Chrom® CF-1 Fraction Collector (Spectrum® Laboratories, Inc.). To determine final concentrations ( $C$ ), 1 ml of effluent was extracted

from each tube, pipetted into scintillation vials, filled with scintillation cocktail and counted in the scintillation counter in the same method used to enumerate initial concentrations. The concentration of tritiated water residual in the influent pool of KCl buffer solution was sampled periodically during the experiment to account for mixing at the inlet. Following the completion of the conservative tracer run, at least 15 PV of KCl buffer solution were flushed through to ensure that the column effluent was devoid of radiolabel.

### **Bacterial Tracer**

A 0.5 PV (~32-35 ml) pulse of  $^3\text{[H]}$ -labeled *E. coli* cells was added to each column, one with “live” cells and the other with “dead”, followed by ~17-23 PV of radiolabel-free KCl buffer. Similar to the procedure for the conservative tracer experiments, average linear velocity in the columns again ranged from 4.0 to 4.5 m d<sup>-1</sup>, porosity ranged from 0.36 to 0.4, and flow rate was again set at 2 ml min<sup>-1</sup>. One PV was equal to ~64-70 ml, therefore based on the flow rate, the time for one PV to pass through the columns was ~30-35 min. Experiments were run for a period of 17-23 PV (8.5-11.5 h) in order to track bacterial tailing over time and were stopped when a decrease in effluent concentrations was no longer observed. Experiments were not run longer than this time period to avoid the affect bacterial death and label loss may have had over longer periods of time.

Initial bacterial concentrations ( $C_0$ ) of the cultures used as the influent pulses were measured in quadruplicate via the scintillation counter using the method described above. Effluent ( $C$ ) was collected every 0.1 PV for the first 4 PV to capture initial breakthrough

and every 0.33 or 0.5 PV for the remainder of each experiment. Effluent samples were counted in the scintillation counter following the procedures described above. The frequency of enumeration eliminated the need to sample from effluent tubes in duplicate. The  $^3\text{[H]}$  concentration in the radiolabel-free KCl buffer influent was monitored periodically following the same procedure used to monitor the conservative tracer influent. Experiments were performed in duplicate for each treatment combination. Columns were disassembled following every experiment and thoroughly cleaned.

The half-life of tritium is 12.3 years, therefore radioactive decay was nonexistent. Incorporation of the label into cells could have changed over time, however. To determine if cell label decreased over the course of the experiments, initial concentrations were enumerated by scintillation counting at the beginning and end of each experiment. To ensure that unincorporated label was not also measured, cultures were filtered in quadruplicate through 0.2- $\mu\text{m}$  pore size, 25-mm diameter, membrane filters. The filters were placed in scintillation vials and counted. Effluent from one experiment of uncoated sand with low ionic strength KCl solution was also enumerated 4 d following the experiment to determine if label loss occurred over an extended period of time.

In order to assure that there was no growth or death throughout the experiment, cell concentrations were enumerated over time by plating (Fontes et al. 2001). “Live” bacteria were plated on TSA at the beginning and end of each experiment. In addition, the effluent peaks and concentrations following the peaks for “live” bacteria were plated on TSA for enumeration for several of the experimental runs. The plates were incubated at 37°C and enumerated within 18 to 24 h. “Dead” cultures were also plated to ensure that no growth occurred.

To enumerate both “live” and “dead” bacteria since “dead” cells did not grow on growth media, initial bacterial pulses and portions of the peak and tail of the bacterial effluent from a coated sands experimental run were stained with LIVE/DEAD<sup>®</sup> *Baclight*<sup>™</sup> stock solution (Molecular Probes), incubated for 15 min, filtered through 0.2- $\mu$ m Nucleopore Track-Etch Membrane filters (Whatman), placed on slides, covered with cover slips, and viewed at 1000x using a Zeiss Axioskop 2 *plus* fluorescent microscope.

To verify that results from scintillation counting represented actual bacterial concentrations, and to determine if cells were intact or lysed (Zhang et al. 2001), the samples used for fluorescent microscopy were viewed and photographed at 1000x magnification using an Olympus BH2-RFCA phase microscope. To determine if formaldehyde fixation had an effect on cells and to differentiate the affect of fixation from the affect of cell death, “live” cells were also boiled for 10 min to render them dead. Boiled cells were then cooled and viewed for comparison with cells rendered “dead” by formaldehyde fixation. Approximately 3  $\mu$ l of each sample were placed on slides, covered with cover slips and sealed with Vaspar.

### **Data Analysis**

Conservative tracer and bacterial breakthrough curves (BTCs) were calculated as  $C/C_0$  versus PV (PVs are dimensionless) and plotted on a semilogarithmic scale, as tailing has been overlooked in BTCs plotted on arithmetic axes (Johnson et al. 1995). Peak  $C/C_0$  were averaged between experiment duplicates and compared between experiments. The fraction recovered, defined as the portion of introduced cells recovered in the column effluent (Fontes et al. 1991), was calculated from the curves by dividing

total mass of bacteria in the effluent by influent mass. A three factor analysis of variance (ANOVA) was performed (SAS Institute, 1999) on experiments including duplicates to determine the effects of the three treatments, bacterial state (“live” versus “dead”), grain coatings (uncoated versus coated sand), and ionic strength (low versus high), on the fraction recovered between the two levels within each treatment. Results below the 0.05 confidence level were considered significant and the mean for each experiment and duplicate was presented. The fraction recovered within just the tail effluent was also calculated. Tailing was considered to begin at 2.5 PV after the beginning of each experiment. A second three factor ANOVA was performed on the experiments including duplicates to examine the effects of the treatments on fraction recovered in bacterial tails.

### **Mathematical Modeling**

Few studies have been successful in accurately simulating all portions of bacterial BTCs, therefore this study compared two models and examined the goodness of fit of each to the observed data. In addition, models can be used to estimate parameters for comparison between experiments. To calculate bacterial attachment and detachment rates, interstitial pore water velocity and dispersion were first estimated by calibrating a one-dimensional advection-dispersion equation (ADE) to observed conservative tracer BTCs using the Crank-Nicolson finite-difference method in Matlab (Bolster 2000). These estimated values were then incorporated into an ADE modified to account for both reversible and irreversible attachment (Eqs. 1 and 2). The ADE was fit to the observed bacterial BTCs using the Crank-Nicolson finite-difference method in Matlab (Bolster 2000). Parameter values were obtained by fitting non-dimensional versions of the model

to the concentrations of cells in the aqueous phase over time using the Levenburg-Marquardt method in Matlab (Bolster 2000). A weighted-sum-of-squares objective function, defined as the sum of the weighted squared residuals between modeled aqueous bacterial concentrations and actual measurements of effluent concentrations, was used in order to account for both the peak and tail of each BTC. The models were coded by C.H. Bolster.

The modified ADE has the following form (Harvey and Garabedian 1991; Hornberger et al. 1992):

$$\frac{\partial c}{\partial t} + \frac{\partial s}{\partial t} = D \frac{\partial^2 c}{\partial x^2} - v \frac{\partial c}{\partial x} - k_3 c \quad (1)$$

$$\frac{\partial s}{\partial t} = k_1 c - k_2 s \quad (2)$$

where  $c$  is the aqueous concentration of bacteria (cells ml<sup>-1</sup>),  $s$  is the concentration of bacteria on the solid phase (cells ml<sup>-1</sup>),  $x$  is the distance from the surface of the column (L),  $t$  is time from initial input of bacteria (T),  $D$  is the dispersion coefficient (L<sup>2</sup> T<sup>-1</sup>),  $v$  is the interstitial pore water velocity (L T<sup>-1</sup>),  $k_1$  is the reversible attachment rate (T<sup>-1</sup>),  $k_2$  is the detachment rate (T<sup>-1</sup>), and  $k_3$  is the irreversible attachment rate (T<sup>-1</sup>). Eq. 2 accounts for first-order attachment and detachment processes (Harvey and Garabedian 1991). Fitted rates were obtained for each column. These estimated parameters are useful for analyzing trends in experiments.

Three parameters, reversible and irreversible attachment and detachment were estimated using the above equations, therefore this model is referred to hereafter as the three-parameter model. A second model that estimated reversible attachment and detachment and did not account for irreversible attachment was also fit to the observed

data using a modified Eq. 1 (the irreversible attachment term,  $k_3$ , was set to zero). The second model is referred to hereafter as the two-parameter model.

Three factor ANOVAs were performed to determine the effects of the three treatments, including bacteria state, grain coatings, and ionic strength, on the parameters of duplicate experiments that were estimated by the two models. The Ryan-Einot-Gabriel-Welsch Multiple Range Test was used for pairwise comparisons. Single factor ANOVAs were performed on parameters derived by the three-parameter model to examine the difference between “live” and “dead” cells for each treatment and to also examine changes in parameters of “live” and “dead” cells separately for each treatment. Results below the 0.05 confidence level were considered significant, and the means of duplicate experiments were reported.

Sensitivity analysis of both models was performed in a method similar to that employed by Zhang et al. (2001). For each simulation, the parameters were held constant while one was altered to demonstrate the effect of individual parameters on BTC shapes.

For both conservative and bacterial tracer experiments, model efficiency ( $E$ ), which is similar to  $R^2$  in linear regression, was calculated in the models to determine goodness of fit of fitted results to observed values (Bolster 2000; Hornberger et al. 1992)

$$E = 1 - \frac{\sum_{i=1}^n (r)^2}{\sum_{i=1}^n (s_i - s_{avg})^2} \quad (3)$$

where  $r$  is the residual between model prediction and observation,  $s_i$  is the  $i$ th observed concentration on the solid phase, and  $s_{avg}$  is the average of the observed concentrations, with efficiencies of 1 indicating a perfect fit and 0 a poor fit of the models to the data.

Model efficiencies were averaged for duplicate experiments and  $E$  values for the two-parameter model fits were compared to experimental duplicate means of  $E$  values for the three-parameter model.

### **Protein Analysis**

To determine if the protein signature of “live” cells was different than that of “dead” cells, the protein profiles of “live” and “dead” cells were compared by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins by molecular weight as described by Laemmli et al. (1970) and Shapiro et al. (1967). This method was chosen because separating proteins by molecular weight using SDS-PAGE is an effective way to determine if there are differences in protein profiles. Proteins of the same molecular weight cannot be differentiated by this method. This level of precision, however, was not necessary to determine if there were general differences between samples. The procedure involves denaturing all proteins to the same linear shape using SDS, a detergent that dissolves cell membranes and covers cell proteins with a negative charge. Samples are injected into wells in an electrophoresis apparatus which creates an electrical current and pulls the negatively charged proteins through a polyacrylamide gel towards a positive charge. Proteins with lower molecular weights migrate through gels faster. The electrophoresis is stopped before proteins reach the bottom of the gel so that when stained later with Coomassie Blue, dark bands will be revealed indicating the distance each protein has migrated. To calculate molecular weights of proteins within each sample, the resulting bands are compared to the bands of known proteins of a simultaneously run molecular weight standard.

To prepare samples for analysis, a set of coated sand column experiments was conducted using unlabeled bacteria. Columns were packed with Fe-coated sands in 0.001 M KCl buffer solution. A bacterial pulse, prepared as described above, of “live” cells was run through one column and “dead” cells through the other. One sample was collected from each of the “live” and “dead” influent solutions for the protein analysis. In addition, approximately 6 effluent tubes from each column were combined and analyzed. To determine if formaldehyde fixation affected protein profiles of “dead” cells, the cell culture rendered “dead” by boiling previously for microscopy observations was also analyzed for comparison.

To remove cellular debris, all samples were centrifuged at 12,000 x g for 20 min, and 0.5 to 1 ml of the supernatant were extracted and combined with 4x SDS Treatment Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, CA). To denature proteins, samples were heated at 90°C for 5 min, and 30 µl from each was pipetted into 0.1 cm x 10 cm x 8 cm sample wells of a 10% Tris-Glycine Pre-cast iGel (Gradipore Limited, Australia) for protein separation by SDS-PAGE using a Mighty Small II SE 250/SE260 (Hoeffer, San Francisco, CA) filled with Tris-Glycine running buffer (Bio-Rad Laboratories, Inc.) at pH 8.3. This procedure separated proteins of whole cell lysate by molecular weight, not by isoelectric point because of the negative charge of the buffer. For molecular weight estimations, the first well was filled with 20 µl of broad molecular weight range unstained SDS-PAGE standard (Bio-Rad Laboratories, Inc.).

The samples were run through the iGel for approximately 90 min at 190-volts and cooled with flow through tap water. The iGel was removed and stained in Coomassie Blue R-250 (Bio-Rad Laboratories, Inc.) for 45 min. The iGel was then rinsed in DIW,

shaken in SDS-PAGE destain (Bio-Rad Laboratories, Inc.) overnight and stored in 10% aqueous glycerol. In order to estimate the molecular weights of the proteins in the samples, the migration of each band was compared to the migration of the protein standards of known molecular weights in the molecular ladder.

### III. RESULTS

#### Column Experiments

The tails and peaks of the breakthrough curves (BTCs) were examined to determine experimental treatment effects on attachment and detachment within columns. BTC tailing, which indicates a lack of irreversible attachment, was observed in all experimental runs (Figures 5a-5d). Higher tailing indicates lower irreversible attachment, greater reversible attachment, and higher concentrations of cells recovered in the effluent.  $C/C_0$  was higher for “live” *E. coli* cells versus “dead” cells in all BTC tails except for one duplicate of uncoated sand at low ionic strength. Tail height varied between some experimental duplicates, especially those using low ionic strength solution or “dead” cells, but duplicates were very similar for most experiments using “live” bacteria. This lack of replication was obvious between duplicates, but there was not much variability observed within individual breakthrough curves from sample point to sample point. Detachment is indicated by the slope of the BTC tails, with steeper slopes indicating greater detachment. Slopes seemed to be similar for all experiments, indicating that detachment did not vary with experimental treatments. Slopes were similar between most experimental duplicates.

In order to verify the accuracy of the radiolabel method as a means for enumeration of BTC tails and to verify that “dead” bacterial concentrations were lower than “live” cells as indicated from scintillation counting, bacterial tail concentrations

were observed under fluorescent and phase microscopy. Concentrations of “live” and “dead” cells within BTC tails were too low to observe under fluorescent or phase microscopy, however. To determine “live” bacterial concentrations during tailing over time, effluent samples were plated. “Live” cell concentrations were nearly 600 colonies  $\text{ml}^{-1}$  after 2 PV, 200 after 3 PV and 10 colonies  $\text{ml}^{-1}$  after 10 PV in coated sand experiments at both high and low ionic strengths. Concentrations were not detected by plating methods after 15 PV. BTC tails were not enumerated by plating in uncoated sand experiments. Some tailing of conservative tracers was observed following breakthrough because low concentrations of the conservative tracer were observed in influent for the first few PVs (Figures 6 and BF-1a to BF-1g). This tailing was minimal compared to bacterial tailing, however (Figures 6 and BF-1a to BF-1g).

BTC peaks were analyzed as they are indicators of cell attachment, with lower peaks indicating greater attachment and lower cell concentrations detected in the column effluent. Detachment is not reflected by peak height. BTC peaks of coated sand experiments were an order of magnitude lower than uncoated sand BTC peaks, with coated sand experiments yielding peak  $C/C_0$  duplicate means from 0.011 to 0.017 and uncoated sand experiments yielding peak  $C/C_0$  means from 0.64 to 0.85 (Table 1 and Figures 5a-5d). Peaks were similar between experimental duplicates. Multiple BTC peaks were observed in coated sands, with a more pronounced second peak at low ionic strength (Figures 5c and 5d). Multiple peaks were observed for duplicate experiments, but the height of the lower peak varied between duplicates. Conservative tracers do not react or interact with media surfaces, therefore, complete breakthrough of conservative tracers was always observed.  $C/C_0$  peaks of  $^3\text{H}$  were always between 0.95 and 1.0.

