PARTITIONING BETWEEN THE SOIL-ADSORBED AND PLANKTONIC PHASES OF *ESCHERICHIA COLI*

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ABSTRACT

A scarcity of comparable research on the transport of bacteria has forced hydrologic models to assume that bacteria travel as dissolved chemicals. In reality, most bacteria preferentially attach to soil aggregates, and behave very differently from planktonic bacteria. The goal of this research project was to identify and evaluate a laboratory method for partitioning between attached and planktonic bacteria that could be used to improve hydrologic modeling.

Attachment was measured indirectly as the difference between total and planktonic bacterial concentration. Planktonic concentration was defined as the concentration of bacteria that could pass through an 8 μm screen. Total concentration was determined by disaggregating attached bacteria through a dispersion treatment. A randomized complete block design was structured to test for the effects of filtering, two dispersion treatment options, and the presence of soil on concentration. Tween-85 surfactant was selected as the best dispersant for use in further studies. About 78% of bovine *E. coli* in the laboratory samples were adsorbed/associated with sterile soil particles.

Twenty samples of different bacteria-soil ratios were analyzed using this method to develop an isotherm equation describing *E. coli* partitioning. The *E. coli* used to inoculate these samples was cultured using a chemostat reactor to control cell growth stage and control variability. A linear isotherm (R^2=0.88) was selected to describe this experimental data; however, future studies characterizing the partitioning behavior of *E. coli* under different environmental conditions are recommended in order to better understand attachment prior to modeling attached and planktonic *E. coli* separately.

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CHAPTER 1: INTRODUCTION

Waterborne human pathogens are microorganisms that are transmitted to people through drinking water or recreational water activities, such as swimming, fishing, or boating. Waterborne pathogens are responsible for a wide variety of diseases, including common gastrointestinal illness and diarrhea, fever, dermatitis, and more severe and potentially deadly diseases such as amebic meningoencephalitis (Rosen, 2001). These diseases are not confined solely to third world countries without modern water and sanitation methods. Between 1999 and 2000, there were 36 outbreaks of waterborne disease in the United States associated with drinking water and 59 outbreaks associated with recreational water use. These outbreaks were responsible for over 4,000 cases of reported illness and six deaths (CDC, 2002). Clearly, it is important to monitor our nation's waters for the presence of these pathogens in order to identify waters that are potentially unsafe, and to identify and eliminate sources of pathogens in order to prevent future illness.

Because it would be difficult, expensive, and time-consuming to test waters designated for human use for the presence of all bacteria, protozoa, viruses, and helminthes known to cause waterborne disease, waters are typically tested for only one or two organisms that are considered indicative of the presence of other common pathogens (Rosen, 2001). Traditionally, the most common indicator organism used to evaluate water quality is fecal coliform bacteria. Fecal coliforms are found in the digestive tract of warm-blooded mammals and are thought to indicate fecal contamination if found in a water body. Recently, there has been a shift toward using a single specific type of fecal coliform, *Escherichia coli* (*E. coli*), as the main indicator of biological water quality, due to *E. coli*'s higher correlation with many common pathogens, such as viruses and protozoa. The Commonwealth of Virginia will require monitoring of all fresh

waters for *E. coli* by 2008. Water bodies with a geometric mean of *E. coli* counts higher than 126 colony-forming units (CFU)/100 mL or with a single sample count higher than 235 CFU/100 mL will be considered impaired for human recreational use (VASWCB, 2003).

Section 303(d) of the 1972 Clean Water Act mandates the development of Total Maximum Daily Loads (TMDLs) for water bodies that do not meet water quality standards in order to protect public and environmental health. A TMDL is a plan that identifies the maximum amount of a pollutant that can be discharged to a water body while still meeting water quality standards for its designated uses. The Virginia Department of Environmental Quality has identified 4,318 miles of Virginian streams that do not meet water quality standards and require TMDL development; of these, over 3000 miles are impaired due to high levels of fecal coliform bacteria (VADEQ, 2002). Nationally, pathogen indicators are the most common pollutant responsible for identified river and stream impairments (USEPA, 2000).

