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THE IMPACT OF MICROBIAL POPULATION DYNAMICS ON THE TRANSPORT AND BIODEGRADATION OF ORGANIC COMPOUNDS

by

Susannah Kathleen Sandrin

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Susannah Kathleen Sandrin entitled The Impact of Microbial Population Dynamics on the Transport and Biodegradation of Organic Compounds

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Mark L Brusseau	17 July 2001
Dr. Mark L. Brusseau	Date
[signature]	7/17/01
Dr. Raina M. Maier	Date
[signature]	7/17/01
Dr. James L. Walworth	Date
	Date
	Date

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Mark L Brusseau	30 July 2001
Dissertation Director Dr. Mark L. Brusseau	Date

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DEDICATION

This work is dedicated to Mrs. Naomi Detty, my grandmother and my hero, who showed me that anything is possible.

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ABSTRACT

The impact of microbial population dynamics on the biodegradation and transport of organic compounds was evaluated in this study. At the laboratory-scale, results from miscible-displacement studies demonstrated that transport and biodegradation behavior in systems with increasing biologic diversity and population density variation was considerably more variable. Biokinetic parameters associated with biodegradation of the target compound were found to be considerably different in batch versus flow-through systems. While growth rates were always higher in the flow-through systems, the impacts on microbial lag and cell yield were opposite in different soils. In homogeneous sand, microbial lag was longer and column cell yields were larger than values reported under batch conditions. However, in more heterogeneous soils, microbial lag was shorter and column yields were smaller in the flow-through systems. This was determined in part using a one-dimensional contaminant transport and biodegradation model that incorporates the effects of microbial lag, inhibition, bacterial transport and nonuniform distribution of microbes, which was developed as a part of this study.

In the second part of this study, a contaminant transport and biodegradation model incorporating linear biodegradation was applied to recovery data from small input pulses of biotracers at the field scale. One field site was low in oxygen and fairly homogeneous. The other had been subjected to a surfactant flush that enhanced oxygen concentrations, and thus microbial population densities, near the injection wells. Application of this model allowed for quantitative determination of the spatial distribution of microbial activity at the field sites.

INTRODUCTION

PROBLEM EXPLANATION

The study of transport and biodegradation of organic contaminants through and in porous media is critical for prediction of contaminant fate in the subsurface and the design of effective, efficient, and inexpensive remediation strategies. Factors physical, chemical, and biological in nature are implicated in influencing these processes at the laboratory and field scale. While many physical and chemical factors have been elucidated, the impact of many microbial factors and growth kinetics are less understood.

There are too many factors to discuss all of them in detail in this text, however, the primary factors and their general effects are included. Physical factors may include soil texture, contaminant size, and heterogeneity of soil, in the form of aggregates, layered porous media, or inclusions of low hydraulic conductivity. Chemical factors may include sorption, and biodegradability (as controlled by the type of compounds present). Biological factors may include microbial growth, microbial lag, transport of bacteria as controlled by adhesion and detachment of microorganisms to the porous matrix, inhibition, and spatial variability of organisms.

Incorporation of the controlling parameters for a system experiencing contaminant transport and biodegradation is critical to the predictive capabilities of a mathematical model. However, biological factors such as metabolic lag, inhibition, and bacterial transport are often not incorporated into contaminant transport and biodegradation

models. In the case of bacterial transport models, our limited understanding of the relative importance of the (numerous) factors that influence this behavior prevents inclusion of a mechanistic model. Other factors have not been included until recently because they were not considered to be important (microbial lag), or the occurrence of the process was uncertain (inhibition). However, the impact of microbial lag was demonstrated to be significant under certain conditions in a previous work by this author (Sandrin et al., 2000).

Predictive capabilities of mathematical models are also highly dependent on utilization of the correct values for controlling factors. Biokinetic values obtained from batch studies are often used in coupled contaminant transport and biodegradation models. The assumption that these parameters are the same in flow-through systems is an area of controversy, and is tested in this body of work. Finally, accurate estimates of the spatial distribution of degrader organisms is critical for obtaining useful modeling results. While this information is often difficult to determine, modeling results presented herein describe a method by which it can be more accurately described.

MICROBIAL GROWTH

Bacterial growth curves are generally divided into 4 phases: 1) the lag phase, 2) the exponential (or logarithmic) growth phase, 3) the stationary phase, and 4) the death phase (Henrici, 1928; Bailey et al., 1986; Maier et al., 2000). These phases can be further subdivided into seven, as summarized by Henrici (1928) into an initial stationary phase

(no increase in cells), the lag phase (positive acceleration of growth rate), the logarithmic growth phase, phase of negative acceleration, the maximum stationary phase, the phase of accelerated death, and the logarithmic death phase. However, as will be discussed later, the transition phases between the four classical phases of lag (no growth), exponential growth, stationary and death are likely due to the variability in the microbial community and are not true phases for describing growth of one bacterial cell.

Generally speaking, the lag phase is the phase of adjustment of a microorganisms when external conditions are altered, or in other words, the phase prior to significant growth of the population of micrrorganisms. The exponential growth phase is the period of maximal growth rate of the microbes, when ample nutrients are available. The stationary phase is a period of no net change in cell numbers that occurs when the rate of increase in cell numbers equals the rate of cell loss due to death. This can be triggered by a lack of nutrients in the growth medium, or due to the buildup of a toxic substance. The death phase describes the phase when death of the microbes exceeds any growth. The phases of primary interest to the research discussed herein are the lag phase and the exponential growth phase. As such, these will be discussed in further detail.

The lag phase

The concept of the lag phase has been around since the early 1900s. The phenomena of microbial growth and the preceding lag phase was discussed in a review article summarizing work by Drs. Waksman, Buchanan and Lane-Claypon (1928). This growth phase is most often studied by food scientists, who are interested in predicting lag

times and in elongating the lag phase to prevent food spoilage (Baranyi et al., 1994). This phase is also studied by microbiologists, biochemists and biochemical engineers in an effort to predict mechanisms responsible for microbial lag. In order to achieve these goals, the impacts of medium composition, cell properties and environmental parameters have been investigated. These parameters include growth substrate, initial substrate concentration, age of the cell culture, starvation time of the culture prior to inoculation, cell volume, initial inoculum density, aeration and CO_2, salt content, temperature, and pH (Henrici, 1928; Walker, 1932; Rabotnova et al., 1959; Duncan et al, 1963; Lankford et al., 1966; Kawashima, 1973; Novitsky et al., 1976; Amy et al., 1983; Smith, 1985; Thayer et al., 1987; Skinner et al., 1994; Davey, 1991). An additional cause of lag in bioengineered systems could be a lag prior to growth due to gene transfer from an introduced, donor organism to an indigenous, recipient microorganism (Di Giovanni et al., 1996).

As early as 1928, work had been performed demonstrating that the lag period of a culture was shortened when the inoculum size was increased (Henrici, 1928). From this, it was concluded that the cause of microbial lag was due solely to cell properties and not medium properties. However, just 4 years later, Walker (1932) investigated the impact of CO_2 on the lag phase of Eschericia coli, based on previous research that showed a buildup of CO_2 in cells prior to cell division. Cultures were aerated with CO_2-free air, causing them to remain in lag phase, while regularly aerated cultures entered exponential phase. Upon aerating the cultures with air containing CO_2, the cultures had a 'normal' lag phase

prior to the onset of growth.

A review article in 1959 by Rabotnova et al. discussed a number of factors that had been found to affect the length of the lag phase of a culture. The primary external factor was stated to be medium composition, in terms of the type of substrates present as well as the concentration of the substrate. This concept is still commonly accepted today, and it is further assumed that if the concentration of a substrate is too low, enzyme induction leading to substrate consumption will not occur at all, such that the cells will not grow on the substrate at all (Rittmann, 1992). Physicochemical factors, such as pH and salt content, were considered to influence the lag phase of a microorganism, as well as other environmental factors of temperature and aeration. Finally, factors corresponding to cell activity were known to affect the length of the lag phase, including the age and growth stage of the cells removed from a culture and added to a new one. It had also been observed that cell dimensions of some microbes increased in the lag phase, and then decreased during the exponential growth phase.

Investigators working with *Salmonella typhimurium* studied the impact of salt content, pH and temperature (Thayer et al., 1987). They found that salt content (NaCl) was the primary factor influencing lag times for the organism over a pH range of 5 to 7, temperatures from 19 to 35°C, and salt contents of 0 to 5%. Additionally, the rate of growth decreased with increasing salt content, with the maximal rate occuring at 0.5% NaCl. However, the range of pH and temperatures studied do not encompass extreme values, so the dominating effect of NaCl content may have been selected for.

The inoculum density of a culture has also been shown to impact the length of the lag phase.. Lankford et al. (1966) determined that inoculum dependent lag is due to the time needed for cells to synthesize enough of a diffusible metabolite necessary for growth to reach.. Thus, growth and division would not proceed in certain cultures until a critical external concentration of was achieved.. They found that in a chemically defined culture (with no metabolites added) the lag time was inversely related to the inoculum density of the culture, but in cultures that were amended with filtrates of cultures (containing metabolites), the lag time was independent of inoculum density.

Another factor, especially important in environmental systems, is the physiologic 'age' of the inoculum. This effect was investigated on *Pseudomonas fragi* (Duncan et al., 1963), where it was found that cells harvested from the exponential phase experienced the shortest lag when reinoculated into a growth medium versus cells in a resting phase (i.e., no carbon source) which had the longest phase. Furthermore, this effect is increased with the length of time that bacteria are subjected to starvation conditions.

The effect of physiologic age is important because of the extreme environments that soil, aquifer, and marine bacteria survive in. Starvation is the most common situation for aquifer bacteria, so the 'dormancy state' of bacteria prior to growth is most likely a protective mechanism. A series of studies by a group of researchers at the University of Oregon attempted to elucidate this behavior and the mechanisms responsible for it. They found that the length of the lag phase of a psychrophilic marine organism (a Vibrio sp., with the same inoculum density) following starvation was linearly related to the

starvation period for periods of up to 6 weeks. However, for periods of 8.5 weeks or more, remaining cell numbers had decreased such that this relationship was no longer valid (Amy et al., 1983).

It was hypothesized that this effect may be due to the fact that average cell size decreases as starvation time increases, and a minimum cell volume is needed prior to cell division. Novitsky et al. (1976) found for the same species that after 1 week of starvation cells shrank to small rods, and after 6 weeks of starvation the cells were small cocci. The size reduction of the cells was most rapid in the first 2 days, but continued for the first three weeks of starvation. In a second study with this organism, Novitsky et al. (1977) found that during the first week of starvation, cell numbers increased up to 800% over the initial number of cells concurrently with a decrease in cell size and a decrease in total DNA. Therefore, it was assumed that the cells had stored up complete nuclear bodies during growth and then packaged them into complete smaller cells upon starvation conditions. Upon reinoculation into a growth medium, cells enlarged prior to dividing.

In engineered bioremediation systems, a potential cause of lag could be due to the transfer of genes from inoculated, donor organisms to indigenous, recipient organisms. This can be particularly significant in co-contaminated (with metals and organics) sites. In one study, soil contaminated with 2,4-dichlorophenoxyacetic acid (2,4-D) and mercury was inoculated with a donor organism which contained a plasmid encoding for mercury resistance. Significant populations of transconjugates arose, composed of indigenous 2,4-D degraders containing the mercury resistance plasmid. At one week, there was not a

significant increase in mercury resistant organisms (due to gene transfer), but by two weeks, the population had increased by an order of magnitude, concurrently with the onset of biodegradation (DiGiovanni et al., 1996). Therefore, the cause of lag in biodegradation can be due to natural causes associated with the system surrounding the organism, the type of organism, and due to transfer of genes from inoculated organisms in engineered sites.

The exponential growth phase

Bacterial growth is described as exponential, or logarithmic, because it occurs via division of cells (from one cell to two smaller cells) such that the increase in cell numbers in a culture during this period is exponential. The rate of increase in cells (specific growth rate) during this growth phase is equivalent to the slope of the line obtained on a plot of time (ordinate) versus the natural log of cell numbers or cell mass (abscissa).

The specific growth rate is also subject to change depending on many of the same factors that influence lag time. These include the cell factors of the size, age, and previous history of the inoculum (Henrici, 1928; Harvey et al., 1967; Skinner et al., 1994). Environmental factors of temperature, pH, salt content, and growth environment (liquid culture versus porous medium) have also been shown to influence growth rate (Henrici, 1928; Smith, 1985). For example, the growth rate of a strain of *Escherichia coli* was shown to be half as fast in porous media and the lag time was twice as long as

compared to growth in liquid culture (Sharma et al., 1993). Finally, the substrate concentration and medium composition (in terms of carbon, oxygen, and other limiting nutrient concentrations) is a crucial variable which influences the specific growth rate of a culture (Henrici, 1928; Rabotnova, 1959; Bailey et al., 1986; Maier et al., 2000).

Microbial growth models

The most general division of growth models can be into empirical versus mechanistic models. A review article by Barford et al. (1978) discussed the general types of growth models and the advantages and disadvantages associated with these types of models. The role of empirical models is to identify the important variables in a system, without including the internal interactions of the variables and how they cause overall behavior. These models can only be used to optimize a system. Mechanistic models postulate as to how system variables interact to produce overall behavior, and are able to predict behavior, rather than just optimizing the model to fit the behavior. The choice of the type of model used should, of course, relate to the knowledge available to the researcher. A stochastic model, is in some sense, a compromise between these two models. It assumes a variation of behavior about a mean value, and therefore requires knowledge of mechanisms, but assumes these parameters are better represented by a distribution than one value.

Other classifications of models include distributed versus segregated, and structured models. Distributed versus segregated models refers to whether mass transfer

of material from the medium to the biomass phase is considered. A segregated model separates phases while a distributed model assumes the cells are part of the culture medium. Structured models assume the cell mass is divided into two of more components cooperating to produce overall cell behavior.

Bacterial growth has been represented by many models and equations. Most simply, bacterial growth during the exponential phase is represented by the basic differential equation

$$\frac{dX}{dt} = \mu X$$

where X is biomass, t is time, and μ is the specific growth rate. The specific growth rate is considered to be a constant for a specific substrate concentration. However, the substrate concentration changes as biodegradation occurs, such that the specific growth rate also changes. The Monod equation, which supplies a relationship between the substrate concentration and the growth rate, coupled with the differential equation for exponential growth is the most widely used kinetic equation to describe bacterial growth (Bailey et al., 1986; Maier et al., 2000). The Monod relationship is an empirical relationship based on experimental observations, but it can also be related to Michaelis-Menten enzyme kinetics equation (mechanistic), assuming these are the limiting mechanism for growth. The Monod equation is

$$\mu = \frac{\mu_{max} S}{K_S + S}$$

where μ_{max} is the maximal growth rate (a constant), Ks is the half-saturation constant describing the substrate concentration for which the specific growth rate is half the maximal rate, and S is substrate concentration. This equation is capable of describing the exponential and stationary phases of the growth curve since the growth rate approaches zero as the substrate concentration decreases to zero.

When coupled with the exponential growth equation, the Monod growth equation is

$$\frac{dX}{dt} = X\frac{\mu_{max}S}{K_S + S}$$

The Monod growth equation is a hyperbolic function that simplifies to

$$\frac{dX}{dt} = \mu_{max}X$$

if $S>>K_S$ and to

$$\frac{dX}{dt} = X\frac{\mu_{max}S}{K_S}$$

if $S<<K_S$. In order to describe substrate loss using this equation, the rate of substrate loss can be related to the rate of cell growth using a yield constant (Y), using the equation:

$$\frac{dS}{dt} = -\frac{1}{Y}\frac{dX}{dt} = -\frac{1}{Y}\frac{\mu_{max}S}{K_S + S}$$

where Y has units of mass cells generated/mass substrate consumed.

The determination of the kinetic parameters of μ_{max} and K_s is generally accomplished by generating growth curves for a range of substrate concentrations and calculating the specific growth rate at each of these concentrations. The Monod equation may be linearized by a number of methods, as described in Bailey et al. (1986) for the Michelis-Menten equation, and the kinetic parameters are determined by the equation of the line generated. The most commonly used plot is the Lineweaver-Burk plot (Bailey et al., 1986; Stryer, 1995; Maier et al., 2000), which plots 1/S (ordinate) versus $1/\mu$ (abscissa). The equation of the line is

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{K_S}{\mu_{max}} \frac{1}{S}$$

such that the intercept with the abscissa equals $1/\mu_{max}$, and the slope equals K_S/μ_{max}. Unfortunately, each linearization method tends to skew the results. The growth rates closest to the maximal growth rate are generally the most accurate, yet on the Lineweaver-Burk plot, these values are clustered near the origin and the slope of the line is influenced primarily by the lower growth rates. Similar problems occur with the other linearization methods (described by Bailey et al., 1986), causing problems in determining the exact values of the kinetic parameters.

Generally, the parameters of cell yield (Y), half-saturation constant (K_S), and maximal growth rate (μ_{max}) are considered to be constants for a specific substrate and microorganism. For example, biodegradation rates of benzene and toluene in soil slurries

were described well by Monod kinetics for a certain concentration range, yielding K_S values of 12.2 and 17.4 mg/L and combined cell yield and maximal growth rate coefficients of 8.3 and 9.9 g substrate/g cells/day for benzene and toluene respectively. However, concentrations above 100 mg/L were inhibitory and above 250 mg/L no growth was observed at all, such that traditional Monod kinetics could not describe biodegradation outside of this concentration range. Additionally, these 'constants' can change under certain conditions (Alvarez et al., 1991). Kelly et al., 2000 found that a *Pseudomonas putida* culture growing on toluene had a cell yield of 0.63 in batch systems, whereas the cell yield was 1.1 in continuous cultures. Additionally, they observed that the yield decreases as the concentration increases.

Similarly, Monod kinetics were also found to vary widely in a phenanthrene system, where volatilization and partitioning were significant (Guha et al., 1996). Phenanthrene biodegradation was studied in a mixed culture of phenanthrene degraders, and a model was proposed to determine the kinetic parameters of μ_{max}, K_s and Y. The model implemented a Monod growth kinetic equation that was fit to the data using a nonlinear regression technique, and it accounted for volatilization and partitioning of the component onto the apparatus and the biomass present (values related to these processes were determined independently). When these processes were not accounted for, the estimated parameters were off by as much as 50%. Furthermore, although the cell yield determined from the model could be determined within a small range (estimated value of 0.392), the values for K_s and μ_{max} (estimated values of 0.0898 mg/L and 0.0011 $hour^{-1}$

respectively) were found to vary widely.

In fact, Monod parameters in the literature are often associated with a large variance. The cause of this variance was examined by Liu et al. (2000). The consensus in this paper is that the variability is due to three primary factors: culture history, kinetic assay procedure, and linear parameter correlation. Generally speaking, the correlation coefficients between the parameters are over 0.9 for all cases, and as the value of this coefficient increases, so does the variability. The relative standard deviation levels off when sample numbers are large (greater than 60). However, obtaining 60 samples (in triplicate) would require 180 growth curves at 60 different initial substrate concentrations, which would be incredibly labor-intensive.

Incorporating the effects of inhibition, toxicity, and multiple substrates

There are numerous cases of inhibition of growth and/or metabolism of microbes by substrates and/or products of biodegradation (Hinshelwood, 1946; Holzberg et al., 1967; Andrews, 1968; Aiba et al., 1969; Bailey and Ollis, 1986; Lenbury et al., 1987; Koesnander et al., 1990; Rittmann, 1992; Belfares et al., 1995; Bainotti et al., 1996; Maier et al., 2000). An inhibitor is defined as a substance that decreases the rate of a biochemical reaction (Mahler, 1966). At high concentrations, practically any substrate can be inhibitory. The process of inhibition often plays a very important role in a cell. Inhibition of cellular functions by products of biodegradation provides a control mechanism by which a relatively constant intracellular environment can be maintained.

From a biochemists' point of view, inhibition can be classified primarily as competitive, uncompetitive or noncompetitive (Mahler, 1966; Parkin and Speece, 1982; Lehninger, Nelson and Cox, 1993). In competitive inhibition, an inhibitor may resemble the substrate sufficiently that it binds to the active site of the enzyme. According to Garcia et al. (2000), competitive inhibition is the most common type of inhibition. This is described by the following equation for specific growth rate

$$\mu = \frac{\mu_{max} S}{K_S(1 + \frac{I}{K_I}) + S}$$

where μ = specific growth rate, μ_{max} = maximal growth rate, S = substrate concentration, K_I = inhibition constant, I = inhibition concentration, and K_s = half-saturation constant for the substrate. The compound formed is called a dead-end inhibitor if the complex cannot be converted to a product, or a competitive inhibitor if the rate of conversion to a product is much slower than would occur in the absence of an inhibitor. For the process of uncompetitive inhibition, the inhibitor binds with the enzyme, effectively lowering the amount of enzyme that is available to bind with the substrate. For the most part, uncompetitive inhibition occurs in systems with more than one substrate. This type of inhibition can be described by the equation

$$\mu = \frac{\mu_{max} S}{K_S + (1 + \frac{I}{K_I})S}$$

where the parameters are the same as in the above equation. The third type of inhibition, noncompetitive inhibition, occurs when the inhibitor binds to the enzyme at a second site on the enzyme structure. This binding slows the conversion of substrate to product, but it does not prevent binding of the substrate to the enzyme. There are three subclasses of noncompetitive inhibition; hyperbolic, partial, and pure noncompetitive. The basic form of the noncompetitive inhibition equation is

$$\mu = \frac{\mu_{max} S}{(K_S + S)(1 + \frac{I}{K_I})}$$

where again, all of the parameters are the same as those in the equations shown above. All of these types of inhibition mechanisms can be described by altering the Monod growth equation to incorporate an inhibition constant and the inhibition concentration. On a Lineweaver-Burk plot (a plot of the reciprocal of reaction rate and the reciprocal of substrate concentration), the slope and/or the intercept are altered by factors of $(1 + I/K_I)$, where I = inhibitor concentration and K_I is a constant (the inhibitor concentration at which the reaction rate is half the rate achieved when the inhibitor is not present).

Substrate inhibition is generally modeled using a function often referred to as the Haldane function (Andrews, 1968; Bailey and Ollis, 1986; Koesnander et al., 1990; Rittmann, 1992; Belfares et al., 1995; Bainotti et al., 1996; Maier et al., 2000).

$$\mu = \frac{\mu_{max}}{K_S + S + \frac{S^2}{K_i}}$$

Andrews (1968) pointed out that the Monod model alone is not valid for substrates which limit growth at low concentrations or are inhibitory at higher concentrations, and discussed the practicality of using the Haldane model for predicting biodegradation of waste streams containing compounds including phenols, thiocyanates, nitrates, ammonia, and volatile acids. Furthermore, Andrews (1968) coupled the Haldane function with the differential growth equations for batch and continuous cultures and performed numerical simulations to determine the effects of substrate inhibition. The primary results of inhibition (as predicted by this model) were an increase in the lag time in a batch system, and process instability in a continuous culture under some conditions. When two substrates are present (such as in co-metabolism), a Michelis-Menten expression incorporating competition of 2 substrates for a single enzyme system may be combined with the inhibition expression (Bailey et al., 1986; Kelly et al., 2000).

Inhibition can also be caused by other substances/compounds in the environment. Metals are a classic example of inhibitory substances that are present in the surroundings and are not created or degraded by the cells. Metals can bind to enzymes and cause a number of different types of inhibition. These systems are generally represented by a competitive, non-competitive or a combination equation to account for inhibition (Jin et

al., 1996; Nakamura et al., 2000).

Product inhibition is a more elusive process than substrate inhibition since the product that is inhibiting growth/metabolism is more difficult to isolate. Additionally, a number of different models are needed to account for the diversity of product inhibition mechanisms. Of the mechanistic models, the most common way to account for inhibition is to multiply the growth rate by a factor of the type $(1 + I/K_I)$, as described above. Bailey et al. (1986) cite a case where anaerobic glucose fermentation by yeast was treated by Aiba, Shoda, and Nagatani with a specific-growth function of the type

$$\mu = \mu_{max}\left(\frac{S}{S + K_S}\right)\left(\frac{K_P}{K_P + P}\right)$$

This is the classical noncompetitive inhibition model, and versions of it have been used by many researchers to fit their experimental data (Aiba et al., 1969; Lenbury et al., 1987; Koesnander et al., 1990; Belfares et al., 1995; Bainotti et al., 1996). Other equations, including linear inhibition models (Hinshelwood, 1946; Holzberg et al., 1967; Belfares et al., 1995) and empirical inhibition models (Aiba et al., 1968; Luong, 1985; van der Meer et al., 1993) have also been used to simulate product inhibition behavior.

Researchers from the University of Minnesota took a different approach to describing inhibitory mechanisms within a microbiological system, and instead concentrated on the fate and concentration of the inhibitor in the system. In one early article (Ramkrishna et al., 1967), they listed four possible interactions between an inhibitor and active biomass; 1) the inhibitor is consumed by the interaction with biomass

(i.e., it becomes permanently associated with the active biomass), 2) the inhibitor remains present in the system indefinitely once formed, 3) the concentration of the inhibitor increases in the system as it is produced by the biomass, or 4) there is a lag between formation of nonviable mass and release of additional inhibitor into the medium. These four cases were all incorporated into the differential equations for cell growth, substrate loss, and inhibitor concentration (using a non-structured model) and solved numerically for a number of different parameters. For example, case 1 was incorporated by including a term describing the rate of deactivation of the cells linearly related to inhibitor concentration. Case 3 was also described using a structured model, in which the cell mass is divided into nucleic acid mass and protein mass. This structured model was able to predict lag phase and was more realistic in terms of the shape of the growth curve. However, there was no experimental data presented in this paper, as well as no examples of actual cases where these mechanisms occur.

In 1970, researchers from the same institution presented a similar paper in which possible cases of inhibition production and cell growth were considered (Fredrickson et al., 1970) and a noncompetitive inhibition model was proposed to describe enzyme kinetics in their system.

Biofilm growth models

Bacteria residing in soil systems generally exist in small colonies attached to soil surfaces (Baveye et al., 1989; Baveye et al., 1992; Rittmann, 1993; Peyton et al., 1995;

Hendry et al., 1997; Maier et al., 2000). The net accumulation of bacteria on the surface of porous media is controlled by 4 processes: growth, deposition, decay, and detachment of bacteria. In bioengineered systems (with high substrate flux), bacterial colonies can spread and grow in films that spread across a matrix, called continuous biofilms. Biofilms are defined as 'microorganisms and extracellular polymers associated with a substratum, or solid surface,' (McFeters, 1984). The formation of continuous biofilms is common in wastewater treatment systems, fixed bed bioreactors, and simulated soil systems (glass beads) containing high levels of nutrients (such as near injection wells).

The growth of bacteria in continuous biofilms has been shown to clog pores and decrease permeability of porous media, including media composed of spherical glass beads (Cunningham et al., 1991) and flat plates (Sharp et al., 1999). In one of the studies using glass beads, researchers inoculated 4 different sizes of glass beads (1 mm, 0.7 mm, 0.54 mm and 0.12 mm) with *Pseudomonas aeruginosa* and subjected the systems to a continuous pulse of glucose media. Porosity in these systems decreased between 50 and 96%, and permeability decreased between 92 and 98%. Furthermore, they determined that the maximum biofilm thickness was related to the initial permeability of the media, and that the final permeability of all the systems was approximately the same.

The primary factors affecting biofilm thickness and activity are investigated in order to optimize engineered systems and bioremediation schemes. Rittmann et al. (1992) compared the effect of substrate flux and biofilm detachment on the active amount of biomass growing in biofilms in a fluidized bed reactor. In this system, the effect of

changing the cell detachment rate (by increasing the shear stress on the biofilm) was found to be negligible compared to substrate flux. As substrate flux was increased in this system, new cells were generated at a faster rate such that the ratio of inactive cells decreased. This was accompanied by an increase in yield and a decrease in oxygen consumption.

Oxygen availability has also been found to affect the bioactive zone in a biofilm. For cells grown on a tube-shaped membrane in a stirred reactor (such that the substrate source was on the 'outside' of the continuous biofilm and the oxygen source was on the 'inside' of the biofilm), the bioactive zone moved with the oxygen source used. When air was injected into the tube, the bioactive zone was in the zone closest to the membrane, but when pure oxygen was injected into the tube, the bioactive zone shifted away from the membrane and closer to the substrate source (Wanner et al., 1994).

Biofilm models have been developed by engineers, predominantly to describe bacterial growth of attached microbes in fixed bed bioreactors, wastewater treatment systems and for drinking water filtration systems. A number of continuous biofilm models have been successfully applied to inoculated porous media systems (Widdowson et al., 1988; Cunningham et al., 1991; Kinzelbach et al., 1991; Rittmann et al., 1992; Furumai et al., 1992; Peyton et al., 1995; Cunningham et al., 1995; Wanner et al., 1995), generally under high substrate loading conditions and in experimental conditions using glass beads or flat surfaces. More recently attempts have been made to apply these models to systems of discontinuous biofilms or colonies of differing geometries

(Rittmann et al., 1999; Noguera et al., 1999).

Biofilm models all account for three predominant processes; substrate consumption, growth (and subsequent expansion) of biomass, and molecular diffusion of substrate into biomass. A majority of these models assume that diffusion is only in 1 direction, either in a planar (Wanner et al., 1985; Widdowson et al., 1988; Taylor et al., 1990; Rittmann et al., 1991; Kinzelbach et al., 1991; Cunningham et al., 1995; Wanner et al., 1995) or radial (Chang et al., 1987) geometry, and that the biofilm is homogeneous (in terms of density and thickness). Monod growth kinetics are assumed in virtually all biofilm models to describe substrate consumption.

The main area of controversy and confusion in biofilm models is the incorporation of bacterial detachment. This process has been neglected in some biofilm models altogether (Cunningham et al., 1995; Wanner et al., 1995). In other models, biomass detachment has been represented as a first order process with respect to cell mass (Chang et al., 1987; Rittmann et al., 1992; Peyton et al., 1995) or using other empirical equations (Wanner et al., 1996). Alternatively, application of the model used by Peyton et al. (1995) to data from an inoculated porous media reactor found that the detachment rate coefficient varied as a function of substrate loading (i.e., influent concentration). Biomass detachment has been incorporated as being a function of shear stress (Taylor et al., 1990), biofilm thickness, and biofilm density in some models, while other models assume intermittent sloughing of biofilm occurs when substrate concentrations in the depth of the biofilm reach a critical minimum (Wanner et al., 1985). Furthermore, the

subsequent reattachment of bacterial cells downstream is often not accounted for.

In addition to cell detachment and attachment, Wanner et al. (1996) addressed the need for inclusion of the processes of turbulent diffusive transport of substrate into the biofilm (due to expansion and shrinking of the film), transport of particulates (i.e., bacteria) in biofilms due to diffusive forces, and changes in biofilm porosity. This is especially important for systems of mixed cultures, where mixing of types of organisms occurs and cannot be accounted for in traditional models. Although these advances are promising for application of continuous biofilm models to soil systems, the main concern with these models is whether soil organisms occur in continuous biofilms, even in bioengineered systems. Soil particles are generally much smaller than the porous media used in the biofilm studies discussed herein, and the substrate loading is rarely as high in natural systems.

The problems concerning the application of biofilm models to bacterial growth and transport in natural soil systems have been addressed in a number of articles (Baveye et al. 1989 and 1992; Rittmann, 1993; Peyton et al., 1995; Reddy et al., 1996; Hendry et al., 1997). A review article by Rittmann (1993) discussed this controversy; that some scientists assume a continuous biofilm coats the porous media and should be modeled as such, while others assume (and have observed) soil bacteria exist in patchy aggregates rather than in biofilms. His research concluded that under low substrate load situations (such as groundwater and drinking water treatment systems) bacteria do not exist in continuous biofilms, but under high-load situations, they do exist in continuous biofilms.

The application of a biofilm model to a flow-through soil-column system under low-nutirent loading was compared to that of a simplified first-order biodegradation model (both coupled to contaminant transport models). Under these conditions, it was concluded that the simplified batch model was more representative. Also, the average cell density in this experiment was calculated to be 1 bacteria per sand grain, shedding doubt on the presence of a biofilm.. Reddy et al. (1996) agreed with the inapplicability of biofilm models to groundwater systems because of low nutrient availability and laminar flow regimes. These researchers additionally cited the work of Vandivivere and Baveye (1992) who used scanning electron micrograph results to show that bacteria do not coat sand surfaces under groundwater conditions.

Some researchers have attempted to visualize soils inoculated with cells to solve the 'biofilm dilemna.' Shales et al. (1987) studied the attachment of Micrococcus luteus (NCIB 8553) to sand in shake flasks. Using different concentrations of cells, the maximum cell density obtained was 2×10^9 cells/g sand, which only covered about 20% of the sand surface. Additionally, sands inoculated with Klebsiella oxytoca were visualized using scanning confocal laser microscopy and although cells were seen in pore throats, less than 0.5% of the surface was covered with cells (Hendry et al.,1997).

Although traditional 'biofilms' may not form in soil systems by expanding to form a continuous mass, Rittmann (1993) discussed that the length of the 'biofilm' can be significantly extended by deposition of detached bacteria downstream. This is a very important process in discontinuous biofilm development, but as discussed above, it is

agreed in the biofilm modeling literature (and other bacterial transport models) that methods for incorporating bacterial deposition and detachment vary and are empirical in nature because the primary controlling mechanisms for these processes are not known (Wanner et al., 1986; Rittmann 1993; Peyton et al., 1995; Wanner et al., 1995).

'Biofilm' models assuming alternate geometries to continuous, flat biofilms have been generated in an attempt to simulate bacterial growth in systems that do not contain 'traditional' biofilms. Rittmann et al. (1999) developed a multi-species bacterial transport and growth model for a cylindrically shaped biofilm attached to a 'flat' surface. They found that sub-cluster niches for slow growing species were reduced in size for this geometry compared to a 1-dimensional biofilm model, with substrate entering from one side only. In another 3-dimensional model developed by Noguera et al. (1999), growth of 2 competing species on a flat surface was simulated. Predictions found different geometries of the biofilm depending on substrate conditions.

Many contaminant transport and biodegradation models assume bacteria are evenly distributed in the soil system, and do not account for the distribution of bacteria in clusters or biofilms. However, research by Rittmann and Peyton et al. (1995) has shown that under low substrate conditions, although bacterial microcosms do not exist in continuous biofilms, they can still often be represented by biofilm models. It is hypothesized that diffusion constraints under low substrate conditions are negligible, since these substrate levels are not adequate to support large populations capable of forming thick biofilms, and thus any microcosms biofilms are fully penetrable. Thus, the

biodegradation capacity of the system is the same, regardless of the geometry and distribution of the bacteria.

Therefore, the problems associated with application of traditional biofilm models to discontinuous biofilms are not as significant in trying to predict substrate utilization (on a large-scale) as they are in accounting for the distribution of bacteria in a system due to deposition of detached organisms downstream. This phenomena was noted by Peyton et al. (1995). These researchers inoculated a column packed with 1-mm glass beads with Pseudomonas aeruginosa and subjected it to low substrate loading (2.3 or 19.7 mg/L) conditions. Using a traditional biofilm model, but including cell transport and attachment and detachment (using first order reactions with respect to biomass present at a location), they were able to predict contaminant destruction and nutrient composition, but not the cell distribution within the system.

Modeling microbial lag

Mathematically speaking, the lag phase is described as the x-axis intercept, on a growth curve, of the line tangent to the inflection point (Zwietering et al., 1990). The modeling of microbial lag in batch systems is most frequently discussed by food scientists/microbiologists and biochemical engineers.

