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The Effect of Bacterial Chemotaxis on
In Situ Bioremediation Rate

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Christopher John O'Lenick
May 2006

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DEDICATION

This dissertation is dedicated to my parents,
Maureen Crost and
Dr. Richard O'Lenick.

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ABSTRACT

The process of sensing an attractant chemical by a motile bacterium and subsequent motion towards that attractant, known as chemotaxis, occurs in *Escherichia coli*, *Pseudomonas putida* G7, *Rhizobium meliloti*, and several other subsurface strains. To date, there have been no widely accepted experimental studies that demonstrate whether bacterial chemotaxis can enhance biodegradation of contaminants in the subsurface. This research investigates the effect of bacterial chemotaxis on degradation rate in an experimental model for *in situ* bioremediation. The novel experimental protocols of this work, developed to investigate bacterial chemotaxis and migration, have provided for the systematic evaluation of the effect of the chemotaxis phenomena in a porous medium. The hypothesis formulated is that a bacterium undergoing chemotaxis in a porous medium will be able to sense an attractant chemical, bias its motion towards it, and subsequently degrade the attractant at a higher rate than a strain exhibiting non-chemotactic behavior.

The experimental model has been developed to measure the degradation rate of serine, a simulated contaminant and chemoattractant. *E. coli* RP437 was used as a representative chemotactic *in situ* bacteria while *E. coli* RP5700, a *tsr*-mutant strain of RP437 that lacks the serine chemoreceptor, was used as the control strain. RP5700 exhibits random motility similar to RP437, regardless of serine gradients. These two strains were highly characterized for this work, a process which was rigorous and performed in more detail than in prior works.

Chemotactic ability of RP437 toward serine was validated via capillary and swarm plate assays. Swimming speeds, run lengths, and turn angles were compared using a tracking microscope and were statistically similar. Serine uptake rates in liquid media were also statistically similar. These results show that these strains are suitable for investigating any enhancing effect of chemotaxis on biodegradation rate.

For *in situ* bioremediation experiments, a model aquifer has been designed to introduce RP437 and RP5700 bacteria to serine in saturated sand via a sharp gradient. The aquifer was used to compare serine degradation rates and migration rates through sand. Results showed that the degradation rate of serine was statistically similar for both strains over a 21 hour period, indicating that enhancement was not detected. The experimental parameters chosen for this study did not elucidate degradation or migration enhancements due to chemotaxis. However, the experimental methodologies developed to acquire these results represent novel contributions to the field of chemotaxis analysis in porous media. These methodologies can easily be extended for the variation of other sets of parameters, such as particle size, cell densities, growth conditions, and selection of chemoattractants.

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NOMENCLATURE

3CB	3-chlorobenzoate
β_0	basal tumbling frequency of <i>E. coli</i> (s^{-1})
$\delta_{\alpha\beta}$	Kronecker delta
ε_p	void fraction
μ	random motility coefficient, (cm^2/s)
v	swimming speed, (cm/s)
σ	constriction factor
τ	tortuosity
χ_0	chemotactic sensitivity coefficient, (cm^2/s)
a	attractant concentration (mM)
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
AMQ	6-aminoquinoline
AWB	Adler wash buffer
AWBS	Adler wash buffer with serine
BCC	bacterial chemotaxis chamber
c	cell concentration, (cells/cm^3)
CFU	colony-forming unit
D	diffusivity of attractants (cm^2/s)
D_{eff}	effective diffusion coefficient for attractants in porous media, (cm^2/s)
GMM	glycerol minimal medium
Δh	grid size for simulation box (cm)

HPLC	high performance liquid chromatograph
ISB	<i>in situ</i> bioremediation
J_c	cell population flux, (cells/(cm·s))
K	turn angle distribution
K_d	dissociation constant for α -methylasspartate, (mM)
L	number of directions in which a particle can travel
MSF	migration sampling frame
n	number of bacteria at position X
n_α	number of bacteria moving in the $\hat{s}_\alpha$ direction
n_β	number of bacteria moving in the $\hat{s}_\beta$ direction
OD ₆₀₀	optical density measured by spectrophotometer, 600 nm
p	general tumbling probability
$p_{\alpha\beta}, p_{\alpha\gamma}$	probability per unit time that a bacterium tumbles and changes direction from lattice direction $\hat{s}_\alpha$ to lattice direction $\hat{s}_\beta$
P00	tumbling probability
R1	sphere radius for mathematical model
$\hat{s}, \hat{s}_\alpha$	initial vector of motion of bacterial density unit
$\hat{s}', \hat{s}_\beta$	vector of motion of bacterial density unit after tumbling
SFDC	stopped flow diffusion chamber
t	time (s)
$T_{\alpha\beta}$	transition matrix containing probability terms
TCE	trichloroethylene
UV	ultraviolet

WBT	Wolfe-Berg tryptone
<i>X</i>	position of bacterial density unit

1 INTRODUCTION

Chemical contamination in the subsurface is a problem of global proportions. Because the use of synthetic chemicals has spread to all regions of the industrialized world, improper disposal and leakage of chemicals has spread as well. Subsurface contamination by hazardous chemicals creates a threat to human and animal health. *In situ* bioremediation (ISB) methods are often used when the volume of the subsurface contaminant plume is too large for treatment by physical processes or excavation. Currently, ISB is actively researched and applied in the field by environmental engineering consulting firms such as URS Corporation, Shaw Environmental and Infrastructure, Inc. and Arcadis, Inc.

Bacterial chemotaxis, the process of sensing an attractant chemical by a motile bacterium and subsequent motion towards that attractant, has been studied as a biological phenomenon that could potentially enhance ISB. Many hazardous chemicals are actually chemical attractants for subsurface bacteria. By increasing the transport rates of degrading bacteria to the contaminant-attractant, it has been theorized that the overall degradation rate of contaminants in the subsurface will be higher in environments that support chemotaxis.

The study of chemotaxis through porous media requires expertise from many disciplines, such as chemical engineering, civil engineering, environmental engineering, microbiology, and geology. One reason that chemical engineering laboratories are involved in chemotaxis research is because of the close parallels between bacterial random motility (unbiased motion in three directions) and

molecular diffusion. Many of the same equations describing molecular diffusion can be modified and directly applied to random motility of bacteria in both aqueous and porous media.

The purpose of this research is to compare and contrast levels of attractant biodegradation by bacteria under chemotactic and non-chemotactic conditions. This comparison and contrast was performed by the utilization of standard chemotaxis assays and by the development of novel assays to elucidate any effect of chemotaxis on biodegradation in a saturated porous medium simulating an ISB environment.

1.1 Experiments in chemotaxis

Experimental chemotaxis studies in porous media by others have yielded conflicting results. These differing conclusions are likely to be the result of the wide range of factors involved with testing chemotaxis in porous media. The major factors in these studies include motility of bacteria, dissolved oxygen content, swimming behaviors, particle size, selection of chemoattractants and growth substrates, and growth conditions in the porous medium. When considering transport in porous media, additional factors such as capillary action, wicking action, surface gliding by bacteria (Frymier 1995), and end effects cannot be ignored. In order to isolate the sole effect of chemotaxis, these factors have to be considered systematically. While prior works have used variable combinations of these factors to draw conclusions about chemotaxis, no one work has been

able to isolate the sole effect of chemotaxis, which in the strictest sense, is biased swimming motion of bacteria.

In order to eliminate the effects of these other factors on degradation and penetration through porous media, all relevant aspects of the control system must be identical, except for the chemotactic ability of the cells. A sound control experiment is essential when comparing chemotactic and non-chemotactic behavior. A unique feature of this work is that for the first time, substrate degradation, by two highly characterized strains, is measured rigorously. Total extent of degradation is of course the most significant parameter in bioremediation projects.

1.2 Current opinion on chemotaxis and ISB

Research programs around the country have been developed based upon the hypothesis that chemotaxis can be used to enhance ISB. Several quotes demonstrate the current thinking on chemotaxis in the subsurface:

“Behavioral sensing of aromatic compounds may give cells a competitive advantage in natural environments by enabling them to locate low concentrations of compounds that can be used as growth substrates. This could be especially important in situations in which pollutants have become dispersed throughout a wide area (Harwood et al., 1990)”

“It has been suggested that chemotaxis may be one mechanism that can be exploited for increasing the overall effectiveness of biodegradation as a treatment process by facilitating contact between bacteria and the contaminant to be degraded (Barton and Ford 1995).”

“...Chemotaxis... plays a critical role in the distribution and dynamic interaction of bacterial populations associated with many microbial

processes such as... biodegradation of groundwater contaminants (Strauss, Frymier et al. 1995)."

"Currently, chemotaxis is not adequately accounted for when determining the rate of contaminant biodegradation in heterogeneous environments because of a lack of knowledge on how chemotaxis affects contaminant biodegradation and removal rates. (Law 2004)"

Experimental studies have shown that chemotaxis, the directed movement of cells towards attractants, significantly enhances transport rates of motile subsurface bacteria in an *aqueous* medium. As shown above, researchers have conjectured that this phenomenon may also enhance bacterial transport rates leading to increased contaminant degradation rates in *porous* media such as that found in subsurface environments. There have not been any definitive studies, comparing motile chemotactic strains with motile non-chemotactic, attractant-blind strains that demonstrate whether bacterial chemotaxis enhances biodegradation of hazardous compounds in the subsurface.

Prior experimental works in chemotaxis in porous media produced conflicting results. Barton and Ford (1995) were unable to demonstrate a significant effect of chemotaxis on transport of a *Pseudomonas putida* strain. Similarly, Reynolds *et al.* (1989) showed that a chemotactic strain of *Escherichia coli* penetrated packed sand cores at slower rates than a non-chemotactic mutant. Contrary to these results, two studies found that chemotaxis did affect penetration rates. The first study (Soby and Bergman 1983) concluded that chemotaxis is required for penetration of *Rhizobium meliloti* through a sandy loam. Secondly, Lopez de Victoria (1989) presented evidence that deep

subsurface bacteria, chemotactic towards trichloroethylene (TCE), penetrated a sand core at higher rates in the presence of a TCE gradient.

1.3 Seeking chemotaxis evidence

Previous studies have attempted to measure the effect of chemotaxis by determining bacterial density profiles in porous media. The errors associated with cell counting in a porous medium, however, were too large to elucidate this phenomenon. In addition, the use of non-metabolizable attractants precludes the measurement of degradation rates by changes in attractant concentration. These difficulties can be overcome by direct measurement of the degradation rates of a metabolizable attractant in two identical systems, differing primarily in the chemotactic ability of the cells.

A suitable control is needed to isolate the sole effects of chemotaxis on transport of cells in a porous medium. A highly rigorous experiment would utilize a control strain that is as similar to the experimental strain as possible, differing only in the chemotactic ability of the cells. Critical aspects of similarity include: (1) tumbling frequency; (2) growth rate; (3) swimming speed (4) percentage of motile cells in a given media; and (5) uptake rate, if metabolizable attractant is used.

While *E. coli* is generally not considered to be a biodegrader of hazardous chemicals, it is used here because of the availability of the control strain which lacks the chemoattractant chemoreceptor, yet exhibits random motility. Studying chemotaxis in *E. coli* is, however, environmentally relevant when one considers the transport of another strain of *E. coli*, the pathogenic strain O157:H7. This

strain can be transported to humans from cattle and dairy farms through subsurface aquifers and surface lakes and streams. When studying chemotaxis for ISB, one would wish to enhance chemotaxis and degradation. However, when considering the transport of pathogenic *E. coli*, one would desire to inhibit the spread of these bacteria due to chemotaxis and reduce the threat to humans. Thus the study of *E. coli* transport as developed in this work is relevant both to systems which could benefit from the enhancement of chemotaxis and systems which would benefit from the inhibition of chemotaxis.

Bacterial chemotaxis is a response to chemical gradients, and a chamber has been developed to generate a steep attractant gradient in saturated sand. The chamber uses a saturated-sand filled rectangular trough. The chamber and chemotaxis medium have been designed to maximize the chemotactic ability and motility of the cells. Additionally, a novel *migration sampling frame* (MSF) has been built to measure penetration of bacteria through the chambers under chemotactic conditions. It has been hypothesized that if chemotaxis does not have a significant effect under the optimal conditions provided in this chamber, then chemotaxis will probably not have a significant effect in the less than optimal conditions found in the subsurface.

One goal of this dissertation is to develop a more systematic approach to chemotaxis studies. An experimental methodology has been developed which will first allow for the isolation and measurement of chemotaxis effects alone. Then the experimental methods developed here can be extended for the systematic addition of other factors involved in chemotactic microbial transport in

porous media. Ultimately, the theoretical and experimental approach used here can be used to determine whether or not there exists a set of parameters which will result in an optimum condition where chemotaxis has the greatest effect on degradation rates.

The concluding results of this work were that the experimental and control strains are an excellent pair of strains for comparing chemotactic behavior with non-chemotactic behavior. However, with the parameters chosen and use of the protocols developed, the chemotactic strain did not consume more of the attractant chemical than the control strain in a 21 hour experiment. In attempt to evaluate possible enhancement of chemotaxis under growth conditions, an intriguing result showed that the stimulant caffeine may enhance migration through a swarm plate. This indicates that chemotaxis in a porous media may be able to be enhanced in a system where growth and/or chemotaxis were stimulated.

This dissertation is organized by the presentation of a background chapter, chapters stating the goals and objectives of the research, discussion of methods and materials, followed by a chapter on the characterization of the bacterial strains used in this work. Experiments in chemotaxis are detailed in the next section, followed by a discussion chapter. In Appendix E, a mathematical model simulating chemotaxis in a porous media via a FORTRAN program is discussed and included in this dissertation for reference. The mathematical model describes a system that is similar to the experiments of this work, but not identical, making comparisons between the two inexact.

2 BACKGROUND

In this chapter, the process of ISB will be introduced. The relevance of bacterial chemotaxis to ISB will be elucidated. Because these two processes occur at different length scales, it is important to understand how these two phenomena are interrelated. By carefully reviewing the literature in both of these areas, one will gain a fuller understanding of the purpose of this research.

2.1 In situ bioremediation

2.1.1 Overview

Environmental remediation processes of the contaminated subsurface can be split into two major categories: physical treatment processes and biological treatment processes. Currently there is much interest in biological treatment processes because of their potential lower cost and environmentally benign outcomes.

2.1.1.1 Physical treatment processes

Physical treatment process consists of treating contaminated soils by physically removing them or reacting them with other chemicals to make them innocuous to the environment. Four representative types of physical treatment processes are excavation, pump and treat, incineration and *in situ* vitrification. Treatment by excavation is possible only if a contaminant plume is small, and it represents a quick fix to the problem. Pump and treat is another common

approach, but is not always a perfect solution because it fails when there is a high level of contaminants adsorbed to the soil (Bouwer 1992). Also, the process is more expensive than ISB (Levin and Gealt 1993). Incineration is an expensive solution, but extremely thorough. A newer, novel physical treatment is *in situ* vitrification, which applies a strong electrical current through contaminated soil, which, after cooling, becomes a solid glassy substance, where contaminants are destroyed or permanently immobilized.

2.1.1.2 Biological treatment processes

Biological treatment processes are quickly becoming an environmentally attractive alternative to expensive physical treatment processes. There are a large number of microorganisms, fungi, and plants that can convert hazardous chemicals into harmless by-products or concentrate them into biomass (phytoremediation). Table 2-1 demonstrates that certain microorganisms can remediate fairly powerful environmental toxins. *Ex situ* processes currently in use include pump and treat coupled with surface bioreactors to degrade contaminants with microorganisms. *In situ* processes include ISB, bioventing, biostimulation, and natural attenuation. On the forefront of these up and coming biological processes is bioaugmentation by genetically engineered microorganisms. When used in conjunction with ISB, and as legal and environmental permits allow, this method holds a lot of promise.

ISB has been used successfully in several cases, particularly at Air Force

Table 2-1: Biodegrading microorganisms and their substrates.

<i>Burkholderia cepacia</i>	2,4,5-trichlorophenoxyacetic acid
<i>Pseudomonas putida</i> G7	naphthalene
<i>Phanerochaete chrysosporium</i>	2,4,6-trinitrotoluene (TNT)
<i>Ralstonia eutropha</i> JMP34(pJP4)	2,4-dichlorophenonxyacetate
<i>Methanotrophs</i>	BTEX, short chain hydrocarbons, trichloroethylene

bases where waste airplane solvents and fuels are a problem such as Moffett Naval Air Station, CA (1,1,1-trichloroethane) and Traverse Coast Guard Air Station, MI (JP-4 jet fuel). Petroleum products are also amenable to biological cleanup *in situ*, and ISB has been applied in an oil spill in the Upper Rhine Valley, Germany and also at a gasoline spill site in Long Island, NY.

Newer ISB technologies include Arcadis' method of adding molasses to the contaminant plume to add harmless nutrients to the subsurface microorganisms to promote growth of biodegrading microorganisms. Vegetable oil has also been used recently for this purpose. One final method, employed by Shaw Environmental, Inc. is to use *Dehalococcoides* strains for reductive anaerobic dechlorination of subsurface regions contaminated with chlorinated solvents.

Research in the area of biological treatment of hazardous waste continues with vigor, due to the high levels of contamination throughout the world. This work represents another step forward into this arena of using microorganisms to degrade difficult to remediate areas of the subsurface.

2.1.1.3 Cost benefits

If one considers costs, bioremediation continues to be attractive, at a cost of \$50 to \$150 per ton of soil. Excavation and subsequent land disposal is more expensive at \$200 to \$300 per ton, while incineration can be cost prohibitive at \$300 to \$1000 per ton (Levin and Gealt 1993). Thus, bioremediation continues to be a viable economic alternative to physical treatment processes.

2.2 Bacterial motility and chemotaxis

2.2.1 Bacterial motility

Bacteria have several means of locomotion, but the type of bacterial motion undergone by *E. coli*, the type that is critical to this work, is locomotion via 8-10 peritrichously arranged flagella (Figure 2-1). These flagella, which are helical in shape and anchored to the cell wall via a unique, biochemical *motor*, coalesce in a bundle when the motor spins counter-clockwise and propel the bacteria forward. This motion is called *swimming* or a *run*. For about a tenth of a second (for *E. coli* bacteria), the motor reverses direction causing the flagella to separate and the cell to tumble in place. The cell then re-orient itself to move in a new direction (see Figure 2-1). This motion is called *tumbling*. If chemotaxis is occurring, the cell typically extends its run in the direction of the chemical attractant. In this study, the term *wild-type* swimming motion is used to describe a chemotactic bacterium (*E. coli* RP437) that exhibits chemotactic behavior if there is a gradient of attractant present, but otherwise exhibits random motility.

2.2.2 Chemotactic behavior

Over the last several years, investigators have established a vocabulary to describe different aspects of the chemotaxis process. However, this vocabulary has not been standardized, particularly when describing non-chemotactic behavior and conditions. In this section, a review of this vocabulary is presented, and confusion about non-chemotactic behavior and its nomenclature is addressed.

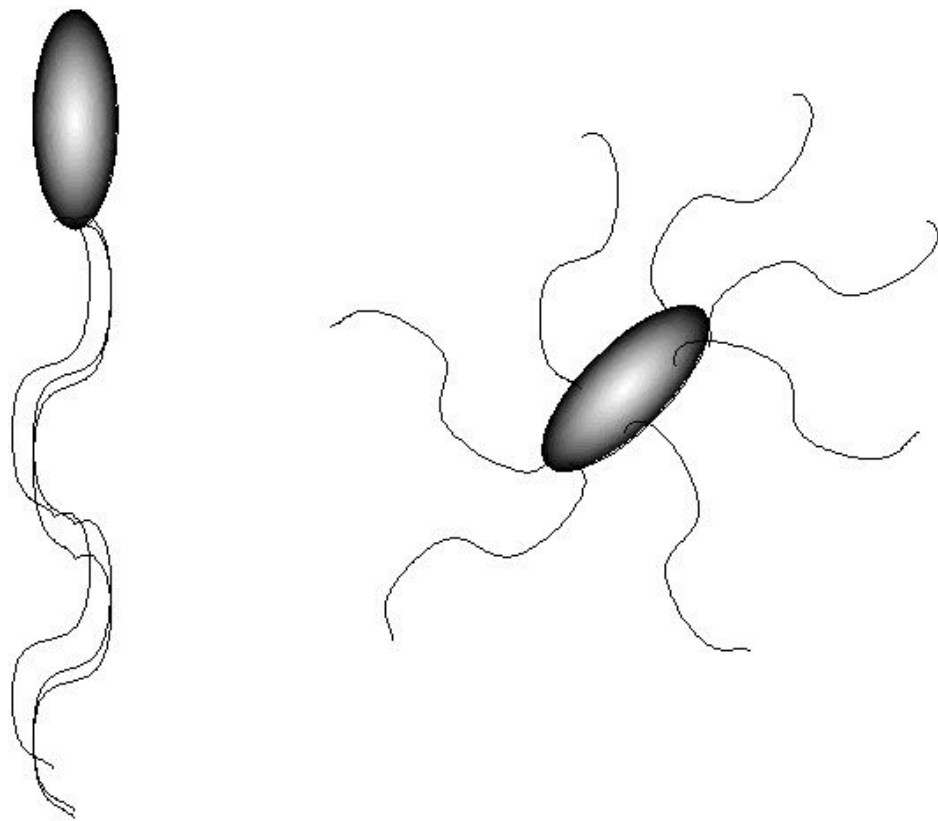


Figure 2-1: Diagram showing the coalesced bundle of flagella driving this bacterium forward. On the right is a tumbling bacterium, with peritrichous flagella splayed in multiple directions.

Chemotaxis is a naturally occurring phenomenon among biological organisms that can be defined as motion toward or away from chemical compounds. The phenomenon occurs in a wide range of mobile organisms, from bacteria to sperm to white blood cells. The chemical compound that affects the organism's motion is called the *chemoeffector*. Chemicals that attract organisms are called *chemoattractants*, and chemicals that repel organisms are called *chemorepellents*. If an organism exhibits chemotaxis in response to a chemical, the organism is said to exhibit a *chemotactic response*. If an organism does not exhibit chemotaxis in response to a chemical due to the properties of the organism or its environment, it is said to be *non-chemotactic*. A bacterium is said to be *specifically non-chemotactic* if it is missing the chemoreceptor for a particular attractant chemical (Adler 1976). If the strain has a damaged chemotactic mechanism and cannot respond chemotactically to anything, it is said to be *generally non-chemotactic* (Hazelbauer, Mesibov et al. 1969).

The chemotactic response is mediated by a *chemoreceptor* (Figure 2-2), which is a transmembrane protein consisting of a periplasmic sensing domain and a cytoplasmic signaling domain (Murphy, Kovacs et al. 2001; Pandey and Jain 2002). When a chemoattractant binds to the chemoreceptor in the periplasmic space, a signal is initialized that is transmitted through the chemoreceptor to the cytoplasm. In the cytoplasm, shown in Figure 2-3, a methylation cascade, initiated by the cellular proteins CheR and CheB alternately methylate and demethylate the cytoplasmic domain of the chemoreceptor (Manson and Segall 1992; Brock, Madigan et al. 1994). If the chemoreceptor is

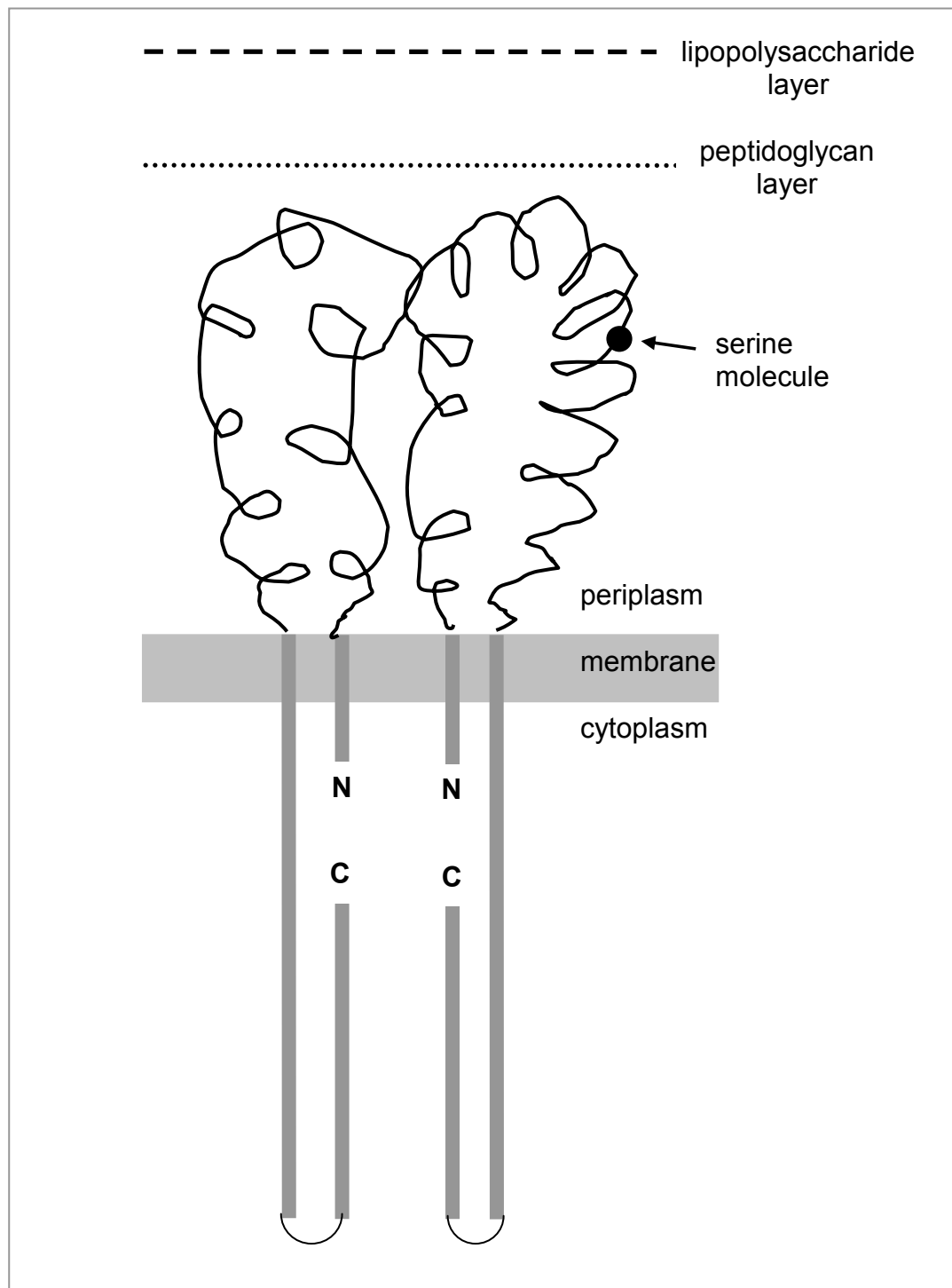


Figure 2-2: Diagram of serine bound to chemoreceptor bridging the cell membrane in *E. coli*. Adapted from Murphy *et al.*(2001).

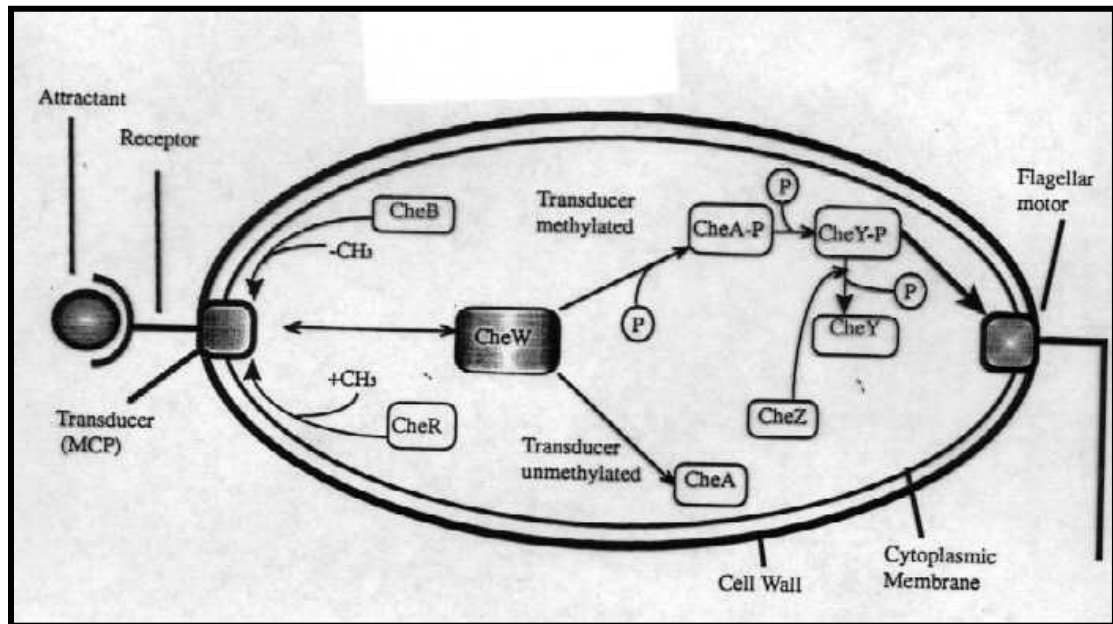


Figure 2-3: Diagram of flagellated bacterium illustrating phosphorylation cascade from receptor to flagellum. Adapted from Brock, *et al.* (1994)

methyated, the bacterium undergoes runs and if demethyated, it undergoes tumbles. Before the signal to change from running to tumbling reaches the flagellar motor, other cellular proteins are phosphorylated and dephosphorylated in the signal transduction reaction (CheW, CheA, and CheY, see Figure 2-3). The phosphorylation state of the flagellar motor determines whether the cell is swimming (counterclockwise rotation) or tumbling (clockwise rotation). In opposite fashion to methylation, phosphorylation of the flagellar motor results in tumbles, rather than runs which are controlled by methylation of the chemoreceptor as described above.

There are certain levels of chemoattractant that elicit a response from a cell behaving chemotactically. If the chemoattractant levels are too high, the chemoreceptor becomes saturated, and the chemotactic response is reduced, as shown by the capillary experiments of this work (see Chapter 7).

As described in a previous section, many types of bacteria such as *E. coli* swim in alternating patterns of *running* and *tumbling*. Running refers to motion in a straight or slightly curved line, while tumbling refers to a short period of spinning in place, after which the bacterium resumes a run in a new direction. The length of a run traveled by a chemotactic bacterium depends upon the concentration of chemoattractant. A concentration *gradient* is said to exist whenever the concentration of chemoattractant in the bacterium's local environment is greater in one direction over the other directions. If there is an increasing attractant gradient sensed during a run, a chemotactic bacterium will continue in that direction for a longer time than if there were no gradient. The

bacterium will continue to tumble occasionally, but the net result of the longer runs moves the bacterium closer to the attractant. In statistical terms, there is a greater distribution of long run lengths in the direction of the chemoattractant. The resulting path of this alternating tumbling and running can be described as *biased random motion*.

One possible explanation why chemotactic behavior has evolved is that bacteria that are genetically predisposed to chemotaxis towards nutrients theoretically have a greater chance for survival. Certain chemicals that attract bacteria are actually nutrients that are essential for the bacteria's survival, and chemotaxis towards these nutrients aids in the growth of the strain. Bacteria that do not exhibit chemotaxis towards nutrients may have a lower chance for survival because they cannot sense where to move to obtain more nutrients.

2.2.3 Non-chemotactic behavior

There are several reasons why bacteria are *non-chemotactic*. For example, if a bacterial strain cannot sense an attractant due to a faulty sensory mechanism or a missing chemoreceptor, it cannot exhibit chemotaxis toward the attractant, and its behavior is described as non-chemotactic.

The term *non-chemotactic* can be used to describe different types of bacterial behavior. The term *non-chemotactic behavior* has been used to imply random motion in the presence of an attractant gradient. Figure 2-4 compares a bacterium swimming in a random pattern with a bacterium exhibiting chemotaxis. The implication is that when bacteria are behaving non-chemotactically,

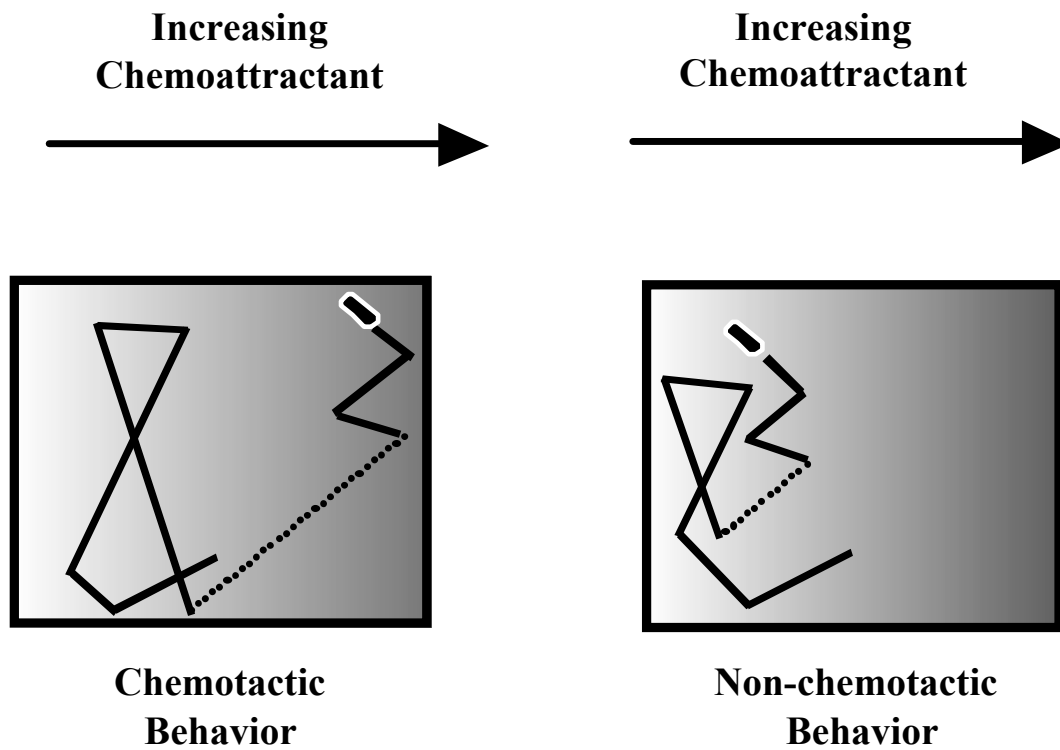


Figure 2-4: Comparison of chemotactic with non-chemotactic (randomly motile) swimming behaviors. The gray regions indicate areas containing attractant.

they do not favor one direction over any of the others. They simply swim about with no regard for the attractant gradient. In statistical terms, the distribution of run lengths in all directions is approximately equal.

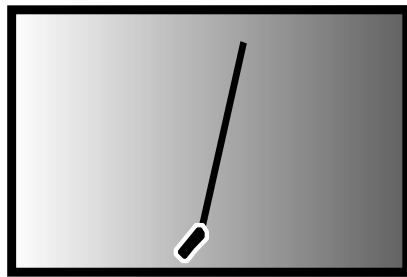
There are several different and distinct conditions that can result in bacteria behaving non-chemotactically. Due to these different conditions, it is critical that a discussion of this behavior includes a description of the swimming motion, the gradient conditions, and sensory response of the bacteria. No one aspect of the bacterial system sufficiently describes non-chemotactic behavior. A description of some of the different cases of non-chemotactic behavior follows:

- A trivial case of non-chemotactic behavior is that the bacteria are non-motile because they are oxygen deprived (oxygen is required for aerobically motile strains) or lack flagella for motility. Because they do not move, they cannot exhibit a chemotactic response.
- Smooth swimmers are non-chemotactic, since they swim in a straight line without regard to attractant gradients.
- Tumbly swimmers are non-chemotactic because they do not make runs at all.
- A chemotactic bacterium that is swimming in a region without a gradient (constant spatial distribution of attractant) can also be said to exhibit non-chemotactic behavior. Because there is no gradient, chemotaxis does not occur.
- Attractant-blind bacteria are non-chemotactic. This type of bacteria cannot sense the attractant and does not alter its run length response to attractant gradients.

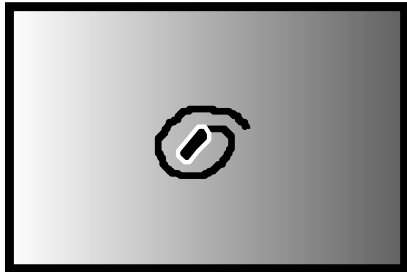
As it has been shown, merely calling a bacterium non-chemotactic is insufficient to properly define the experimental system of interest. In much of the literature, any cells with non-wild type tumbling frequencies were called non-chemotactic, when the cells may actually have a chemotactic mechanism.

2.2.4 Tumbling frequency

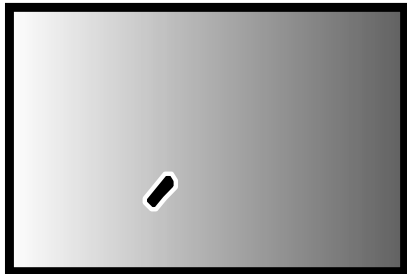
Several different types of bacterial swimming behaviors have been identified. Figure 2-5 shows bacteria in a fluid medium with a chemoattractant gradient. Four types of non-chemotactic behavior are shown. These types of behaviors can be distinguished by considering the tumbling frequency of each case, that is, the number of tumbles a bacterium performs as compared to the number of runs it performs in a given period of time. The first case shown is a *straight-line* swimming bacterium. Straight-line swimming behavior is also called *smooth* swimming behavior. A smooth swimming bacterium has a tumbling frequency of zero, because it swims in only one direction without tumbling. The second case shown is a *continuously tumbling* swimming bacterium. This bacterium tumbles constantly, so while mathematically defining a tumbling frequency is not logical, it could be said that the frequency is essentially infinite. The third case shown is a non-motile bacterium, and it is another example of zero tumbling frequency. There are several reasons why a bacterium may be non-motile. It may lack energy sources, structures for locomotion, or it may simply be dead. Thus it is important to differentiate between cell motility and cell viability. The fourth case shown is an *attractant-blind* bacterium. Standard microbiological screening techniques along with genetic engineering have been applied to create strains which lack the chemotaxis receptors for a given attractant (Callahan, 1987) so that the strains lack the ability to sense the



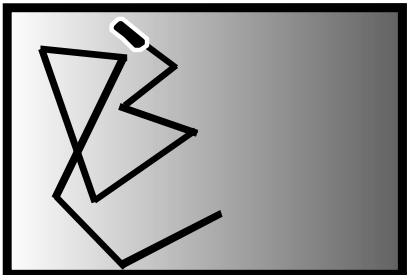
Straight line swimming:
zero tumbling frequency.



Continuous tumbling:
very high/infinite tumbling frequency.



Non-motile: describes cells lacking locomotion apparatus and dead cells.



Attractant-blind: wild-type tumbling frequency. Truly non-chemotactic. Used as control in this work.

Figure 2-5: Comparison of non-chemotactic swimming behaviors. The gray regions indicate areas containing attractant.

attractant and move towards it. These bacteria exhibit completely random motion in the chemoattractant gradient because the signal transduction system for run and tumble behavior remains intact.

An attractant-blind bacterium is used as a control in the experimental system of this work and is compared with a chemotactic bacterium. The run lengths of the chemotactic bacterium are longer in the direction of the chemoattractant, resulting in biased random motion, as shown in the dashed line in Figure 2-4. This motion results in a relocation of the bacterium into regions of higher attractant concentration.

In Figure 2-4 it is interesting to note that the tumbling frequencies in the presence or absence of a chemoattractant gradient are more similar to each other when compared to the cases shown in Figure 2-5. That is, in the time period shown in Figure 2-4, both cells tumbled eight times. The chemotactic bacterium moved a bit further towards the attractant due to the extension of one of the run lengths in the direction of the attractant. If these runs took two seconds, the frequency would be four tumbles per second. If these runs for the non-chemotactic strain took one second, its tumbling frequency would be eight tumbles per second. Thus, the tumbling frequency was higher for the non-chemotactic strain, and the bacterium did not move as far.

Lower tumbling frequencies under chemotactic conditions are a key feature of the experimental system of this work. Prior studies have compared a chemotactic bacterial strain with smooth swimmers, tumbly swimmers, or possibly non-motile, energy deprived bacteria. The tumbling frequencies of the

control strains were different from the chemotactic strains. An attractant blind bacterium with a tumbling frequency similar to a chemotactic bacterium makes a good choice for a control strain, because it allows the effects of biased, chemotactic motion to be compared with purely random motion.