In order to develop TMDLs, sources of the pollutant must be identified and decreased to a level that will keep the water body in compliance with water quality standards. The primary tools used to determine the required pollutant source reductions are nonpoint source (NPS) computer simulation models. Because there is a large amount of uncertainty in bacterial modeling, many models may oversimplify bacterial transport. To the author's knowledge, no existing models account for bacteria-sediment interactions, bacteria resuspension from sediments, or bacteria aggregation, all of which can heavily influence the bacteria concentrations in water. For example, the Hydrologic Simulation Program-Fortran (HSPF) model, which is supported by the USEPA for bacteria modeling, models indicator bacteria as dissolved pollutants (USEPA, 2001). The over-simplicity of the models and the lack of sufficient monitoring data can make it very difficult to accurately predict bacteria levels in a stream, even when all possible

bacteria sources are known. If predictions of bacteria levels contain a great deal of uncertainty, it is very difficult to accurately determine the source reductions required to meet applicable water quality standards. Improvements in understanding and modeling bacteria fate and transport will improve the accuracy of NPS computer model predictions and make the process of TMDL development and implementation more manageable.

1.1 Objectives

The overall goal of this research project is to characterize fecal bacteria attachment to soil particles in order to more accurately simulate bacteria in both planktonic and adsorbed forms. Specifically, the objectives were to:

1. Investigate the accuracy and reliability of different methods used to differentiate between planktonic and adsorbed bacteria.
    a. Identify candidate methods from the literature.
    b. Test promising methods using samples of known composition.
    c. Evaluate and select the best method for differentiation between planktonic and adsorbed bacterial forms.
2. Use the selected method(s) to develop isotherm equations describing the partitioning of *E. coli* bacteria between planktonic and sediment-adsorbed forms.

1.2 Research Hypotheses

1. A significant proportion of *E. coli* will become attached to topsoil after mixing.
2. There is no difference in total concentration obtained from a sample after treatment with a dispersion technique and an untreated sample.
3. The planktonic concentration of *E. coli* in a water sample is related to the soil-adsorbed concentration of *E. coli* in the sample and can be described using an isotherm model.

CHAPTER 2: LITERATURE REVIEW

2.1 Bacteria and Sediment Interactions

While bacteria are currently modeled as dissolved pollutants in widely used nonpoint source pollution models, considerable evidence exists indicating that bacteria actually preferentially attach to sediment particles. Clay particles and aggregates, though overall negatively charged, have positive edges to which negatively charged bacteria may attach (Marshall, 1975). Studies of sediment underlying rivers have shown a positive correlation between the amount of bacteria present in the sediment and decreasing average particle size of sediment (Albinger, 1993) and studies focusing on bacterial attachment have shown that most particle-associated bacteria were associated with fine (<10 μm) particles (Auer and Niehaus, 1993).

The potential for bacterial attachment to particles varies both by species and by species phenotype. Bacteria prone to attachment have the potential to develop a layer of surface polymers on their cell walls, called an exocellular polysachharide layer or slime layer, or cell wall attachment structures, such as fimbrae or pili. These characteristics may only be exhibited in the presence of potential attachment surfaces, so the environment in which bacteria grow may greatly influence their attachment abilities. LeChavallier et al. (1984) conducted a study on the ability of microorganisms to withstand chlorination when attached to particles of granular carbon from a filter. When the microorganisms were grown in the presence of granular carbon particles for several days, they exhibited growth of slime layers and strong attachment; however, when exposed to granular carbon particles for only 20 minutes prior to chlorination, the microorganisms exhibited some attachment, no growth of slime layers, and less resistance to disinfection. A study by Schillinger and Gannon (1985) determined that pure culture bacteria

grown in a nutrient broth developed fimbrae and exhibited greater attachment to sediment aggregates than cultures grown on a nutrient agar, which did not develop fimbrae.

Cell surface hydrophobicity, or attraction to water, strongly influences whether bacteria will become particulate-attached. Huysman and Verstraete (1993) studied the migration of hydrophobic and hydrophilic bacteria through soil columns as a result of irrigation practices. Hydrophobic strains, like *E. coli*, experienced significantly less migration than hydrophilic strains, like streptococci. This lack of movement through the column was attributed to attachment and retention by soil particles. A study by Zita and Hermansson (1997) confirmed that increases in cell surface hydrophobicity of *E. coli* increased the bacteria's adhesion to sludge flocs in wastewater treatment processes.

2.2 Effects of Bacterial Attachment

Attachment to particles may also affect the fate and transport of bacteria. A plot study investigating the efficiency of filter strips at improving the water quality of discharges from fields spread with poultry manure found that bacterial numbers were decreased by the use of filter strips, suggesting that while some bacteria infiltrate the soil profile, some cells probably settle out with sediments (Coyne and Blevins, 1995). Huysman and Verstraete's (1993) study on the irrigation of soil columns inoculated with fecal bacteria found that not all the bacteria were leached out of the soils, and the addition of clay to the columns decreased the transport of all bacteria through the columns. Because these experiments took place over a 24-hr period, the lack of movement was attributed solely to bacterial adhesion to sediment particles, and die-off and regrowth were assumed to be negligible.