The Arrhenius (linear and non-linear) equation has been used frequently to incorporate the impact of environmental factors on microbial lag time. This model is empirical, and takes the form of a polynomial. The linear Arrhenius model has the

general form:

$$T_{lag} = C_0 + \frac{C_1}{x} + \frac{C_2}{x^2} + C_3 y$$

When the linear Arrhenius model was applied to a number of published data sets to predict the effect of temperature and water activity on lag times, the model explained about 80% of variation in results for both gram-positive and gram-negative bacteria (Davey, 1991). Broughall et al. (1983) used the non-linear Arrhenius relationship to define lag time as a function of temperature and water activity. In a later model, they used an inverse square-root relationship to correlate lag times with temperature, and applied the non-linear Arrhenius relationship to relate pH and water activity (Broughall et al., 1984). In 1989, a similar form of the modified Arrhenius equation was applied to 7 sets of published growth curve data, for which the variables of temperature and water activity were examined, and the model was found to explain about 90% of the variability in the results (Davey et al., 1989). This is an empirical model, and requires 5 coefficients, as compared to 3 for the linear model.

There are a number of other empirical fitting equations of different forms that have been used to relate environmental parameters to the length of lag time. For example, the Square Root model was described (Ratkowsky et al., 1983; Smith, 1985; Adair et al.,1989) and compared with the nonlinear Arrhenius equation. This simple model contains two fitting parameters, b and T_o:

$$\sqrt{r} = b(T - T_o)$$

where r is the growth rate (1/generation time) or the inverse of the lag time, T is the temperature, T_o is a theoretical temperature at which lag time becomes infinite (a fitting parameter), and b is a regression coefficient. The nonlinear Arrhenius model was found to provide a better fit than the Square Root model for all cases (Adair at al., 1989), but the Square Root relationship was found to be superior to the linear Arrhenius relationship (Ratkowsky et al., 1983). Similarly, a quadratic function was used to predict kinetic parameters of maximal growth rate, lag time, and maximum population density as a function of pH, NaCl concentration, and temperature. These values were used to predict growth, and then compared with other empirical growth models, and found to provide an equally good fit (McClure et al., 1993).

Additionally, numerous empirical growth models have been proposed that incorporate most or all of the traditional growth phases. In food microbiology, the most commonly used empirical models are those of Baranyi and Gompertz (Buchanan et al., 1997). The Gompertz equation

$$y = a\exp[-\exp(b - ct)]$$

is in a class of sigmoidal functions which including the logistic (Wolf et al., 1992), Gompertz, Richards, Schnute, and Stannard equations. Predictive capabilities of these functions were compared with each other and with that of other empirical models by Zwietering et al. (1990). The Gompertz equation provided a better fit than the logistic,

linear, quadratic, *n*th power, and exponential models. In cases with more data, a 4-parameter sigmoidal function (the Schnute model) provided a better fit than the Gompertz function, but this function also had trouble converging at times. Additionally, the three parameters in the Gompertz equation can all be converted to a biological parameter, but the fourth parameter in the Schnute equation is a shape parameter and has no biological meaning. In a second evaluation, Garthright (1991) also determined that the Gompertz equation provided a better fit than the logistic model.

In order to use the Gompertz equation to predict growth curves for varying environmental conditions, models have been developed to relate the environmental parameters to the Gompertz variables. Gibson et al. (1988) derived a polynomial model to generate the three Gompertz parameters for different pH, temperature, and NaCl concentration. Likewise, Buchanan et al. (1990) developed a response surface analysis to generate a cubic model to generate the Gompertz parameters for the variables of temperature, pH, $NaNO_2$, and atmosphere (aerobic versus anaerobic). Alternatively, a neural network model was applied to the anaerobic growth of Shigella flexneri in order to predict modified Gompertz growth parameters (Najjar et al., 1997; Hajmeer et al., 1997). This empirical model produced correlation coefficients close to 1.0, and found that temperature was the most important environmental factor, but it did not predict actual growth curves.

The model proposed by Baranyi et al. (1993 and 1994) is an unstructured, nonsegregated growth model which describes the lag phase using an adjustment function

of the form

$$\alpha_n(t) = \frac{t^n}{\lambda^n + t^n}$$

where α describes the transition of the culture when it is transferred from pre-inoculation conditions to post-inoculation conditions, λ is the lag fitting parameter, t is time, and n is a fitting parameter to describe the curvature of the growth curve after the lag phase. This adjustment function describes the situation of the culture going from one environment to a new environment, which causes the organisms to enter the lag phase prior to biodegradation. When n was equal to 4 (meaning that the adjustment function is of the fourth order), the model fit the growth curve data better than the Gompertz function for 35 data sets. Additionally, this equation has more of a mechanistic basis. For the case where growth is described by Michaelis-Menten type kinetics, this equation becomes

$$\alpha(t) = \frac{P(t)}{K_p + P(t)}$$

which is essentially equivalent to the Monod growth equation.

Numerous mechanistic models have been shown to predict the lag phase of microbial growth for a specific organism growing on a specific substrate (Pamment et al., 1978; Andrews, 1968). These models are praised for the insight they give into the

processes that are occuring in the system, and their ability to predict behavior, rather than just be 'fitted' to the data. However, they are very specific as to the cause of lag in a system, and generally only describe the behavior of one population. While these models are very useful in predicting behavior of growth of one organism in bioreactors, they are not easily applied to environmental systems composed of numerous bacterial species and with varying mechanisms.

A deterministic model proposed by Pamment et al. (1978) suggested that the lag phase of *Saccharomyces cerevisiae* was due to the time needed to synthesize adequate levels of respiratory and glycolytic enzymes. Therefore, they described metabolism as occuring in two stages, respiration of glucose followed by the process of glycolysis accompanied by ethanol production.

Due to the complexity of environmental microbial communities and the fairly static environmental parameters (with regard to pH, temperature, salt concentration, etc.) in subsurface environments, the models described above are not generally applicable to these systems. Furthermore, a three-phase linear growth model (incorporating the lag, exponential and stationary phases of 'growth') in which the lag period of the cells was assumed to be described by a normal distribution was shown to fit the growth curves of numerous microorganisms approximately as well as the models by Baranyi and Gompertz (Buchanan et al., 1997). This model requires an input of 4 parameters: initial biomass concentration, final biomass concentration, lag time of a single cell, and the time at which the exponential phase ends for a single cell. It is proposed that the non-abrupt transition

between the lag and exponential phase is due to (or a reflection of) the biological variation of the bacterial population. This large population of cells has a lag time with a variation that can be described by a normal distribution. When tested on the growth curves of 30 or so microbe/substrate systems, the simulations from this model compared well with the real data and with simulations using the models of Gompertz and Baranyi, although the transitions between growth phases were slightly more abrupt. It is assumed that this problem could be solved by decreasing the size of the time steps in the model. This model takes into account certain physiologic behaviors of cells, and accounts for the behavior of individual cells and bacterial populations.

The idea that the growth of individual bacterial cells is variable within a culture has been tested and noted many times in the literature (Kelly et al., 1932; Kubitschek, 1966; Harvey et al., 1967; Trueba et al., 1982; Liou et al., 1997). The successful application of a normal (regular or skewed) distribution to the biological variability of bacterial cell cycles has been demonstrated previously by many researchers (Kubitschek, 1966; Harvey et al., 1967; Bremer, 1982; Trueba et al., 1982; Koch et al., 1982; Keasling et al., 1995).

BACTERIAL TRANSPORT

Bacterial transport is studied by scientists and engineers interested in the fields of pathogen immobilization in soils, introduction of microorganisms for the purpose of enhancing bioremediation in-situ, in modeling of bioreactor performance, and in

enhanced oil recovery. Therefore, the extent of literature discussing this topic is vast. However, despite the large volume of literature discussing the effect of different variables on bacterial transport through porous media, a mechanistic model that describes bacterial transport in a natural environmental setting (incorporating the many factors present in these systems) has yet to be generated. The additive, or often conflicting, effects of the many variables affecting attachment/detachment and movement of microbes makes the elucidation of the primary factors difficult in-situ and therefore makes prediction of bacterial transport even more difficult.

Bacterial transport through porous media is affected by numerous factors including cell properties, soil characteristics, and environmental factors. Soil texture, referring to the size fractions of the soil particles, is a critical factor in determining the amount of physical straining an organism will experience as it is being transported through the soil matrix. The composition of a soil is also important in influencing adhesion/attachment of microbes. Cell properties such as cell surface hydrophobicity or charge also influence the adhesion of microbes (physicochemical effects), and the shape and size of the bacterial cell being transported determines the degree of physical straining the cell is subjected to. This is further complicated by the fact that the physiologic state of a microorganism can influence these properties. Some environmental factors that have been discussed to influence bacterial transport include flow rate of the aqueous phase through the porous media, salt content, and presence of surfactants. Finally, transport of bacteria by sedimentation (density driven transport downward) may be a mechanism in

some subsurface environments.

Reversible microbial adhesion is generally thought to be influenced by three primary factors: electrostatic interactions, hydrophobic interactions, and van der Waals forces. The electrostatic force between microbes and soil particles is generally repulsive as described by Coulomb's Law:

$$F \propto \frac{q_1 q_2}{\varepsilon}$$

where q_1 is the (generally negative) charge of the microbe and q_2 is the (generally negative) charge of the soil particle, r is the distance between the two, and ε is the dielectric constant. Hydrophobic interactions occur between the microbes and the hydrophobic portions of the soil, specifically the organic matter in the porous medium. This is due to the lower energy state obtained by the system by the partitioning of the hydrophobic cell surfaces from the polar water molecules. However, the degree to which this interaction influences bacterial adhesion depends on the bacterial cell surface, the properties of the porous medium, and the solution present. Finally, van der Waals forces are generally attractive and occur due to temporary charge distributions that favor interactions of neutral molecules. Irreversible microbial adhesion refers to stronger and more permanent attachment of bacteria to surfaces. This attachment can be achieved by production of extracellular polymers that promote attachment to a surface, or with the aid of extracellular fimbriae.

In addition to these mechanisms, sedimentation of bacteria may also be a factor in

bacterial transport (Wan et al., 1995). The densities of 25 subsurface bacterial strains were measured and all were found to be greater than that of water. A mathematical model incorporating sedimentation in a water column showed that the rate of bacterial transport is almost 90% of the rate of water flow, and can be a significant mechanism for transport over the length of a few microbial generations in soil systems.

These factors have been studied extensively and are the subject of many publications summarized herein. Additionally, numerous methods have been studied to enhance microbial transport, including the use of ultramicrobacteria, introduction of surfactants, and the introduction of a substrate pulse. These methods are also discussed further. Finally, the need for more studies of bacterial transport under conditions that simulate environmental systems is discussed.

Effect of soil characteristics

A large percentage of bacterial transport experiments are conducted in saturated systems packed with clean sand or glass beads. While these experiments are useful for the isolation of non-soil effects, they are not representative of soil conditions in a vast majority of aquifers or other subsurface environments. Indeed, sorption of bacteria is minimized in sand systems compared to mixed soil systems. Mills et al. (1994) found that when sand was mixed with particles coated with iron (III), adhesion of bacteria increased. Similarly, van Schie et al. (1999) found that of 4 species, adhesion of one to glass coverslips increased when the surface was coated with iron (III), but a coating of

silicon hydrophobic groups decreased the overall number of attached cells, despite the fact that two of the four species had some hydrophobic cell properties.

Particle size, and its consequent effect on pore size, influences the rate of bacterial transport even within sand systems. Generally, this is due to increased filtration and physical straining in media containing smaller pores. In a flow-through column experiment with different sand textures, Fontes et al. (1991) demonstrated that coarse sands allowed for increased bacterial transport relative to fine-grained sands. Furthermore, they found this to be the most important variable compared to ionic strenth of the solution, cell size, and heterogeneity of porous media (in reference to existence of preferential flow paths). Similar results were reported by Hornberger et al. (1992), who conducted bacterial transport studies in two different sands, and by Sharma et al. (1994), who conducted studies in five different sand grain sizes. This effect is true also for soil systems where motility was studied, and there was no bulk flow of liquid. Barton et al. (1995) found that the rate of movement of motile bacteria was decreased by a factor of 20 when the grain size of the sand was decreased from 800 to 80 μm. Similarly, in the study by Sharma et al. (1994), penetration of non-motile and motile strains increased linearly with grain size, but above a certain cutoff value, penetration of motile strains became independent of grain size.

In addition to particle size, physical variability of soil influences bacterial transport. Harvey et al. (1993) compared laboratory scale column studies with field scale transport studies. In the column studies, bacterial transport was directly related to particle

size, and physical straining was found to be a significant factor in bacterial transport, possibly due to a migration of fine materials in the columns. From these results, it was determined that physical variability over short distances such as column lengths are enough to affect dispersion greatly, and thus, column work should not be extrapolated to field predictions due to differences in scales of heterogeneities.

In porous media containing significant quantities of clays and minerals, there are the additional factors of an increased surface area and the presence of charged surfaces, which imparts a greater potential for adhesion. The adhesion of hydrophilic bacteria was found to be enhanced in soil columns with higher clay contents by Cattaneo et al. (1997). This effect was attributed to interactions between negatively charged bacteria and positively charged clay particles and the presence of divalent cations. Huysman et al. (1993a) conducted unsaturated soil column experiments using different bulk densities and amounts of clay. An increase in the bulk density of the soil (causing a decrease in the volume of the pores by 80%) caused a significant decrease in transort of incoulated bacteria. When this sandy soil was mixed with 20% clay, transport of bacteria decreased 40 to 80%. In another paper published by these authors (Huysman et al., 1993b), it was stated that adhesion of bacteria to clay soils was greater than to sand soil in the presence of divalent ions, indicating that electrostatic interactions played a role in adhesion to clay particles.

In fractured rock, or gravel, the opposite effect has been seen. Champ et al. (1988) compared breakthrough curves for *Eschericia coli* with those of bromide and

radioactive tracers through fractured rock and found that bacteria arrival was before or concurrent with the conservative tracers, although there was significant loss of organisms. Thus, it was concluded that significant underestimates of microbial contamination exposure could occur at recovery wells located in fractured media. Bacterial transport of a *Bacillus* strain was tested in sandstone containing nutrients (Jenneman et al., 1985). Penetration rates decreased rapidly with decreasing permeability below a certain cutoff permeability. In more permeable sandstone, penetration rates were approximately constant, indicating that cells could move through the material in a sort of piston-type flow.

The water content, or degree of saturation, of a soil greatly influences the transport of bacteria through the soil. This effect is caused by a number of factors present in an unsaturated system, but two primary reasons were identified by Powelson et al.(1998); increased interaction between cell surface and sand grains due to flow through smaller pores in unsaturated flow and due to bacterial adsorption at the gas-water interface. These researchers conducted transport studies using two indigenous bacteria collected from an aquifer on clean sand columns. There was enhanced removal of both organisms in unsaturated columns compared to saturated columns. Similar results were reported by Schafer et al. (1998), who studied transport of 2 bacteria (*Rhodococcus* sp. C125 and *Pseudomonas putida* mt2) in sand columns with varying degrees of saturation. The effect of the gas-water interface on colloid and bacterial transport has also been investigated by Wan and Wilson (1992, 1994a, 1994b, 1994c) in a number of

manuscripts. They reported that the gas-water interface caused increased retention of colloids and bacterial cells, and that the degree of sorption was controlled mainly by cell surface hydrophobicity, solution ionic strength, and particle charge sign.

Research conducted by Devare et al. (1995) on unsaturated soil and sand columns examined biodegradation in columns inoculated at the top that received only intermittent water flow. Only about 1% of the bacteria passed through soil columns and 4% passed through sand columns after 1.5 pore volumes, and biodegradation of the substrate (phenanthrene) at the bottom of the column was negligible. Balkwill et al. (1998) hypothesized that filtration of bacteria dominates bacterial movement in these systems, because water flow is due to capillary force, and this does not allow for bacterial movement. In natural systems, Kieft et al. (1998) discussed the origin of bacteria in unsaturated, buried sediments. It was hypothesized that in shallow sediments, communities are of the approximate age of pore water and systems are dominated by tranpsort of organisms, whereas in deep sediments, transport is severely restricted such that bacteria are considerably older than pore water and the systems are succession dominated.

Jenneman et al. (1986) examined sterilization by dry heat versus autoclaving of a model soil (Berea sandstone) to see the effect of autoclaving on bacterial penetration. Both sterilization methods caused a decrease in bacterial penetration time, or an increase in rate of transport, but autoclaving caused bacteria to penetrate the soil 3.5 to 6.0 times faster. The porosity and pore entrance size did not appear to be significantly affected by

either method. The primary effect of autoclaving was to cause the breakdown of clay particles (as evidenced by a reduction in levels of silica, aluminum, or potassium), although not enough to change particle porosity and cementation. Additionally, autoclaving caused a large increase in soluble chloride ion, which caused the increase in negative charge at the mineral surface, and thus, a greater electrostatic repulsion between the mineral surface and the bacterial surface.

Effects of cell properties

Cell surface hydrophobicity is considered to be a primary factor influencing cell adhesion to porous media, and thus, transport of bacteria. Huysman et al. (1993) found that migration of hydrophobic bacteria was 2-3 times slower than for hydrophilic species, and also confirmed the visualization of more hydrophobic species on the soil surface. The mechanisms responsible for hydrophobic adhesion of bacteria to soil surfaces were investigated by Williams et al. (1996) by evaluating transport of mutant strains of Pseudomonas fluorescens through quartz sand columns. Mutant cells with altered surface proteins were selected and found to exhibit enhanced retention in soil columns compared to the non-mutant strains. Results from polyacrylamide gel electrophoresis showed that these mutants were lacking a polysaccharide (O antigen) that causes increased exposure of the lipid moiety of the lipopolysaccharide. This makes the cells more hydrophobic and increases adhesion and retention of bacteria in porous media.

The transport of two different bacterial populations through a uniform porous

media was studied by Baygents et al. (1998) by looking at bacterial distributions as a function of depth in the column. The affinity of cells for the porous media (glass beads) was found to decrease with distance the cells had traveled through the column. Batch results indicated intrapopulational differences in the surface charge density of the bacteria, explaining why some cells traveled farther than others. Additionally, surface charge has been shown to influence retardation of microspheres in aquifer material (Harvey et al., 1989).

The relative importance of hydrophobic interactions versus electrostatic interactions has been investigated by a number of researchers. It is generally agreed that hydrophobicity is much more important than cell surface charge, which is often found to have a negligible effect on adhesion and transport (Huysman et al., 1993) Fletcher et al. (1979) found that bacterial attachment (to a variety of surfaces) was maximal for hydrophobic surfaces with little or no charge. Less bacteria were attached to hydrophilic surfaces with positive or neutral charges, and the least degree of attachment was apparent for hydrophilic surfaces with negative charges. The hydrophobic forces in the systems were dominant over electrostatic. Additionally, van Loosdrecht et al. (1987) compared electrostatic interactions and hydrophobic interactions of 23 bacterial species with styrene, and found that electrostatic forces were more important for cells that were less hydrophobic (more hydrophilic). These results indicate that bacterial cells generally adhere the most to hydrophobic surfaces that are not negatively charged, which is logical given the hydrophobic nature and negative charge of most cell surfaces.

In one study (Camesano et al., 1996), cell motility was found to cause an increase in cell transport and a decrease in cell attachment at low velocities, compared to non-motile cells. For motile cells, fractional retention of cells decreased by 65% when fluid velocity decreased 2.5 orders of magnitude, while retention of nonmotile cells increased with decreased fluid velocity. It was assumed that 'swimming' cells were able to avoid detachment at low velocities. At high velocities, cell motility did not affect attachment. In sandstone, cell penetration due to slow water movement was found to be 3 to 8 times faster for a motile strain than for a nonmotile strain (Jenneman et al., 1985).

The relative impact of biological factors, including motility, growth, chemotaxis and gas production, was evaluated in clean sand columns containing a growth medium, under anaerobic conditions (Reynolds et al., 1989). Motile strains were found to penetrate the sand medium four times faster than the mutant non-motile strains. Furthermore, when growth rates were higher (in the presence of higher substrate concentrations), penetration rates of motile strains increased, indicating that penetration rate is regulated by in-situ growth rate. For non-motile strains, gas-producing strains penetrated four to six times faster than non-gas producing strains. Chemotaxis was not found to play a significant role in penetration rate.

The effect of chemotaxis by motile bacteria was evaluated in porous media of different pore size and in the presence or absence of a chemical attractant by Barton et al. (1995). Bacterial distributions were not found to be significantly different in any of the systems, and thus, chemotaxis was deemed to not affect bacterial migration in general.

However, transport of chemotactic cells was found to be significantly different from nonchemotactic strains under growth conditions in the presence of a chemical gradient, but in the absence of advective flow (Sharma et al., 1993). A motile, chemotactic strain moved through a soil column in a band-like fashion (cells would build up in one section of the column before moving to the next section), while cells of a nonchemoctactic strain moved through the column in a diffuse manner.

The physiologic state of an organism is a function of what growth stage the organism is in. This state has been shown to influence the adhesion of cells and consequently, their transport through porous media. For example, Grasso et al. (1996) demonstrated that *Pseudomonas aeruginosa Olin* adhered more to a dolomite surface during the stationary growth phase. Cell surface properties changed as a function of growth state such that cells became more hydrophobic during the stationary phase. These authors cited other cases that were consistent with this one, but contrary to those of other researchers, who had noted decreased adhesion during stationary phase. Thus, the effect of the different phases on cell surface properties may be organism specific.

The transport of 19 different strains of bacteria was tested under no-growth conditions in a loam soil (Gannon et al., 1991) to determine the effect of cell size on transport. The soil columns were inoculated at the top with cells following saturation, and then water was flushed through the columns. There was a significant correlation between percentage of cells eluted and cell size. This correlation was attributed to mechanical filtration. However, in comparison, there was no correlation found between

cell transport and hydrophobicity, net surface charge, and capsule formation. In comparing the transport of two cells of similar hydrophobicity under no-growth conditions, Fontes et al. (1991) reported that the larger cells were transported to a lesser degree and they experienced more dispersion. Likewise, it was reported by Balkwill et al. (1998) that bacteria capable of producing spores would be transported more efficiently in unsaturated environments due to the smaller size of spores relative to bacterial cells.

Transport of 14 isolated bacterial strains through quartz sand was examined and evaluated based on their cell shape, under non-growth, advective flow conditions (Weiss et al., 1995). This parameter was quantified as a ratio of cell width to cell length. Cells present in the column effluent were always smaller and rounder than those in the column influent. Rod shaped cells had the largest change in cell shape.

Environmental factors

The pH of groundwater has been shown to significantly affect the transport of bacteriophage, but has a lesser effect on bacteria (Bales et al., 1995). For example, at a pH of 5.7, a bacteriophage was highly attenuated, but upon introduction of solutions of pH 6 to 8, bacteriophage were remobilized in soil. This effect was not found to occur with bacteria tested, which were highly sorbed independent of pH, indicating that bacteriophage are not good indicators of bacterial transport. However, in another paper by these researchers (Kinoshita et al., 1993), it was reported that bacterial adhesion was higher at pH of 7.0 than at a pH of 5.5 in a column packed with silica beads, although

adhesion was substantial in all systems.

Ionic strength of the aqueous phase is generally a secondary effect on bacterial transport, although under some conditions it has been shown to be significant. In an experiment performed evaluating the transport of two bacterial species in sand columns under non-growth conditions, an increase in ionic strength of the solution being pumped through the sand caused a decrease in the transport of the two bacterial species (Fontes et al., 1991). It was hypothesized that the increase in ionic strength decreased the double layer around the porous media, allowing the cells to get closer to the mineral surface of the sand. An increase in bacterial adsorption due to an increase in ionic strength was also noted by Hornberger et al. (1992) and Mills et al. (1994).

Flow conditions have been shown to increase bacterial adhesion, when compared to batch systems. Bacterial adhesion was compared using both hydrophilic and hydrophobic cells in column and static batch conditions (Rijnaarts et al., 1993). Bacterial adhesion was two to four times higher in column conditions compared to batch conditions, possibly due to an increase in collision frequency with the porous media. It was only at high velocities that the two cells exhibited different behaviors; stronger adhesion was reported for hydrophobic strains whereas the opposite was true for hydrophilic strains. However, Huysman et al. (1993a) reported an increase in bacterial transport at higher irrigation (flow) rates compared to lower rates for all cell types and in sand and clay soils. Additionally, they reported that transport of the inoculated organisms was significantly greater if the flow began immediately after flushing. If cells were

allowed to sit on the soil surface even 10 to 60 minutes, cell transport was decreased and applicability of a colloid filtration model was not valid (as it was if flow began immediately).

Surfactants and biosurfactants can alter the cell surface properties or soil properties to enhance microbial transport. Bai et al. (1996) performed a series of flow-through column experiments examining the movement of three different bacterial strains (of different hydrophobicity) and found that the presence of an anionic biosurfactant (rhamnolipid) enhanced transport of all three bacteria. It was determined that the rhamnolipid caused an increase in the negative surface charge of the porous media, but it did not alter the cell surface charge.

Growth of microorganisms has also been shown to influence transport of bacteria in porous media. This behavior is assumed in biofilm models, which relate the detachment rate to the number of organisms present, such that at higher growth rates, the detachment of bacteria from the biofilm increases. In one study (Clement et al., 1997), the transport of bacteria and nutrients in an anaerobic system was monitored in soil columns containing different levels of nitrates (to serve as alternate terminal electron acceptors, the limiting compound). Overall detachment and transport rates were shown to be higher in systems with higher nitrate levels and increased growth, although the rates were still much lower than in aerobic systems. Additionally, Sharma et al. (1994) reported increased penetration rates of motile and non-motile strains of E. coli in sands under growth conditions.

Besides the fact that more organisms are present during bacterial growth, causing an increase in the overall number of organisms being transported, growth of bacteria can also alter the cell surface to cause enhanced, or decreased, transport. A study of 23 different bacterial strains found that bacteria became more hydrophobic at high growth rates, causing increased adhesion to polystyrene (van Loosdrecht et al., 1987).

The presence of a carbon source does not always indicate increased survival and transport. In a study performed by Blackburn et al. (1994), the effect of the presence of a chlorinated phenols on the survival of introduced bacteria (capable of degrading the contaminants) was examined. They found that introduced bacteria survived better in remediated soils at lower temperatures than in soils with high levels of contaminants. This is likely due to competition with the indigenous degraders already in the soil, but no explanation was offered in the manuscript.

The effect of starvation on bacterial adhesion and transport has also been studied. Starvation was found to decrease adhesion of two of four anaerobic bacteria studied (van Schie et al., 1999). A similar study was performed using a marine Pseudomonad sp., and it was discovered that starvation induced the synthesis of proteins that form peripheral extrapolysaccharide (EPS) to induce detachment of microorganisms by reducing flagellar movement (Wranstadh et al., 1990).

Just as adhesion is affected by microbial growth, growth of microorganisms is also affected by adhesion. The growth of a marine Vibrio sp. DW1 was studied in the aqueous phase, at an air-water interface, and at a solid-water interface (Kjelleberg et al.,

1982). Cells at an air-water interface grew faster in the presence of nutrients than free-floating cells, and they were able to grow and divide at this interface at nutrient concentrations less than that required in the aqueous phase.

Comparisons with non-biological tracers or colloids

The transport behavior of bacteria (that had been collected from an aquifer and stained) was compared with that of conservative tracers (bromide and chloride) and bacteria-sized microspheres with carboxylated, carbonyl or neutral surfaces (Harvey et al., 1989) and with Rhodamine WT dye (Pang et al., 1998). Breakthrough of bacteria was early compared to the tracers and the dye, presumably due to size exclusion, and the transport of the bacteria was significantly different from that of the microspheres. Transport of the microspheres was affected by surface charge and size. In another study by Harvey et al. (1993), transport of bacteria at the field scale was compared to conservative tracers and microspheres, and behavior was similar at 2 depths within the aquifer, but not at another depth. In comparing infiltration of viruses and bacteria at a wastewater land application site, 48% of the viruses were shown to reach the aquifer within 48 hours, while bacteria remained concentrated at the soil surface (Schaub et al., 1977). In yet another study by Harvey et al. (1995), transport of flagellated protozoa and flagellate-sized carboxylated microspheres was evaluated. There was a higher rate of immobilization for flagellates than for bacteria, and this rate decreased with residence time in the aquifer because the flagellates decreased in size over time. Unlike with

bacteria, transport of 2 μm carboxylate spheres mimicked that of flagellates.

Transport of a bacteria (E. coli) and 'non-reactive' particles through fractured rock was compared with bromide and radioactive tracers at a field site. The bacteria and 'non-reactive' particles appeared prior to, or at the same time as, the conservative tracers, although there was significant removal of bacteria overall and at a similar rate as in gravel systems. Therefore, it was concluded that significant underestimates of microbial contamination exposure could occur at recovery wells located in fractured rock.

Microbial transport models

Transport of bacterial cells in the aqueous phase through porous media is mathematically described by the advection-dispersion equation in a vast majority of models (Corapcioglu et al., 1984; Corapcioglu et al., 1985; Harvey et al., 1991; Hornberger et al., 1992; Lindqvist et al., 1994; Zysett et al., 1994; Tan et al., 1994; Corapcioglu et al., 1995; Reddy et al., 1996; Hendry et al., 1997; Murphy et al., 1997; Baygents et al., 1998; Bolster et al., 1998). However, some researchers have accounted for bacterial transport by assuming it is a function of shear detachment, and assume that detached bacteria are swept into the next flow regime (MacDonald et al., 1999b). Common behaviors represented in these models are bacterial growth (Corapcioglu et al., 1984 and 1985; Lindqvist et al., 1994; Zysett, et al., 1994; Corapcioglu et al., 1995; Reddy et al., 1996; Murphy et al., 1997; MacDonald et al, 1999b), partitioning of cells to the solid phase (through sorption/adhesion, partitioning into organic matter, partitioning

to the solid-water interface), and release of cells from the solid phase. When included in the model, bacterial growth is generally described by Monod growth kinetics (Corapcioglu et al., 1984 and 1985; Zysett et al., 1994; Corapcioglu et al., 1995; Reddy et al., 1996; Murphy et al., 1997; MacDonald et al., 1999b). However, since many bacterial transport experiments were conducted under non-growth conditions, many models do not include a growth term. Partitioning of cells is described by a number of methods and is an area of controversy.

Microbial transport models (in the absence of biofilms) have the same trouble addressing the mechanisms responsible for bacterial attachment, detachment and partitioning in general as biofilm models. As discussed previously, the large number of mechanisms influencing bacterial adhesion and transport and uncertainty of their relative importance makes development of a mechanistic bacterial transport model very challenging. Thus, most bacterial transport models account for the rates of attachment and detachment as being related to aqueous and sorbed concentrations, rates of collision with the porous media (such that the rate of attachment is a function of flow velocity), fluid shear, or a combination of these.

The transport models developed by Homberger et al. (1992), Tan et al. (1994) Lindqvist et al. (1994), Zysett et al. (1994), Murphy et al. (1997) and Bolster et al. (1998) incorporated a kinetic model to mathematically describe the rates of adsorption and desorption as being linearly related to aqueous and sorbed concentrations respectively. A model developed by Hendry et al. (1997) was similar to the kinetic models described

above except that it also contained a term for irreversible sorption, that was correlated to the aqueous concentration of cells in the system. The 'colloid filtration model' described by Harvey et al. (1991) included many of the same parameters as the kinetic models, except that it contains an extra collision term relating the aqueous concentration and the pore water velocity to the rate of bacterial adsorption.

Other mathematical models for incorporating adsorption into a bacterial transport model have been developed. For example, Baygents et al. (1998) represented 'collision efficiency' by a probability density function. Additionally, an equilibrium model for bacterial adsorption and desorption (in equilibrium with the aqueous phase concentration) was compared with a kinetic model of the type described above by Reddy et al. (1996). When coupled with a contaminant transport model, both models were able to fit contaminant breakthrough curves, but they produced significantly different bacterial distributions and breakthrough curves. However, because bacterial distributions were not monitored in the experiments, it was not possible to discern which model provided a better representation of mechanisms. Lindqvist et al. (1994) compared his kinetic model with a 2-site model (commonly used in contaminant transport) and achieved similar results.

It was reported by Hornberger et al. (1992) that the basic kinetic model did not fit the early part of experimental bacterial breakthrough curves and that "there are clearly serious gaps in our knowledge of what controls the values of the phenomological coefficient". Further limitations of all these models were described by Harvey et al.

(1991) as not incorporating processes such as motility, range of buoyant densities of cells, heterogeneites, variations in cell size, variation of bacterial attachment with species, solution conditions, and nature of solid surfaces, and they assume bacterial species are spherical, even though many bacteria are rod-shaped. Corapcioglu et al. (1984) addressed cell factors of chemotaxis, decay and growth, and the physical factor of sedimentation, and discussed the need for incorporation of these phenomena in bacterial transport models as well.

BIODEGRADATION IN NATURAL SYSTEMS

Bioremediation of contaminated sites is a very promising technique because it is easy to implement and is extremely cost-effective. However, an understanding of the dominant factors that affect biodegradation at a site is important for proper use of the technique. Biodegradation in natural systems is influenced by environmental factors, substrate interactions, and the distribution of microbial populations, just to name a few factors.

Environmental factors

Biodegradation is affected by environmental conditions, just as bacterial growth is impacted by system surroundings. However, the impact of environmental parameters on biodegradation in subsurface systems is generally hard to determine, since many factors may be acting at once and due to the difficulty in adjusting environmental parameters. A

number of studies, have focused on various environmental parameters such as electron acceptor and nutrient content, rate of groundwater flow, temperature, salt content, pH, soil texture, and soil heterogeneity.

In general, an increase in temperature causes an increase in growth rate, and thus in the biodegradation rate, within the growth range of an organism. In a study examining the biodegradation of phenol by Acinetobacter radioresistens, the rate was found to be twice as fast at 30° C than at 20° C (for pH between 7-8), but there was no biodegradation observed at 40° C (Pessione et al., 1996). Biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) was significantly faster in flow-through systems at 20° C compared to those conducted at 4° C (Shaw et al., 1998). Additionally, the rate of biodegradation was increased at a contaminated site in Eielson Air Force Base, Alaska by circulating heated groundwater through a contaminated cell, as compared to a control cell and a cell covered with a solar heating panel (Leeson et al., 1993). However, biodegradation rates are not necessarily slower in colder climates, due to the differences in optimal temperature ranges that exist in the microbial world. Intrinsic biodegradation rates were found to be comparable for benzene at an arctic site in Barrow, AK to more temperate sites (Braddock et al, 1996). Likewise, rates of mineralization of toluene by microcosms isolated from contaminated sites were twice as high in those from Adak, AK compared to Hannahan, SC, when incubated at their respective in-situ temperatures (Bradley et al., 1995). Therefore, the slow rate of biodegradation in colder climates has been attributed to the lack of a lengthy thaw season, rather than to the temperature during this thaw

season (Pessione et al, 1996 and Bradley et al., 1996).