2.2.5 Environmental relevance of chemotaxis

Because bacterial chemotaxis occurs in wild-type bacteria, it has been conjectured to occur in natural subsurface environments. Chemicals hazardous to humans can be found in some subsurface environments, particularly in those around gasoline stations, chemical plants, and airfields. There is considerable uncertainty as to whether or not bacterial chemotaxis plays a significant role in affecting the rates at which these hazardous chemicals are degraded in the subsurface. This question has been addressed in this work by using a model experimental system to simulate subsurface conditions. The attractant-blind type of non-chemotactic behavior described above is the major focus of this work, and has been compared with a bacterial strain exhibiting chemotactic behavior.

2.2.6 Why do chemical engineers care about chemotaxis?

Chemical engineers study and apply theories of transport phenomena. Chemical engineering is the study of the transport of mass, momentum, heat and the chemical reactions that affect these transport processes. There are three primary reasons why the expertise of chemical engineering is applicable to the

study of microbial transport and microbial decontamination of chemicals in the subsurface:

1. Random motility and chemotaxis are mathematical analogues of molecular diffusion.
2. Cells are essentially microscopic bioreactors for degradation processes, and theories of chemical reactor analysis apply to these miniature biochemical powerhouses.
3. Chemical engineers study how to control reactions, and it may be possible to use chemotaxis as a control mechanism for these reactive processes, i.e. via enhancement of transport in some cases and inhibition in others.

For these reasons, chemical engineers are firmly established in the study of chemotaxis in subsurface bacteria.

2.3 Reviews on chemotaxis

Chemotaxis in bacteria has been reviewed several times since the 1970s. Classic reviews were written by two key investigators in this field, H. Berg and J. Adler (Berg 1975; Adler 1976). A critical review of ecological impacts of chemotaxis was written in 1976 (Chet and Mitchell 1976), and environmental aspects of chemotaxis towards hazardous compounds were reviewed more recently (Parales and Harwood 2002). Reviews on the biochemical aspects of the chemotaxis process have also been written (Stock, Lukat et al. 1991; Parkinson 1993).

2.4 Microbial transport in liquid media

In the 1800s, W. Pfeffer first identified and coined the term chemotaxis (Pandey and Jain 2002). He first observed a formation of chemotactic cloud of bacteria in a fluid-filled test tube. In this section, transport and chemotaxis of motile bacteria in fluid is explored for comparison and contrast with experiments that have been performed in fluid in which a porous medium was added, such as sand or glass beads. These liquid media experiments were preliminary to the study of chemotaxis in porous media and have served as a basis for the experiments performed in this work.

2.4.1 Capillary methods

For many years, bacterial chemotaxis has been observed by filling a small capillary tube with an attractant chemical. Formation of bands of bacteria is observed with the naked eye, a microscope, or a densitometer, which quantifies the amount of cells that accumulate in regions of the capillary containing chemoattractants.

A novel method for submerging one end of a capillary in a reservoir was developed in the 1960's (Adler 1966). Figure 2-6 illustrates this method. A capillary is inserted into a small pool of cell suspension contained on a microscope slide and beneath a microscope coverslip. Surface tension contains the fluid underneath the microscope slide on the inside of the U-tube. A capillary containing an attractant chemical is inserted into the pool, and cells are allowed

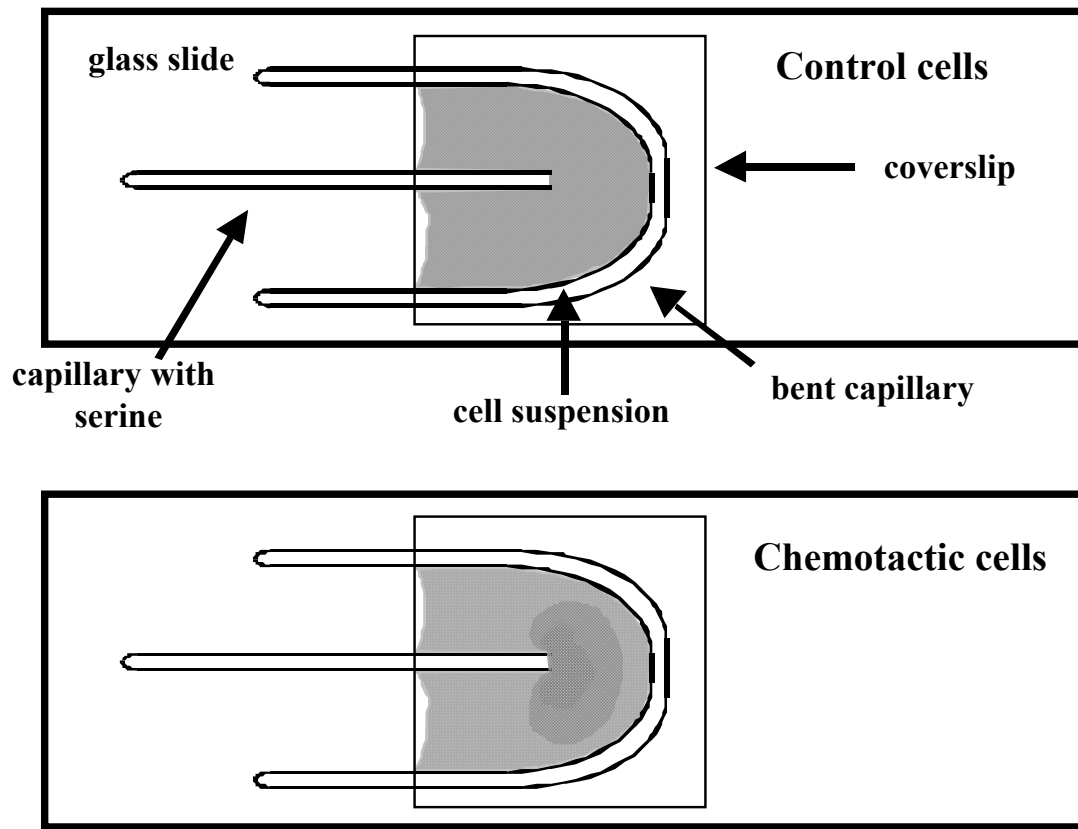


Figure 2-6: Classic capillary method of Adler for testing chemotaxis. The upper microscope slide shown contains the control cells, which are not attracted to the capillary. The lower slide shown contains chemotactic cells, which are attracted to and subsequently swim up the capillary.

to migrate into the tube. Emptying of the capillary tube onto plates allows for the enumeration of cells migrated for different conditions and attractant chemicals. This experiment has been used and modified several times in chemotaxis work (Mazumder, Phelps et al. 1999). The method is introduced here since it relates to microbial transport study in liquid media and is described in more detail in Section 7.3 where it is used as an assay this work.

2.4.2 Stopped flow diffusion chamber

It is important to understand this next apparatus in detail, since the concept, scale, dimensions and methodology of this apparatus were used in the design of the apparatus of this work.

An apparatus and procedures for measuring bacterial density profiles in aqueous media has been developed and used to gather data on parameters relative to bacterial chemotaxis (Ford, Phillips et al. 1991). This apparatus, called the stopped-flow diffusion chamber (SFDC), provides a reproducible method of measuring chemotaxis effects in aqueous media. An excellent example of the use of the SFDC to study chemotaxis is given by Barton (1994). Barton used the system to characterize the behavior of the chemotactic strain *Pseudomonas putida* PRS2000 and the non-chemotactic *P. putida* strain, PRS2000 NTG.

Figure 2-7 shows a top view of the experimental apparatus. The apparatus used consists of two microscope slides aligned with a small gap between them and sealed to prevent leakage of the media. The slides are illuminated from below to allow visualization and capturing of the slide image. The slides are

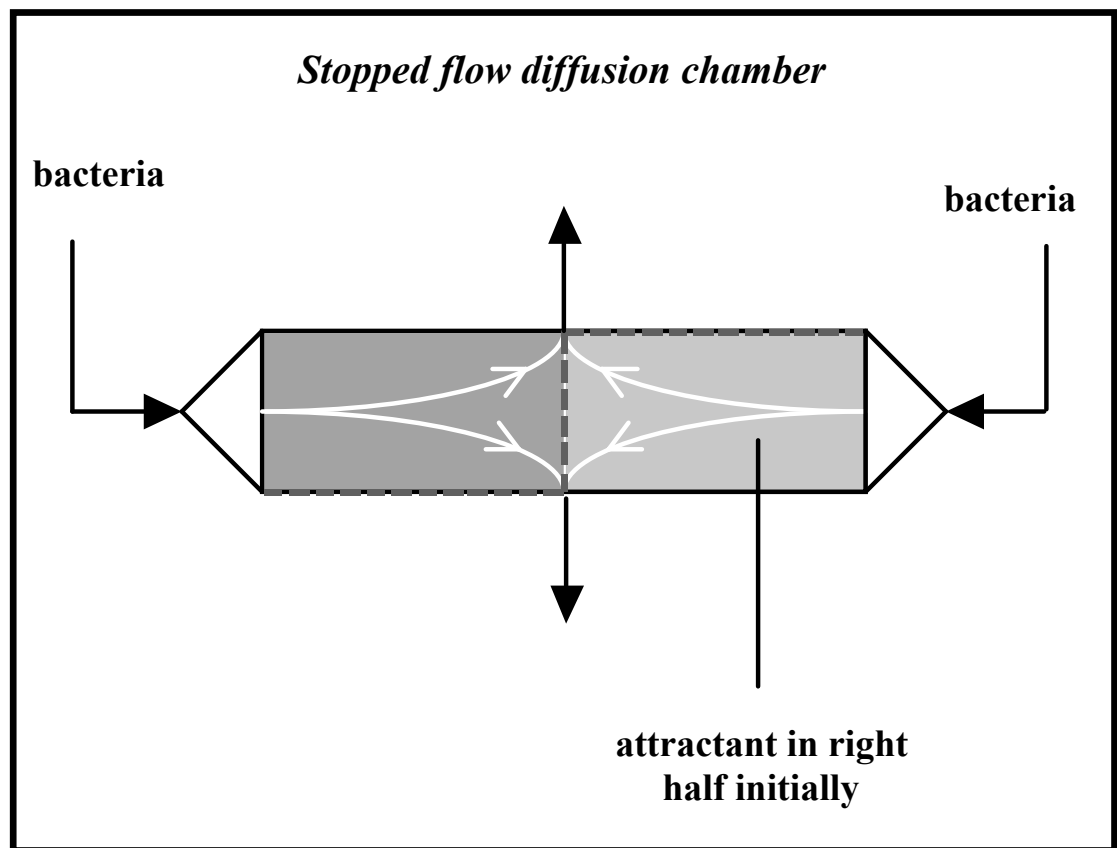


Figure 2-7: Stopped flow diffusion chamber. The white flow lines illustrate the impinging flow of the fluid. In this figure, the attractant is illustrated by the light grey area. Adapted from Barton (1994).

represented by the gray rectangle in Figure 2-7. Syringe pumps are connected to the left and right ends of the slides, through which chemoattractants and bacteria are introduced to the chamber. Before entering the chamber, the fluids flow through triangular regions filled with nylon beads. The beads help to disperse the fluid and any momentum associated with the flow, so that reproducible, well defined gradient conditions can be created. The step gradient is created by the impinging flow, shown by the curved lines in Figure 2-7, that results from the flow configuration of the chamber. The attractants and bacteria are pumped into the right side of the chamber shown in Figure 2-7, through the apparatus, and out through ports on the top and bottom of the slides. Bacteria alone are pumped into the left hand side, at the same flow rate as the inputs to the right side. The grey dashed line in Figure 2-7 shows the attractant concentration profile at the start of the experiment. The impinging flow resulting from this configuration allows a sharp gradient to be formed between the left and right side fluids. This sharp profile becomes more curved as the attractant diffuses during the course of the experiment.

The sampling procedure consists of digitally capturing images of the light scattering profile of the chamber, and subsequently analyzing the light intensity of the image. The light intensity is then calibrated with bacterial density, and density profiles can be obtained. From these density profiles, mathematical parameters relating to chemotaxis and bacterial diffusion can be calculated, such as the random motility coefficient (μ_0) and the chemotactic sensitivity coefficient (χ_0).

The results of the SFDC analysis yield a bacterial density profile similar to the one shown in Figure 2-8. Shown here is an image from an experiment where there is initially a uniform distribution of bacteria ($t=0$). The attractant is located in the left half only. Over time, the bacteria migrate towards the attractant, causing a region of higher density immediately to the left of the impingement point (the center on this image), and forming a white band in the chamber. The migration of bacteria toward the attractant leaves a depletion zone immediately to the right, forming a dark band. The bands shown in Figure 2-8 represent only a few millimeters in length. As will be shown later, it is this very small length scale which makes measurements of profiles in porous media difficult.

SFDC analysis has not been shown to be effective in the visualization of the profiles when a porous media is added to the chamber. The difficulty is that any porous medium tends to cause background light-scattering noise, which obscures the light scattering caused by the bacterial migration. Other labs, such as R. Ford's group at the University of Virginia, are currently attempting to overcome this difficulty by modifying the SFDC chamber to include a porous medium that will not affect the light scattering.

2.5 Microbial transport in porous media

Since ISB is a process occurring in porous sands and soils, the focus turns to a review of major experimental works investigating chemotaxis in porous media. The relationship between ISB and chemotaxis will be discussed, and the experimental study of this work will be introduced.

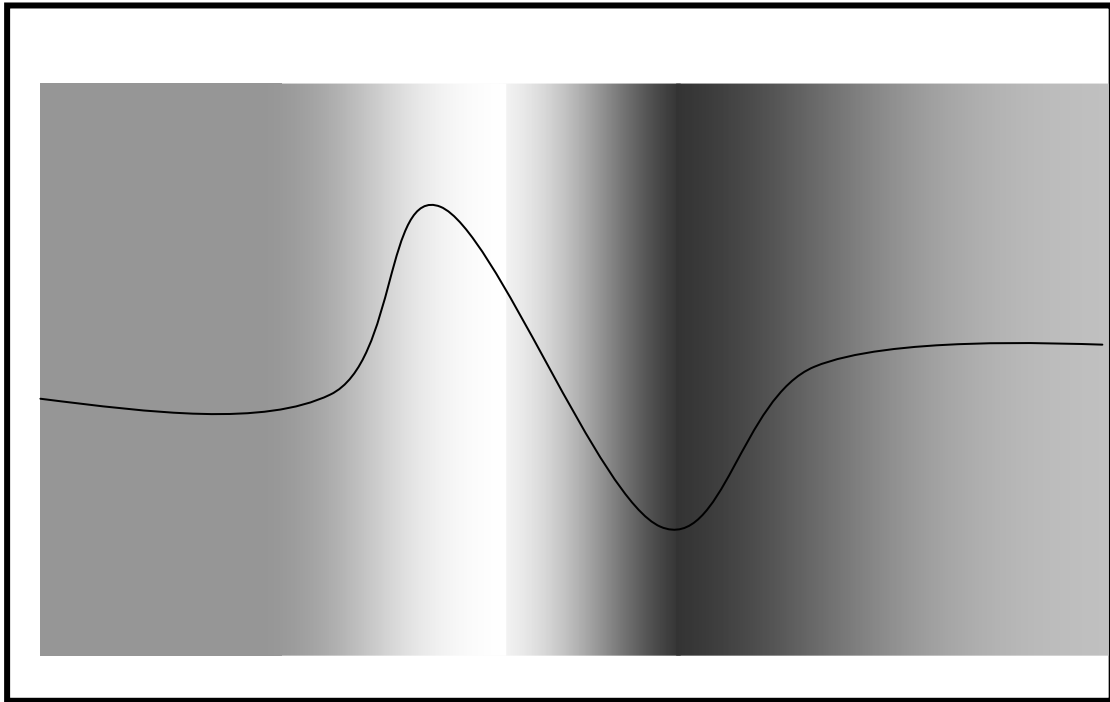


Figure 2-8: A simulated bacterial density profile obtained from the SFDC. The black line represents the cell density data at each point along the image. The white area represents the cells, and the black area represents the depletion zone. Adapted from Barton (1994).

Few works on chemotaxis in porous media have definitively demonstrated that chemotactic bacteria penetrate a porous media at faster rates than non-chemotactic bacteria when exposed to an attractant gradient. An early work on transport of bacteria through a model aquifer conjectured that chemotaxis towards di- and tri- chlorobenzenes (Van-der-Meer, Roelofsen et al. 1987) was influential on penetration rates of *Pseudomonas* P51 through their model aquifer. This work sparked interest in chemotaxis as a phenomenon that could enhance ISB.

A fundamental premise of most prior work has been the measurement of bacterial density profiles, which are the number of colony forming units (CFU) as a function of a characteristic length of the experimental system. It is theorized that as bacteria move chemotactically through a medium, either liquid or porous, they form a front of high bacterial density. This motion results in a depletion zone behind the high density front. In the SFDC, this can be seen as a white band of bacteria (Barton 1994). Several investigators (Lopez-de-Victoria 1989; Sharma, McInerney et al. 1993; Barton 1994; Kunkler 1994) attempted to sample this band of bacteria that develops as the bacteria move through the porous media. The band is often sampled by a slicing procedure that can mix the porous media, giving errors in bacterial counts. Samples have also been taken by withdrawing fluid from the interstices of the porous media (Reynolds, Sharma et al. 1989). However, the band is easily disturbed by the sampling procedure because it may only be a millimeter or so in length. These disturbances, coupled with the errors

inherent in bacterial counting procedures, may have resulted in obscuring any effects of chemotaxis on microbial transport rates.

Along with experimental methodology, the oxygen content of the experimental system is another important factor in motility and chemotaxis studies in porous media. Oxygen provides energy for motility indirectly via the proton motive force (Brock, Madigan et al. 1994). The proton motive force is increased via electron transport through the electron transport system (Rebbapragada, Johnson et al. 1997). The increase in proton motive force thus increases the energy available for motility. Prior studies made no specific provisions for oxygen transport, so it is possible that the bacteria were lacking energy for motility. If the bacteria were non-motile, then the experiments were not testing truly chemotactic bacteria, but bacteria which were transported by diffusion alone. This current work utilizes an aerobically prepared chamber that allows the system to maintain aerobic conditions via an open-air design.

Table 2-2 summarizes several important previous studies of bacterial chemotaxis in porous media. The table compares two important variables used in prior studies, and compares them with the study described in this work. The *Attractant* column lists the chemoattractants used in the studies. It is desirable to use an environmentally relevant contaminant for chemotaxis studies for bioremediation, to simulate the contaminated subsurface as closely as possible. However, chemotaxis mutants that respond to environmental contaminants are scarce. As shown in the Table, Barton (1994) used 3-chlorobenzoate, a model compound for chlorinated aromatic hydrocarbon pollutants, in his chemotaxis

Table 2-2: Summary of prior work on chemotaxis in porous media.

<i>Investigators</i>	<i>Attractant</i>	<i>Attractant utilized</i>	<i>Chemotaxis Evidence</i>
Reynolds <i>et al.</i> (1989)	galactose	no	Distal MPN measurement
Lopez de Victoria (1989)	TCE	no	Bacterial density profiles
Sharma <i>et al.</i> (1993)	galactose-peptone	yes	Distal MPN measurement
Kunkler (1994)	benzoate	yes	MPN measurement
Barton and Ford (1994)	3-chlorobenzoate	in some cases	Bacterial density profiles
Marx (2000)	naphthalene	yes	Capillary enumeration
This work	serine	yes	Substrate degradation

studies. Lopez de Victoria (1989) utilized a relevant environmental contaminant from degreasing and dry cleaning operations, trichloroethylene (TCE). Other studies used attractants that are not contaminants such as galactose, and this study utilizes the benign amino acid serine. The *Attractant utilized* column in the table shows which cases utilized a chemoattractant that could be degraded by the bacteria chosen in the study.

While prior studies did not measure substrate degradation, this current work measures substrate degradation as an indicator of the extent of migration into an attractant region. This substrate utilization based assay complements the need to measure bacterial density profiles, which have large error bars associated with their determination, as explained above. The following sections provide details of the prior works on chemotaxis in porous media which have been outlined in Table 2-2. Materials and methods are outlined briefly, as are important results. Emphasis is placed on technique for these pages, since the fine scale of this work requires a refined, reproducible sampling method. Commentary is also provided on the how the results relate to the current work.

2.5.1 Reynolds *et al.*

Reynolds *et al.* (1989) studied the penetration of *E. coli* through anaerobic, nutrient-saturated sand packed cores. In this work, the penetration rates of chemotactic strains and non-chemotactic strains through porous media were compared. The chemotactic bacteria used were *E. coli* RP437 and RP487. Strain RP487 exhibits chemotaxis towards galactose but does not degrade it. This

strain was utilized in some of the experiments to eliminate the creation of gradients by substrate uptake into the cells. Three types of non-chemotactic mutants were used in this study:

- non-motile *E. coli* lacking flagella (RP2912)
- smooth swimming *E. coli* (RP5232)
- tumbling swimming *E. coli* (RP1616)

In addition to galactose, the chemoattractants peptone and methionine were also used in the study. The porous media was a commercially available sand of a particle size ranging from 75 to 150 μm . The experimental apparatus consisted of specially modified test tubes, 8 to 10 cm in length, with septa and caps at both ends. Figure 2-9a shows a schematic of the sand core. *E. coli* were introduced into the test tubes, which were packed with sand into one end of the test tube, the proximal end, by insertion of a 22-gauge syringe saturated with anaerobically prepared liquid media. Bacteria were introduced via a syringe containing bacteria. Care was taken during the insertion process so that air pockets were not created. The bacteria were then allowed to migrate into the sand core, and samples were taken at the opposite end of the core, the distal end, at appropriate time intervals.

The oxygen conditions were anaerobic in the columns used for the study. The gradient conditions for the chemotaxis studies were created by altering the composition of galactose, peptone, and methionine in the distal syringe and the sand core. For example, in one of the studies using RP487, the sand core contained liquid media lacking in galactose, peptone, and methionine, while the

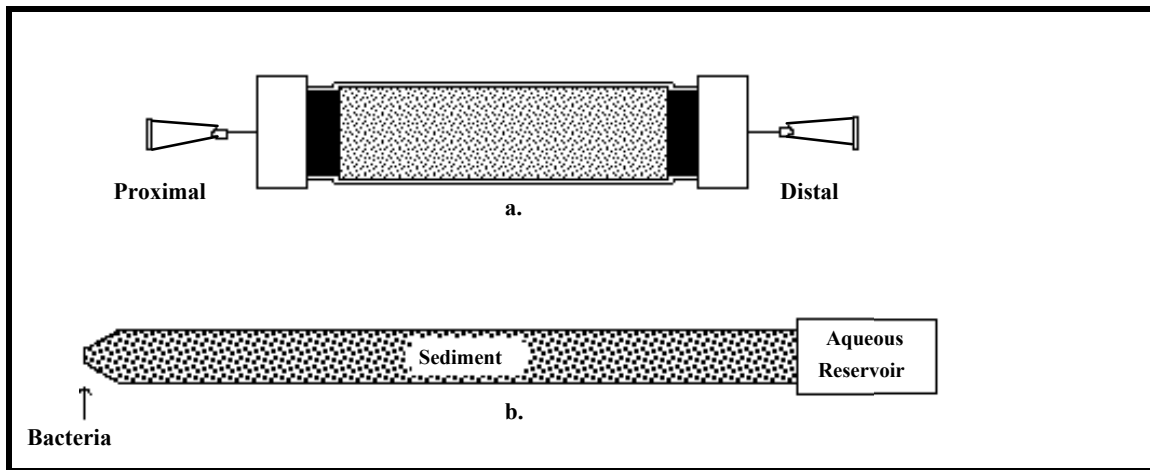


Figure 2-9: a. Core system used by Reynolds *et al.* (1989) and Sharma *et al.* (1993). b. Core system used by Lopez de Victoria (1989). Adapted from Barton (1995)

distal syringe contained media with all of these components. The complete configuration consisted of bacteria at the proximal end, a core of sand containing no chemoattractant, and a syringe at the distal end containing chemoattractant. The gradient that was formed was a type of step gradient, with attractant localized only in the distal syringe.

The sampling procedure consisted of careful withdrawal and re-insertion of the distal syringe at appropriate time intervals. Samples from the distal syringe were inoculated into sterile tubes containing growth media and incubated at 35°C. The tubes were examined to determine if growth had occurred after 48 hours. Penetration rates, shown in Table 2-3, were calculated by measuring the time required for viable cells to reach the distal syringe and dividing by the length of the core.

The results of these chemotaxis studies demonstrated that RP487 penetration rates were independent of attractant (galactose) concentration in the distal syringe. Statistically similar bacterial penetration rates, which were essentially zero, were obtained both for cases where RP487 was introduced into cores with a distal syringe containing galactose and for cases where the distal syringe contained no galactose. From this result, Reynolds *et al.* concluded that chemotaxis may not be required for bacterial penetration through porous media. Barton (1994) pointed out that in this particular case, although galactose was not present in the sand core, peptone was present and may have served as a growth substrate. He hypothesized that because there was sufficient peptone to support bacterial growth, metabolites may have been produced that can induce negative

Table 2-3: Comparison of specific growth rates and penetration rates for strains used in Reynolds *et al.* study.

<i>E. coli strain</i>	<i>Specific growth rate (h⁻¹)</i>	<i>Penetration rate with galactose (cm/h)</i>
RP437 - chemotactic	0.27 ± 0.020	0.073 ± 0.03
RP2912 - non-motile	0.37 ± 0.037	0.086 ± 0.02
RP5232 - smooth swimmer	0.5 ± 0.030	0.28 ± 0.02
RP1616 - tumbling swimmer	0.35 ± 0.026	0.16 ± 0.007
Data from Reynolds <i>et al.</i> (1989)		

chemotactic responses. One other explanation for the lack of penetration by RP487 was that it is possible the concentration of galactose in the proximal end may have been too low to elicit a chemotactic response from the bacteria. Because Reynolds *et al.* did not publish studies on the amount of galactose that penetrated the sand core from the distal end to the proximal end, it is difficult to show with confidence that the bacteria were actually exhibiting chemotaxis.

The data of Reynolds *et al.* also showed that the non-chemotactic *E. coli* mutants RP1616, RP5232, and RP2912 penetrated at faster rates than the chemotactic strain RP437. The reason given for this result is that the chemotactic strains sense a nutrient rich environment in all directions and thus do not grow throughout the entire volume of the core, concentrating growth and motility only in one section before moving to the next section. The non-chemotactic strains move without regard to the attractant gradient, and are thus able to swim freely and penetrate the core at a faster rate. Another explanation given by Reynolds *et al.* points out the differences in specific growth rates of the chemotactic and non-chemotactic strains. Table 2-3 shows the specific growth rates of the strains used in the study along with the penetration rates. As the table shows, the specific growth rates of the non-chemotactic strains are higher than the specific growth rate of the chemotactic strains. The penetration rates of the non-chemotactic strains are also higher than the chemotactic strain. The data suggest that both motility and growth rate are important factors in subsurface transport of bacteria. However, because no additional oxygen was added to the cores after the initial

charging of the tubes with bacteria, it is uncertain whether or not the bacteria maintained motility in the cores after they were sealed from ambient air.

2.5.2 Lopez de Victoria

Lopez de Victoria (1989) studied the penetration rates of several deep subsurface bacteria through sand cores using an experimental apparatus similar to Reynolds *et al.* (1989). The chemotactic bacteria used in the study were subsurface co-metabolic TCE degraders. The non-chemotactic bacteria used were a non-identified species referred to as strain C0464. Barton (1994) showed that this strain exhibited no chemotaxis response towards TCE. TCE was not degraded in the studies because no additional carbon source was added. The porous medium was a loose-pack Middendorf sediment. The experimental apparatus consisted of a 30 cm long glass column fitted with an aqueous water column on one end. A schematic of the apparatus is shown in Figure 2-9b. The gradient conditions were created by this water column, which provided a constant source of dissolved TCE via diffusion. Penetration rates were compared in cases both with and without a TCE attractant gradient. The sampling procedure was similar to that of Reynolds *et al.*, except that cores were cut into sections in the cases where no bacteria were detected at the distal end after 9 days. The results of this study demonstrated that an enhancement of the penetration rate occurred in the presence of TCE gradients. However, penetration rates were two orders of magnitude larger than the *E. coli* penetration rates measured by Reynolds *et al.*, and are similar to the rates measured by flagellar bacteria in pure aqueous

media. Barton (1994) suggested that this intriguing result may be due to bacterial migration along a fissure in the porous matrix or undetectable convective currents. The existence of either of these anomalies would possibly amplify the enhancement of penetration rate due to chemotactic effects, because Lopez de Victoria may have had a partial aqueous phase in her apparatus.

2.5.3 Sharma et al.

A study similar to the work of Reynolds et al. (1989) was carried out using the same apparatus and technique (Sharma, McInerney et al. 1993). Sharma *et al.* studied the penetration of *E. coli* through anaerobic, nutrient-saturated sand packed cores using an experimental apparatus similar to Reynolds *et al.* (1989). The chemotactic bacteria that was used was *E. coli* RP437. The non-chemotactic bacteria used in the study were straight-line swimming *E. coli* RP5232 and also the non-motile strain RP2912. The attractant chosen for the study was a galactose-peptone mixture. The porous medium was a fine Ottawa sand. The experimental apparatus was similar to that of Reynolds *et al.*, shown in Figure 2-9a, and the sand cores were 10 cm in length. The oxygen conditions were anaerobic in the columns used for the study. The gradient was created only by consumption of substrate by bacteria as they moved through the sand cores. The sampling procedure was also similar to that of Reynolds *et al.*, which consisted of withdrawing samples at the distal end with a syringe. Sharma *et al.* also sectioned the column into 2 cm increments and measured bacterial density profiles.

The results of this study demonstrated that the non-chemotactic bacteria used in the study, the straight-line swimming RP5232, migrated through the column faster and in a more diffuse manner than the wild type swimming RP437. The faster migration of the straight-line swimmer was attributed to differences in its mode of growth in the porous media over the chemotactic strain. The non-motile strain RP2912 also penetrated the cores, and it was suggested that the mechanism was actually a physical displacement of bacteria along the length of the core, caused by high cell density in the proximal direction. These results are similar to the results of Reynolds *et al.* (1989), which were explained in Section 2.5.1.1, in terms of differences in specific growth rates. In order to compare penetration rates, bacteria with similar growth rates should be used, to eliminate differences in penetration rates caused by differences in growth rates. One possibility is to use the same bacteria under gradient and non-gradient conditions, which is the technique used by Barton and Ford (1994).

2.5.4 Barton and Ford

Barton and Ford (1995) studied the migration of *P. putida* through carefully assembled vertical sand cores. The chemotactic bacteria that was used in the study was the strain *P. putida* PRS2000. One of the non-chemotactic bacteria used in the study was a non-motile strain *P. putida* PRS2000 NTG. However, for the experiments in which non-chemotactic behavior was required, the chemotactic PRS2000 bacteria was used without any attractant. The attractant chosen in Barton's chemotaxis studies was 3-chlorobenzoate (3CB). The porous

medium consisted of a commercially available, acid washed sand with average particle size of 194 μm . The experimental apparatus consisted of 2.5 cm diameter polypropylene tubes filled with sand, bacteria, and media to a depth of 6 cm. The oxygen conditions were initially aerobic because the media and tubes were prepared under aerobic conditions. No further oxygen was introduced into the system after the tubes were filled. The gradient conditions were created by filling one half of the tube with bacteria and the other half with a 5 mM 3CB solution. To fill the tubes, sand and bacteria were carefully introduced in 2.5 mm layers until the appropriate depth was achieved. The sampling procedure consisted of slicing the columns into 1 cm sections and analyzing the sections for bacterial penetration. Dilution series were performed to determine the most probable number (MPN) of bacteria in each section. The MPN was then converted into a bacterial density in the units of cells/ml and plotted as a function of distance along the column.

The results of the study demonstrated that there were no statistically significant differences in bacterial density profiles between the cases where attractant was present (chemotactic behavior) and the cases where there was no attractant (non-chemotactic behavior). Barton and Ford concluded that any chemotaxis effects on the extent of bacterial migration into the column may be below the detection limits of the assay used in the study. They suggested that the bacterial transport mechanism may have been affected by the porous media. Specifically, they pointed out that the porous media may have interfered with runs undertaken by the bacteria during migration through the column.

2.5.5 Marx and Aitken

In a recent study (Marx and Aitken 2000), chemotaxis towards naphthalene by *Pseudomonas putida* G7 was studied. *P. putida* G7 is a common subsurface bacteria that is frequently studied in ISB due to its ability to sense and degrade naphthalene, a common environmental contaminant. This study elucidated a difference in the amounts of naphthalene degraded by *P. putida* G7 and a non-chemotactic control strain *P. putida* C1 (pHG100) in liquid filled capillaries.

Marx and Aitken also worked with capillaries filled with a porous medium. They compared accumulation in the capillary using *P. putida* G7, but they only determined the number of cells accumulated in the capillary when naphthalene was present versus when naphthalene was not present. This result showed that more cells accumulated in the capillaries, but it did not show whether these additional cells contributed to enhanced naphthalene degradation. They did measure naphthalene degradation by *P. putida* G7 in the glass beads, but they only compared degradation with losses in uninoculated systems. They did not compare degradation due to chemotactic cells versus degradation due to non-chemotactic cells, as the work of this dissertation does.

2.5.6 Experimental system of this work

Along with the problems of non-metabolizable attractants, there were several inconsistencies in the experimental approaches of these previous works, primarily concerning the selection of the non-chemotactic control strain. Often,

when the prior works appeared to be comparing transport differences due to chemotaxis alone, they were actually comparing cells with different chemotactic sensitivity coefficients as well as different tumbling frequencies and growth rates. Thus, transport differences due strictly to chemotaxis alone may not have been isolated.

The experimental system for this work has been designed to overcome these difficulties of strain swimming behavior, wet sand sampling, and lack of rigorous measurement of contaminant degradation. The system is described in detail in the Methods section, but highlights are included here for comparison to the earlier works. For this work, a chemotactic and attractant blind strain of *E. coli* are introduced to a saturated sand chamber and allowed to migrate and consume a chemoattractant. Chemoattractant concentrations are then measured and compared. The experimental system consists of a rectangular sand chamber, prepared aerobically. The sampling procedure consists of mixing the chamber contents and measuring final serine concentrations for the two cases. Penetration rate measurements were also taken with the system using a novel sampling apparatus.

3 RESEARCH GOALS AND OBJECTIVES

The goal of this dissertation is to investigate a hypothesis about bacterial chemotaxis and whether it has a statistically significant effect on the ISB process.

Several objectives have been accomplished in this work:

- Selection and characterization of experimental and control bacterial strains which are similar in nearly all aspects except chemotactic response towards serine
- Selection of experimental parameters to test such as particle size, chemoattractant concentration, bacterial concentration, migration temperature and migration time
- Design of novel apparatus and assays to measure chemotactic effects on microbial transport

A specific null hypothesis, relevant to the experimental work of the dissertation, has been developed:

Chemotactic *E. coli*, when exposed to a gradient of metabolizable serine in a porous medium consisting of liquid saturated sand, will degrade the same amount of serine during a given period of time even if one of the strains is lacking the serine chemoreceptor.

The goal of this dissertation is thus to determine whether this null hypothesis can be rejected, demonstrating that chemotaxis does have an effect on degradation of serine in the experimental system.

This investigation has proceeded via the measurement of both the degradation rate of a model contaminant and the penetration rate of bacterial strains through a saturated porous media under conditions of chemotaxis. An experimental system that models a saturated, porous aquifer with a

chemoattractant gradient and a chemotactic organism has been designed for this research and used to test the above hypothesis, by measuring the degradation of the chemoattractant at low cell densities and non-growth conditions.

Experimental parameters have been selected in order to optimize the ability of cells to swim vigorously in a chemotactic manner.

4 MATERIALS

4.1 Bacterial strains

The strains used in this study were *E. coli* RP437 and *E. coli* RP5700 and were obtained from J. S. Parkinson from The University of Utah. Genotypes are shown in Table 4-1. RP5700 is a spontaneous deletion mutant of RP437 that lacks the chemotaxis receptor to detect serine (*tsr*), and thus cannot exhibit chemotaxis towards serine. However, it has a wild-type swimming pattern highly similar to RP437 despite the loss of the serine chemoreceptor, making it an ideal strain for the study (Callahan, Frazier et al. 1987; Parkinson 1997). Both strains lack the ability to synthesize the amino acids histidine, leucine, methionine and threonine (Parkinson 1996). This inability of the strains to synthesize these amino acids has been useful in this work, as this allowed experiments to be performed in which cells could consume serine without growth, simply by omitting these amino acids in the growth media. This is essential because this work studies consumption of a chemoattractant without growth. Strains were grown on Wolfe-Berg tryptone (WBT) media [(Wolfe and Berg 1989), see Appendix A] and frozen to -80° C under 50% glycerol. Both strains are resistant to streptomycin, which was used in all experiments to assist in the maintenance of pure cultures. Chemotactic ability of frozen stocks was confirmed by inoculating WBT swarm plates (see Appendix A), which is WBT with 0.2% agar added and poured onto Petri dishes. Ring growth on the swarm plates was consistent. RP437

Table 4-1: Genetic properties of strains used in this study.

<i>E. coli</i> strain	Relevant genotype
RP437	<i>thr</i> (Am)-1 <i>leuB6</i> <i>his-4</i> <i>metF</i> (Am)159 <i>eda-50</i> <i>rpsL</i> 136 [<i>thi-1</i> <i>ara-14</i> <i>lacY1</i> <i>mtl-1</i> <i>xyl-5</i> <i>tonA31</i> <i>tsx-78</i>] / <i>jsp</i> /
RP5700	(<i>tsr</i>) DE7028 <i>thr</i> (Am)-1 <i>leuB6</i> <i>his-4</i> <i>metF</i> (Am)159 <i>eda-50</i> <i>rpsL</i> 136 [<i>thi-1</i> <i>ara-14</i> <i>lacY1</i> <i>mtl-1</i> <i>xyl-5</i> <i>tonA31</i> <i>tsx-78</i>] / <i>ac</i> /

Source: J. S. Parkinson, 1996, personal communication

stocks always demonstrated a fast growing serine ring, while RP5700 stocks formed rings much more slowly.

For the majority of the experiments, cultures were grown by introducing 100 μ l frozen stocks, thawed for 10 minutes, into 250 ml foam stoppered baffled flasks containing 25 ml WBT. An abiotic control flask containing no cells was used in every experiment, and contamination was not ever detected. After inoculation, cultures were placed in an incubator set to shake at 145 rpm (Adler and Dahl 1967) and 35 °C, a temperature used frequently in *E. coli* chemotaxis experiments as one which insures proper growth of flagella (Adler and Templeton 1967).

Cells were grown overnight for 11 hours to an optical density at 600 nm (OD_{600}) of approximately 1.1, as measured with a *Beckman-Coulter* DU640B spectrophotometer. After 11 hours, 500 μ l of the overnight culture was transferred to 50 ml fresh WBT. Cells were incubated under the same conditions of the overnight culture, and allowed to grow for about 5 hours to an optical density (OD_{600}) of ~ 0.5 , a density representative of mid-exponential growth, which is an optimum range for cell viability, motility, and chemotaxis (Adler 1973). This *working culture* was then prepared further according to the experiment that was being performed (see Cell preparation, Section 5.2).

4.2 Sand

The sand used in the study is Unimin Corporation's 20/30 Accusand. It is 99.7% silicon dioxide and has a tight particle size distribution between

600-710 μm : 41.3% 600 μm and 56.4% 710 μm .

Sand was purified by washing with 1 N H_2SO_4 (Barton 1994) using an apparatus designed to wash the sand with minimal damage to the sand particles (Figure 4-1). Purification was necessary to remove any possible chemoattractants or nutrients which may have supported growth. Other methods employed, i.e., mixing with an impeller, caused sand particles to break into fine particles. The sand washing apparatus consisted of a Masterflex peristaltic pump connected to three vertical PVC pipes containing a total of 1200 ml sand. Glass wool was used inline to prevent entrainment of sand particles into the pump. Acid was introduced in an upflow manner to ensure that all acid contacted all regions of the sand. Acid was recirculated in the apparatus for one hour to remove organic contamination. After one hour, the system was flushed with 2 liters of a sand phosphate buffer consisting of 6.15 mM K_2HPO_4 and 3.85 mM KH_2PO_4 (see Appendix A, sand phosphate buffer). This buffer was chosen because its pH is nearly 7.0, the pH for all of the experiments in this work. After washing, sand was placed in small cups and autoclaved on the dry cycle for 20 minutes. Bacterial contamination of sand was never observed.