Sediment-attached bacteria can aggregate into larger particles that can settle out of the water column faster (Schillinger and Gannon, 1985). Numerous studies of sediments underlying

surface waters have found bacterial concentrations significantly higher than in the overlying waters. Higher sediment concentrations of bacteria have been documented in both freshwater (Irvine and Pettibone, 1993; Stephenson and Rychert, 1982; Matson et al., 1978) and marine waters (Ferguson et al., 1996; Erkenbrecker, 1981), indicating that bacteria may be deposited with sediments, where they can accumulate over time.

The presence of sediments may reduce bacterial die-off and increase regrowth, resulting in the maintenance of large bacterial populations near sediment deposition areas in stream bottoms. Sherer et al. (1992) tested the die-off rates of fecal coliforms and fecal streptococci in suspension in waters with higher suspended sediments, waters with no suspended sediment, and waters with no suspended sediment overlying settled sediments. Die-off in waters with suspended sediment was lowest, especially when the suspended sediment was mostly composed of fine particles. Gerba and McLeod (1976) tested the effect of adding sediments to suspensions of *E. coli* in marine waters and also found that prolonged survival was associated with the addition of sediments. Howell et al. (1996) studied the die-off rates of fecal coliforms and fecal streptococci in manure-amended sediments of different particle size distributions. Die-off rates for both species decreased with decreasing sediment particle size.

The addition of sediments to bacterial suspension may actually induce regrowth of the population in addition to preventing die-off. Desmarais et al. (2002) added river water containing fecal bacteria to varying amounts of sterilized river sediments. The addition of sediment resulted in a significant increase in numbers of *E. coli* and enterococci within 24 hours. The magnitude of the increase in growth was directly related to the amount of sediment added, indicating that the observed regrowth was not due only to the absence of predators, but also the presence of the sediment. LaLiberte and Grimes (1981) observed that the addition of both sterile and non-sterile

sediments to lake water containing *E. coli* resulted in an increase in the *E. coli* population. The extended survival and regrowth of bacteria in sediments or sediment-laden waters demonstrated in these lab-based experiments may explain the significant presence of bacteria in sediments even when bacteria levels in overlying waters are relatively low.

Increases in bacterial survival when associated with sediment may be due to physical protection by the sediments and additional nutrient sources. Roper and Marshall (1978) determined that attachment to clays and other fine particles by fecal bacteria provides a physical barrier between these bacteria and predators, resulting in increased survival. Physical protection through particle attachment similarly also reduces die-off of fecal bacteria exposed to different forms of disinfection, including chlorine (Herson et al., 1987; LeChevallier, 1984) and ultraviolet radiation (Qualls et al., 1983). Sediments can also provide bacteria with required nutrients, resulting in regrowth and maintenance of a higher population (Desmarais et al., 2002; LaLiberte and Grimes, 1981).

Because sediment-attached bacteria may settle out in underlying sediments and accumulate, if these sediments are resuspended by human activity or storm surges, overlying waters may become recontaminated long after the original pollution event and "disappearance" of the bacteria. Resuspension of fecal bacteria from bottom sediments has been observed in the field and induced experimentally. Pettibone et al. (1996) observed an increase in fecal coliform and total suspended solids concentration following a ship's passage. Increases in fecal bacteria concentration have also been observed in waters after the dredging of bottom sediments (Grimes, 1975) and after cow crossings (Sherer et al., 1988).

Researchers have also purposely disturbed sediments expected to contain large bacteria populations to quantify the amount of bacteria that can be potentially re-released to the overlying

waters. In order to simulate a storm event without the addition of new bacteria from overland sources, McDonald et al. (1982) created artificial storm surges by releasing water from a reservoir into a river. Observed concentrations of total coliforms and *E. coli* following this artificial storm surge were more than ten times their original values. Stephenson and Rychert (1982) simulated resuspension of *E. coli* from a stream bottom near a cow pasture by raking an area of 4 m^2 of bottom sediment vigorously for 30 s using a garden rake. Water samples from the resulting plume were collected at 10 s intervals at a point 5 to 10 m downstream. The concentrations in the collected samples were 2-760 times the original concentration of *E. coli* observed in the overlying waters before induced resuspension. A similar technique to induce sediment and bacterial resuspension used by Sherer et al. (1988) determined that between 1.8 and 760 x 10^6 fecal coliforms and 0.8 to 5610 x 10^6 fecal streptococci could be resuspended from 1 m^2 of a creek bottom adjacent to a cow pasture.