Other environmental factors have been proven to be just as important for the survival of target organisms. Biodegradation of phenol was observed to be quick in cultures of Acinetobacter radioresistens for solutions of pH 7 to 8, while the process took 10 times as long at a pH of 6 (Pessione et al., 1996). Additionally, acclimation period was found to be crucial to growth/biodegradation kinetics in this study. If cultures were acclimated for 3 days to increasing concentrations of phenol (from 50 to 200 mg/L), there was a lag phase followed by complete biodegradation within 3 hours (under optimal environmental conditions). In non-acclimated cultures, an initial stage of cellular death was observed. Similarly, successive pulses of 2,4-D were degraded to a greater extent in soil columns than the initial pulse (Shaw et al., 1998). Salt (NaCl) has also been discussed as an inhibitory substance to biodegradation, particularly in wastewater or marine settings (Kapley et al., 1999). Finally, Harms et al. (1994) demonstrated that while attached cells of Sphingomonas sp. were relatively equal to that of suspended cells in a batch system, the half-maximum uptake rate concentration (affinity constant) was higher for attached cells due to diffusion limitations for these cells as compared to suspended cells. Thus, as agitation or flow rate increased, so did the affinity constant.

In the vadose zone, inorganic nutrients are often a limiting component. While these components can also be limiting in the saturated zone, oxygen limitations are usually the overwhelming constraint under high contaminant loads because of the low solubility of oxygen. When organic materials are added to the soil, such as in the case of

hydrocarbon spills or landfarming of petroleum wastes, the high concentrations of carbon in the system often cannot be degraded in a reasonable time scale because nitrogen becomes a limiting nutrient in the system and slows bacterial growth dramatically.

Although it is commonly accepted that resident levels of nitrogen are inadequate to support biodegradation of a pollutant (in a reasonable time) at most sites, the recommendations of how much nitrogen should be added to the subsurface can vary greatly from source to source. In general, the C:N ratio concept is used to predict how much nitrogen should be added to a site to optimize the rate of biodegradation at a site. Many studies have reported an increase in the rate of biodegradation when nitrogen was added to their systems, however, the optimal C:N ratios reported by these studies varies significantly from 9:1 to 124:1 (Brown et al., 1983), to 60:1 (Dibble and Bartha, 1979) and greater. Due to this variance in recommended values, the U.S. Environmental Protection Agency suggests a ratio between 10:1 and 100:1 (Braddock et al., 1997).

The reasons for the variations in the optimal C:N ratio have been shown to be caused by a number of environmental and contaminant parameters (Braddock et al., 1997). The optimal C:N ratio has been reported to be drastically different for different contaminants (Brown et al., 1983) in the same soil under the same conditions (water content, temperature, pH, etc.). The soil type, and the absence or presence of significant levels of lignin, were also found to affect the optimal C:N ratio (Vigil and Kissel, 1991). Walworth et al. (1997) found that the optimal C:N ratio for a soil was a function of the water content of the soil. This relationship also helps explain why coarser-textured soils

(which retain less water than finer-textured soils) are more susceptible to overfertilization. Finally, Alexander (1994) reports that in some cases high concentrations of nitrogen are required to increase the rate of biodegradation. This was accredited to a high K_s value (affinity constant) for nitrogen for the organisms at the site rather than the amount of nitrogen that is needed for incorporation into cell mass. From these results, it is apparent that an optimal C:N ratio should be determined independently for each site by performing lab-scale studies under the environmental conditions that exist at the site.

Oxygen availability is often cited as the primary limiting factor for in-situ biodegradation of a contaminant plume (Borden et al., 1986 a and b; Kindred et al., 1989; MacQuarrie et al., 1990; Kinzelbach et al., 1991). Reduction of oxygen provides more energy than reduction of other electron acceptors, and thus, aerobic biodegradation is the fastest if the compound can be degraded aerobically (Borden et al., 1995). The rate and extent of biodegradation of BTEX compounds was shown to be strongly influenced by the electron acceptor type and availability at a gasoline contaminated site. Biodegradation of all compounds was fastest at the leading edge of the contaminant plume, where oxygen and ferric iron were present (Borden et al., 1995).

Because the solubility of oxygen is very low, about 10 mg/L depending on temperature (Kinzelbach et al., 1991) it is utilized before all of the contaminant is used up (for a vast majority of contaminant plumes). In fact, depletion of oxygen in groundwater is often used as an indicator of intrinsic bioremediation (Borden et al., 1995; Hunkeler et

al., 1999; Kao et al., 1999). Thus, remediation technologies have evolved to introduce oxygen into the subsurface and enhance bioremediation. These include bioventing (vadose zone) and biosparging (saturated zone).

In the saturated zone, the solubility of oxygen limits its introduction in large quantities, so alternative electron acceptors can be introduced instead. Examples are peroxide or nitrate. In soil column experiments containing an indigenous population of BTX degraders, BTX biodegradation was significantly increased when peroxide was introduced, but the introduction of nitrate only enhanced biodegradation of toluene (Anid et al., 1990). The presence of soil heterogeneities also influences bioremediation, at the microscale and at the field scale. The effect of the presence of micropores in a porous matrix on substrate biodegradation and microbial growth was emphasized in a paper by du Plessis et al. (1998). In short, the presence of these sites causes increased biodegradation of a miscible-phase compound. The presence of micropores provides niches for microbial growth. Also, substrate enters micropores due to diffusion, allowing the concentration changes in the micropores to be less abrupt so that cells can adjust. The residence time of the substrate is greater in micropores than in the bulk pore volume of the system, which leads to more contact time between cells and substrate. Additionally, a slower velocity in the micropores causes less shear stress and straining on the cells. In unsaturated soil conditions, smaller (micro-) pores retain more water under tension, providing bacteria with more niches for growth in an unsaturated soil system. Finally, a porous medium containing micropores has a higher surface area than one without, and

this allows more cells to be retained within the medium. At the field scale, the presence of clay lenses and regions of lower conductivity can serve as long-term sources of contamination. This phenomenon will be addressed in the section relating to contaminant transport and bioremediation.

It is generally assumed that bacteria consume substrate from the aqueous phase, such that contaminants associated with the soil phase are not available to degraders. The term bioavailability describes the amount of contaminant that is available to a microorganism for consumption and growth. This property is influenced by solubility and hydrophobicity of the compound, soil texture and percent organic matter. A decrease in the bioavailability of a compound causes a subsequent decrease in the biodegradation rate of a compound. For example, the biodegradation of naphthalene and phenanthrene by an organism isolated from a domestic waste-water treatment plant was studied in the presence of sludge, to determine effects of sorption (Volkering et al., 1992). The degrader was introduced to a contaminated sludge slurry, following a period of equilibration to allow for sorption. Linear bacterial growth was observed rather than exponential. Experimental results were shown to fit a simple mathematical model that assumed that growth was limited by mass transfer from the solid to the aqueous phase. A field study evaluating the biodegradation of atrazine by an inoculated degrader found that when the field was irrigated with treated waste water rather than control water shortly after atrazine application (within 24 hours), biodegradation was retarded (Masaphy et al., 1997). This was determined to be due to the presence of dissolved organic matter in the

waste water, that served as a source of adsorption for atrazine.

The physico-chemical properties of a soil are often altered in laboratory-scale systems when a field soil is autoclaved prior to inoculation with a model degrader organism or re-inoculated with indigenous organisms. While these experiments are generally meant to mimic environmental conditions, autoclaving can significantly affect a number of important soil properties. Shaw et al. (1999) conducted studies wherein a silt loam soil was autoclaved and then reinoculated with indigenous organisms isolated from the soil. Autoclaving the soil significantly decreased the pH of the soil (from 7.2 to 6.0), and increased the water-soluble organic carbon (WSOC). When these soils were subsequently pre-incubated prior to addition of a contaminant (2,4-dichlorophenol), the observed lag phase was reduced from seven days in non-autoclaved soils to less than three days in autoclaved soils. Additionally, the overall amount of biodegradation in the autoclaved, reinoculated soil after 21 days was almost double that of the non-autoclaved soil.

Substrate interactions

The presence of multiple substrates can have positive or negative effects on the biodegradation of the mixture, depending on the compounds and the concentrations to which they are present. In the case of co-metabolism, the presence of a primary substrate allows for the biodegradation of harmful contaminants that may not otherwise be degraded. However, in the case of some mixed plumes, contaminants may be degraded

preferentially, leaving the constituents with larger molecular weights or that were less biodegradable.

Biodegradation of a compound does not always cause growth of the degrading population, as in the case of secondary utilization. In a review article by Rittmann (1992), the interaction between primary substrates and secondary substrates was discussed. A secondary substrate is one which can be degraded by the bacterial population, but cannot support growth or cell maintenance of the population. This may occur because the substrate is available at too low a concentration, or the reaction itself may not supply energy to the cells. In either case, the presence of a primary substrate that can support the active biomass is required before biodegradation commences. Often contaminants remain at a site at 'residual levels' below which biodegradation is not feasible, but above permissible maximum contaminant levels (Nocentini et al., 2000), and this is a prime example of when secondary utilization is required. Co-metabolism refers to the case where degradation of the secondary substrate does not release energy to the cells, but is performed as a result of the lack of specificity of an enzyme causing biodegradation of the primary substrate.

Batch and flow-through soil column studies were performed to investigate the use of secondary utilization to biodegrade chlorinated organic compounds (Bouwer et al., 1983 and 1984). Acetate was added as a primary substrate to systems containing very low concentrations (<100 ug/L) of 1- and 2-carbon halogenated aliphatic compounds under methanogenic conditions. Degradation of the target compounds was 90% complete

or more in the flow-through systems after two days (Bouwer et al, 1983). In a similar system, acetate was utilized to examine secondary utilization of chlorinated benzenes, halogenated aliphatics and non-chlorinated aliphatic compounds at low concentrations and similar results were achieved (Bouwer et al., 1984). Microcosm studies performed by Kao et al. (1999) indicated that chlorobenzene could act as a primary substrate to enhance biodegradation of trichloroethylene in soil from a contaminated site in Georgia. This biodegradation was performed by a methanogenic population.

Secondary utilization of what is often the 'target compound' in engineered systems can be inhibited by the primary substrate, as observed by Semprini et al. (1991). Methane and oxygen were injected into a field site contaminated with chlorinated ethenes to act as primary substrates. While they increased the rate of biodegradation of vinyl chloride (VC), trans-1,2-dichloroethylene (t-DCE), cis-1,2-dichloroethylene (c-DCE), and trichloroethylene (TCE), methane was also found to competitively inhibit the biotransformation of VC and t-DCE, such that transformation rates of these compounds were increased temporarily when methanol and formate were substituted for methane and oxygen. However, after a few days, the concentration of the target compounds increased rapidly, indicating the cessation of transformation, due to the lack of substrate for the methanotrophic population. Therefore, the effect of inhibition was secondary to the necessity for a primary substrate, and the amount of primary substrate (methane in this case) injected should be monitored to optimize these two conflicting effects.

When multiple substrates are present at significant concentrations at a site, a

number of behaviors have been observed. In one study, soil microcosms were 'contaminated' with petroleum mixtures. The lighter components of diesel fuel were preferentially degraded, but the mineral oil components were all degraded at about the same rate (Nocentini et al., 2000). The biodegradation rates of several dissolved alkylbenzenes were found to depend on the molecular structure of the compound in a study by Angley et al. (1992). The first-order biodegradation rate constants decreased with a decrease in the number of C in the alkyl groups, and compounds with substituents in the ortho position had the smallest rate constant of all the isomers. In another set of batch studies, biodegradation of BTX compounds (together and separately) was examined using two pure cultures and two aquifer slurry populations (Alvarez et al., 1991). The presence of toluene enhanced the biodegradation of benzene and xylene by one isolated strain, and one isolate only degraded toluene and xylene when benzene was present. Conversely, the presence of xylene increased the lag time prior to biodegradation of benzene and toluene in both aquifer slurry populations and in one of the isolates.

Utilization of genetically engineered microorganisms (GEMs)

Genetically engineered microorganisms refer to any microorganism which has been altered genetically (via insertion of a plasmid or by insertion of a gene into the chromosomal sequence) to confer the organism with an ability that it did not previously have. There are a plethora of review articles discussing the advances that have been made in this area of research (e.g. Pieper et al., 2000; Sayler et al., 2000; Chen et al., 1999),

however, they are out of the scope of this text. These organisms often are conferred with the ability to degrade particular compounds, compete with indigenous populations in severe climates, and/or serve as biosensors of the presence or biodegradation of a contaminant. As an example of a potential application of a GEM to aid in bioremediation, Kapley et al. (1999) genetically transformed four isolated hydrocarbon degraders such that they were tolerant to solutions with high salt concentrations. Use of this technique would allow for faster clean-up of marine estuaries or other high-salt environments (such as waste-water streams) if these bacteria were introduced. An example of GEMs used as biosensors are those that have been transformed to luminesce when a contaminant is present or during the onset of biodegradation. These organisms have been useful in determining the distribution of the introduced bacteria in-situ and particularly, in determining the location of active populations (Ripp et al., 2000; Yocubal, 2001).

Intermediate-scale studies

In this research area, there appears to be a lack of intermediate-scale studies that can bridge the gap between lab-scale batch and column studies and field-scale remediation studies. However, a set of lysimeters were converted to allow for the study of in-situ biodegradation at Oak Ridge National Laboratory (Cox et al., 2000). This system is particularly useful for introduction of genetically engineered microorganisms into systems that are closer to field scale, but are self-contained. Other advantages of this

system include the ability for aeration, multiple means of adding nutrients, electron acceptors and electron donors, monitoring instrumentation is installed, sampling ports are available for liquid, soil, and gas samples. Also, because there are six identical units, experiment replication can be performed and/or technologies can be compared. This system was used in a biodegradation study for the compounds naphthalene, phenanthrene and anthracene using an inoculated luminescent GEM (Pseudomonas fluorescens HK44). Although there was significant die-off of the inoculated organism, an adequate population remained to detect luminescence by the GEM during biodegradation of the target compounds (Ripp et al., 2000).

Distribution of microbial populations in-situ

The distribution of bacteria in the subsurface is often affected by the presence of contamination. At a sewage infiltration site in Cape Cod, MA, the total bacterial counts in the aqueous phase and the degradation capacity of the soil (indicating total bacteria present in all phases) were found to be linearly correlated with distance from the source of contamination (Harvey et al., 1984). A majority of these organisms were associated with small particulates (less than 60 um), not in the aqueous phase or on large soil particles. The distribution of bacteria capable of degrading benzene, toluene, and xylene (BTX) was evaluated at a 'natural attenuation' site using gene probes to target genotypes associated with biodegradation of BTX compounds and naphthalene. More organisms

with these genes were detected within the contaminant plume than in the uncontaminated area of the site (Stapleton et al., 2000).

In addition, the association of bacteria has also been shown to be influenced by available carbon source. Harvey et al. (1992) demonstrated that the relative number of bacteria existing as 'free-living bacteria' (FLB) or in the aqueous phase, was directly related to the levels of dissolved organic carbon and distance from a contaminant source in a contaminated aquifer. Near the contaminant source (0.25 km away), as many as 31% of the bacteria existed as FLB, but 2 km downgradient from the source less than 7% of bacteria existed as FLB. The decline in FLB as a function of distance from the contaminant source fit an exponential decay function. Similar findings were reported by Krueger et al. (1998), who were studying the transport of a biodegradable surfactant. The surfactant was not degraded in an uncontaminated portion of an aquifer and the number of free-living bacteria was constant, but there was an increase in the number of FLB in a sewage contaminated area as well as partial degradation of the surfactant.

In the absence of contamination, microbial abundance and activity (as defined by the number of substrates that could be oxidized in a Biolog assay) were shown to decline with depth at two sites in Washington state (Kieft et al., 1998). Additionally, when populations from different depths were grown on glucose, lag times increased with sediment age and depth. This may be due to the fact that populations in older sediments were less dense and/or had been dormant. Similarly, a study performed by Severson et al. (1991) indicated that counts of culturable heterotrophs at two sites decreased

coincidentally with total organic carbon as a function of depth in the vadose zone. It was additionally determined that the number and variation in colony type of organisms was increased at the site with more TOC.

The method of enumeration of indigenous populations generally leads to different numbers. Viable counts from 60 soil cores at a contaminated site in Mississippi were two to three orders of magnitude less than estimates obtained using DNA hybridization with 16S rDNA genes. Additionally, population estimates using viable counts indicated that microbial counts were an order or magnitude lower in fine-grained soils compared to coarse, while other methods did not indicate this trend (Stapleton et al., 1998). However, viable counts can only enumerate certain organisms, depending on the substrate used and the environmental conditions (e.g. aerobic or anaerobic), so these results are not unexpected.

COUPLED CONTAMINANT TRANSPORT AND BIODEGRADATION SYSTEMS

Contaminant transport and biodegradation began to be extensively studied during the 1980s, as evidenced by a large body of literature that emerged during this time. This research can be divided into laboratory-scale miscible-displacement studies, field-scale studies, and model development aimed at simulating these results. The laboratory-scale studies performed to date have been useful in isolating factors that contribute to contaminant transport and biodegradation, so that predictive models can more accurately describe these processes. Field-scale studies, on the other hand, are useful in elucidating

the dominant factors that influence contaminant transport and biodegradation.

Non-biologic effects

Transport of contaminants is affected by non-biological parameters of sorption or partitioning (linear or nonlinear, and instantaneous or rate-limited) contaminant to the solid phase, solute size, pore water velocity and soil heterogeneities. At the field-scale, these factors can also include presence of non-aqueous phase liquids, structured media, clay lenses, and spatial variability of sorption properties, hydraulic conductivity, and/or hydraulic gradient. There is a plethora of articles discussing these processes in the literature, and they are out of the scope of this text. However, a couple of exceptional review articles have been published that summarize these processes (e.g. Brusseau, 1994).

Sorption of contaminants to the solid matrix causes retardation of the contaminant. Thus, the contaminant emerges later than a non-reactive tracer. Nonlinear sorption causes assymetrical breakthrough curves and the retardation factor is dependent on concentration (not constant). Sorption of a contaminant is never instantaneous, but it can be approximated as such if the process is fast compared to transport processes. Otherwise, sorption is considered to be rate-limited. Rate-limited sorption causes early breakthrough and tailing of contaminant breakthrough curves.

If the porous media is aggregated or contains regions of lower hydraulic conductivity, intraparticle dispersion or mass transfer from the regions of low

conductivity can become significant. At the lab-scale, solutes of different sizes were shown to have different apparent dispersivities in aggregated media. This was attributed to the variance in the time required for diffusion out of small pores in the aggregates for different sized compounds. Thus, it was concluded that tracers of similar size to the target compound should be used when experiments are performed in media of this type (Brusseau, 1993; Hu et al., 1994). This effect was discussed at the field-scale by Goltz et al. (1986), and models to represent this behavior were compared. These are summarized in the following text.

Laboratory-scale experiments

The effect of flow rate and/or path length, and thus residence time, has been discussed in a number of experimental and modeling articles. The transport and biodegradation of p-nitrophenol (PNP) was analyzed in unsaturated soil column experiments. Biodegradation increased with an increase in path length or a decrease in flow rate, both of which cause an increase in residence time and contact time between the soil microorganisms and the contaminant. Interestingly, PNP degraders were found to be concentrated in the top 1/4 of the column despite the presence of PNP at greater depths (Kelsey et al., 1995). Similar results relating amount of biodegradation and residence time were noted by Brusseau et al. (1999) using benzoate as the contaminant. Conversely, Langner et al. (1998) reported that while the first order biodegradation rate

coefficient for 2,4-D (1 mg/L) in unsaturated soil columns increased with a decreasing pore water velocity, this value remained constant with varying residence times.

Batch biodegradation studies are often used to determine biodegradation parameters that are then used in transport and biodegradation models. However, this is somewhat controversial, since it is uncertain if these parameters remain the same under the flow conditions not present in batch systems. Batch and column experiments were performed to compare the biodegradation rate parameters of batch versus column systems for 2,4-dichlorophenoxyacetic acid (2,4-D) in saturated and unsaturated systems (Estrella et al., 1993). The lag phases for 2,4-D biodegradation were similar under batch and column conditions, suggesting that batch experiments may be predictive for this parameter, but possibly not for other parameters. When a transport model, incorporating growth kinetics and sorption, was optimized to fit the data from the column experiments, the optimized values for the column systems were much higher than values estimated from batch studies. Additionally, when an updated model (to include inhibition) was applied to this data, the values estimated from model calibration for cell yield was lower than that observed in batch, and the value estimated for the half saturation constant was larger (Maier et al., 1997). Therefore, it was concluded that the values determined for these parameters in batch were not appropriate for column experiments because of the different conditions (e.g., mixing, aeration, solid/water ratio) associated with the two systems and the resulting impact that these processes have on biodegradation. Shaw et al. (1998) observed a similar increase in biodegradation rate for 2,4-D in column systems

compared to batch systems. They observed a downward transport of degraders in soil columns but not in batch systems, and hypothesized that this was the cause of the discrepancy between the two systems.

Likewise, laboratory column experiments were performed by Kuhn et al. (1985) to simulate the transport and transformation of trace organics under saturated flow conditions and to compare the laboratory results with field data. Although degradation of the compounds studied (xylenes, dichlorobenzenes, and tetrachloroethylene) was qualitatively the same in the lab as in the field (i.e., they did or did not degrade under aerobic or denitrifying conditions in either setting), the rate constants observed in the lab were not transferable to those observed in the field.

The relative impacts of microbial growth and lag were examined by Sandrin et al. (2000). Miscible-displacement experiments were conducted to monitor the transport and biodegradation of salicylate in a homogeneous sand inoculated with *Pseudomonas putida* RB1353 (a GEM). While both processes were credited for causing nonsteady transport, results from these experiments demonstrated that microbial lag was important at early time, but microbial growth was the dominant influence at later times. Similar results were reported by Yolcubal et al. (2001).

Soil heterogeneities and preferential flow paths influence the transport, and thus the biodegradation of, contaminants in the subsurface. The transport and biodegradation of quinoline was monitored in a flow cell packed with 2 soil layers of different hydraulic conductivities that were subjected to flow parallel to the layers. Quinoline and oxygen

arrived 10 to 100 times faster in the low hydraulic conductivity layer near the interface than away from the interface between the two soils, indicating that interlayer mass transfer is more important than advection in this region. As a result of this phenomenon, growth of bacteria in this region was approximately three times that of other areas in the flow cell (Szecsody et al., 1994). When this flow cell was packed with an inoculated sand with clay inclusions, and a benzoate solution was supplied to the column, microbial growth was again increased in the boundaries between the two porous media. There was a five-fold increase in bacteria over the course of the experiment (Murphy et al., 1997). Similarly, biodegradation of PNP was found to be faster in a macropore present in a soil column as compared to the overall soil column. This increase in activity was attributed to more favorable conditions in the channel for biofilm development, such as increased oxygen concentrations (Pivetz et al., 1996).

Field-scale experiments

Transport and biodegradation of contaminants has been studied at many different field sites. The natural gradient solute transport experiment conducted in Borden, Ontario is probably the most studied solute transport and biodegradation experiment. The initial results from this experiment were published in 1986 (Mackay et al.), and little analysis had been performed on the raw data. In a separate paper, biodegradation was discussed, as there was a net decline in mass of organic compounds over time (Roberts et al., 1986).

The exact biodegradation mechanism is often not known at a site, and it often varies with relative location within a plume. At another field site, an organic rich aqueous phase was detected downstream from a creosote contaminated site. There was more aqueous contaminant lost that could be attributed to dispersion and/or dilution. This site was determined to be devoid of oxygen, but high in methane and hydrogen sulfide. Additionally, methanogenic microbes were found at the site. Thus, methanogenesis was implicated at this site (Godsy et al., 1992).

Mathematical models

Transport of dissolved compounds through porous media is described mathematically using the advection-dispersion equation (ADE), also referred to as the convection-dispersion equation, in a vast majority of cases (Borden et al., 1986; Goltz et al., 1986; Molz et al., 1986; Widdowson et al., 1988; Kindred et al., 1989; MacQuarrie et al., 1990; Kinzelbach et al., 1991; Angley et al., 1992; Brusseau, 1993; Brusseau et al., 1993; Estrella et al., 1993 ; Brusseau, 1994; Hu et al., 1994; Wood et al., 1994; Zysett et al., 1994; Corapcioglu et al., 1995; Wood et al., 1995; Maier et al., 1997; Murphy et al., 1997; Hu et al., 1998; Langner et al., 1998; Tebes-Stevens et al., 1998; Brusseau et al., 1999; Lee et al., 2000; Miralles-Wilhelm et al., 2000). The ADE is written in most general terms as

$$\frac{\partial(C\theta)}{dt} = \nabla \bullet \left[-\vec{q}C + D\theta\nabla C\right]$$

where C = contaminant concentration, t = time, D is the dispersion coefficent, q = Darcy's velocity, θ= the porosity or water content. This equation is often simplified to 1-D and assumes incompressible fluid with no sinks or sources. It is written

$$\frac{\partial C}{\partial t} = -v\frac{\partial C}{dx} + D\frac{\partial^2 C}{dx^2}$$

where v = pore water velocity. This equation is then coupled with the appropriate equations to describe processes occuring in a system.

The ADE is traditionally solved numerically utilizing the operator splitting approach, in which the transport and reactive portions of the model are solved sequentially. An alternative to this approach is the sequential iterative approach, which involves repetitively solving the transport and reactive steps until convergence is achieved. The operator splitting method is subject to mass-balance errors and numerical dispersion, problems which can be addressed by using a smaller time step. In most cases, the operator splitting method is more computationally efficient. However, this advantage diminishes significantly if the time step has to be very small to avoid numerical dispersion, and the sequential iterative approach is a better option (Tebes-Stevens et al., 1998).

Alternative flow models have been developed which do not utilize the ADE. One such model is a stochastic-convective reaction (SCR) model, which represents the subsurface as an ensemble of 1-D stream tubes of varying velocities and reaction rates.

This program was extended to incorporate nonlinear biodegradation due to microbial growth using the Monod equation (Ginn et al., 1995). When the stream tube solution is averaged over travel time and representative reaction properties, this allows for up-scaling of the model to represent chemical and physical heterogeneities. Utilizing the Monte Carlo approach, this method was stated to be more accurate that the ADE solution.

Sorption or partitioning into organic matter is represented using a linear sorption equation (Brusseau, 1994; Brusseau et al., 1999), or nonlinear sorption equation such as the Freundlich equation (Brusseau, 1994; Brusseau, 1995; Hu et al., 1998). These are incorporated into a kinetic rate model if sorption is rate-limited (Brusseau et al., 1993; Brusseau, 1994; Brusseau, 1995; Hu et al., 1998). In the case of aggregated media or heterogeneous media with lenses of low conductivity, the 'two-site' model or the mobile-immobile region model (Goltz et al., 1986; Brusseau et al., 1993). This model represents the media as consisting of two flow regimes; one in which transport is described by advection and dispersion and another that has negligible advection such that transport is dominated by diffusion. Two mobile-immobile region models were compared by Goltz et al. (1986). One model was a first-order kinetic model that described transfer between the two zones while the other was a spherical diffusion model with sperical immobile zones. The kinetic model relied on parameter fitting, while the spherical diffusion model was more predictive because it allowed for independent parameter estimation. However, the kinetic model required much less computation.

Biodegradation is described in these systems by the 1st order, or linear biodegradation equation (Angley et al., 1992; Langner et al., 1998), or the Monod equation, which is also referred to as Michaelis-Menten equation, coupled to substrate, electron acceptor and/or nutrient loss (Borden et al., 1986; Molz et al., 1986; Widdowson et al., 1988; Kindred et al., 1989; Kinzelbach et al., 1991; Estrella et al., 1993; Wood et al., 1994; Zysett et al., 1994; Corapcioglu et al., 1995; Wood et al., 1995; Maier et al., 1997; Murphy et al., 1997; Brusseau et al., 1999; Lee et al., 2000; Miralles-Wilhelm et al., 2000). Indeed, the linear biodegradation model is simply the Monod equation for the special case of low substrate concentrations ($C << K_S$).

A methodology was developed by Brusseau et al. (1999a) to determine when the transport equation should be coupled with linear biodegradation, or when the nonlinear Monod equation for growth should be used to incorporate an increase in substrate demand by microorganisms over time. A nondimensional A-D equation coupled with linear, instantaneous sorption, nonlinear biodegradation, biomass growth and decay, and electron acceptor availability was derived, and three controlling dimensionless parameters from this equation were used to generate a 'type' curve. The location of these parameters on the type curve was successful in predicting whether nonlinear biodegradation would occur (Brusseau et al., 1999b). Simulations performed using this model showed that nonsteady transport behavior was observed when the input substrate concentration was larger relative to the half saturation constant, because in these cases the specific growth rate was closer to the maximal specific growth rate. Also, at higher initial biomass

concentrations, the increase in substrate was more significant (and nonsteady transport was achieved more rapidly), because the same relative increase in biomass causes a much larger absolute change in cell numbers. However, if the initial biomass concentration was too high relative to the input substrate concentration, there was not be an appreciable relative increase in cell numbers, and steady state transport was not observed. Finally, as the maximal growth rate or residence time (contact time) increased, simulations showed that the substrate demand increased.

The effects of field-scale heterogeneity have been simulated, and its importance emphasized, in a number of publications. Two separate research teams have developed stochastic models coupled with the ADE and Monod growth kinetics. One model was used to compare 'clean-up times' in a simulated homogeneous aquifer versus a simulated heterogeneous aquifer containing clay lenses (Lee et al., 2000). While the model predicted a 7-year clean-up time for the homogeneous aquifer, the heterogeneous aquifer only achieved a theoretical 60% removal after 10 years. The presence of clay lenses in the model acted as sources of contaminants, due to the relatively slow rate of diffusion out of these regions. This behavior is observed commonly at field sites, but the numerical model is useful in quantifying the effect. In a more complex stochastic model, Miralles-Wilhelm et al. (2000) examined the effect of spatial variation of physical and biological factors. They associated variations with the contaminant decay coefficient (a coefficient composed of the yield and maximal growth rate), microbial death coefficient, contaminant retardation coefficient, and macrodispersion. While the same initial biomass

was assumed to be distributed throughout the site, the microbial population soon became spatially variable due to the variability of the growth kinetic parameters. The spatial variability of these growth parameters was also found to influence solute transport and biodegradation predictions.

The effect of soil heterogeneities in layered porous media has been simulated using a few different models. Transport and biodegradation of an organic compound (quinoline) in a 2-layered porous media system were studied and compared with simulations from a layered transport and biodegradation model incorporating the microbial kinetics involved (Wood et al., 1994). Monod growth equations for substrate and oxygen utilization and metabolic lag were used to describe biodegradation kinetics. Results from a model using biofilm principles were compared with those from a single microbial phase model. When compared to experimental results, the single phase model was more appropriate. Also, the inclusion of metabolic lag proved to be very important in the performance of the model. In an extended layered model similar to the last, many more processes were incorporated and model simulations were compared to experimental results (Murphy et al., 1997). This model included terms for bacterial transport, metabolic lag, induction threshold concentration of the substrate prior to biodegradation, endogenous respiration and its consumption of oxygen, and buoyancy effects associated with small solution density variations. It was discussed that the microbial parameters of endogenous respiration, bacterial transport (specifically the coupling of detachment with growth of bacteria) and buoyancy effects were particularly significant in providing

greater predictive abilities. At the field-scale, application of a layered model incorporating biodegradation led to very different conclusions. The primary factor influencing contaminant transport and biodegradation was the mixing of the organic and dissolved oxygen in the groundwater between individual soil beds, not the microbial kinetic parameters (MacQuarrie et al., 1990).

Sorption of a contaminant to the porous media retards transport and decreases bioavailability of a contaminant. The effect of sorption on the biodegradation of alkylbenzenes in a transport system was investigated by Angley et al. (1992), and results were compared with a transport model that incorporated rate-limited, linear sorption, and first-order biodegradation (i.e., no growth). The model accurately predicted transport behavior. Therefore, it was concluded that first-order biodegradation is a good assumption for this type of system because sorption of a compound significantly limits its bioavailability, such that the concentration of the compound in aqueous phase is often too low to support growth. This model was later amended to incorporate nonlinear, rate-limited sorption as described by the Freundlich isotherm (in general, $S=K_f C^n$). Simulations performed using this model demonstrated that a linear sorption model cannot accurately simulate transport and biodegradation for solutes that have $n < 0.9$ (Brusseau, 1995).

Incorporation of microbial parameters other than bacterial growth and bacterial transport is fairly novel. The process of bacterial decay has been included in a number of models utilizing a first-order decay equation (Borden et al., 1986; Wood et al., 1994;

Zysett et al., 1994; Murphy et al., 1997; Brusseau et al., 1999 a and b; Miralles-Wilhelm et al., 2000). A transport model that included aerobic and denitrification biodegradation was presented by Molz et al. (1986), and amended to include inhibition of the denitrifying enzyme by oxygen. This was represented by altering the Monod equation to account for basic noncompetitive inhibition. A coupled transport and biodegradation model presented by Estrella et al. (1993) incorporated substrate inhibition using the Haldane inhibition modification to the Monod equation. The process of microbial metabolic lag is emerging as an important variable, and has been incorporated using a piecewise-linear metabolic potential functional (Wood et al., 1994 and 1995; Murphy et al., 1997). Application of the model developed by Wood et al. (1995) to results from a set of column experiments was compared to application of a similar developed by Chen et al. (1992) that did not incorporate microbial lag. Wood et al. (1995) reported a better fit to the data set than that obtained by Chen et al. (1992). Finally, the importance of the incorporation of endogenous respiration (and its impact on oxygen consumption) and an induction threshold substrate concentration has been presented (Murphy et al., 1997).

A broad conceptual subsurface contaminant transport with biodegradation model which incorporated many different microbial parameters was developed by Kindred et al. (1989). It incorporates aerobic and anaerobic degradation, including several different models for the anaerobic case. The basic conceptual model incorporates Michaelis-Menten uptake kinetics, and options for competitive and noncompetitive uptake inhibition. The growth equation reduced to a first-order biodegradation equation as a

function of the linear sorption coefficient (which dictates bioavailability). Simulations were conducted for the cases of aerobic biodegradation, co-metabolism, uptake of multiple organic substrates, aerobic biodegradation with nitrate reducing metabolism, and aerobic and anaerobic fermentative metabolism.

Finally, the effect of facilitated contaminant transport by sorption of the contaminant onto mobile bacteria was examined by Corapcioglu et al. (1995). They developed a model composed of the ADE coupled with the Monod growth equation, bacterial transport and equilibrium partitioning between the aqueous and solid phase, and equilibrium partitioning of the contaminant between the mobile and immobile bacteria. When simulations were compared with experimental results from inoculated soil columns subjected to a phenanthrene solution, it was determined that while the presence of bacteria caused enhanced transport of the contaminant, biodegradation by these organisms caused attenuation of the contaminant. Thus, these effects seemed to cancel out each other.

DISSERTATION FORMAT

This dissertation is composed of a review of relevant literature, and two manuscripts which are included as appendices. The first manuscript (Appendix A) includes results from batch sorption, batch biodegradation, and miscible-displacement experiments. Additionally, it describes the development of a contaminant transport and biodegradation mathematical model, and its application to all of the miscible-

displacement experiments described above. I designed, performed, and analyzed the results for all of the batch experimental systems. The miscible-displacement experiments were performed jointly with Dr. Fiona L. Jordan, a co-author on that manuscript. However the experimental design for those experiments was based off my previous work, also conducted with Dr. Jordan, which has since been published (Sandrin et al., 2001).

The mathematical model developed for this study is a modification of a model previously developed, as described in the manuscript. The idea of incorporating microbial lag and inhibition were my own, and the idea for incorporating bacterial transport stems from conversations with my advisor Dr. Brusseau, and colleague, Ms. Li Li. I decided how to implement the parameters of microbial lag and inhibition, and that we could not use a mechanistic model to incorporate bacterial transport. However, Ms. Li did most of the FORTRAN coding and troubleshooting of the code. I ran all of the model simulations presented myself, and determined and coded the individual bacterial transport and distribution equations for each system.