4.3 Chemicals

The following chemicals were obtained from Fisher, Inc.: KH_2PO_4 , K_2HPO_4 , H_2SO_4 , NaCl, tryptone, HPLC water and acetonitrile, L-histidine, L-Leucine, L-methionine, and L-threonine. Agar was obtained from Becton-Dickinson, Inc. L-serine and was obtained from Aldrich, Inc., streptomycin sulfate

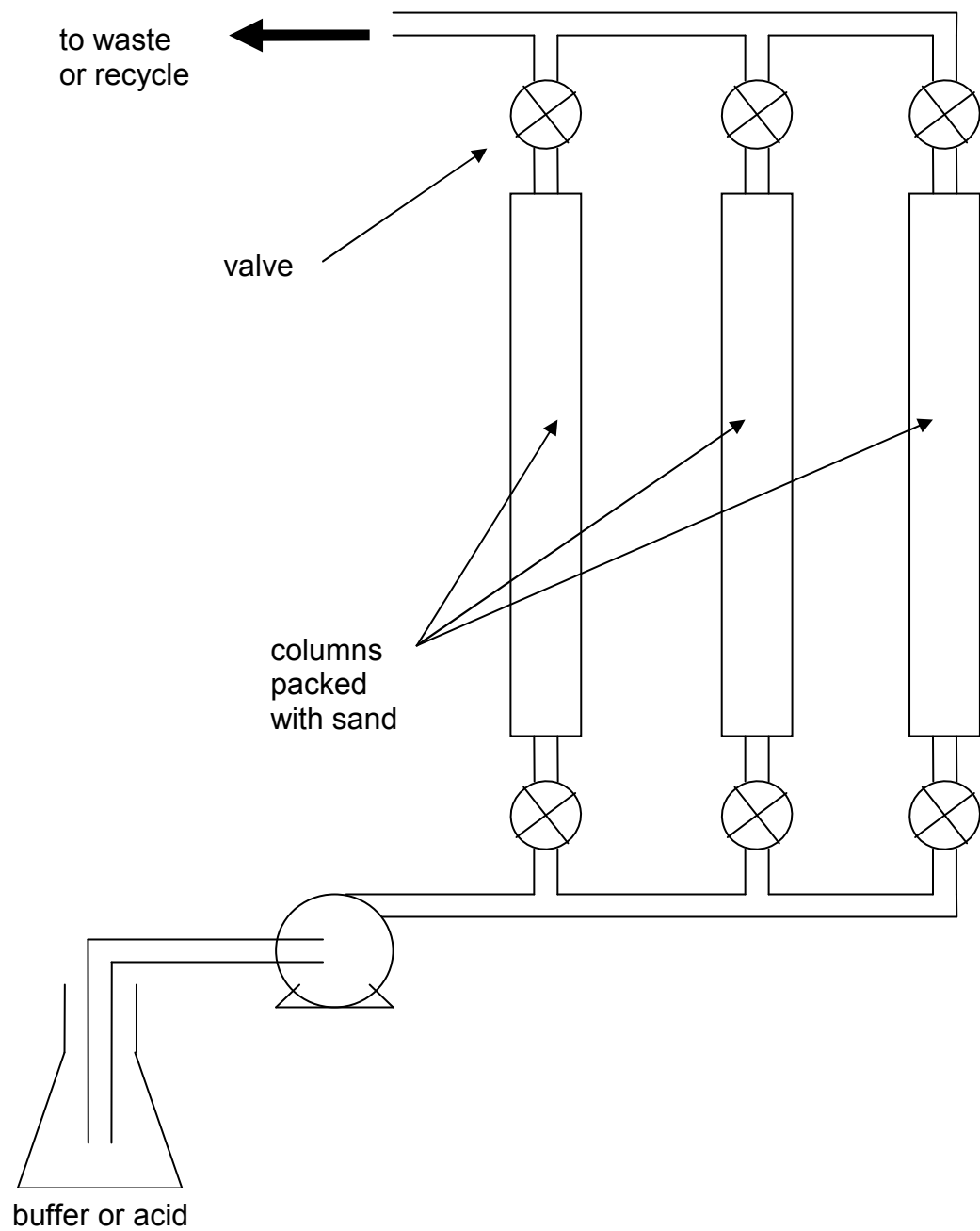


Figure 4-1: Sand washing apparatus. Three PVC pipes were connected to globe valves to regulate flow.

and ethylenediamine tetraacetic acid, disodium salt (EDTA) from Sigma, Inc., and glycerol was purchased from Mallickrodt. Derivatization reagents and proprietary HPLC eluents were purchased from Waters, Inc.

5 METHODS

5.1 High performance liquid chromatography

Samples were analyzed for serine concentration using high performance liquid chromatography (HPLC). Because amino acids do not give a very strong ultraviolet (UV) absorbance or a fluorescent signal, derivatization is often used to link the amino acids with a molecule that will give a UV or fluorescent response. A method has been developed for derivatization of amino acids (Cohen and Michaud 1993) using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), which is a member of a new class of amine-derivatizing compounds (Figure 5-1 and Figure 5-2).

This method has been tested and is sold as a chemistry package by the Waters Company under the name *AccQTag*[®]. The method consists of dilution of the sample containing the amino acids with HPLC grade water. 10 µl of diluted sample is then combined with 70 µl of a borate buffer, followed by 20 µl of an AQC solution prepared in acetonitrile. The sample is vortexed and heated to 55°C for ten minutes, at which time it is ready for injection to the HPLC column. The column used is a *Waters AccQTag*[®] C18 reversed phase 15 cm column. Three eluents are used with the pump. Eluent A consists of an aqueous solution of 1.73% weight percent sodium acetate trihydrate, 0.59% phosphoric acid, 0.136% triethylamine, and 0.009% sodium azide (prevents microbial growth in the solvent bottles). Eluent B consists of pure acetonitrile,

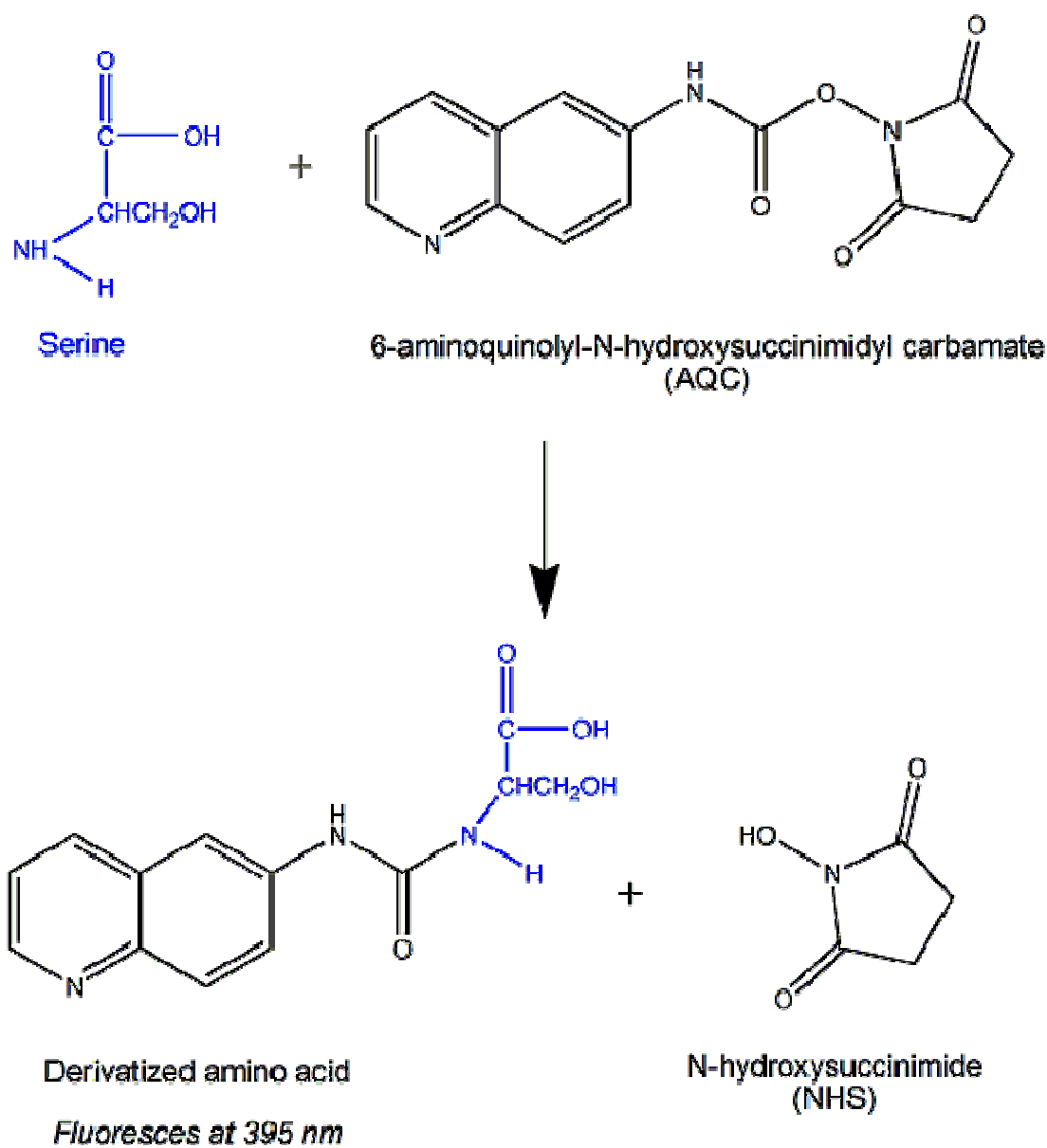


Figure 5-1: Reaction of a primary or tertiary amino acid with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) to yield a derivatized amino acid plus the byproduct N-hydroxysuccinimide.

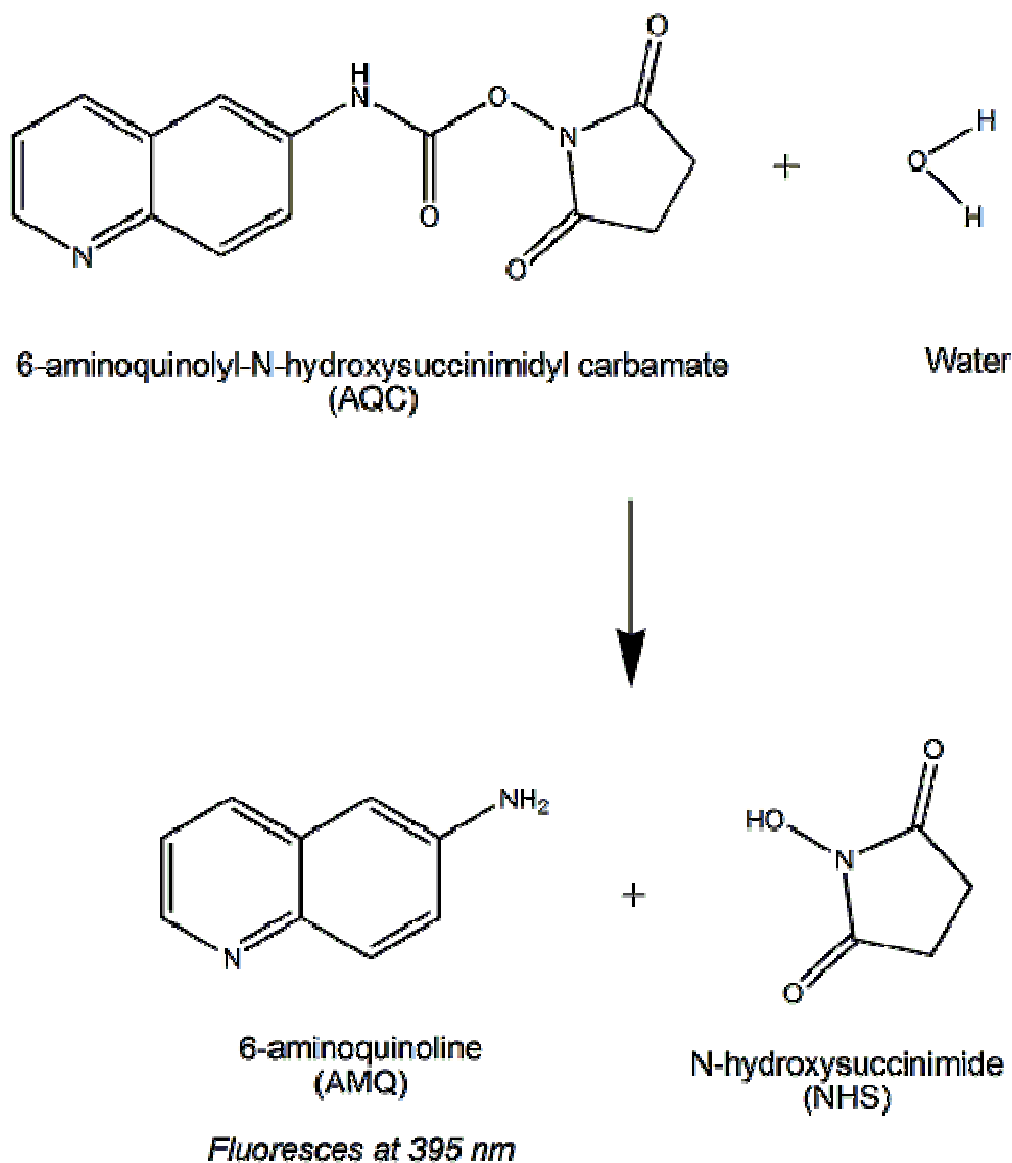


Figure 5-2: Hydrolysis reaction of AQC to 6-aminoquinoline (AMQ) and NHS. This reaction produces AMQ in excess to confirm that there was enough AQC in the reaction to consume the serine and produce this by-product.

and Eluent C consists of HPLC grade water.

For this study, a *Waters* 2690 HPLC was used along with a *Perkin Elmer* LC240 fluorescence detector. A *Waters busSAT/IN*[®] module was used to convert the analog signal from the fluorescence detector to a digital signal that could be read by the accompanying data acquisition computer (See Figure 5-3 for a schematic of the apparatus). An amino acid calibration standard, provided by the *Pierce* chemical company, was used for peak identification. The gradient program is shown in Appendix B. All seventeen amino acids provided in the calibration standard were resolved with the system described above. To insure that the reaction of all amino acids in a mixture has gone to completion, a byproduct from the reaction of AQC, 6-aminoquinoline (AMQ), can be used to monitor the reaction (Figure 5-2, and see Figure 5-4 for an example chromatogram). AMQ is resolved chromatographically, and does not interfere with the analysis. If no AMQ is present, then it is possible that underivatized amino acids may be undetected. Inspection of a multitude of HPLC runs indicates that AMQ was always present. Derivatization blanks were prepared and used to identify the AMQ and ammonia peaks, along with derivatization artifacts. A 6-point calibration curve was performed, and a correlation coefficient (R^2) value of 0.999 was obtained. The curve is shown in Appendix B, Figure B-1. The errors in generating this curve include errors in sample preparation chemistry along with errors in HPLC Analysis. This serine detection limits using these procedures ranged from 1.0 mM to 0.05 mM.

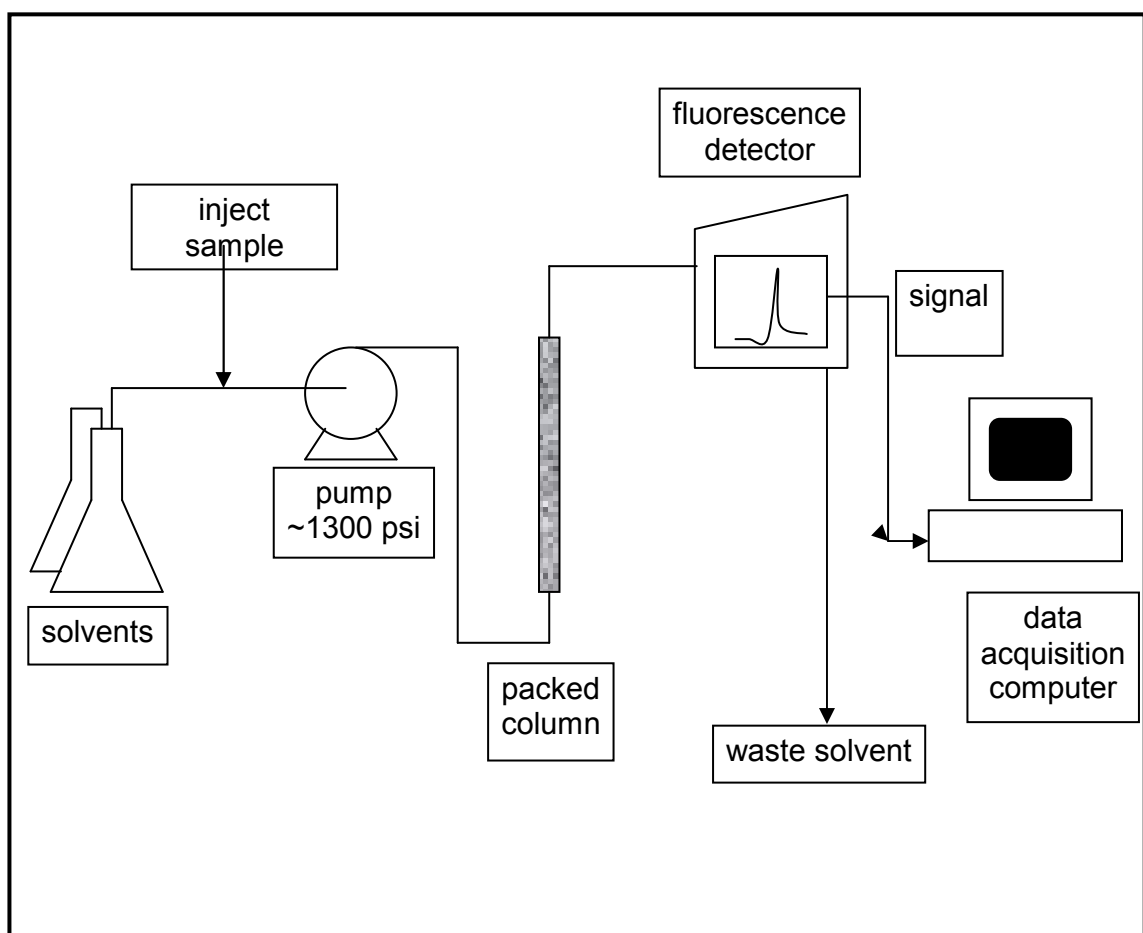


Figure 5-3: Schematic of high performance liquid chromatography (HPLC) system used in the quantification of serine.

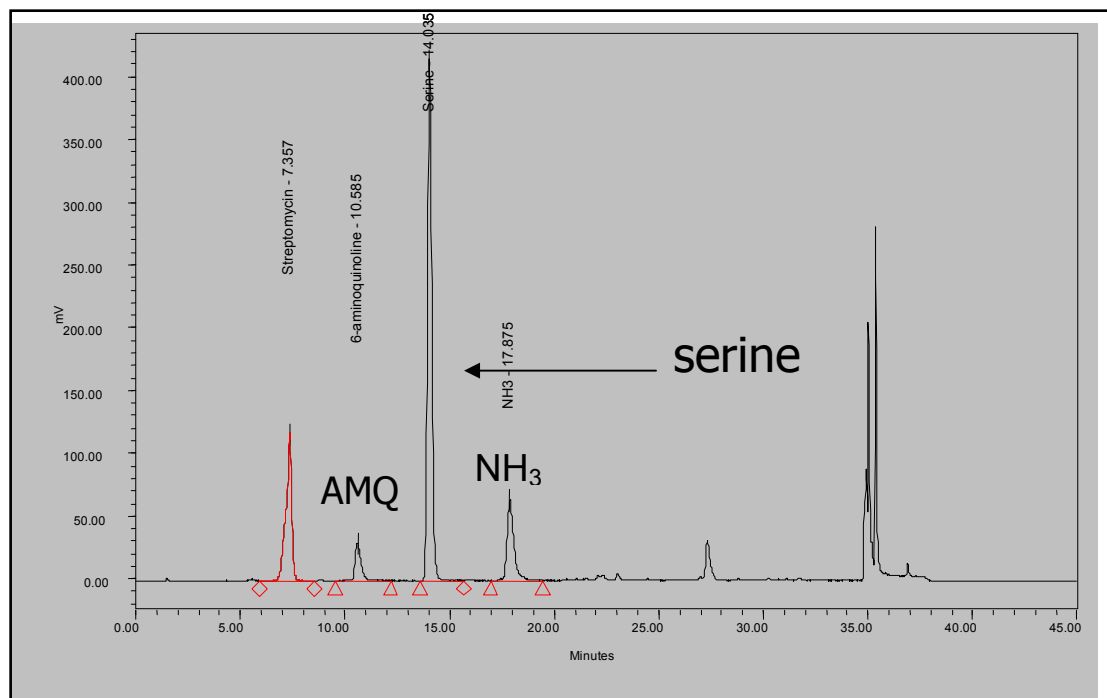


Figure 5-4: HPLC chromatogram showing components of derivatized sample. Peaks shown, with good baseline resolution are streptomycin (7.357 min), 6-aminoquinoline (10.585 min), derivatized serine (14.053 min), ammonia (17.875 min) and derivatization byproducts (27.5 min and 35 min). Red diamonds and triangles indicate peak integration endpoints.

5.2 Cell preparation

Cultures for the experiments were prepared as described in Section 4.1. After the working culture was prepared, two different methods were used to wash cells for chemotaxis experiments: Method 1 used a centrifuge to precipitate cells and resuspend them while Method 2 used a filtration technique. In both methods, cells were washed with Adler wash buffer (AWB, see Appendix A), a buffer with pH 7.0 that is based upon a solution created by Adler (Adler and Dahl 1967). Methionine was added to the buffer because it is required for the chemotaxis mechanism. Cells are smooth swimmers if no methionine is present, not run and tumble swimmers (Adler and Dahl 1967). EDTA is added because it chelates metal ions, which are inhibitory to chemotaxis (Adler 1973). Streptomycin is also added, since RP437 and RP5700 both have a resistance to this antibiotic.

5.2.1 Cell Wash Method 1: Centrifugation

To begin the centrifuging procedure, 43 ml of cell suspension in WBT was transferred to a 50 ml sterile polypropylene centrifuge tube. A Beckman Allegra 6 benchtop centrifuge was used at 3000g for the cell wash. The culture was centrifuged for 12 min. at 3000g and the supernatant decanted. The pellet was then resuspended in 30 ml of AWB using a vortex. The suspension was again centrifuged for 12 minutes and resuspended 2 more times. The final pellet was resuspended in 40 ml of AWB and at this point was ready for experiments. The washing procedure cleaned the cells of tryptone and metabolite by-products,

leaving no detectable serine which resides in the WBT, as verified via HPLC. It was observed that cells were not very motile after this procedure. By visual inspection, cells were 10 – 20% motile. Thus, this method was used only for experiments not requiring motility, such as the serine liquid uptake experiments. For experiments requiring motility, an alternate filtration method was developed.

5.2.2 Cell Wash Method 2: Filtration

For the filtration method, 43 ml of working cell suspension in WBT was transferred to the funnel of a Corning disposable sterile filter unit with a 250 ml capacity and a 0.2 μm nylon membrane. The vacuum was turned on and the suspension was filtered through the membrane. At the end of the filtration, just as a small section of the membrane began to be visible, 35 ml of AWB was introduced into the filter funnel. The membrane was not allowed to become dry to reduce clogging of the membrane with cells and to prevent cells from being damaged by becoming trapped in the membrane pores.

After the first 35 ml was passed through the filter, another 35 ml AWB was added and again allowed to filter though without allowing the membrane to dry. After this 35 ml passed through, the vacuum was turned off and the cells were resuspended a final time with 35 ml AWB. The filter was then swirled to raise any cells on the membrane into the suspension, and transferred to a 50 ml sterile polypropylene centrifuge tube. The cells were then ready for motility experiments. 80% motility was typically achieved for this method.

6 CHARACTERIZATION OF BACTERIAL STRAINS

6.1 Introduction

Physiological data on the two strains of *E. coli* used in this work have been collected for comparison. It was critical that the strains be as similar as possible, so that the only difference between them is chemotactic ability. Data on growth on serine, a standard curve, serine uptake rates in liquid media, and swimming parameters have been collected and are presented in this section.

6.2 Growth on serine

If a batch of subsurface bacteria was migrating through an aquifer consuming contaminants, this flux could be caused by two reasons, assuming no flow of subsurface fluid: moving bacteria and cells that are moving and growing as they migrate. In this study, it was desirable to decouple migration due to chemotaxis from migration due to chemotaxis and growth. Analysis of the effect of chemotaxis alone in the absence of other phenomena is the focus of this work. RP437 and RP5700 are missing genes for synthesis of four amino acids as mentioned earlier, yet they will degrade the amino acid serine. This lack of growth in the absence of these amino acids is advantageous to this study, as it allows for experimentation with swimming cells that are not growing. Figure 6-1 shows the results of the inoculation of RP437 (chemotactic) and RP5700

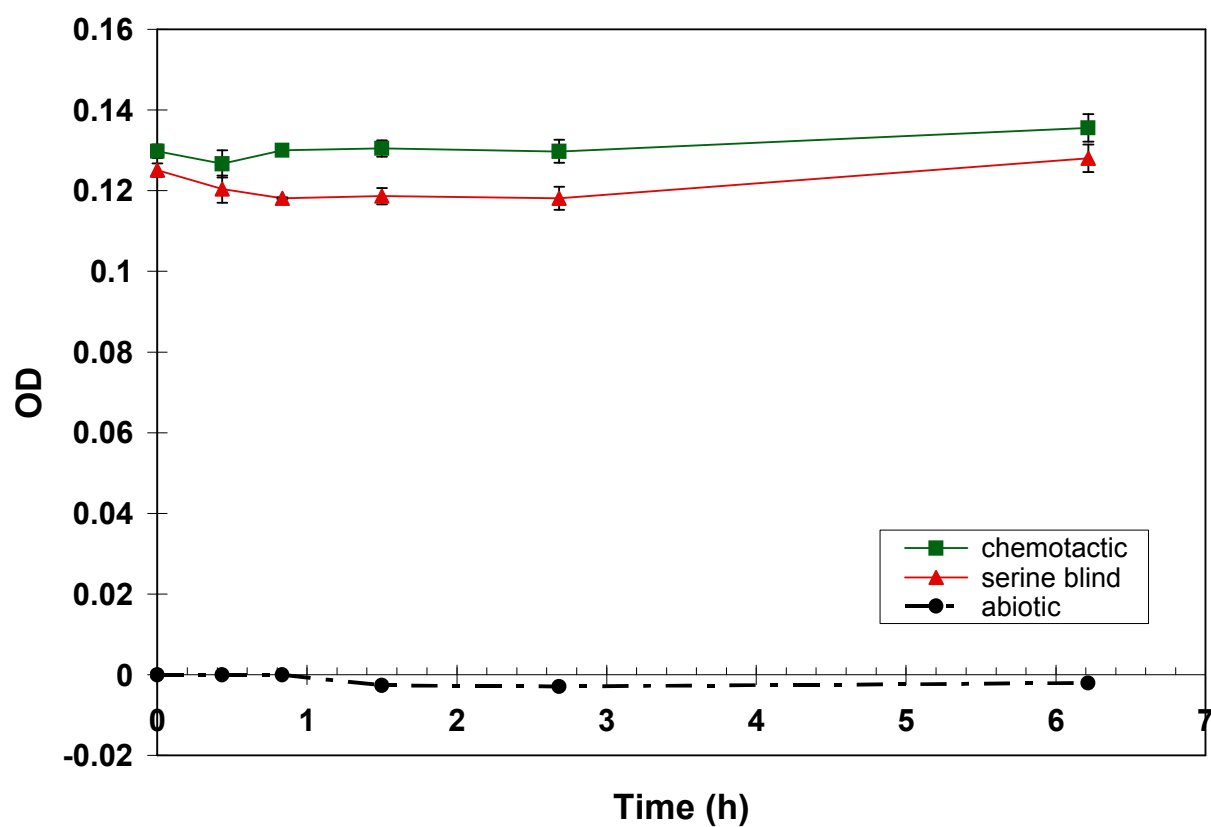


Figure 6-1: Average optical density (600 nm) vs. time for *E. coli* RP437 strain (chemotactic) and *E. coli* RP5700 (serine blind) grown on a serine motility buffer, similar to AWB, lacking the four required amino acids. No growth is observed. Error bars indicate one standard deviation, and a duplicate experiment yielded similar results.

(serine-blind) into serine motility buffer, a buffer similar to AWB, with 1 mM serine. The vertical axis represents the OD₆₀₀ readings. The experiment was run for 6 hours in triplicate flasks. An abiotic case was run concurrently and shows that there was no contamination in the flasks that were used. The results show that while the chemotactic cells were at a slightly higher density, neither strain demonstrated any growth over this time period. Thus, this lack of growth is a characteristic that allows experimentation without the effect of further degradation by new cells that may grow in the model aquifer of this work.

6.3 Standard curve

The standard (viable) plate count method is used to quantitatively determine the number of live cells, or, colony forming units (CFU) that correspond to a given optical density (CFU/ml vs. OD). The determination of the number of live cells is important in this work so that differences in the number of live bacteria present in a given sample can be determined and used to report data in the unit of CFU, a unit that is consistent with literature values of parameters such as uptake rates and most probable number (MPN) enumerations.

The procedure used for the determination of the standard curve is similar to a published method (Harley and Prescott 1996). To begin the standard curve experiments, an overnight culture was started according to the method described in Section 4.1. The culture was transferred to a working culture, and, for the standard curve determination, the wild type cells were grown to an OD₆₀₀ of 0.53

and the serine blind cells were grown to an OD₆₀₀ of 0.55. The cells were then washed via Method 1, centrifugation. The final pellet was re-suspended in 14.3 ml of AWB, which served to concentrate the cells for further dilution. After re-suspension, the final OD₆₀₀ of the cell suspension was 1.03 for the wild type cells and 1.05 for the serine blind cells. The cells were then diluted as shown in Table 6-1.

Dilutions were performed in 50 ml sterile centrifuge tubes with AWB. Each dilution tube was vigorously shaken 25 times with the forearm making 90° arcs. Each dilution level was then further diluted for plating, from the range of 10⁻² to 10⁻⁹. 1.0 ml and 0.1 ml aliquots were transferred to 100 mm x 15 mm sterile plastic Petri dishes. Liquid WBT containing 1.5% agar, situated in test tubes heated at 50 °C, was then poured into the plates containing the cell suspension. Plates were gently swirled to mix the cells with agar, the plates were then left overnight for growth at 35 °C, and the resulting colonies counted after 18 hours. Only plates containing 25-250 colonies were counted as per the method of Harley (Harley and Prescott 1996).

For both strains, colony counts in the 25-250 range were encountered only in the dilution levels of 10⁻⁶ and 10⁻⁷. Figure 6-2 shows the resulting standard curve. The horizontal axis represents the OD₆₀₀ reading and the vertical axis represents the number of CFU per milliliter of solution. A correlation coefficient (R²) of 0.95 for a linear fit was achieved for the wild-type strain, and 0.97 for the serine blind strain. Data was scattered in the higher OD₆₀₀ range of 0.9, likely due to errors in counting plate containing nearly 250 colonies. Most of the

Table 6-1: Cell suspension dilutions used to generate standard curve. Care was taken to ensure that OD's were as similar as possible

Wild type dilutions (OD₆₀₀)	Serine blind dilutions (OD₆₀₀)
0.89	0.91
0.74	0.76
0.57	0.58
0.36	0.37
0.13	0.14

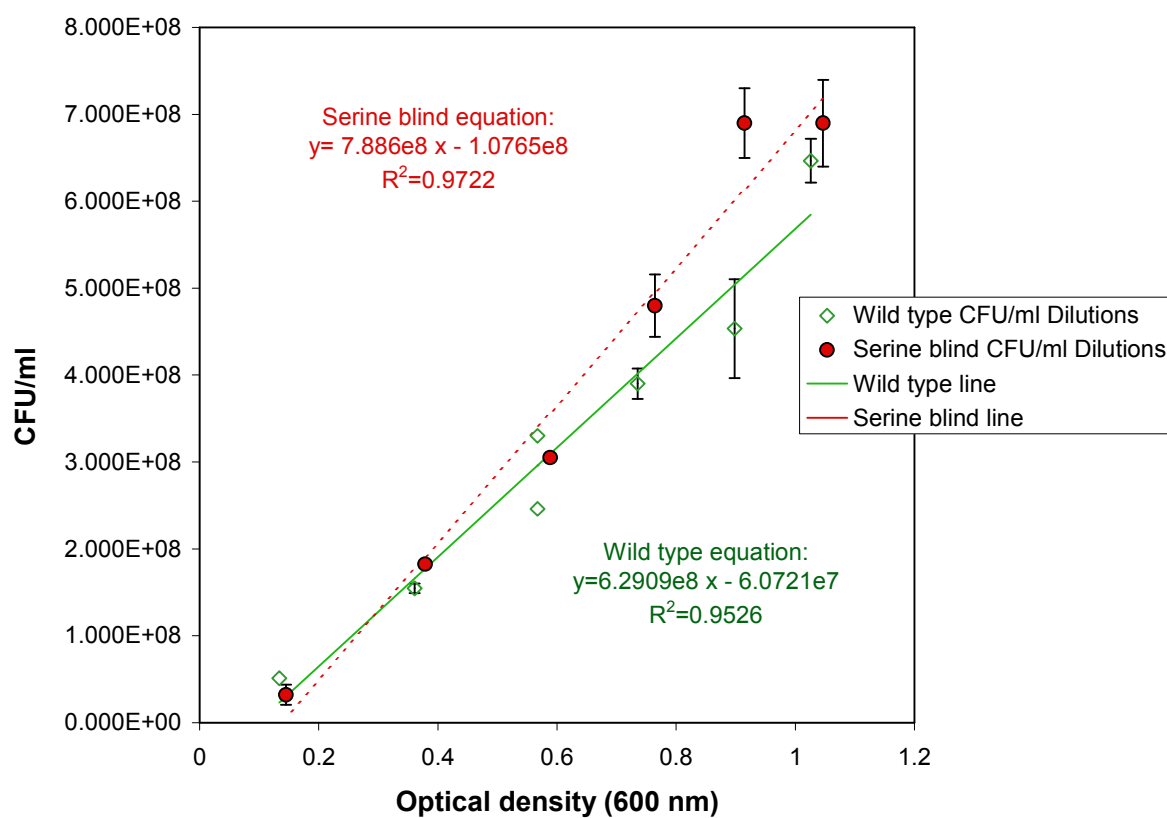


Figure 6-2: Standard curve, wild type *E. coli* (RP437) and serine blind *E. coli* (RP5700). This curve was used to convert optical density measurements into CFU/ml measurements, to ensure that the same number of cells was used in each experiment.

experiments of this work used cells in the OD₆₀₀ range of 0.2 to 0.6, where the data fits a straight line more exactly.

6.4 Serine liquid uptake experiments

6.4.1 Objectives

The goal of this experiment is to determine whether or not chemotaxis can lead to higher bioremediation rates in an *in situ* bioremediation system containing a saturated porous medium. In order to prove the hypothesis that a system containing actively chemotactic bacteria will yield a faster bioremediation rate, entire porous media samples containing chemotactic RP437 in one case, and non-chemotactic RP5700 *E. coli* in another case were assayed for serine consumption. Because serine consumption rate is the parameter of interest in the bulk porous media, one must consider the fact that the individual strains may have different intrinsic consumption rates. The question thus becomes:

Are the differences in consumption rate really due to chemotaxis-enhanced penetration into the porous media or do the strains have intrinsic differences in consumption rate?

This question can be answered by measuring consumption rates in a homogeneous, unstirred buffer solution without a porous media. In this experiment, the rates of consumption of serine by the two strains, via the enzyme L-serine deaminase, have been measured. The flasks used for these measurements were unstirred, simulating static conditions that exist in the porous media experiments of this work and conditions that may exist in an ISB

system. By-products of the reaction consist of the products pyruvate and ammonia (NH_3), and an approximate measurement of ammonia production has been generated.

6.4.2 Methodology

To begin the uptake experiments, a working culture was prepared (Section 4.1) and cells were washed using cell wash Method 1 (Section 5.2.1). For these uptake experiments, the washed cells were normalized by addition of AWB to a final OD_{600} of 0.4 (2×10^8 CFU/ml), a typical concentration for chemotaxis experiments. (Adler 1966).

After the suspension was normalized, an 875 μl aliquot was withdrawn from the flask containing 35 ml cell suspension and sterile filtered with a Millipore *Steriflip*[®] vacuum filtration unit (see Figure 6-3). The unit consists of a 50 ml capacity centrifuge tube and is constructed with a 0.22 μm polyether-sulfone, membrane. This sample, taken after the cells were washed free of the serine-containing WBT media, was analyzed for background serine and none was detected.

The next step consisted of the timed addition of serine to the cells in the flask. An 875 μl aliquot of a 40 mM serine concentrate of Adler wash buffer with serine (AWBS, see Appendix A), was added to the 34.125 ml in the flask using a 1000 μl pipetter. This raised the starting volume to 35 ml and lowered the serine concentration to 1mM. It has been shown that serine concentrations higher than

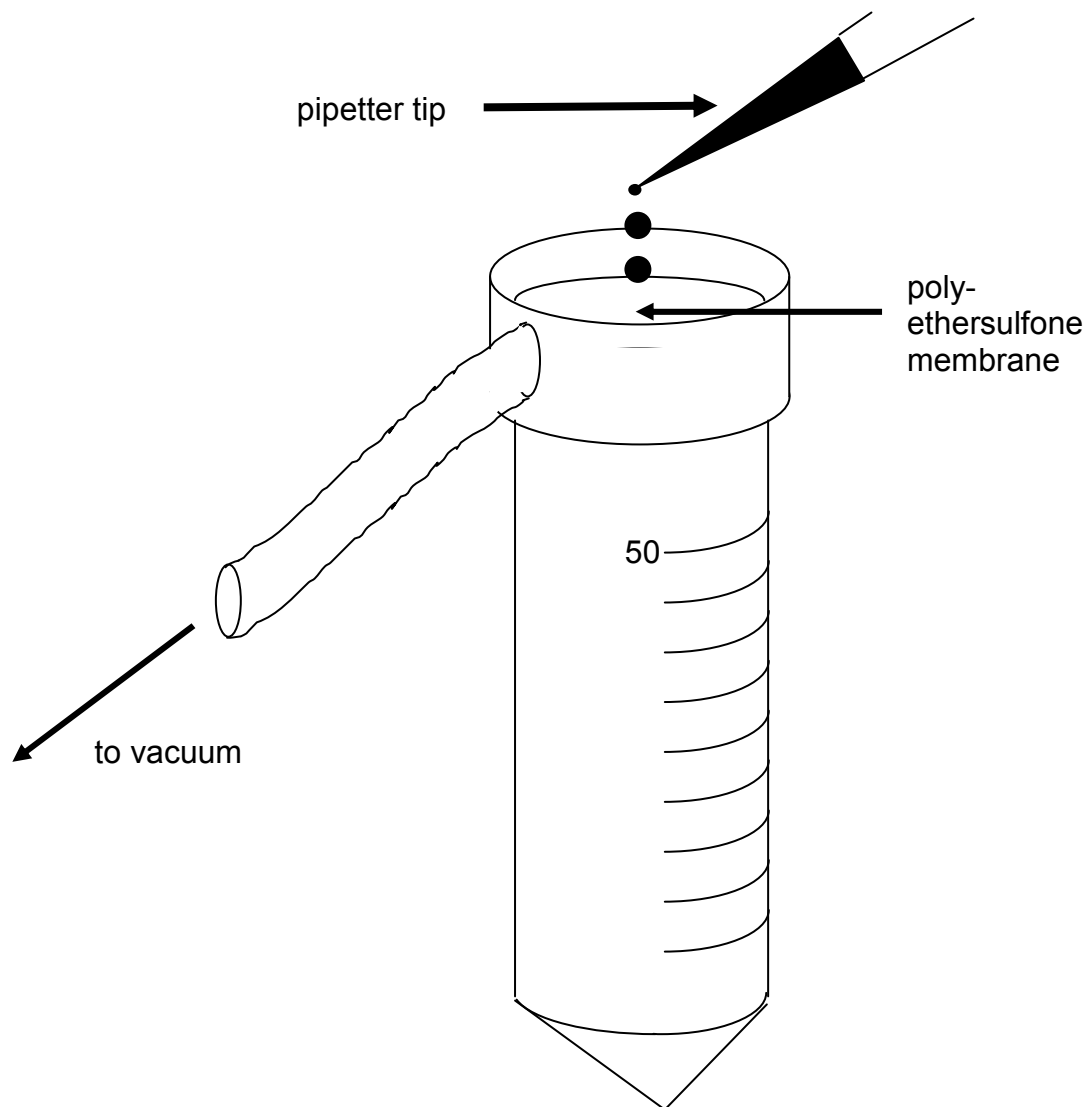


Figure 6-3: *Steriflip*[®] filtration unit. Figure illustrates addition of drops of serine-bacteria suspension to the membrane.