2.3 Partitioning Between Attached and Planktonic Bacteria

While there is currently no accepted standardized method for separation and enumeration of planktonic and sediment-attached bacteria in water, several different techniques have been used to quantify sediment-attached bacteria, including chemical and/or ultrasonic dispersion of bacteria from soils, filtration using screens, and centrifugation.

2.3.1 Filtration

Filters of uniform porosity or a series of filters of differing porosity can be used to partition between sediment-attached and planktonic bacteria. Those bacteria retained on the screen after filtration are considered attached, while those passing through the screen are considered planktonic. Schillinger and Gannon (1985) passed stormwater samples through a series of four screens of decreasing porosity (52 μm, 30 μm, 10 μm, and 5 μm) to determine the

relative attachment of different bacteria isolates to different particle size classes. Bacteria were removed from the screens for enumeration by aseptically removing each screen, placing it in a tube of 0.02% polysorbate 80, and shaking the tube vigorously. Eluted bacteria were then enumerated using the MPN technique. Results from this study indicated that the majority of bacteria, especially gram-negative bacteria, were associated with particles retained on the 52µm and 30 µm screens. Auer and Niehaus (1993) used a similar serial screening technique to study fecal coliform attachment to particles and sedimentation rates in a lake. This study used seven screens of decreasing porosity (102 µm, 53 µm, 20 µm, 10 µm, 6 µm, 1 µm, and 0.45 µm) to partition sediment size classes. Those particles passing through the 0.45 µm screen were considered dissolved. To remove the bacteria from the screens for enumeration, the screens were aseptically removed and added to a buffered solution with an added surfactant, Tween 85, to disperse the bacteria from the screens. In contrast to the Schillinger and Gannon study, this study found that the majority of bacteria were associated with particles between 6 µm and 10 µm (retained on the 6 µm screen).

Some studies investigating the behavior of particle-attachment operationally define attached bacteria as those bacteria retained on an 8 µm screen (Qualls et al., 1983; Mahler et al., 2000). Since most bacteria are only 1 µm to 2 µm in size, it was assumed an 8 µm screen would only allow single bacteria or bacteria attached to very small particles to pass through. Mahler et al. (2000) tested this hypothesis by passing sediment-planktonic suspensions of *E. coli* and *S. aureus* of known concentration through 8 µm screens to determine how many planktonic bacteria were retained on the screen. Over 99% of *E. coli* and 97% of *S. aureus* passed through the screen, indicating that very few planktonic bacteria failed to pass through the filter.

2.3.2 Dispersion from particles: ultrasonic treatment and surfactants

Rather than culturing the actual sediment-attached bacteria retained on a screen, in some techniques attached bacteria are determined by taking the difference of the total sample bacterial concentration and the bacteria passing through a screen since more than one bacteria may attach to a sediment particle yet one sediment particle will only produce one colony forming unit when cultured. Before the total concentration of bacteria in an unknown sample can be determined, the attached bacteria must be dispersed from the particles. Dispersion can be achieved by ultrasonic treatment or surfactants.

Mahler et al. (2000) used ultrasonic treatment of groundwater samples to determine a total bacterial concentration. Water samples were split, half for total bacteria analysis, and half for planktonic bacteria analysis to determine the amount of attached bacteria in their samples. Half of the sample was passed through a filter and bacteria in the filtrate were defined as planktonic. The other half of the sample was sonicated at 45 kHz, 20 W for 5 minutes to disperse bacteria from sediment particles and then analyzed to obtain a total bacterial concentration. Results indicated that at times over 99% of the bacteria in groundwater were sediment attached. Ferguson et al. (1996) also used sonication to disperse bacteria from estuarine sediments for enumeration. Sediment samples were diluted 1:10 with sterile water and then sonicated at 100 W for 30 s. Only total bacterial counts were recorded, rather than relative amount of attached and planktonic bacteria. Pike et al. (1972) tested the lethal effects of ultrasonic treatment on *E. coli* by exposing a known bacterial concentration to varying times of treatment and found that the die-off rate during treatment was very small.