Additionally, conservative tracer peaks occurred at the same time and were the same width as bacterial BTCs, which indicated that early or late bacterial breakthrough was not a factor in these experiments (Figures 6 and BF-1a to BF-1g).

Results from a three factor ANOVA indicated that the fraction of *E. coli* cells recovered in column effluent was significantly lower in coated sands than in uncoated sands by an order of magnitude ( $p < 0.0001$ ) (Table 2). This supports the observation that BTC peaks of coated sand experiments were noticeably lower. The fraction of cells recovered in effluent from coated sand experiments ranged from 0.01 to 0.06 and in uncoated sand experiments from 0.64 to 1.0. There was no significant difference between the fraction of “live” or “dead” cells recovered ( $p > 0.05$ ) or between fractions recovered from experiments using solutions of high or low ionic strength ( $p > 0.05$ ). Although this contradicts observations that tailing of “live” cells was higher than “dead” cells in BTCs, this can be explained by the fact that the fraction recovered in the column effluent accounted for the bacterial concentrations in both the peaks and tails of BTCs. The fraction recovered within BTC tails was therefore analyzed separately using a second three factor ANOVA. Fraction recovered within BTC tails of “live” cells was significantly higher than “dead” cells ( $p < 0.05$ ), which supports the observation that tailing was generally higher for “live” cells in BTCs. There were no significant differences in the fraction of tailing *E. coli* cells recovered from coated and uncoated sand experiments or experiments using low or high ionic strength ( $p > 0.05$ ) (Table 3). There were no significant interactions found between the three treatments for either ANOVA ( $p > 0.05$ ).

The ability of the bacteria cells to retain the radiolabel throughout the duration of experiments was tested by quantifying the amount of cell-associated label at the beginning and end of each experiment. Over the course of 12 hours, the “live” and “dead” cultures used as influent pulses both lost radiolabel over the course of some of the experimental runs (Table 4). “Live” cells lost between 0 to 16% and “dead” cells lost from 0 to 24% of the radiolabel. Note that the cultures used as the influent bacterial pulses were measured to determine label loss, and that these high cell concentrations were only injected into columns during the first 0.5 PV of experiments. When effluent samples from an experiment using uncoated sand in low ionic strength solution were measured immediately and again four days later, label loss did not always occur (Table 5). Therefore, it was not clear if label loss was a factor in BTC results.

<b>Grain coating</b>	<b>Ionic Strength</b>	<b><u>"Live" <i>E. coli</i></u> C/C<sub>0</sub></b>	<b><u>"Dead" <i>E. coli</i></u> C/C<sub>0</sub></b>
Uncoated	Low	0.85 (0.04)	0.80 (0.17)
	High	0.64 (0.05)	0.66 (0.06)
Coated	Low	0.015 (0.005)	0.011 (0.001)
	High	0.015 (0.005)	0.017 (0.004)

Table 1. Mean peak C/C<sub>0</sub> (standard errors) of experiment duplicates. Peak C/C<sub>0</sub> was an order of magnitude higher in experiments using uncoated sands then coated sand experiments. “Dead” cells were fixed with 0.5% final concentration formaldehyde prior to injection.

<b>Treatment</b>	<b>Fraction recovered</b>	<b>p-value</b>
"Live" cells	0.42	0.55
"Dead" cells	0.39	
Uncoated grains	0.78	<b>&lt;0.0001</b>
Coated grains	0.03	
Low IS	0.45	0.15
High IS	0.37	

Table 2. Average fraction of *E. coli* cells recovered in column effluent analyzed by a three factor ANOVA for all treatments. P-values in bold indicate significant differences ( $p < 0.05$ ). The fraction of cells recovered was significantly lower for experiments using coated sands than for uncoated sand experiments.

<b>Treatment</b>	<b>Fraction recovered</b>	<b>p-value</b>
"Live" cells	0.022	<b>0.02</b>
"Dead" cells	0.008	
Uncoated grains	0.016	0.69
Coated grains	0.014	
Low IS	0.015	0.90
High IS	0.015	

Table 3. Average fraction of *E. coli* cells recovered in tail effluent analyzed by a three factor ANOVA for all treatments. P-values in bold indicate significant differences ( $p < 0.05$ ). The fraction of cells recovered was significantly higher for experiments using “live” *E. coli* cells than for experiments using “dead” cells.

<b>Grain Coating</b>	<b>Ionic Strength</b>	<b>“Live” <i>E. coli</i></b>	<b>“Dead” <i>E. coli</i></b>
Uncoated	Low	0.88 (0.23)	1.01 (0.090)
	High	0.84 (0.025)	0.76 (0.005)
Coated	Low	1.0 (0.090)	0.90 (0.29)
	High	1.0 (0.025)	0.78 (0.21)

Table 4. Means for duplicate experiments for fraction (standard errors) of radiolabel retained by influent pulses of “live” and “dead” *E. coli* cells over time. Some label loss over time was observed.

<b>"Live"</b> <b><i>E. coli</i></b>	<b>"Dead"</b> <b><i>E. coli</i></b>	<b>Pore</b> <b>Volumes</b>
0.78	1.00	1
1.00	1.38	6

Table 5. The fraction of radiolabel retained by effluent concentrations of “live” and “dead” *E. coli* cells transported through uncoated sand at low ionic strength. Effluent was sampled immediately and again after 4 d. Samples were removed and enumerated 1 PV and 6 PV after experiment commencement.

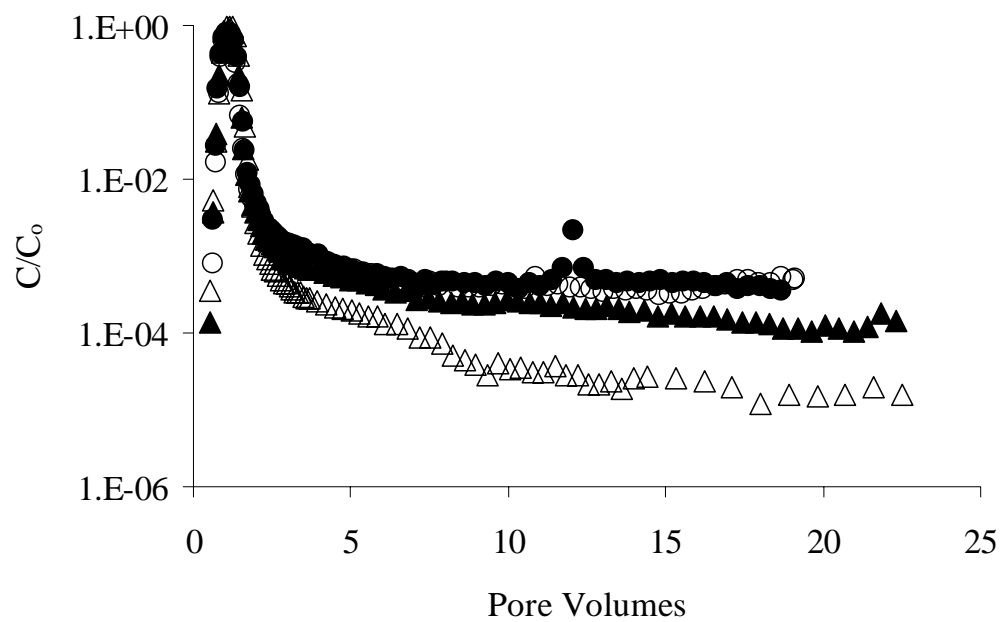


Figure 5a. Observed duplicates (circles versus triangles) of breakthrough of “live” (closed symbols) and “dead” (open symbols) *E. coli* HCB 136 cells through laboratory columns packed with uncoated quartz sand in low ionic strength buffer solution. “Dead” cells were fixed with 0.5% final concentration formaldehyde prior to injection. Concentrations of “dead” cells were often lower than that of “live” cells within the tail.

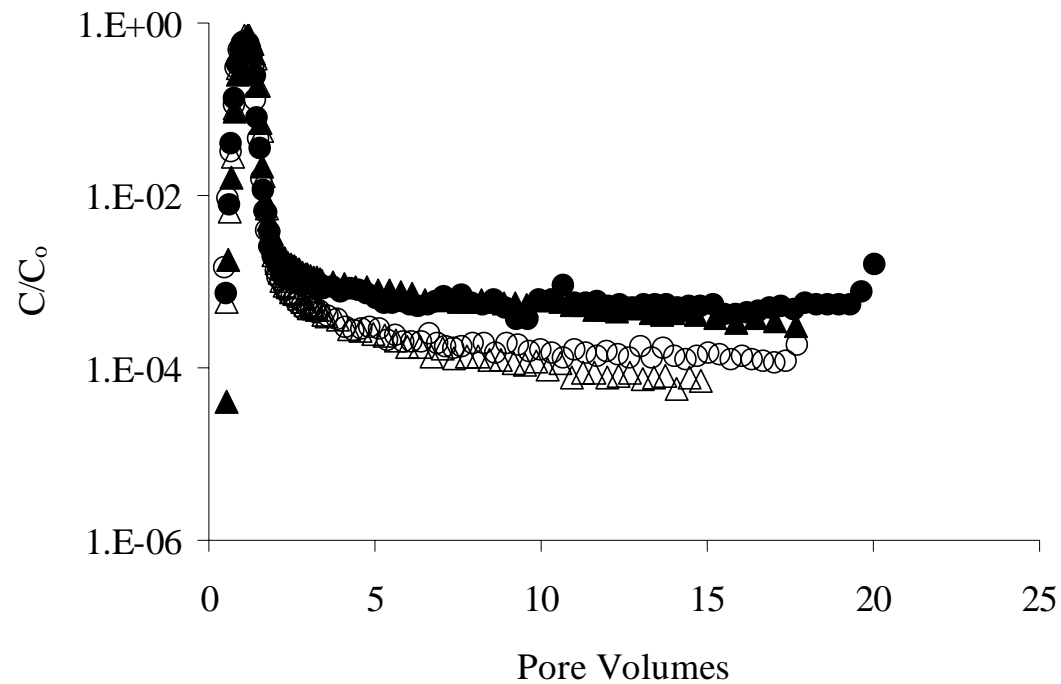


Figure 5b. Observed duplicates (circles versus triangles) of breakthrough of “live” (closed symbols) and “dead” (open symbols) *E. coli* HCB 136 cells through laboratory columns packed with uncoated quartz sand in high ionic strength buffer solution. Concentrations of “dead” cells were often lower than that of “live” cells within the tail.

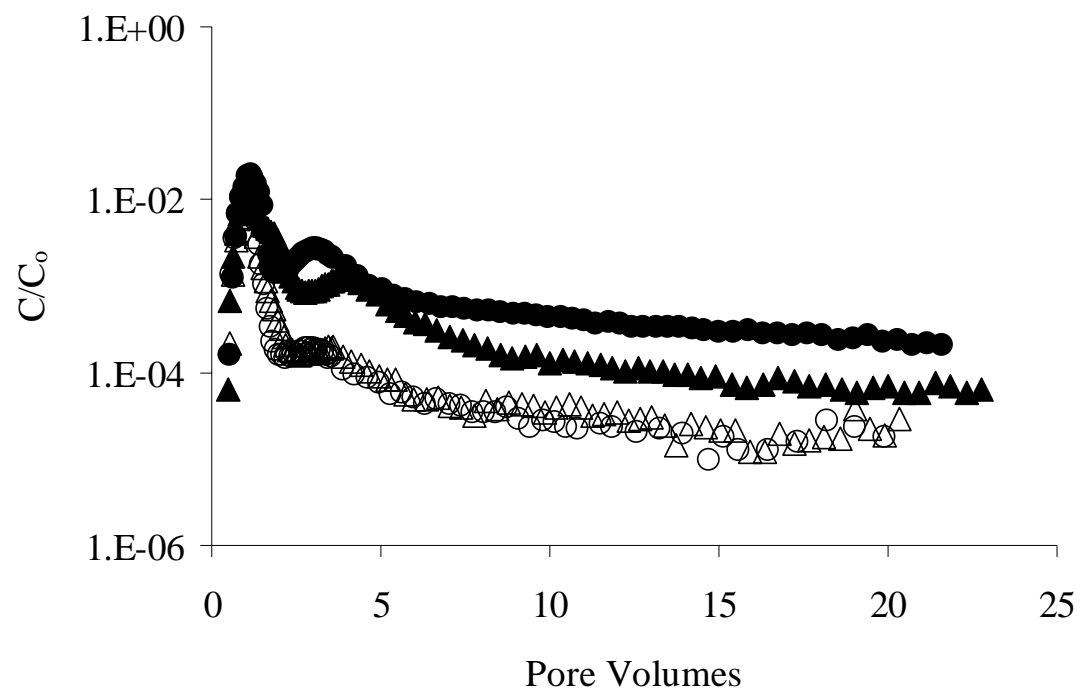


Figure 5c. Observed duplicates (circles versus triangles) of breakthrough of “live” (closed symbols) and “dead” (open symbols) *E. coli* HCB 136 cells through laboratory columns packed with coated quartz sand in low ionic strength buffer solution. Concentrations of “dead” cells were often lower than that of “live” cells within the tail. Multiple peaks were observed in coated sands.

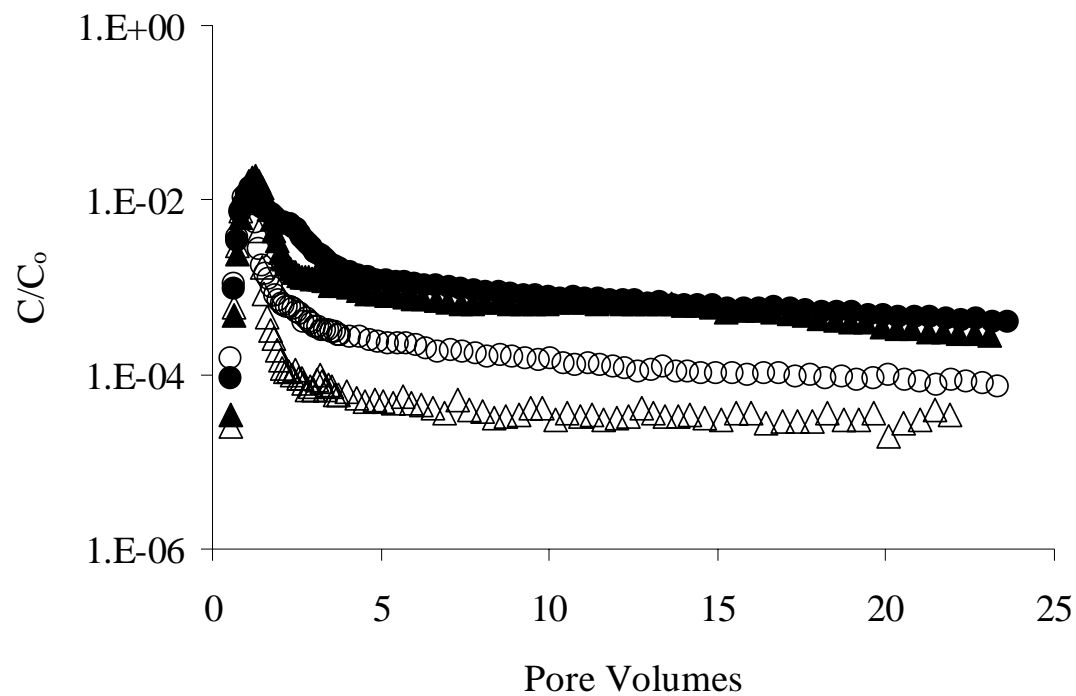


Figure 5d. Observed duplicates (circles versus triangles) of breakthrough of “live” (closed symbols) and “dead” (open symbols) *E. coli* HCB 136 cells through laboratory columns packed with coated quartz sand in high ionic strength buffer solution. Concentrations of “dead” cells were often lower than that of “live” cells within the tail. Multiple peaks were observed in coated sands.