1 mM yield no additional chemotactic response in *E. coli* (Adler 1966) and this concentration was tested via a capillary method in this work (see Section 7.3). The results indicated that a 1 mM serine concentration was an optimum level and that a higher 10 mM concentration yielded a lower response, likely due to saturation of the serine chemoreceptors in the bacteria. After the addition of serine, the flask was shaken briefly to distribute the serine, but otherwise, the flask was unstirred.

The following steps required the use of a vacuum manifold custom designed to allow vacuum to be delivered to several *Steriflip*[®] sterile filters in sequence. The manifold allowed for the filtration of several samples in rapid succession (Figure 6-4) and was built of polyvinyl-chloride piping with plastic valves and connected to the laboratory vacuum outlet at 15 in Hg vacuum. A plastic dessicator was used as a vacuum “holdup” tank to increase the vacuum capacity as the valves were opened. Shelf-purchased manifolds such as this one cost hundreds of dollars, but at \$4.00 each, the disposable *Steriflip*[®] filters represented significant cost and time savings when assembled into a manifold. Another essential piece of equipment was a notebook computer running a custom made timer program (Chemotaxis Sample Timer, Douglas Ingram, Applied Computing Technology, Knoxville, TN) to track 10 different samplings over a time course of 12 seconds to 3 hours and sound alarms when a sample is required. The timer was started exactly at the point of introduction of the serine solution to the flask. As quickly as possible, the foam stopper sealing the flask was lifted and a 1000 µl aliquot was removed using a 1000 µl pipetter. A rubber

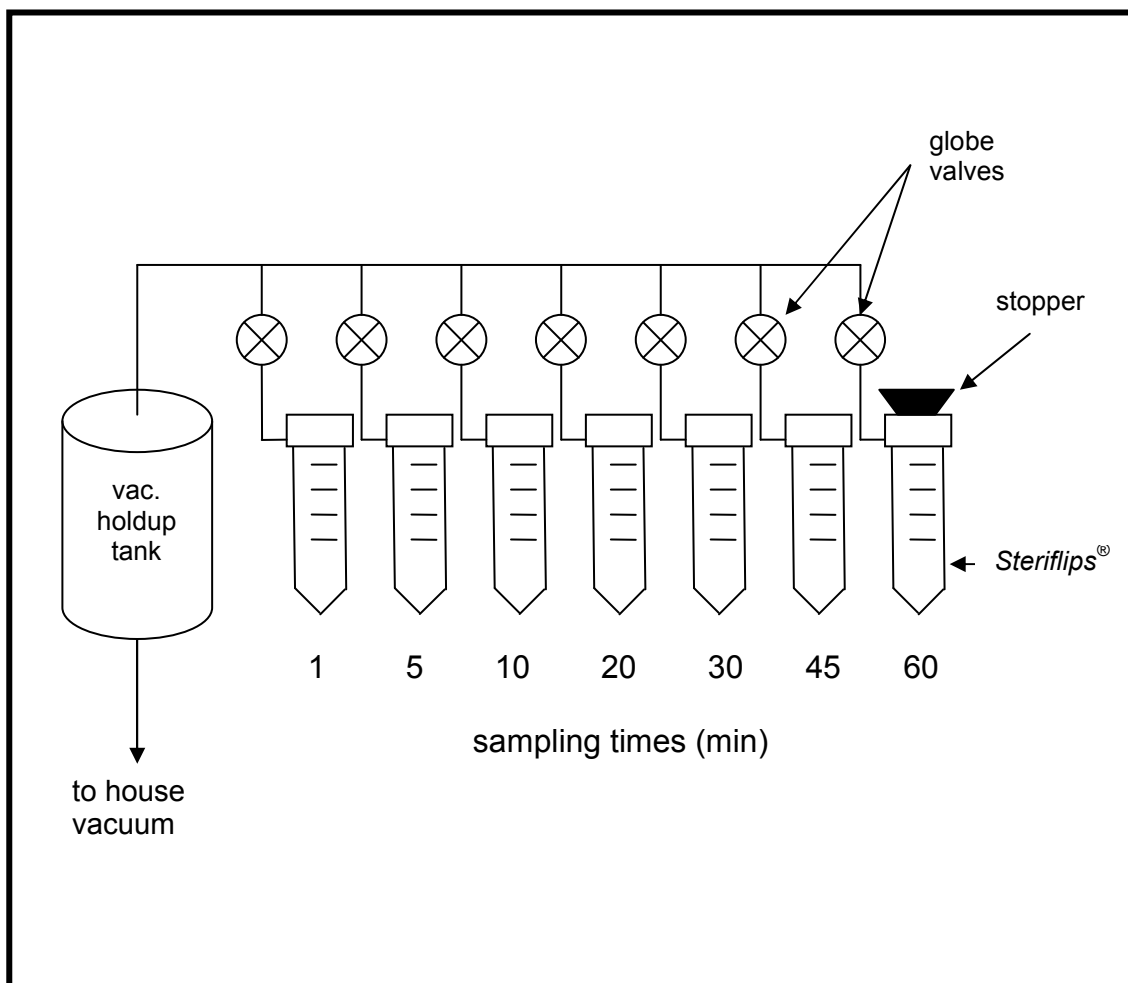


Figure 6-4: *Steriflip*[®] filter vacuum manifold. Seven filters are shown connected by tubing to globe valves to pull vacuum on the filter of the desired sample time. The vacuum holdup tank allowed more than one filter to be opened at a time without loss of negative pressure. A stopper is shown at the 60 minute filter, which did a suitable job of sealing the manifold.

cork, which sealed the *Steriflip*[®] filters and helped maintain vacuum, was lifted to expose the filter inlet. At the point where the timer program sounded an alarm (12 seconds for the first time point) the aliquot was carefully pipetted into the filter inlet. The sample was sucked into the filter, sterilizing the sample and simultaneously stopping the serine consumption reaction by separating the bacteria from the serine. The sample took approximately two seconds to filter. Immediately upon pipetting samples into the filter, the spacebar on the notebook computer was tapped and the exact sampling time was recorded. The sample was then transferred into a cryovial and stored at -80 °C until HPLC analysis could be performed. Further samples were taken at one minute, five minutes, 10 minutes, 20 minutes, 30 minutes, 45 minutes and 60 minutes. All serine was shown to be consumed by 75 minutes, so the experiment was stopped after that duration.

The assumptions made in this experiment are that all of the serine disappears via consumption only by live bacteria. Losses due to adhesion of serine-containing solutions to laboratory equipment are neglected as these losses were found to be small. Finally, it was assumed that the freezing process of samples did not cause the concentration of the samples to change in any way.

6.4.3 Results

Figure 6-5 shows raw data collected by HPLC analysis of the serine-containing samples. On the horizontal axis is time of reaction of the cells in serine (in minutes), and on the vertical axis is integration area (mV·min).

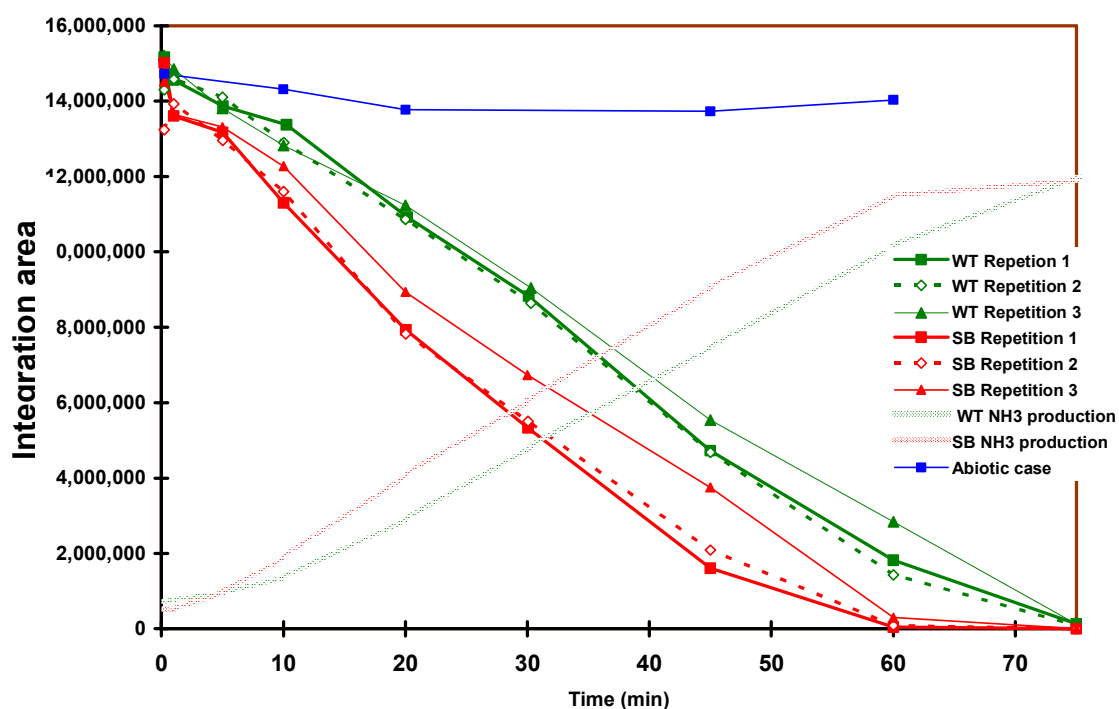


Figure 6-5: Serine uptake by wild type swimming (WT) and serine-blind (SB) *E. coli*. Concentration units for this curve are represented by chromatogram integration area on the y axis since ammonia levels were not converted to concentration. A calibration for ammonia was not performed, although the uncalibrated values show a trend in increasing ammonia output as time progressed. An abiotic case, containing serine only without any of its reaction by-products, demonstrated that there were no losses in serine during the course of the experiment.

This area represents the area under the curve of the peaks in the chromatograms which is converted to concentration via the calibration curve (see Figure B-1 in Appendix B).

Experiments were performed in triplicate, as shown by the three individual curves for each strain. One replicate time course was performed each day, to accommodate accurate data acquisition and to maintain the integrity of the experiments since the timing of each sampling was critical.

The data shows that after 75 minutes, for both strains, all of the serine is consumed. The slopes of the lines are similar, indicating that serine degradation rates are likely the same for both strains. The data in Figure 6-5 is shown in its raw form from the HPLC analysis, to demonstrate the interesting fact that over time, the amount of ammonia is increasing. The derivatization procedure for the serine described in Section 5.1 enables the detection of ammonia, which is a by-product of the degradation of serine. The curves shown in the figure demonstrate that the serine is being degraded into an end product of ammonia. This data is semi-quantitative in that a calibration curve for ammonia was not generated. Ammonia is also a small end-product of the derivatization chemistry, so actual quantitation would require a separate calibration of both ammonia sources.

Also shown in Figure 6-5 is an abiotic case. In this validation experiment, no cells were added to the reaction flask, and it can be seen that after 1 hour, nearly all of the serine is recovered from the reaction flask.

It has been suggested (Parkinson 1997) that uptake rate experiments of this type be conducted at the time scale as close to instantaneous as possible,

that is, on the order of magnitude of several seconds. Literature reported values of uptake rates are also calculated in this manner (Leveau, Zehnder et al. 1998; Marx 2000). However, as Figure 6-6 demonstrates, a close-up of serine disappearance data, suggests that the data is nonlinear and noisy initially, indicating the need to carry out the sampling at later times. The reaction appeared to take some time getting started, possibly due to lag time in either the transport of serine or the activation of the serine deaminase enzyme. Thus, longer time scales were used to measure rates, since the time scale of the experiments of this work are on the scale of several minutes to several hours.

The cumulative amount of serine consumed over time is converted to a per CFU basis using the standard curve (Section 6.3). The resulting data is plotted with the units

$$\frac{pmol}{min \cdot 10^7 CFU}$$

Figure 6-7 shows the degradation data converted into actual uptake of serine into the cells. Uptake data was obtained from disappearance data by measuring the different amounts of serine in the flasks over time. After accounting for losses due to sampling, the differing amounts in the flask over time were assumed to be due to uptake into the cells. The conversion of the data into uptake rates requires careful consideration, as volume changes due to sample withdrawal have to be accounted for. The sample volumes of 1 ml each are not negligible when compared to 35 ml in the reaction flask. Repeated

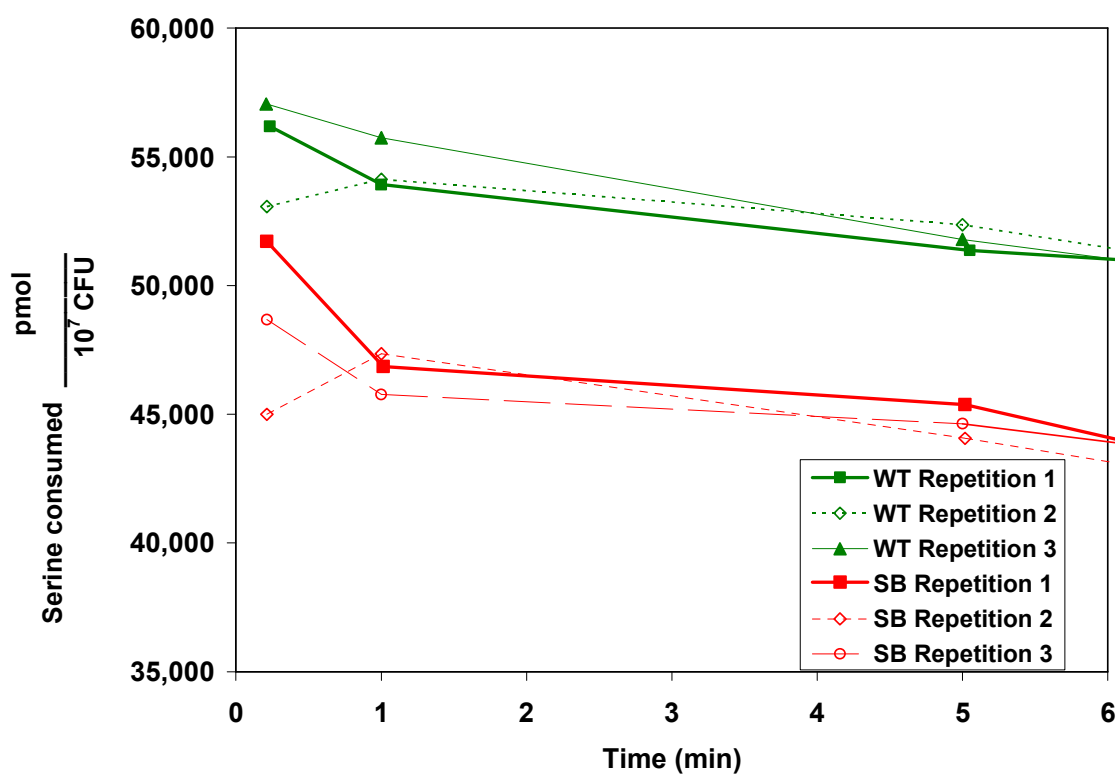


Figure 6-6: Serine disappearance vs. time, data from time points less than 5 minutes. Typically uptake experiments are performed at times from a few seconds to a few minutes, but for this experiment data at 14 seconds is scattered, possibly due to experimental error. Thus, data was collected at longer times, up to 1 hour, and then linearized

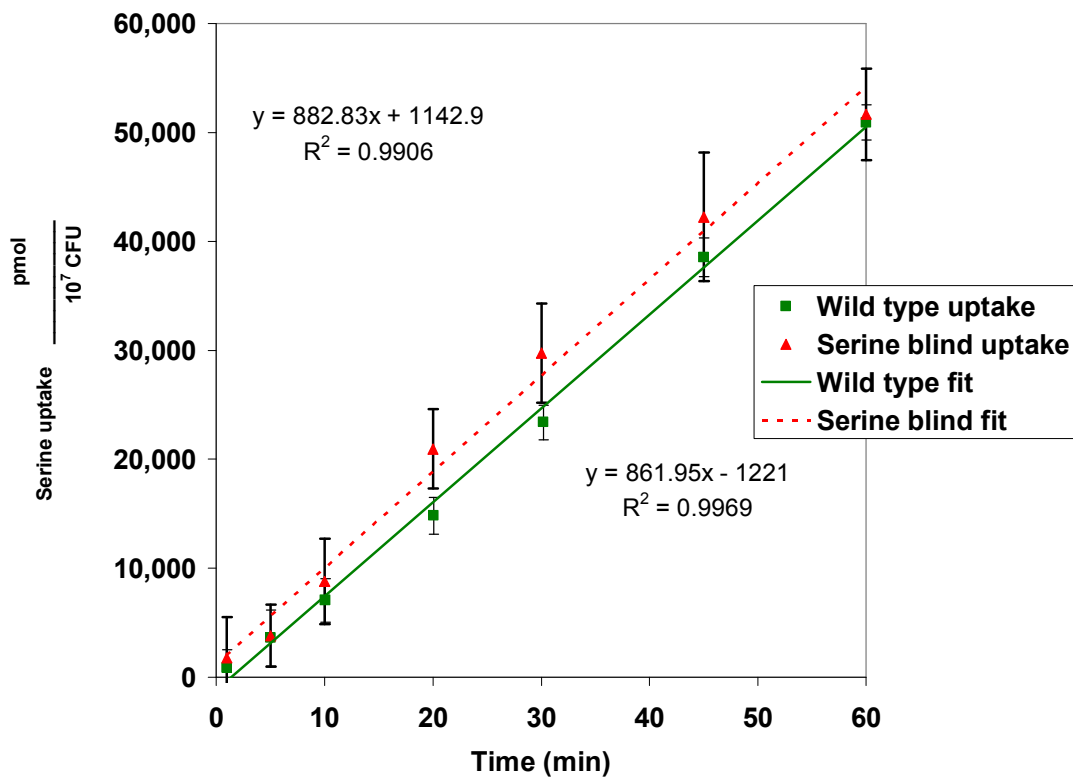


Figure 6-7: Comparison of uptake rates between *E. coli* RP437 (wild-type swimmers) and RP5700 (serine-blind swimmers). Standard deviation bars have a high degree of coincidence in almost all cases.

samples deplete the volume in the flask, so at each time point the total volume used for calculating concentrations in the flask must be adjusted. By taking these factors into consideration, the results can be converted mathematically from disappearance in flasks to uptake into cells.

In Figure 6-7 serine uptake over one hour is plotted, after adjustments for changing volumes in the flask. By performing a linear regression on the triplicate data, uptakes rates were calculated for the two strains. The fitted lines are slightly offset vertically, possibly due to variations in the number of cells used in each experiment. Figure 6-8 illustrates mean values of the uptake rates. Three rates were calculated from the regressed line for each strain, and then the mean of the three rates was calculated for a value of $861.94 \pm 39 \text{ pmol}/(\text{min} \cdot 10^7 \text{ CFU})$ for the wild type strain and $882.84 \pm 44 \text{ pmol}/(\text{min} \cdot 10^7 \text{ CFU})$ for the serine blind strain. The mean uptake rates of each strain differ by only 2%. The spread of this rate data with corresponding standard deviations is less than 5% in either direction. This spread could have been lessened with more repetitions, as only three slopes (rates) were used per strain. However, in the following discussion section literature is cited which states that from a biological perspective, *tsr*-mutants such as RP5700 should not have defects in serine uptake. Finally, the uptake data fit the linear model well, generating R^2 values of 0.99.

6.4.4 Discussion

Because the serine-blind mutant strain in this study had not been previously tested, it was critical to design and execute an experiment which

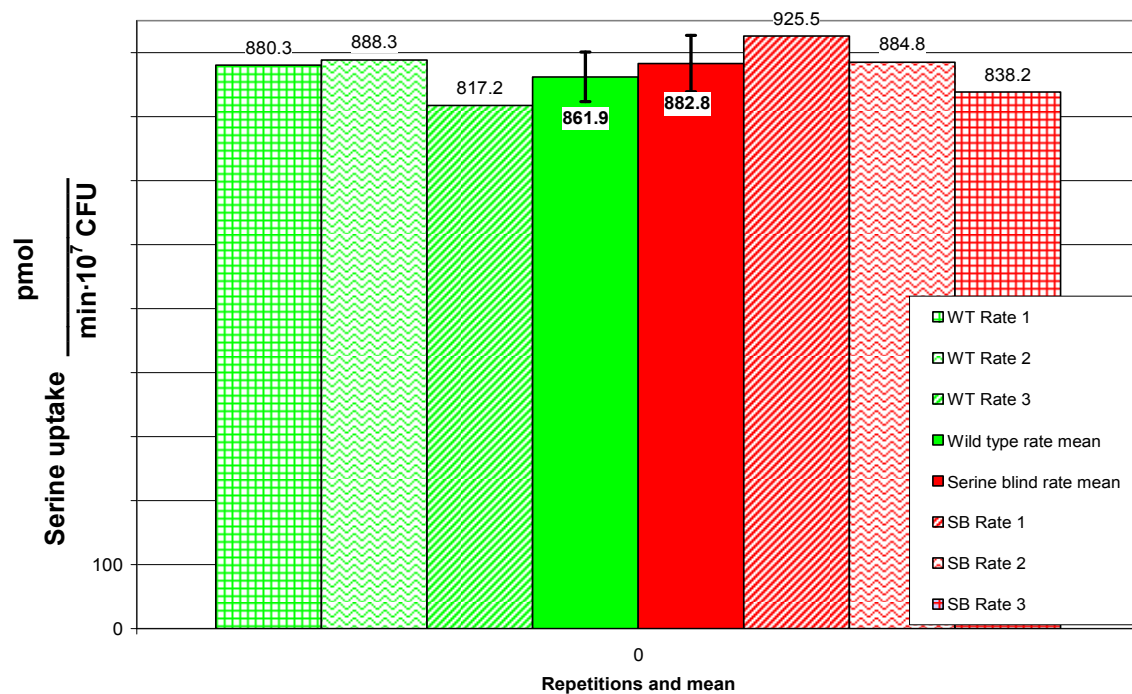


Figure 6-8: Bar chart depicting uptake rate data from the slopes of serine concentration vs. time. Values of individual repetitions are shown to the right and left of the mean values for the wild type and serine blind uptake rates.

would quantify long term uptake behavior, rather than simply instantaneous behavior. The results indicate that RP437 and RP5700 are ideal for use in degradation experiments due to their similar uptake rates. Because the intrinsic serine uptake rates of each strain are the same over a one hour time scale, no mathematical or other correction is needed when quantifying uptake rates in the long run times of the porous media experiments of this work. No change in rate over time is expected because the cells continuously encounter a serine concentration of 1mM as they swim through the porous media. These results are in agreement with three prior works (Hazelbauer, Mesibov et al. 1969; Clarke and Koshland 1979; Hedblom and Adler 1980) which state that *E. coli* that are defective in L-serine chemotaxis are not deficient in the uptake of this compound. The works state that the serine chemoreceptor is not a serine transporter and that the transport system for serine is separate.

6.5 Swimming parameters

6.5.1 Objectives

The objective of this experiment was to measure swimming parameters of the two strains used in this work. The parameters of interest in this section are swimming speed, run length, and turn angle.

Swimming speed is the scalar speed of the bacteria as they swim through a liquid medium. The run length, measured in time, is the length of time that a bacterium swims without executing a tumble. Turn angle is the angle formed when a bacterium swims in one direction, tumbles, and then swims in a new

direction. If these parameters are different between the two strains, the goal of measuring degradation between a cell exhibiting chemotaxis and one exhibiting random motility may not be achieved. Similarity of as many characteristics of the strains as possible is necessary for this study. Cases for non-equality of these parameters are listed below:

- If swimming speed is different, one strain may penetrate faster not due to chemotaxis, but due to swimming speed.
- If run length is different, one strain may be a smooth swimmer and could get jammed in the porous media
- If turn angle is lower for a strain, that strain may not spread in the same way as the other, exhibiting very tight turns in a small area if the turn angle is small.

In this section, the use of a three-dimensional tracking microscope to collect these parameters and compare them statistically is discussed.

6.5.2 Tracking microscope

6.5.2.1 Design

The tracking microscope was built in the early 1970s and has been described thoroughly in the literature (Berg 1971; Berg and Brown 1972). An illustrative schematic of the apparatus can be found in Berg (1971). Currently, the microscope resides at the University of Virginia in R. Ford's laboratory and data collection on swimming parameters was kindly provided by members of her lab.

Figure 6-9 shows a simplified schematic of the microscope. The microscope consists of box containing a fluid medium and several bacteria. The box is mounted on a stage which is moved by three drive coils that move in such

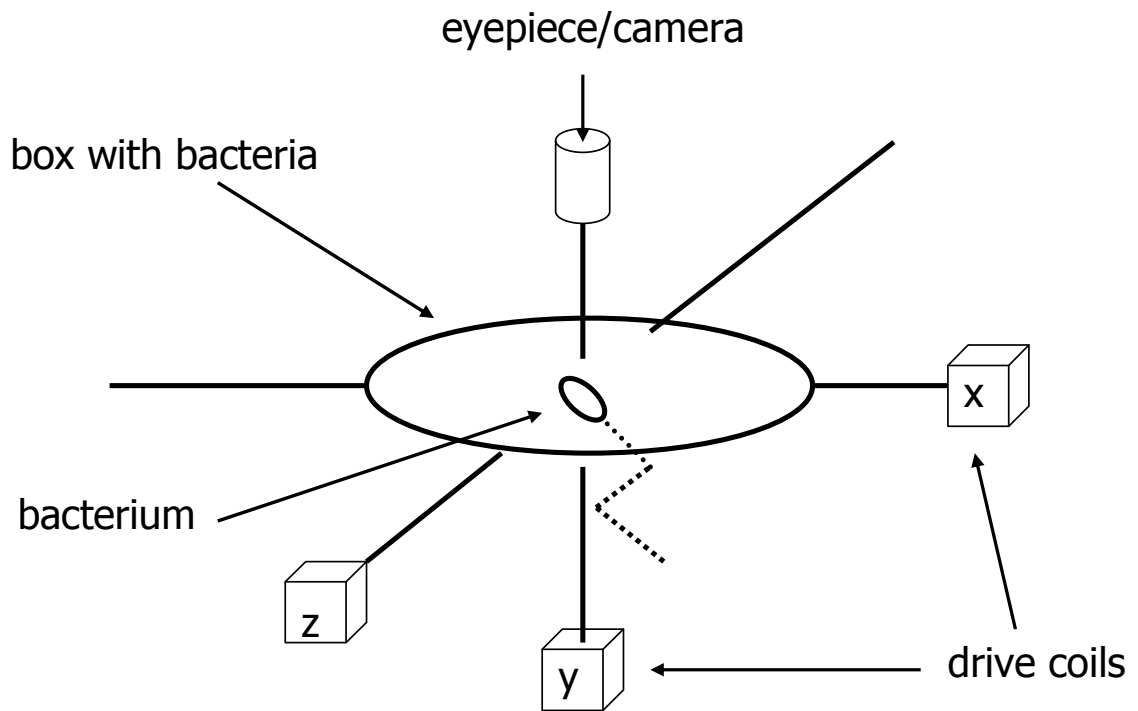


Figure 6-9: Conceptual schematic of tracking microscope. Shown are three axes, a box filled with medium and bacterium, and drive coils, which move the box while position data is collected with a computer. Adapted from Berg (1971).

a way that the bacterium of interest is kept in focus. As the stage is moved, data on the time and position of the bacterium in three directions is collected and stored on a computer. The data can then be analyzed to determine swimming speeds and run lengths. From the data, turn angles can also be calculated.

6.5.2.2 Tracking microscope methodology

For the tracking experiments, a working culture of each strain was prepared and the cells were washed via cell wash Method 1. Two types of growth media were used to grow cells: (i) a minimal media containing a phosphate buffer, glycerol, and the four critical amino acids for growth (glycerol minimal medium, see Appendix A), and (ii) WBT (tryptone). Upon washing, cells were resuspended in AWB to a level of OD₆₀₀ of 0.2 and then placed in the microscope chamber for analysis.

6.5.3 Results

Table 6-2 shows data collected on swimming parameters. The table is divided into three sections: speeds, runs, and turn angles. Under these headings, the data is further divided according to the type of media that was used to grow the cells: tryptone or minimal media (GMM). For each strain, the parameter value is shown as well as the number of data points that were collected by the tracking microscope for each parameter. Data points do not necessarily come from a single bacterium. A number of bacteria may have been tracked for a given set of data points. The number of data points varied from 17 to 193. This variation was caused by the ease at which the microscope was able to stay locked on a cell.

Table 6-2: Results of microscopic swimming measurements.

Strain	Speeds ($\mu\text{m/s}$)		Runs (s)		Turns (?)	
	<i>Tryptone</i>	<i>Minimal media</i>	<i>Tryptone</i>	<i>Minimal Media</i>	<i>Tryptone</i>	<i>Minimal media</i>
Wild type (RP437)	22.0 ± 6.7	13.8 ± 2.2	1.19 ± 1.09	1.88 ± 1.37	83.3 ± 49.7	55.2 ± 32.3
<i>Number of data points:</i>	<i>30</i>	<i>17</i>	<i>153</i>	<i>149</i>	<i>145</i>	<i>148</i>
Attractant blind (RP5700)	23.9 ± 6.4	12.5 ± 2.3	1.39 ± 1.18	0.716 ± 0.85	66.8 ± 34.7	77.9 ± 45.5
<i>Number of data points:</i>	<i>54</i>	<i>27</i>	<i>172</i>	<i>193</i>	<i>169</i>	<i>193</i>

Occasionally, cells would drift out of the range of the microscope.

Similarity of the data between strains is excellent. Figure 6-10 shows the data of Table 6-2 in graphical format. For the swimming speed measurements with cells grown on WBT, the difference between measurements is indistinguishable. The standard deviations, depicted on the data bars, have a high degree of coincidence. Swimming speed measurements in the minimal media are also highly correlated, however, it is interesting to note that the swimming speeds were higher in WBT than in minimal media. Discovery of this led to a switching of growth media from the minimal media to WBT for the remainder of the experiments of this work, because it was desirable to have cells that were as active as possible. Run lengths for the two strains in WBT are also very highly correlated. Run lengths, or times, are represented with a Poisson distribution. For this distribution, the variance is equal to the mean of the data (Walpole and Myers 1993), and standard deviations were calculated by take the square root of the mean, or variance. For data distributed by Turn angles, which affect the way bacteria are distributed as they swim, were similar among both media and strain types.

Further evidence that swimming speeds of the two strains are higher when grown in WBT is shown in Figure 6-11. These histograms show mean swimming speeds that are higher when cells were grown in tryptone media. The histograms were generated by sorting each of the given swimming speeds into bins. It can be seen that bins for cells grown in WBT had a greater number of points at

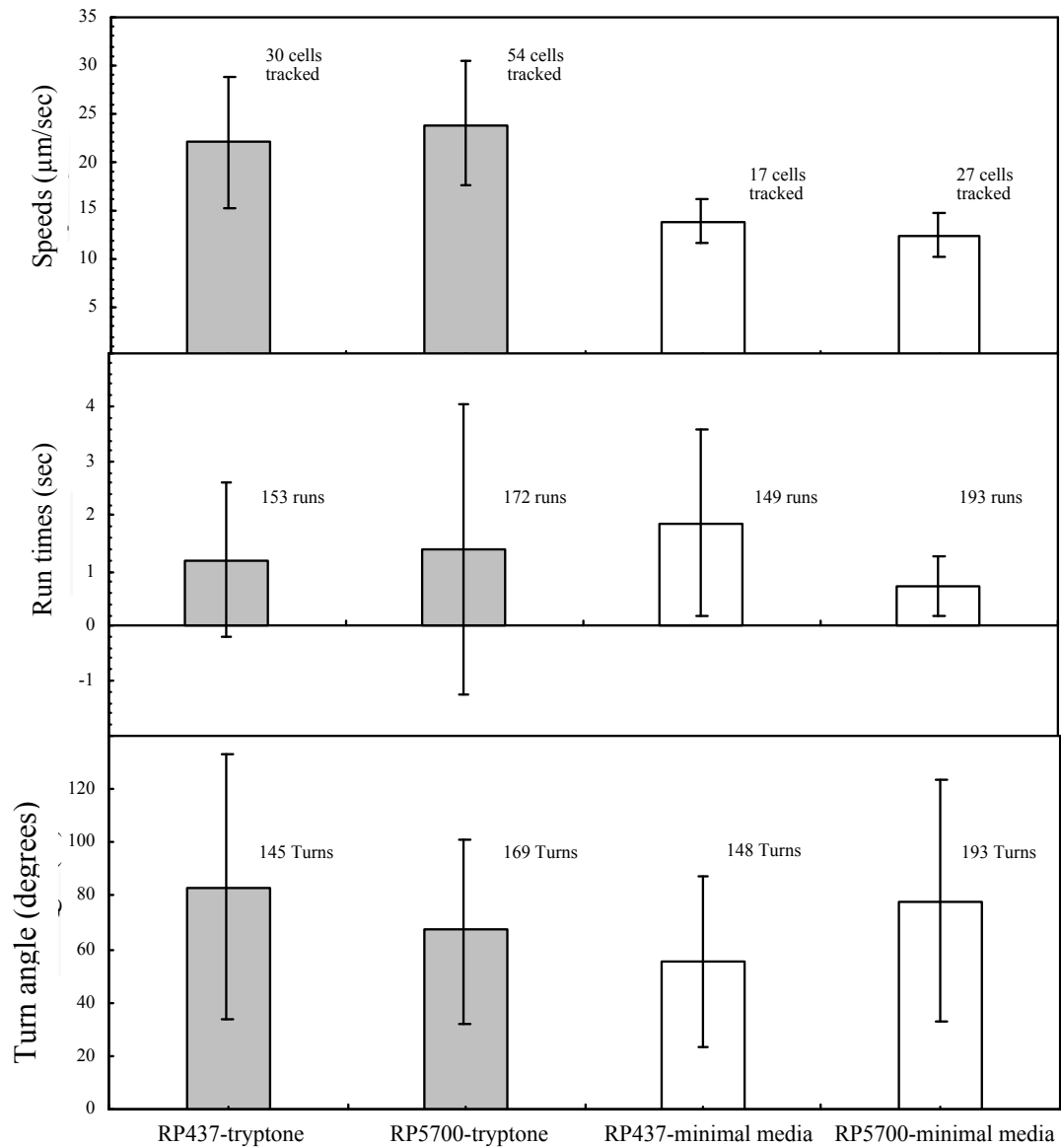
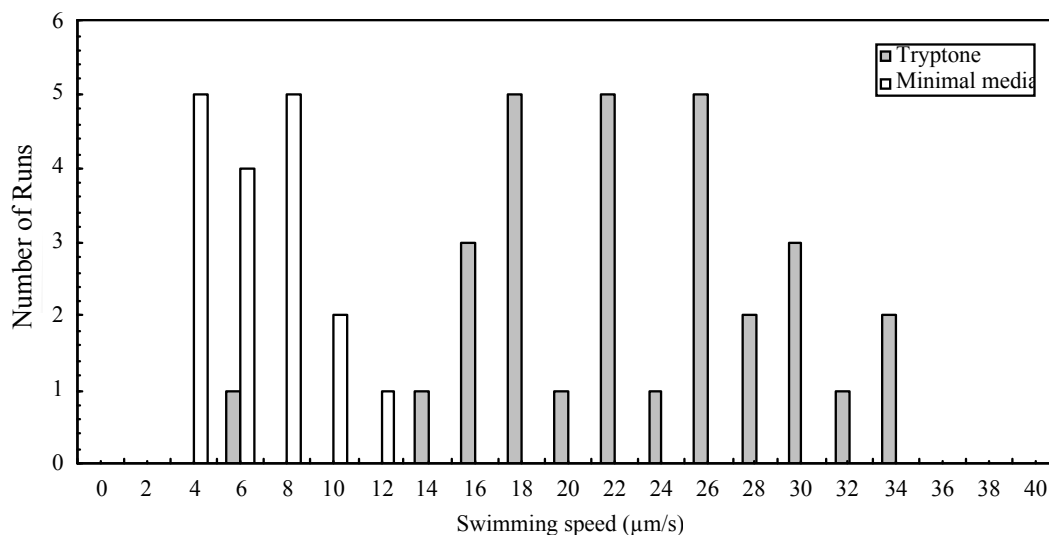


Figure 6-10: Results of microscopic swimming measurements of swimming speed, run times, and turn angles for two different growth media: tryptone and a glycerol minimal media containing the four necessary amino acids for growth

a. Swimming speed distribution for chemotactic *E. coli* RP437:



b. Swimming speed distribution for attractant blind *E. coli* RP5700:

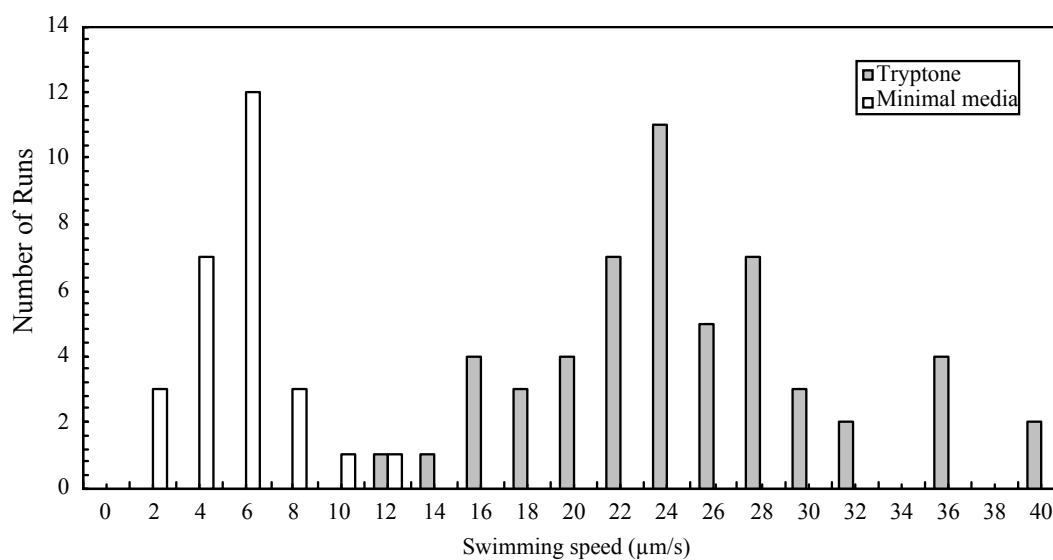


Figure 6-11: Swimming speeds for *E. coli* strains - comparison of growth media. Results show that cells were more active in the tryptone media. This led to a change in growth media to supply more active cells.

higher swimming speed bins than the number of data points in the bins for cells grown in minimal media.

6.5.4 Discussion

It has been shown in this section that the swimming parameters of speed, run time, and turning angle are statistically similar for both strains. This provides further evidence that this pair of strains is appropriate for experimentation in comparing chemotactic behavior with random motility in bacteria. It was also observed that cells grown in WBT have higher swimming speeds, and are generally more vigorous than cells grown on a minimal medium. One reasoning for this difference in behavior is that the mutations in the strains affected some other aspect of the motility machinery that requires a nutrient other than that provided by the minimal media. Although the strains grow in the minimal media, they are not as energetic as those grown in WBT.

6.6 Conclusion

The various experiments presented in this characterization section demonstrate that the two strains have similar optical properties (standard curve), serine uptake rates, swimming speeds, run lengths and turning angles. This is reasonable considering the fact that RP5700 is mutant of RP437. Apparently, the mutation in RP5700 was not significant enough to alter properties relevant to the testing of random motility and chemotaxis.

7 CHEMOTAXIS EXPERIMENTS

7.1 Introduction

This chapter presents experiments which address the fundamental hypothesis of this dissertation: Does chemotaxis make a difference for ISB? Four critical experiments are presented:

- Swarm plate chemotaxis assays to elucidate increased migration due to serine.
- Capillary experiments to determine the optimum concentration of serine to use and confirm chemotactic differences between the strains.
- Novel model aquifer degradation experiments to compare serine degradation in a porous medium.
- Novel model aquifer migration experiments to explore migration rate differences.