The second manuscript (Appendix B) is a collaboration of work. The actual field experiments were conducted by other students in my research group (including John McCray, Brent Cain, William Blanford, Nicole Nelson, and many other helpers) and by my advisor, Dr. Brusseau. The results from this experiment are published elsewhere. Ms. Stacie Alter compiled much of the recovery data from the Hill site. Dr. Joe Piatt, a co-author on this manuscript, compiled the recovery data and ran simulations for the Tucson site. Dr. Brusseau had the initial idea to determine the effect of the surfactant

flush on the biodegradation rate constant. My contribution was to run all the simulations for the Hill site, including determination of transport parameters using breakthrough data for bromide, and determination of the linear biodegradation coefficients for the biotracers. Finally, I was responsible for writing most of the text presented, organizing the data into a cohesive paper, and generating the figures presented.

In addition to the work presented here, there was a significant amount of preliminary work that went into this study. A large portion of this work, which describes the relative impacts of microbial lag and bacterial cell growth on transport and biodegradation behavior, has already been published in a manuscript that I authored (Sandrin et al., 2001). This text was not included in this dissertation because a portion of it was included in my Master's work. Finally, I also contributed to a manuscript (still in review), which is based on the same miscible-displacement experiments discussed herein, but focuses on bacterial transport in these systems. In addition to contributing to all of these experiments, Dr. Jordan and I conducted numerous non-reactive tracer experiments utilizing the same soils, and I provided editing for the manuscript.

PRESENT STUDY

The methods, results, and conclusions of this study can be found in the manuscripts included as appendices. The following summary discusses the most important findings presented in these papers.

Transport and biodegradation of organic contaminants in the subsurface has been studied extensively in an effort to determine the ultimate fate and distribution of contaminants that may threaten local groundwater supplies. Research aimed at tackling this problem has ranged from static batch, biodegradation studies to laboratory scale miscible-displacement systems, and all the way up to large field scale ranging over many square kilometers. While variability and scaling effects of geologic and soil properties have been discussed frequently, it is only recently that variability and scaling effects of biologic processes have been recognized.

In this study, four specific objectives were met by conducting experiments and model simulations. These objectives are as follows; 1) determine the effect of increasing both biologic diversity and population density variability on biodegradation at the laboratory-scale, 2) determine applicability of batch derived biokinetic parameters to non-static systems, 3) elucidate the relative importance of certain biologic parameters in different soil systems, and 4) present a method for evaluating the spatial distribution of microbial activity in-situ.

Objective 1

Batch biodegradation and miscible-displacement experiments were conducted to evaluate the impact of increasing both biologic diversity and population density variability on the variability of biodegradation behavior in batch and flow-through systems. Batch biodegradation studies were performed utilizing a microbial community composed of a pure culture and two indigenous soil communities. Miscible-displacement systems ranged from homogeneous sand inoculated with a pure culture community to heterogeneous soils containing indigenous communities. Enhanced variability was exhibited between trials of the miscible-displacement systems with greater biologic diversity and variations in population density. Additionally, the variability associated with microbial lag was larger for batch and miscible-displacement systems containing indigenous populations. This caused a more gradual decrease in the observed concentration of the target compound in the column effluent for the flow-through systems.

Objective 2

Biokinetic parameters were found to be significantly different in batch versus continuous flow systems. Biokinetic parameters related to biodegradation of the target compound were determined in a suite of batch experiments for a single isolate, *Pseudomonas putida* RB1353, and for two indigenous soil populations. Additionally, a transport and biodegradation model incorporating microbial lag, bacterial cell elution,

inhibition, and initial nonuniform spatial distributions of bacteria was developed and calibrated to determine the biokinetic parameters from miscible-displacement experiments containing the same populations.

The change in biokinetic parameters from batch to continuous flow systems was not the same in the homogeneous sand versus the heterogeneous soil, except with respect to the maximal growth rate. The maximal growth rate was larger in the miscible-displacement experiments than in the batch experiments in all cases. However, microbial lag was longer in homogeneous sand, but shorter in heterogeneous soil for inoculated and indigenous populations as compared to batch values. Finally, the observed cell yield in the miscible-displacement systems was larger in sand and smaller in heterogeneous soil as compared to batch values.

Objective 3

Model simulations were performed to quantify effects of certain biologic behaviors in different flow-through soil systems. In the inoculated sand systems, simulations were significantly affected by the incorporation of both cell elution and microbial lag. Conversely, incorporation of cell elution did not significantly alter simulation results for the inoculated or indigenous heterogeneous soil systems, which retained significantly more bacteria. Model simulations conducted for the indigenous, heterogeneous systems were markedly affected by the incorporation of microbial lag, but

the inoculated soil systems had such short lag phases that this parameter was not as important.

Objective 4

A contaminant transport model incorporating linear biodegradation model was applied to recovery data from short input pulses of biotracers at two field sites to determine the spatial variability of biodegradation rates in-situ. At one field site, the system was undisturbed and had low levels of oxygen, such that the distribution of microbial activity was fairly uniform. However, the other field site had recently been subjected to a surfactant flush, thereby increasing oxygen levels near the injection wells and causing a subsequent increase in microbial population densities (confirmed by viable counts obtained from soil cores at the site). This increase was evident in the spatial distribution of the calculated biodegradation rates, which were significantly higher near the injection well. Thus, this method is valuable and relatively simple for determining spatial variability of microbial activity and biodegradation potential.

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APPENDIX A:

APPLICATION OF A BIODEGRADATION AND TRANSPORT MODEL INCORPORATING MICROBIAL LAG TO SYSTEMS OF INCREASING HETEROGENEITY AND BIOLOGICAL DIVERSITY

Susannah K. Sandrin[1], Fiona L. Jordan[1], Li Li[1], Raina M. Maier[1], and Mark L. Brusseau[1]

[1]Department of Soil, Water and Environmental Sciences
University of Arizona
Tucson, Arizona

ABSTRACT

A biodegradation and transport model incorporating bacterial growth, microbial lag, and cell transport was developed, and its predictive capability was examined by comparing simulation results to an array of experimental results obtained from systems of increasing complexity. The first and least complex system consisted of a sterilized, homogeneous quartz sand inoculated with a pure culture of *Pseudomonas putida* RB1353, a strain of bacteria capable of degrading salicyate, the model organic compound. A somewhat more complex system consisted of a sandy loam soil inoculated with the pure culture used above. The most complex systems comprised soils (of differing textures) containing indigenous communities capable of degrading salicylate. Batch mineralization studies were conducted for all systems, using the same communities as in the miscible-displacement experiments, to determine biodegradation and bacterial growth kinetic parameters (including lag times). Kinetic parameters determined from batch studies and hydrodynamic parameters obtained from miscible-displacement experiments performed using conservative tracers were used as input to examine the independent prediction capabilities of the model.

When simulations were performed utilizing the batch biokinetic parameters, the simulation results were significantly different from experimental results. Therefore, the biokinetic parameters of maximal growth rate, mean lag time, and lag time variance were calibrated for simulations utilizing either the cell yield observed for that community in the batch or miscible-displacement experiments. According to model calibrations for the

miscible-displacement systems, the maximal growth coefficient was larger in all systems compared to batch values. Additionally, lag times for the inoculated homogeneous sand system were longer than those reported in batch systems, but shorter in the inoculated and indigenous heterogeneous soil systems. The intermediate system was least affected by the incorporation of microbial lag and transport, while the simulation for the inoculated sand system was significantly affected by the incorporation of both. Finally, there appeared to be increased variability in terms of cell elution, substrate biodegradation and transport, and bacterial growth in the most complex systems. The least variability, in terms of these behaviors, was observed in the inoculated sandy loam soil system.

INTRODUCTION

Contaminants in the subsurface are subjected to many processes, including the predominant processes of transport, degradation (biological, chemical or radioactive decay), and sorption. Biodegradation has been studied from the pore-size scale to the field scale, in batch systems and in flow-through systems. Contaminant transport has also been examined at all scales. As scale increases, both geologic heterogeneities and biologic diversity have an increasing influence on contaminant fate.

Transport models have evolved to incorporate biodegradation. The simplest models incorporate biodegradation as a 1st order process, such that biodegradation is assumed to occur at a constant rate by a population of invariant size. More advanced models have incorporated microbial growth, generally by assuming Monod growth kinetics (Borden et al., 1986; Molz et al., 1986; Widdowson et al., 1988; Kindred et al., 1989; Kinzelbach et al., 1991; Estrella et al., 1993; Wood et al., 1994; Zysett et al., 1994; Corapcioglu et al., 1995; Wood et al., 1995; Murphy et al., 1997; Brusseau et al., 1999; Lee et al., 2000; Miralles-Wilhelm et al., 2000). Finally, other microbial processes have been included into even more advanced contaminant transport and biodegradation models, or into biochemical engineering substrate consumption biofilm models, in recent years. These processes include microbial lag (Wood et al., 1994 and 1995; Murphy et al., 1997), bacterial transport and the influence of growth and metabolism on bacterial transport (Corapcioglu et al., 1984 and 1985; Chang et al., 1987; Rittmann et al., 1992; Lindqvist et al., 1994; Zysett, et al., 1994; Corapcioglu et al., 1995; Peyton et al., 1995;

Wanner et al., 1996; Reddy et al., 1996; Murphy et al., 1997; MacDonald et al, 1999b), and inhibition (Molz et al., 1986; Estrella et al., 1993; Murphy et al., 1997).

Microbiological systems are generally composed of large numbers of organisms. For example, one gram of soil can easily contain a million bacteria or more. Due to these large numbers of organisms and potential for genetic exchange and mutation events, a great deal of variability exists among the physiologies of individual microorganisms, even in a pure culture, which leads to variance in observed behaviors of individual cells. The idea that the growth of individual bacterial cells is variable within a culture has been tested and noted many times (Kelly et al., 1932; Kubitschek, 1966; Harvey et al., 1967; Trueba et al., 1982; Liou et al., 1997). Furthermore, the biological diversity of natural systems, composed of mixed populations, leads to increased variance in these behaviors. Yet it is generally very difficult to account for this phenomenon in transport and biodegradation models.

Most coupled transport and biodegradation models utilize composite or lumped values for microbial growth parameters, and fail to acknowledge that each population present has a unique growth behavior for a particular substrate and soil system. This is not for lack of knowledge that soils are composed of a dynamic population of microorganisms, rather, it represents a limitation of resources. It is currently not possible to identify and account for all the individual populations that may participate in biodegradation. Therefore, the assumption is made that the community can be described by use of parameters that represent average behavior, along with a representation of the

deviation from this average. This method was applied to account for microbial lag in the model described herein.

In addition to natural variation in microbial populations, contaminant transport and biodegradation models need to take into account the impact of non-uniform spatial distributions of microorganisms. Population densities of microorganisms have been shown to be spatially variable as a function of soil texture and/or carbon or oxygen availability at the field-scale (Harvey et al., 1984; Severson et al., 1991; Harvey et al., 1992; Kieft et al., 1998; Krueger et al., 1998; Stapleton et al., 1998 and 2000) and at the laboratory scale (Wood et al., 1994). Miralles-Wilhelm et al.(2000) demonstrated model simulations that incorporate spatially-variable microbial parameters produce significantly different results from model simulations that assume uniform distributions.

Finally, previous researchers have observed that certain biokinetic parameters were different for batch and flow-through systems (Kuhn et al., 1985; Estrella et al., 1993; Shaw et al., 1998). While it is common to perform biodegradation studies in batch systems, these types of systems may not be indicative of field situations. In batch experiments, the microbial community is subjected to a single instantaneous 'pulse' of substrate, rather than a constant supply of substrate. Additionally, these systems are not subject to removal of bacteria due to elution of bacterial cells from the system. However, many of the transport models in the studies mentioned above did not account for the biologic parameters included in the model presented herein (microbial lag, bacterial transport, and non-uniform initial distributions of bacterial cells). Thus, for these studies,

it is unknown if the discrepancy in biokinetic parameters between batch and flow-through systems was an effect of scaling, or an artifact of the lack of consideration of certain processes.

Experimental results from a set of batch biodegradation and miscible-displacement systems are presented in this paper. These systems were composed of different combinations of three microbial communities and three soil types. The purpose of these experiments was to elucidate the effects of population density, cell elution and influence of soil texture, and microbial lag and its variability on biodegradation in batch and flow-through systems. The three microbial communities studied in the batch biodegradation experiments consisted of one pure culture and two indigenous communities present in two soils. Four experimental miscible-displacement systems were studied, including one system composed of a well-sorted quartz sand inoculated with a single degrading species, a soil system inoculated with the same single degrading species as above, and two soils (a loamy sand and a mixed soil from two sites) with diverse, indigenous degrading populations.

In order to quantitatively compare biokinetic parameters from batch and miscible-displacement systems and to evaluate the relative effects of certain biologic behaviors, a coupled transport and biodegradation model incorporating growth and microbial lag was applied to this experimental data. Model simulations were performed utilizing microbial growth parameters obtained from batch studies and compared with experimental data to determine the predictive capability of the model using these parameters. Additionally,

simulations were performed utilizing calibrated growth parameters (maximum growth rate coefficient, mean lag time, and lag time variance) to provide a comparison for these parameters in static and flow-through systems. Finally, simulations that did not incorporate subroutines for cell elution or microbial lag were performed to determine the relative effect of these behaviors in these systems.

MATERIALS AND METHODS

Materials. The same soils and substrate were used in both the batch and column experiments so the results could be used to compare independent model predictions, obtained using batch data, with column data. The simplest system consisted of a well-sorted sand (a 20/30 mesh Accusand, Unimin Corp.) inoculated with a pure culture of *Pseudomonas putida* RB1353. This particular species contains the NAH plasmid that encodes genes for the degradation of napthalene and salicylate, as well as a selectable antibiotic (Kanamycin) resistance marker. This culture was kindly provided by Robert Burlage (Oak Ridge National Laboratories, TN). The intermediate, or somewhat more complex system consisted of a loamy sand (referred to hereafter as Hayhook soil) inoculated with *P. putida* RB1353 at the same population density as the sand system. The two most complex systems were composed of loamy sands containing indigenous salicylate degrading communities of multiple populations. These soils were Hayhook, a soil with relatively low organic matter (0.4%), and an 80:20 mixture of Vinton and Mt. Lemmon soil of higher organic matter content of 1% (referred to hereafter as Vinton).

The chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, Mo). A phosphate-buffered mineral salts solution (MSB, composed of 1.5 g/L KH_2PO_4, 0.5 g/L Na_2HPO_4, 0.2 g/L $MgSO_4$-$7H_2O$, 2.5 g/L NH_4Cl, 0.3 mg/L $FeCl_3$, 0.0132 g/L $CaCl_2$-$2H_2O$) was used as the background solution for all aqueous solutions used in the batch and miscible-displacement experiments (including salicylate and the non-reactive tracers). Radiolabeled ^{14}C-salicylic acid (ring-labeled), with a measured specific activity of 5.9 mCi/mmol (> 95% purity), was also purchased from Sigma Chemical Co.

General methods. All system components and materials (except for incubated soils) were sterilized prior to each experiment to eliminate biodegradation by microbes other than the inoculated or indigenous soil bacteria. Specifically, the sand was autoclaved for 60 minutes, whereas the soil for the inoculated experiments was autoclaved 30 minutes a day for four consecutive days. The exception to this technique was for the batch sorption studies. Serial dilutions of the sterilized porous medium were plated on nutrient agar to determine that the medium was sterile. The tubing, pump, and other components that could not withstand autoclaving were sterilized by treatment with a 2% bleach solution for a minimum of 30 minutes, followed by treatment with sterile 0.01% Na thiosulfate solution, used to neutralize the bleach, and a final treatment with sterile distilled, deionized water. All glassware and solutions were sterilized by autoclaving, except for the salicylate solutions, which were filter sterilized (0.2 um filter).

Batch Biodegradation Studies. Batch studies were performed to determine the microbial growth kinetic parameters half-saturation constant (K_S), maximal growth rate (μ_{max}), yield ($Y_{X/S}$), and the lag parameters of mean lag time (t_{lag}) and the associated standard deviation (σ_{lag}). These studies were performed for a range of salicylate concentrations and for each microbial community used (*P. putida* RB1353 and the two indigenous communities). Salicylate solutions were mixed with inoculum in 250 mL Erlenmeyer flasks with threaded caps lined with rubber septa. The threads were also wrapped with teflon tape to insure that the flasks were airtight. Flasks were opened in a fume hood when aqueous samples were removed, to prevent any ^{14}C-CO_2 from being vented into the laboratory. Samples were taken to monitor the decline in aqueous concentration of salicylate over time. Biodegradation experiments were performed three times, in triplicate for each community z(for a total of nine degradation curves for each concentration) over the range of salicylate concentrations.

The *P. putida* RB1353 solution was prepared by growing a 24-hour pre-culture from one isolated colony on a streak plate, followed by a 48-hour culture in Luria Broth (Atlas et al., 1993) amended with 100mg/L Kanamycin (LBK). Kanamycin was added to select for *P. putida* RB1353. The cultures were harvested in late stationary phase. The cells were pelleted by centrifugation, resuspended in saline to dissolve any attached carbon, repelleted, and finally resuspended in a phosphate-buffered mineral salts solution. The soil inoculums were prepared by saturating the soil with MSB to the desired water content, followed by a 96 hour incubation period to allow the indigenous community to

equilibrate. The single-isolate batch systems were inoculated by adding the concentrated inoculum of *P. putida* RB1353 to the salicylate solution to achieve the final desired concentration of salicylate and population density of bacteria (10^7 CFU/mL). For the 'inoculum' of indigenous populations present in the soils used, wet soil equivalent to 10 g dry soil was added to flasks followed by enough salicylate solution to achieve a final aqueous volume of 20 mL and the desired final concentration of salicylate (dilution by the soil moisture was taken into account).

The aqueous concentration of salicylate was analyzed by two methods, depending on the initial aqueous concentration, and if dissolved organic carbon was present to interfere with spectrophotometer readings (as in the soil batch and column experiments). Aqueous concentrations from experiments with low dissolved organic carbon and initial concentrations of 20 mg/L or greater (aqueous batch experiments and sand columns with *P. putida* RB1353) were analyzed using a UV spectrophotometer (Hitachi, Model U-2000, Danbury, CT) at an absorbance wavelength of 296 nm, subtracting the background absorbance of the MSB solution. Otherwise, salicylate solutions were spiked with ^{14}C-salicylate. Samples from batch slurry systems or soil column effluent were centrifuged for 10 minutes at 10,000 x G to separate suspended soil particles and bacterial cells, including those containing ^{14}C. It was assumed that any remaining ^{14}C in the supernatant was ^{14}C-salicylate. A 1 mL sample of the supernatant was analyzed for radioactivity using a liquid scintillation counter (Model 1600TR Packard Instrument Company, Meriden, CT).

The cell yield for these populations growing on salicylate was determined differently for the indigenous populations versus *P. putida* RB1353. For the slurry studies, ^{14}C-salicylate spiked salicylate solutions were used to track the fate of carbon in the system. Evolved CO_2 (including ^{14}C-CO_2) was collected in upright tubes of 1N potassium hydroxide (KOH) sealed in the flasks. When there were non-detectable quantities of aqueous salicylate remaining in the flask, it was assumed that it had all been consumed to produce new cells or CO_2. Samples were taken from the KOH solution and analyzed for ^{14}C-CO_2 using a liquid scintillation counter.

Determination of cell yield for *P. putida* RB1353 growing on salicylate was accomplished separately from the smaller scale (250 mL) biodegradation studies. A concentrated solution of cells was inoculated into 1 L of a 50 mg/L salicylate solution in a 2.8 L flask and placed on a shaker. An initial cell mass was determined by centrifugation of the same volume of inoculum in a microcentrifuge for 10 minutes at 10,000 x G, and decanting of the supernatant. The remaining pellet was dried and weighed. Salicylate was added to the experimental flask two more times, such that the total mass of salicylate consumed was 150 mg. Small samples were taken periodically to monitor salicylate loss. After degradation of the third addition of salicylate, the solution was centrifuged in aliquots in a 250 mL centrifuge bottle, and the supernatant was decanted after each centrifugation event. The pellet remaining after the 4 centrifugation events was dried and weighed. As a control for growth due to residual carbon remaining in the cells, the same volume of cells was inoculated into MSB, and the cell mass at the

end of the incubation time for the experimental flask was determined. All experiments (control and experimental) were performed in duplicate. The (slight) average increase in mass observed in the control flasks was subtracted from the results obtained for the experimental flasks.

Batch Sorption Studies. Sorption studies were performed in batch systems containing sterilized soil slurries (1 part soil: 2 parts aqueous solution by mass) for five aqueous concentrations of salicylate (1, 5, 20, 50 and 100 mg/L) spiked with ^{14}C-salicylate. Soil was sterilized by oven-drying at 105° C for three days. One gram of sterilized soil was added to a sterilized test tube, followed by addition of two mL of salicylate solution. These systems were allowed to equilibrate for 24 hours on a shaker table at 120 rpm, with the capped tubes at an approximate 45° angle for maximum agitation. Following this incubation, tubes were placed upright for 15 minutes to allow for settling of the large soil fractions, and a 1.5 mL aqueous sample from each tube was vortexed in a microcentrifuge at 14,000 x G for 10 minutes to increase the rate of sedimentation of any remaining soil particles. The supernatant was analyzed for aqueous ^{14}C-labeled salicylate as described above.

Miscible-displacement experiments. Salicylate transport was evaluated in sterile and non-sterile systems to determine the effect of biodegradation on the transport of this compound. However, the sterile salicylate transport experiments were not used to determine sorption parameters, because autoclaved soils were used for these studies, and this procedure may have affected their chemical properties (Shaw et al., 1999).

Additionally, miscible-displacement experiments were performed with non-reactive tracers to characterize the hydrodynamic properties of the systems.

For the systems inoculated with *P. putida* RB1353, the sterilized porous media were inoculated with10^7 CFU/g dry soil. Ten mL of inoculum were slowly aliquoted onto 200 g of dry soil as the mixture was being stirred to insure uniform distribution. The indigenous soil systems were prepared as described for the batch studies. The porous media were subsequently packed into glass columns (15 cm length, 2.5 cm diameter) under a laminar flow hood using aseptic techniques.

The packed column was connected to a piston-displacement pump using teflon tubing. For all experiments, excluding the control, a phosphate buffered mineral salts broth (MSB) was pumped through the column for approximately 22 hours (~15 pore volumes), followed by a 24 hour pulse of salicylate solution (C_o=20 mg/L). The substrate reservoir was sparged with oxygen for all experiments to eliminate oxygen constraints within the system. All experiments were conducted using a flowrate of 0.3 ml/min, which is equivalent to an average pore water velocity of approximately 10 cm/hr.

Breakthrough curves were obtained for salicylate and elution curves were obtained for cells for each system by analyzing column effluent samples. For salicylate, effluent samples were collected continuously (at a rate of 1 sample every 20 minutes) in culture tubes using a fraction collector. In order to prevent degradation of the compound in the tubes, each tube was amended with 1N sodium hydroxide to achieve a final, post collection concentration of 0.3 N in each tube. Bacterial cell samples were also taken

every 2 or 3 hours by allowing column effluent to drip into a sterile microcentrifuge tube. Serial dilutions followed by plating on a nutrient agar were performed immediately.

Bacterial cell enumeration. Viable cells were enumerated for all biodegradation and coupled biodegradation and transport experiments. These cell counts were obtained by plating serial dilutions of samples, in triplicate, on nutrient agar of LBK for the inoculated systems, or on both salicylate nutrient agar (100 mg/L salicylate, 100 mg/L cycloheximide, MSB, and noble agar) and R2A for the indigenous systems. Two nutrient agars were utilized for the indigenous columns to obtain estimates for heterotrophic bacteria, and the subset of those capable of degrading salicylate. Plate counts were chosen to provide a conservative estimate of the active population.

Samples of the porous media were collected prior to each biodegradation experiment to determine the cell numbers associated with the porous media. For the miscible-displacement experiments, soil samples were collected following the experiment as well. A 9.5 ml aliquot of a 0.85% saline solution was added to approximately 1.3 g of wet sand or soil, and the slurry was vortexed for 1 minute to transfer the cells into solution. Serial dilutions of this solution were plated on nutrient agar. Prior to each experiment, three subsamples were collected from the porous media. Following each miscible-displacement experiment, soil samples were taken from both ends of the column and at two locations, 5 cm and 10 cm from the influent end of the column, in duplicate (i.e., a total of eight samples were taken at four locations within the column).

Cell mass balance calculations were performed for all of the miscible-displacement experiments. The initial number of cells present in the soil column was calculated by multiplying the average viable counts/g dry soil by the mass of dry soil packed into the column. At the end of the experiment, the average viable counts/ g dry soil for each region were multiplied by 1/4 the mass of dry soil in the column, and these values were summed. Total cells eluted, and cells eluted pre- and post-salicylate pulse were determined by numerically integrating under the cell elution curve. The increase in cells due to growth from consumption of salicylate (column yield) was calculated as the difference between the sum of the number of cells eluted post-salicylate pulse ($E_{post\text{-}pulse}$) and remaining in the column (R) and the difference in the initial number of cells in the column (I) and the number of cells eluted pre-salicylate pulse ($E_{pre\text{-}pulse}$). In equation form, the number of new cells due to growth resulting from salicylate biodegradation is

$$G = (R + E_{post-pulse}) - (I - E_{pre-pulse})$$

An estimate of the theoretical biomass produced based on the amount of substrate degraded was also calculated using the cell yield coefficient obtained from batch experiments. This cell mass was converted to cell numbers assuming an individual cell mass of 9.5×10^{-13} g (Neidhardt et al., 1990). The mass of salicylate degraded was determined for each column by comparing the total mass of salicylate eluted with the total input mass.

MODEL DEVELOPMENT

A one-dimensional advective-dispersive solute transport model with biodegradation was amended to incorporate microbial lag, inhibition, and cell transport. This transport model, which is coupled with linear, instantaneous sorption, nonlinear biodegradation and consumption of electron acceptors, biomass growth (as described by the Monod equation) and first-order bacterial decay, was previously presented by Brusseau et al. (1999). The equations used in this model were presented as:

$$(\theta + \rho K_d)\frac{\partial C}{\partial t} = -q\frac{\partial C}{dx} + D\frac{\partial^2 C}{dx^2} - \frac{\mu M \theta}{Y}$$

$$\frac{dM}{dt} = \mu M - b(M - Mo)$$

with the parameters described in Appendix A. In addition to microbial growth, the processes of microbial lag, product and substrate inhibition, cell elution and non-uniform initial cell distribution are incorporated in this model to more accurately account for the biological processes occurring in the miscible-displacement experimental systems.

The phenomenon of microbial lag is a widely acknowledged phenomenon by microbiologists, food scientists and biochemical engineers. Numerous models have been proposed for batch growth systems, including empirical, mechanistic, and segregated models. The largest contributor to microbial lag models is the field of food science, (Baranyi et al., 1994), however most of these models are empirical models relating lag times of microorganisms to salt content, temperature, and pH (Rabotnova et al., 1959;

Lankford et al., 1966; Kawashima, 1973; Smith, 1985; Thayer et al., 1987; Skinner et al., 1994; Davey, 1991). These parameters are generally not adjustable in environmental systems. The field of biochemical engineering has provided model advancements, many of which are mechanistic. While these models are very useful in predicting microbial lag based on the activities performed by the cell, they are also generally specific to one organism under certain conditions (Ramkrishna et al., 1967; Fredrickson et al., 1970).

Therefore, a more general model is necessary for modeling microbial growth and biodegradation for biologically diverse systems. A microbial lag model proposed by Buchanan et al. (1997) lends itself well to this field because of its ability to be used for biologically diverse systems. Buchanan's three-phase linear growth model assumes the lag period of the population is described by a normal distribution, and it was demonstrated to fit the growth curves of numerous microorganisms approximately as well as the models by Baranyi and Gompertz. In this manner, the transition from microbial lag to exponential growth can be represented by values for an average and a standard deviation associated with the lag time of the population.

This three-phase linear growth model is based on the proposition that the non-abrupt transition between the lag and exponential phase is due to (or a reflection of) the biological variation of the bacterial population. As discussed previously, the idea that the growth of individual bacterial cells is variable within a culture is widely accepted (Kelly et al., 1932; Kubitschek, 1966; Harvey et al., 1967; Trueba et al., 1982; Liou et al., 1997). Furthermore, the successful application of a normal (regular or skewed) distribution to

the biological variability of bacterial cell cycles has been demonstrated previously by many researchers (Kubitschek, 1966; Harvey et al., 1967; Bremer, 1982; Trueba et al., 1982; Koch et al., 1982; Keasling et al., 1995).

Therefore, this general technique was incorporated into the transport model. Once a threshold concentration (C_{ind}, required for induction of metabolic processes) at any node within the 1-D system is achieved, a 'timer' is turned on for that node. The input parameters of mean and standard deviation are then used to calculate the number of cells emerging from the lag phase at any one time step, using the following equation to represent the normal distribution:

$$M_{ti} = \int_{t_{i-\Delta t}}^{t_i} \frac{1}{\sqrt{2\pi}\sigma} \exp\left(- \frac{(t_i - (t_{lag})_i)^2}{2\sigma_i^2} \right) dt + M_{(t-\Delta t)i}$$

These cells are added to the number of metabolizing cells at that node and then substrate consumption is computed.

The process of inhibition was incorporated into substrate growth kinetics using a simpler modification. Substrate inhibition was represented by the Haldane inhibition model, and product inhibition was represented by competitive inhibition kinetics, yielding an overall equation of the form

$$\mu = \mu_{max} \left(\frac{1}{K_S(1+(I/K_I)) + C + (C^2/K_i)} \right) \left(\frac{O}{K_o + O} \right).$$

where μ is used to describe the effective growth rate as it is affected by these parameters.

Finally, cell elution was accounted for explicitly, rather than by a mechanistic model. This means that the measured cell elution curves were fit with an equation, which was used to represent cell loss from the transport system. A similar technique was used to describe non-uniform initial cell distributions in the system that resulted from irregular cell elution during saturation. The reasoning for this method over a mechanistic model is two-fold. First of all, there was a great deal of variability in the elution behavior of the cells from the soil columns, particularly for the indigenous columns. A detailed description of the cell elution behavior is described in Jordan et al. (2000). Secondly, there is a great deal of controversy surrounding bacterial transport modeling.

Bacterial transport has been shown to be influenced by soil properties, cell properties, and environmental conditions. Soil properties that have been shown to affect bacterial transport include soil composition, texture, degree of heterogeneity, and water content (Fontes et al., 1991; Hornberger et al., 1992; Harvey et al., 1993; Huysman et al., 1993 a and b; Mills et al., 1994; Sharma et al., 1994; Barton et al., 1995; Devare et al., 1995; Cattaneo et al., 1997; Balkwill et al., 1998; Kieft et al., 1998; Powelson et al., 1998; Schafer et al., 1998; van Schie et al., 1999). Likewise, cell properties include cell surface hydrophobicity and/or charge density, cell size, physiologic state, motility, chemotaxis, gas production, and whether or not they are growing (Fletcher et al., 1979; Jenneman et al., 1985; van Loosdrecht et al., 1987; Harvey et al., 1989; Reynolds et al., 1989; Fontes et al., 1991; Gannon et al., 1991; Huysman et al., 1993; Sharma et al., 1993; Barton et al., 1995; Camesano et al., 1996; Grasso et al., 1996; Williams et al., 1996;

Balkwill et al., 1998; Baygents et al., 1998). Environmental factors that have been implicated in influencing bacterial transport are ionic strength, presence of carbon source, and flow conditions (Fontes et al., 1991; Hornberger et al., 1992; Huysman et al., 1993a; Rijnaarts et al., 1993; Mills et al., 1994).

There is not one transport model that includes all of these effects and that can 'predict' bacterial transport and attachment/detachment behavior for a range of conditions, primarily because of the lack of identification of the primary mechanisms responsible for this behavior, and this problem has been addressed by a number of authors (Corapcioglu et al., 1984; Wanner et al., 1986; Harvey et al., 1991; Hornberger et al., 1992; Rittmann 1993; Peyton et al., 1995; Wanner et al., 1995). Therefore, empirical models, such as first-order equilibrium (with respect to aqueous or attached concentrations), or kinetic models are often used to model bacterial transport (Hornberger et al., 1992; Tan et al., 1994; Lindqvist et al., 1994; Zysett et al., 1994; Reddy et al., 1996; Hendry et al., 1997; Murphy et al., 1997; Bolster et al., 1998). While these models may be adequate for some cases, they may not be adequate with soils containing indigenous bacterial populations and higher levels of clay and/or organic carbon. Therefore, empirical equations were generated for each miscible-displacement experiment to describe cell elution from the column, as well as cell distribution in the column, based on experimental data. The cell elution equation subtracted the appropriate amount of metabolizing biomass from each node in the column at each time step.

RESULTS AND DISCUSSION: EXPERIMENTAL SYSTEMS

Sorption and biodegradation batch studies. Sorption of salicylate to the homogeneous sand was shown to be negligible from comparisons of salicylate transport with that of a non-reactive tracer, pentafluorobenzoic acid, or PFBA (Sandrin et al., 2001) in sterilized sand. Sorption of salicylate to Hayhook and Vinton soils was adequately represented by a linear sorption isotherm over the range of concentrations used in these studies (Figure 1). These isotherms yielded linear sorption coefficient (K_d) equal to 0.078 and 0.28 [mg sorbed salicylate/kg soil)/(mg salicylate/L aqueous solution] for the Hayhook and Vinton systems respectively.

Biokinetic growth parameters were determined for all three systems in the same manner. Growth curves were obtained for the three communities for five or more salicylate concentrations, and data from these curves were used to determine all of the batch biokinetic parameters except for cell yield. These curves (Figure 2) were generated by calculating the mass of cells generated at each sampling time from the amount of salicylate degraded and the final cell yield (mass cells generated/ mass salicylate degraded). Growth rates at each concentration were computed as the slope of the linear portion of the growth curve when the natural log of the viable counts (ordinate) was plotted versus time (abscissa). This slope was calculated from a linear regression analysis of data from all three triplicate flasks. Standard error and standard deviation of the specific growth rates were also determined for the triplicate flasks by this regression. The maximal growth rate was estimated by the Lineweaver-Burk method (Bailey et al., 1986),

a linearized Monod growth equation. The half-saturation constant was considered to be the substrate concentration corresponding to half the maximal growth rate on the Monod-type plot (Figure 3).

The maximal growth rates reported for each system are similar in magnitude, however, the calculated maximal growth rates were smaller for the indigenous communities. A similar trend was observed for the half-saturation constant. Thus, while the maximal growth rate of the indigenous communities may be lower, they will achieve this maximal rate at a lower substrate concentration. Therefore, the indigenous communities will grow faster at low substrate (salicylate) concentrations, but at higher concentrations, the inoculated community will grow faster.

All of these populations exhibited Monod-type growth, as demonstrated by the asymptotic shape on a graph of specific growth rate versus initial substrate concentration (Figure 3). The Hayhook indigenous community experienced inhibition at 100 mg/L, as shown by a decrease in specific growth rate at this initial substrate concentration. For the Vinton indigenous community, the trend of the mean specific growth rate was to decrease from an initial substrate concentration of 50 to 100 mg/L. However, the presence or absence of inhibition could not be confirmed due to the variance associated with this value. At a contaminate field site, inhibition due to substrate, products, or toxic by-products is likely to occur near the center of a plume, where concentrations of these compounds are high. However, as evidenced by the data presented herein, inhibition can

occur at fairly low concentrations (for example, 100 mg/L), and will depend on the substrate and community present.