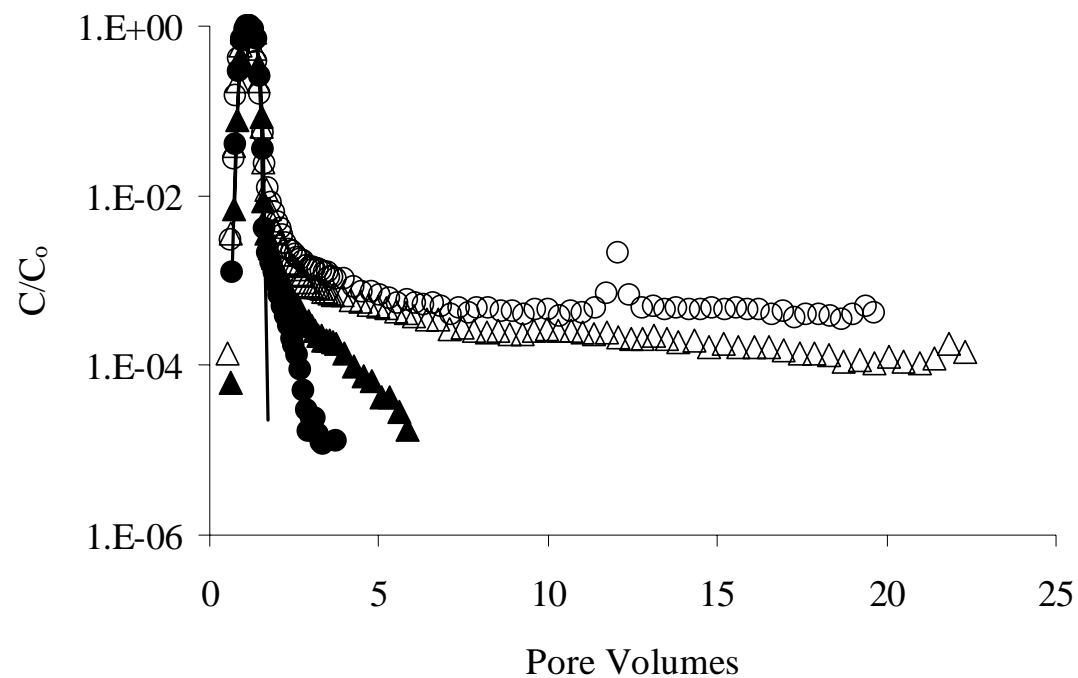
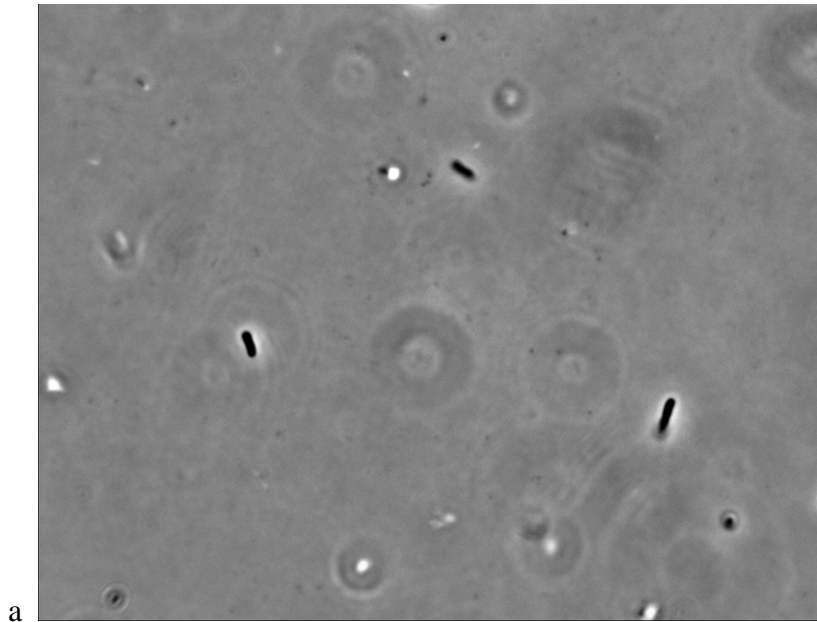


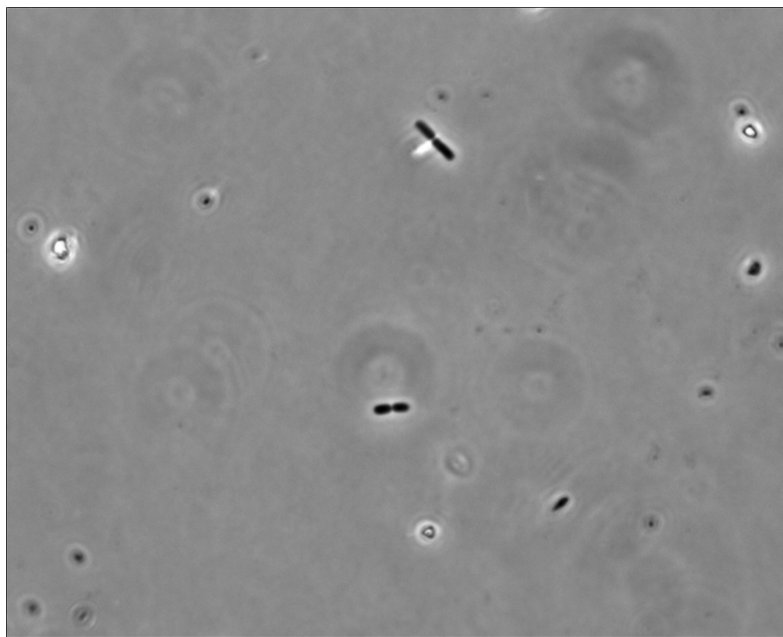
Figure 6. Observed breakthrough curves for duplicates (circles versus triangles) of conservative tracer experiments (closed symbols) and duplicate bacteria experiments (open symbols) using “live” *E. coli* cells in uncoated quartz sand at low ionic strength. The solid line represents the best fit of the model to the conservative tracer duplicates. Tailing of the conservative tracer was noticeably minimal compared to the extended tailing of bacteria over time.

In order to determine if either growth or death occurred throughout the duration of experiments, cell concentrations of the cultures used as influent pulses were enumerated at the beginning and end of each experiment. Bacterial concentrations did not increase or decrease during column experiments, therefore it was assumed that growth or death did not affect results. Concentrations determined by plating “live” *E. coli* cells on TSA were approximately  $1-2 \times 10^7$  colonies  $\text{ml}^{-1}$  at the beginning and end of each experiment. “Dead” cell concentrations were similar to “live” concentrations. Although “dead” cells did not grow on TSA and therefore could not be enumerated via plating, enumeration using fluorescent microscopy did reveal similar initial “live” and “dead” cell counts.

To determine if formaldehyde fixation affected cells, bacterial cultures used as influent pulses were viewed by phase microscopy. Neither “live” nor “dead” cells appeared to have lysed (Figures 7a and 7b). Fixing bacteria with formaldehyde may have had some effect on cell sizes, although it was not possible to verify this by observing cells with phase microscopy (Figures 7a and 7b).



a



b

Figure 7. “Live” *E. coli* cells viewed by phase microscopy at 1000x (a) and “dead” *E. coli* cells at 1000x (b). “Dead” cells may have been larger than “live” cells.

### **Model Fits**

Velocity and dispersion were estimated by fitting the advection-dispersion equation to conservative tracer breakthrough curves in order to later incorporate the two parameters into the equations used to model bacterial breakthrough. Estimated parameter values derived by fitting the model to the conservative tracers and averaged for experiment duplicates spanned a mean velocity of 0.31 to 0.32 cm min<sup>-1</sup> and covered dispersion values of 0.011 to 0.015 cm<sup>2</sup> min<sup>-1</sup> with standard errors 0 to 0.01 for velocity and 0.001 to 0.004 for dispersion (Table 6). Model efficiencies (*E*) were calculated in order to determine goodness of fit of the model to the observed conservative tracer BTCs. Model efficiency ranged from 0.99 to 1.0 on a scale of 0 to 1 for conservative tracer experiments. Zero represents a poor fit and 1 represents a perfect fit.

The two- and three-parameter models were used to estimate attachment and detachment rates. Model efficiencies were calculated in order to determine goodness of fit of the fitted data to the observed bacteria BTCs for both models. Model efficiency (*E*) was averaged between experiment duplicates and the means ranged from 0.89 to 0.95 in the three-parameter model and from 0.75 to 0.93 in the two-parameter model (Table 7). The three-parameter model provided slightly superior fits to the observed data versus the two-parameter model for experiments using either “live” or “dead” *E. coli* cells (Table 7; Figures 8a-8h). Examination of Figures 8e-8h reveals that neither model accounted for the multiple peaks observed in experiments using coated sands. In addition, Figures 8a-8h reveal that both models did not accurately simulate the slope of the BTCs immediately following curve peaks, the portion of the curve that represents elution of the initial concentration of bacteria after breakthrough (Figure 3, Section D).

Attachment and detachment rates were first derived by the two-parameter model and the results were averaged between experiment duplicates. The resulting reversible attachment rate ( $k_1$ ) means ranged from 0.02 to 0.48 min<sup>-1</sup> in uncoated sands and from 3.9 to 4.9 min<sup>-1</sup> in coated sands (Table 8). Standard errors were 2 to 100% of mean  $k_1$  values. Detachment rate ( $k_2$ ) duplicate means ranged from 0.05 to 0.14 min<sup>-1</sup> in uncoated sands and from 0.001 to 0.01 min<sup>-1</sup> in coated sands for  $k_2$  with standard errors 20 to 100% of  $k_2$  means.

A three factor ANOVA was performed on  $k_1$  and  $k_2$  values that were derived using the two-parameter model. Means of  $k_1$  were significantly lower for “live” than “dead” cells ( $p < 0.05$ ) (Table 9). The two-parameter model estimations for  $k_1$  were higher in coated versus uncoated sand experiments ( $p < 0.0001$ ), yet there was no significant difference in  $k_1$  means between experiments using high or low ionic strength solutions ( $p > 0.05$ ). There were no significant differences among all treatments for  $k_2$  means ( $p > 0.05$ ). Additionally, there were no significant interactions found between the three treatments ( $p > 0.05$ ).

Attachment and detachment rates were then derived by the three-parameter model and the results were again averaged between experiment duplicates. Averages of  $k_1$  ranged from 0.01 to 0.06 min<sup>-1</sup> in experiments using uncoated sands and from 0.25 to 1.2 min<sup>-1</sup> in coated sands (Table 10). Standard errors were 6 to 100% of mean  $k_1$  values. Mean  $k_2$  ranged from 0.07 to 0.18 min<sup>-1</sup> in uncoated sands and from 0.10 to 0.21 min<sup>-1</sup> in coated sands, with standard errors 5 to 56% of mean  $k_2$  values. Lastly, irreversible attachment rate ( $k_3$ ) means ranged from 0.23 to 0.5 min<sup>-1</sup> in uncoated sands and from 2.9 to 4.8 min<sup>-1</sup> in coated sand experiments, with standard errors 1 to 96% of mean  $k_3$  values.

Results of the three-parameter model analyzed by a three factor ANOVA indicated that “live” cell  $k_1$  means were significantly higher than  $k_1$  for “dead” cells ( $p < 0.05$ ). This contradicts the result derived using the two-parameter model which indicated reversible attachment rates were significantly higher for “dead” cells than for “live” cells. Alternatively,  $k_3$  means for “dead” cells were significantly higher than for “live” cells ( $p < 0.05$ ) (Table 11). No significant differences were found in  $k_2$  means between “live” and “dead” cells ( $p > 0.05$ ). Although  $k_1$  and  $k_3$  means were significantly higher in coated versus uncoated sand experiments ( $p < 0.0001$ ), there was no difference between coated and uncoated sands for  $k_2$  means ( $p > 0.05$ ). Average  $k_1$  was significantly lower at low versus high ionic strength ( $p < 0.05$ ). This contrasts results from the two-parameter model in which there were no differences in  $k_1$  because of changes in ionic strength. Overall, there were no significant differences in  $k_2$  or  $k_3$  means between experiments using high or low ionic strength ( $p > 0.05$ ). Significant interactions were found between bacteria and sand coatings ( $p = 0.007$ ) and between bacteria and ionic strength ( $p = 0.04$ ) for  $k_1$ . Significant interactions were also found between bacteria and sand coatings ( $p = 0.006$ ) for  $k_3$ .

Single factor ANOVAs were performed to compare each treatment effect separately on “live” cell attachment and “dead” cell detachment rates. Statistical analysis only determined significant differences between “live” and “dead” cells for  $k_1$  and  $k_3$  means within coated sand experiments ( $p < 0.05$ ) (Table 12). As a general trend, however,  $k_3$  means were generally lower for “live” versus “dead” cells for all treatments. The single factor ANOVA comparing differences of “live” and “dead” cells separately on attachment and detachment rates indicated that  $k_1$ ,  $k_2$  and  $k_3$  means for “live” cells, were

always significantly higher in coated versus uncoated sand experiments ( $p < 0.05$ ), while for “dead” cells,  $k_1$  and  $k_3$  means were significantly higher in coated sand experiments ( $p < 0.05$ ) (Table 13). There were no treatment effects of “live” or “dead” cells for any calculated parameter at high or low ionic strengths.

Grain Coating	Ionic Strength	"Live"		"Dead"	
		Velocity (cm min <sup>-1</sup> )	Dispersion (cm <sup>2</sup> min <sup>-1</sup> )	Velocity (cm min <sup>-1</sup> )	Dispersion (cm <sup>2</sup> min <sup>-1</sup> )
Uncoated	Low	0.32 (0.010)	0.011 (0.001)	0.32 (0.005)	0.012 (0.003)
	High	0.32 (0.005)	0.012 (0.004)	0.32 (0.005)	0.011 (0.001)
Coated	Low	0.31 (0.005)	0.015 (0.001)	0.32 (0.005)	0.014 (0.003)
	High	0.32 (0.000)	0.014 (0.001)	0.32 (0.005)	0.013 (0.001)

Table 6. Mean fitted values (standard errors) for velocity and dispersion for the conservative tracer experiment duplicates run for columns to which a pulse of “live” or “dead” *E. coli* cells was later introduced.

Grain Coating	Ionic Strength	"Live" <i>E. coli</i>		"Dead" <i>E. coli</i>	
		2-parameter	3-parameter	2-parameter	3-parameter
Uncoated	Low	0.91 (0.010)	0.91 (0.010)	0.88 (0.005)	0.89 (0.010)
	High	0.93 (0.000)	0.94 (0.000)	0.89 (0.020)	0.92 (0.005)
Coated	Low	0.78 (0.045)	0.91 (0.035)	0.75 (0.005)	0.90 (0.020)
	High	0.85 (0.015)	0.95 (0.015)	0.87 (0.030)	0.95 (0.005)

Table 7. Mean model efficiencies ( $E$ ) (standard errors) for duplicate experiments demonstrating goodness of fit of the two- and three-parameter models to observed data. The three-parameter model provided slightly superior fits than the two-parameter model, particularly for experiments using Fe-coated sands.

Grain Coating	Ionic Strength	<b>"Live" <i>E. coli</i></b>		<b>"Dead" <i>E. coli</i></b>	
		$K_1$ (min <sup>-1</sup> )	$k_2$ (min <sup>-1</sup> )	$k_1$ (min <sup>-1</sup> )	$k_2$ (min <sup>-1</sup> )
Uncoated	Low	0.02	0.08	0.48	0.14
		(0.01)	(0.02)	(0.48)	(0.14)
	High	0.31	0.05	0.33	0.08
		(0.28)	(0.05)	(0.32)	(0.08)
Coated	Low	4.2	0.01	4.8	0.001
		(0.39)	(0.002)	(0.16)	(0.0002)
	High	3.9	0.01	4.9	0.004
		(0.16)	(0.002)	(0.08)	(0.002)

Table 8. Mean fitted values (standard errors) for duplicate experiments for reversible attachment ( $k_1$ ) and detachment ( $k_2$ ) rates derived using the two-parameter model.