These experiments will provide guidance into future research on chemotaxis effects on ISB.

7.2 Swarm plate chemotaxis assays

Rates of formation of swarms on swarm plates served as a confirmation assay for chemotaxis by the two strains used in this study. This assay was an initial test for chemotaxis by *E. coli* RP437 and has been used before with *tsr*-strains such as *E. coli* RP5700 (Parkinson 1980). Figure 7-1 shows chemotaxis

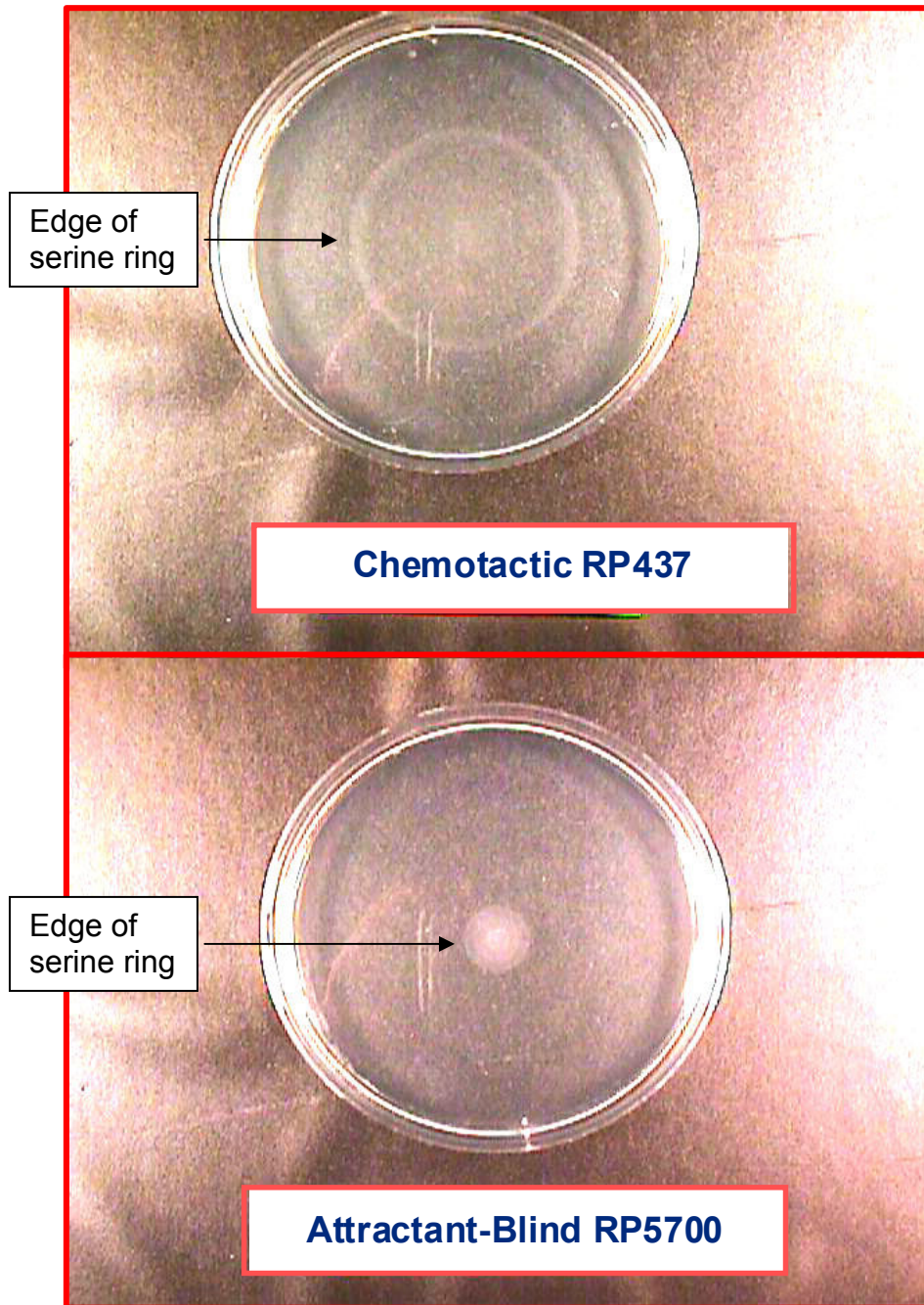


Figure 7-1: Chemotaxis ring formation on swarm plates. These rings compare motility and growth by *E. coli* strains RP437 and RP5700. Rings are formed by swimming and growing cells. As shown, serine causes significant migration of the chemotactic cells when compared to the non-chemotactic RP5700 cells.

ring formation on swarm plates. These rings compare motility and growth by RP437 (chemotactic to serine) and RP5700 (serine blind – lacks serine chemoreceptor).

These plates consist of a light tryptone agar (see Appendix A) containing fluid-filled pore spaces for migration. The plates are inoculated by adding 5 µl of cell suspension in the center of the plate and incubating the plate as the ring forms. Rings are formed by swimming and growing cells. The cells are attracted to several components in the tryptone, but the larger ring in the top photo indicates enhanced migration and growth due to the component serine, which is found in tryptone and is the model “contaminant” of this study. It is also the cause of the outermost ring on the RP437 swarm in Figure 7-1, as the cells consume the serine first (Armstrong, Adler et al. 1967). As shown, serine causes significant migration of the chemotactic RP437 cells over the non-chemotactic RP5700 cells and verifies that growth-coupled chemotaxis is occurring with RP437.

Figure 7-2 shows the swarm diameter versus time. After 10 hours the ring size of the RP437 cells were 53% larger than the RP5700 cells. Cells were grown in glycerol minimal medium (GMM, see Appendix A). Ring data from a prior work (Wolfe and Berg 1989) for RP437, is shown for comparison. It can be seen that the rate curves have similar shapes also that ring formation was faster when cell cultures were grown on tryptone media. Thus, the assay successfully confirmed chemotaxis by RP437.

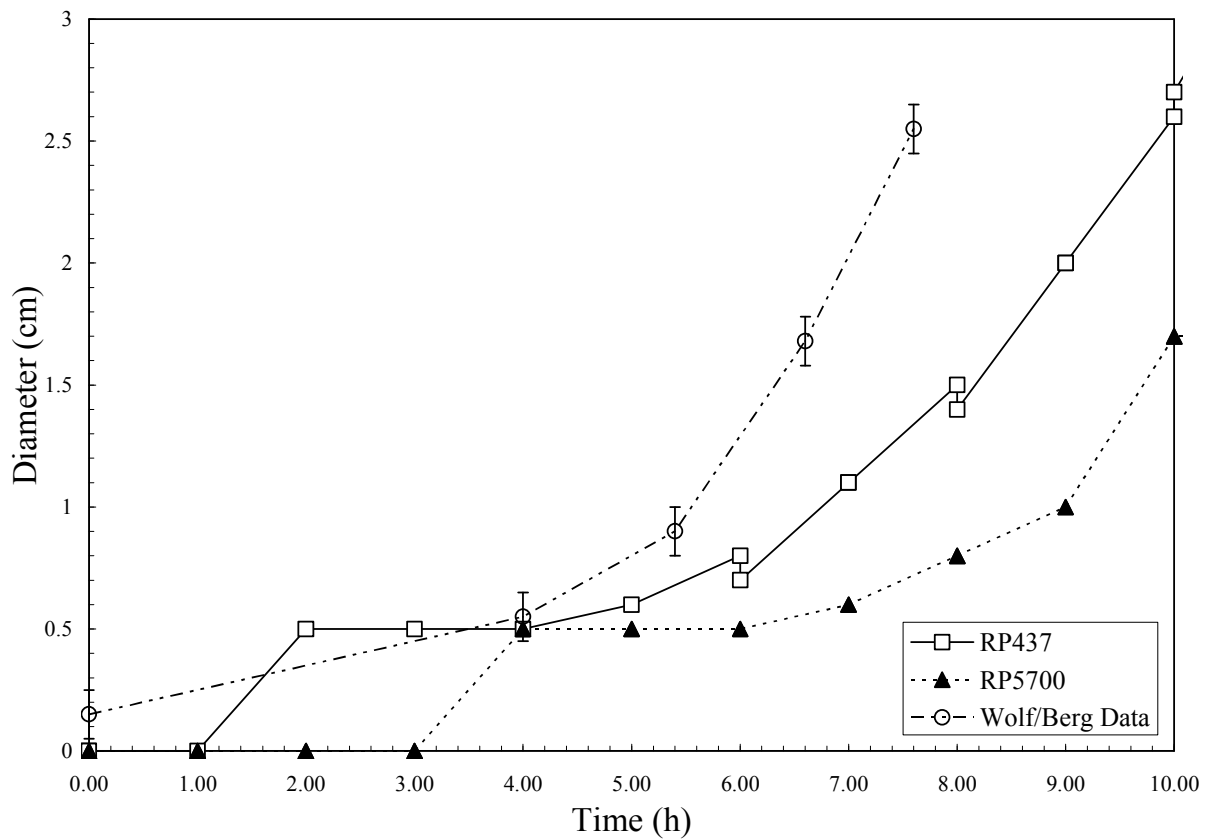


Figure 7-2: Swarm diameter vs. time. Data on ring growth rates of RP437 from Wolf and Berg (1989) is shown for comparison. The higher rate in the Wolf and Berg study is likely due to the fact that cells were grown on tryptone in that study.

7.3 Capillary experiment

The capillary assay is a method for enumerating the response of a bacterium to a chemoattractant under a wide range of conditions. Figure 2-6 shows a schematic of the classic Adler experiment. The goal of this experiment was to determine an optimum serine concentration to use for model aquifer experiments.

Guided by the serine concentration level of 1 mM, above which no additional cells are attracted into a capillary that has been reported (Adler 1966; Adler 1969), serine concentrations in AWB were chosen at 0.1 mM, 1 mM and 10 mM. To begin the experiments, 500 μ l of bacterial frozen stock was grown overnight in 25 ml GMM to mid-exponential phase (0.3-0.8 OD₆₀₀). A working culture was then grown with fresh GMM to an OD₆₀₀ of 0.4. U-tubes, microscope slides, and microscope coverslips were then flame sterilized and the U-tubes were set up. One end of the 1 μ l capillaries (Drummond Scientific) was then flame sealed. Cells were then washed using cell wash Method 1 at 8000g. The final culture was then normalized to 5×10^7 CFU/ml (OD₆₀₀=0.1). Sterile test tube caps were then filled with AWBS at 0.1 mM, 1 mM, or 10 mM. The capillary tubes were then passed 10 times over a flame, and plunged into the caps containing the AWBS. The tubes were allowed to cool for 10 minutes, and as they cooled, the AWBS was drawn into the bore of the capillaries by the cooling, contracting air inside. At this point, the microscope slides containing the U-tubes were prepared with the cell suspension and the capillaries were inserted into the U-

tubes. The capillaries were allowed to sit for one hour, after which time the capillaries were removed from the U-tubes, washed with sterile deionized water, and broken at the sealed end with a pair of pliers. A small rubber bulb was then used to squirt the contents into dilution tubes. The contents of each tube were then properly diluted and spread on Luria broth plates. Plates were allowed to grow overnight at 37°C until colonies were large enough to be counted.

Results are shown in Figure 7-3. On the horizontal axis is shown three different concentrations for RP437, RP5700 (control) and an abiotic case. On the vertical axis is CFU/capillary which is the number of CFU which were counted after the contents of the capillary tube were emptied and enumerated via streaking on LB plates. The results shown are triplicate runs, and standard deviations are shown. The highest response was observed for the chemotactic strain at 1 mM serine (16,500 CFU), and this concentration was used in the remainder of the experiments in this work. A decrease in bacterial accumulation is observed at the 10 mM concentration of serine. This is likely due to a high level of binding of serine to the chemoreceptors. If too many of the chemoreceptors are bound to serine, the cell is unable to respond chemotactically. It becomes blinded to the gradient of the serine, and does not decrease its tumbling frequency in the direction of the increasing gradient.

These results also verify that the control strain is deficient in chemotactic ability, as do the swarm plate results in the previous section. The abiotic cases, performed exactly as the others but without live cells, demonstrate that sterile conditions were achieved in the experiment.

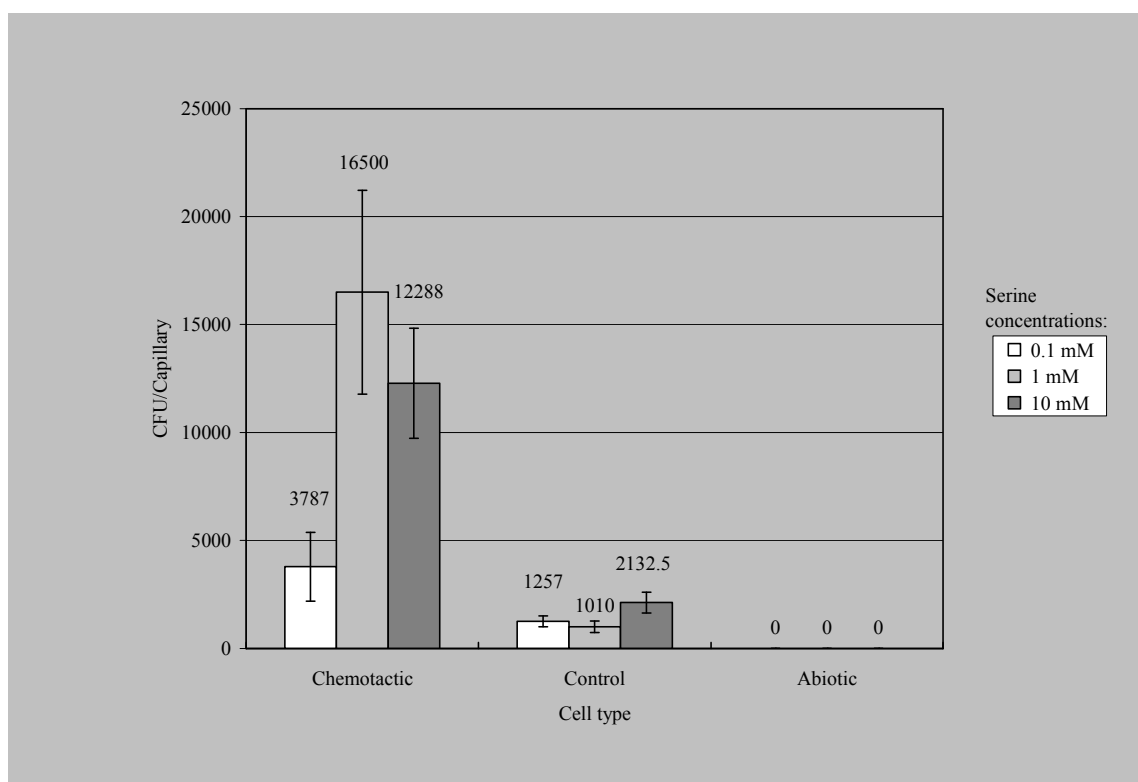


Figure 7-3: Capillary assay results for chemotactic RP437 and serine blind RP570 (control) strains. The results shown are triplicate runs, and are presented in terms of CFU/capillary. The highest response was observed for the chemotactic strain at 1 mM serine.

7.4 Chamber degradation experiments

In this section, the model aquifer of this work, the bacterial chemotaxis chamber (BCC), will be discussed. Development of the chamber will be described briefly, followed by a discussion of the experimental methodology used to perform serine degradation experiments. Results and implications of those results will also be discussed.

7.4.1 Objectives

The objectives of these chamber degradation experiments are fourfold:

- Rigorously measure biodegradation in a porous medium.
- Develop a method for creating a sharp gradient in wet sand that can be modeled mathematically.
- Develop a reproducible experimental methodology.
- Develop a consistent sampling and analytical methodology.

As explained in the Background section, much experimentation has been done in chemotaxis in porous media, but only a few studies have actually measured the extent of biodegradation by chemotactic bacteria. When discussing ISB, level of degradation is a key variable, and this section addresses this issue.

Working with saturated porous media has posed problems. It is difficult to handle, and attempts to form gradients have failed because it is very easy to entrain air when working with wet sand. Over the course of four designs, a

method which creates a sharp gradient has been developed. The experiments in this section also have high reproducibility, as evidenced by the standard deviation bars on data collected. The experiments described here include a reproducible sampling and analytical methodology. It is hypothesized (Chapter 3) that the chemotactic bacteria will be able to sense the serine and bias their motion towards it, thus degrading more serine than the serine-blind strain as the front of bacteria penetrates the sand.

7.4.2 Development of Bacterial Chemotaxis Chamber

The BCC represents an evolution of several ideas developed over time. There are several characteristics that a chamber for degradation measurements in a saturated porous media must include. The most important objective in the design of the chamber and of the experiment as a whole is to create conditions that will optimize chemotaxis and motility so that chemotactic behavior can be readily compared with non-chemotactic conditions.

Because it is desirable to model the chamber mathematically, the ability to create a sharp gradient of chemoattractant and bacteria in wet sand is required. A step gradient is easy to model mathematically, so different designs were used to find the most effective way to generate a sharp gradient. The chamber must be simple enough to assemble and use to allow for several replications. It is also desirable to maximize chemotaxis and motility of the bacteria by providing enough oxygen by aerobic assembly to sustain viability and motility. Evaporation control is also required, to maintain the mass balance for serine quantification.

Two different prototypes were used prior to the final design of the BCC. The first design used an oxygen permeable film to contain the bacteria, liquid media, and sand. It was thought that the oxygen permeable film would result in more rigorous swimming due to oxygen transfer. Although serine degradation was achieved in this prototype, it was difficult to assemble and was abandoned for the second prototype, a simple polymer trough which contained the components of model aquifer in an open air design. This design was also abandoned because of difficulty creating the gradient. A support for the acetate partition, used to separate the bacteria from serine initially, was needed, and this can be found in the most current design, the BCC.

Figure 7-4 shows a schematic of the BCC, the culminating design of this work. The chamber was constructed from a recycled polymer box used for packaging molecular biology reagents. The height of the box was reduced by milling, and the final dimensions of the chamber were 4 $\frac{1}{8}$ " long, $\frac{3}{4}$ " wide, and $\frac{3}{4}$ " tall. The figure shows that one side of the chamber holds the serine and the other side contains bacteria. The figure also shows a raised acetate partition, which in the lowered position served to segregate the bacteria and serine sides of the chamber during loading. In the original box before milling, there was a partition in the middle of the box that was milled out to create a frame to hold the acetate partition in place. This milled out *frame* can be seen in the photograph of Figure 7-5. The acetate partition is also showed in this photograph, along with a string used to lift the partition at the beginning of the run. The partition is held in place and sealed by Vaseline administered via a needle and syringe prior to the run.

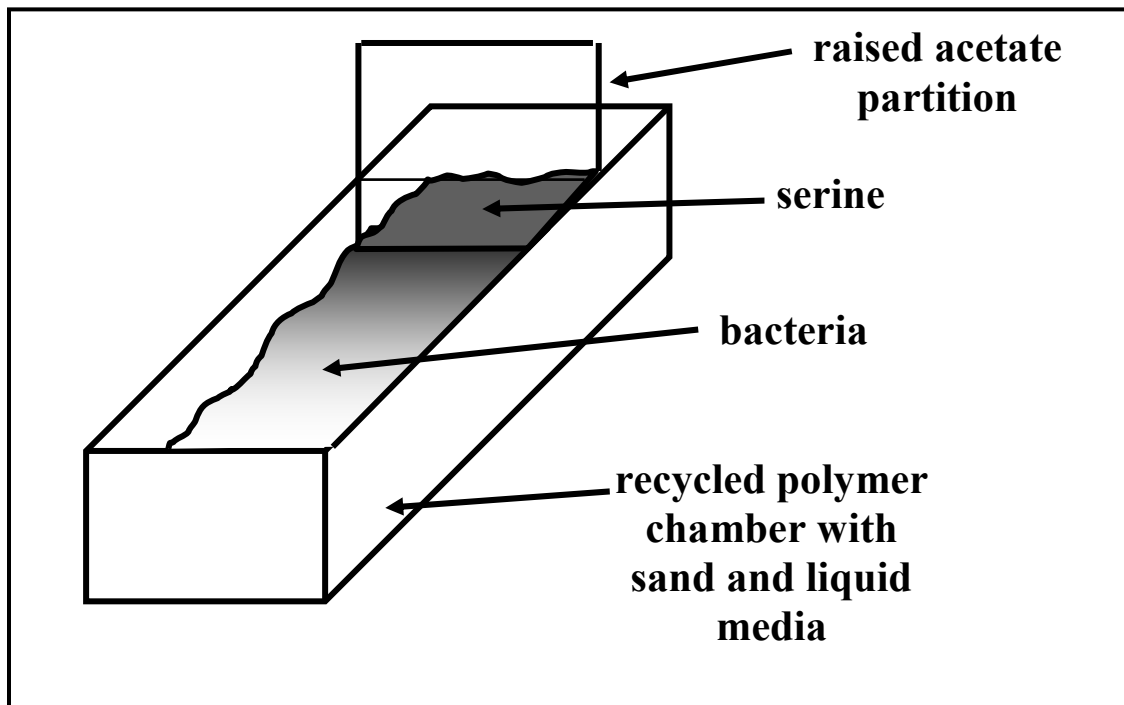


Figure 7-4: Bacterial chemotaxis chamber schematic. The chamber is filled with 600 – 710 μm sand. The partition frame is not shown in this schematic.

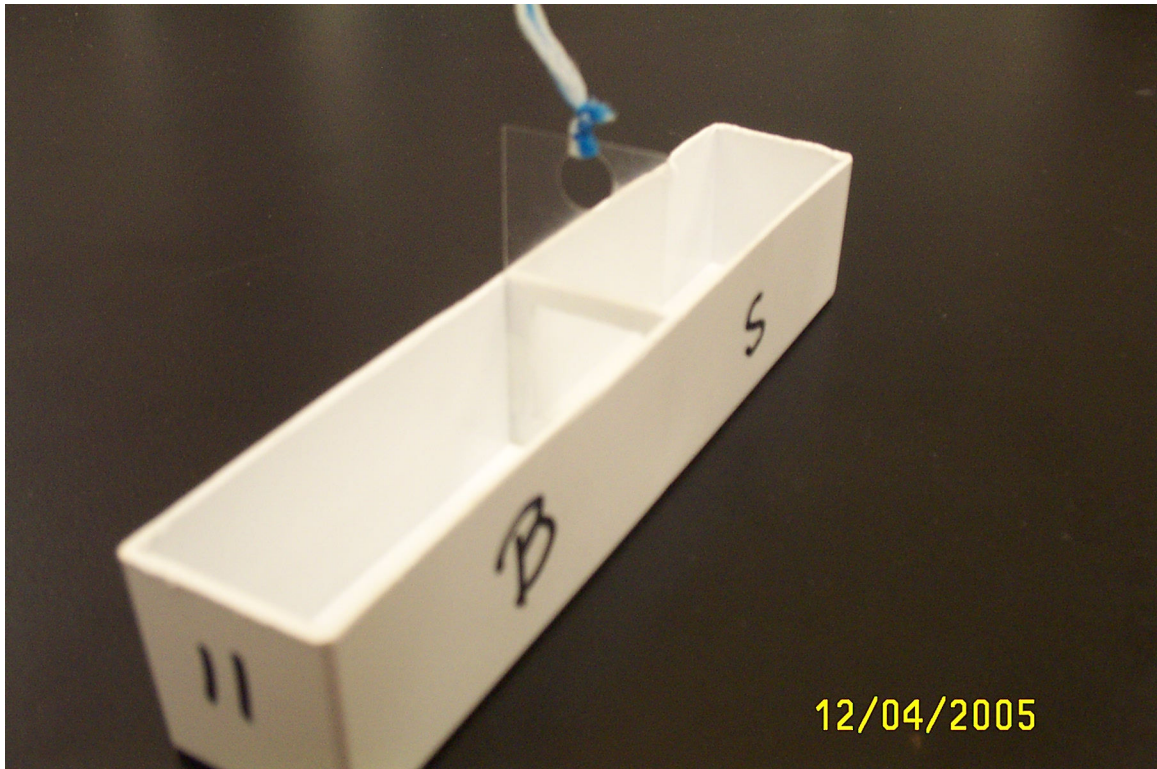


Figure 7-5: Bacterial chemotaxis chamber (BCC). The partition is shown attached to the partition frame. The string is used to gently raise the partition while reducing torques that would disturb the sand.

Below is a summary of the experimental details:

- Porous media is silica sand, 600-710 μm particle size (see Section 4.2). This particle size was chosen as an optimum because it represents a diameter over 100 times larger than the bacteria themselves, providing a minimum level of interference to bacterial motility.
- Bacteria are two strains of *Escherichia coli* - one chemotactic (RP437) and one serine-blind (RP5700).
- Chemoattractant is a model chemical contaminant, the amino acid serine.
- The gradient is formed in the sand filled BCC, saturated with bacteria in AWB on one side and AWBS without bacteria in the other.

The partition is then removed and degradation of the serine begins as the serine diffuses towards the bacteria and the bacteria diffuse and swim towards the serine.

7.4.3 Bacterial Chemotaxis Chamber Methodology

The experimental procedure for running the BCC experiments is presented here. The complexity of the experimental details required the use of detailed logsheets for each run. A sample logsheet is included in Appendix C for reference. Critical details of the procedure are outlined below.

7.4.3.1 Preparation of BCCs

To prepare the BCCs for a run, they were first cleaned and sterilized. The cleaning procedure consisted of washing the chamber with Sparkleen laboratory glass cleaner and deionized water, followed by rinsing with pentane to remove any residual Vaseline from the sealing of the partitions. The chambers were then sterilized by dipping into a 70:30 ethanol:water bath and dried under vacuum.

The acetate partitions were washed in a similar manner, and they were applied to the partition frame on the chamber using Vaseline and gentle pressure under aseptic conditions (see Figure 7-5 for a view of the acetate partition in place). Abiotic runs showed that minimal serine disappearance occurred after 21 hours, indicating that the procedure was satisfactory and there was minimal absorption of the serine to the sand or the chamber.

7.4.3.2 Loading of BCCs

Figure 7-6 shows a flowchart of the chamber loading procedure. A working culture of cells was grown and washed using cell wash method 2, filtration. The suspension was normalized to a concentration of 2×10^8 CFU/ml. The *bacteria side* of the chamber was loaded first with 6 ml washed cells. Sterile sand was then added and the chamber was checked for any air pockets to maintain saturated conditions. Any such pockets would produce irreproducible irregularities and generate an undesirable three-phase system.

After the chamber was filled to the top with sand and liquid media, the *serine side* of the chamber was filled with 6.2 ml AWBS at 1 mM serine. Again sand was added to the top of the chamber and air pockets removed. The chamber was tapped lightly to settle the sand until all of the sand particles were wet. At the start of the experiments, the partitions were raised by grasping the string and pulling gently to avoid disturbing the sand. After a few more taps to settle the sand and fill and voids that may have been left by removal of the partition, the chambers were placed in a humidified incubator at 30 °C for the

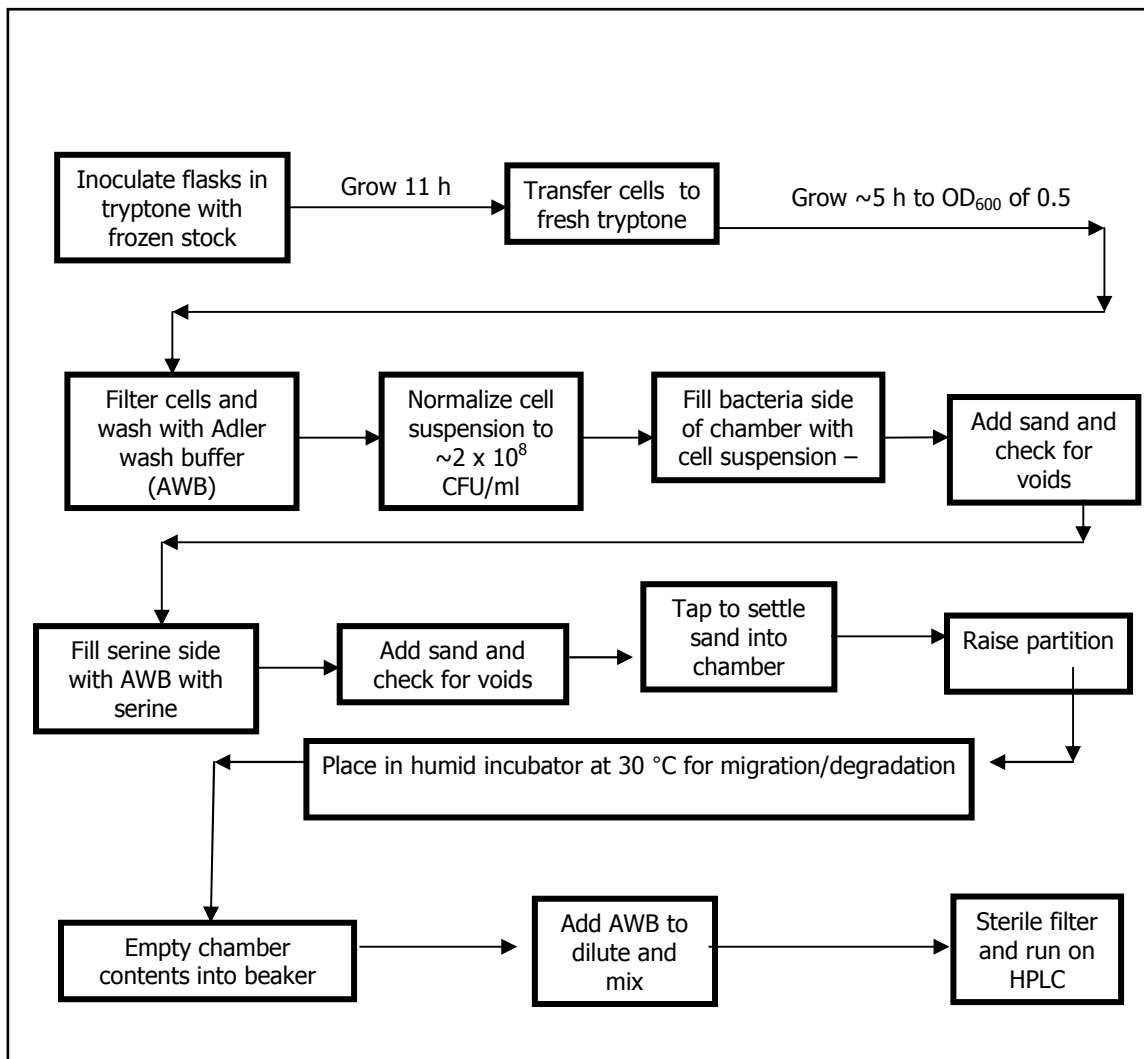


Figure 7-6: Bacterial chemotaxis chamber serine degradation sampling protocol.

desired degradation time. This temperature was chosen because it was reported that an increase in temperature from 20 °C to 30 °C resulted in a twenty-fold increase in chemotaxis response (Adler 1973). The incubator was humidified by placing the chambers first in a dessicator filled with water beneath a holding stage, followed by insertion of the dessicator into an incubator. This setup was satisfactory, as it led to an evaporation loss of less than 0.5% in the chambers.

7.4.3.3 Sampling of BCCs

After weighing the chambers for evaporation loss, the entire contents of the chamber were emptied into a beaker and 8 ml of AWB was added to provide a wetter environment for mixing of the chamber contents. An important feature of this method is the determination of the bulk serine content, as opposed to sampling by slices as other works have done (Barton 1994; Kunkler 1994). This allows one to determine total serine loss due to biodegradation, irregardless of the concentration profile of the bacteria or serine.

Upon addition of the AWB, the beaker contents were stirred for 30 seconds. At this point, a 3 cc syringe was used to withdraw a sample of the contents. The needle was then removed, and a Whatman 0.2 µm pore size, 13mm polysulfone syringe filter was attached, and the sample filtered into a cryovial. The sample was then stored at -80 °C until HPLC analysis could be performed.

7.4.4 Results

Figure 7-7 shows the results of the BCC chamber degradation over a 21 hour time course. On the vertical axis is shown concentration of serine in mM. Samples were taken at zero, nine, 15 and 21 hours. Each time point represents quadruplicate repetitions. After 21 hours, a degradation level of 18% percent was achieved for both strains. At 15 and 21 hours, degradation levels of both strains have a high degree of coincidence in the amount of serine that each degraded and in the standard deviations calculated for each run. At nine hours, there was more offset in data, and the wild type strain seemed to degrade more serine. The degradation curves seem to have the shape of an enzymatic reaction (Shuler and Kargi 1992). The enzyme in this case for conversion of serine to pyruvate is L-serine deaminase, assuming aerobic conditions. The experiment was halted after 21 hours because microscopic observations showed that the cells had a low motility, indicating that the chambers may have gone anaerobic. *E. coli* can degrade serine anaerobically, however, so differences in degradation due to motility of cells would not be a factor if the cells stopped moving. Triplicate abiotic time points were taken at 21 hours, demonstrating only a 2% loss of serine due to the experimental procedure.

7.4.5 Discussion

The parameters chosen for this experiment such as particle size, starting serine concentration, temperature, aerobic chamber preparation, and cell density were

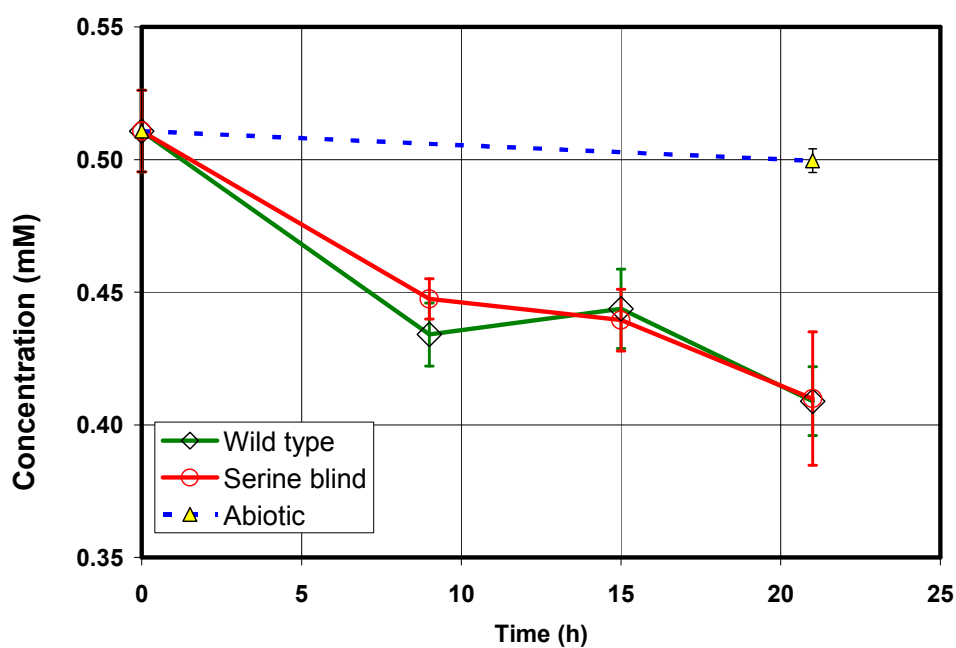
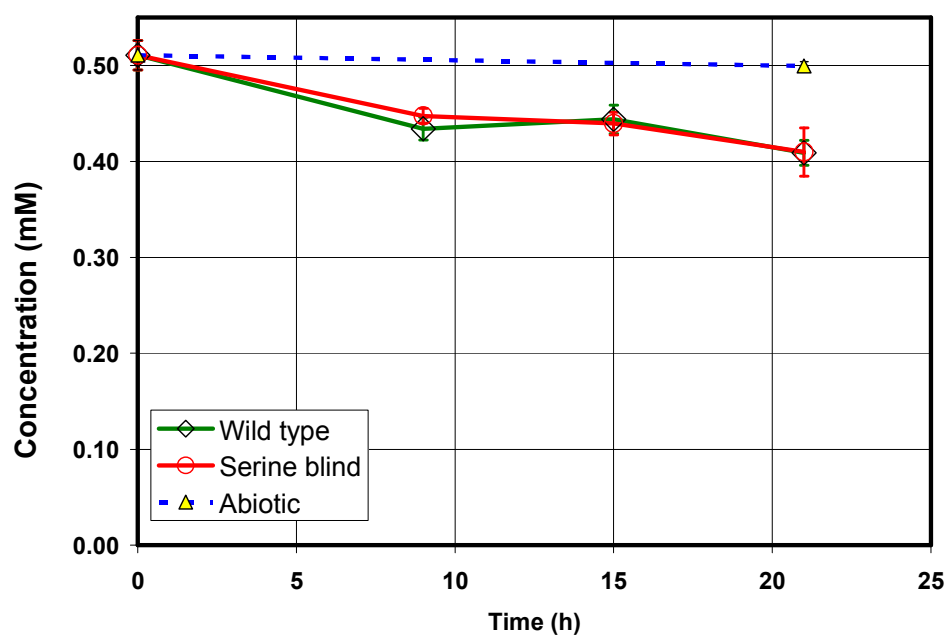


Figure 7-7: Serine degradation in the bacterial chemotaxis chamber. A full scale image of the data is shown in the top figure, and higher resolution image is shown in the bottom. The green lines represent the wild-type runs and the red lines represent the serine blind runs. Error bars shown on the time points are standard deviations for the quadruplicate runs.

all chosen to optimize the potential for chemotaxis and motility of the cells.

Overall, it appears as though there is not a difference in the amount of serine degraded by the two strains, given the parameters chosen for this study. However, the four objectives of this experiment have been achieved. First, biodegradation of a model contaminant was rigorously measured. Secondly, a repeatable method for generating a sharp gradient in wet sand was developed. Thirdly, reproducibility was achieved as evidenced by the standard deviations in Figure 7-7. And finally, a consistent sampling and analytical (HPLC) methodology was developed.

7.5 Chamber migration experiments

In support of the BCC chamber degradation experiments on effects of chemotaxis on degradation rate, an additional experiment was developed to complement this work and measure the rates of migration of *E. coli* through the sand under chemotactic and non-chemotactic conditions.

7.5.1 Objectives

The objectives of these chamber migration experiments were threefold:

- Develop a novel sampling system for migration in the BCCs.
- Calculate penetration rates of cells through the porous media.
- Compare results with literature values of penetration rates.

It was hypothesized (Chapter 3) that the chemotactic bacteria will be able to sense the serine and bias their motion towards the serine side of the chamber, thus penetrating the porous media at a higher speed than the serine-blind strain.

7.5.2 Migration sampling frame

The migration sampling frame (MSF) was developed to allow reproducible bacterial sampling of liquid contained in the pore spaces of the sand in the BCC. In the development of the MSF, an early prototype used a wooden toothpick array. This array was dipped by hand into the wet sand with the idea that fluid absorbed by the wood could be transferred to a medium which would allow the growth of cells, giving a picture of how far the cells traveled by counting the number of toothpicks that supported growth.

This toothpick array led to inconsistent results and large spreads of data in penetration distance, possibly due to disturbances in the sampling of the sand. Static conditions during sampling are necessary to prevent false readings of bacterial presence. During sampling with the toothpicks, large holes were formed which disturbed the static fluid. This may have caused the toothpicks to contact bacteria by convection of fluid, rather than by contacting bacteria in a static fluid. A more stable, less-intrusive method was called for, which is why the MSF was developed.

7.5.2.1 Design

Figure 7-8 shows a schematic and photograph of the MSF apparatus. The schematic shows a cross section of the BCC during sampling. At the center of the photograph is the BCC, the white chamber labeled “B B S.” The BCC is resting in the chamber platform, which is a six compartment holding stage that can hold up to six BCCs at a time. Above the BCC is the syringe bank. The syringe bank consists of 10 1cc syringes, milled to fit tightly together and held together by a blue clip. This blue clip provided stability which allowed all 10 plungers, encased and epoxied in a wooden clip at the top of the syringe bank, to be moved simultaneously. This clip was held in place by metal hold-down bars to the left and right of the blue clip. The syringe bank is shown immediately before a sample is to be taken.

The benefits of this design are severalfold. The frame provides stability to generate repeatable samples without producing hand-sampled sideways torques that may disturb the static chamber contents. The small syringe needles also minimize disturbances to the sand, and the tight packing of them gives the ability to sample 10 different points in the BCC at a time. The syringe bank also provides easy sterilization via pumping 70:30 ethanol:water solution repeatedly throughout the needle and syringe bodies.