Dispersing agents, or surfactants, can be used to separate bacteria from activated sludge flocs (Gayford and Richards, 1970), vegetation, sediments (Velji and Albright, 1986), and

polystyrene (Paul and Jeffrey, 1985). Velji and Albright (1986) compared chemically treating sediment-attached marine bacteria with deflocculants to dispersing them using sonication. They used a formaldehyde solution to strengthen attached cells before dispersing them using pyrophospate, sonication at 100 W for 30 s, and treating the cells with pyrophosphate prior to sonication. Since the formaldehyde killed the cells, bacteria were enumerated microscopically by counting the number of cells present in 20 randomly chosen microscopic fields. The resultant mean number of bacteria after treatment had the lowest coefficient of variation for the combination treatment. Relative amounts of attached and planktonic bacteria were not calculated.

2.3.3 Centrifugation

Centrifuging bacteria and sediment samples can be used to determine the amount of bacteria that are sediment-attached, though this technique may be less precise without accurately determining the total concentration. Huysman and Verstraete (1993) added a clay mixture to a bacterial suspension of known concentration, mixed the sample slowly to promote attachment, and then centrifuged at 120g for 30 s. Bacteria in the supernatant were considered planktonic, and the difference between this concentration and the original concentration was assumed to be the amount of bacteria that had attached to the clay. The clay-bacteria mixture was allowed to mix for varying times to determine the optimum time required for adhesion. While there was measurable adhesion to the clay after only 30 s, adhesion to the particles was not complete for 15-20 minutes.

A study of ammonia-oxidizing bacteria in soils by Aakra et al. (2000) separated bacteria using a centrifuging technique called dispersion-density-gradient centrifugation. Soil samples were diluted and dispersed using a blender at full speed (22,000 rpm) for 10 min. After

dispersion, a 200 mL sample of the soil dilution was centrifuged for 3 hours at 7,000 rpm on top of a 40 mL cushion of Nycodenz solution, a density-gradient material. After the centrifugation procedure, the soil still containing attached bacteria had settled below the Nycodenz cushion, while planktonic bacteria remained suspended above the cushion and could be sampled using a syringe.

2.3.4 Microscopic methods

Attachment to particulates may be verified microscopically. Most procedures involve staining the bacterial cells and then enumerating the number of cells present in a randomly selected field. Huysman and Verstraete (1993) transferred a few drops of their clay-bacteria solution to a slide, allowed it to air dry, and then stained the cells with crystal violet. The slide was then analyzed microscopically by an automated image analysis system that determined the number of bacteria attached to twelve randomly selected sediment aggregates. Other common stains used for direct counts include acridine orange (AO) and 4,6-diamidino-2-phenylindole (DAPI). Unlike crystal violet, which stains the cell's cytoplasm, these stains bind to the DNA or RNA of cells and fluoresce when excited by certain wavelengths, making the cells easy to count (Porter and Feig, 1980). Most microscopic techniques for evaluating microbial attachment concern attachment to surfaces that are relatively large as compared to sediment particles (Paul and Jeffrey, 1984; Morisaki et al., 1999). Bacteria are introduced to a surface, such as a glass slide, and allowed a period of time to develop attachment mechanisms. After this time period, the surface is washed in a salt and mineral medium to remove unattached bacteria. The surface is then stained and the bacteria present in 10 different microscopic fields enumerated to determine a concentration of attached cells.

2.4 Calculation of Retention Coefficients

In order to better understand and model the transport of fecal bacteria, Reddy et al. (1981) used the Freundlich isotherm, simplified to a linear isotherm, to partition between sediment-attached and planktonic bacteria:

$$MORT = K*MOSOL \qquad \textit{(Eq. 1)}$$

where MORT = the number of attached bacteria per gram of soil; K = retention coefficient; and MOSOL = number of planktonic bacteria per mL of soil solution. Retention coefficient values for total coliforms, fecal coliforms, and fecal streptococci were estimated from data obtained from a study by Matson et al. (1978). Estimated retention coefficients ranged from 261 mL/g for fecal streptococci to 1909 mL/g for fecal coliforms. The study by Matson et al. recorded only amounts of bacteria present in river sediment and overlying water; therefore, the bacteria concentrations considered attached may have been influenced by sedimentation, die-off, and regrowth.

2.5 Bacterial Growth Stage

2.5.1 Growth stage in the natural environment

The desired growth stage of the bacteria tested is an important consideration in any lab experiment, regardless of the partitioning methods later used on the culture. Different growth stages result in different physiological states, influencing the bacteria's exhibited metabolic capabilities and morphological characteristics, which can cause variation in laboratory results.