Treatment	$k_1$ (min <sup>-1</sup> )	p-value	$k_2$ (min <sup>-1</sup> )	p-value
"Live" cells	2.1	<b>0.02</b>	0.04	0.63
"Dead" cells	2.6		0.06	
Uncoated grains	0.29	<b>&lt;0.0001</b>	0.09	0.09
Coated grains	4.5		0.006	
Low IS	2.4	0.95	0.06	0.64
High IS	2.4		0.04	

Table 9. Mean fitted values for reversible attachment ( $k_1$ ) and detachment ( $k_2$ ) rates derived using the two-parameter model and analyzed using a three factor ANOVA for all experimental treatments. P-values in bold indicate significant differences (p<0.05).

Grain Coating	Ionic strength	<b>"Live" <i>E. coli</i></b>			<b>"Dead" <i>E. coli</i></b>		
		$k_1$ (min <sup>-1</sup> )	$k_2$ (min <sup>-1</sup> )	$k_3$ (min <sup>-1</sup> )	$k_1$ (min <sup>-1</sup> )	$k_2$ (min <sup>-1</sup> )	$k_3$ (min <sup>-1</sup> )
Uncoated	Low	0.03	0.08	0.23	0.03	0.18	0.47
		(0.01)	(0.02)	(0.02)	(0.03)	(0.10)	(0.45)
	High	0.06	0.07	0.50	0.01	0.13	0.45
		(0.01)	(0.03)	(0.05)	(0.004)	(0.04)	(0.19)
Coated	Low	0.77	0.21	3.5	0.25	0.20	4.8
		(0.04)	(0.05)	(0.42)	(0.07)	(0.01)	(0.16)
	High	1.2	0.14	2.9	0.55	0.10	4.5
		(0.19)	(0.02)	(0.03)	(0.25)	(0.03)	(0.28)

Table 10. Mean fitted values (standard errors) for duplicate experiments for reversible attachment ( $k_1$ ), detachment ( $k_2$ ), and irreversible attachment ( $k_3$ ) rates derived using the three-parameter model.

Treatment	$k_1$ (min <sup>-1</sup> )	p-value	$k_2$ (min <sup>-1</sup> )	p-value	$k_3$ (min <sup>-1</sup> )	p-value
"Live" cells	0.52	<b>0.005</b>	0.13	0.46	1.8	<b>0.003</b>
"Dead" cells	0.21		0.15		2.5	
Uncoated grains	0.03	<b>&lt;0.0001</b>	0.11	0.17	0.41	<b>&lt;0.0001</b>
Coated grains	0.70		0.16		3.9	
Low IS	0.27	<b>0.04</b>	0.17	0.11	2.2	0.40
High IS	0.46		0.11		2.1	

Table 11. Mean fitted values for reversible attachment ( $k_1$ ), detachment ( $k_2$ ), and irreversible attachment ( $k_3$ ) rates derived using the three-parameter model and analyzed using a three factor ANOVA for all experimental treatments. P-values in bold indicate significant differences (p<0.05).

<b>Treatment</b>	<b>Parameter</b>	<b>"Live" <i>E. coli</i></b>	<b>"Dead" <i>E. coli</i></b>	<b>p-value</b>
Uncoated Sand	$k_1$	0.04 (0.01)	0.02 (0.01)	0.28
	$k_2$	0.07 (0.01)	0.15 (0.05)	0.15
	$k_3$	0.37 (0.08)	0.46 (0.20)	0.67
Coated Sand	$k_1$	1.0 (0.16)	0.40 (0.14)	<b>0.03</b>
	$k_2$	0.18 (0.03)	0.15 (0.03)	0.50
	$k_3$	3.2 (0.25)	4.6 (0.15)	<b>0.003</b>
Low IS	$k_1$	0.40 (0.21)	0.14 (0.07)	0.29
	$k_2$	0.15 (0.04)	0.19 (0.04)	0.53
	$k_3$	1.9 (9.6)	2.6 (1.3)	0.66
High IS	$k_1$	0.65 (0.35)	0.28 (0.18)	0.39
	$k_2$	0.10 (0.03)	0.12 (0.02)	0.80
	$k_3$	1.7 (0.69)	2.5 (1.2)	0.59

Table 12. Mean fitted values for “live” and “dead” cell reversible attachment ( $k_1$ ), detachment ( $k_2$ ), and irreversible attachment ( $k_3$ ) rates derived using the three-parameter model and analyzed using a single factor ANOVA for each treatment. P-values in bold indicate significant differences ( $p < 0.05$ ). Units are  $\text{min}^{-1}$ .

		Grain Coatings			Ionic Strength		
Bacteria		Uncoated	Coated	p-value	Low IS	High IS	p-value
"Live" <i>E. coli</i>	$k_1$	0.043 (0.011)	1.0 (0.16)	<b>0.0009</b>	0.40 (0.21)	0.65 (0.35)	0.56
	$k_2$	0.074 (0.015)	0.18 (0.030)	<b>0.02</b>	0.15 (0.043)	0.10 (0.026)	0.44
	$k_3$	0.37 (0.080)	3.2 (0.25)	<b>&lt;0.0001</b>	1.9 (0.96)	1.7 (0.69)	0.88
"Dead" <i>E. coli</i>	$k_1$	0.022 (0.013)	0.40 (0.14)	<b>0.03</b>	0.14 (0.069)	0.28 (0.18)	0.50
	$k_2$	0.15 (0.047)	0.15 (0.031)	0.90	0.19 (0.043)	0.11 (0.021)	0.17
	$k_3$	0.46 (0.20)	4.6 (0.15)	<b>&lt;0.0001</b>	2.6 (1.3)	2.5 (1.2)	0.94

Table 13. Mean fitted values for reversible attachment ( $k_1$ ), detachment ( $k_2$ ), and irreversible attachment ( $k_3$ ) rates derived using the three-parameter model and analyzed by a single factor ANOVA for “live” or “dead” cell treatments. P-values in bold indicate significant differences ( $p < 0.05$ ). Units are  $\text{min}^{-1}$ .

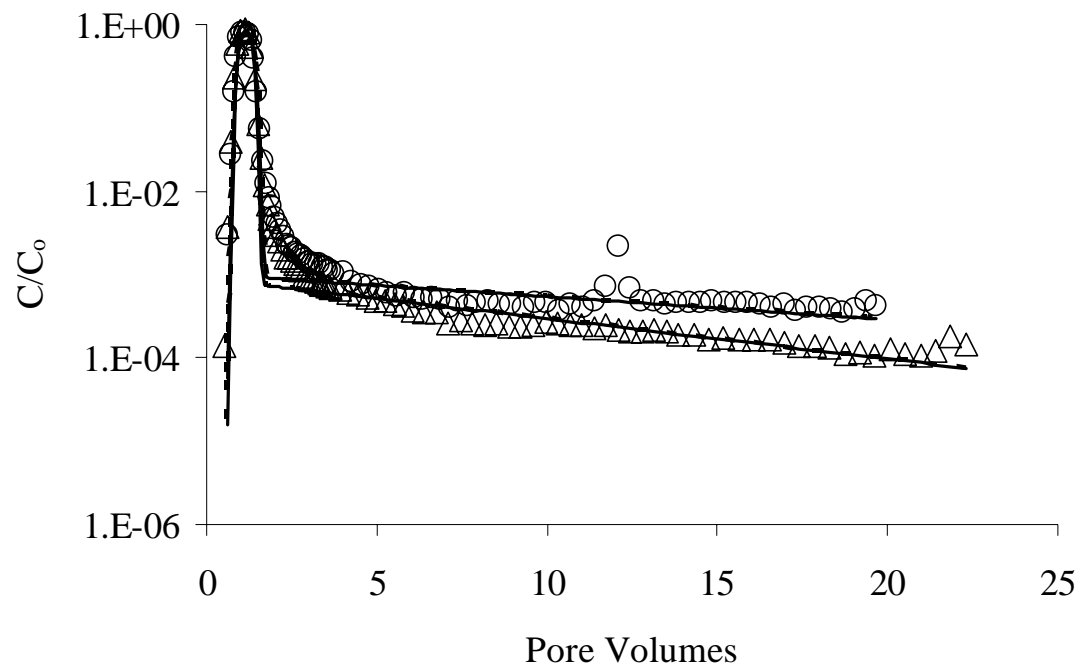


Figure 8a. Observed (symbols) and fitted (lines) breakthrough curves for duplicate experiments (circles versus triangles) using “live” *E. coli* cells in uncoated quartz sand at low ionic strength. The solid line represents the best fit of the three-parameter model and the dashed line represents the best fit of the two-parameter model.

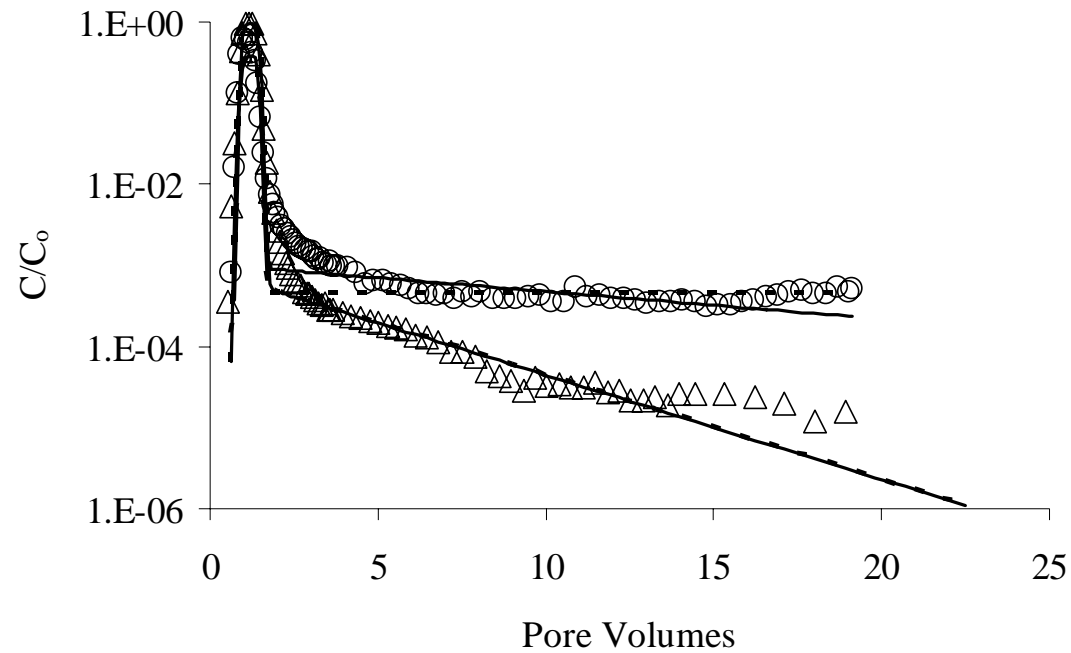


Figure 8b. Observed (symbols) and fitted (lines) breakthrough curves for duplicate experiments (circles versus triangles) using “dead” *E. coli* cells in uncoated quartz sand at low ionic strength. The solid line represents the best fit of the three-parameter model and the dashed line represents the best fit of the two-parameter model.

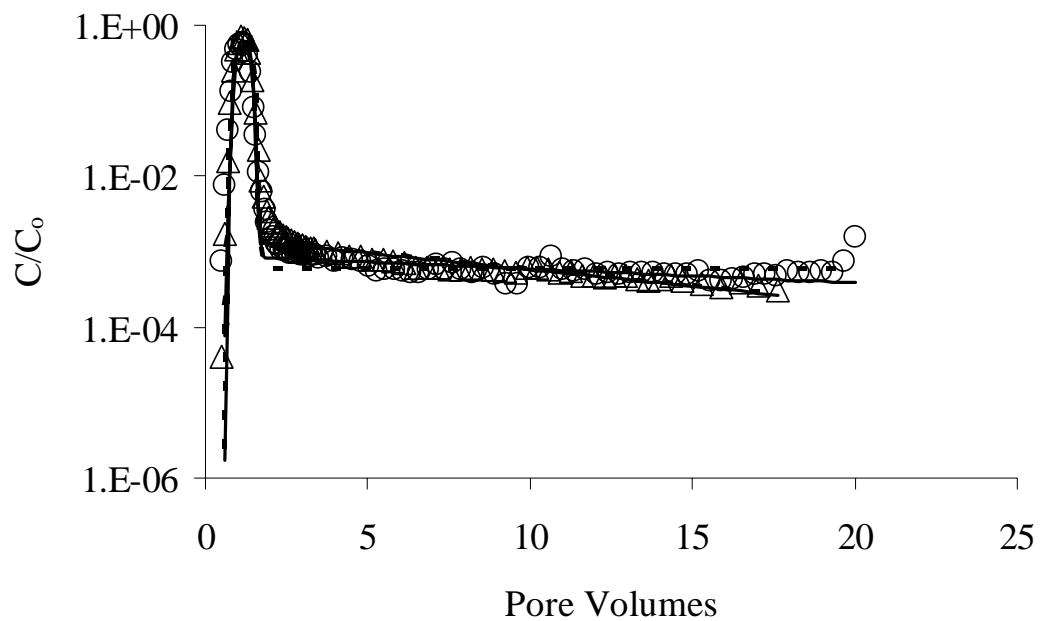


Figure 8c. Observed (symbols) and fitted (lines) breakthrough curves for duplicate experiments (circles versus triangles) using “live” *E. coli* cells in uncoated quartz sand at high ionic strength. The solid line represents the best fit of the three-parameter model and the dashed line represents the best fit of the two-parameter model. The three-parameter model was found to provide slightly superior fits to the observed data than the two-parameter model.

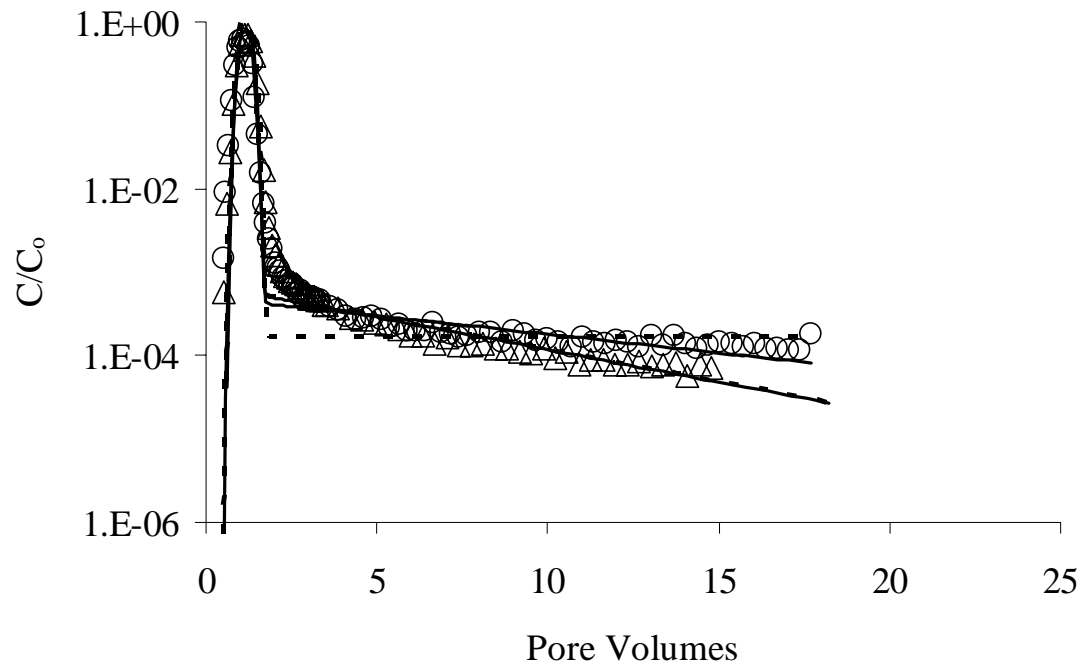


Figure 8d. Observed (symbols) and fitted (lines) breakthrough curves for duplicate experiments (circles versus triangles) using “dead” *E. coli* cells in uncoated quartz sand at high ionic strength. The solid line represents the best fit of the three-parameter model and the dashed line represents the best fit of the two-parameter model. The three-parameter model was found to provide slightly superior fits to the observed data than the two-parameter model.