7.5.2.2 Sampling methodology

The experimental procedure for running the MSF experiments is presented here. Preparing the BCCs for MSF experiments was very similar to the

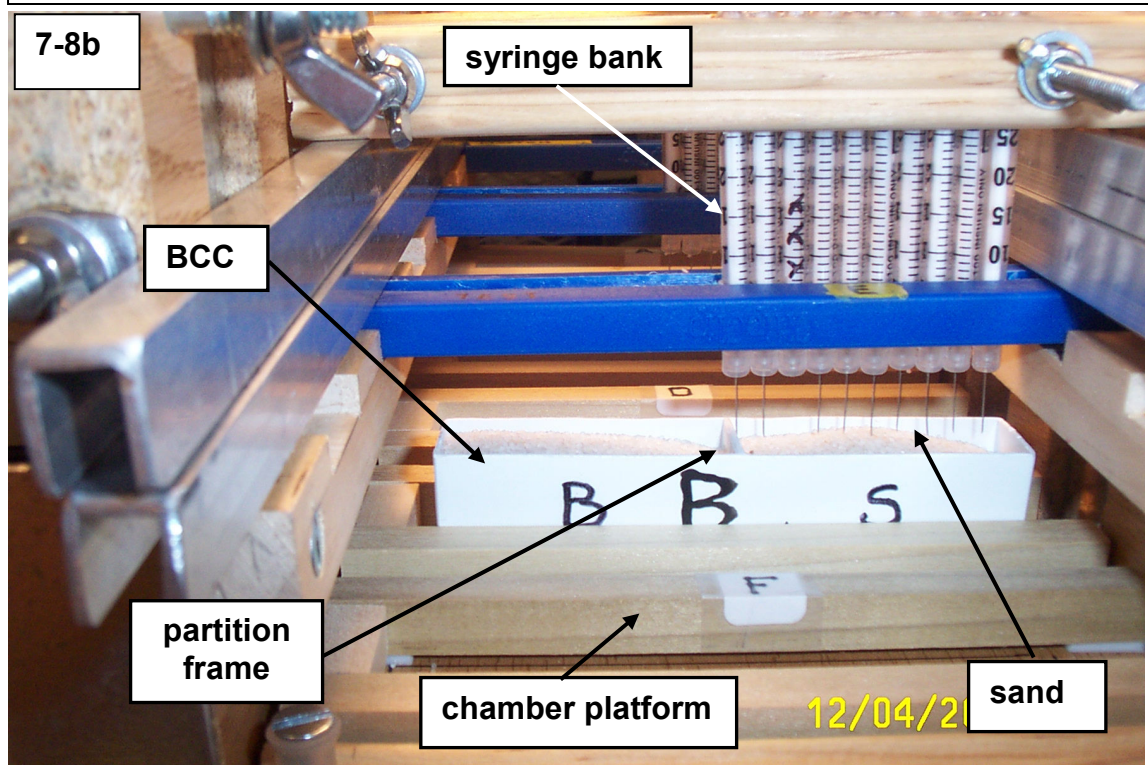
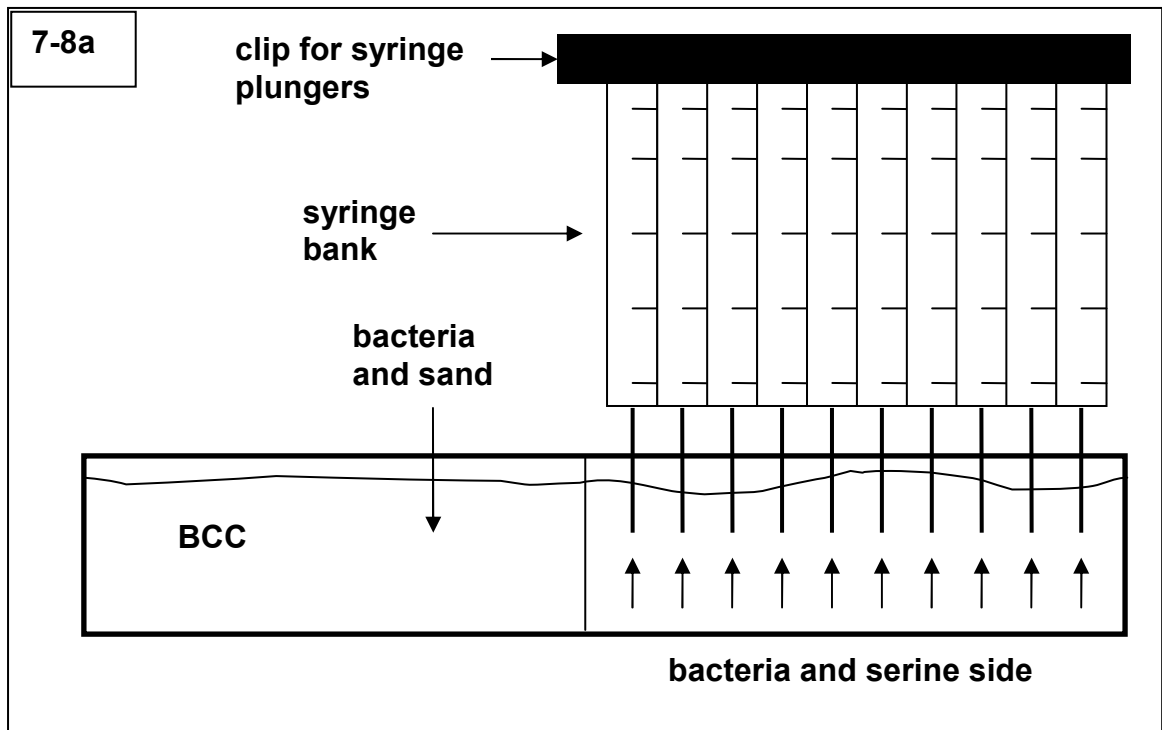


Figure 7-8: (a) Migration sampling frame, schematic. The arrows indicate flow into the syringes. **(b)** Interior view of BCC about to be sampled with the frame.

method used for the chamber degradation experiments (see Section 7.4.3.1 and Section 7.4.3.2). Prior to each run, all parts of the MSF were carefully sterilized with a 70:30 ethanol:water mixture. After the BCCs were prepared, loaded and incubated, the MSF sampling procedure was begun. This procedure began by the insertion of the BCCs into the chamber platform, which was designed to lock each BCC in place for sampling. Typically, four BCCs were inserted and sampled at a time. Upon securing the BCCs in place and aligning them with the needles of the syringe banks, the chamber platform was slowly raised via a built-in, screw-type laboratory jack, until the syringe bank needles penetrated the sand. The serine side of the BCCs were sampled to determine the extent of bacterial migration through the BCC into the sand containing AWBS. Penetration was measured by positive growth of bacteria in a subsequent incubation step. Each needle was roughly 0.5 cm apart, so, for example, if three syringes immediately to the right of the partition frame (see Figure 7-8) contained enough bacteria to grow a cloudy culture, then it was assumed that the bacteria traveled 1.5 cm. If the remaining seven syringes did not contain enough bacteria to support growth, then it was assumed that bacteria had not traveled to that region of the BCC. To sample, the plungers in the syringe banks were raised to withdraw approximately 300 μ l of chamber fluid into the syringe bodies. There was little disturbance of the sand using this procedure.

The next step involved lowering the chamber platform, removing the BCCs and replacing the platform with a different one containing machined, sterile polyetherimide well banks. Each well bank contained 10 compartments, or wells,

which aligned properly with each syringe needle of the syringe banks. Each well also contained sterile WBT for bacterial growth testing. The well bank platform was then raised until each syringe needle was submerged into the well fluid. The syringes were then withdrawn 1 cm to fill the syringes with WBT and flush out any bacteria that might be present into the well fluid. In this fashion, fluid was withdrawn and flushed two more times to capture as many bacteria as possible.

Upon flushing the syringes, the well banks were removed and incubated for 24 hours at 35 °C. The contents of each well were then transferred to a cuvette, and OD₆₀₀ readings were taken to test for bacterial growth. If the OD₆₀₀ was greater than 0.1, the syringe was considered to contain bacteria and the distance traveled was calculated.

7.5.3 Results

Figure 7-9 shows the results of the chamber migration studies. On the vertical axis are six different cases. Penetration distance is on the horizontal axis. The flooded case is one where the chamber was filled with bacteria throughout its entire volume. Each syringe produced growth in this flooded case, indicating proper functioning of the syringes. The abiotic case consisted of samples without added bacteria, indicating that the preparation of the MSF was sterile. Four time-courses are represented here. At 3 hours and 15 hours, the standard deviations had a high degree of coincidence. There is a large spread in the remaining data rendering results on the effects of chemotaxis on migration rates inconclusive. However, two generalizations can be made. First, a trend in migration distance

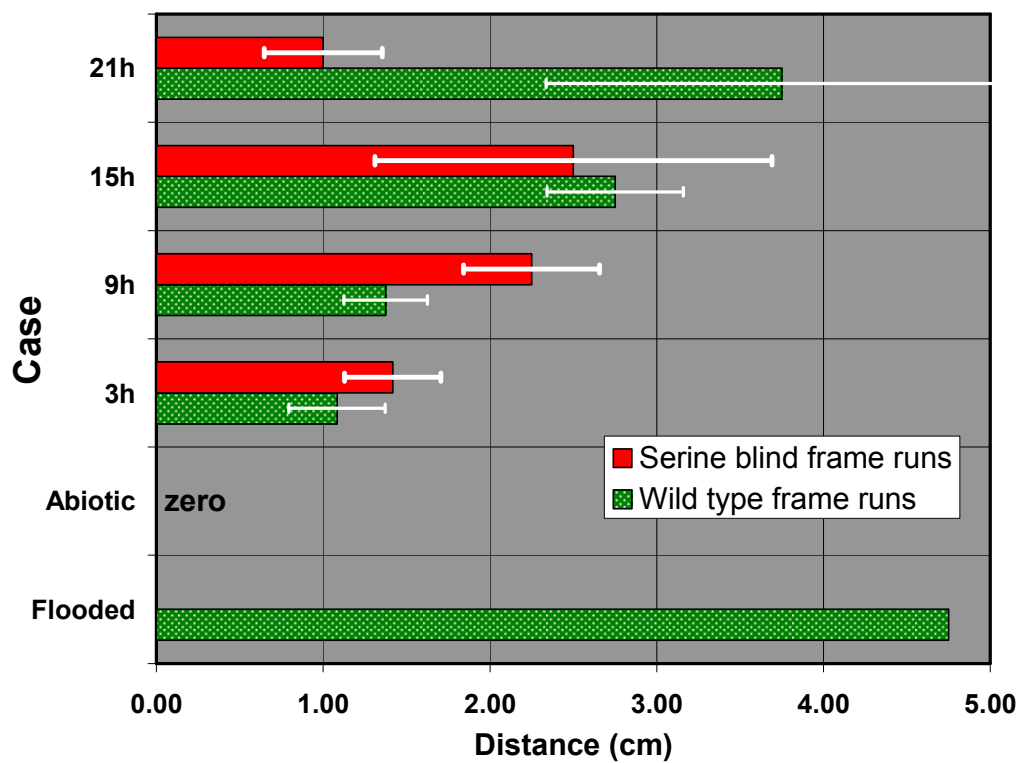


Figure 7-9: Migration distances for six different cases. The abiotic case showed that the sampling procedure was sterile. The flooded case shows that when the entire chamber was filled with cells, all of the syringes were functioning properly.

over time is apparent. Over time, for the majority of cases, bacteria migrated further into the chamber. Secondly, neglecting the spread of the data and using mean values, penetration rates of 0.06 cm/h to 0.56 cm/h were calculated, the same order of magnitude of penetration rates for *E. coli* in a prior study (Reynolds, Sharma et al. 1989) of 0.073 cm/h to 0.28 cm/h (Table 2-3).

7.5.4 Discussion

The MSF experiment is one that can benefit from some refinements. There are some possibilities which may have led to the high degree of spread in the data. It is possible that the zone of influence of the syringes may have been high enough to interfere with neighboring syringes, causing convection to disperse the bacteria in several syringes that would have not otherwise withdrawn bacteria. End effects may have played a role, meaning that cells may have migrated along the bottom and sides of the BCCs. One refinement that might help mitigate this problem would be to use a larger chamber size.

7.6 Chemotaxis for control

Chemical engineers study methods of controlling processes. ISB is a process that is subject to control via addition of nutrients, non-native degradative bacteria, oxygen, etc. Another way to control ISB could be by the addition of chemical enhancers of chemotaxis. Enhanced chemotaxis may lead to faster biodegradation rates. Conversely, there may be a situation in an aquifer where

chemotaxis inhibition may be required, for example, in an aquifer contaminated with coliform bacteria or pathogenic *E. coli* O157:H7.

The goal of this work was to detect any enhancement by chemotaxis for an experimental model of ISB. While there was not strong evidence for the enhancement of chemotaxis in the experiments of this work, perhaps if chemotaxis was enhanced further, a difference could be detected, leading to experimentation at the lab scale which could eventually result in chemotaxis enhancement in a full scale ISB situation. A step in the enhancement of chemotaxis was taken by the addition of a possible chemotaxis enhancer to a porous media.

7.6.1 Enhancers and inhibitors

In order to use chemotaxis as a control mechanism for subsurface processes, one may wish to speed up or slow down the ability of microorganisms to undergo chemotaxis. The stimulating drugs DL-amphetamine and epinephrine have been shown to enhance chemotaxis of *Pseudomonas fluorescens* in capillary studies (Chet, Henis et al. 1973). Caffeine has been shown to be an enhancer of motility in human spermatozoa (Ralt, Manor et al. 1994). Inhibitors of chemotaxis include acetylcholine and tetrahydrocannabinol (Chet, Henis et al. 1973) along with ether, chloroform, and ethanol (Chet and Mitchell 1976). These enhancers and inhibitors represent opportunities to study chemotaxis as a control mechanism. In this next experiment, caffeine was used as a potential enhancer of chemotaxis in a porous medium.

7.6.2 Caffeine swarm experiment

An experiment was performed in enhancing *E. coli* chemotaxis using caffeine in WBT swarm plates. Despite the lack of literature on the effect of caffeine on chemotaxis, it was chosen due to its ready availability over the other stimulants of DL-amphetamine and epinephrine. Figure 7-10 shows swarm diameter vs. time. Data points are triplicates with standard deviations shown. 10 μ l of a fresh culture of chemotactic *E. coli* RP437 cells at mid-exponential phase were inoculated on swarm plates containing four different levels of caffeine: 0, 0.1 μ g/ml, 1.0 μ g/ml, and 10 μ g/ml. These orders of magnitude were chosen based on the level used in tests with amphetamines (Chet, Henis et al. 1973). After 8 hours, the plates inoculated with 1.0 μ g/ml and 10 μ g/ml contained rings which had an 8.5% larger diameter, which translates into a 16% larger area, and thus 16% more biomass under the stimulated conditions caused by the caffeine.

When compared to the swarm rings presented in Figure 7-2 where no caffeine was added, it is evident that larger rings formed during the same time interval when caffeine was added. This may be due to the effect of the caffeine, or the fact that the inoculation volume used in the rings of Figure 7-2 was 5 μ l instead of 10 μ l as was used in the caffeine experiment.

The mechanisms of enhancement due to caffeine and amphetamines are uncertain. The enhancement effect may be similar to the stimulating effects of these drugs in humans. There may be a motility enhancement such as the motility enhancement found in human spermatozoa. This motility enhancement

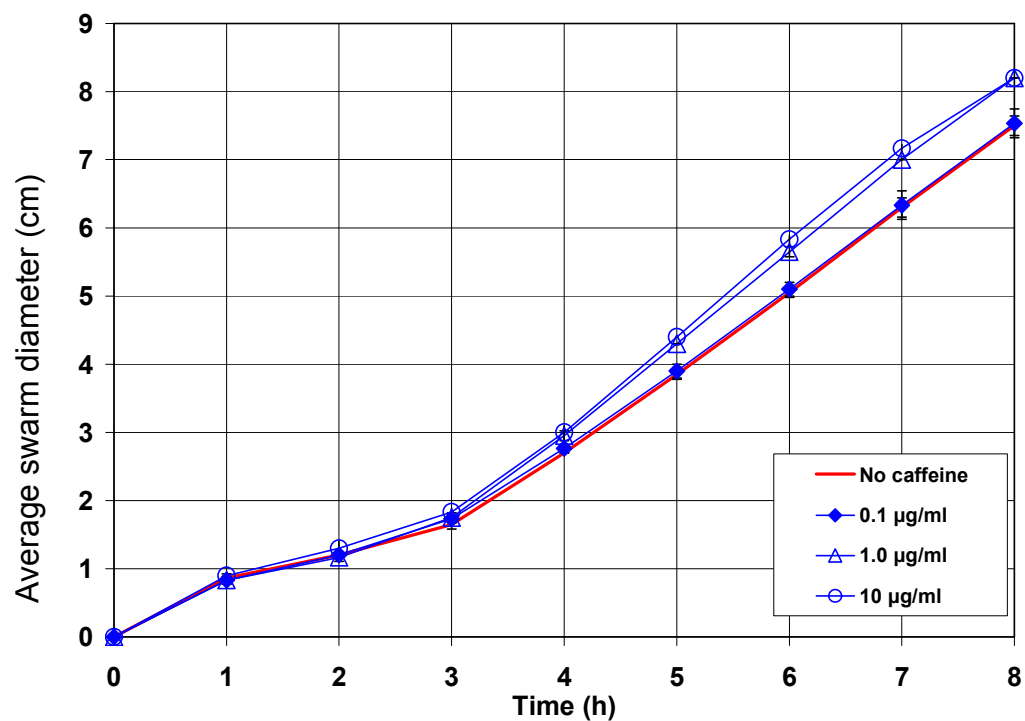


Figure 7-10: Caffeine experiments, swarm diameter vs. time. Data points are triplicates with standard deviations shown. Four different levels of caffeine are shown. After 8 hours, the plates inoculated with 1.0 µg/ml and 10 µg/ml contained rings which had an 8.5% larger diameter.

could be an increase in swimming speed. The caffeine may be an additional energy source for the bacteria, or perhaps the chemotactic sensitivity coefficient is increased by the presence of caffeine. Further research is needed in this area. For example, one may wish to add stimulants (or others) to the BCCs of this work to increase chemotaxis and subsequent degradation rates.

8 DISCUSSION

8.1 Interpretation of experimental results

The results from the serine degradation experiments in the BCCs show there was no difference in degradation rates when the chemotactic strain was introduced to a serine gradient versus the case where the serine-blind strain was introduced to the gradient. The levels of degradation in both cases were statistically similar. That is, standard deviations in the quadruplicate experiments had a high degree of coincidence. The reasons for the lack of difference may have been because the additional degradative power that was achieved by the addition of a small number of chemotactic cells may not have been high enough to result in a higher level of serine degradation. Also, the ratio of cells to serine may have been too low to show a difference statistically. On inspection, this implies that chemotaxis is insignificant in the ISB process – the degree of additional degradation may be too small to be of interest. However, one must consider the additional data from this study to get a better understanding of whether or not chemotaxis is beneficial to ISB. Chemotaxis may yet have an effect on degradation, if the proper experimental parameters can be uncovered.

8.2 A perspective on chemotaxis and ISB

In this section, prior studies in chemotaxis in porous media will be compared with the results of this work, and a recommendation will be made regarding the continuance of research in this area.

One of the most important parameters to consider in porous media chemotaxis studies is cell density. At low cell densities, degradation of contaminants is minimal due to lack of cell volume. At high cell densities, migration of cells could become limited, reducing the ability for cells to migrate to different areas of the contaminant plume. Migration limitations at high cell densities include factors such as biofilm growth and cellular wastes. Biofilm growth due to adhesion of cells to particles and each other would effectively reduce pore sizes and restrict the mobility of the cells. Cellular waste products could serve as chemorepellents which may prevent a homogeneous dispersion of cells in the contaminated region.

Prior experiments have been conducted under variable conditions of cell density and growth conditions, and some of the works suggested that chemotaxis had some effect. This evidence supporting chemotaxis coupled with the existence of limitations at both the high and low range of cell densities suggests that there may be an optimum set of conditions in the porous media where chemotaxis has the greatest effect.

The goal of chemotaxis studies should be to find this optimum combination of parameters to maximize chemotaxis effects. If this optimum combination of

parameters shows an insignificant enhancement due to chemotaxis, then it can be concluded that further chemotaxis studies for ISB would be fruitless.

The following hypothesis has been generated to examine the question of enhancement of degradation rates due to chemotaxis in porous media and to guide future work in this area:

There exists a set of conditions of cell density, particle size, and growth conditions where the enhancement of biodegradation of contaminants in the subsurface due to bacterial chemotaxis is a maximum.

The consideration of prior experimental work on penetration rate enhancement due to chemotaxis provides some support for this hypothesis.

Barton and Ford (1995), by operating at non-growth conditions, were operating at the low cell densities, which may be why they reported no effects of chemotaxis on penetration rates. Reynolds *et. al* may have operated under growth conditions, since growth substrate was available. Lopez de Victoria claimed to uncover some chemotaxis effect, but the sand column used may have had fractures which could have channeled cells towards her TCE gradient, altering the results that were reported.

After reviewing these results and the experimental results of this study, it is recommended that research in this area continue. This study described fully the swimming and degradation ability of the RP437/RP5700 combination. It is hoped that this pair finds use in further chemotaxis studies in porous media.

9 FUTURE WORK

There are several directions in which experimentation in chemotaxis effects of ISB can proceed, based upon the groundwork of this dissertation.

The BCCs used require a difficult step in attaching the acetate partitions to the frame. A two piece, autoclaveable Lexan prototype, shown in Figure 9-1, has been developed to ease the assembly of the chamber. The two pieces are held together with a clamp in which variable force is used to seal the acetate partition to the chamber body. Utilization of this prototype may lower the preparation time needed for the chambers.

It would be interesting to study the effects of higher cell concentrations in the BCC, to determine whether there is a concentration dependence upon the level of degradation which is achieved. Additionally, the testing of chemotaxis and degradation under conditions of growing bacteria may show an enhancement of degradation, due to the amplification of degradative power by the addition of new bacteria during migration.

Refinements in the MSF design and sampling procedure may give a better look into the patterns of migrating bacteria in the chambers. Larger BCCs may be required to reduce end effects, and sampling at multiple chamber heights may give an improved picture of the bacterial profile.

Finally, this system lends itself to testing of other subsurface, aerobic bacteria. The use of such bacteria will bring the experimental system closer to

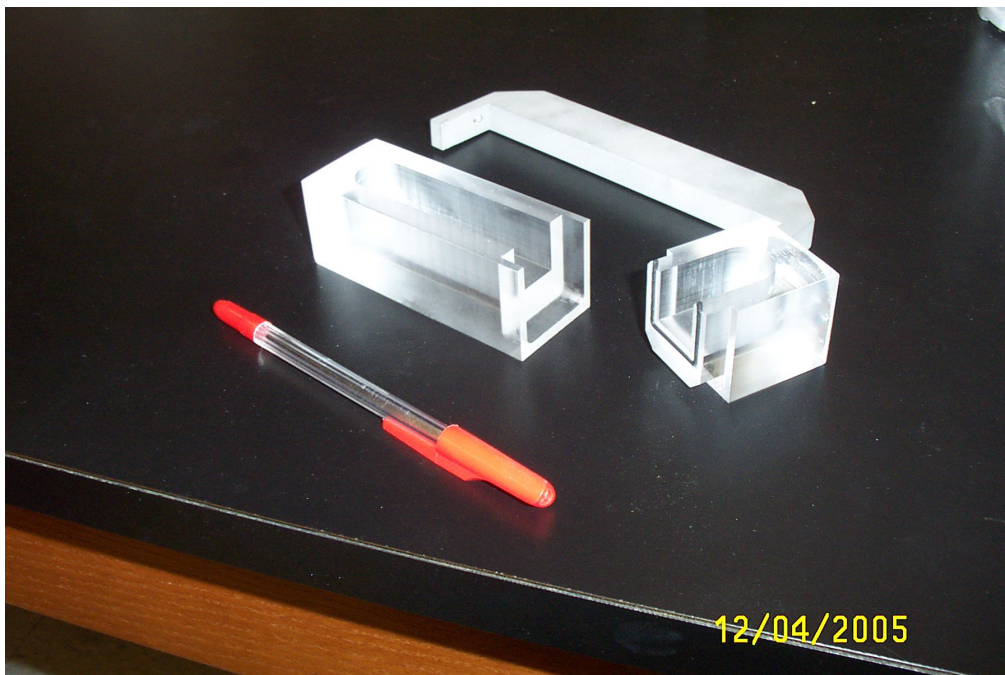
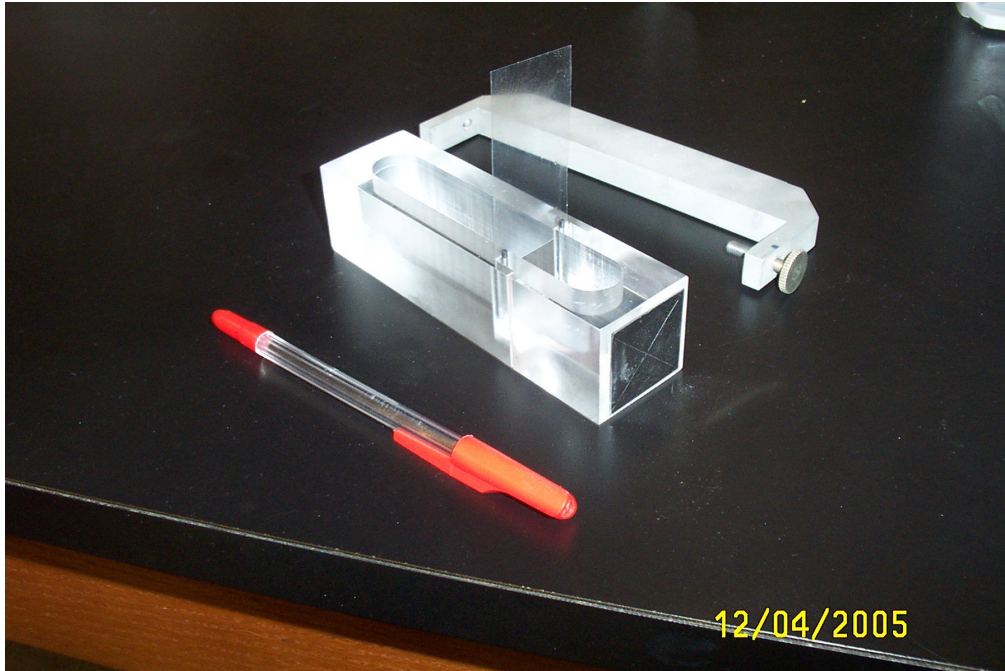


Figure 9-1: Prototype four made with autoclaveable Lexan. The two part prototype separates to allow easy insertion of the acetate partition.

the real world parameters of ISB.

10 CONCLUSION

The results of these studies reveal the effects of chemotaxis of bacteria on degradation rates of an attractant chemical in porous media. These rigorous measurements, which have attempted to consider many parameters not accounted for in any single prior work, have related the microscopic phenomenon of bacterial chemotaxis to the macroscopic measurement of degradation rate. Investigations into the literature have revealed that previous studies used a wide range of bacterial species and strains, porous and liquid media, growth conditions, and gradient formation techniques. Such wide variance in these works makes comparison among experiments nearly impossible. The literature also revealed a scarcity of physiological mutants available to conduct these studies. As a result, the continued use of model experimental systems using *E. coli* and other non-subsurface bacteria is needed until isolation of appropriate chemotaxis mutants of subsurface bacteria is achieved.

This work contributes to the knowledge base of chemotaxis for ISB in several ways. The protocols for sampling of the bacterial chemotaxis chambers for degradation and penetration rate represent a methodology for the systematic evaluation of the phenomena of chemotaxis and migration of bacteria through porous media that is easily amenable to modification for further study. The protocols represent an evolution of ideas improving upon earlier works in this area after careful scrutiny of prior experimental protocols.

A pair of strains, *E. coli* RP437 and RP5700, were highly characterized and were found to be an excellent choice for comparing chemotactic behavior with random motility. For the first time, the swimming parameters of swimming speed, run length, and turn angle were measured rigorously via the tracking microscope prior to experimentation in porous media, ensuring that the desired swimming behavior was achieved.

Optimal conditions for chemotaxis were ensured in other ways, that is, cells were harvested in the exponential phase where motility is an optimum, a proper chemotaxis buffer was developed and used, an optimum concentration of serine was determined, and a growth medium which provided faster swimming speeds, WBT, was used. The accomplishments of this work will assist in guiding further work in chemotaxis for ISB.

A standardized hypothesis about chemotaxis in porous media was developed and directly addressed. The hypothesis that was developed to guide this work, restated from Chapter 3, is:

Chemotactic *E. coli*, when exposed to a gradient of metabolizable serine in a porous medium consisting of liquid saturated sand, will degrade the same amount of serine during a given period of time even if one of the strains is lacking the serine chemoreceptor.

This is the null hypothesis of this work. Based upon the experimental results of this work, there was insufficient evidence to reject this hypothesis. In other words, the claim that the chemotactic bacteria would degrade more of the serine was not supported with the experimental parameters chosen for this study.

Migration experiments provided only a qualitative trend in increasing distance traveled over time for both strains.

Chemotaxis, possibly coupled with growth, may have an effect on degradation rates for ISB at some length and time scale due to its ability to shunt biological degradative power toward regions of higher contaminant-attractants. However, with the set of experimental parameters chosen in this study, the enhancement in degradation rates was not revealed. As described in the Background section, other researchers failed to elucidate any strong evidence that chemotaxis has any effect on degradation and transport rates. Nonetheless, noting that 18% of the serine was degraded in the BCC degradation studies demonstrates that this methodology can be used with different experimental parameters such as particle size, addition of a growth medium, higher cells densities, or different chemoattractants. Extending this work may yield conditions where a measurable chemotactic response can be observed, at which point work in enhancing chemotaxis in a porous medium can begin.

REFERENCES

References

- Adler, J. 1966. Effect of amino acids and oxygen on chemotaxis in *Escherichia coli*. *Journal of Bacteriology*. 92: 121-129.
- Adler, J. 1969. Chemoreceptors in bacteria. *Science*. 166: 1588-1597.
- Adler, J. 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *Journal of General Microbiology*. 74: 77-91.
- Adler, J. 1976. The sensing of chemicals by bacteria. *Scientific American*. 234(40): 40-47.
- Adler, J. and M. M. Dahl. 1967. A method for measuring the motility of bacteria and for comparing random and non-random motility. *Journal of General Microbiology*. 46: 161-173.
- Adler, J. and B. Templeton. 1967. The effect of environmental conditions on the motility of *Escherichia coli*. *Journal of General Microbiology*. 46: 175-184.
- Armstrong, J. B., J. Adler, et al. 1967. Nonchemotactic mutants of *Escherichia coli*. *Journal of Bacteriology*. 93: 390-398.
- Barton, J. 1994. Determination of effective coefficients for bacterial migration in sand columns. Dissertation. University of Virginia.
- Barton, J. W. and R. M. Ford. 1995. Determination of effective transport coefficients for bacterial migration in sand columns. *Applied and Environmental Microbiology*. 61: 3329-3335.
- Berg, H. C. 1971. How to track bacteria. *The Review of Scientific Instruments*. 42(4): 868-871.
- Berg, H. C. 1975. Chemotaxis in bacteria. *Annual Review of Biophysics and Bioengineering*. 4: 119-136.
- Berg, H. C. and D. A. Brown. 1972. Chemotaxis in *Escherichia coli* analyzed by three-dimensional tracking. *Nature*. 239: 500-504.
- Bouwer, E. J. 1992. Bioremediation of organic contaminants in the subsurface. *Environmental Microbiology*. R. Mitchell. New York, Wiley-Liss.
- Brock, T. D., M. T. Madigan, et al. 1994. *Biology of microorganisms*. Englewood Cliffs, NJ, Prentice Hall.

Brosilow, B. J., R. M. Ford, et al. 1996. Numerical solution of transport equations for bacterial chemotaxis: Effect of discretization of directional motion. *SIAM Journal on Applied Mathematics*. 56(6): 1639-1663.

Callahan, A. M., B. L. Frazier, et al. 1987. Chemotaxis in *Escherichia coli*: Construction and properties of λ tsr transducing phage. *Journal of Bacteriology*. 169: 1246-1253.

Chet, I., Y. Henis, et al. 1973. Effect of biogenic amines and cannabinoids on bacterial chemotaxis. *Journal of Bacteriology*. 115(3): 1215-1218.

Chet, I. and R. Mitchell. 1976. Ecological aspects of microbial chemotactic behavior. *Annual Review of Microbiology*. 30: 221-239.

Clarke, S. and D. E. Koshland. 1979. Membrane receptors for aspartate and serine in bacterial chemotaxis. *The Journal of Biological Chemistry*. 254: 9695-9702.

Cohen, S. A. and D. P. Michaud. 1993. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. *Analytical Biochemistry*. 211: 279-287.

Fogler, H. S. 1992. *Elements of chemical reaction engineering*. Englewood Cliffs, NJ, Prentice Hall.

Ford, R. M., B. R. Phillips, et al. 1991. Stopped-flow chamber and image analysis system for quantitative characterization of bacterial population migration: Motility and chemotaxis of *Escherichia coli* K12 to fucose. *Microbial Ecology*. 22(2): 127-138.

Frymier, P. D. 1995. Bacterial migration and motion in a fluid phase and near a solid surface. Dissertation. University of Virginia.

Harley, J. P. and L. M. Prescott. 1996. *Laboratory exercises in microbiology*. Dubuque, Wm. C. Brown Publishers.

Hazelbauer, G. L., R. E. Mesibov, et al. 1969. *Escherichia coli* mutants defective in chemotaxis toward specific chemicals. *Proceedings of the National Academy of Sciences USA*. 64: 1300-1307.

Hedblom, M. L. and J. Adler. 1980. Genetic and biochemical properties of *Escherichia coli* mutants with defects in serine chemotaxis. *Journal of Bacteriology*. 144: 1048-1060.

Keller, E. F. and L. A. Segel. 1971. Traveling bands of chemotactic bacteria: A theoretical analysis. *Journal of Theoretical Biology*. 30: 225.

- Kunkler, J. 1994. Modeling and experimental study of bacterial migration and growth in porous media. Masters Thesis. University of Virginia.
- Law, A. M. J. 2004. Bacterial chemotaxis to naphthalene enhances naphthalene removal from a nonaqueous liquid, but is attenuated by oxygen depletion. Dissertation. The University of North Carolina at Chapel Hill.
- Leveau, J. H., A. J. B. Zehnder, et al. 1998. The *tfdK* gene product facilitates uptake of 2,4-dichlorophenoxyacetate by *Ralstonia eutropha* JMP134(pJP4). *Journal of Bacteriology*. 180: 2237-2243.
- Levin, M. A. and M. A. Gealt. 1993. Biotreatment of industrial and hazardous waste. New York, McGraw Hill.
- Lopez-de-Victoria, G. 1989. Chemotactic behavior of deep subsurface bacteria toward carbohydrates, amino acids, and a chlorinated alkene. Masters Thesis. University of Puerto Rico.
- Manson, M. D. and J. E. Segall. 1992. Chemotaxis. *Encyclopedia of microbiology*. San Diego, CA, Academic Press: 501-512.
- Marx, R. B. 2000. The relationship between chemotaxis and naphthalene biodegradation by *Pseudomonas putida* G7. Dissertation. The University of North Carolina at Chapel Hill.
- Marx, R. B. and M. D. Aitken. 2000. A material-balance approach for modeling bacterial chemotaxis to a consumable substrate in the capillary assay. *Biotechnology and Bioengineering*. 68(3): 308-315.
- Mazumder, R., T. J. Phelps, et al. 1999. Determining chemotactic responses by two subsurface microaerophiles using a simplified capillary assay method. *Journal of Microbiological Methods*. 37: 255-263.
- Mesibov, R., G. W. Ordal, et al. 1973. The range of attractant concentrations for bacterial chemotaxis and the threshold and size of response over this range: Weber law and related phenomena. *Journal of General Physiology*. 62(2): 203-223.
- Murphy, O. J., F. A. Kovacs, et al. 2001. Site-directed solid-state NMR measurement of ligand-induced conformational change in the serine bacterial chemoreceptor. *Biochemistry*. 40: 1358-1366.
- Pandey, G. and R. K. Jain. 2002. Bacterial chemotaxis toward environmental pollutants: Role in bioremediation. *Applied and Environmental Microbiology*. 68(12): 5789-5795.

- Parales, R. E. and C. S. Harwood. 2002. Bacterial chemotaxis to pollutants and plant-derived aromatic molecules. *Current Opinion in Microbiology*. 5: 266-273.
- Parkinson, J. S. 1980. Novel mutations affecting a signaling component for chemotaxis of *Escherichia coli*. *Journal of Bacteriology*. 142: 953-961.
- Parkinson, J. S. 1993. Signal transduction schemes of bacteria. *Cell*. 73: 857--871.
- Parkinson, J. S. 1996. Genetic descriptions of *Escherichia coli* RP437, RP5700, and RP2361. Personal communication.
- Parkinson, J. S. 1997. Information on *Escherichia coli* RP5700. Personal communication.
- Ralt, D., M. Manor, et al. 1994. Chemotaxis and chemokinesis of human spermatozoa to follicular factors. *Biology of Reproduction*. 50: 774-785.
- Rebbapragada, A., M. S. Johnson, et al. 1997. The Aer protein and the serine chemoreceptor Tsr independently sense intracellular energy levels and transduce oxygen, redox, and energy signals for *Escherichia coli* behavior. *Proceedings of the National Academy of Sciences USA*. 94: 10541-10546.
- Reynolds, P. J., P. Sharma, et al. 1989. Mechanisms of microbial movement in subsurface materials. *Applied and Environmental Microbiology*. 55: 2280-2286.
- Sarman, S., P. T. Cummings, et al. 1994. Lattice boltzmann model simulations of chemotaxis in bacterial populations. Unpublished.
- Sharma, P. K., M. J. McInerney, et al. 1993. *In Situ* growth and activity and modes of penetration of *Escherichia coli* in unconsolidated porous materials. *Applied and Environmental Microbiology*. 59: 3686-3694.
- Shuler, M. L. and F. Kargi. 1992. *Bioprocess engineering*. Englewood Cliffs, NJ, Prentice Hall.
- Soby, S. and K. Bergman. 1983. Motility and chemotaxis of *Rhizobium meliloti* in soil. *Applied and Environmental Microbiology*. 46(5): 995-998.
- Stock, J. B., G. S. Lukat, et al. 1991. Bacterial chemotaxis and the molecular logic of intracellular signal transduction networks. *Annual Review of Biophysics and Biophysical Chemistry*. 20: 109-136.
- Strauss, I., P. D. Frymier, et al. 1995. Analysis of bacterial migration: II. Studies with multiple attractant gradients. *American Institute of Chemical Engineering Journal*. 41(2): 402-415.

Van-der-Meer, J. R., W. Roelofsen, et al. 1987. Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 in nonsterile soil columns. FEMS Microbiology Ecology. 45: 333-341.

Walpole, R. E. and R. H. Myers. 1993. Probability and statistics for engineers and scientists, Macmillan.

Wolfe, A. J. and H. C. Berg. 1989. Migration of bacteria in semisolid agar. Proceedings of the National Academy of Sciences USA. 86: 6973-6977.