The lag time prior to biodegradation (t_{lag}) was quantitatively determined by calculating the intercept of the tangent line to the exponential growth curve (as determined from the linear regression aforementioned) with the value of the initial cell mass. The lag time variance (σ_{lag}) is a quantitative measurement of the variability of the mean lag parameter between the three triplicate flasks and among the community in each flask. This parameter was calculated from the errors associated with the slope and intercept of the regression line through data from the triplicate flasks.

The mean lag times for all communities tended to increase with increasing substrate concentration (Figure 4). This effect of initial substrate concentration has been observed by other researchers (Rabotnova et al., 1959; Rittmann, 1992). For the pure culture of *P. putida* RB1353, differences in lag times as a function of concentration were significant (at two standard deviations from the mean). However, differences between lag times were not significant (within two standard deviations or approximately 95% confidence) for the indigenous communities due to the wide variability in their lag times. This phenomenon of biological variability with respect to bacterial growth has been discussed previously. Finally, lag times of different communities growing at the same substrate concentration were observed to be different (Table 1). Thus, at a contaminated field site with spatially variable contaminant concentrations, the mean microbial lag time (t_{lag}) may also vary spatially and should not be considered constant. The variability of the

microbial community will also increase at the field scale, causing the parameter of lag time variation (σ_{lag}) to increase. As evidenced by the increase in this parameter from the pure culture community and the indigenous communities, lag time variance may be more important than the change in mean lag times due to spatial variations in concentration.

The indigenous communities appeared to have a lower overall affinity for salicylate, as evidenced by the smaller observed maximal growth rate, an increase in the lag times prior to biodegradation, and potential inhibition effects at 100 mg/L. The *P. putida* RB1353 community did not experience inhibition at concentrations of up to 500 mg/L salicylate, while the indigenous Hayhook community experienced inhibition at 100 mg/L. Additionally, the differences in mean lag times of the indigenous communities were significantly different from the mean lag time of *P. putida* RB1353 by more than two standard deviations at a salicylate concentration of 20 mg/L. Conversely, the cell yield for the indigenous populations were high in comparison with that of *P. putida* RB1353. This effect is likely due to the differences in the conditions of the batch experiments. The biodegradation experiements for the pure culture of were conducted in a sterile salicylate solution, while the biodegradation experiments utilizing the indigenous communities were conducted in a soil slurry. Therefore, the presence of dead cells and other carbon sources in the soil during the batch experiments may have influenced the cell yields in these systems. This effect may also be due to the other characteristics conferred upon this organism with the NAH plasmid, including luminescence during

degradation and antibiotic resistance, although this effect is likely negligible in comparison.

Transport experiments. Biodegradation of salicylate was observed in all experimental columns as compared to sterilized control systems. Salicylate recovery was greater than 99% in the control systems, and was comparable to that of the non-reactive tracers (bromide or pentafluorobenzoic acid). Transport parameters (Peclet number and the dispersion coefficient) were determined from moment analysis and use of an advective-dispersive transport model coupled with a least squares optimization program to fit the experimental transport data (Table 2). Biodegradation of salicylate during transport was preceded by a lag in biodegradation or maximal activity in all cases (Figure 5). This lag effect was more pronounced in the indigenous columns, whereas the shortest lags were observed in the inoculated Hayhook columns.

There were differing degrees of variability observed between breakthrough curves of different trials for the four different miscible-displacement systems (Figure 5). Overall, the inoculated systems demonstrated less variability between trials than the systems containing indigenous communities. Maximal variability was observed between the indigenous Hayhook tirals, while minimum variability was observed between the inoculated Hayhook trials. Additionally, variability between trials was considerable in terms of the cell yields observed in the miscible-displacement experiments.

The cell yields observed in the miscible-displacement systems were significantly different from the cell yields observed by the same communities in batch systems for all

systems except the inoculated sand. This cell yield was calculated by numerically integrating under the salicylate breakthrough curve to determine the amount of salicylate degraded, and dividing the increase in cell mass (since the introduction of the substrate pulse, as described previously) by the mass of salicylate degraded. The observed cell yields in the inoculated sand columns were approximately the same or greater than that obtained for the batch experiments for the same community. Conversely, the cell yields observed for the indigenous experiments were much lower than those obtained in batch for all systems (Table 3). Thus, in general, the cell yield parameter was shown to be different for batch versus flow-through systems. These results indicate that utilization of cell yield values determined in batch experiments may not accurately represent the amount of new cells generated at the field-scale.

The trend in lag times observed in the miscible-displacement experiments corresponded with batch results. The soil columns containing indigenous populations were subject to the longest lag periods prior to growth, whereas the inoculated columns had the shortest. The inoculated Hayhook systems experienced the shortest lag periods. Although the Hayhook soil and sand were inoculated to the same population density prior to column saturation, the Hayhook soil retained more cells during saturation such that it had a larger population density than the inoculated sand columns upon introduction of salicylate. Shorter microbial lag times have been associated with larger initial cell densities in the literature (Henrici, 1928; Lankford et al., 1966). This effect is attributed to the build-up of external metabolites prior to the onset of biodegradation, which occurs

faster when more cells are present. This effect should not be confused with an "apparent" cell-density dependent 'lag' period that can be observed if the amount of biodegradation is initially below detection levels due to biodegradation by low cell numbers. This type of 'lag' is accounted for in the transport and biodegradation model.

Variability between and among miscible-displacement experiments was attributed primarily to variability in initial cell numbers and population diversity. Columns were packed in the same manner for all experiments, and transport of non-reactive tracers and salicylate (in sterile columns) was repeatable with negligible variation (as reported in Jordan et al., 2000). Among the two inoculated systems, there was less variation in the number of viable bacteria remaining in the inoculated Hayhook columns at the introduction of the salicylate pulse. Additionally, more cells were retained during the salicylate pulse in this system. This caused the biodegradation and transport behavior in the loamy-sand soil to be less variable than that exhibited for the homogeneous sand.

Conversely, Hayhook and Vinton soil containing indigenous populations retained a large percentage of their salicylate degrading population, but transport and biodegradation behavior among the three 'replicates' demonstrated increased variability. This is assumed to be due to the increase in variation in population density and population diversity in these systems since hydrodynamic soil effects were not found to cause this behavior in the inoculated Hayhook system. Indeed, samples taken from the source of the soil used in the Hayhook experiments (a 12 liter reservoir of soil) showed that culturable microbial counts ranged over 2.5 orders of magnitude depending on the

location of the sample in the container (Figure 6), and that different types of microorganisms were present in different samples. This variability was also present in the populations in the three column experiments.

RESULTS AND DISCUSSION: MODELING COMPARISONS

Model simulations utilizing the biokinetic parameters obtained from the batch experiments were not able to predict the results obtained from the column experiments, even with the inclusion of microbial lag and bacterial transport (Figure 7). Therefore, values for the maximal growth rate (μ_{max}) and lag parameters (t_{lag} and σ_{lag}) were calibrated to fit the data. These calibrated systems required the use of larger maximum growth rate coefficients to successfully simulate the experimental breakthrough curves (Table 4).

Calibrated lag parameters from the model simulations were different from those determined by batch studies. Specifically, calibrated lag times of the bacterial population were longer in the homogeneous sand, while lag times were shorter in all of the soil systems (inoculated and indigenous) compared to batch values. The calibrated lag time variance parameter represents the variance in the mean lag time for the microbial community present in the column. In terms of its effect on the breakthrough curves, systems containing communities with larger lag time variances had a more gradual transition from the peak relative concentration to the minimal relative concentration. For the batch experiments, the lag time variance was a quantitative determination of the

variance between and among the mean lag time of the communities in three separate flasks at a specific initial concentration. Thus, these values are determined differently.

As noted above, the cell yield calculated using data obtained from the miscible-displacement experiments was different than that determined from the batch biodegradation studies. Thus, the effect of this parameter was investigated by conducting an additional set of simulations wherein the observed cell yield for each miscible-displacement system was used rather than the batch yield value. Once again, the maximal growth rate and lag parameters were calibrated to fit the experimental data. The values of these three calibrated parameters were closer to the batch values (for most cases) than the calibrated values obtained from the simulations conducted utilizing the batch cell yield value. However, the calibrated maximum growth rate coefficients were still larger than those observed in batch systems, and the lag times were still different from the batch values. Therefore, use of the cell yield from the miscible-displacement experiments was shown to provide calibrated parameters (of μ_{max}, t_{lag} and σ_{lag}) closer to those observed in batch systems, as compared to simulations conducted using the batch cell yield.

In order to demonstrate the impact of the maximum growth rate coefficient on model simulation results, simulations were conducted for different values of this coefficient keeping all other parameters constant. Simulations were conducted utilizing values of μ_{max} that were 20% and 50% larger and smaller than the calibrated values (from the batch cell yield simulations). Varying this parameter had the largest influence on the indigenous systems (Figure 8). This is despite the fact that the inoculated systems had

the largest μ_{max} values to begin with, such that they had the largest numerical increase/decrease in the coefficient for these simulations. In short, the simulations were shown to be sensitive to this parameter, with a 20% difference causing a noticeable difference in simulated results, and the calibrated values were shown to provide a good fit. Therefore, the calibrated parameters are in good agreement within a 20% error margin.

Finally, the impacts of incorporating cell elution and microbial lag into the simulations were investigated by conducting simulations without utilizing the subroutines accounting for these behaviors. These simulations used the calibrated biokinetic parameters from the simulations conducted using the batch cell yield value. However, in the simulations performed without incorporation of microbial lag, the lag parameters (t_{lag} and σ_{lag}) were not used. The parameters used in the simulations conducted are listed in Table 5.

The deletion of the microbial lag process significantly affected the simulated results of the sand system, while little effect was observed for the inoculated Hayhook system, even though the same microorganism was used for both systems. Thus, the lag time of this organism was affected by the difference in porous media. The incorporation of lag was significant in both indigenous systems.

Cell elution was of secondary importance to lag in all systems. Inclusion of cell elution in simulations produced larger deviations from the calibrated transport results in the sand system as compared to the soil systems. This is expected due to the increased

cell elution from the sand system. Conversely, in the inoculated Hayhook systems, simulation results were not significantly affected by inclusion of cell elution or microbial lag.

In a field setting, soils are even more heterogeneous and microbial communities are even more variable. Therefore, these simulations demonstrated that the impact of cell elution on contaminant biodegradation and transport is likely to be negligible at most sites, excluding those composed primarily of sandy soils with low organic matter. Additionally, microbial lag may be of importance when modeling biodegradation and transport at the front of a contaminant plume, where microbes are just coming into contact with a new substrate. The importance of this behavior will be influenced by the microbial community present, spatial variability of the community and its population density, the type and concentration of the contaminant, and other environmental parameters (such as temperature, soil composition and texture, and pH).

The biodegradation and transport model presented herein incorporated numerous biologic phenomena that are not included in traditional models. However, even with the inclusion of microbial lag and its variability, cell elution, non-uniform initial cell distribution, and threshold induction concentration, batch parameters were not able to predict biodegradation and transport behavior. For example, the calibrated maximum growth rate coefficient for the miscible-displacement experiments was significantly larger compared to the observed batch value. This scale-dependence of parameters has been noted previously in other research (Kuhn et al., 1985; Angley et al., 1992; Estrella et al.,

1993; Shaw et al., 1998) and may be due to the soil conditions, presence of dissolved organic carbon (DOC) supplied by the soil (more probable with the soils), or the exposure of the cells to a constant influx of substrate rather than declining levels as experienced under batch conditions. Mixing effects are unlikely, as batch studies performed at the different shaker speeds produced identical biodegradation curves (data not shown).

An increase in lag time from batch to column systems can be explained by research demonstrating that a build-up of external metabolites is often required prior the emergence of bacterial cells from the lag phase (Henrici et al., 1928; Lankford et al., 1966). In a flow-through system, the external metabolites can be transported away from the cells, thus causing it to take longer for the external concentration to achieve adequate levels. However, in the Hayhook soil, the opposite effect was seen for both bacterial populations. The decrease in lag time in this soil may be due to an increased presence of DOC (compared to the homogeneous sand). The bacteria in the Hayhook and Vinton columns were exposed to DOC for approximately 22 hours during column saturation prior to the introduction of the salicylate pulse. This exposure may have caused the decrease in lag times observed in these systems, because cells were already metabolizing other organic compounds present in the DOC in the soil.

CONCLUSIONS

This study has demonstrated that the use of biokinetic parameters determined from batch systems may not be applicable to flow-through systems, and utilization of

these parameters in current transport and biodegradation models may lead to inaccurate predictions of experimental data. Additionally, differences in population densities and diversities were found to be the primary factor in variability between trials of miscible-displacement experiments.

The development of a coupled contaminant transport and biodegradation model amended to incorporate the effects of microbial lag, bacterial cell elution, and non-uniform initial cell distribution was presented. Despite the incorporation of these effects, model simulations utilizing batch parameters were still not able to predict experimental data. Differences in the biodegradation conditions between the batch and miscible-displacement systems are likely to influence biodegradation and bacterial growth. Furthermore, a number of specific reasons (presence of DOC, flow conditions, constant influent concentration) to explain these differences were presented.

Finally, simulations were performed to determine the relative importance of incorporating the effects of microbial lag and bacterial cell elution. Microbial lag was demonstrated to have a significant impact, especially in soils containing indigenous populations. Conversely, incorporation of cell elution was influential on the simulation conducted for the sand system, but not for the soil systems, which tend to retain more bacteria.

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MODEL PARAMETERS

b = first -order decay coefficient [h^{-1}]

C = substrate (or contaminant) concentration [mg/L]

C_{ind} = substrate concentration required to induce biodegradation [mg/L]

D = dispersion coefficient [cm^2-h^{-1}]

i = iteration step

I = product concentration [mg/L]

K_I = product inhibition constant [mg/L]

K_i = substrate inhibition constant [mg/L]

K_s and K_O = half-saturation constants for substrate and oxygen, respectively [mg/L]

O = dissolved oxygen concentration [mg/L]

M = active (metabolizing) biomass [mg]

M_o = the initial biomass [mg]

q = Darcy's velocity [cm/hr]

t = time, with t = 0 when $C > C_{ind}$

t_{lag} = mean lag time [h]

Y = cell yield [g cells produced/g substrate consumed]

ρ = bulk density of the soil [g-cm^{-3}]

θ = soil porosity [cm^3 pores-cm^{-3} total]

u_{max} = maximum growth rate coefficient [h^{-1}]

σ_{lag} = variance associated with the mean lag time [h]

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Table 1: Biokinetic parameters from batch experiments

	P. putida RB1353	Hayhook indigenous	Vinton indigenous
μ_{max} (h^{-1})	0.25	0.15	0.105
K_S (mg/L)	70	4.5	2.5
t_{lag} (h)*	0.83	16.83	7.08
σ_{lag} (h)	0.25	4.17	3.33
Yield (g/g)**	0.22 (0.19-0.25)	0.52 (0.44-0.60)	0.68 (0.62-0.75)

*The lag parameters reported are for a substrate concentration of 20 mg/L (the concentration used in all miscible-displacement experiments).

**Values in parentheses represent 2 standard deviations from the mean (approximate 95% confidence intervals), except for *P. putida* column, which represents range of duplicate samples.

Table 2. Transport Parameters.

Soil	Peclet number	Kd ((mg/kg)/(mg/L))	Dispersion coefficient (cm^2/hr)
Sand	160	0	1
Hayhook	15	0.078	10
Vinton	15	0.28	8.5

Table 3. Cell yield for miscible-displacement experiments

	New Cells: Total CFU	% Relative Increase	Yield (g/g)
Sand A	1.65×10^9 ($1.08\text{-}2.22 \times 10^9$)	96.2 (50.6 - 173)	0.236 (0.155-0.316)
Sand B	5.10×10^9 ($3.76\text{-}6.45 \times 10^9$)	448 (209-1347)	0.631 (0.465-0.797)
Sand C	6.58×10^9 ($4.03\text{-}9.13 \times 10^9$)	223 (111-402)	0.734 (0.449-1.02)
Hayhook A	3.71×10^8 ($0\text{-}8.65 \times 10^8$)	83.8 (0-869)	0.0628 (-0.00137-0.146)
Hayhook B	5.77×10^8 ($2.08\text{-}9.91 \times 10^8$)	372 (71-712)	0.151 (0.0545-0.259)
Hayhook C	1.68×10^8 ($9.99 \times 10^7\text{-}2.34 \times 10^8$)	2440 (763-10500)	0.112 (0.0664-0.156)
Vinton A	1.64×10^9 ($1.25\text{-}2.12 \times 10^9$)	354 (229-725)	0.196 (0.149-0.254)
Vinton B	1.63×10^9 ($1.27 - 1.99 \times 10^9$)	1453 (681-7140)	0.221 (0.173-0.270)
Vinton C	5.01×10^8 ($1.40 - 8.83 \times 10^8$)	147 (24.2-1090)	0.0863 (0.0242-0.152)
Inoculated. Hayhook A	5.05×10^8 ($-1.05 \times 10^9 - 2.46 \times 10^9$)	16.9 (-26.3-154)	0.0600 (-0.125-0.292)
Inoculated Hayhook B	6.84×10^8 ($-1.19 \times 10^9 - 2.56 \times 10^9$)	23.4 (-28.2-158)	0.0799 (-0.139-0.299)
Inoculated Hayhook C	1.57×10^8 ($-8.45 \times 10^8 - 1.03 \times 10^9$)	7.95 (-34.9-62.3)	0.0190 (-0.102-0.125)
Inoculated Hayhook D	-6.31×10^8 ($-2.36 \times 10^9 - 1.45 \times 10^9$)	-19.7 (-55.5-80.2)	-0.0771 (-288-0.177)

Values in parentheses denote 95% confidence intervals.
A, B, C, D designations denote separate trials.

Table 4. Calibrated biokinetic parameters from miscible-displacement experiments.

	μ_{max} (hr^{-1})		Lag, ave (hr)		Lag, stdev (hr)	
System	Calibrated w/ batch yield	Calibrated w/ column yield	Calibrated w/ batch yield	Calibrated w/ column yield	Calibrated w/ batch yield	Calibrated w/ column yield
Sand B	0.9	0.95	5.0	3.5	2.5	1.5
Inoculated Hayhook A	3.6	2.5	0	0.83	0	0.25
Hayhook A	0.7	0.55	5.0	7.5	2.0	2.5
Vinton A	0.23	0.15	2.8	4.5	0.85	1.7

Table 5. Transport and biologic parameters used in simulations presented.*

System	Sand B	Inoculated Hayhook A	Hayhook Indigenous A	Vinton Indigenous A
Peclet	160	15	15	15
q (cm/hr)	3.67	3.45	2.81	4.45
K_d (mg/kg)/(mg/L)	0	0.08	0.08	0.3
θ (cm^3/cm^3)	0.36	0.31	0.31	0.43
ρ (g/cm^3)	1.79	2.11	2.06	1.75
K_S (mg/L)	70	70	4.5	2.5
M_o (mg/L)	111	127	18.2	14.1
Biokinetic parameters for simulations using batch values				
Y(g/g)	0.22	0.22	0.52	0.68
μ_{max} (h^{-1})	0.25	0.25	0.15	0.105
t_{lag} (h)	0.83	0.83	16.83	7.08
σ_{lag} (h)	0.25	0.25	4.17	3.33
Calibrated biokinetic parameters for simulations using batch cell yield values				
Y (g/g)	0.22	0.22	0.52	0.68
μ_{max} (h^{-1})	0.9	0.7	3.6	0.23
t_{lag} (h)	5.0	5.0	0.0	2.8
σ_{lag} (h)	2.5	2.0	0.0	0.85
Calibrated biokinetic parameters for simulations using column cell yield values				
Y (g/g)	0.402	0.0464	0.0628	0.196
μ_{max} (h^{-1})	0.95	0.55	2.5	0.15
t_{lag} (h)	3.5	7.5	0.83	4.5
σ_{lag} (h)	1.5	2.5	0.25	1.7

*L = 15 cm (all columns)

FIGURE CAPTIONS

Figure 1. Sorption isotherms for salicylate to soils used in experiments. The correlation coefficients (R^2) for the linear regressions are 0.97 and 0.96 for the Hayhook and Vinton soils respectively.

Figure 2. Growth curves obtained from batch biodegradation studies for the three populations A) *Pseudomonas putida* RB1353, B) Hayhook indigenous, and C) Vinton indigenous. Cell concentrations were calculated based on amount of salicylate degraded at each sampling time and the observed cell yield from batch experiments.

Figure 3. Monod growth plots for the three populations A) *Pseudomonas putida* RB1353, B) Hayhook indigenous, and C) Vinton indigenous. Two standard deviations about the mean growth rate are shown.

Figure 4. Lag time as a function salicylate concentration for the three microbial populations A) *Pseudomonas putida* RB1353, B) Hayhook indigenous, and C) Vinton indigenous as a function of initial substrate concentration. Two standard deviations about the mean lag time are shown.

Figure 5. Salicylate transport in experimental miscible-displacement systems A) sand inoculated with *P. putida* RB1353, B) Hayhook soil inoculated with *P. putida* RB1353,

C) Hayhook soil with indigenous population present, D) Vinton soil with indigenous population present.

Figure 6. Viable counts associated with Hayhook soil in reservoir as a function of location where 't' denotes top of reservoir and 'i' denotes inner reservoir and A, B, C, and D refer to quadrants delineated on the surface.

Figure 7. Model simulations compared with experimental data for systems A) inoculated sand, Trial B, B) inoculated Hayhook Trial A, C) indigenous Hayhook Trial A, and D) indigenous Vinton Trial A. Transport and biologic parameters for each simulation are listed in Table 5.

Figure 8. Sensitivity analysis of the effect of varying μ_{max} on model simulations for all systems.

Figure 1.

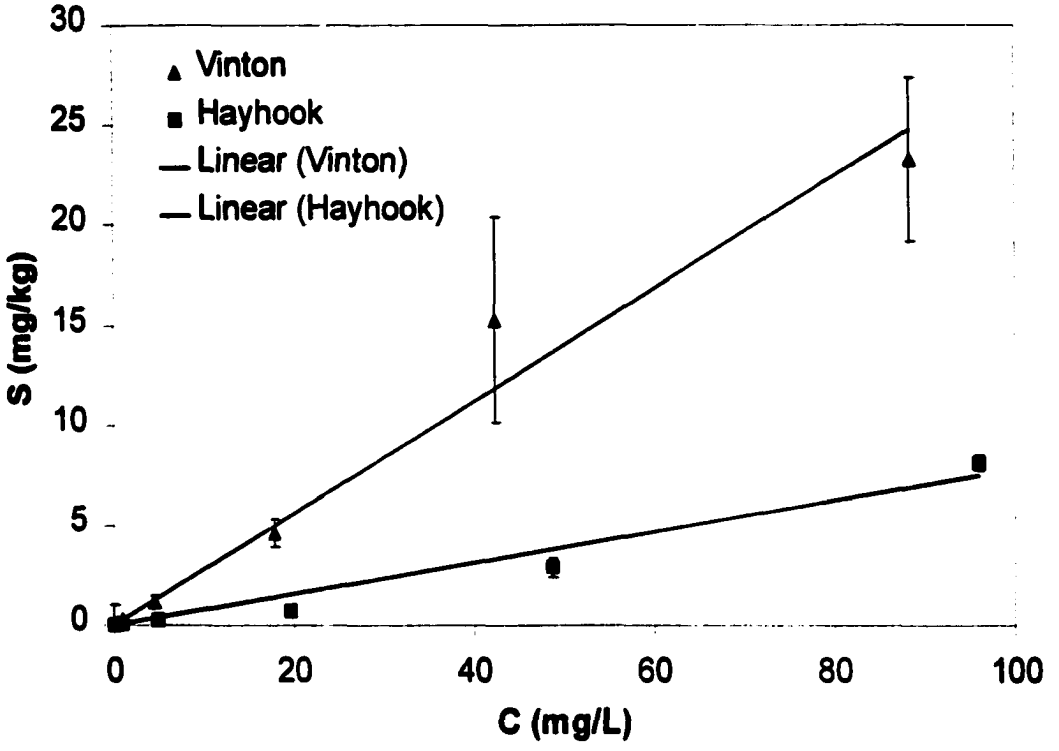

30
25
20
15
10
5
0
S (mg/kg)
Vinton
Hayhook
Linear (Vinton)
Linear (Hayhook)
0
20
40
60
80
100
C (mg/L)

Figure 2.

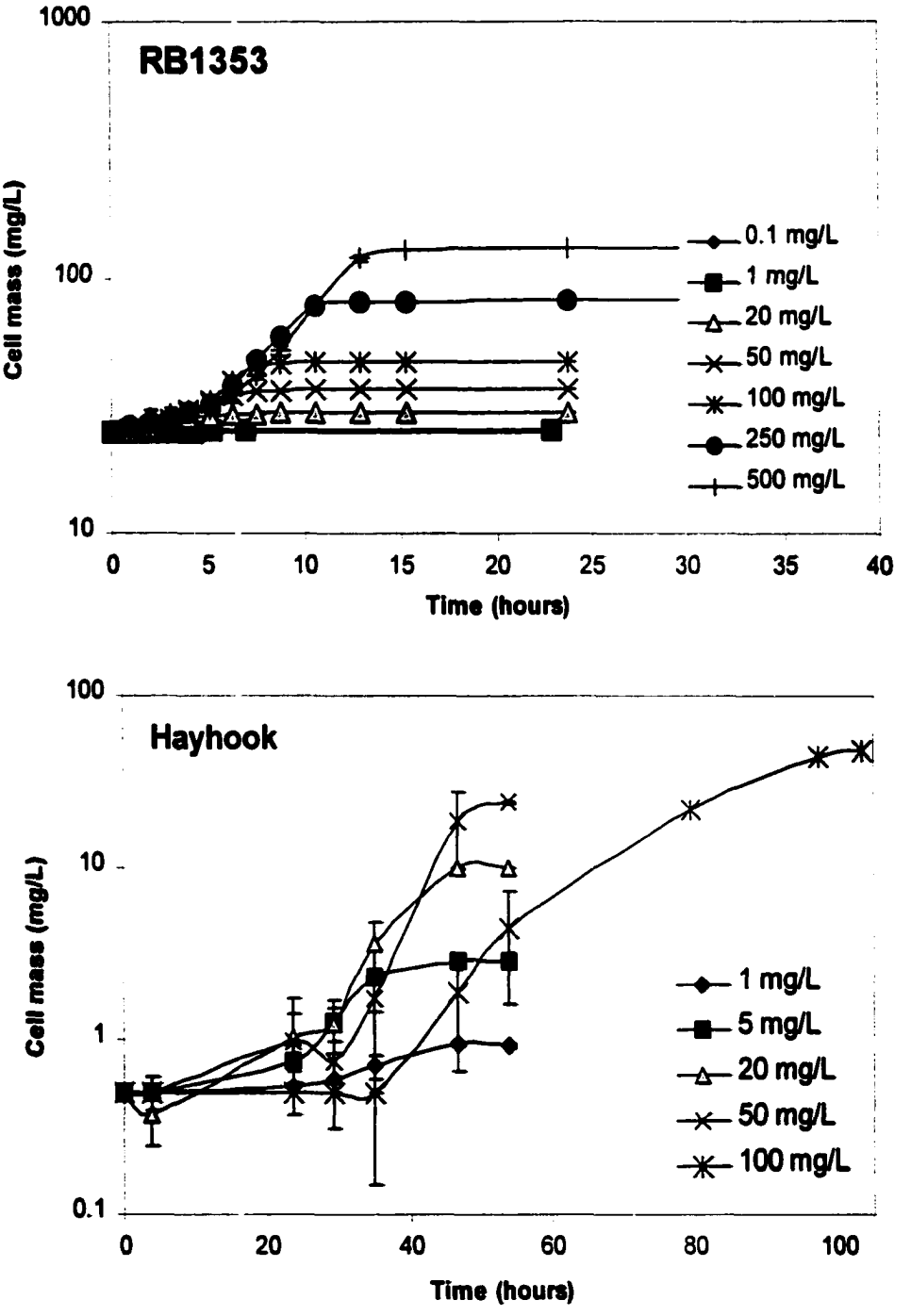

RB1353
Cell mass (mg/L)
1000
100
10
0
5
10
15
20
25
30
35
40
Time (hours)
0.1 mg/L
1 mg/L
20 mg/L
50 mg/L
100 mg/L
250 mg/L
500 mg/L
Hayhook
Cell mass (mg/L)
100
10
1
0.1
0
20
40
60
80
100
Time (hours)
1 mg/L
5 mg/L
20 mg/L
50 mg/L
100 mg/L

Figure 2, continued.

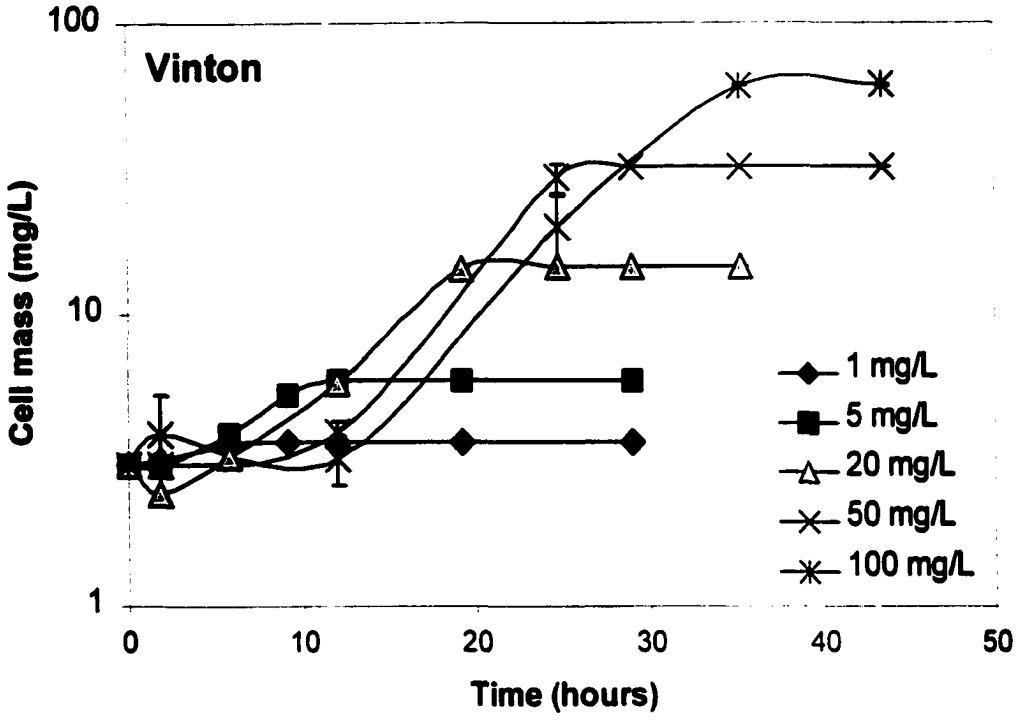


Figure 3.

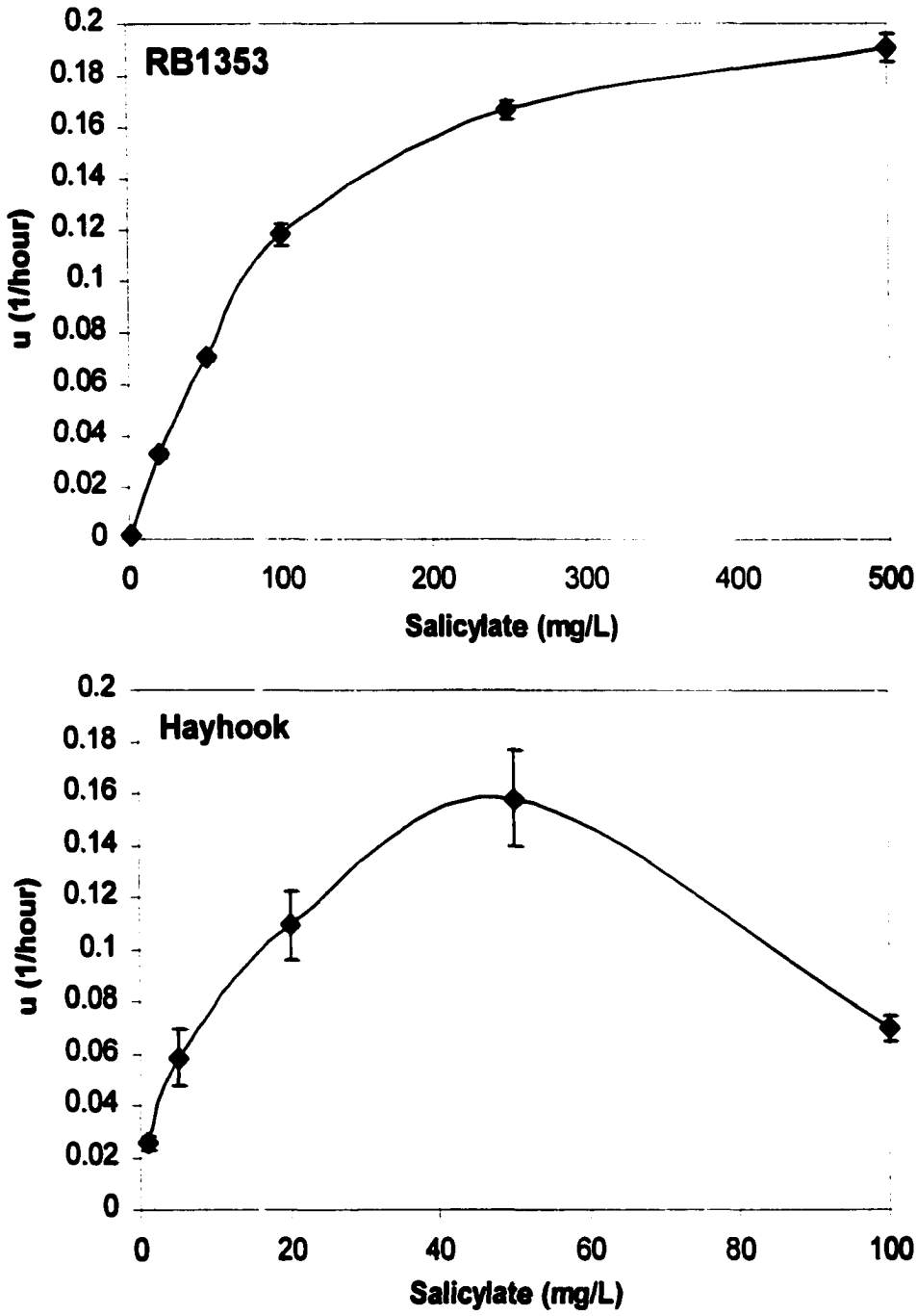

RB1353
u (1/hour)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
100
200
300
400
500
Salicylate (mg/L)
Hayhook
u (1/hour)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
20
40
60
80
100
Salicylate (mg/L)

Figure 3, continued

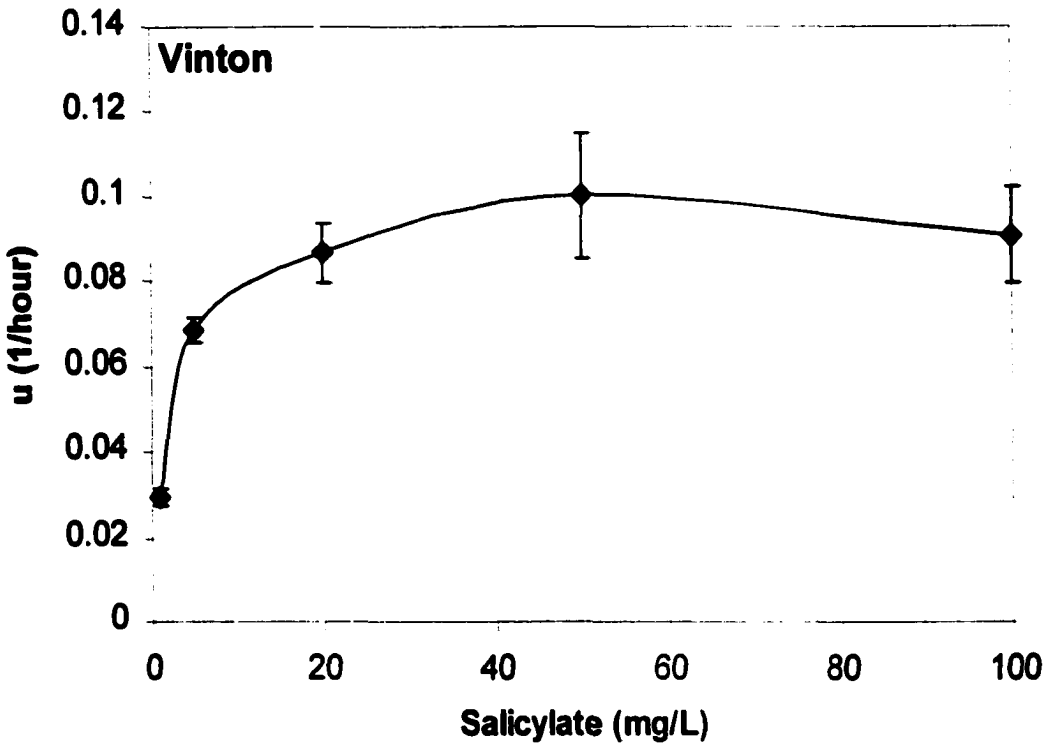


Figure 4.