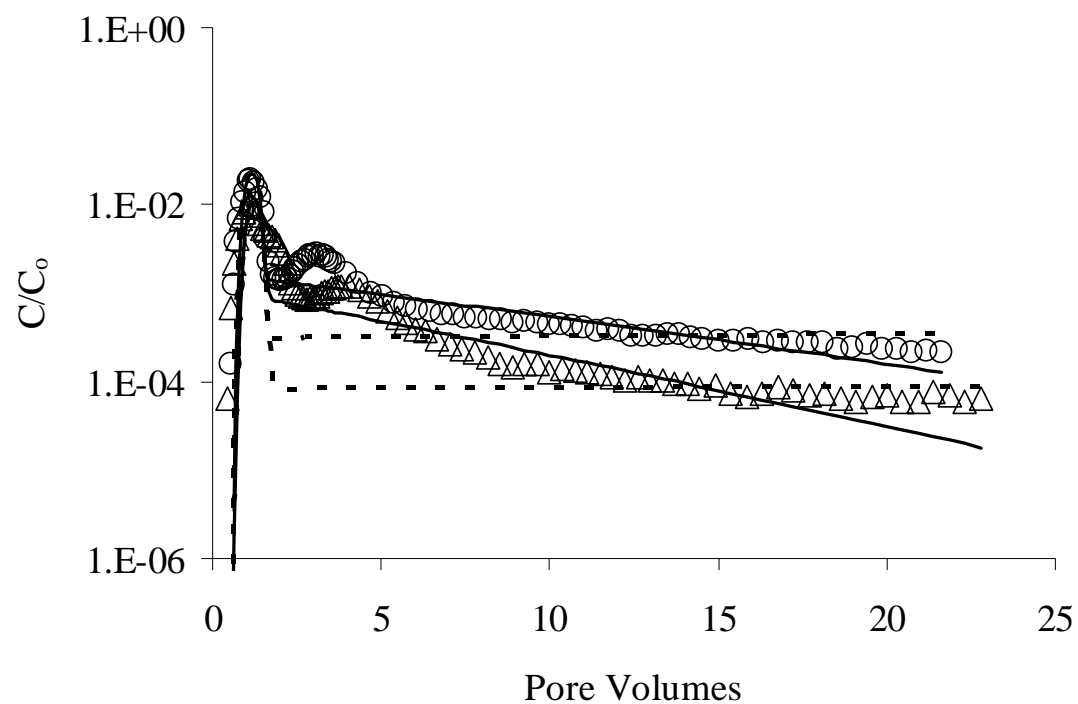


Figure 8e. Observed (symbols) and fitted (lines) breakthrough curves for duplicate experiments (circles versus triangles) using “live” *E. coli* cells in coated quartz sand at low ionic strength. The solid line represents the best fit of the three-parameter model and the dashed line represents the best fit of the two-parameter model. The three-parameter model was found to provide slightly superior fits to the observed data than the two-parameter model.

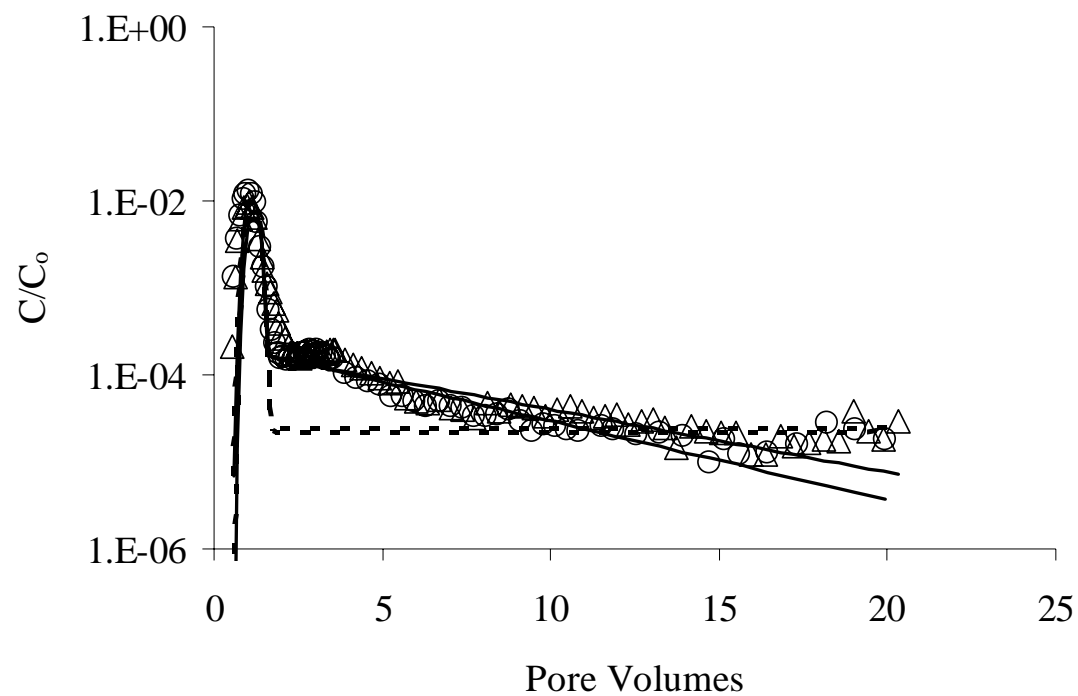


Figure 8f. Observed (symbols) and fitted (lines) breakthrough curves for duplicate experiments (circles versus triangles) using “dead” *E. coli* cells in coated quartz sand at low ionic strength. The solid line represents the best fit of the three-parameter model and the dashed line represents the best fit of the two-parameter model. The three-parameter model was found to provide slightly superior fits to the observed data than the two-parameter model.

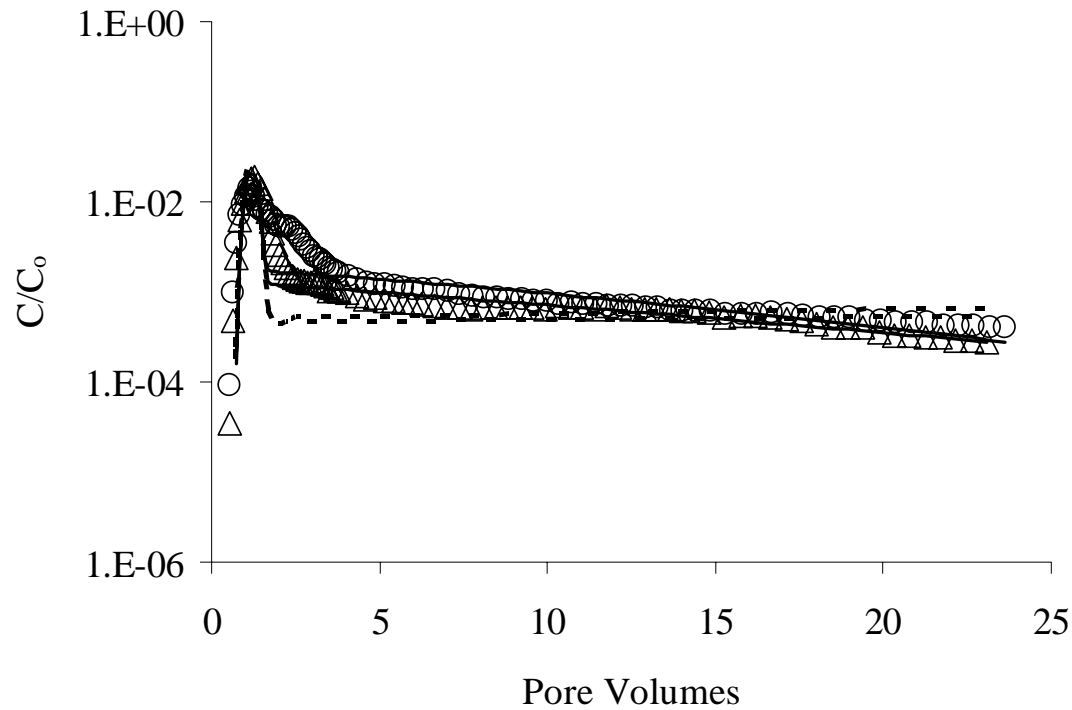


Figure 8g. Observed (symbols) and fitted (lines) breakthrough curves for duplicate experiments (circles versus triangles) using “live” *E. coli* cells in coated quartz sand at high ionic strength. The solid line represents the best fit of the three-parameter model and the dashed line represents the best fit of the two-parameter model. The three-parameter model was found to provide slightly superior fits to the observed data than the two-parameter model.

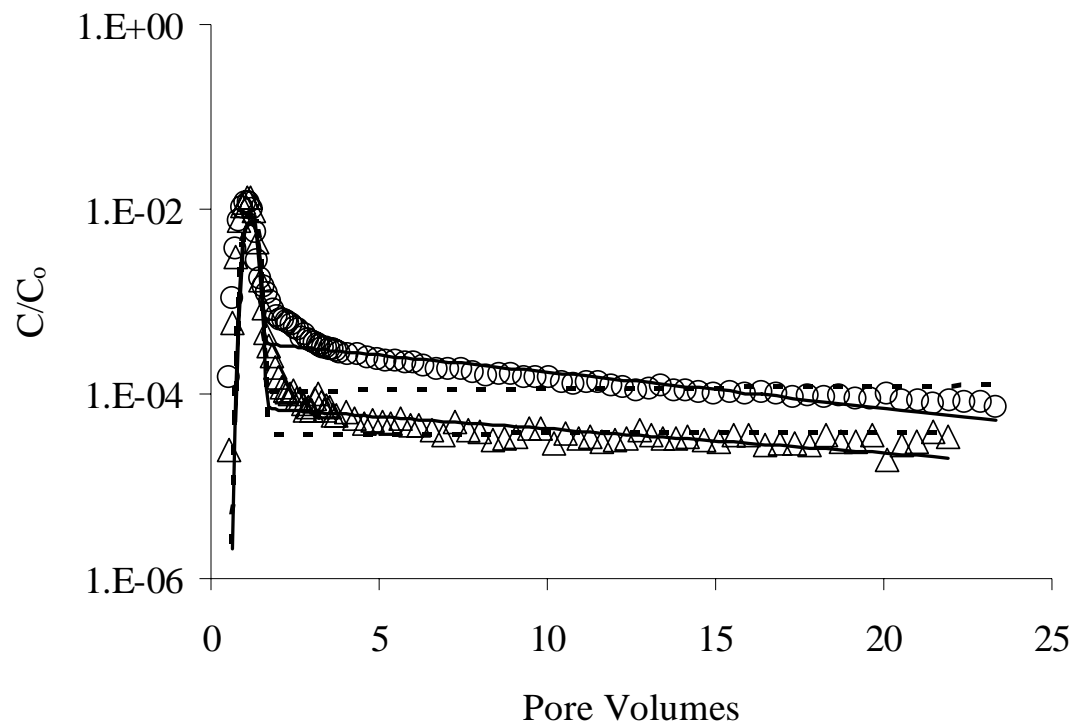


Figure 8h. Observed (symbols) and fitted (lines) breakthrough curves for duplicate experiments (circles versus triangles) using “dead” *E. coli* cells in coated quartz sand at high ionic strength. The solid line represents the best fit of the three-parameter model and the dashed line represents the best fit of the two-parameter model. The three-parameter model was found to provide slightly superior fits to the observed data than the two-parameter model.

## Sensitivity Analysis

Sensitivity analysis of the two- and three-parameter models indicated that higher reversible attachment rates,  $k_1$ , and irreversible attachment rates,  $k_3$ , lowered the height of curve peaks and altered the height of curve tails; higher  $k_1$  increased the height of the tail while higher  $k_3$  lowered the height (Figures 9 and 10). At the highest simulated value for  $k_3$ , tailing was no longer observed. As the detachment coefficient,  $k_2$ , increased, the slope of BTC tails increased (Figure 11). The detachment coefficient had no effect on curve peaks.

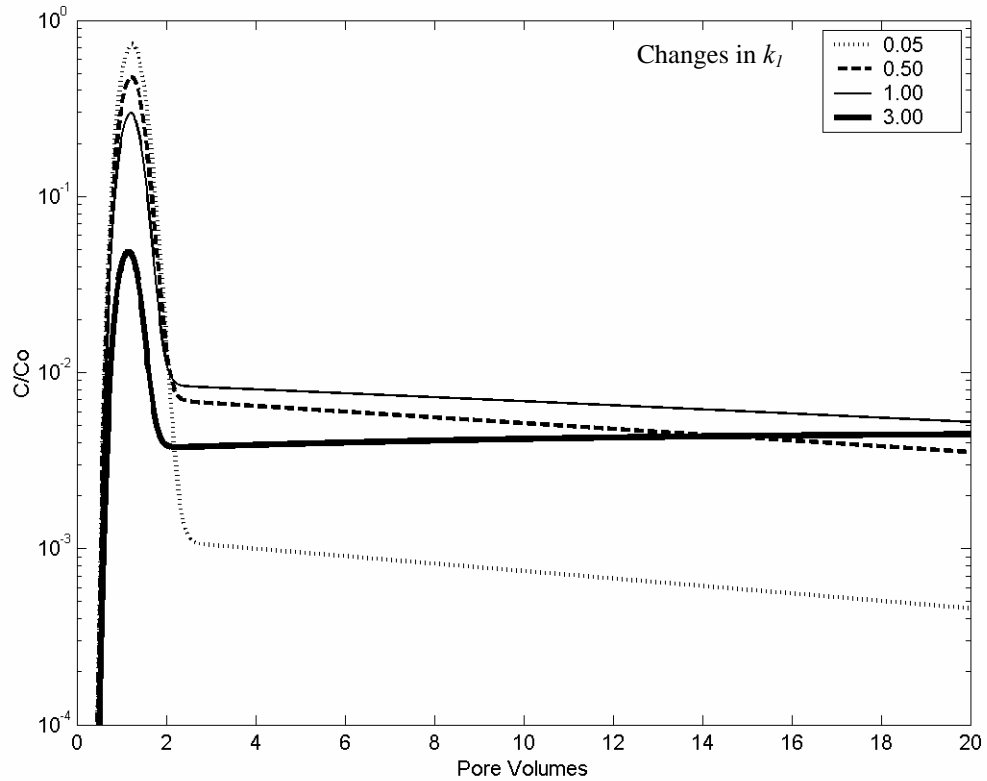


Figure 9. Model simulations of the three-parameter model demonstrating the effect of changing reversible attachment rates,  $k_1$ , on breakthrough curve peaks and tailing when detachment rates,  $k_2$ , and irreversible attachment rates,  $k_3$ , were held constant at 0.05. Removing  $k_3$  to simulate effects of the two-parameter model yielded the same curves.