APPENDIX

Appendix A: Media Formulations

Table A-1: Wolfe & Berg Tryptone Media (WBT)

Recipe to make 1800 ml solution

<i>Component</i>	<i>Amount according to Wolfe & Berg</i>	<i>Amount to add</i>
tryptone	1% w/w	18 g
NaCl	0.5% w/w	9 g
streptomycin sulfate, 10 mg/ml solution	125 µg/ml	22.5 ml
deionized water	n/a	1800 ml
<i>For Wolfe & Berg Tryptone Swarm Plates:</i>		
agar	0.2% w/w	3.6 g

Table A-2: Adler Wash Buffer (AWB)

Recipe to make 1000 ml of solution

<i>Component</i>	<i>Concentration of stock solution</i>	<i>Final concentration</i>	<i>Amount to add</i>
K ₂ HPO ₄	n/a	1.07 g/L	1.07 g
KH ₂ PO ₄	n/a	0.52 g/L	0.52 g
EDTA disodium salt stock solution	0.5 M	200 µl of 0.5 M solution	200 µl
methionine stock solution	1x10 ⁻⁴ M	10 ml/L	10 ml
streptomycin stock solution	10 mg/ml	125 µg/ml	12.5 ml
deionized, reverse osmosis treated water	n/a	n/a	978 ml

Table A-3: Adler Wash Buffer with Serine (AWBS)

Recipe to make 200 ml solution

<i>Component</i>	<i>Final concentration</i>	<i>Amount to add</i>
AWB	n/a	200 ml
L-serine	1 mM	0.021 g

Table A-4: Sand Phosphate Buffer (SPB)
Recipe to make 2000 ml solution

<i>Component</i>	<i>Final concentration</i>	<i>Amount to add</i>
K ₂ HPO ₄	1.071 g/L	2.14 g
KH ₂ PO ₄	0.523 g/L	1.048 g
deionized, reverse osmosis water	n/a	2000 ml

Table A-5: Glycerol Minimal Media (GMM)

Recipe to make 1000 ml of solution

<i>Component</i>	<i>Amount to add</i>
K ₂ HPO ₄	11.2 g
KH ₂ PO ₄	4.8 g
EDTA disodium salt, 0.5 M stock solution	200 µl
(NH ₄) ₂ SO ₄	2 g
MgSO ₄ ·7H ₂ O	0.25 g
glycerol	1.924 ml
histidine	0.155 g
leucine	0.131 g
threonine	0.119 g
methionine	0.149 g
thiamine	0.001 g
streptomycin sulfate	0.1 g
Fe(SO ₄) deionized, reverse osmosis water	0.001 g 1 L

Appendix B: HPLC Calibration curve and gradient program

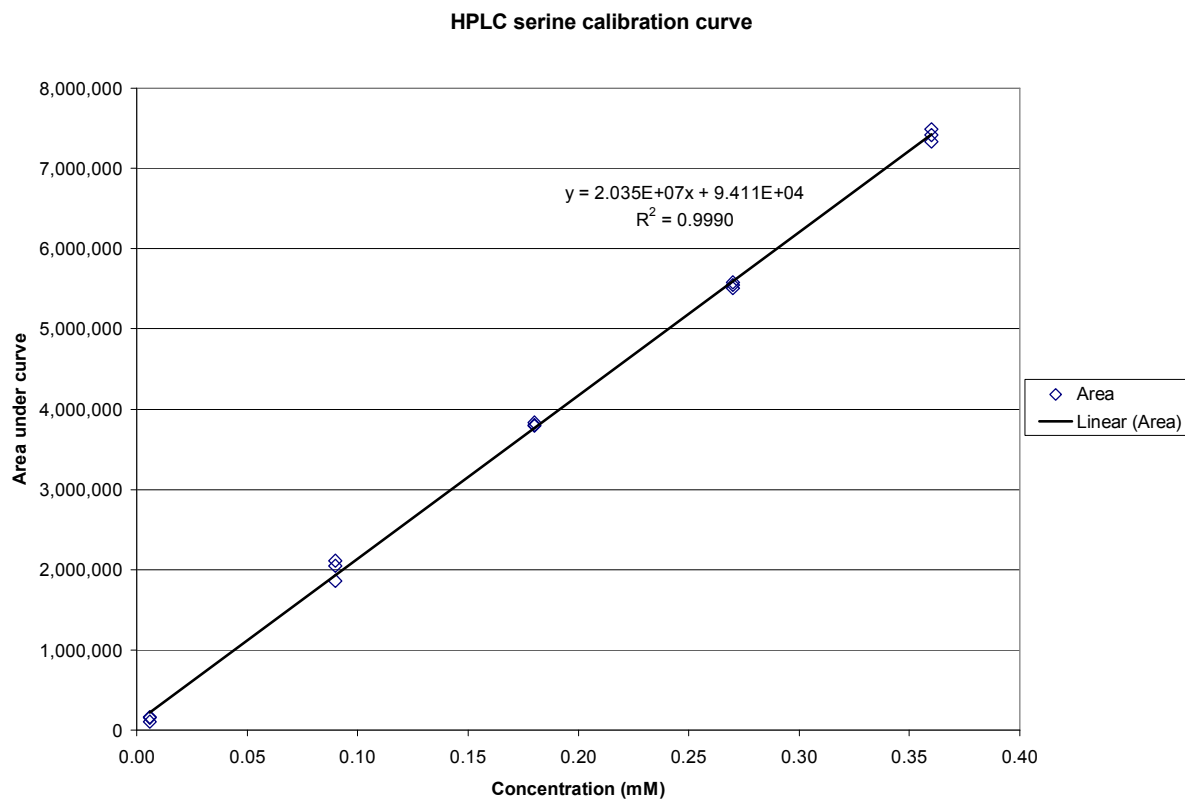


Figure B-1: Calibration curve used for converting integration area to serine concentration (mM).

Table B-1: HPLC gradient program

<i>Elapsed time (min)</i>	<i>%Solvent A</i>	<i>% Solvent B</i>	<i>% Solvent C</i>
0	100	0	0
0.5	99	1	0
18	95	5	0
23.5	0	60	40
26.5	100	0	0
41	100	0	0

Appendix C: Logsheet for serine degradation experiments

Chamber Log Sheet - Migration Distance Experiments (Serine degradation)		Name:	
Logsheet Updated: 5/23/2005		Date:	
Filename: Chamber_log_sheet&data_L.xls, Tab Migration_Log_Ver_L			
Reference: See Word document trough_experiments.doc			
Experiment Code:			
Comments on experimental details:		Strain:	WT / SB
A. Lab access		Run #	
1 Prop open BOTH doors to Lab 719		Rep #	
2 Don lab coat & safety glasses			
B. Check solutions -1 or 2 Days before chamber run:			
1	☐ Sterile Adler Wash Buffer (AWB, E-6-9 and 4-42B, see notebook 6 page 9 and notebook 4-42B) for washing cells, found in cold room , 120 ml per chamber run. This solution is the mother liquor for Adler Wash Buffer with Serine, below.	Expired?	Y / N
2	☐ Adler Wash Buffer with serine (AWBS, E-6-9-S, prepared from E-6-9 above, see notebook 6 page 9), 1 mM serine, found in cold room attached to AWB bottle , 6 ml per chamber.	Expired?	Y / N
3	☐ Wolfe-Berg tryptone for growing cells, code 4-37B, found in cold room, 400 ml per experiment .	Expired?	Y / N
4	☐ Sterile 1.5 ml Eppendorf tubes containing WBT, found in room 722 on top shelf , 7 tubes per chamber. Schedule a time for piercing and labeling.		
5	☐ 1 L sterile water for dessicator	Sterile?	Y / N
6	☐ Sterile sponge for dessicator	Sterile?	Y / N
7	☐ Autoclave wooden probes, dry cycle, 20 minute sterilization followed by 10 minute drying cycle.	Sterile?	Y / N
8	☐ Ensure availability of dry, sterile Unimin 20:30 sand in foil-covered sand filled plastic cups. Autoclave on dry cycle, 20 minute sterilization followed by 10 minute drying cycle.	Sterilized?	Y / N
9	☐ 6 x sterile 250 ml baffled flasks.	Sterile?	Y / N
C. Check supplies - Night before chamber experiment:		Time started:	
1	☐ Lab notebook, place on <i>bookshelf at desk</i> , current notebook is #7.		
2	☐ Clipboard, found somewhere in the lab , place at Chamber Preparation area.		
3	☐ Ball-point pen, place in syringe drawer near chamber prep area.		
4	☐ Fine-point Sharpie, place in syringe drawer near chamber prep area.		
5	☐ 70:30 denatured ethanol:water mix in squeeze bottles, one at area 51 and one at Culture Preparation Area. Fill if needed from stock solution below West lab hood in 719.		
6	☐ Paper towels or cheesecloth for sterilizing work area, place stack behind Chamber Preparation		
7	☐ 1 CONTAMINATED waste container to contain used filters & pipette tips, bags found above Chamber Preparation Area, place in wire frame at Area 51.		

Logsheet for serine degradation experiments

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8	☐	1 UNCONTAMINATED waste disposal container to contain miscellaneous soggy waste, <i>place trash can near Chamber Preparation Area</i>		
9	☐	GREEN Tape for WT strain; RED tape for AB strain, <i>place above Chamber Preparation Area, tape found in 722 drawer next to lab hood.</i>		
10	☐	2 x 50 ml sterile plastic centrifuge tubes labeled WORKING and CHAMBER , with GREEN tape for WT or RED tape for SB , cracked open and gently resealed. <i>Tubes above Chamber Preparation Area, place on CRASH CART.</i>		
11	☐	20 Microcuvettes in cuvette box with lid, arrow to the left.		
12	☐	2 Cuvette boxes, <i>place one with lid near incubator for OD readings and the other at Area 51.</i>		
13	☐	Pipet filler labeled "Frymier", <i>placed at Area 51</i>		
14	☐	250 ml sterile filtration unit, found under North sink or Room 731-A , <i>place at Area 51</i>		
15	☐	Calculator ON CRASH CART.		
16	☐	5 x 5 ml pipets, found in pipet drawer and <i>place 5 for crash cart.</i>		
17	☐	15 x 10 ml pipets, found in pipet drawer and <i>place 5 for crash cart and 10 for Chamber Preparation Area in tall beaker.</i>		
18	☐	10 x 25 ml pipets, found in pipet drawer , and <i>place 5 on crash cart and 5 for Chamber Prep. Area in tall beaker.</i>		
19	☐	Restock pipet drawer with pipets from shelves in 719 or room 731-A across the hall, 15 each of 5 ml,		
21	☐	2 x 1000 ☐ pipetter (Eppendorf GREY body w/ BLUE Button Top) Set at: 1000 ☐ , <i>place one in crate behind Derivatization Prep. Area and the other at the Culture Preparation Area.</i>		
22	☐	4 x boxes of 1000 ☐ pipetter tips, UNTOUCHED STERILE (check tape for black stripes), in a BLUE box. <i>Place in Area 51, Culture Prep. Area, incubator, and crash cart. Extra tips found in Room 722. Use gloves to load.</i>		
23	☐	Vaseline syringe, <i>place in drawer next to Chamber Preparation Area.</i>		
24	☐	6 x ethanol sterilized chambers and partitions, <i>leave in dessicator until ready for use. (1 extra in case of loading mishap)</i>	Sterilized?	Y / N
25	☐	Logsheet for Eppendorff tube OD readings, (Filename Chamber_log_sheet&data.xls), found on CJ's notebook computer , <i>attach to clipboard.</i>		
26	☐	Chamber experiment startup log (Filename chamber_Exp_Startup_Ver_0.xls), found on CJ's notebook computer , <i>attach to clipboard.</i>		
27	☐	5 x 250 ml beakers, found above North sink for emptying and mixing chambers, rinsed with House DI and <i>placed at Chamber Preparation Area.</i>	Washed?	Y / N
28	☐	6 x labeled cryovials clear for wild type run and red insert for serine blind run, found in syringe drawer and <i>placed in white rack at Chamber Preparation Area. Label cryovials F, 0, 1, 2, 3, 4 on top and experiment code + date on side (see CJ for experiment code)</i>	Labeled?	Y / N
29	☐	2 microscope slides, <i>placed at microscope</i>		

Logsheet for serine degradation experiments

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30	☐	6 x coverslips, Corning No. 1 1/2, 22 mm sq., placed at microscope.		
31	☐	Sample timer, placed at Chamber Preparation Area.		
32	☐	5 x 3 ml syringes found in 722 and placed in syringe drawer.		
33	☐	Spatula, found near analytical balance drawer and placed in syringe drawer.		
34	☐	5 x 0.2 µm, 25 mm sterile syringe filters: Whatman 0.2 µm PSU, 13 mm. #6780-1302, found in box above Chamber Preparation Area.		
35	☐	5 x 1" 23g syringe needles, found in 722 and placed in syringe drawer.		
36	☐	Sharps disposal container, placed at Chamber Preparation Area.		
37	☐	Tray for chambers, sand-free and placed in Chamber Preparation Area.		
38	☐	Dessicator, 70:30 Denatured EtOH:H ₂ O sterilized and placed in Chamber Sterilization Area.	Sterilized?	Y / N
39	☐	5 sterilized chamber caps, placed in Chamber preparation area.	Sterilized?	Y / N
40	☐	6 x partitions, sterilized and placed in sterile dessicator until ready for use.	Sterilized?	Y / N
41	☐	Sterilized sponge, placed in dessicator for humidity.	Sterilized?	Y / N
42	☐	Aquamarine tray for sterilizing chambers, containing 70:30 200° EtOH:Barnstead DI Water, placed at Chamber Sterilization Area.		
43	☐	1000 ml beaker for drying containing 200° EtOH, placed at Chamber Sterilization Area.		
44	☐	Tweezers, found in syringe drawer.		
45	☐	Working E. coli cell suspension, transferred from overnight culture and regrown in WBT, (WT or SB), 43 ml per chamber run.		
D. Experiment preparations - Night before experiment				
1	☐	Arrange for overnight culture inoculation and morning transfer. Overnights are typically started at 10 PM and transferred at 9 AM, allowing for 11 hours of growth to an OD of roughly 1.1. For reference, see file Log Sheet Overnight Ver 1.3 and Log Sheet Transfer		
2	☐	Sterilize dessicator with 70% ethanol.		
3	☐	Set up CHAMBER STERILIZATION AREA on benchtop.		
4	☐	Clean and sterilize chambers, caps and partitions . Use pentane to degrease chambers, then submerge in 70% ethanol solution, followed by 200 proof ethanol to assist in drying. Dry in vacuum dessicator 1 hour.		
5	☐	Line bottom and sides of chambers with a line of Vaseline.		
6	☐	Store chambers in sterile dessicator until needed.		
7	☐	Pierce, label, and sterilize Eppendorff tubes for migration samples.	Sterilized?	Y / N
8	☐	Fill tubes with 1.5 ml WBT.	Tubes filled?	Y / N
9	☐	Prepare cuvettes in cuvette box for migration OD readings.		

Logsheet for serine degradation experiments Page 4

E. Equipment accessibility - Day of experiment		
1	☐ MEDIA WARMING: Remove AWB/AWBS from cold room and place at Area 51.	
2	☐ Spectrophotometer: 600 nm.	
3	☐ Balance (100g).	
4	☐ Incubator, room 719, set to 0 rpm and 30 °C for chamber migration.	
5	☐ Sterilize Chamber Prep. Area, using tray and/or screen for chamber assembly.	
F. Preparation for chamber experiment: FILTRATION of cells		
1	☐ Open 250 ml sterile filtration unit and clamp to ringstand at Area 51.	
2	☐ Transfer 43 ml E. coli working culture in WBT to funnel of 250 ml sterile filtration unit, 30 ml then	Time started:
3	☐ Set sample timer to zero, count up.	
4	☐ Open vacuum and press start on timer.	Elapsed time: 0:00
5	☐ Immediately fill 25 ml plastic pipet with 35 ml AWB.	
6	☐ As soon as dry filter becomes visible, add 35 ml AWB to funnel. Swirl gently and record elapsed	Elapsed time:
7	☐ Wait 3 minutes and fill 25 ml plastic pipet with 35 ml AWB.	
8	☐ As soon as dry filter becomes visible, add 35 ml AWB to funnel. Swirl gently and record elapsed	Elapsed time:
9	☐ Wait 4 minutes and fill 25 ml plastic pipet with 35 ml AWB.	
10	☐ As soon as dry filter becomes visible, add 35 ml AWB to funnel and TURN OFF VACUUM . This is the final re-suspension of the cells. Swirl gently and record elapsed time.	Elapsed time:
11	☐ Swirl gently and thoroughly and record elapsed time when swirling is finished.	Final elapsed time:
12	☐ Transfer funnel contents into 50 ml centrifuge tube labeled "Working."	Time transferred:
13	☐ Withdraw sample w/syringe and sterile filter into cryovial labeled "F" for serine filtration	
14	☐ Check OD, using AWB as control and update Chamber Startup Logsheet	
15	☐ Normalize suspension to an OD of 0.4 for WT cells and 0.3788 for SB cells in 50 ml centrifuge tube labeled Chamber .	
16	☐ Check cells for motility: % MOTILE: _____ % RUN AND TUMBLE: _____	Time checked:
G. Preparation for chamber experiment: Loading chambers		
1	☐ Fill bacteria side of all 5 chambers with 6 ml cell suspension. Wait 2 minutes for any leaks to	
2	☐ Drop sand into cell suspension of all 5 chambers until sand is level with top of frame. Level sand using sterile spatula, but do not spill any wet sand. Make sure there is no 'puddle' of cell suspension on surface. Surface should consist of peaks and valleys of sand particles.	
3	☐ Fill serine end of all 5 chambers with 6.2 ml Adler Wash Buffer with 1 mM serine, code E-6-9-S (see notebook 6 page 9).	
4	☐ Drop sand into serine solution in all 5 chambers until sand is level with top of frame. Level sand using sterile spatula, but do not spill any wet sand. Make sure there is no 'puddle' of cell suspension on surface. Surface should consist of peaks and valleys of sand particles.	

Logsheet for serine degradation experiments

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5	☐ Check that incubator is set at 30 °C and that the rpm is set to ZERO .					
6	☐ Raise partitions gently on 4 of the 5 chambers using attached string and remove from chamber. Tap chambers 3-5x gently with spatula to settle sand.	<table border="1" style="width: 100%;"> <tr> <td style="width: 70%;">Start time of partition raising:</td> <td></td> </tr> </table>	Start time of partition raising:			
Start time of partition raising:						
7	☐ Sprinkle sand on surface of all 4 chambers to dry.	<table border="1" style="width: 100%;"> <tr> <td style="width: 70%;">End time of partition raising:</td> <td></td> </tr> </table>	End time of partition raising:			
End time of partition raising:						
8	☐ Weigh chambers to determine evaporation loss later (g): Chamber 1: Chamber 2: Chamber 3: Chamber 4: Chamber 5: Chamber 6: Chamber 7: Chamber 8:	<table border="1" style="width: 100%;"> <tr> <td style="width: 50%;">Run Time:</td> <td style="width: 50%;">Sample time:</td> </tr> <tr> <td></td> <td></td> </tr> </table>	Run Time:	Sample time:		
Run Time:	Sample time:					
9	☐ Attach caps and place chambers in incubator set at 30 °C . Add beaker of hot tap water to incubator and let stand desired length of time.	<table border="1" style="width: 100%;"> <tr> <td style="width: 50%;"></td> <td style="width: 50%;"></td> </tr> </table>				
10	☐ Raise partition on time zero chamber, scrape out sand into beaker, add 8.13 ml AWB, stir for 30 seconds and sample with syringe filter.	<table border="1" style="width: 100%;"> <tr> <td style="width: 70%;">Time in incubator:</td> <td></td> </tr> <tr> <td style="width: 70%;">Time zero sample filtered:</td> <td></td> </tr> </table>	Time in incubator:		Time zero sample filtered:	
Time in incubator:						
Time zero sample filtered:						
11	☐ Bring filtration ("F") and time zero samples to -80 C freezer.					
12	☐ Prep beakers, syringes, needles and filters for sampling after migration/degradation.					
H. Chamber experiment: Sampling chambers						
1	☐ Weigh chambers to determine evaporation losses: Chamber 1: Chamber 2: Chamber 3: Chamber 4: Chamber 5: Chamber 6: Chamber 7: Chamber 8:	<table border="1" style="width: 100%;"> <tr> <td style="width: 70%;">Time started:</td> <td></td> </tr> </table>	Time started:			
Time started:						
2	☐ To measure migration into sand, using wooden combs, dip into chamber to the bottom, withdraw, and dip into WBT filled, 1.5 ml sterile Eppendorff tubes. Incubate tubes at 35 °C for 24 hours and					
3	☐ For degradation experiments, gently scoop contents of chamber into a 250 ml glass beaker.					
4	☐ Add 8.13 ml room temperature Adler Wash Buffer (AWB) to beaker and stir for 30 seconds					
5	☐ Use a 3 ml syringe with a 1" x 23g gauge needle to sample liquid in beaker. Use 0.2 µm 13 mm sterile syringe filter into labeled cryovials.					

Logsheet for serine degradation experiments

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6	☐	Go to step 3 and repeat for 3 other chambers.	Time completed:	
7	☐	Store at -80 °C until analysis for serine via HPLC.		
8	☐	Check cells for motility: % MOTILE: _____ % RUN AND TUMBLE: _____	Time completed:	
9	☐	Clean work areas.		
10	☐	Return AWB/AWBS to coldroom.		

Appendix D: Safety Considerations

HPLC solvents

Acetonitrile is a flammable, toxic solvent and is stored in appropriate explosion proof solvent cabinets. The buffer solution contains toxic sodium azide, and all HPLC solvents are disposed of in accordance with University of Tennessee hazardous waste disposal policy.

HPLC equipment

Safety glasses are worn when working with HPLC pumps and fittings. Care is taken to ensure that solvents do not contact the electronic components of the pump and the detector.

***E. coli* bacteria**

The *E. coli* bacteria used in the study are non-pathogenic. However, all materials which have contacted *E. coli* are autoclaved at 121 °C or sterilized using a 70% ethanol/water wash solution.

Acid procedures

The concentrated sulfuric acid used to wash the sand is stored in an acid cabinet. Gloves, goggles, and a laboratory coat are worn when working with concentrated sulfuric acid. All acid/base solutions are neutralized to a pH between 5 and 9 before disposal down the laboratory drains.

Appendix E: A Mathematical Model for Bacterial Transport

Introduction

The first equation developed to mathematically model the chemotaxis process (Keller and Segel 1971) is given by:

$$J_c = -\mu \nabla c + \chi_0 c \nabla a \quad \text{E-1}$$

where

J_c = cell population flux, cells/(cm·s)

μ = random motility coefficient, cm²/s

c = cell concentration, cells/cm³

χ_0 = chemotactic sensitivity coefficient, cm²/s

a = attractant concentration, mM

This equation is illustrative in that terms in the equation define a contribution to dispersion of bacteria via two terms – random motility and chemotaxis. This equation is theoretically sound but is difficult to solve. Another approach, a Lattice-Boltzmann approach, allows for solution by simple finite differences and discretization of bacterial motion into six individual directions. This approach is used in this mathematical model. Following are the assumptions of the mathematical model:

- One dimensional gradient in cells and the attractant α -methylaspartate.
- Motion in six discretized directions, straight line runs only.
- No collisions between bacteria.

- Bacterial volume neglected.
- 4,000 second simulation time, 2 mm simulation box.
- Instantaneous tumble time.
- Consumption of chemoattractant neglected.

The attractant α -methylaspartate was used for the mathematical model due to the lack of available parameters for *E. coli* such as chemotactic sensitivity coefficient for serine and dissociation constant K_d .

Governing equations

The governing integro-differential equation for the simulation is the cell balance equation of Alt-Stroock (Sarman, Cummings et al. 1994):

$$\frac{\partial n(\mathbf{X}, \hat{\mathbf{s}}, t)}{\partial t} = -v\hat{\mathbf{s}} \cdot \nabla[n(\mathbf{X}, \hat{\mathbf{s}}, t)] + \int p(\hat{\mathbf{s}}', \hat{\mathbf{s}}, \mathbf{X}, t)n(\mathbf{X}, \hat{\mathbf{s}}', t)d\hat{\mathbf{s}}' - \int p(\hat{\mathbf{s}}, \hat{\mathbf{s}}', \mathbf{X}, t)n(\mathbf{X}, \hat{\mathbf{s}}, t)d\hat{\mathbf{s}}' \quad \text{E-2}$$

where

n = cell density at position $\mathbf{X}$, cells/cm³

v = swimming speed, $\mu\text{m/s}$

p = tumbling probabilities

$\hat{\mathbf{s}}$ = initial vector of motion

$\hat{\mathbf{s}}'$ = vector of motion after tumbling

The first term on the left hand side represents the rate of change in the cell density over time. The first term on the right hand side represents the change

in density due to cells swimming in and out of the population. The second and third terms on the right hand side of the equation represent the change in cells tumbling into the population and tumbling out the population, respectively.

Tumbling probabilities can be calculated by (Sarman, Cummings et al. 1994; Brosilow, Ford et al. 1996):

$$p(\mathbf{r}, \hat{\mathbf{s}}, \hat{\mathbf{s}}', t) = \min \left[\beta_0 \exp \left[-\frac{\chi_0}{\nu} \frac{K_d}{[K_d + a(\mathbf{r}, t)]^2} \hat{\mathbf{s}} \bullet \nabla a(\mathbf{r}, t) \right] K(\hat{\mathbf{s}}, \hat{\mathbf{s}}'), \beta_0 \right] \quad \text{E-3}$$

where

β_0 = basal tumbling frequency, s^{-1}

K = turn angle distribution

K_d = dissociation constant between α -methylaspartate and its chemoreceptor on the bacterial surface, mM

p = tumbling probability

K_d is essentially an equilibrium constant (Mesibov, Ordal et al. 1973) describing the interaction between a chemoattractant and its corresponding chemoreceptor. The trend of equation E-3 shows that decreasing K_d , corresponding to an increase in the degree of binding of the chemoreceptors. This results in an increase of the probability that a cell will tumble and eliminate long runs which are the typical result of chemotaxis. If binding of all chemoreceptors occurs, chemotaxis will be greatly reduced until a lower concentration of chemoattractant is encountered. This was shown in the capillary

experiments of this work (Figure 7-3) where a reduction in cells accumulating in the capillary was observed for a serine concentration of 10 mM.

Applying a finite difference approximation in time and position $\mathbf{X}$ to the Alt-Stroock equation above yields:

$$\frac{n_{\alpha}(\mathbf{X}, t + \Delta t) - n_{\alpha}(\mathbf{X}, t)}{\Delta t} = -v \frac{n_{\alpha}(\mathbf{X}, t) - n_{\alpha}(\mathbf{X} - \Delta \mathbf{x}_{\alpha}, t)}{\Delta \mathbf{x}} + \sum_{\beta=1}^L \mathbf{T}_{\alpha\beta}(\mathbf{X}, t) n_{\beta}(\mathbf{X}, t) \quad \text{E-4}$$

where

$$\mathbf{T}_{\alpha\beta}(\mathbf{X}, t) \equiv p_{\beta\alpha}(\mathbf{X}, t) - \delta_{\alpha\beta} \sum_{\gamma=1}^L p_{\alpha\gamma}(\mathbf{X}, t) \quad \text{E-5}$$

and

$\delta_{\alpha\beta}$ = Kronecker delta

L = number of directions (6 in this case)

The equation above is the equation for the transition matrix. This term contains the tumbling probabilities, which vary depending upon the direction of the attractant gradient. There is a lower tumbling probability if the bacteria encounter increasing levels of chemoattractant, keeping the cells swimming in a straight line.

In the experiments performed in this work, the chemoattractant was consumed by the bacteria and was dispersed by diffusion. For the diffusion of the chemoattractant α -methylaspartate, the diffusion coefficient must be altered to allow for the porous medium. An effective diffusion coefficient can be calculated by (Fogler 1992):

$$D_{eff} = \frac{D \varepsilon_p \sigma}{\tau} \quad \text{E-6}$$

where

D = diffusion coefficient for α -methylaspartate, cm^2/s

D_{eff} = effective diffusion coefficient for α -methylaspartate corrected for porous medium, cm^2/s

ε_p = porosity

τ = tortuosity

The diffusion of α -methylaspartate is assumed to be governed by Fick's law of diffusion (Strauss, Frymier et al. 1995). To account for the fate of α -methylaspartate, the following equation can be used:

$$\frac{\partial a(\mathbf{X}, t)}{\partial t} = D_{eff} \frac{\partial^2 a(\mathbf{X}, t)}{\partial x^2} \quad \text{E-7}$$

The term on the left is disappearance of the α -methylaspartate over time. The term on the right calculates the spatial distribution of the α -methylaspartate in one direction.

These equations have been formulated into a FORTRAN code, which is included in Appendix F.

Parameter selection

Simulation parameters were chosen for *E. coli* and the chemoattractant α -methylaspartate. The parameter containing the chemotactic sensitivity coefficient

is KSI. This parameter is varied in different simulation runs between zero and one, zero representing attractant-blind bacteria and one being the upper end on chemotactic sensitivity that was tested. The program creates a simulation box with solid spheres of a radius of 0.035 cm. The step size used in time is 0.4s. Additional parameter values are listed in Table E-1.

Results

Figure E-1 shows profiles in the simulation box. On the vertical axis is dimensionless bacterial concentration. On the horizontal axis is grid point, showing the length in the simulation box. The 100 grid points represent a length of 8.75 mm. The blue line shows the initial α -methylaspartate gradient, which is a step function with all of the chemoattractant on the right hand side of the simulation box. The red and green lines show the bacterial density profiles, initially step functions on the left side of the simulation box. The black dashed line indicates the value of the solid fraction, showing in one dimension where particles are on the grid. The green curve represents the profile for chemotaxis, with KSI=1, the highest value tested in the simulation.

Humps can be seen in this green curve, between 30th and 50th grid points. The humps in the green curve follow a pattern similar to the solid fraction

Table E-1: Critical parameters for the mathematical model.

<i>Parameter</i>	<i>Symbol</i>	<i>Value</i>	<i>Source</i>
α -methylaspartate diffusivity	D	$8.6 \times 10^{-6} \text{ cm}^2/\text{s}$	(Strauss, Frymier et al. 1995)
α -methylaspartate diffusivity, adjusted for porous media	D_{eff}	$1.089 \times 10^{-6} \text{ cm}^2/\text{s}$	calculated
basal tumbling frequency	β_0	2.87/s	(Sarman, Cummings et al. 1994)
chemotactic sensitivity coefficient	χ_0	$4.1 \times 10^{-4} \text{ cm}^2/\text{s}$	(Strauss, Frymier et al. 1995)
chemotaxis term	$KSI = \chi_0 / v$	0, 0.186, 0.372, 1 cm	calculated
constriction factor	σ	0.8	(Fogler 1992)
dissociation constant	K_d	0.125 mM	(Mesibov, Ordal et al. 1973)
grid size	Δh	0.00875 cm	(Sarman, Cummings et al. 1994), adjusted
running speed	v	0.0022 cm/s	(Strauss, Frymier et al. 1995)
sphere radius	$R1$	0.035 cm	experimental work
time step	Δt	0.4 s	calculated
tortuosity	τ	3.0	(Fogler 1992)
tumbling probability	$P00$	0.6827	calculated
void fraction	ϵ_p	0.4749	calculated

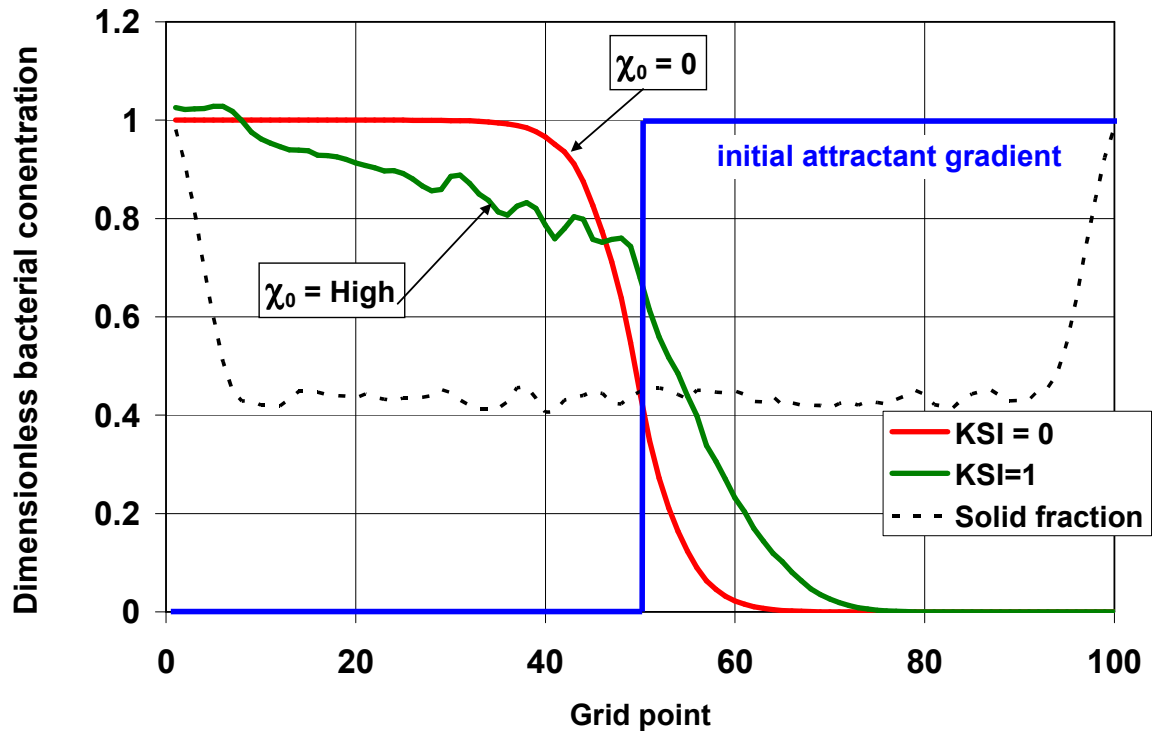


Figure E-1: Effect of chemotactic sensitivity coefficient on migration. The blue line indicates the initial α -methylaspartate attractant gradient. The red line represents diffusion of bacteria without chemotaxis. The green line represents diffusion with a high chemotactic sensitivity coefficient. The black dashed line represents the solid fraction of the porous medium.

indicating a possible coincidence between the bacterial accumulation and the porous medium. These humps in the green curve may indicate formation of a layer of bacteria due to the high chemotactic sensitivity coefficient. Bacteria may be getting pinned in the medium because they have a strong tendency to move towards the chemoattractant. Humps are not visible for the non-chemotactic case ($KSI=0$), demonstrating that this lack of a chemotaxis driving force does not result in pinning of the bacteria into the porous medium.

Figure E-2 shows the development of the bacterial profile from 800 seconds to 4,000 seconds. A congregation of bacteria, or band, can be seen developing just to the right of grid point 50. This band is likely due to chemotaxis of the bacterial cells toward the α -methylaspartate gradient. The chemotactic parameter used for this simulation run is $KSI=0.372$, representing a doubling of the chemotactic sensitivity coefficient for *E. coli* toward α -methylaspartate. This band is similar to band formation in the SFDC seen in Figure 2-8, although the bacteria in the SFDC are dispersed on both sides of the chamber. In this simulation, cells are placed only on the left side of the grid.

Figure E-3 shows the effect of increasing KSI on bacterial profiles. The α -methylaspartate profile is shown after 4,000 seconds, indicating that the chemotactic band at $KSI=0.372$ forms at the steepest part of the attractant gradient, again, similar to the SFDC profile.

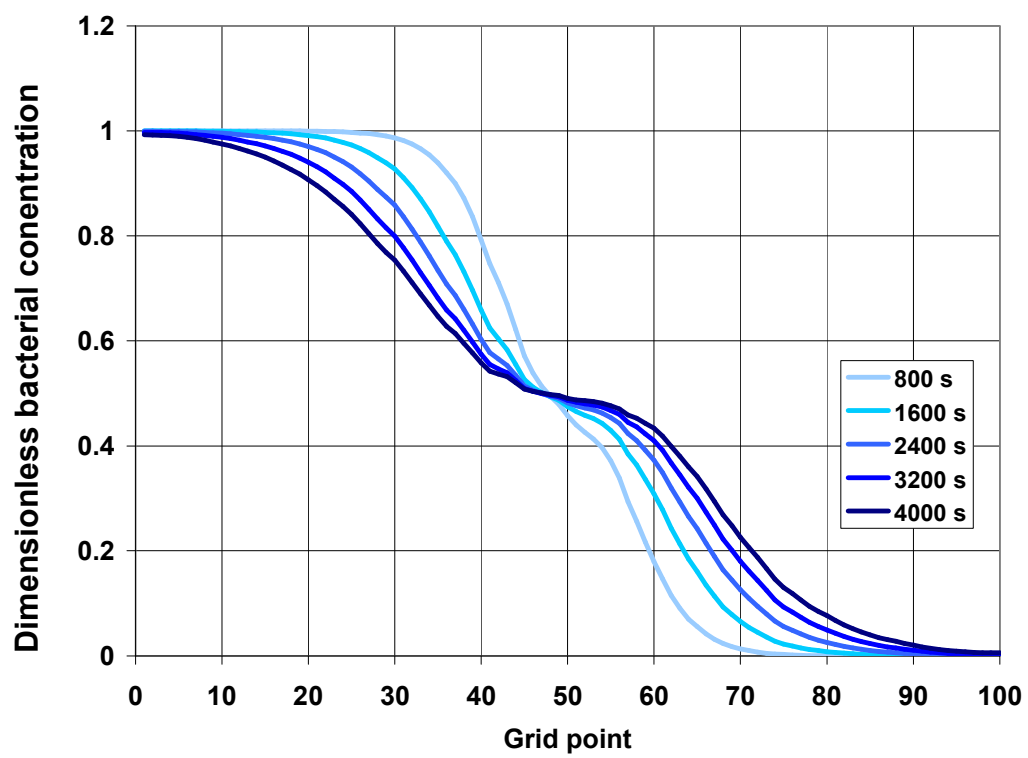


Figure E-2: Development of bacterial profile over 4000 seconds for a chemotactic bacterium. Formation of a band of bacteria is visible.

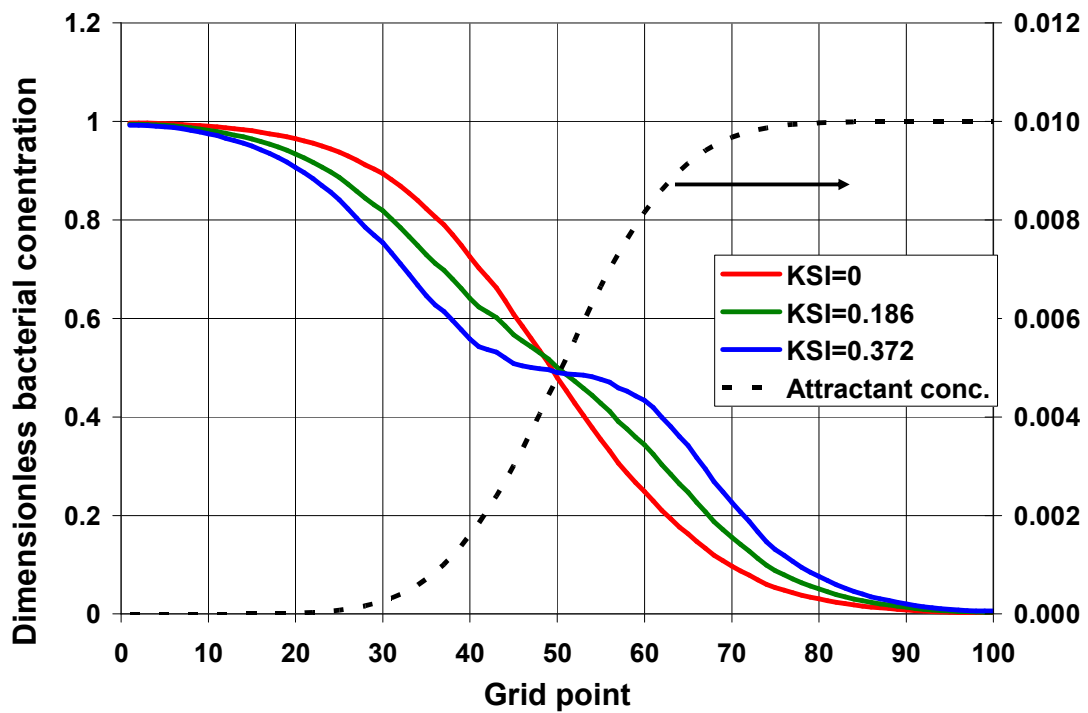


Figure E-3: Effect of increasing chemotactic sensitivity. Bacterial density and attractant profiles are shown for 4,000 second runs. A chemotactic band can be seen in the case for chemotaxis parameter $KSI=0.372$ representing a high chemotactic sensitivity coefficient.

Interpretation of mathematical model results

The results from the mathematical model showed that a chemotactic band begins to form given a particular set of parameters. The variations in KSI and corresponding chemotactic sensitivity coefficient used to elucidate this band are within the limits of chemotactic sensitivities that have been measured experimentally (Barton 1994). In the simulation, chemotaxis had an effect on the number of bacteria that populated a given region of the porous matrix. While this result contradicts the results of BCC experiments which showed no increased degradation due to chemotaxis, it is the result expected theoretically. Other parameters were varied to achieve the simulation results, such as particle size and run time. One must use these parameters systematically to achieve a simulation that is as close to experimental conditions as possible.