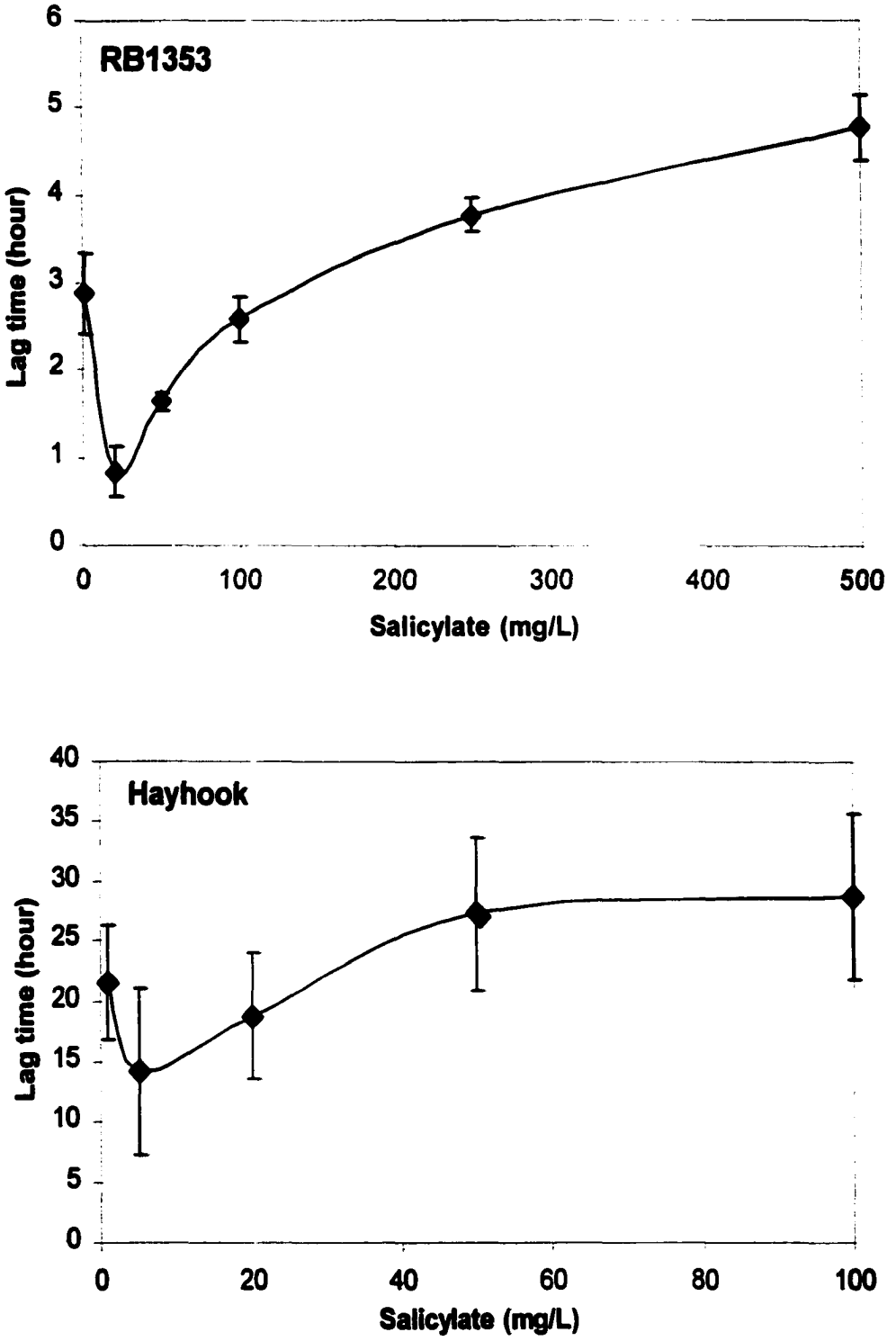

RB1353
Lag time (hour)
0
1
2
3
4
5
6
0
100
200
300
400
500
Salicylate (mg/L)
Hayhook
Lag time (hour)
0
5
10
15
20
25
30
35
40
0
20
40
60
80
100
Salicylate (mg/L)

Figure 4, continued.

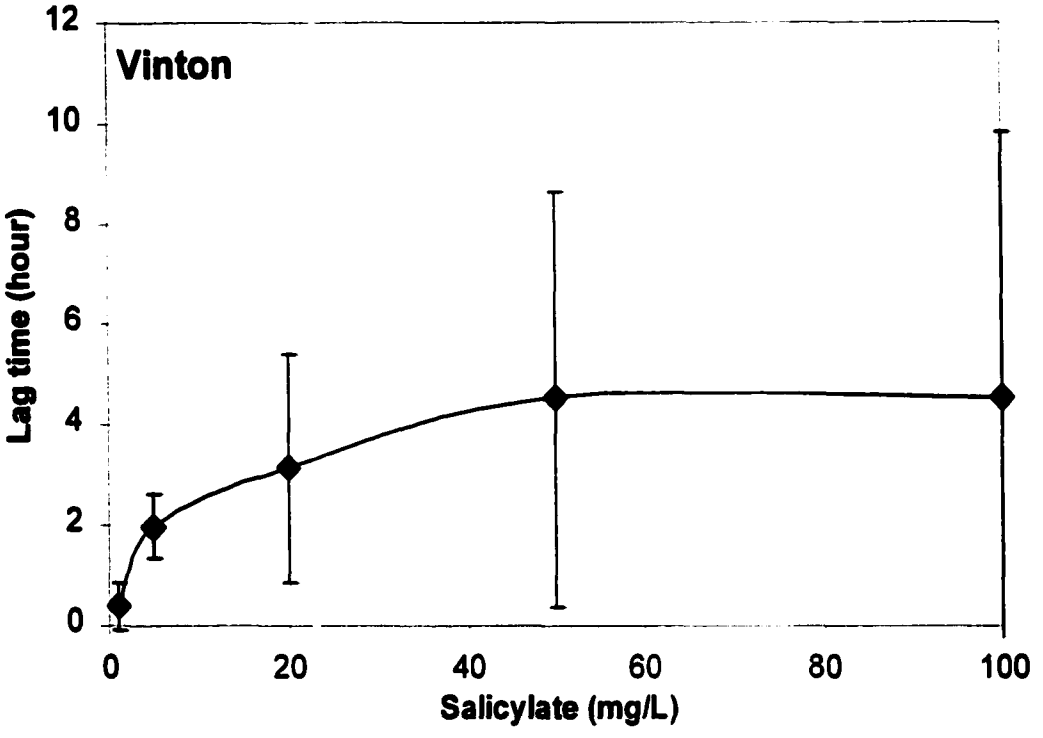
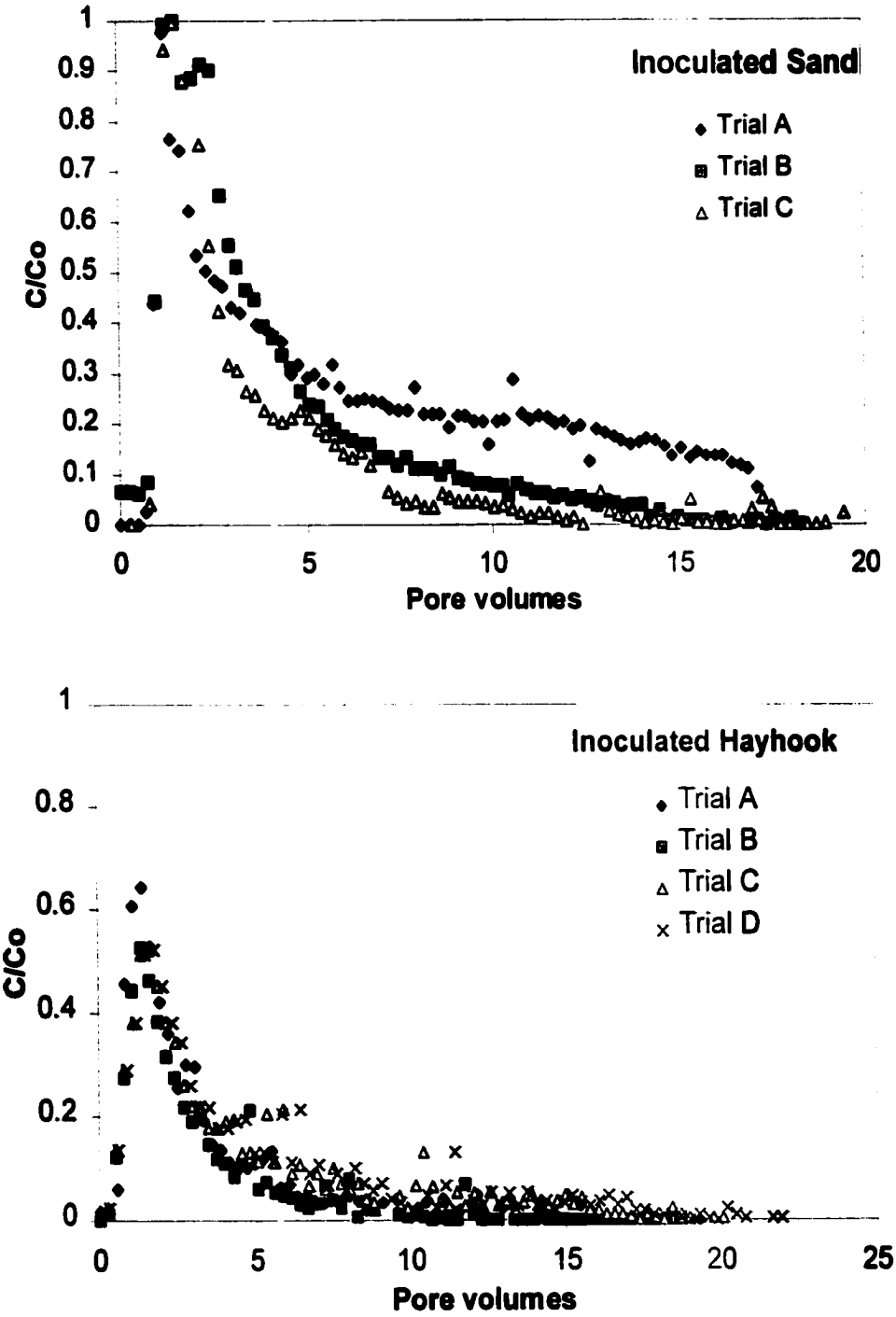


Figure 5.


Inoculated Sand
Trial A
Trial B
Trial C
C/Co
Pore volumes
Inoculated Hayhook
Trial A
Trial B
Trial C
Trial D
C/Co
Pore volumes

Fig

Figure 5, continued.

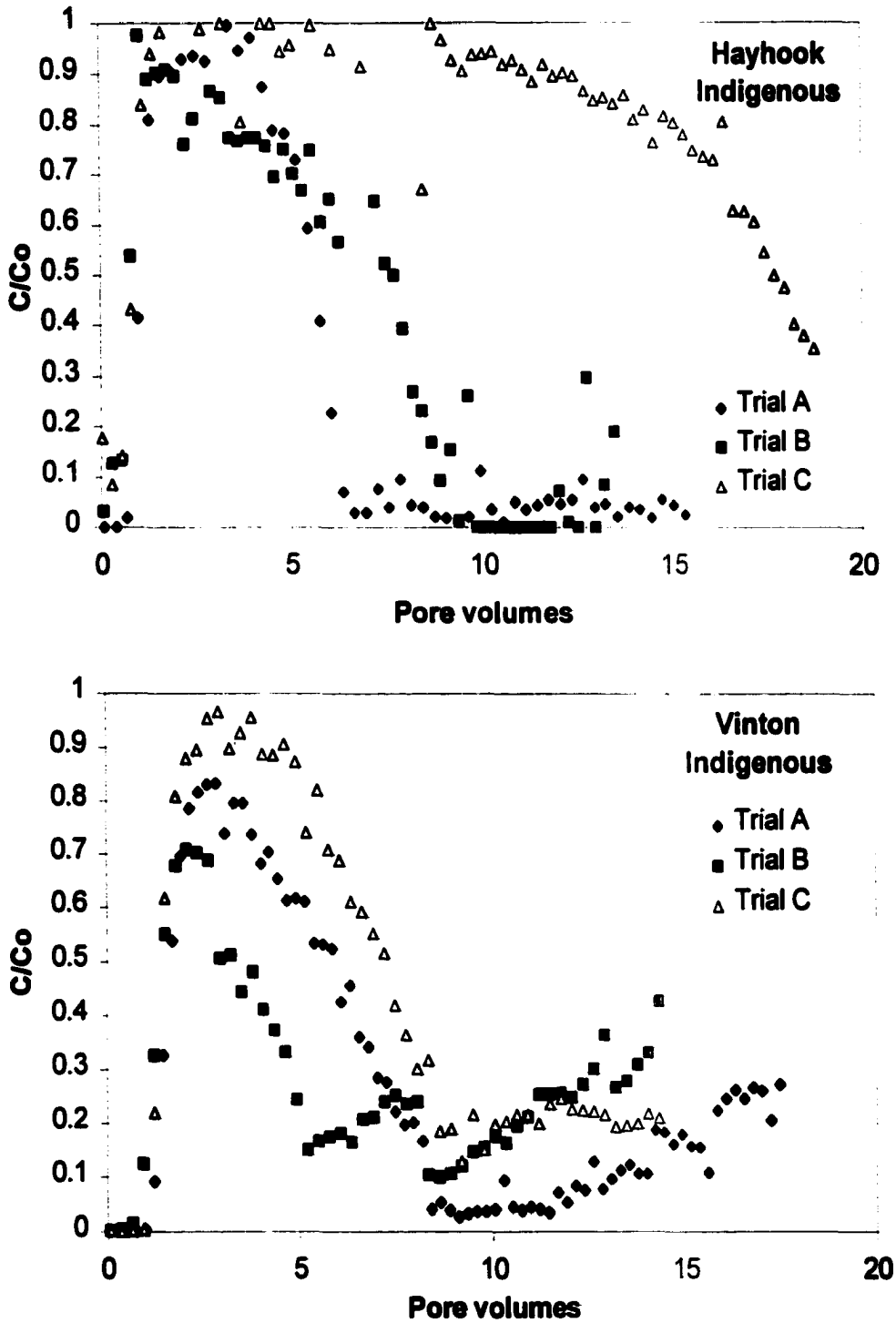


Figure 6.

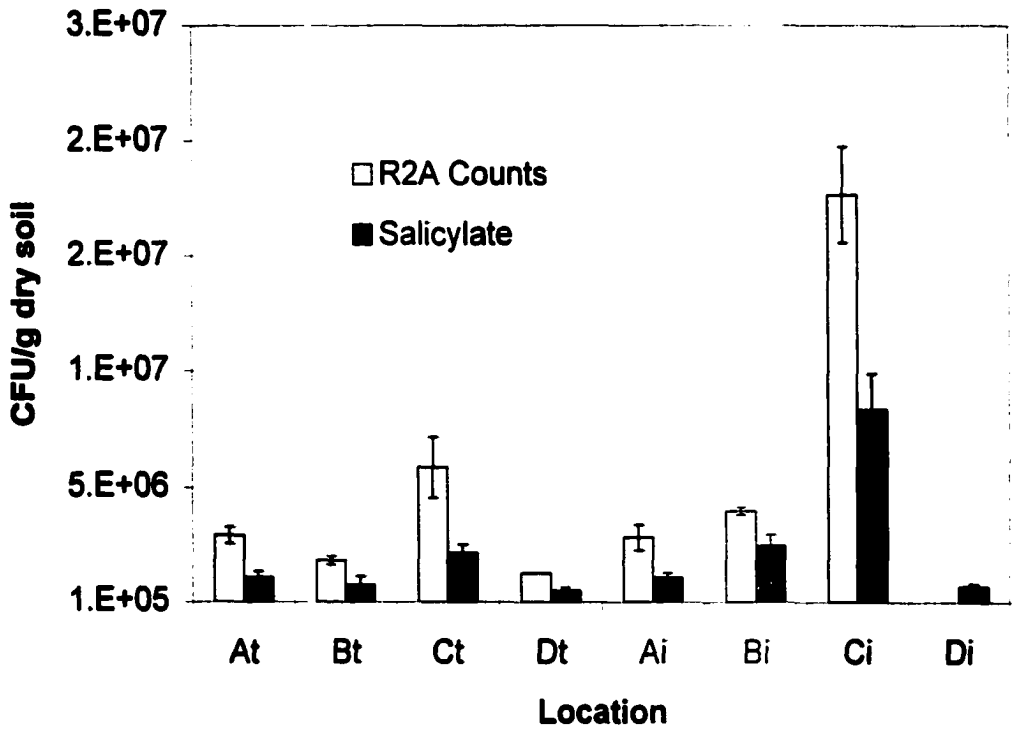

3.E+07
2.E+07
2.E+07
1.E+07
5.E+06
1.E+05
CFU/g dry soil
R2A Counts
Salicylate
At
Bt
Ct
Dt
Ai
Bi
Ci
Di
Location

Figure 7.

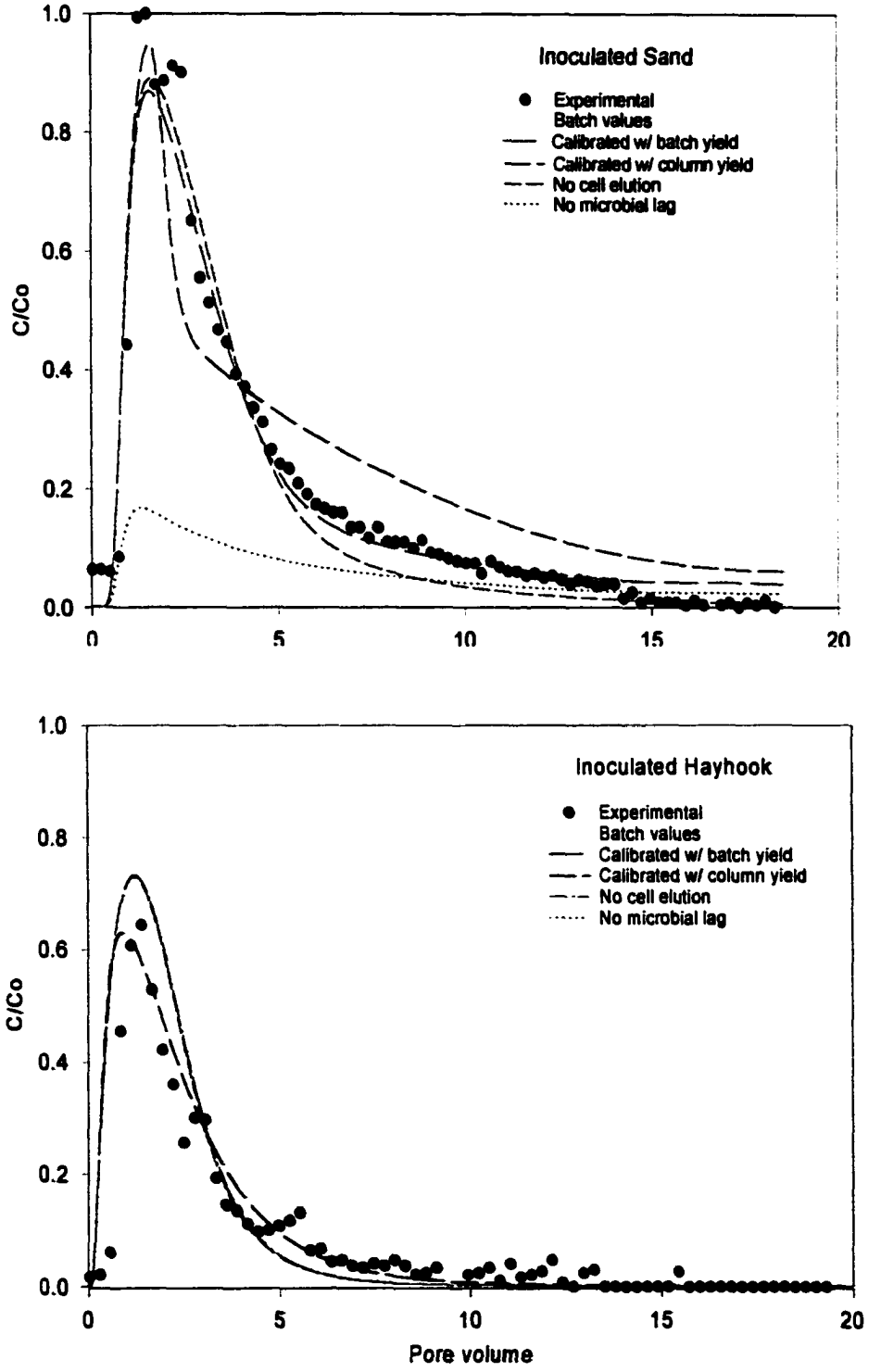

Inoculated Sand
Experimental
Batch values
Calibrated w/ batch yield
Calibrated w/ column yield
No cell elution
No microbial lag
C/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
Inoculated Hayhook
Experimental
Batch values
Calibrated w/ batch yield
Calibrated w/ column yield
No cell elution
No microbial lag
C/Co
Pore volume

Figure 7, cqntinued

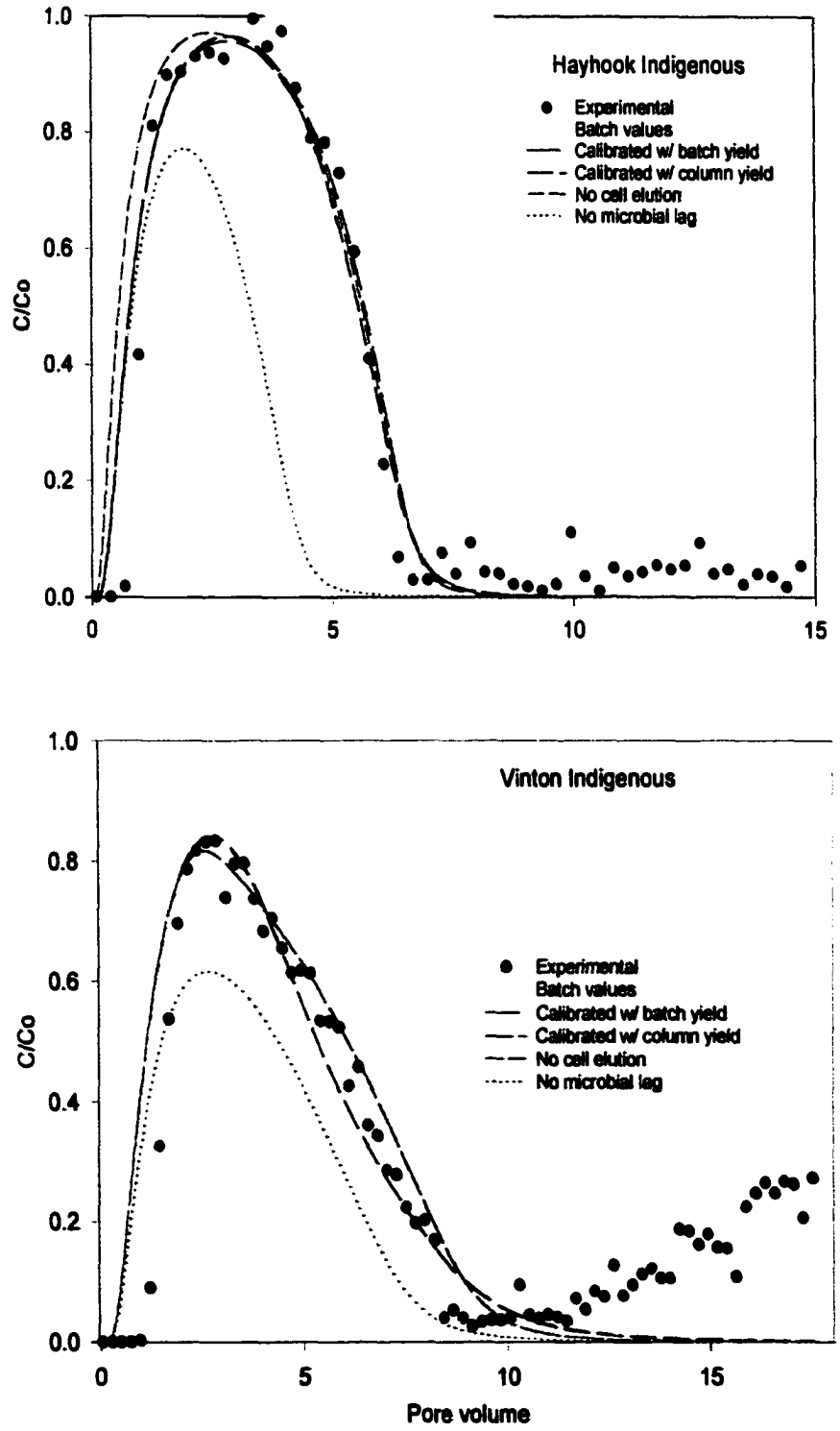
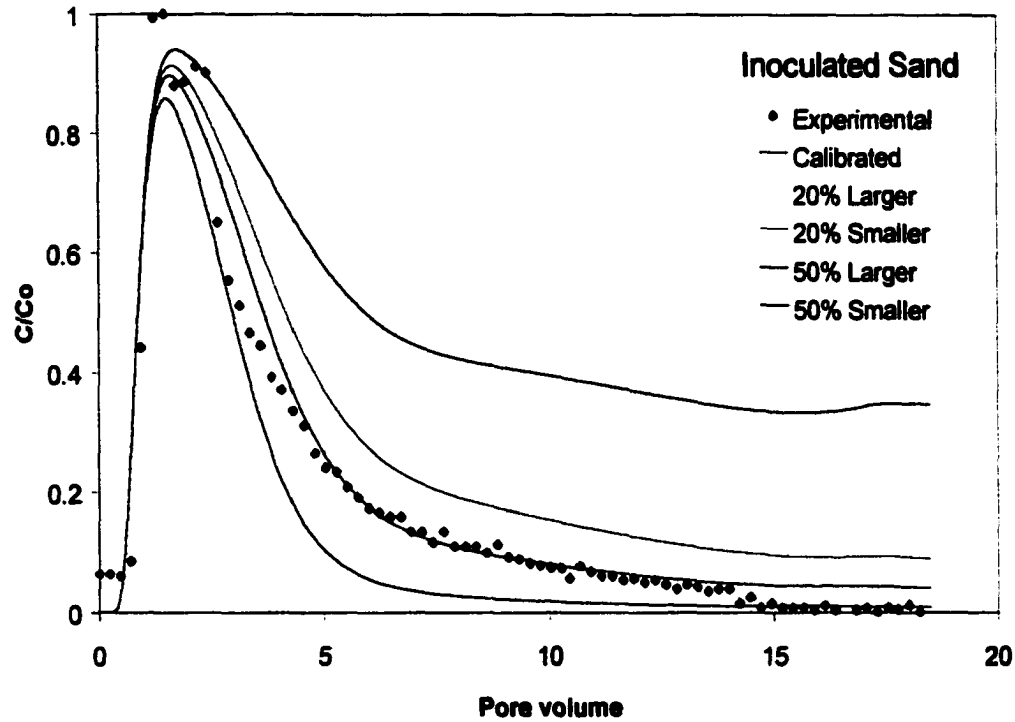


Figure 8.


Inoculated Sand
Experimental
Calibrated
20% Larger
20% Smaller
50% Larger
50% Smaller
C/Co
Pore volume
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20

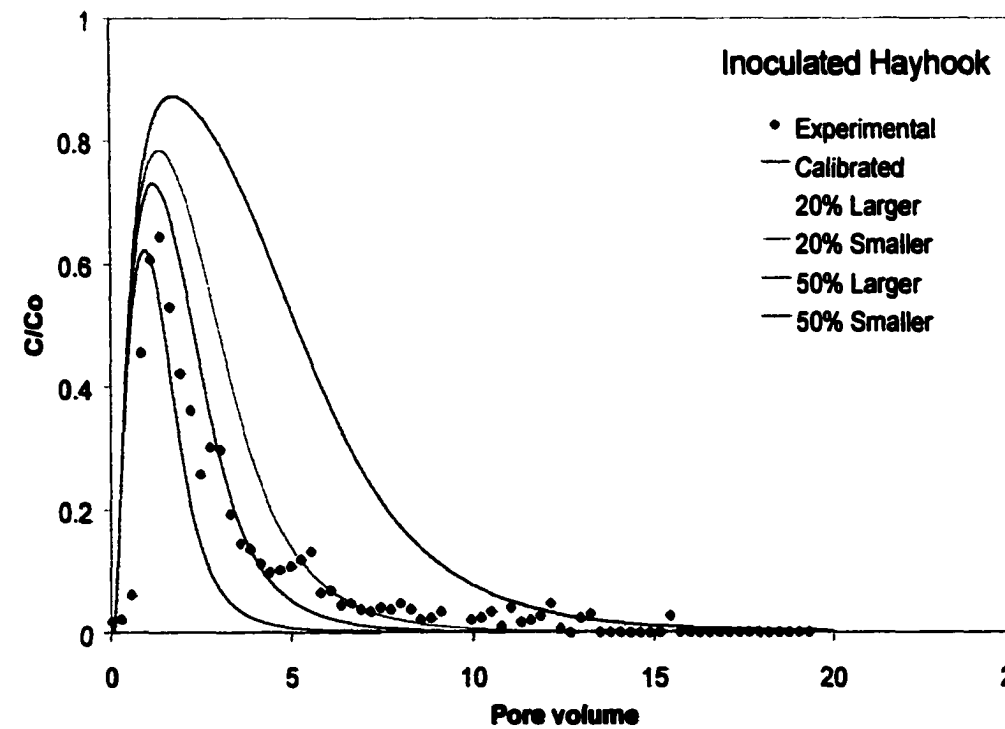
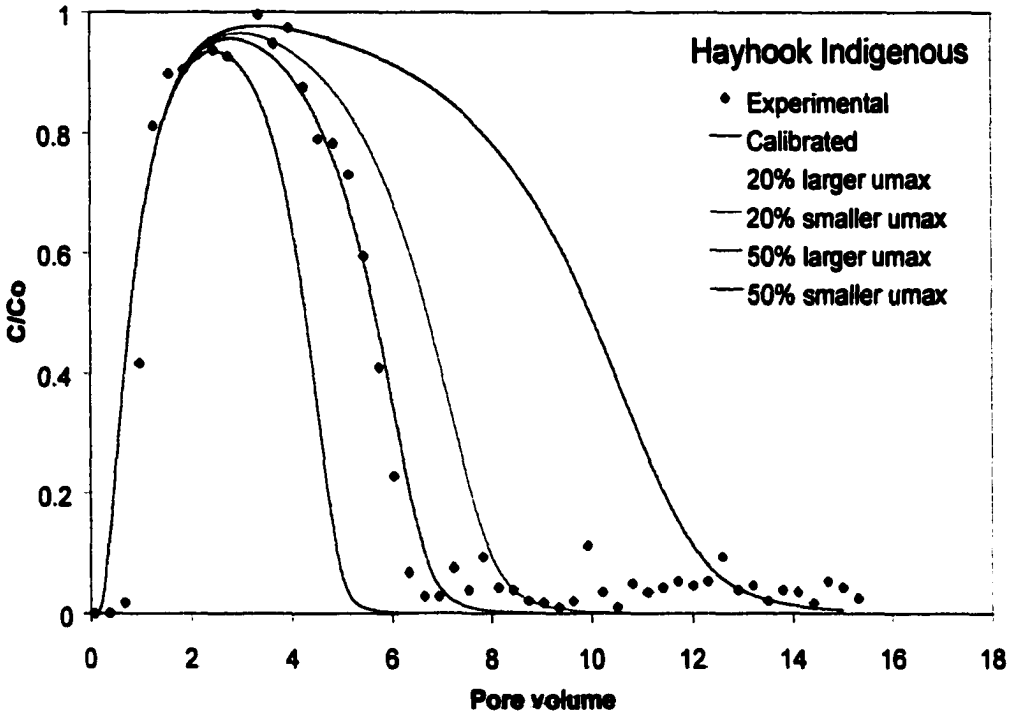
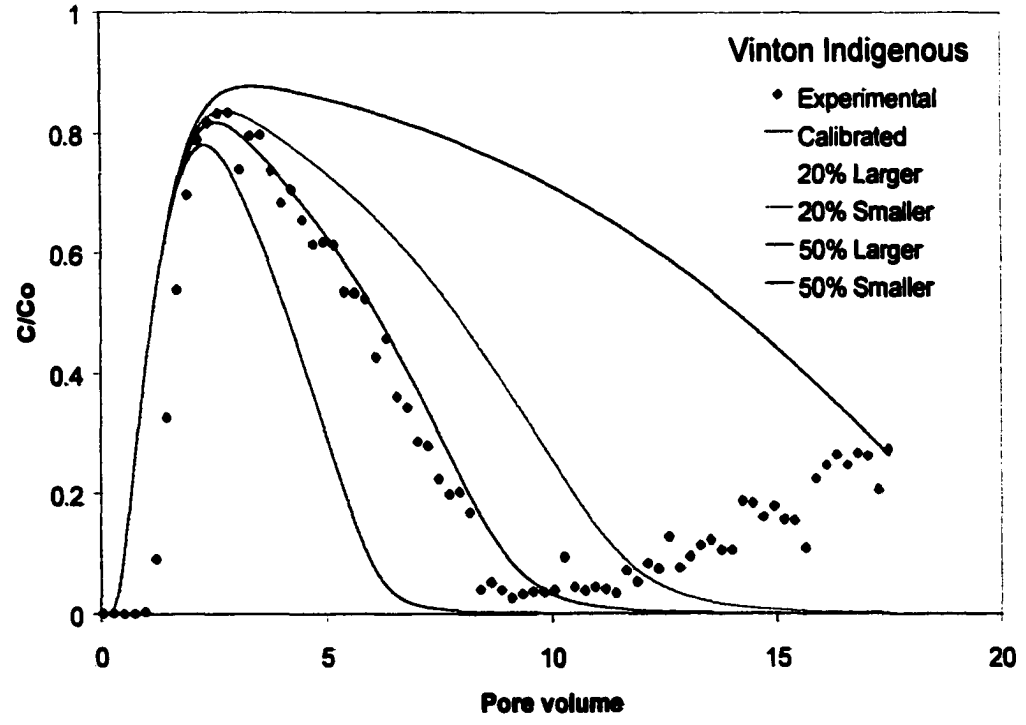

Inoculated Hayhook
Experimental
Calibrated
20% Larger
20% Smaller
50% Larger
50% Smaller
C/Co
Pore volume
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25

Figure 8, continued





APPENDIX B:

CHARACTERIZING VARIABILITY AND SPATIAL DISTRIBUTION OF IN-SITU MICROBIAL ACTIVITY USING BIOTRACER TESTS

Susannah K. Sandrin[1], Joseph J. Piatt[1,3], Adria A. Bodour[1] and Mark L. Brusseau[1,2,*]

[1] Soil, Water and Environmental Science

[2] Hydrology and Water Resources

The University of Arizona, 429 Shantz, Tucson, Arizona 85721

[3] Carroll College, 100 North East Avenue, Waukesha, WI 53186

ABSTRACT

Tracer tests were conducted using biodegradable solutes or "biotracers" to investigate the effects of spatially variable microbial activity and physical heterogeneity on biodegradation and transport of organic solutes at two field sites: a chlorinated-solvent contaminated regional aquifer in Tucson, AZ, and a mixed-waste contaminated surficial aquifer in Utah. The Tucson site is a desert environment, with low organic matter and deep water table, leading to low inherent microbial activity. Conversely, the Utah site has higher overall activity due to more favorable conditions. Additionally, biotracers were injected at the Utah site following an oxygenated cyclodextrin flush, which may have enhanced the density of microbial populations near the injection wells. The extent and rate of in-situ biodegradation of nontoxic, biotracers, was determined using residence time and mass recovery data from these well-controlled field experiments.