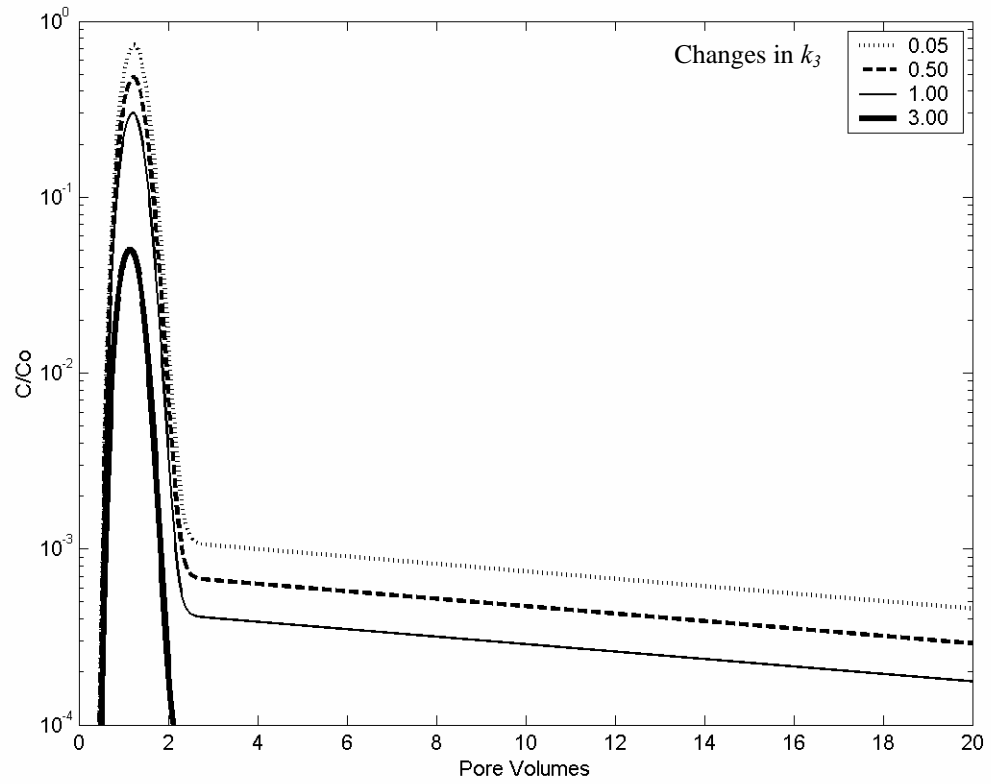


Figure 10. Model simulations of the three-parameter model demonstrating the effect of changing irreversible attachment rates,  $k_3$ , on breakthrough curve peaks and tailing when reversible attachment rates,  $k_1$ , and detachment rates,  $k_2$ , were held constant at 0.05. Increasing  $k_3$  to 3.00 eliminated the BTC tail.

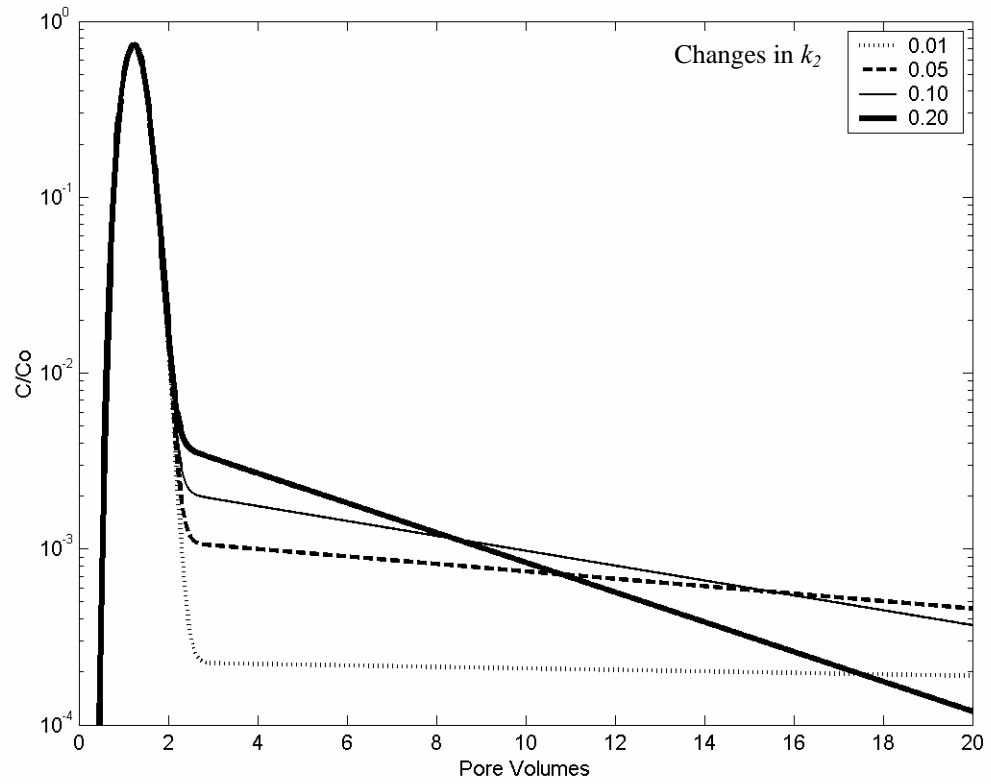


Figure 11. Model simulations of the three-parameter model demonstrating the effect of changing detachment rates,  $k_2$ , on the slope of breakthrough curve tailing when reversible attachment rates,  $k_1$ , and irreversible attachment rates,  $k_3$ , were held constant at 0.05. Removing  $k_3$  to simulate effects of the two-parameter model yielded the same curves.

### **Protein Analysis**

Differences in protein profiles between “live” and “dead” *E. coli* cultures used as the influent bacterial pulses were observed using SDS-PAGE (Figure 12). Bands, which represent the migration of proteins within each sample, with molecular weights of ~42 and ~46 kDa were observed in both lanes 3 and 4 of the “dead” *E. coli* cells (formaldehyde fixed and boiled). These bands were not observed in lane 2 of the “live” cell sample. Concentrations of “live” and “dead” tails were too low to observe using SDS-PAGE, therefore protein profile of cells within the effluent from the columns could not be determined.

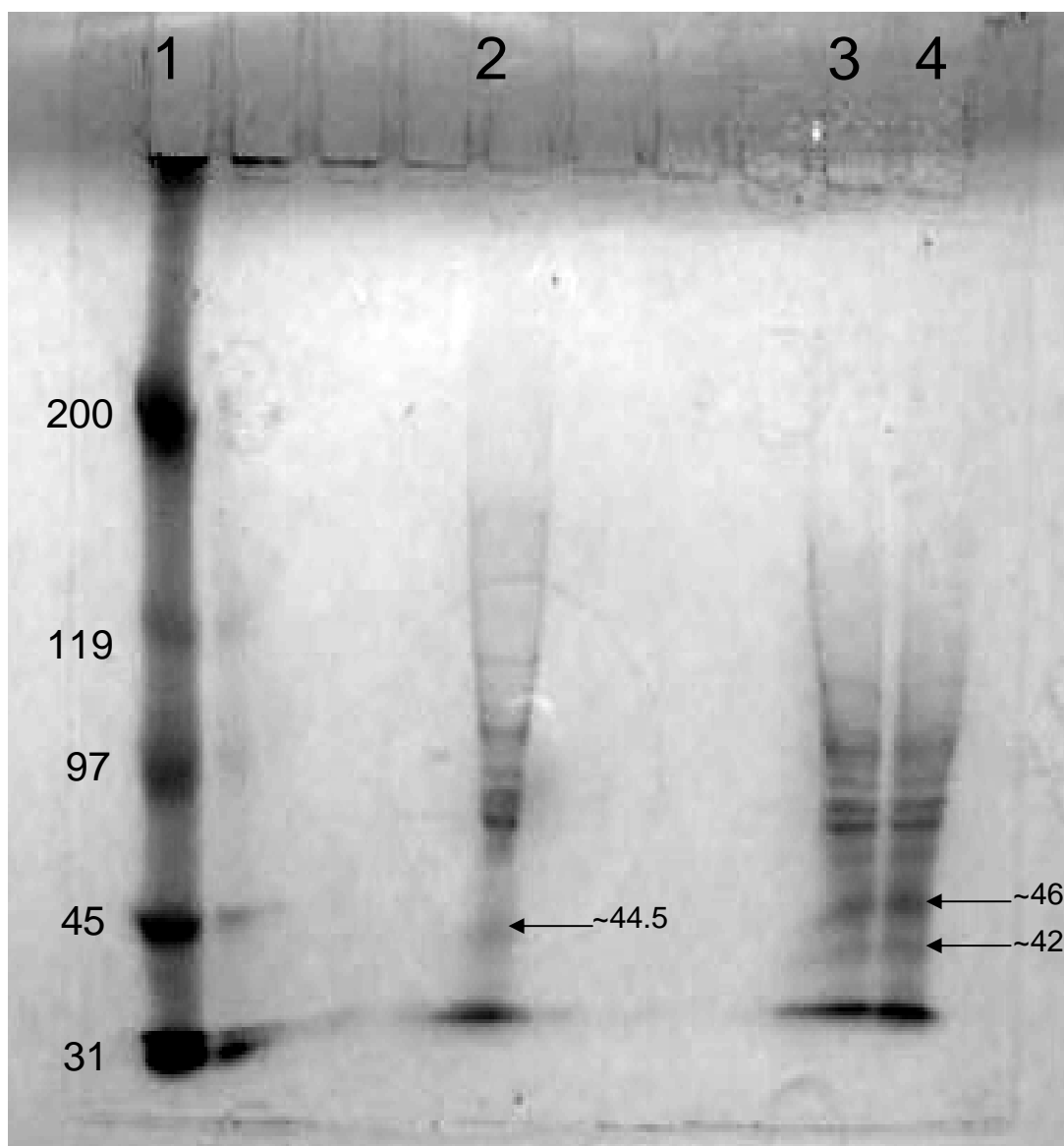


Figure 12. Results of SDS-PAGE of *E. coli* cells. Differences between lane 2 and nearly identical lanes 3 and 4 are indicated with arrows. Lane 1: Molecular mass standards (kDa). Lane 2: “Live” *E. coli* cells. Lane 3: *E. coli* cells fixed with 0.5% final concentration formaldehyde prior to injection. Lane 4: *E. coli* cells boiled in a water bath for 15 min rendering them “dead”. Bands with molecular weights of ~42 and ~46 kDa were observed in lanes 3 and 4 but not in lane 2.

## **IV. DISCUSSION**

### **Column Experiments**

It was interesting that there were no observed differences in detachment rates estimated by either model or in BTC tail slopes, which would indicate differences in detachment rates, between any of the experiment treatments. It was expected that detachment rates would be lower in “live” cells than “dead” cells because the “live” cells would be actively involved in the attachment process, that detachment would be higher at low ionic strength because attractive van der Waals forces at high ionic strength would limit detachment, and that detachment would be lower in uncoated sands because uncoated sands are negatively charged and therefore are repulsive to Gram-negative bacteria. The lack of difference in detachment rates between experiments may be because detachment is negligible under steady state conditions and is only a factor when conditions are altered. This is important as it indicates that in a homogeneous aquifer system, detachment rates will be similar at steady state regardless of differences in the physicochemical properties tested here.

As mentioned previously, the slow release of bacteria over time increases the potential for bacterial contamination of aquifers. If detachment is indeed negligible at steady state, managers should consider limiting disturbances, such as aquifer pumping, in areas near septic systems or other systems that introduce bacteria into the subsurface in order to avoid perturbations that may foster detachment. More research is necessary to

determine if detachment rates change in the presence of larger sized porous media or other strains of bacteria under steady state conditions. At the field scale, perturbations in aquifer systems do occur and the subsurface is usually heterogeneous, therefore it is also necessary to understand the effect of system perturbations and heterogeneity on attachment and detachment. In addition, the difference in kinetics between attachment and detachment rates may explain the failure of short term laboratory experiments to detect differences in detachment rates between experimental treatments, with time scales on the order of minutes and hours for attachment and days for detachment rates. Short term laboratory experiments may not be able to detect the slower rates. Attachment rates seemed to better explain the variability observed between experiments than detachment rates.

The higher tailing in breakthrough curves (BTCs) of “live” bacteria observed for almost all experiments was opposite than expected. Tailing indicates that portions of the cells retained were not irreversibly attached (Fontes et al. 1991), therefore this would indicate that less “live” cells attached irreversibly than “dead” bacteria. Since greater tailing also indicates greater reversible attachment, this may also indicate that “live” cells are actively involved in reversibly attaching to surfaces. Examination of the reversible and irreversible attachment rates as derived by the three-parameter model also indicated that  $k_1$  was significantly higher and  $k_3$  was significantly lower for experiments using “live” cells versus “dead” cells. In addition, the fraction of “live” cells recovered from bacterial tailing was significantly higher than recovered “dead” cells. In comparison to the observations of other studies, it was surprising that reversible attachment was higher for “live” cells and that less “live” cells irreversibly attached than “dead” cells. Navarro

et al. (2002) observed that cell-bead binding properties were similar for both live cells and cells fixed with formaldehyde or Prefer fixative. Hanein et al. (1995) examined attachment of live and paraformaldehyde fixed epithelial cells to calcium tartrate tetrahydrate crystals and conversely found that the attachment of fixed cells was weaker than for living cells. Several possible explanations for higher reversible attachment rates and lower irreversible attachment rates for “live” cells include “live” cell nutritional requirements, and differences in “live” and “dead” cell surfaces and sizes.

One possible explanation why fewer “live” cells remained irreversibly attached is that reversible attachment is a mechanism to combat starvation. Van Loosdrecht et al. (1987b) similarly hypothesized that during starvation, detachment will reintroduce cells into pore water to increase the probability of cells reaching nutrient rich waters. Hanein et al. (1995) reported that starved cells often increased surface stickiness in order to promote attachment, however. Kjelleberg and Hermansson (1984) also observed increases in irreversible attachment to glass for several strains of bacteria under starvation conditions. Even in the carbon-limited subsurface, cells can utilize stored carbon to produce extracellular polymers (Harvey 1991). Additionally, van Schie and Fletcher (1999) reported that starved cells remain attached even after long periods of time. In carbon-limiting environments, most bacteria will be associated with surfaces and the remainder of the population within the aqueous phase will be transported by physical processes (Murphy and Ginn 2000). DeFlaun et al. (1990) found greater attachment when bacteria were removed from growth medium, indicating that attachment may indeed be related to nutrient conditions. It was not clear if nutrient requirements were factors in these experiments.

The observed differences in the cell protein profiles of “live” and “dead” cells could illustrate why variations in transport were found between the two. As previously mentioned, proteins in the outer membrane can be involved in attachment. Özkanca and Flint (2002) found changes in the amounts of various outer membrane proteins in *E. coli* under extended periods of starvation. Changes in outer membrane proteins because of the continued starvation of the “live” cells may explain why differences were observed in the protein profiles between “live” and “dead” cells and why differences were not observed between “dead” cells that were fixed or boiled. The inability of “dead” Gram-negative bacteria to undergo cell wall turnover, defined as the shedding of peptidoglycan during metabolism (Doyle and Sonnenfeld 1989), is another possible explanation for variations in cell protein profiles. As “live” cells grow, cell walls are “shed”, and this process would not occur in “dead” cells.

Others have speculated that fixation may affect cell surface properties. The similarity in protein profiles between fixed and boiled cells, however, indicates that formaldehyde did not alter cell protein profiles in the current experiments. In support of this, Hanein et al (1995) reported that cell surface molecules involved in attachment were not affected by fixation. Conversely, Camesano and Logan (1998) observed that *Pseudomonas fluorescens* P17 rendered nonmotile via fixation transported farther than motile P17 at high velocities. They speculated that this difference in transport might have been because fixation using acridine orange solution containing 2% formaldehyde could have changed the surface properties of P17. In addition, Metz et al. (2004) reported that peptides and proteins underwent a range of chemical changes when treated with formaldehyde.

Hanein et al. (1995) speculated that the difference in adsorption between fixed and living cells was the inability of the fixed cell surfaces to interact in attachment. They also reported, however, that fixation did not prevent cell attachment. Becker et al. (2004) observed that percent recovery of Gram-positive bacteria actually increased in the presence of bead coatings, while recovery decreased for Gram-negative bacteria in column experiments. They suggested that cell wall/surface interactions were affected by grain coatings.