The hump in the green curve shown in Figure E-1 may represent the formation of a zone of locally high cell density. It has been reported that cells which have long run lengths tend to get stuck when migrating through a swarm plate (Wolfe and Berg 1989). Thus, the extended runs caused by the high chemotactic sensitivity coefficient may have caused the cells to become trapped in the porous media, causing the formation of this simulated zone of locally high density.

The next step in the computer simulation is to increase the simulation box size to allow higher run times and further elucidation of the chemotactic band of bacteria. The simulation may then be used as a guide for further experimentation

in chemotaxis in this system, as well as serve to predict real world consequences of variations in its different parameters. In addition, aligning the mathematical model with the use of serine parameters versus α -methylaspartate may provide further guidance in experimentation.

Relationship between experimental and model results

While the results of the BCC serine degradation runs did not show a statistically different level of degradation, the computer simulation showed that a chemotactic band did begin to form (with the α -methylaspartate attractant), as in the SFDC.

Appearance of the band indicates that there may yet exist a set of parameters experimentally, that are yet to be discovered, that will elucidate chemotaxis as a beneficial part of the ISB process. One limitation of the model as it is now is that the bacteria migrate to the edges of the simulation box, causing problems due to end effects. The experiments were performed at a run time of 21 hours, while the simulation was run for only 1.11 hours before the bacteria migrated to the edge of the simulation box. In addition, the chemoattractant used in the mathematical model is α -methylaspartate while the chemoattractant used in the experiments is serine. Both chemicals have different chemotactic sensitivity and diffusion coefficients making comparisons difficult, but once chemotactic sensitivity coefficients and dissociation constants become available for *E. coli* and serine the model can be easily adapted.

Appendix F: FORTRAN Code for the Mathematical Model

Input file:

```
10000 10000 10 0 5 6 1
0.6827 0.37 0.30 0.0001 0.0001 0.1006
0.155 0.01 160400.D0 0
0.01 4.356e-7 0.125 0.186 0.00875 0.035
```

```
NSTEP,KPROC,KCHECK,NBOUND,KPROP,NTYPE,NTRAN
P00,P11,PII,DELTA1,DELTA2,DMP
P90,P180,PART,ISHIFT
C0,DIFF,KD,КСI,DELTAH,R1
```

Program file:

```
c filename: lcg.f
c This version, MN2-92, returns to a 100x100x100 grid, changes back lines
c 443-445 for cube
c This version also accesses coord.ascii, the original input file
program lm
implicit none
include 'Imp.inc'
integer itmp1,itmp2,itime,mclock,kprop,kproc,kprint

c Lines to begin counting CPU time, CJO 5/9/05
REAL time_begin, time_end
CALL CPU_TIME(time_begin)

open(2,file='lm.in',status='old')

read(2,*) nstep,kproc,kcheck,nbound,kprop,ntype,ntran
read(2,*) p00,p11,pii,delta1,delta2,dmp
read(2,*) p90,p180,part,ishift
read(2,*) c0,diff,kd,ksi,deltah,r1
part=npix*ncpy*npz
close(2)
c kproc=nstep
kprint=1000000

call init
do 785 kb0=1,nstep
```

```

        kstep=kstep+1
        call castep
c      if (mod(kb0,10).eq.0)
c      $  write(6,900) ' propagation:',kb0
        if(mod(kb0,kprop).eq.0) then
            kav=kav+1
c      write(6,900) ' statistics:',kb0,mclock()-itime
        endif

c      if (mod(kb0,kcheck).eq.0) call test
        if (mod(kb0,kprint).eq.0) call out
        if (mod(kb0,kproc).eq.0) call process2
785  continue

900  format(a,i4,i10)
902  format(4i10)

        CALL CPU_TIME(time_end)
        PRINT *, 'Time of operation was ',time_end-time_begin,' seconds'
        PRINT *, 'Simulation time was ', NSTEP*0.4,' seconds'
        end

subroutine castep
implicit none
include 'Imp.inc'
double precision nue(6),neq(6),dro,tmp,tmp1,tmp2,den,den2,cx,dcx,
&fac,dmp0,dmp1, derf
integer i,j,k,l,m,n,itime,mclock

c      itime=mclock()

        do 100 k=1,npz
        do 100 j=1,npj
        do 100 i=1,npx
        if (.not.void(i,j,k)) then
            do 101 l=1,6
                g2(l,i,j,k)=g1(l,i,j,k)
101      continue
            endif
100  continue

c      write(6,992) fac

```

```

c   write(6,*)
c   write(6,990) ((tranmat(i,j),j=1,6),i=1,6)
c   write(6,*)

c   write(6,900) ' tumbling:',mclock()-itime
c   itime=mclock()

c   if (epsilon.ne.0.d0) call force

      if (nbound.le.1) then

        do 400 j=1,npj
        do 400 k=1,npz
        do 400 l=0,6
          g2(l,0,  j,k)=g2(l,npx,j,k)
          g2(l,npx+1,j,k)=g2(l,1,  j,k)
400    continue

c     if (nbound.ge.1) call flip

        do 410 k=1,npz
        do 410 l=0,6
          g2(l,0,  0, k)=g2(l,0,  npy,k)
          g2(l,npx+1,npy+1,k)=g2(l,npx+1,1, k)
          g2(l,npx+1,0, k)=g2(l,npx+1,npy,k)
          g2(l,0,  npy+1,k)=g2(l,0,  1, k)
410    continue

        do 411 j=1,npj
        do 411 l=0,6
          g2(l,0,  j, 0)=g2(l,0,  j,npz)
          g2(l,npx+1,j,npz+1)=g2(l,npx+1,j, 1)
          g2(l,npx+1,j, 0)=g2(l,npx+1,j,npz)
          g2(l,0,  j,npz+1)=g2(l,0,  j, 1)
411    continue

      elseif (nbound.eq.2) then

        do 403 j=1,npj
        do 403 k=1,npz
        do 403 l=0,6
          g2(l,0,  j,k)=g2(l,npx,npy-j+1,k)
          g2(l,npx+1,j,k)=g2(l,1,  npy-j+1,k)
403    continue

```

```

c      call flip

      do 420 k=1,npz
      do 420 l=0,6
      g2(l,0, 0,k)=g2(l,0, npy,k)
      g2(l,npx+1,npy+1,k)=g2(l,npx+1,1 ,k)
      g2(l,npx+1, 0,k)=g2(l,npx+1,npy,k)
      g2(l,0, npy+1,k)=g2(l,0, 1 ,k)
420    continue

      do 421 j=1,npy
      do 421 l=0,6
      g2(l,0, j,0 ) = g2(l,0, j,npz)
      g2(l,npx+1,j,npz+1) = g2(l,npx+1,j, 1)
      g2(l,npx+1,j, 0) = g2(l,npx+1,j,npz)
      g2(l,0, j,npz+1) = g2(l,0, j, 1)
421    continue

      endif

      do 401 i=1,npx
      do 401 k=1,npz
      do 401 l=0,6
      g2(l,i,0,k)=g2(l,i,npy,k)
      g2(l,i,npy+1,k)=g2(l,i,1,k)
401    continue

      do 402 i=1,npx
      do 402 j=1,npy
      do 402 l=0,6
      g2(l,i,j,0)=g2(l,i,j,npz)
      g2(l,i,j,npz+1)=g2(l,i,j,1)
402    continue

      do 412 i=1,npx
      do 412 l=0,6
      g2(l,i,0,0)=g2(l,i,npy,npz)
      g2(l,i,npy+1,npz+1)=g2(l,i,1,1)
      g2(l,i,npy+1,0)=g2(l,i,1,npz)
      g2(l,i,0,npz+1)=g2(l,i,npy,1)
412    continue

```

```

c  write(6,900) ' periodic boundary conditions:',mclock()-itime
c  itime=mclock()

    if (nbound.eq.0) then

        do 414 j=1,npj
        do 414 k=1,npz
        do 414 l=0,6
            g2(l,0, j,k)=g2(l,1, j,k)
            g2(l,npx+1,j,k)=g2(l,npx,j,k)
414  continue

        endif
        call test2

        den2=4.d0*diff*dble(kstep+ishift)
        den=dsqrt(den2)
        fac=c0/den/sqpi
c      Line added to print out a-methylaspartate profile, CJO 8/21/05 1:15 pm
        open(unit=10,file='ama_profile.out')
c      write(10,*)'C0 = ',c0

        do 10 i=1,npx
            cx=0.5d0*c0*(1.d0+ derf(x0(i)/den))
c      Line added to flip initial chemoattractant profile. CJO, 4/18/05 8:34 pm
c      cx=-(cx-0.5)+0.5
c      Above line did not result in flipping chemoattractant profile CJO, 4/21/05
c      Negative sign added below to flip initial chemoattractant profile. CJO,
4/18/05 8:35 pm
c      Negative sign removed from below CJO, 4/21/05
            dcx=fac*dexp(-x2(i)/den2)
            ptx(i)=dexp(-ksi*kd*dcx/((kd+cx)*(kd+cx)))
c      write(6,*) i, (-ksi*kd*dcx/((kd+cx)*(kd+cx))), ptx(i)
            if (kstep.eq.10000) write(10,*) kstep, i, cx
c      write(10,*)i, cx
            ptxi(i)=1.d0/ptx(i)
            ptx(i)=dmin1(ptxi(i),1.d0)
            ptxi(i)=dmin1(ptxi(i),1.d0)
10  continue

c990  format(6f10.6)
c992  format(1pe12.4)

        fac=0.d0

```

```

tmp1=0.d0
tmp2=0.d0
do 300 k=1,npz
do 300 j=1,npj
do 300 i=1,npx
if (.not.void(i,j,k)) then

    tranmat(2,2)=tranmat0(2,2)
    tranmat(3,3)=tranmat0(3,3)
    tranmat(5,5)=tranmat0(5,5)
    tranmat(6,6)=tranmat0(6,6)

    do 302 m=1,6
    tranmat(1,m)=ptx(i)*tranmat0(1,m)
    tranmat(4,m)=ptxi(i)*tranmat0(4,m)
302    continue

    tranmat(1,1)=-p00*ptx(i)*(1.d0-p11)
    tranmat(2,2)=-p00*(1.d0-p11)
    tranmat(3,3)=-p00*(1.d0-p11)
    tranmat(4,4)=-p00*ptxi(i)*(1.d0-p11)
    tranmat(5,5)=-p00*(1.d0-p11)
    tranmat(6,6)=-p00*(1.d0-p11)

c    do 310 l=1,6
c    tmp=0.d0
c    do 311 m=1,6
c    tmp=tmp+tranmat(l,m)
c311 continue
c    fac=dmax1(fac,dabs(tmp-1.d0))
c310 continue

    do 306 l=1,6
    tmp=0.d0
    do 308 m=1,6
    tmp=tmp+tranmat(m,l)*g1(m,i,j,k)
308    continue
    nue(l)=tmp
306    continue

    if (void(i+1,j,k)) then
        dmp0=dmp
    else

```

```

    dmp0=dmp
endif
if (void(i-1,j,k)) then
    dmp1=0.d0
else
    dmp1=dmp
endif

tmp1=tmp1+g1(1,i,j,k)
g1(1,i,j,k)=(1.d0-dmp0)*g1(1,i,j,k)+dmp1*g2(1,i-1,j,k) + nue(1)
$      +(dmp-dmp1)*g2(4,i,j,k)
tmp2=tmp2+g1(1,i,j,k)

if (void(i,j+1,k)) then
    dmp0=dmp
else
    dmp0=dmp
endif
if (void(i,j-1,k)) then
    dmp1=0.d0
else
    dmp1=dmp
endif

tmp1=tmp1+g1(2,i,j,k)
g1(2,i,j,k)=(1.d0-dmp0)*g1(2,i,j,k)+dmp1*g2(2,i,j-1,k) + nue(2)
$      +(dmp-dmp1)*g2(5,i,j,k)
tmp2=tmp2+g1(2,i,j,k)

if (void(i,j,k+1)) then
    dmp0=dmp
else
    dmp0=dmp
endif
if (void(i,j,k-1)) then
    dmp1=0.d0
else
    dmp1=dmp
endif

tmp1=tmp1+g1(3,i,j,k)
g1(3,i,j,k)=(1.d0-dmp0)*g1(3,i,j,k)+dmp1*g2(3,i,j,k-1) + nue(3)
$      +(dmp-dmp1)*g2(6,i,j,k)
tmp2=tmp2+g1(3,i,j,k)

```

```

if (void(i-1,j,k)) then
    dmp0=dmp
else
    dmp0=dmp
endif
if (void(i+1,j,k)) then
    dmp1=0.d0
else
    dmp1=dmp
endif

tmp1=tmp1+g1(4,i,j,k)
g1(4,i,j,k)=(1.d0-dmp0)*g1(4,i,j,k)+dmp1*g2(4,i+1,j,k) + nue(4)
$      +(dmp-dmp1)*g2(1,i,j,k)
tmp2=tmp2+g1(4,i,j,k)

if (void(i,j-1,k)) then
    dmp0=dmp
else
    dmp0=dmp
endif
if (void(i,j+1,k)) then
    dmp1=0.d0
else
    dmp1=dmp
endif

tmp1=tmp1+g1(5,i,j,k)
g1(5,i,j,k)=(1.d0-dmp0)*g1(5,i,j,k)+dmp1*g2(5,i,j+1,k) + nue(5)
$      +(dmp-dmp1)*g2(2,i,j,k)
tmp2=tmp2+g1(5,i,j,k)

if (void(i,j,k-1)) then
    dmp0=dmp
else
    dmp0=dmp
endif
if (void(i,j,k+1)) then
    dmp1=0.d0
else
    dmp1=dmp
endif

tmp1=tmp1+g1(6,i,j,k)

```

```

    g1(6,i,j,k)=(1.d0-dmp0)*g1(6,i,j,k)+dmp1*g2(6,i,j,k+1) + nue(6)
$      +(dmp-dmp1)*g2(3,i,j,k)
    tmp2=tmp2+g1(6,i,j,k)

endif
300 continue
c commented 3/31/95 pdf2f    write(6,*) tmp1,tmp2,tmp1-tmp2
    call test2
c    call rescale
c    write(6,900) ' propagation:',mclock()-itime
900 format(a,i6)

return
end

subroutine init
implicit none
include 'Imp.inc'
double precision aij(-2:2),fac,tmp,xmin,xmax,ymin,ymax,zmin,zmax
integer ici(6,3),iscprod,i,ii,j,k,l

ici(1,1)= 1
ici(1,2)= 0
ici(1,3)= 0

ici(2,1)= 0
ici(2,2)= 1
ici(2,3)= 0

ici(3,1)= 0
ici(3,2)= 0
ici(3,3)= 1

ici(4,1)=-1
ici(4,2)= 0
ici(4,3)= 0

ici(5,1)= 0
ici(5,2)=-1
ici(5,3)= 0

ici(6,1)= 0
ici(6,2)= 0
ici(6,3)=-1

```

```

do 15 j=1,3
do 15 i=1,6
ci(i,j)=dble(ici(i,j))
15 continue

dflip=0
pn=0
kav=0
py0=0
kb0=0
fac=(8.d0*datan(1.d0))**(-1.5)
sqpi=dsqrt(4.d0*datan(1.d0))

do 7 i=0,npx
c  x0(i)= dble(i)*deltah
x0(i)=(-npx*deltah)*(2*i - npx - 1)/dble(2*(1 - npx))
x2(i)= x0(i)*x0(i)
7 continue

do 8 j=0,npj
c  y0(j)=dble(j)*deltah
y0(j)=(-npj*deltah)*(2*j - npy - 1)/dble(2*(1 - npy))
8 continue

do 9 k=0,npz
c  z0(k)=dble(k)*deltah
z0(k)=(-npz*deltah)*(2*k - npz - 1)/dble(2*(1 - npz))
9 continue

c  do 333 i= 1, npx
c  write(6,*) x0(i)
c 333 continue
c  do 334 j= 1, npy
c  write(6,*) y0(j)
c 334 continue
c  do 335 k= 1, npz
c  write(6,*) z0(k)
c 335 continue

open(2,file='coord.ascii')
do 5 i= 1, np
read(2,*) r0(1,i), r0(2,i), r0(3,i)
5 continue

```

```

close(2)
  write(6,*)'r0 from ascii.coord = ',r0(1,1),r0(2,1),r0(3,1)
xmin=1000000.d0
xmax=0.d0
ymin=1000000.d0
ymax=0.d0
zmin=1000000.d0
zmax=0.d0

c   fac=npix*deltah/(2048/0.8442)**(1.d0/3.d0)
  fac= 2.d0*r1/1.085
  do 10 i=1,np
c   Returned lines for square box, CJO 6/28/05
  r0(1,i)=(r0(1,i) - .5*(2048)**(1./3.))*fac
  r0(2,i)=(r0(2,i) - .5*(2048)**(1./3.))*fac
  r0(3,i)=(r0(3,i) - .5*(2048)**(1./3.))*fac

c   Changed lines below to compensate for rectangular box, 6/24/05
c   r0(1,i)=(r0(1,i) - .5*2*(2048)**(1./3.))*fac
c   r0(2,i)=(r0(2,i) - .5*.5*(2048)**(1./3.))*fac
c   r0(3,i)=(r0(3,i) - .5*.5*(2048)**(1./3.))*fac

c   r0(1,i)=(r0(1,i))*fac - 2.*r1
c   r0(2,i)=(r0(2,i))*fac - 2.*r1
c   r0(3,i)=(r0(3,i))*fac - 2.*r1
10  continue

  write(6,*)'r0 after adjustment = ',r0(1,1),r0(2,1),r0(3,1)
  nr=int(r1/deltah)+1

do 500 i=1,np
xmin=min(xmin,r0(1,i))
xmax=max(xmax,r0(1,i))
ymin=min(ymin,r0(2,i))
ymax=max(ymax,r0(2,i))
zmin=min(zmin,r0(3,i))
zmax=max(zmax,r0(3,i))
500  continue

write(6,*) xmax,ymax,zmax
write(6,*) xmin,ymin,zmin

```

```

      call blackout
c    call plate
      if (ntype.eq.0) then

        do 30 i=1,npx
        do 30 j=1,npj
        do 30 k=1,npz
        tmp=fac*dexp(-0.5d0*(dble(i-13)**2+dble(j-13)**2+dble(k-13)**2))
        g1(0,i,j,k)=tmp*p00
        g2(0,i,j,k)=tmp*p00
        do 30 l=1,6
        g1(l,i,j,k)=tmp*(1.d0-p00)/6.d0
        g2(l,i,j,k)=tmp*(1.d0-p00)/6.d0
30      continue

      elseif (ntype.eq.1) then

        do 29 i=1,npx
        do 29 j=1,npj
        do 29 k=1,npz
        do 29 l=0,6
        g1(l,i,j,k)=0.d0
        g2(l,i,j,k)=0.d0
29      continue

        g1(0,npx/2+1,npj/2+1,npz/2+1)=1.d0
        g2(0,npx/2+1,npj/2+1,npz/2+1)=1.d0

      elseif (ntype.eq.2) then
        call read
        do 32 i=1,npx
        do 32 j=1,npj
        do 32 k=1,npz
        do 32 l=0,6
        g2(l,i,j,k)=g1(l,i,j,k)
32      continue
      elseif (ntype.eq.3) then
        do 33 i=1,npx
        do 33 j=1,npj
        do 33 k=1,npz
        do 33 l=0,6
        g2(l,i,j,k)=0.d0
        g1(l,i,j,k)=0.d0
33      continue

```

```

do 31 l=1,6
g1(l,npx/2+1,npj/2+1,npz/2+1)=1.d0/6.d0
g2(l,npx/2+1,npj/2+1,npz/2+1)=1.d0/6.d0
31  continue

elseif (ntype.eq.4) then

do 70 j=1,npj
do 70 k=1,npz

do 72 i=1,npx/2+1
do 72 l=1,6
g2(l,i,j,k)=1.d0/6.d0
g1(l,i,j,k)=1.d0/6.d0
72  continue

do 74 i=npx/2+2,npx
do 74 l=1,6
g2(l,i,j,k)=0.d0
g1(l,i,j,k)=0.d0
74  continue

70  continue

elseif (ntype.eq.5) then

do 76 j=1,npj
do 76 k=1,npz
do 76 i=1,npx
do 76 l=1,6
g2(l,i,j,k)=1.d0/6.d0
g1(l,i,j,k)=1.d0/6.d0
76  continue

elseif (ntype.eq.6) then

part=0.d0
do 78 j=1,npj
do 78 k=1,npz
do 78 i=1,npx
c To flip bacterial profile, change "le" below to "ge" CJO, 4/18/05
  if (void(i,j,k).or.i.ge.npx/2) then
c    if (void(i,j,k).or.i.le.npx/2) then

```

```

      do 80 l=1,6
      g2(l,i,j,k)=0.d0
      g1(l,i,j,k)=0.d0
80      continue
    else
      part=part+1.d0
      do 82 l=1,6
      g2(l,i,j,k)=1.d0/6.d0
      g1(l,i,j,k)=1.d0/6.d0
82      continue
    endif
78    continue
c commented 3/31/95 pdf2f      write(6,*) part
    endif

    if (ntype.ne.3) kstep=0

    if (ntran.eq.1) then

      do 34 j=1,6
      do 34 i=1,6
      tranmat0(i,j)=p00*p90
      tranmat(i,j)=p00*p90
34      continue

      do 35 i=1,6
      tranmat0(i,i)=p00*p11
      tranmat0(i,mod(i+2,6)+1)=p00*p180
      tranmat(i,mod(i+2,6)+1)=p00*p180
      tranmat(i,0)=0.d0
      tranmat(0,i)=0.d0
35      continue

      tranmat(0,0)=0.d0

    elseif (ntran.eq.2) then

      do 40 i=0,6
      tranmat(i,0)=1.d0-2.d0*(p00+p11+pii)
      tranmat(i,1)=p00+delta1
      tranmat(i,2)=p11
      tranmat(i,3)=pii
      tranmat(i,4)=p00-delta1
      tranmat(i,5)=p11

```

```

    tranmat(i,6)=pii
40    continue

    elseif (ntran.eq.3) then

        do 50 j=1,6
        do 50 i=0,6
        tranmat(i,j)=p00
50    continue

        tranmat(1,1)=p00+delta1
        tranmat(1,4)=p00-delta1

        do 52 i=2,6
        tranmat(i,i)=p00+delta2
        tranmat(i,mod(i+2,6)+1)=p00-delta2
52    continue

        do 54 i=0,6
        tranmat(i,0)=1.d0-6.d0*p00
54    continue

    endif
c **commented 3/30/95 pdf    write(6,100) ((tranmat0(i,j),j=1,6),i=1,6)

100 format(6f10.6)

c    fac=-dble(npix/2)*deltah
c    do 60 i=1,npix
c    x0(i)=fac
c    x2(i)=fac*fac
c    fac=fac+deltah
c60    continue

    return
end

subroutine conserve
implicit none
include 'Imp.inc'
integer i,j,k,l

px=0.d0
py=0.d0
pz=0.d0

```

```

an=0.d0

do 10 i=1,npx
  pxl(i)=0.d0
  pyl(i)=0.d0
  pzl(i)=0.d0
  anl(i)=0.d0
10  continue

do 20 k=1,npz
do 20 j=1,npj
do 20 i=1,npx

do 22 l=1,6
  pxl(i)=pxl(i)+ci(l,1)*g1(l,i,j,k)
  pyl(i)=pyl(i)+ci(l,2)*g1(l,i,j,k)
  pzl(i)=pzl(i)+ci(l,3)*g1(l,i,j,k)
22  continue

do 24 l=0,6
  anl(i)=anl(i)+g1(l,i,j,k)
24  continue

20  continue

do 30 i=1,npx
  px=px+pxl(i)
  py=py+pyl(i)
  pz=pz+pzl(i)
  an=an+anl(i)
30  continue

c  write(6,100) ipx1,ipy1,ipz1,ipw1,n1
c  write(6,100) ipx2,ipy2,ipz2,ipw2,n2
c  write(6,100) ipx1+ipx2,ipy1+ipy2,ipz1+ipz2,
c  &ipw1+ipw2,n1+n2
c100 format(7i10)

return
end

subroutine average
implicit none

```

```

include 'Imp.inc'
integer i

acc(1)=acc(1)+px
acc(2)=acc(2)+py
acc(3)=acc(3)+pz
acc(4)=acc(4)+pw
acc(5)=acc(5)+an

do 10 i=1,npx
accpx(i)=accpx(i)+pxl(i)
accpy(i)=accpy(i)+pyl(i)
accpz(i)=accpz(i)+pzl(i)
accpw(i)=accpw(i)+pwl(i)
accn(i)=accn(i)+anl(i)
10  continue

return
end

subroutine process
implicit none
include 'Imp.inc'
integer i,j,k,l
double precision fac,tmp,den,den2

c  fac=dble(kav)
c  den2=4.d0*diff*dble(kstep+ishift)
c  den=dsqrt(den2)
c
c  do 10 i=1,100
c  av(i)=acc(i)/fac
c10  continue

do 20 k=1,npz
do 20 j=1,npj
do 20 i=1,npx
tmp=0.d0
if (.not.void(i,j,k)) then
do 22 l=1,6
tmp=tmp+g1(l,i,j,k)
22  continue
endif
endif

```

```

    profx(i)=tmp
20  continue

c    do 24 j=1,npj
c    tmp=0.d0
c    do 26 l=1,6
c    tmp=tmp+g1(l,npx/2+1,j,npz/2+1)
c26  continue
c    profy(j)=tmp
c24  continue
c
c    do 28 k=1,npz
c    tmp=0.d0
c
c    do 30 l=1,6
c    tmp=tmp+g1(l,npx/2+1,npj/2+1,k)
c30  continue
c    profz(k)=tmp
c28  continue

c    "profiles after..." output occurs in 2 places - added line
c    number to help determine which one is used.
    write(6,104) '735: profiles after',kstep,'time steps'
c    write(6,101) (profx(i),profy(i),profz(i),i=1,npj)
    write(6,103) (i,profx(i),i=1,npx)
    write(6,*)

100  format(a10,1pe18.10)
101  format(1pe12.4,2e12.4)
102  format(/a,i9,a/)
103  format(i4,f15.5)
104  format(///a15,i6,a12//)
    return
    end

subroutine process2
implicit none
include 'Imp.inc'
integer i,j,k,l, number
double precision fac,tmp,den,den2
character*1 tab

tab= char(9)
do 10 i=1,npx

```

```

        number= 0
        tmp=0.d0
        do 20 j=1,npj
            do 30 k=1,npz
                if (.not.void(i,j,k)) then
                    number = number + 1
                    do 40 l=1,6
                        tmp=tmp+g1(l,i,j,k)
c                        if (i.eq.60) write(6,*) l,i,j,k,g1(l,i,j,k)
40                    continue
                    endif
30                continue
20            continue
        profx(i)=tmp/number
10    continue

    write(6,104) 'Profiles after',kstep,'time steps'
    write(6,103) (i,tab,profx(i),i=1,npx)
    write(6,*)

c    12/3/04 CJO: Insert line to print profiles to a file
    open(unit=7,file='profile.out')
    write(7,104) 'Profiles after',kstep,'time steps'
    write(7,103) (i,tab,profx(i),i=1,npx)
    write(7,*)

100  format(a10,1pe18.10)
101  format(1pe12.4,2e12.4)
102  format(/a,i9,a/)
103  format(i4,a1,f15.7)
104  format(///a15,i6,a12//)
    return
    end

c    subroutine test
c    implicit none
c    include 'Imp.inc'
c    double precision msqx,msqy,msqz,msq,antot
c    integer i,j,k,l
c
c    px=0
c    py=0
c    pz=0
c    an=0

```

```

c    antot=0.d0
c    msqx=0.d0
c    msqy=0.d0
c    msqz=0.d0
c    msq=0.d0
c
c    do 20 k=1,npz
c    do 20 j=1,npj
c    do 20 i=1,npx
c
c    an=0.d0
c    do 22 l=0,6
c        px=px+ci(l,1)*g1(l,i,j,k)
c        py=py+ci(l,2)*g1(l,i,j,k)
c        pz=pz+ci(l,3)*g1(l,i,j,k)
c        an=an+g1(l,i,j,k)
c22    continue
c    antot=antot+an
c    msqx=msqx+an*dble((i-npx/2-1)**2)
c    msqy=msqy+an*dble((j-npy/2-1)**2)
c    msqz=msqz+an*dble((k-npz/2-1)**2)
c20    continue
c    msq=msqx+msqy+msqz
c    write(6,*)
c    write(6,100) antot,px,py,pz,msq,msqx,msqy,msqz
c100  format(1pe14.4,e9.2,2e9.2,4e11.3)
c    write(6,*)
c
c    return
c    end

```

```

subroutine out
implicit none
include 'Imp.inc'

```

```

open(2,file='lm.unit2',form='unformatted',status='unknown')
rewind 2
write(2) g1,kstep
close(2)

```

```

return
end

```

```

subroutine read
implicit none
include 'Imp.inc'

open(2,file='lm.unit2',form='unformatted',status='old')
rewind 2
read(2) g1,kstep
close(2)

c   call test
py0=py

return
end

subroutine test2
implicit none
include 'Imp.inc'
integer i,j,k,l
double precision an1,an2

an1=0
an2=0

do 20 k=1,npz
do 20 j=1,npj
do 20 i=1,npx
if (.not.void(i,j,k)) then
do 22 l=1,6
an1=an1+g1(l,i,j,k)
an2=an2+g2(l,i,j,k)
22   continue
endif
20   continue

c commented 3/31/95 pdf2f   write(6,100) an1,an2
100 format(1pe24.16,e24.16)
return
end

subroutine rescale
implicit none
include 'Imp.inc'

```

```

integer i,j,k,l
double precision tmp1,tmp2,tmp3,scfac

tmp1=0.d0
tmp2=0.d0

do 20 k=1,npz
do 20 j=1,npj
do 20 i=1,npx
tmp3=0.d0
do 21 l=1,6
tmp3=tmp3+g1(l,i,j,k)
21  continue
if (.not.void(i,j,k)) then
tmp1=tmp1+tmp3
else
tmp2=tmp2+tmp3
endif
20  continue

c commented 3/31/95 pdf2f      if (mod(kb0,10).eq.0) write(6,*) tmp1,tmp2,(part-
tmp1)/part

scfac=part/tmp1

do 40 k=1,npz
do 40 j=1,npj
do 40 i=1,npx
if (.not.void(i,j,k)) then
do 41 l=1,6
g1(l,i,j,k)=scfac*g1(l,i,j,k)
41  continue
endif
40  continue

c commented 3/31/95 pdf2f      if (mod(kb0,kcheck).eq.0) write(6,100) ' number of
bacteria:',tmp1

100 format(///a20,1pe16.8/)

return
end

subroutine plate
implicit none

```

```

include 'Imp.inc'
double precision xt,yt,zt,dist2,r2
integer i,j,k,i0,j0,k0,ib,jb,kb,is,js,ks,n
character*1 itemp(101,101)
open(unit=8,file='grid.out')

do 10 k= 2,npz-1
  do 10 j= 2,npj-1
    do 10 i=1,npx
      void(i,j,k)=.false.
10 continue

  do 20 j= 2, npy-1
    do 20 k= 2, npz-1
      i= npx/2
      void(i,j,k)= .true.
20 continue

  do 90 k= 1, npz
    do 95 i=1, npx
      do 95 j= 1, npy
        itemp(i,j)= ' '
        if (void(i,j,k)) itemp(i,j)='X'
95  continue
    write(8,1000) ((itemp(i,j), i=1, npx), j=1, npy)
    write(8,*) ' '
90 continue
1000 format(100a1)

return
end

subroutine blackout
implicit none
include 'Imp.inc'
double precision xt,yt,zt,dist2,r2
c      line added for evaluation of porous media, see MN2-24, CJO 5/11/05
      real voidfrac, voidsum
c      declaration of voidsum added as per MN2-24, CJO 5/11/05
integer i,j,k,i0,j0,k0,ib,jb,kb,is,js,ks,n, isolid
character*1 itemp(npx+1,npj+1)
open(unit=8,file='grid.out')
  open(unit=9,file='voids.out')
r2=r1*r1

```

```

    part=0.d0
c commented 3/31/95 pdf2f    write(6,*) deltah,r1
    do 30 k=1,npz
    do 30 j=1,npj
    do 30 i=1,npx
    void(i,j,k)=.false.
30  continue

    do 10 n=1,np

    xt=r0(1,n)
    yt=r0(2,n)
    zt=r0(3,n)

c    i0=int(xt/deltah)
c    j0=int(yt/deltah)
c    k0=int(zt/deltah)

    i0= float(npz - 1)/(npz*deltah) *xt + float(npz+1)*.5
    j0= float(npj - 1)/(npj*deltah) *yt + float(npj+1)*.5
    k0= float(npz - 1)/(npz*deltah) *zt + float(npz+1)*.5

    ib=max( 1,i0-nr)
    is=min(npz,i0+nr)
    jb=max( 1,j0-nr)
    js=min(npj,j0+nr)
    kb=max( 1,k0-nr)
    ks=min(npz,k0+nr)

    do 20 k=kb,ks
    do 20 j=jb,js
    do 20 i=ib,is
    dist2=(x0(i)-xt)**2+(y0(j)-yt)**2+(z0(k)-zt)**2
    if (dist2.lt.r2.and..not.void(i,j,k)) then
        void(i,j,k)=.true.
        part=part+1.d0
    endif
20  continue
10  continue

c commented 3/31/95 pdf2f    write (6,*) part
    part=0.d0

    do 40 k=1,npz
    do 40 j=1,npj

```

```

do 40 i=1,npx
  if (.not.void(i,j,k)) part=part+1.d0
40  continue

c commented 3/31/95 pdf2f    write(6,*) nr,part

do 45 j=1,npj
do 45 k=1,npz
  void(0,  j,k)=void(npx,j,k)
  void(npx+1,j,k)=void(1, j,k)
45  continue

do 50 k=1,npz
  void(0, 0, k)=void(0, npy,k)
  void(npx+1,npj+1,k)=void(npx+1,1, k)
  void(npx+1,0, k)=void(npx+1,npj,k)
  void(0, npy+1,k)=void(0, 1, k)
50  continue

do 60 j=1,npj
  void(0, j, 0)=void(0, j,npz)
  void(npx+1,j,npz+1)=void(npx+1,j, 1)
  void(npx+1,j, 0)=void(npx+1,j,npz)
  void(0, j,npz+1)=void(0, j, 1)
60  continue

do 70 i=1,npx
do 70 k=1,npz
  void(i,0,k)=void(i,npj,k)
  void(i,npj+1,k)=void(i,1,k)
70  continue

do 75 i=1,npx
do 75 j=1,npj
  void(i,j,0)=void(i,j,npz)
  void(i,j,npz+1)=void(i,j,1)
75  continue

do 80 i=1,npx
  void(i,0,0)=void(i,npj,npz)
  void(i,npj+1,npz+1)=void(i,1,1)

```

```

void(i, npy+1, 0)=void(i, 1, npz)
void(i, 0, npz+1)=void(i, npy, 1)
80  continue

isolid= 0
do 90 k= 1, npz
  do 95 i=1, npx
    do 95 j= 1, npy
      itemp(i,j)= ' '
      if (void(i,j,k)) itemp(i,j)='X'
      if (void(i,j,k)) isolid= isolid + 1
95  continue
  write(8,1000) ((itemp(i,j), i=1, npx), j=1, npy)
  write(8,*) ' '
90 continue
  write(6,*) 'solid fraction is', float(isolid)/float(npx*npy*npz)
1000 format(100a1)

```

c lines added to code to evaluate porous media, see MN2-24 for code, CJO
5/11/05

```

      do 100 i=1,npx
        voidsum=0
        do 200, j=1, npy
          do 300 k=1, npz
            if(.not.void(i,j,k)) voidsum=voidsum+1
300 continue
200  continue
      voidfrac=voidsum/(npy*npz)
      write(6,*)'void fraction = ',i,voidfrac
      write(9,*)voidfrac

100  continue

return
end

```

```

FUNCTION derf(x)
double precision, intent(in) :: x
double precision :: derf
CU  USES gammp
double precision gammp
if(x .lt. 0.d0)then
  derf=-gammp(0.5d0,x**2.d0)
else

```

```

    derf=gammp(0.5d0,x**2.d0)
endif
return
END

FUNCTION gammp(a,x)
double precision, intent(in) :: a, x
double precision :: gammp
CU  USES gcf,gser
double precision :: gammcf,gamser,gln
if(x .lt. 0.d0 .or. a .le. 0.d0) pause 'bad arguments in gammp'
if(x .lt. a + 1.d0) then
    call gser(gamser,a,x,gln)
    gammp=gamser
else
    call gcf(gammcf,a,x,gln)
    gammp=1.d0 - gammcf
endif
return
END

FUNCTION gammq(a,x)
double precision, intent(in) :: a, x
double precision :: gammq
CU  USES gcf,gser
double precision :: gammcf,gamser,gln
if(x .lt. 0.d0 .or. a .le. 0.d0) pause 'bad arguments in gammq'
if(x.lt. a+1.d0)then
    call gser(gamser,a,x,gln)
    gammq=1.d0 -gamser
else
    call gcf(gammcf,a,x,gln)
    gammq=gammcf
endif
return
END

SUBROUTINE gcf(gammcf,a,x,gln)
double precision, intent(in) :: a, x
double precision, intent(out) :: gammcf, gln
double precision, parameter :: EPS = 3.d-7
double precision, parameter :: FPMIN = 1.d-30
integer, parameter :: ITMAX = 100
CU  USES gammln
INTEGER i

```

```

double precision an,b,c,d,del,h,gammln
gln=gammln(a)
b=x+1.d0-a
c=1.d0/FPMIN
d=1.d0/b
h=d
do i = 1,ITMAX
  an=-i*(i-a)
  b=b+2.d0
  d=an*d+b
  if(abs(d).lt.FPMIN)d=FPMIN
  c=b+an/c
  if(abs(c).lt.FPMIN)c=FPMIN
  d=1.d0/d
  del=d*c
  h=h*del
  if(abs(del-1.d0).lt.EPS)goto 1
enddo
pause 'a too large, ITMAX too small in gcf'
1  gammcf=dexp(-x+a*dlog(x)-gln)*h
return
END

```

```

SUBROUTINE gser(gamser,a,x,gln)
double precision, intent(in) :: a, x
double precision, intent(out) :: gamser, gln
double precision, parameter :: EPS = 3.d-7
integer, parameter :: ITMAX = 100
CU  USES gammln
  INTEGER n
  double precision :: ap,del,sum,gammln
  gln=gammln(a)
  if(x.le.0.d0)then
    if(x.lt.0.d0)pause 'x < 0 in gser'
    gamser=0.d0
    return
  endif
  ap=a
  sum=1.d0/a
  del=sum
  do n=1,ITMAX
    ap=ap+1.d0
    del=del*x/ap
    sum=sum+del
    if(abs(del).lt.abs(sum)*EPS)goto 1
  enddo
  gamser=sum

```

```

        enddo
        pause 'a too large, ITMAX too small in gser'
1   gamser=sum*dexp(-x+a*dlog(x)-gln)
        return
        END

        FUNCTION gammln(xx)
        double precision, intent(in) :: xx
        double precision :: gammln
        INTEGER :: j
        DOUBLE PRECISION :: ser,stp,tmp,x,y,cof(6)
        SAVE cof,stp
        DATA cof,stp/76.18009172947146d0,-86.50532032941677d0,
        *24.01409824083091d0,-1.231739572450155d0,.1208650973866179d-2,
        *-.5395239384953d-5,2.5066282746310005d0/
        x=xx
        y=x
        tmp=x+5.5d0
        tmp=(x+0.5d0)*dlog(tmp)-tmp
        ser=1.000000000190015d0
        do 11 j=1,6
            y=y+1.d0
            ser=ser+cof(j)/y
11   continue
        gammln=tmp+dlog(stp*ser/x)
        return
        END

```

VITA

Christopher John “CJ” O’Lenick was born in Livingston, New Jersey on March 18, 1970. He attended primary school in Nutley, New Jersey. He attended high schools in Piscataway, New Jersey, Bayonne, New Jersey and Montclair, New Jersey where he graduated in 1988. In May, 1992 he earned his Bachelor of Science in Chemical Engineering from Rutgers University in New Brunswick, New Jersey. In May, 1994 he earned his Master of Science Degree in Chemical Engineering from the same school. In 1995, he entered the doctoral program at the University of Tennessee, Knoxville. He earned his Doctor of Philosophy in Chemical Engineering in May, 2006.