The transport of the biotracers was compared to that of conservative tracers. Mass recovery of the biotracers decreased approximately linearly with increasing residence time for the Tucson site. Similar behavior was observed at the Utah site, except in the region adjacent to the injection zone. Likewise, the first-order biodegradation rate coefficients obtained from model calibration were generally independent of residence time for both field sites. At the Tucson field site, the biodegradation rate coefficients were approximately constant in space. However, they were significantly larger near the injection wells for the Utah site, consistent with a larger cell density measured for that region. These results illustrate the utility of the biotracer method for characterizing in-situ microbial activity.

INTRODUCTION

In-situ remediation technologies, such as in-situ bioremediation, have become preferred methods for cleaning up contaminated sites (Macdonald et al., 1994). In-situ bioremediation is generally practical, cost-effective, and causes minimal perturbation of the environment at the contaminated site (NRC, 1993; Atlas, 1995; Hart, 1996). In-situ bioremediation applications range from natural attenuation, in which biodegradation is not enhanced, to more engineering-intensive techniques, which may range from fertilizer additions to oxygen sparging. The use of monitored natural attenuation, often synonymous with intrinsic bioremediation, is increasing in popularity as the number of identified contaminated sites increases and as it becomes more commonly accepted (NRC, 1993). However, the feasibility of using in-situ bioremediation at a site requires a determination of the in-situ biodegradation potential for the target contaminants and the spatial distribution of this potential at the site.

Characterizing biodegradation at the field-scale is not a trivial task (Madsen, 1991; Sims et al., 1993; McAllister et al., 1994). Determination of such information at the field scale is achieved by monitoring parameters associated with biodegradation, such as contaminant concentration profiles, electron acceptor concentrations (such as O_2, NO_3^-, SO_4^{2-}, Fe^{3+}), contaminant metabolite concentration profiles, degradation products (CO_2, CH_4), and isotope ratios (Klecka et al., 1990; Borden et al., 1995; Chapelle et al., 1996). However, the complexity of typical field sites makes it difficult to conclusively correlate changes in these parameters to biodegradation. Furthermore, the initial mass of contaminant released into the

subsurface is not known at most sites. Thus, it is not possible to quantify the magnitude and rate of biodegradation. Although mass balances have been obtained for controlled experiments conducted at a few field sites, allowing for the determination of in-situ biodegradation rates (Barker et al., 1987; Roberts et al., 1990; MacIntyre et al., 1993; Reinhard et al., 1997), this approach is not available for most contaminated sites.

Another approach often used to evaluate biodegradation potential is to conduct laboratory biodegradation studies using soil samples collected from the field. In-situ microcosms (ISM), which isolate a small volume of the aquifer, have also been used to measure rates of biodegradation (Nielsen et al., 1996; Shaw et al., 1998). Again, the complexity of field sites, including spatial variability of physical and chemical properties and of microbial populations, often precludes scaling from batch experiments even to laboratory column systems, much less to the field scale (e.g., Kuhn et al., 1985; Angley et al.,1992; Estrella et al., 1993; Shaw et al., 1998; Sandrin et al., 2001). Despite the problems of verifying magnitudes and rates of biodegradation, this information is critical to the planning and implementation of biodegradation-based remediation systems.

Finally, the presence of subsurface heterogeneities and the variability in spatial distribution of microbial populations and activity can significantly impact biodegradation and transport at the field scale. Areas of low hydraulic conductivity may limit biodegradation due to mass transfer constraints (MacQuarrie et al., 1990; Lee et al., 2000). Transport of oxygen into these regions, and transport of the contaminant out of these regions is dominated by diffusion, which becomes the limiting factor for remediation near these

regions. Microbial distributions are spatially variable in the subsurface, and at some sites this effect can be quite significant (Harvey et al., 1984; Severson et al., 1991; Harvey et al., 1992; Kieft et al., 1998; Krueger et al.,1998; Stapleton et al., 2000). Stochastic models incorporating this factor yielded significantly different results from a model which assumes a uniform distribution (Miralles-Wilhelm et al., 2000). Unfortunately, determination of the spatial distribution of the population would require extensive and costly field sampling.

The use of solutes as 'biotracers' is useful in evaluating the biodegradation capability, relative rates, and spatial distribution of indigenous microbial communities (Istok et al., 1997; Schroth et al., 1998; Brusseau et al., 1999; Hageman et al., 2001). Biotracers may include any compound that is utilized by a microbial population, including organic compounds, oxygen, nitrate, sulfate and more. Consumption of these compounds in-situ is then monitored over the spatial coordinates of the aquifer. More specifically, biotracers of structures similar to the contaminants present at the site, but more readily biodegradable, can be used to estimate the likelihood and rate of biodegradation by the existing population (Brusseau et al., 1999; Hageman et al., 2001). For example, Hageman et al. (2001) used trichloroflouroethene as an analogue for transport and biodegradation of trichloroethene. Finally, biotracers can be used to determine the change in population density in response to a perturbation, such as a substrate pulse or a flush with a toxic substance. The data obtained regarding soil heterogeneity and the spatial distribution of microbial activity and/or the biodegradation potential (as influenced by oxygen or other nutrient availability) can then be used in prediction models for the contaminants present at the site.

In this study, field experiments were conducted at two sites to evaluate the utility of the biotracer method for characterizing spatial variability of microbial activity. The transport of biodegradable solutes was compared to that of bromide and/or pentafluorobenzoic acid, conservative, non-degradable tracers. The transport data for these biotracers was used to measure the amount and rate of in-situ biodegradation for selected organic compounds, and to determine the relationship between these values and the residence time and spatial location of the samples taken.

MATERIALS AND METHODS

Compounds. The selection of the biotracers involved several criteria. First, the tracers had to be nontoxic to ensure approval from regulatory agencies for injection into the subsurface. In addition, the compounds needed to be easy to analyze, have low volatility and a large aqueous solubility, non-sorptive (to minimize problems with mass balance and mass transport), and, preferably, inexpensive. Sodium benzoate, ethanol, pentanol, and hexanol met all these requirements. Another criterion was that the solutes had to be degradable under both aerobic and anaerobic conditions by commonly occurring soil microorganisms because the field sites contain both types of environments. These biotracers are all readily mineralized under aerobic (Holder and Vaughan, 1987; Budavari, 1989; Grund *et al.*, 1990; van Beilen et al., 1992; Altenschmidt *et al.*, 1993; Wales et al., 1994; Nosova et al., 1997; Hirata et al., 2000;) and anaerobic (Horowitz *et al.*, 1983; Battersby and Wilson, 1989; Kobayashi *et al.*, 1989, van Iersel et al., 1997) conditions.

Field Site 1: Utah. This field site is contaminated with a complex mixture of water-immiscible organic compounds. The zone in which the biotracer test was conducted is part of an unconfined aquifer that consists of fine to coarse sand, interbedded with gravel and silt layers. An isolated cell was created by driving sheet-piles into a clay unit located approximately 8 meters below ground surface. The cell was approximately 3x5 meters and contained 4 fully screened injection wells and 3 fully screened extraction wells. A 60-point, three-dimensional, multi-level sampling array (3x4 locations, 5 depths) connected to a vacuum system was used to collect depth-specific water samples. Prior to the biotracer test, a 10% cyclodextrin solution was pumped through the aquifer for 10 days (a total of 65,500 L), followed by a flushing of the cell with water free of cyclodextrin (about 40,000 L). This pumping introduced oxygenated water into the cell for a significant period of time (greater then 15 days), thereby causing an enhancement of the aerobic micribial population near the injection wells (Figure 9).

The solutes were injected into the subsurface at 4.5 Lpm for a total pulse of 1,325 liters. The initial concentrations of the biotracers injected into the cell were 304 mg/L for benzoate, 1081 mg/L for ethanol, 871 mg/L for hexanol and 797 mg/L for pentanol. The biotracers were injected into the subsurface along with $CaBr_2$ (300 mg/L), the nonreactive tracer. Approximately 15 ml of groundwater was sampled into 20 ml polyethylene vials and immediately sterilized by addition of $HgCl_2$. Samples were then refrigerated at 4°C and shipped overnight to the University of Arizona.

Samples were taken from cores at three drilling locations in the cell to enumerate

heterotrophic bacteria and bacteria capable of degrading jet fuel (JP8). These locations corresponded to the injection end, middle, and extraction end of the cell (Figure 9). Drilling occurred to 13 meters bgs and cores were collected between the depths of 13 and 29 meters bgs. Four meter cores were obtained, with an average recovery of two meters. These samples were also refrigerated for a maximum of 24 hours until they were processed.

Field Site 2: Tucson, AZ. This TCE-contaminated field site is in Tucson, AZ. The tracer experiment was conducted in a semi-confined, 6 meter thick aquifer located 36 meters below ground surface. Cores showed the aquifer material to be mostly sand with some clay lenses and cobble with an organic carbon content of 0.03%. The tracer solution was injected into the subsurface at 170 Lpm for 56 hours for a total pulse of 620,000 liters. Sodium benzoate and calcium bromide were injected together at concentrations of approximately 45 mg/L. Sampling was conducted using an array of ten fully screened piezometer wells and one monitoring well equipped with a 4-port, multi-level sampler (MLS). The MLS allowed ground water samples to be collected from depth-specific locations within the same well. The distance from the injection well to the MLS was approximately 12 meters. Additional details regarding the field site are available in Brusseau et al., 1999b.

Approximately 5 to 15 ml of groundwater was sampled into 20 ml polyethylene vials with screw caps (Research Products International, #121043). The samples were autoclaved and stored under chilled conditions until analysis to prevent degradation of benzoate in the vials. Laboratory studies showed that autoclaving does not affect the concentration of benzoate in the samples (i.e., no occurrence of thermal degradation, volatilization, or

concentration due to evaporation of water).

Laboratory studies were conducted to examine the magnitude of benzoate degradation during storage. Ground water was bailed from wells M72 (close to the injection well and on the centerline flow path) and P10 (distant from the injection well and off the centerline flow path) at the Tucson site. A fraction of this ground water was autoclaved to provide a sterile control for the experiments. High (40 mg/L) and low (4 mg/L) benzoate concentration sets were prepared by spiking 100 ul of a concentrated stock solution into 10 ml of non-steriled and steriled ground water using 20 ml polyethylene vials. The samples were stored at room temperature and analyzed after 7, 14, and 21 days for benzoate. Recall that the field samples were refrigerated, which means that the laboratory-prepared samples are under more optimal conditions for biodegradation. The results indicated that negligible loss of benzoate occurred for the high concentration samples, even after 21 days. The low concentration samples showed negligible loss of benzoate at 14 days, but measurable losses (>5%) by 21 days. Thus, because all samples were refrigerated and sterilized, the biodegradation of benzoate during storage was negligible.

Laboratory Column Experiments. Column experiments were conducted to determine the applicability of the linear biodegradation model (no growth) to systems with small input pulses that may be experiencing bacterial growth. Hill aquifer material was packed into columns, and subjected to input pulses of toluene and PFBA. Samples from the column effluent were collected, and the concentrations of toluene and PFBA were analyzed by the same techniques used for the field data.

Chemical Sample Analysis. Prior to analysis, the samples were filtered through 0.2 um syringe filters (Gelman, #4602) to remove particulate matter. The filtered samples for benzoate detection were transferred directly to 1 ml glass autosampler vials (Alltech #98210) and capped with starburst snap plugs (Alltech #98264). Samples were analyzed with an automated HPLC system using a mobile phase of 40% acetonitrile and 60% pH 3.6 phosphate buffer (0.01 M) and detected using an ultra-violet spectrophotometer at 235 nm. An Adsorbosphere UHS C18 column (Alltech #288118) was outfitted with a prefilter and guard cartridge (Alltech #96080). The detection limits for benzoate was <500 ug/L. The alcohols were analyzed using a gas chromatograph (Shimadzu GC-14A) equipped with a flame ionization detector (FID), with a resultant detection limit of approximately 0.5 mg/L. Bromide was analyzed using a colorimetric technique (Alpkem).

Microbiological Sample Analysis. Two grams of soil from each subsample were diluted in 9 mL of sterilized water and vortexed for one minute. Serial dilutions were plated on 2 different media: R2A (Becton and Dickinson Company, Cockeysville, MD) and a 0.05% jet fuel (JP8) solution in MSM (0.4mg/ml Na2HPO4, 0.1 mg/ml KH2PO4, 0.1 mg/ml NH4Cl, 0.2mg/ml MgSO4, 0.0005 mg/ml yeast extract, and 15 g/L agar) amended with sterile solutions of ammonium iron (III) citrate (5 μg/L) and $CaCl_2$ (100 μg/L). These media were used to enumerate heterotrophic bacteria and jet fuel degrading bacteria, respectively. Plating was performed in triplicate, and plates were counted after incubating at room temperature for one week.

Data Analysis. Mass recoveries and travel times for the nonreactive tracers and for

the biodegradable solutes were calculated by analyzing the breakthrough curves using moment analysis. The zeroth moment is a measure of the mass recovered for a given tracer:

$$M_o = \int Cdt$$

The first moment measures the mean arrival of the solute pulse:

$$M_1 = \int Ctdt$$

The mean travel time (MTT) of the tracers is the time required for the mean position of the tracer pulse to move from the injection well to a given monitoring location:

$$MTT = M_{1,norm} - 0.5T_o$$

where $M_{1,norm}$ is M_1 divided by M_o, and T_o is pulse-injection time normalized by MTT of the non-reactive tracer. The percent recovery of the biodegradable solutes was calculated as the ratio of the mass of the biotracer recovered (Mo) to the mass of the biotracer injected (the product of the pulse width, the injection flowrate and the concentration):

$$\%\text{Recovery} = \frac{M_{o,biotracer}}{Mass_{input,biotracer}}$$

and the normalized time (pore volume) for the biotracers was calculated as the ratio of time for the biotracer to the mean travel time of the non-reactive tracer.

Moment analysis of the breakthrough curves for the nonreactive tracers determined the M_o, or recovered pulse width in terms of pore volume, for each well location. This value was utilized instead of the input pulse width to account for the lack of 100% tracer recovery.

The recovery pulse width for the non-reactive tracer was then input with the corresponding breakthrough curve into a transport model to calibrate values for the Peclet number, which is a measure of the relative contributions of advection and dispersion to transport. For the Tucson-site data, this was done using a 1-D advective-dispersive transport model that utilizes a nonlinear, least-squares optimization program (CFITIM; van Genuchten, 1981). For the Utah-site data, a dual-porosity advective-dispersive transport model was employed, and values for β, ω, and Peclet number were calculated using a different nonlinear, least-squares optimization program (CFITIM3). This model was used to account for heterogeneity effects on transport (e.g., diffusion into and out of layers of lower hydraulic conductivity).

Following independent determination of the values for Peclet number, β, ω, retardation factor, and pulse width using the non-reactive tracer data, first-order biodegradation constants were calculated for the biotracers. This was performed by fitting the breakthrough curves for the biotracers using a model incorporating rate-limited sorption and linear biodegradation (van Genuchten and Wagenet, 1989), or more specifically, the nonlinear, least-squares optimization program FITNLNED (Jessup et al., 1987). This program contains four fitting parameters (P, R, T_o, X_c), for which only X_c (the nondimensional degradation constant) and the retardation factor (UT data only) was optimized. The nondimensional degradation constant is defined as follows:

$$X_C = \frac{\mu L}{v}$$

where μ (t^{-1}) is the first-order biodegradation constant, L is the length of the system, and v

(Lt^{-1}) is the pore water velocity. It is assumed that a linear biodegradation model can be applied to this data because small input pulses of biotracers were utilized (less than 1 pore volume), and the amount of microbial growth that is likely to occur during these pulses is negligible.

RESULTS AND DISCUSSION

Laboratory column studies. Results from laboratory column experiments were used to test the applicability of the linear biodegradation model. Columns were packed with aquifer material collected from the Utah site and subjected to toluene inputs of two different pulse lengths. The assumption that the linear biodegradation model could be applied to experiments with short input pulses (<1 PV) was tested by applying the model to this laboratory column data. A model fit was achieved for the breakthrough curve for the short toluene pulse (0.27 pore volumes) using hydrodynamic parameters typical of laboratory column experiments (Figure 1). However, the model would not converge using the same hydrodynamic parameters (or others) with data from an experiment with a longer pulse of 49.6 pore volumes. Therefore, the model was able to fit transport and biodegradation data for small input pulses and provide a linear biodegradation rate constant. However, this model was not able to fit breakthrough data from an experiment with a larger input pulse and provide a linear biodegradation constant, because significant growth occurred in this experimental system causing the biodegradation rate to increase with time.

Site 1: Utah. Representative breakthrough curves of the nonreactive tracers and biotracers are shown for two wells, one closer to the injection wells and one near the extraction wells (Figure 2). Results of the moment analysis for the non-reactive tracer provided values for the zeroth moment (Table 1), in terms of dimensionless values (pore volumes). The Peclet number values ranged from approximately 3 to 51 (with an average of 20). The optimized simulations for the advective/dispersive transport equation fit the data well for all cases. The mass recovered, or M_0, ranged from 80 to 112 percent, with the largest value obtained at a monitoring location closest to the injection wells.

The recoveries of biotracers were less than or equal to the recoveries of bromide for all monitoring locations. Batch biodegradation studies using ^{14}C-benzoate (Wang, 2000) and samples taken from the soil cored from the site confirmed that the indigenous microflora was capable of degrading the biotracer compounds used. Other mass loss processes such as volatilization, sorption, and hydrolysis were negligible at this site. The mass recoveries ranged from 17 to 130 percent depending on the compound and location (Figure 3).

The percent recovery was found to be approximately linearly related to the residence time of the compound in the system for all of the biotracers when data points near the injection wells were omitted (Figure 3). However, percent recovery was not related to distance from the injection wells due to heterogeneities in the cell. The extent of the heterogeneity in the cell is apparent from inspection of the contour plot relating residence time and distance from the injection well (Figure 4). Therefore, residence times are generally larger at the extraction end of the cell, although there are regions corresponding to

high residence times near the injection wells. This is presumably due to the presence of low hydraulic conductivity regions in the cell.

When the linear biodegradation rate coefficient (μ) was plotted versus residence time, it was not found to be vary as a function of residence time when samples near the injection wells were excluded (those with short residence times) for all compounds. Thus, the compounds were not biodegraded at the same rate at all locations within the cell. Spatial distributions of the rate constant for the biotracers showed the biodegradation rate coefficient was much larger near the injection wells for all compounds (Figure 6), and distribution of this parameter was similar for all compounds. As discussed previously, the Utah site was subjected to a cyclodextrin flush which enhanced mixing in the cell, and infused regions near the injection wells with increased oxygen levels. This stimulated microbial populations and increased the population density of JP-8 degraders in the vicinity of the injection wells. The bacterial population densities of the three soil coring locations on the site were an order of magnitude higher, in terms of both heterotrophic and JP-8 degrader densities, near the injection well (Figure 7) compared to values from the extraction well. Moreover, the decrease in bacterial densities followed a general trend, decreasing with distance from the injection point. The population density of jet fuel degraders was approximately 10^7 CFU/g soil, not even an order of magnitude less than the overall density of heterotrophs.

The corresponding non-uniform spatial distributions of the biodegradation rate coefficients for the biotracers are apparent on contour plots of the linear biodegradation rate coefficient (Figure 6). These calculated rate coefficients were much higher near the injection

wells overall, in some cases by an order of magnitude. However, one of these sampling wells near the injection wells was in an area of apparent low conductivity near the injection well, as indicated on the travel time distribution contour plot. Thus, the biodegradation rate coefficient was lower at this well than the at two adjacent wells. The presence of this region of lower conductivity most likely limited the effect of the flush due to reduced flow in this region, such that oxygen levels were not enhanced as dramatically.

Site 2: Tucson. Representative breakthrough curves of the nonreactive tracers and biotracers are shown for wells M73-B and P8 (Figure 8). The Peclet number values ranged from approximately 3 to 15, and the normalized pulse widths ranged from 0.3 to 1.3 pore volumes (Table 2). This range in pulse widths is a result of the heterogeneous nature of the aquifer system. Not all portions of the aquifer will have equal volumes of flow due to hydraulic conductivity variations. The breakthrough curves for wells with larger normalized pulses reached larger maximum relative concentrations.

For all monitoring locations, the recovery of benzoate was less than the recovery of bromide (Table 2) and the concentrations of benzoate returned to zero much earlier than for bromide. The mass recoveries for benzoate ranged from 14 to 81 percent, and they decreased as the residence time (MTT_{br}) to a given well increased (Figure 9). The residence time is a function of the distance from the injection well and the permeability of the flow domain. Results of batch biodegradation experiments using ^{14}C-benzoate and aquifer material obtained from cores from the site confirmed that the indigenous microflora were capable of degrading benzoate (Wang, 2001). Other mass loss processes such as volatilization,

sorption, and hydrolysis were not operable for these experiments. Thus, it is reasonable to conclude that the mass loss of benzoate was due to biodegradation.

The biodegradation rate constants were similar throughout the Tucson site despite the presence of heterogeneity in the subsurface, ranging from 0.20 to 0.48 d^{-1} (Table 2). This small range of rate constants might be the result of populations of microflora present only in the more permeable regions of the aquifer. It is also possible that dissolved oxygen concentrations, which were in the range of 1 to 3 mgL^{-1}, limited the rate of biodegradation. Conversely, dissolved oxygen concentrations at the Utah site ranged from 8 mg/L (near injection wells) to 2 mg/L (extraction wells). A wider range of rate coefficients might have been expected, considering the heterogeneous nature of the subsurface. However, this effect was mitigated by the low overall microbial activity at this site.

In general, biodegradation rate coefficients remained relatively constant as a function of residence time at both field sites (Table 2 and Figure 5), when the samples near the injection wells for the Utah site were excluded. However, inclusion of these data from the Utah site decreased the correlation coefficient drastically, indicating that the biodegradation behavior is not the same in this region. Not only were the biodegradation rate constants similar within a given site, the rate constants for the same compound (benzoate) were similar between the two field sites. The average rate constants were 0.27 d^{-1} and 0.32 d^{-1} for the Tucson and Utah sites respectively. On a plot of percent recovery of benzoate versus residence time, the data points from both sites scatter around the same line (when points near the injection wells at Utah are omitted). This is explained by the correlation between mass

recovery and residence time at the two field sites, even though the benzoate solution injected at the Utah site (304 mg/L) was 6.8 times more concentrated than at the Tucson site (45 mg/L). Finally, benzoate is likely degraded by a wide variety of bacteria common to many soil types, which would lessen the differences between sites.

Overall, the biodegradation rate constants for benzoate varied from 0.20 to 0.48 d^{-1} for the Tucson site while most values for the biotracers (including data from all 4 compounds) at the Utah site fell in the range of 0.1 to 0.6 d^{-1}. These rate constants are similar in magnitude to those measured for alkylbenzenes, PAH, and chlorinated benzenes using in-situ microcosms (0.2 to 0.9 d^{-1}) (Nielsen et al., 1996). However, they are 20 to 50 times larger than the rate constants reported for benzene (0.007 d^{-1}), p-xylene (0.011 d^{-1}), naphthalene (0.0064 d^{-1}), and o-dichlorobenzene (0.0046 d^{-1}) for a field experiment conducted at Columbus Air Force Base (MacIntyre et al., 1993). They are also 20 times larger than the rate constant reported for toluene (0.0075 to 0.03 d^{-1}) for a field experiment conducted at Hanahan, South Carolina (Chapelle et al., 1996). Thus, these values can vary greatly from one site to the next and for different compounds.

The results of the field and laboratory transport experiments indicate that the biodegradable solutes were, in fact, degraded and that the extent of mass loss was generally correlated to residence time. These results are consistent with expected results from a site with a spatially uniform distribution of microbial activity (constant linear biodegradation rate). For this scenario, an increase in residence time should allow more time for biodegradation and a decrease in mass recovery. However, at the Utah site, recovery of the

biotracers is not a function of residence time when data from the wells near the injection wells are included. This is most likely due to the increased population density of JP-8 degraders in the vicinity of these well, as evidenced by the microbiological sample analysis. Microbial distributions in the subsurface are influenced by a number of environmental factors, such as carbon, oxygen or nutrient availability, soil texture, or heterogeneous conditions. Non-uniform spatial distributions of bacteria in the subsurface as a function of these factors has been reported by a number of researchers (Harvey et al., 1984; Severson et al., 1991; Harvey et al., 1992; Kieft et al., 1998; Krueger et al.,1998; Stapleton et al., 2000). Additionally, the impact of spatially variable microbial parameters has been demonstrated to influence contaminant transport and biodegradation behavior significantly (Miralles-Wilhelm et al., 2000). However, traditional techniques of determining the distribution of subsurface microorganisms, which include obtaining core samples, enumerating bacteria, and conduction microcosm studies to determine biodegradation potential, are tedious.

CONCLUSIONS

The two field sites discussed in this text differ in a number of ways, leading to differences in transport and biodegradation behaviors of the non-reactive tracers and the biotracers. At the Utah site, residence times did not increase linearly with distance from the injection well, as is apparent from a contour plot showing the 2-D spatial distributions of residence times in the cell. Rather, a 'center' of relatively high travel times exists near the injection well, indicating an area of low hydraulic conductivity compared to the rest of the

cell. Conversely, the data from the Tucson site was less affected by these nonideal conditions. Additional differences between the two sites include the size of the swept zone, and the type and concentrations of the contaminants. The swept zone of the Tucson site is lightly contaminated as compared to the swept zone of the Utah site. The Tucson site contains low (sub mg/L) concentrations of TCE, while the Utah site contains high (mg/L) concentrations of mixed organic contaminants (jet fuel, degreasing solvents), as well as NAPL phases.

Analysis of the data obtained in these experiments shows the potential of biotracers to evaluate the biodegradation capability of indigenous populations, and to examine the spatial distribution of bacteria or biodegradation potential at a site. Additionally, similar experiments conducted by other researchers using different compounds have shown similar results, as discussed previously. This application is useful in providing quantitative comparisons of biodegradation potential at different spatial locations within a field site.

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Table 1. Non-reactive Tracer Recovery andTransport Characteristics: Utah site.

Well 34-	% Rec.	MTT (days)	M_{O^3Br} (PV)	Peclet #	β	ω
11 Red	112	0.364	0.626	44	0.822	0.178
21 Red	102	1.23	0.168	13	0.23	0.36
31 Red	86.3	0.362	0.483	13	0.489	0.124
12 Red	102	0.992	0.208	11.8	0.589	0.133
22 Red	89.5	0.522	0.348	9.4	0.62	0.18
32 Red	79.4	0.606	0.266	31.5	0.809	0.293
13 Red	95.2	0.728	0.265	17.6	0.917	0.016
23 Red	98.2	0.466	0.426	13.8	0.792	0.094
33 Red	79.7	0.624	0.212	38.5	0.816	0.267
24 Red	96.2	0.737	0.264	51	0.604	0.157
51 Ext	106	1.41	0.153	4.3	0.623	2.74*
52 Ext	110	1.21	0.185	12	0.445	0.989
53 Ext	107	1.23	0.177	2.8	0.934	0.020

*Data was double-peaked - did not fit well.

Table 2. Recoveries, Transport Characteristics, and Biodegradation Rate Constants: Tucson site.

Well	% Recovery$_{,Br}$	MTT_{Br} (hr)	$M_{O,Br}$ (hr)	Peclet Number[1]	% Bzt Recovery	μ (d^{-1})
M73-A	101	71	56.5	2.9 (2.4, 3.5)	57	0.24
M73-B	96.4	78	54	6.5 (6.2, 6.8)	55	0.20
M73-C	103	64	57.7	3.7 (3.4, 4.0)	63	0.21
M73-D	105	61	59	3.1 (2.9, 3.3)	66	0.21
M72	84.8	60	47.5	13 (11, 15)	32	0.48
P8	80.3	34	45	3.4 (2.2, 4.7)	81	0.21
P10	83.2	150	46.6	15 (13, 16)	14	0.33

[1] Values in parenthesis are 95% confidence intervals.

FIGURE CAPTIONS

Figure 1. Experimental data and simulation results for a miscible-displacement experiment using a 0.27 pore volume pulse of toluene in a laboratory column packed with Hill aquifer material. The values used in the simulation were Peclet number = 40, R =1.99, $\beta = 0.435$, $\omega = 8.04$, and X_{ic} of 0.253 (all parameters are calibrated except Peclet number).

Figure 2. Representative breakthrough curves and simulation results for two wells at the Tucson field site.

Figure 3. The percent of mass recovered for the biotracers used at the Utah site as a function of residence time. Data points from sampling locations near the injection wells excluded. A) ethanol, $R^2 = 0.86$; B) pentanol, $R^2 = 0.83$; C) hexanol, $R^2 = 0.70$; D) benzoate, $R^2 = 0.51$.

Figure 4. The spatial distribution of residence time for the conservative tracer (bromide) at the Utah site.

Figure 5. The relationship between residence time and the biodegradation rate constant. These values were not found to be correlated and thus the least squares linear fit is not shown; R^2 for ethanol is 0.12, R^2 for pentanol is 0.31,R^2 for hexanol is 0.33 and R^2 for benzoate is 0.28.

Figure 6. The spatial distribution of biodegradation rate constants at the Utah site. Values were higher at locations close to the injection wells.

Figure 7. Microbial distribution plots from three soil corings. Viable counts for total heterotrophs and JP-8 degraders are shown.

Figure 8. Representative breakthrough curves and simulation results for two wells at the Utah Site.

Figure 9. The percent of mass of benzoate recovered as a function of residence time for Tuscon. The correlation coefficient for the linear least squares fit is 0.69.

Figure 1.

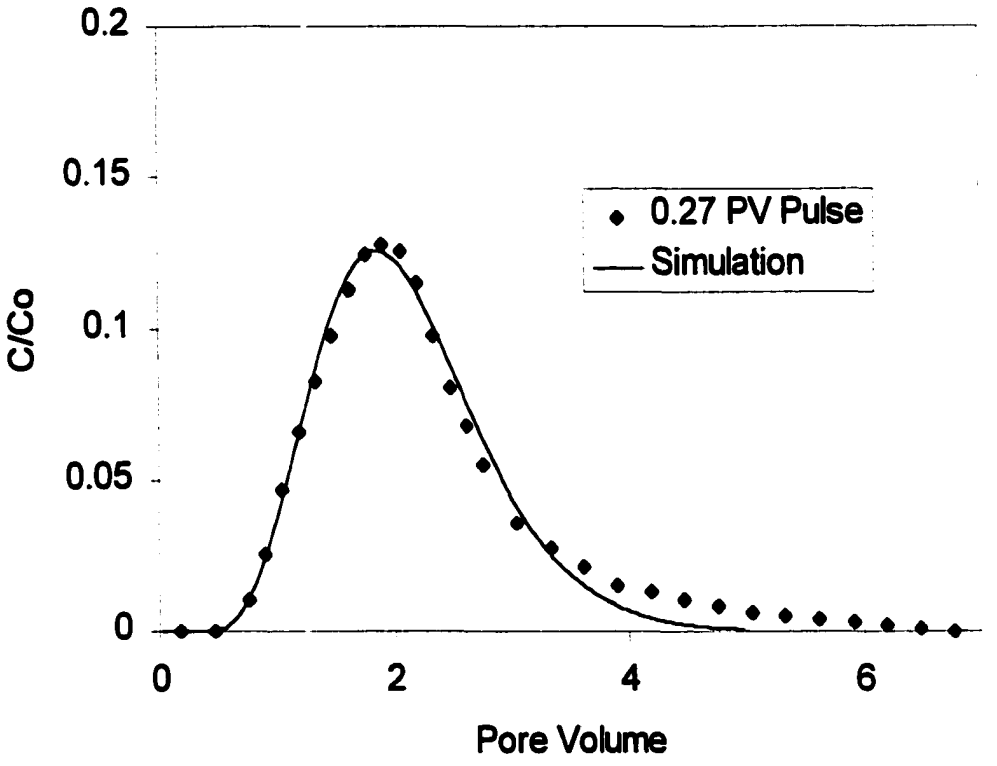


Figure 2.