Differences between the sizes of “live” and “dead” cells may also explain differences in retention and detachment. Although this could not be confirmed by viewing cells under microscopy, “live” cells could have become smaller the longer they remained without nutrients, while the “dead” cells remained the same size they were at the time of fixation. Kjelleberg and Hermansson (1984) and Özkanca and Flint (2002) reported that cell volumes decreased as starvation time periods increased. If the cells were different sizes, however, size exclusion effect would most likely have been observed. Size exclusion occurs when larger cells breakthrough quicker as they move through the larger pore spaces where pore-water velocity is incidentally the highest (Fontes et al. 1991). Early breakthrough was not observed, however, as demonstrated in Figures 6 and BF-1a to BF-1g. Conservative tracer and bacterial breakthrough occurred simultaneously. Additionally, when examining the transport of two sizes of bacteria, Fontes et al. (1991) observed broader BTC peaks for experiments with larger cells. BTC peak widths again did not seem to differ between “live” and “dead” cells in the current experiments based on examination of Figures 6 and BF-1a to BF-1g. Early breakthrough

and differences in peak widths were not observed. Cell sizes, therefore, were probably similar and did not explain the difference between “live” and “dead” cell tailing.

It was an unexpected finding that tailing was prevalent in all experiments using iron coated sands. It was expected that almost complete removal of bacteria from the water column would occur because of oppositely charged grain surfaces, as this has often been reported in the literature (Mills et al. 1994; Scholl et al. 1990). Indeed, the fraction of cells recovered was significantly less for column effluent of coated sand experiments than for uncoated sand experiments. There was no difference in the fraction of cells recovered in BTC tails, however, indicating no relationship between tailing and grain coatings. In addition, detachment rates were similar between coated sand and uncoated sand experiments. Becker et al. (2004) also observed lower than expected effects of iron-oxyhydroxide coated glass beads on hindering bacterial breakthrough in comparison with previous experiments from their laboratory. It may be possible that grain surfaces used in the current experiments were not uniformly coated with iron. Some sites may have therefore remained uncoated and therefore negatively charged, creating a heterogeneous surface on to which bacteria attached at different rates. Grains were not magnified to verify coating uniformity. Silliman et al. (2001) performed experiments using heterogeneous media of varied sizes and coatings and also observed bacterial tailing, therefore partial coatings may explain the observed tailing in coated sand experiments.

Lower BTC peaks for coated sand experiments have often been noted in the literature. The observed multiple peaks in BTCs for coated sand filled columns were unexpected and resemble BTCs observed in column experiments filled with heterogeneous porous media (Fontes et al. 1991). Fontes et al. (1991) packed laboratory

columns with a vein of coarse sand within fine sand. They suggested multiple peaks were partially due to differences in transmissivity, with the coarse sand more transmissive than the fine sand. Grain sizes were relatively homogeneous in the current experiments throughout the packed columns, with diameters ranging from ~350 to ~500  $\mu\text{m}$ , however. It was not clear why multiple peaks were observed, and why the two peaks were observed only in coated sands and not in uncoated sand experiments.

As expected, higher ionic strength increased bacterial reversible attachment. When estimated using the three-parameter model, average  $k_I$  was significantly lower at low versus high ionic strength. Numerous studies have found that high ionic strength increases bacterial attachment to quartz sands due to van der Waals attraction (Bolster et al. 2001; Fontes et al. 1991). In the presence of coated sands, ionic strength has been observed to have minimal effect on attachment (Bolster et al. 2001).

The observed variability between experiment duplicates, especially in experiments using low ionic strength solution or “dead” cells, is important to consider for future column experiments. This variability was probably because experimental conditions could have changed from experiment to experiment because of the variable nature of column packing or of using bacteria as the study organism. Although the laboratory environment simplifies aquifer systems, repacking columns and regrowing new cultures of bacteria for each experimental duplicate are two factors that alone could have introduced variability or heterogeneity into experiments. According to Brown et al. (2002), the preparation of porous media may be the problem if duplicate experiments are not identical. There was not much variability between samples taken from the column effluent for each experiment, as they were taken frequently throughout the duration of

experiments, therefore it was not likely that there were experimental artifacts during individual experiments. Variability may be representative of field conditions, however, as natural conditions are naturally heterogeneous. Many labs find repeatability between duplicates and therefore do not always duplicate column experiments, however, evidence here supports the need for duplication. It was not clear what caused the differences in tail heights between duplicates, but the difference in results underscores the fact that is important to run duplicates or triplicates when performing column experiments.

### **Model fits**

It was expected that adding the third parameter to the modified advection dispersion equation would provide better model fits than using only two parameters. Indeed, incorporating an irreversible attachment rate into the three-parameter model better explained variations in BTC tailing, including higher reversible attachment and lower irreversible attachment rates for “live” cells than “dead” cells. Model efficiency ( $E$ ) was higher for the three-parameter model than the two-parameter model. Standard errors of estimated parameters were high for both models, however, indicating that neither model estimated the parameters extremely well, as can be seen by comparing BTCs to fitted curves. Standard errors were very low for parameters estimated for the conservative tracer indicating that estimates of interstitial pore water velocity and the dispersion coefficient were good.

Curve-fitting when using the three-parameter model seemed to fit reasonably well to all sections of BTCs except the elution portion of the curves (Figure 3, Section D). Johnson et al. (1995) were also unable to account for the elution portion of their BTCs

when using an exponential model or a two-rate model. They found that by incorporating irreversible attachment rates into their model they could account for elution portion, peaks and tails of BTCs. For the current experiments, however, the inclusion of irreversible attachment rates did not enable all three curve sections to be well estimated. Johnson et al. (1995) concluded that it was necessary to account for heterogeneous sorption sites, allowing for fast initial bacterial detachment followed by slow release, in order to describe attachment and detachment.

### **Conclusion**

The main hypotheses were that (1) “live” bacterial cells will have lower detachment rates because “live” cells will be actively involved in the attachment/detachment process, (2) iron coatings will generate lower detachment rates than uncoated sand due to the attraction of negatively charged bacteria to positively charged iron coated sand grains, and (3) higher ionic strength will reduce detachment rates because of stronger attractive van der Waals forces. Detachment rates did not vary between any of the treatments, therefore none of the hypotheses were supported. It was speculated that for the conditions tested here, detachment rates will be similar regardless of the composition of the aquifer if it is at steady state. Detachment may occur mainly when system perturbations are introduced.

Detachment rates were not different between “live” and “dead” cells, therefore first hypothesis was not supported. It was surprising that rendering cells “dead” and therefore not biologically active did not seem to alter detachment rates. “Live” *E. coli* cells were actively involved in the attachment process, however, and this may have been

because of nutritional requirements or cell protein modifications. Greater tailing, and therefore greater reversible attachment rates, of “live” cells may have been due to the low carbon content within columns, as “live” cells would remain weakly attached in order to increase the potential for transport to more optimal growth conditions. This may have important management implications. For example, bacteria used for bioremediation should be starved prior to introduction into the subsurface to ensure that cells would remain mobile and transport more easily to nutrient rich areas, which for bioremediation purposes would be the areas of organic contamination. More research should be done to investigate the biological factors affecting bacterial detachment, including examining other bacteria strains and investigating the involvement of outer membrane proteins in detachment.

The second hypothesis was also not supported by experimental results. Detachment rates were not lower in coated sand than in uncoated sand experiments. Additionally, nearly complete adsorption of *E. coli* cells to Fe-coated sands has been frequently documented (Mills et al. 1994; Scholl et al. 1990) therefore the tailing observed in experiments using Fe-coated sands was unexpected. It was speculated that this was because of the sand grains may have not have been uniformly coated with iron. Reversible and irreversible attachment rates were an order of magnitude higher in iron coated sand experiments, which indicates the removal of bacteria from the aqueous phase. Using coated sands for aquifer filtration, such as in septic systems, may therefore improve aquifer filtration. The results indicate, however, that coated sands would not be provide complete removal of bacteria from the aqueous phase and therefore would need to be combined with other treatments.

The third hypothesis was also not supported by the experiments. Changes in ionic strength did not significantly affect detachment rates. Attachment rates were lower in at low ionic strength, however. Supporting this finding, Mills et al. (1994) observed an order of magnitude increase in attachment when ionic strength was increased by one order of magnitude.

The attachment and detachment rates compared here were derived by two models. Other models may have estimated the parameters differently. Model fits to the observed data, especially tail height and slope, were improved by adding a parameter into the ADE to account for irreversible attachment. Neither model simulated all portions of the BTCs, specifically the elution portion of the curves. The current research underscores the need to develop better models to describe the detachment process under steady state conditions.

The factors affecting detachment of bacteria from saturated porous media are not well understood. Detachment rates are slower than attachment rates and can occur over a period of days. The slow release of bacteria from the solid phase into the aqueous phase increases the opportunity for bacterial growth should cells be transported to areas rich in nutrients. Understanding the conditions in which detachment occurs is necessary for the prevention of groundwater contamination.

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## APPENDIX A

### **Bacteria Preparation**

Several preliminary tests were performed to determine the most efficient method for labeling bacteria with  $^3\text{[H]}$  for use in column experiments. Cultures were prepared using the backwashing procedure (see Materials and Methods section) employed by Camesano and Logan (1998) and were compared to cultures that were not backwashed. Briefly, backwashing of cells consisted of capturing  $^3\text{[H]}$ -labeled bacteria on 0.2- $\mu\text{m}$  pore size, 47-mm diameter membrane filters to remove any label that was not incorporated into the bacterial cells. The filters were then flipped over and buffer water was passed through the filters via vacuum filtration to dislodge the captured bacteria. Cells that were not backwashed were filtered through 0.2- $\mu\text{m}$  pore size, 25-mm diameter, membrane filters immediately before enumeration in order to remove the unincorporated label. The filters were placed in scintillation vials and enumerated. Label loss over time was calculated and results between treatments were compared.

Although concentrations of incorporated label were lower when the backwashing procedure was used, less label appeared to be lost over time using this method (Figure AF-1). This procedure was also less time consuming since backwashing essentially pre-filters the culture, removing excess label prior to the experiment in order to avoid the need to filter every effluent sample to remove the excess.

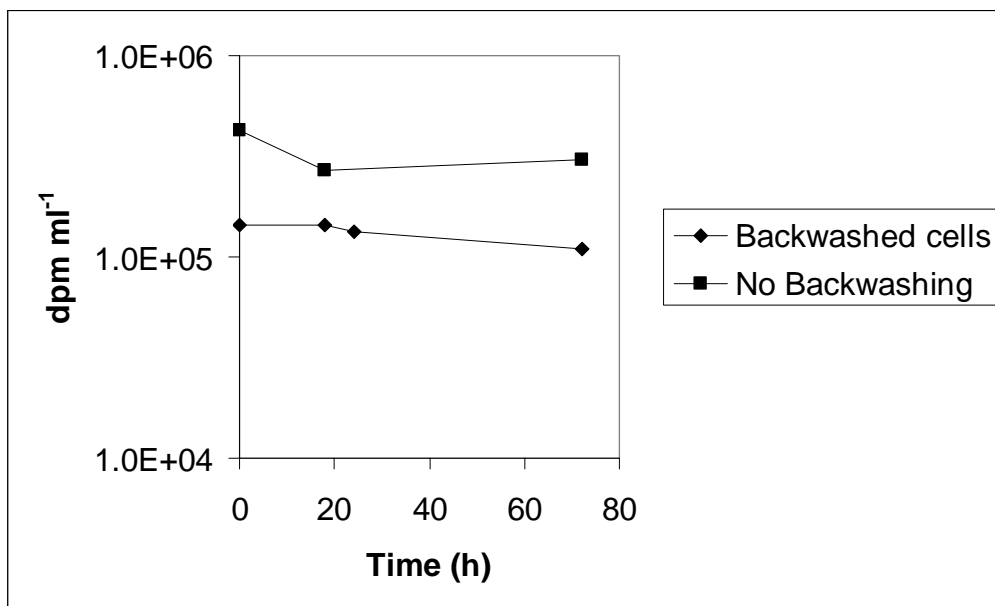


Figure AF-1. Comparison of two methods for enumeration of <sup>3</sup>[H]-labeled *E. coli* cells and respective label loss over time. Unincorporated label was removed prior to the experiment with the backwashed cells whereas unincorporated label was removed at each sampling time by collecting cells on 0.2  $\mu$ m filters and counting the radioactivity on the filters.

In another preliminary test, glucose was added to a  $^3\text{[H]}$ -labeled culture and compared to a glucose free  $^3\text{[H]}$ -labeled culture in order to determine if glucose enhanced label uptake. Both cultures were filtered through 0.2- $\mu\text{m}$  pore size, 25-mm diameter, membrane filters and enumerated over the course of 24 h. *E. coli* cell concentrations with or without glucose amendment over time were within the same order of magnitude (Table A-1), therefore it was deemed unnecessary to amend cultures with glucose during the course of this study.

Elapsed time (h)	Glucose addition (dpm ml <sup>-1</sup> )	No glucose (dpm ml <sup>-1</sup> )
0	8.1E+05	1.1E+06
1	1.8E+06	1.3E+06
2.5	2.3E+06	3.8E+06
4.5	2.0E+06	1.8E+06
24	2.9E+06	2.3E+06

Table AT-1. Comparison of *E. coli* cell  $^3\text{[H]}$ -labeling methods over time. Glucose was added to one culture to determine if glucose encourages label uptake and retention.

## APPENDIX B

### Breakthrough Curves for Conservative Tracer and Bacteria Experiments

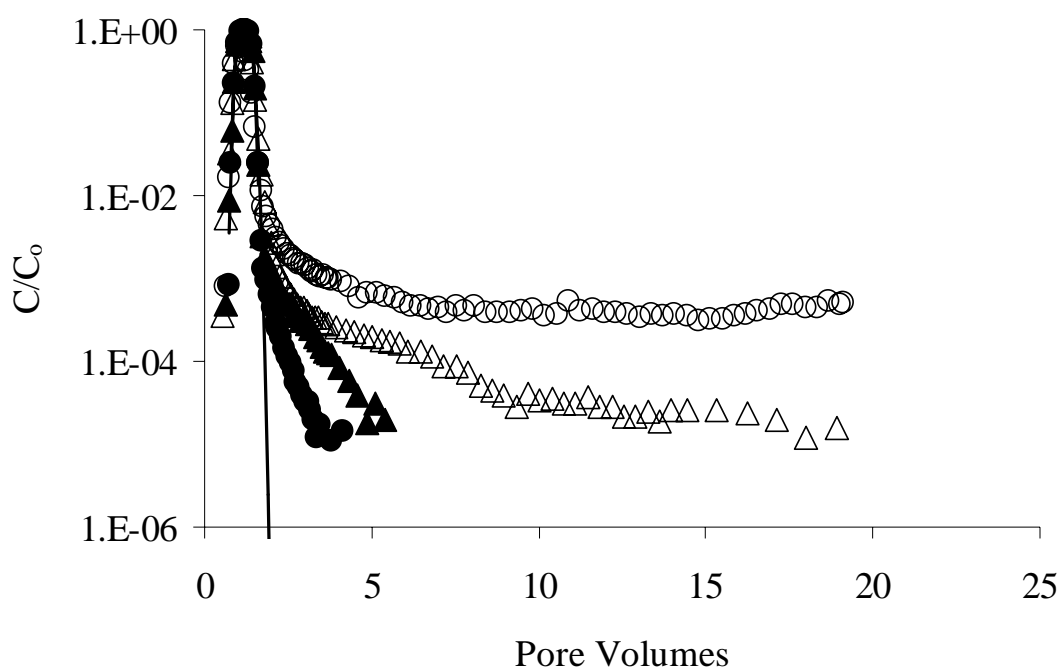


Figure BF-1a. Observed breakthrough curves for duplicates (circles versus triangles) of conservative tracer experiments (closed symbols) and bacteria experiments (open symbols) using “dead” *E. coli* cells in uncoated quartz sand at low ionic strength. The solid line represents the best fit of the model to the conservative tracer duplicates. Tailing of the conservative tracer is noticeably minimal compared to the extended tailing of bacteria over time.