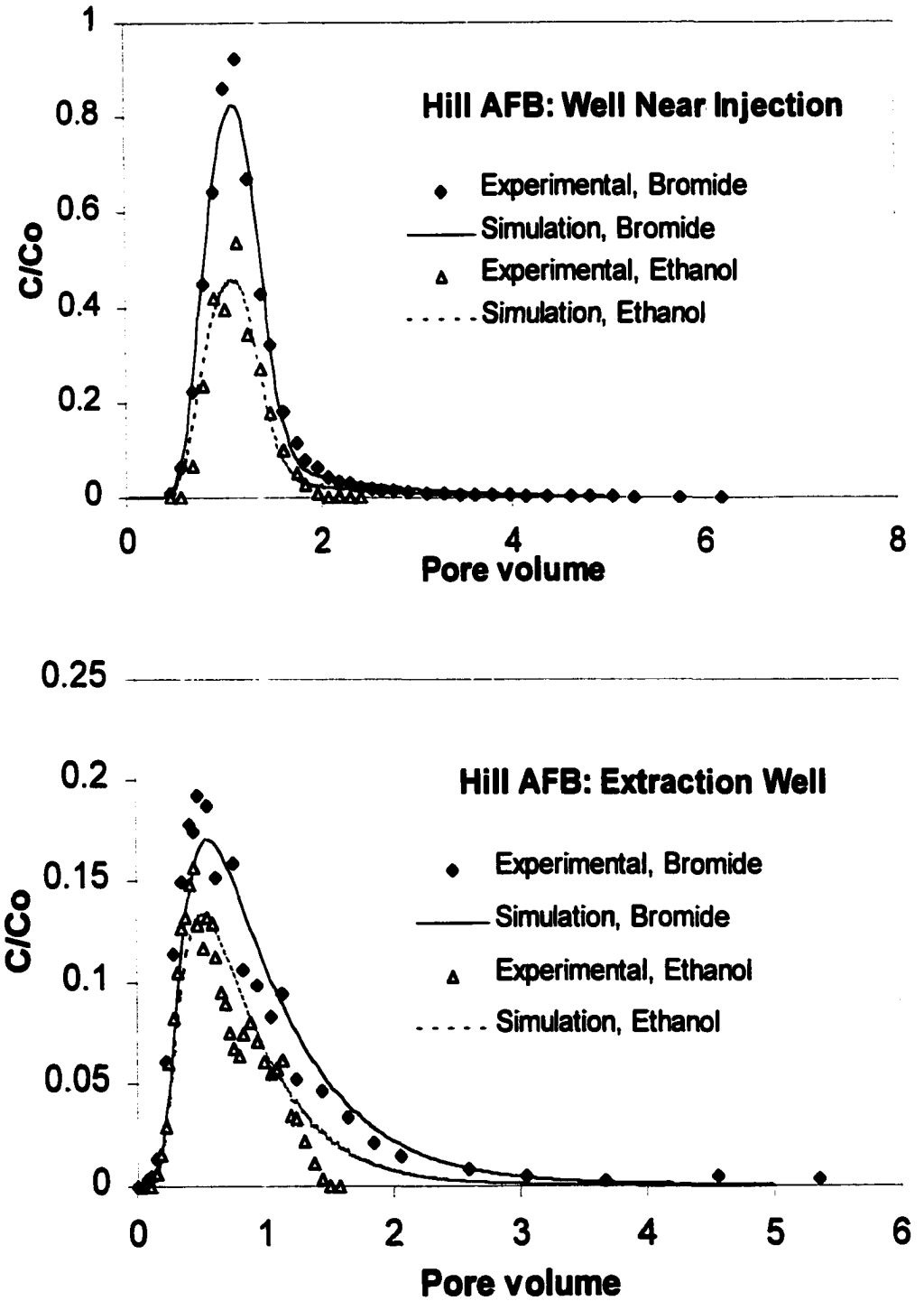

Hill AFB: Well Near Injection
Experimental, Bromide
Simulation, Bromide
Experimental, Ethanol
Simulation, Ethanol
C/Co
Pore volume
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
Hill AFB: Extraction Well
Experimental, Bromide
Simulation, Bromide
Experimental, Ethanol
Simulation, Ethanol
C/Co
Pore volume
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
6

Figure 3.

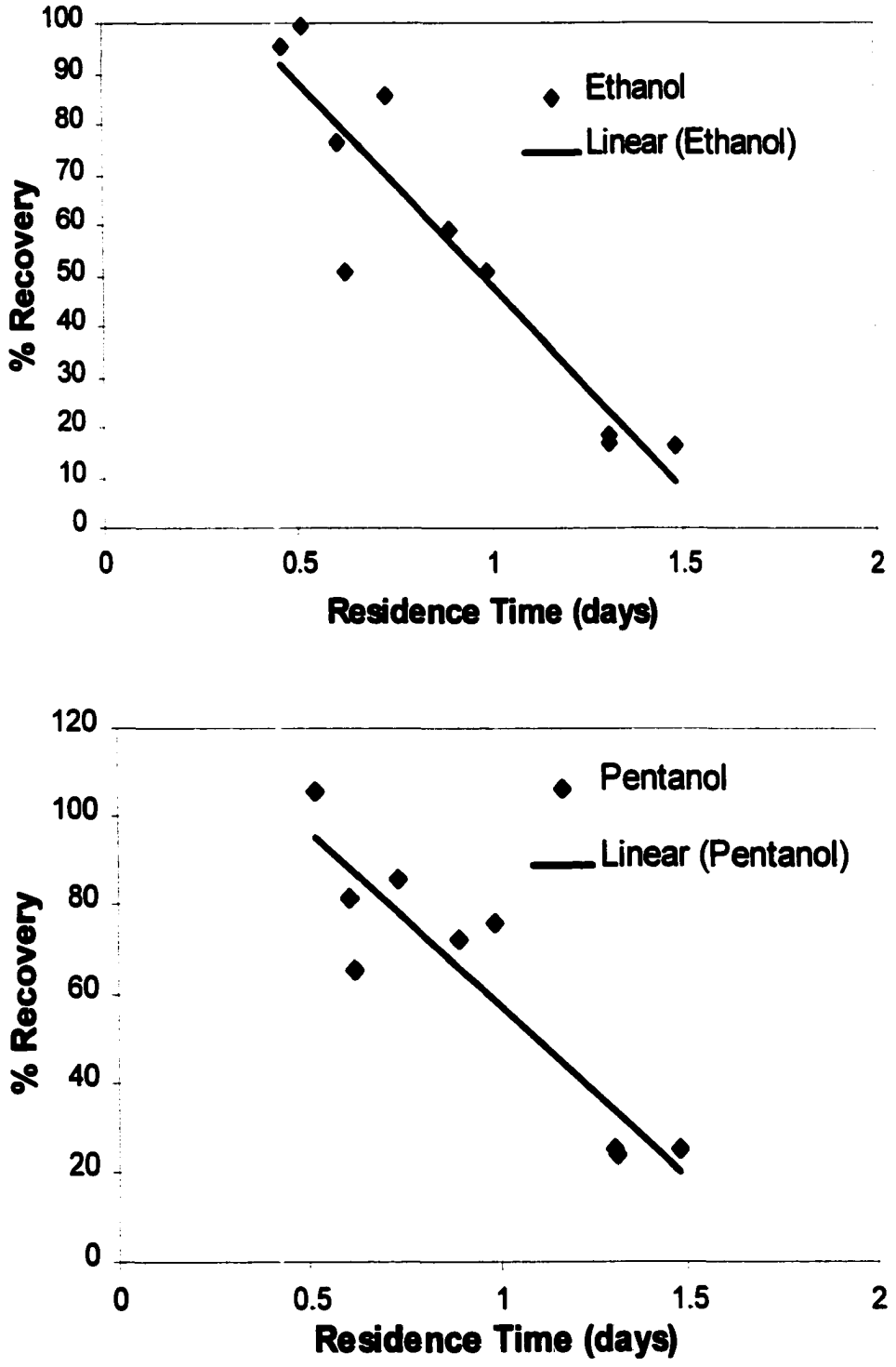

100
90
80
70
60
50
40
30
20
10
0
% Recovery
Ethanol
Linear (Ethanol)
0
0.5
1
1.5
2
Residence Time (days)
120
100
80
60
40
20
0
% Recovery
Pentanol
Linear (Pentanol)
0
0.5
1
1.5
2
Residence Time (days)

Figure 3, continued.

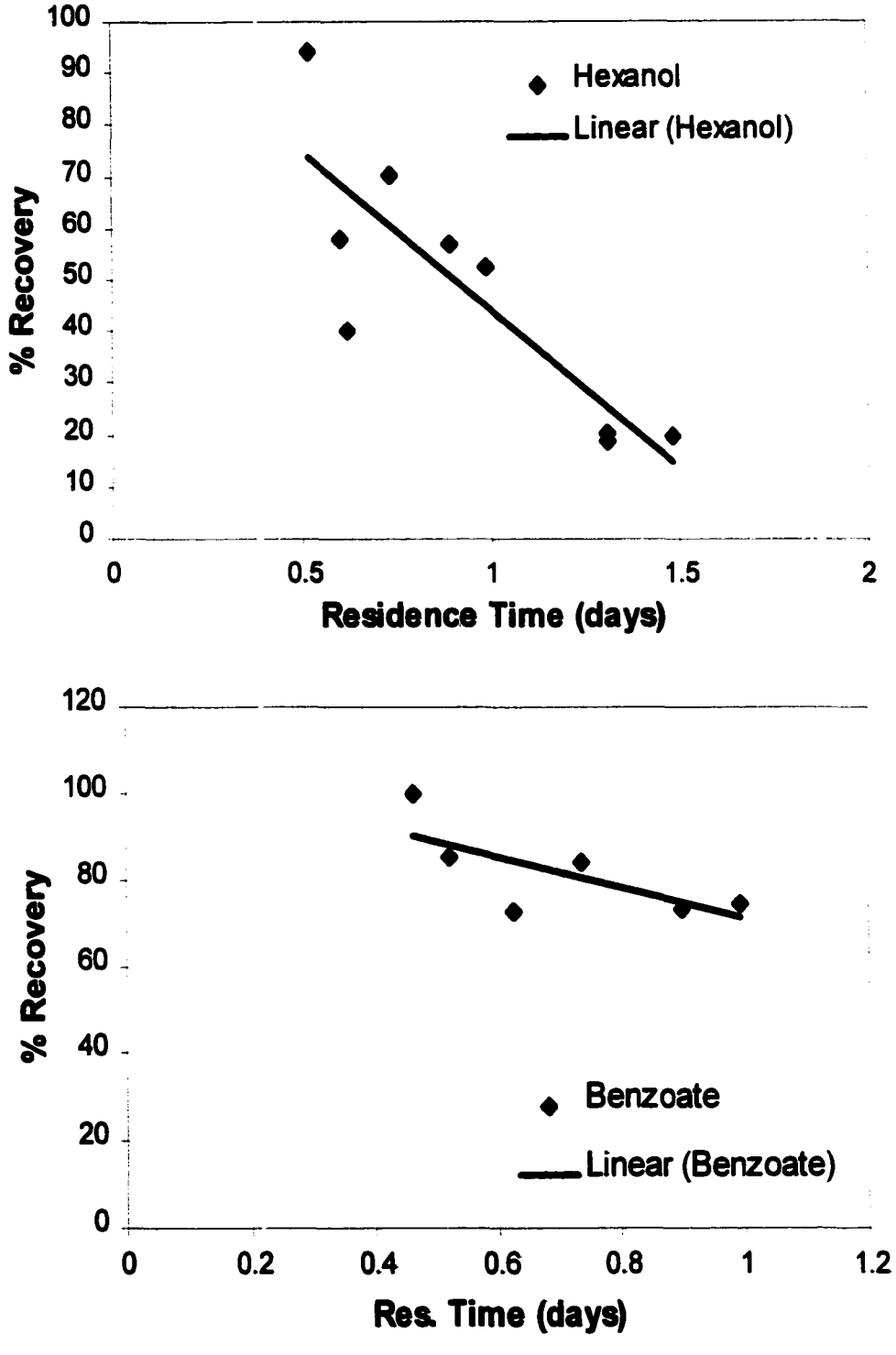


Figure 4.

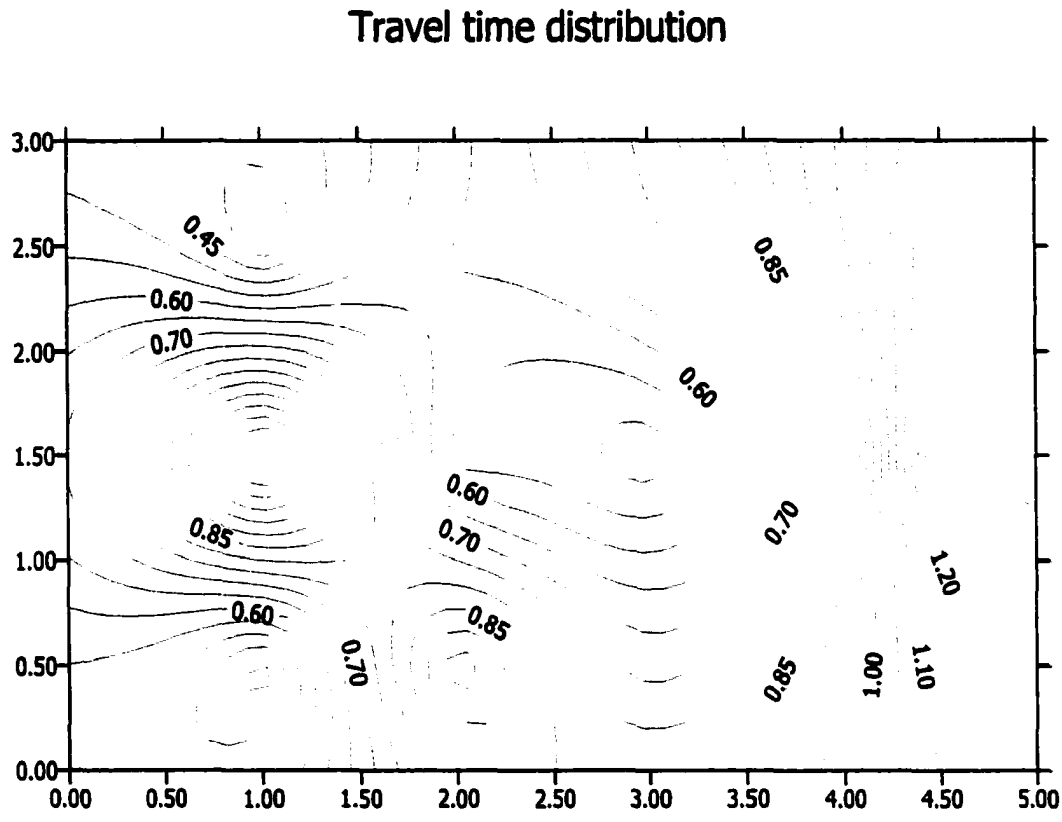

Travel time distribution
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
0.45
0.60
0.70
0.85
0.60
0.60
0.70
0.85
0.60
0.70
0.85
0.70
0.85
1.00
1.10
1.20

Figure 5.

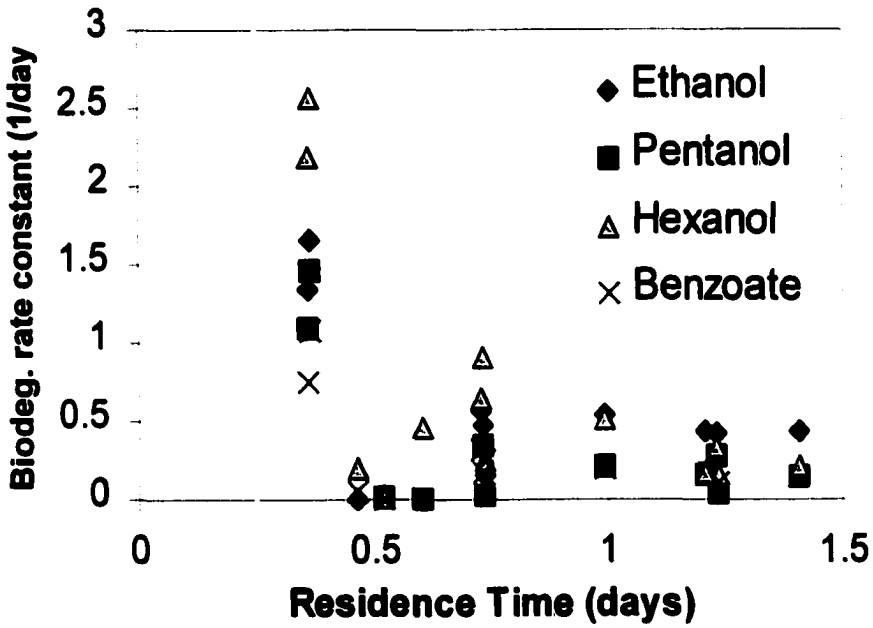

Biodeg. rate constant (1/day
3
2.5
2
1.5
1
0.5
0
0
0.5
1
1.5
Residence Time (days)
Ethanol
Pentanol
Hexanol
Benzoate

Figure 6.

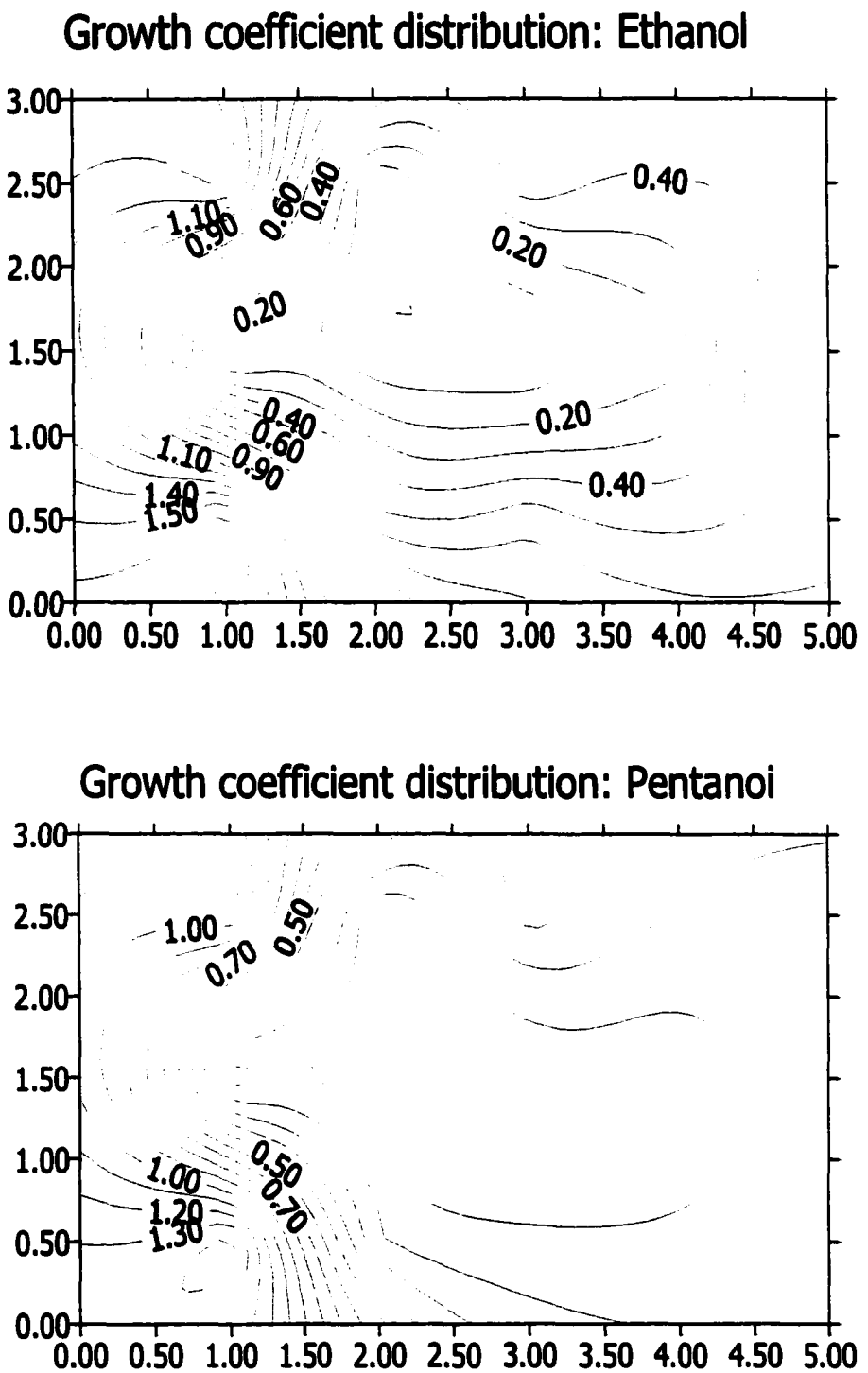
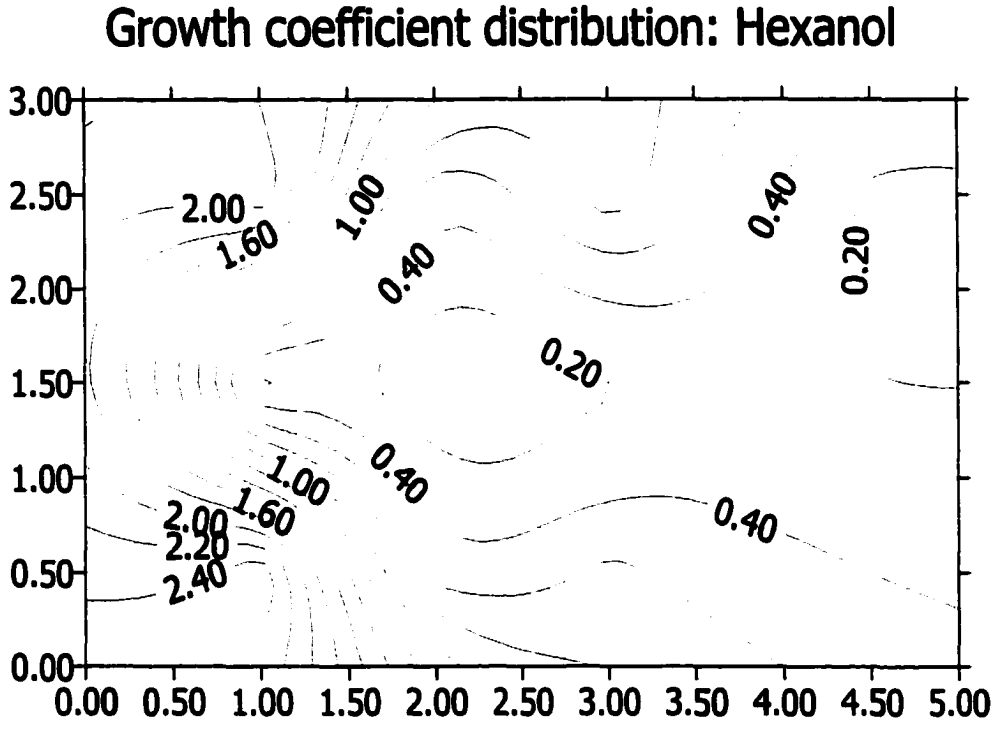

Growth coefficient distribution: Ethanol
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00
1.10
0.90
0.60
0.40
0.20
0.40
0.20
0.40
0.60
1.10
0.90
1.40
1.50
0.20
0.40
Growth coefficient distribution: Pentanoi
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00
1.00
0.70
0.50
1.00
1.20
1.30
0.50
0.70

Figure 6, continued



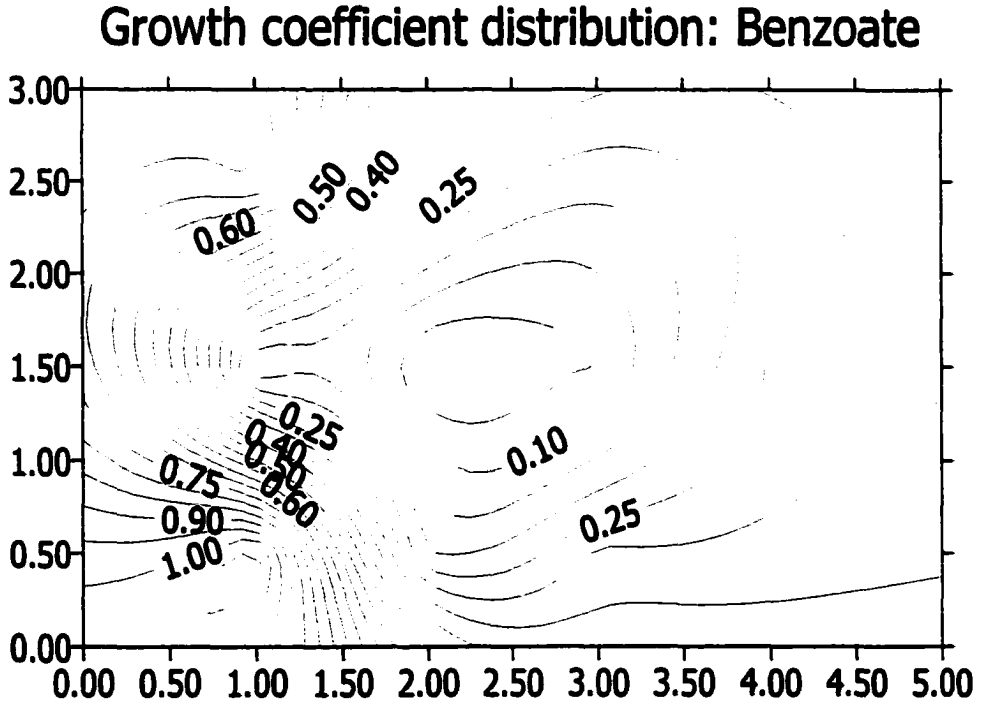
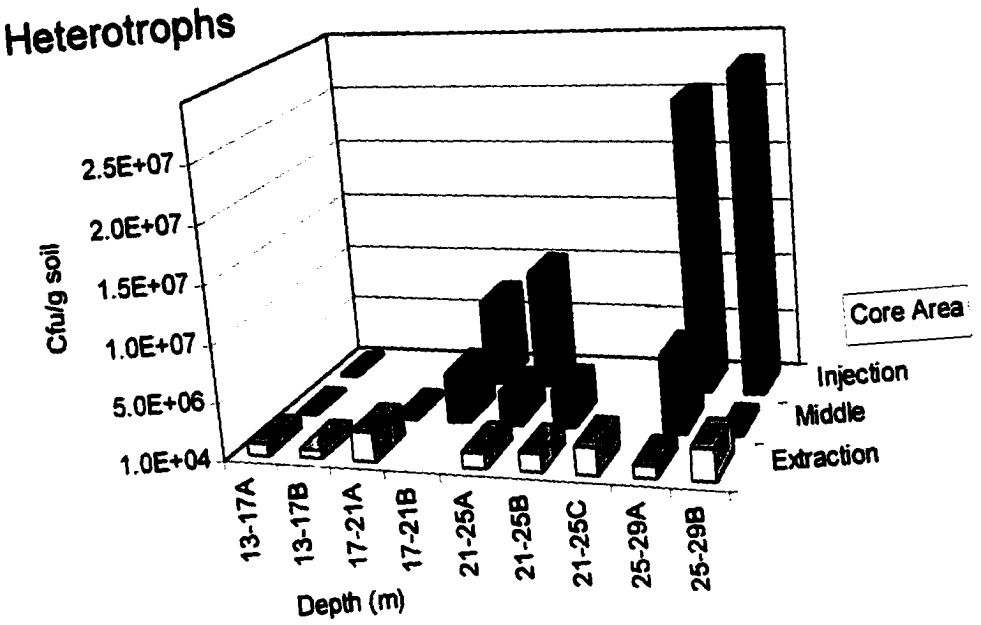


Figure 7.


Heterotrophs
2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.0E+06
1.0E+04
Cfu/g soil
13-17A
13-17B
17-21A
17-21B
21-25A
21-25B
21-25C
25-29A
25-29B
Depth (m)
Core Area
Injection
Middle
Extraction

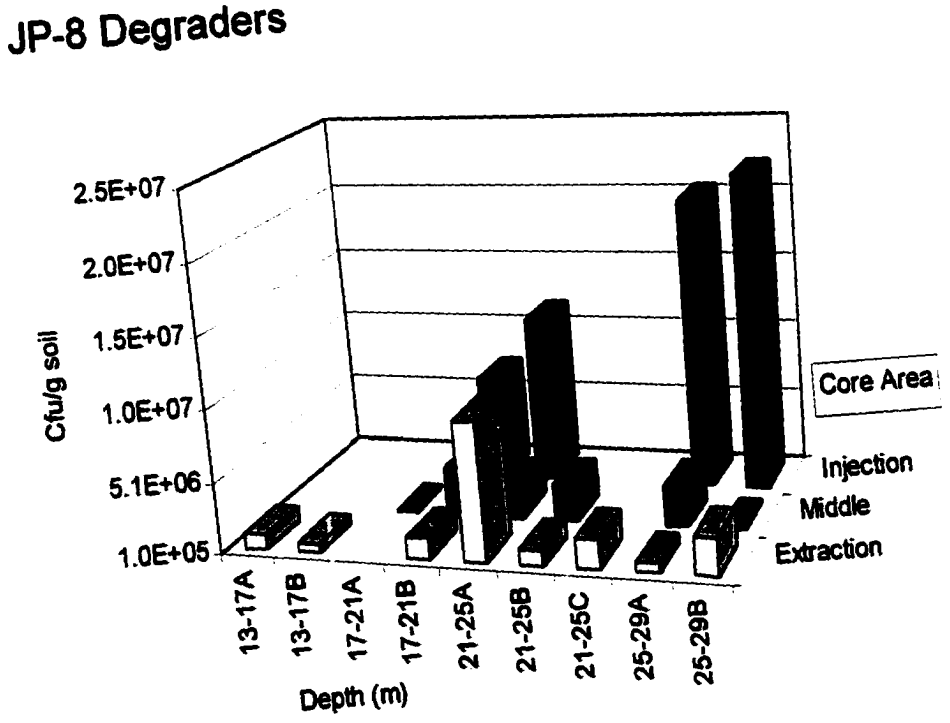

JP-8 Degraders
2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.1E+06
1.0E+05
Cfu/g soil
13-17A
13-17B
17-21A
17-21B
21-25A
21-25B
21-25C
25-29A
25-29B
Depth (m)
Core Area
Injection
Middle
Extraction

Figure 8.

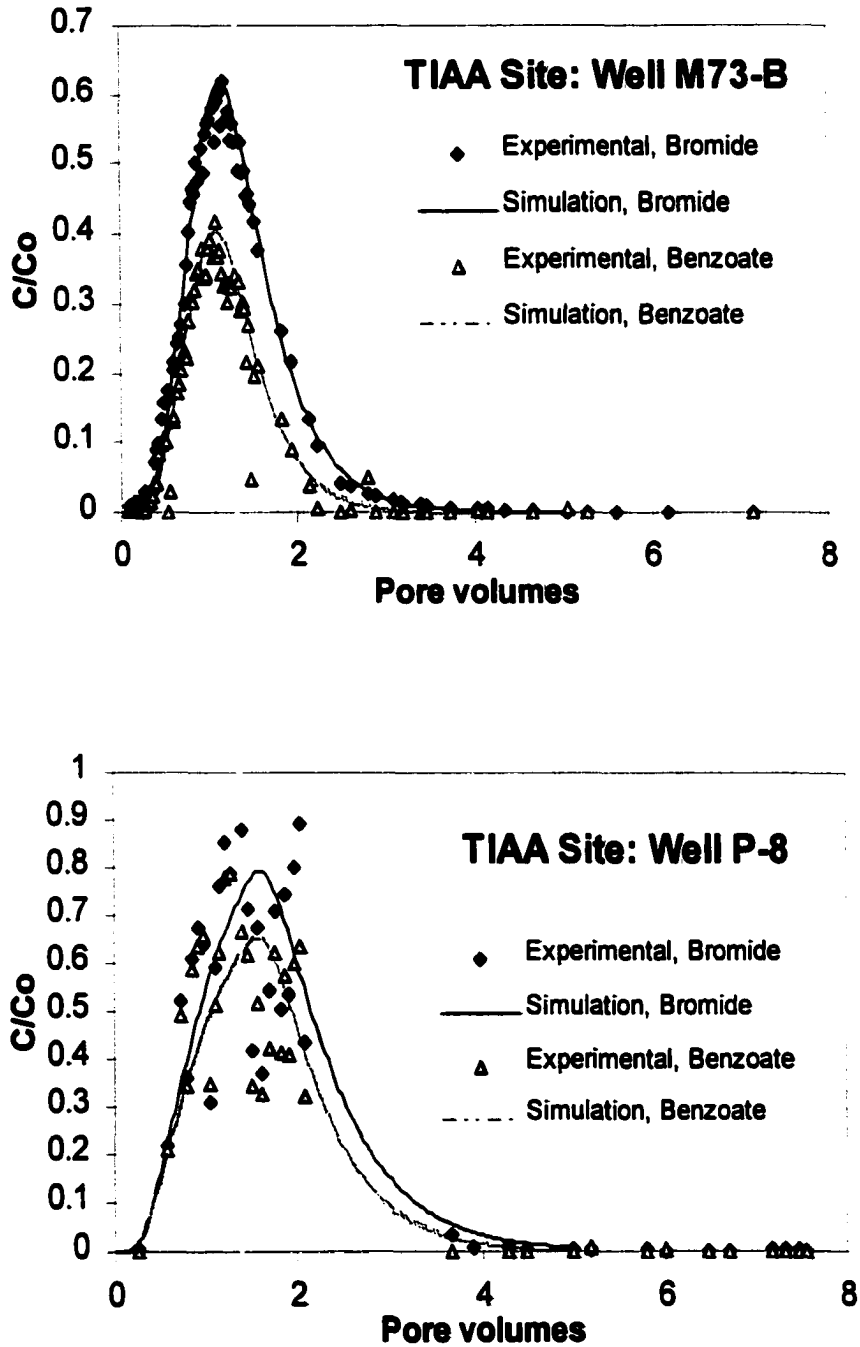
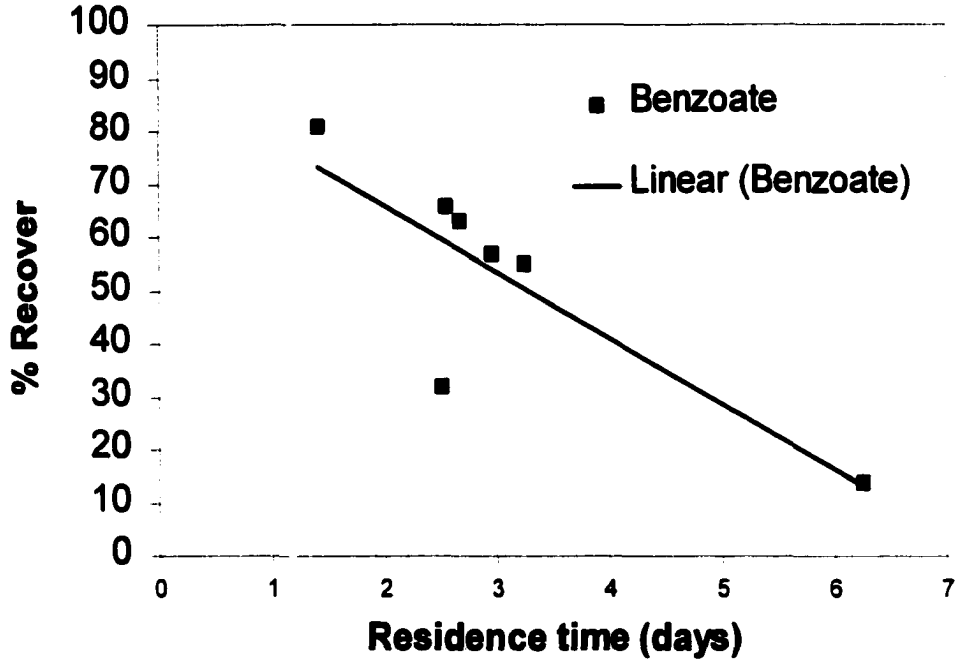

TIAA Site: Well M73-B
Experimental, Bromide
Simulation, Bromide
Experimental, Benzoate
Simulation, Benzoate
C/Co
Pore volumes
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
TIAA Site: Well P-8
Experimental, Bromide
Simulation, Bromide
Experimental, Benzoate
Simulation, Benzoate
C/Co
Pore volumes
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8

Figure 9.


100
90
80
70
60
50
40
30
20
10
0
% Recover
0
1
2
3
4
5
6
7
Residence time (days)
Benzoate
Linear (Benzoate)