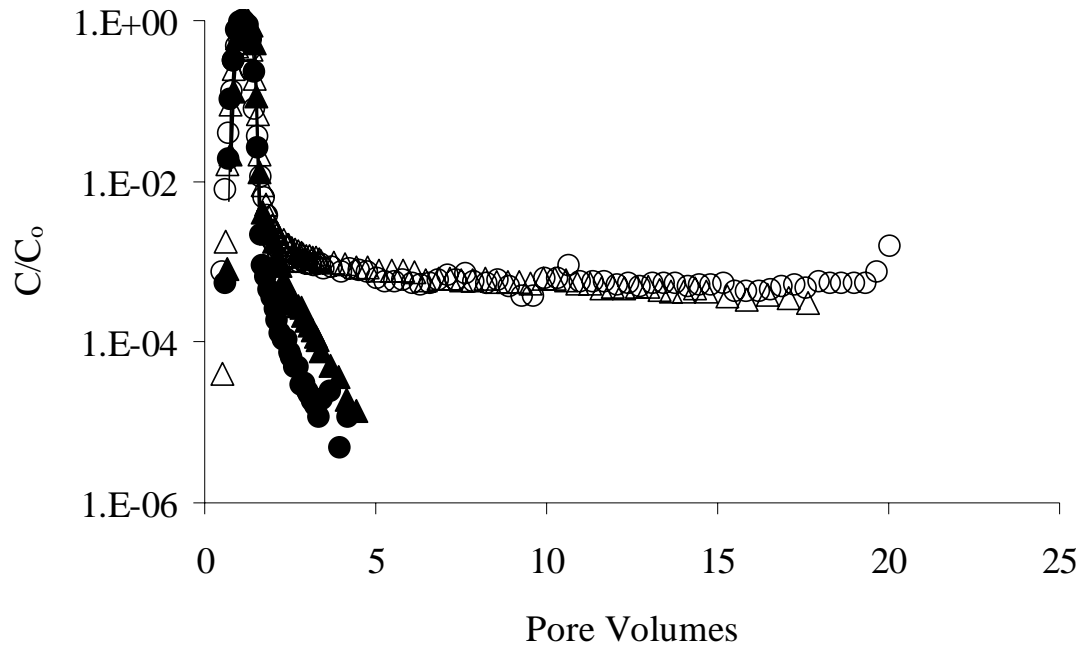


Figure BF-1b. Observed breakthrough curves for duplicates (circles versus triangles) of conservative tracer experiments (closed symbols) and bacteria experiments (open symbols) using “live” *E. coli* cells in uncoated quartz sand at high ionic strength. The solid line represents the best fit of the model to the conservative tracer duplicates.

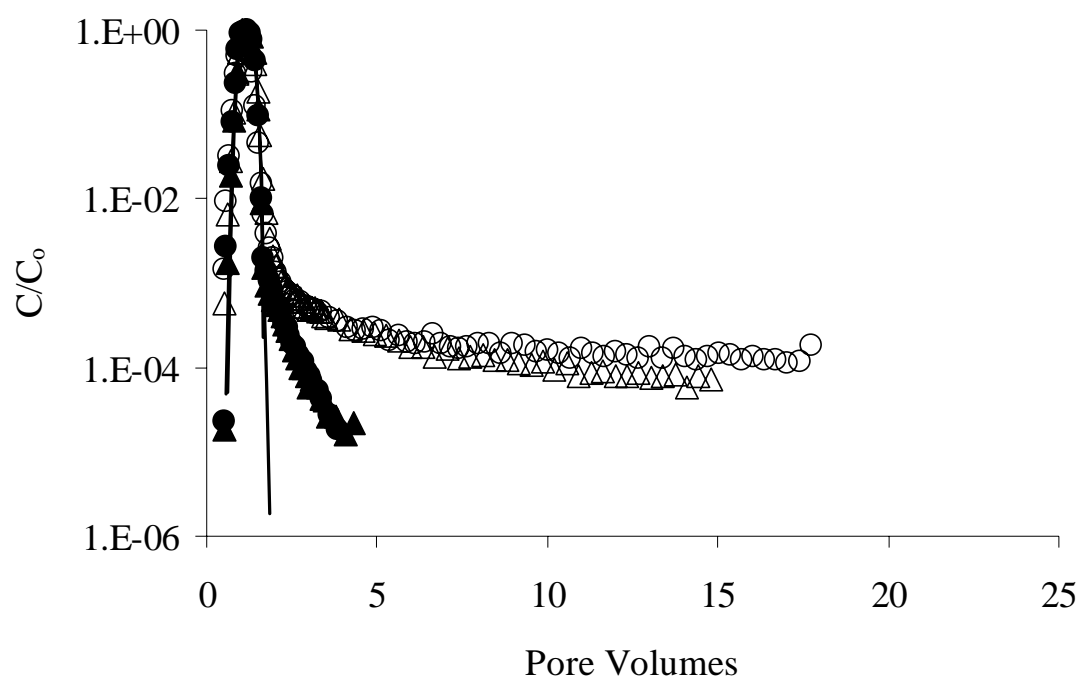


Figure BF-1c. Observed breakthrough curves for duplicates (circles versus triangles) of conservative tracer experiments (closed symbols) and bacteria experiments (open symbols) using “dead” *E. coli* cells in uncoated quartz sand at high ionic strength. The solid line represents the best fit of the model to the conservative tracer duplicates.

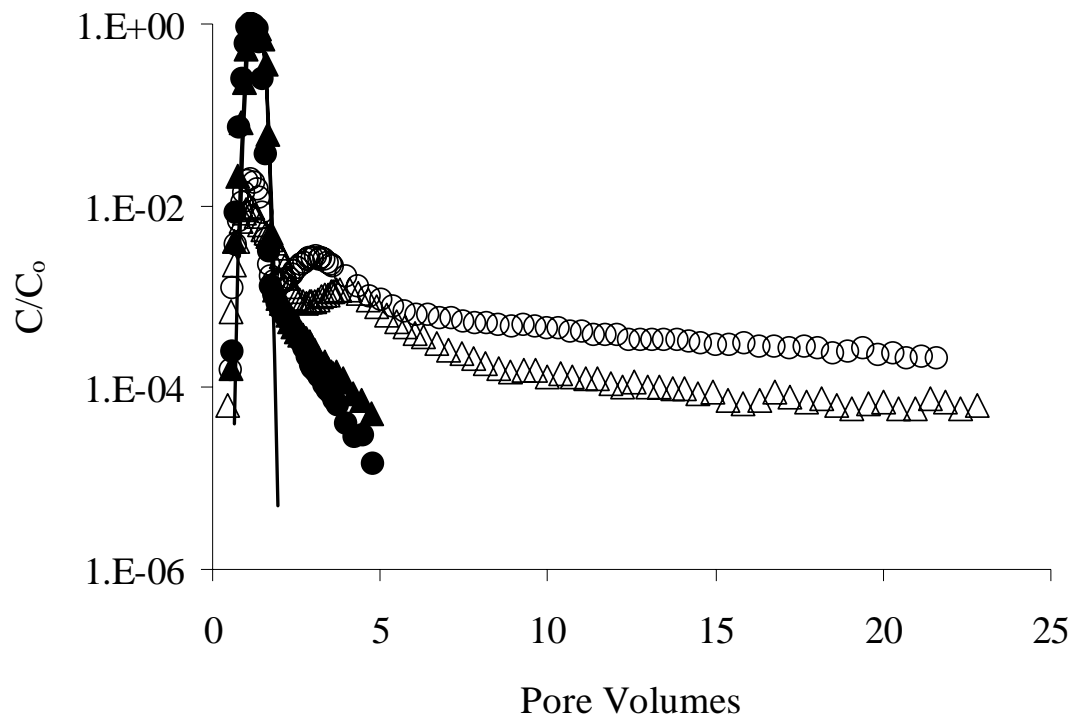


Figure BF-1d. Observed breakthrough curves for duplicates (circles versus triangles) of conservative tracer experiments (closed symbols) and bacteria experiments (open symbols) using “live” *E. coli* cells in coated quartz sand at low ionic strength. The solid line represents the best fit of the model to the conservative tracer duplicates.

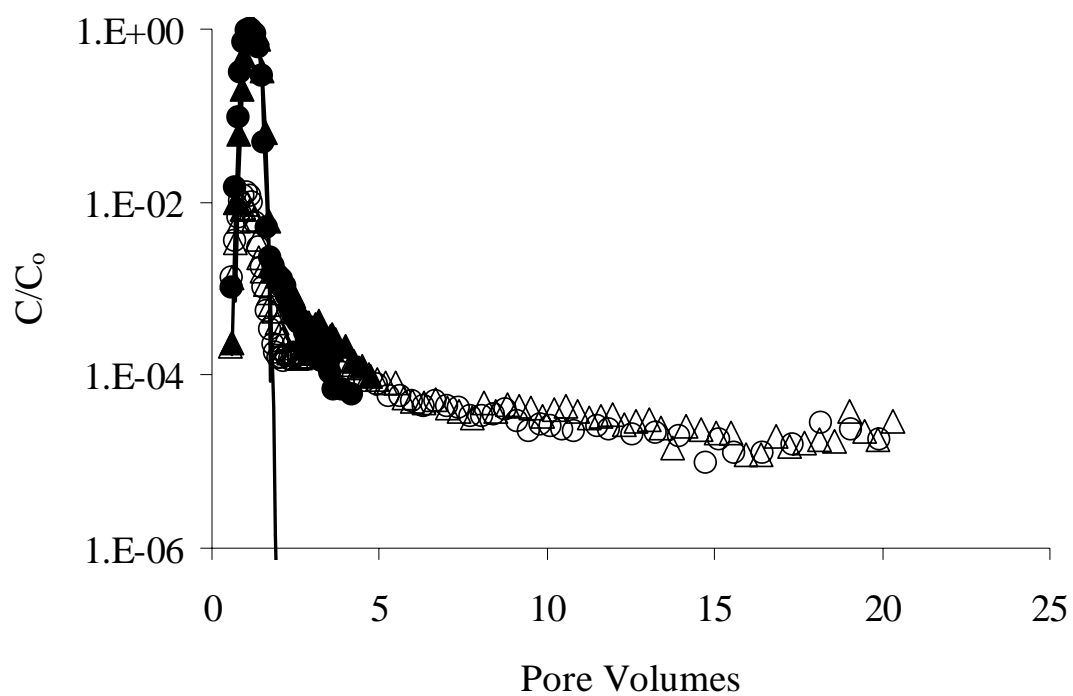


Figure BF-1e. Observed breakthrough curves for duplicates (circles versus triangles) of conservative tracer experiments (closed symbols) and bacteria experiments (open symbols) using “dead” *E. coli* cells in coated quartz sand at low ionic strength. The solid line represents the best fit of the model to the conservative tracer duplicates.

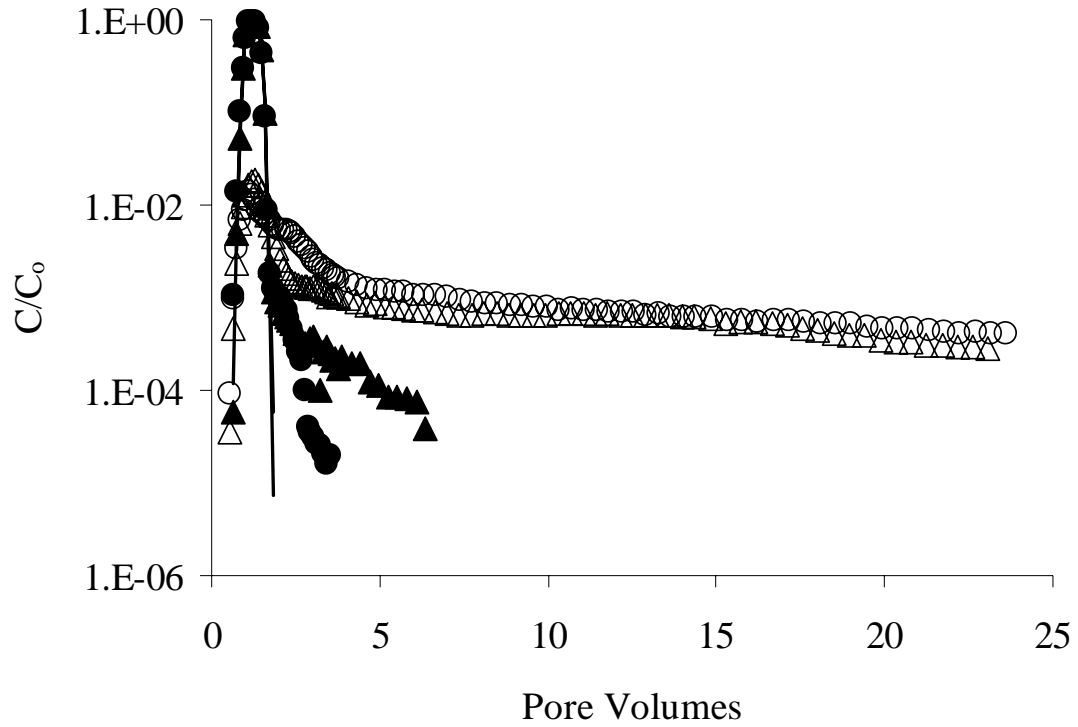


Figure BF-1f. Observed breakthrough curves for duplicates (circles versus triangles) of conservative tracer experiments (closed symbols) and bacteria experiments (open symbols) using “live” *E. coli* cells in coated quartz sand at high ionic strength. The solid line represents the best fit of the model to the conservative tracer duplicates.

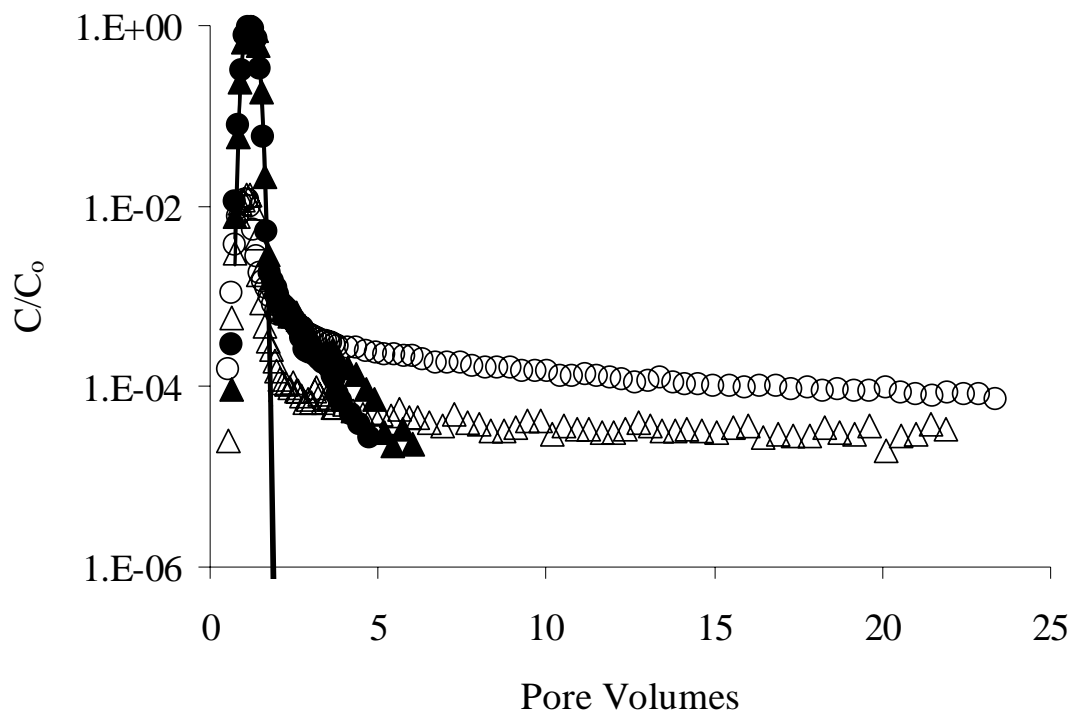


Figure BF-1g. Observed breakthrough curves for duplicates (circles versus triangles) of conservative tracer experiments (closed symbols) and bacteria experiments (open symbols) using “dead” *E. coli* cells in coated quartz sand at high ionic strength. The solid line represents the best fit of the model to the conservative tracer duplicates.