As the cells consume available nutrients, *E. coli* growth follows a typical bacterial growth curve consisting of four main phases: lag phase, log phase, stationary phase, and die-off (Jones, 1997). Cells begin to divide in lag phase, moving quickly into log phase as the cells consume nutrients. The cells are very stress-sensitive in log phase because they are not substrate-limited

and so have not had to adjust to a stressful environment. Log phase is considered "balanced growth"; while the number of cells is increasing, the ratio of DNA to RNA or proteins is constant, indicating a constant level of activity. As the bacteria population grows and begins to consume available nutrients, the cells move into stationary phase. During this period the cells detect the lack of substrate and begin to undergo metabolic and physiological changes in order to adjust to the substrate-limited environment. Changes occur at the molecular level as the cells activate dormant genes or suppress currently active genes in an effort to maximize their survival under this more stressful situation. Cells adapt individually and use highly variable methods of survival; consequently the population as a whole during stationary phase is very heterogeneous. During die-off, all nutrients have been consumed and the population begins to decline (Jones, 1997).

As with nutrient limitation, when confronted by environmental stresses like temperature changes, pH changes, or toxic shocks, bacteria undergo metabolic and morphological changes in order to survive. Cells may produce appendages to maximize attachment, clump together, reduce in size, or shut down certain metabolic pathways (Jones, 1997). Many cells resort to a vegetative state in which they do not grow or divide in response to environmental stresses. These metabolically inert cells are commonly called viable but not culturable, or VBNC, and cannot be detected through current standard water quality monitoring methods as they do not produce growth on agar plates; however, these cells can be detected through other procedures like the fluorescent antibody test or through direct counts (Barcina et al. 1990; Colwell et al., 1985). Levels of VBNC bacteria in exceedance of water quality standards have been found in waters in which standard lab methods found no indicator bacteria (Colwell et al., 1985).

It is likely that most pathogen indicator bacteria in the natural environment exist in a stressed state. A study on the effects of illumination on enteric bacteria in fresh and seawater determined that after less than 24 hours of exposure to visible light, the majority of surviving *E. coli* resorted to a "somincell", or VBNC, state that could not be detected by standard spread plate methods (Barcina et al., 1990). Instead, direct counts were used to quantify the number of cells present. Glucose uptake measurements revealed that these VBNC bacteria had stopped assimilating glucose and so growth had ceased. A study of the survival of enteric bacteria in waters from a sewage outfall by Flint et al. (1987) found that competition, predation, and nutrient depletion forced *E. coli* cells into a non-culturable state that could only be detected by direct counts. Current plating techniques therefore do not provide a true concentration of bacteria present since these VBNC cells are not detected. However, even when in a VBNC state, pathogenic bacteria retain their virulence and can cause illness. Colwell et al. (1985) inoculated a membrane chamber suspended in seawater with *E. coli* and *V. cholerae*. Even after a week, when no cells were culturable by standard methods, harvested VBNC cells of both species produced virulence responses in rabbit ileal loops. Despite the stress on these bacterial cells, the bacteria also retained all plasmid DNA encoding virulence factors. Flint et al. (1987) concluded that even after reaching a VBNC state, enteric bacteria continue to retain plasmids encoding for antibiotic resistance and so do not become less harmful to humans.

2.5.2 Laboratory techniques for attaining balanced growth

The simplest method of maintaining a bacterial population for use in laboratory experiments is a batch culture. A closed flask of growth media of inoculated with growth from a stock agar culture and then incubated for a period of time while the bacteria grow. As time

progresses, the bacteria population grows along a typical growth curve, reaching stationary phase and becoming nutrient-limited as the media is consumed, and eventually dying off.

The main alternative to batch cultures is a chemostat culture. A chemostat is a continuous flow through reactor that allows the researcher to maintain the bacterial population indefinitely at a state of balanced growth. This balanced growth state is different from the previous description of exponential phase. During exponential phase, the cells are dividing and growing at the maximum growth rate, while in a chemostat the growth rate is less than the maximum. Rather than providing an initially nutrient-rich environment in which nutrients are eventually consumed and become scarce, a chemostat provides a constant substrate concentration. Cell physiology is very different between the substrate-limited balanced growth of a chemostat culture and non-substrate-limited balanced growth of exponential phase. Bacteria populations within the chemostat are more representative of the natural environment as the cells are maintained at a nutrient-limited, or starved, state.

A chemostat consists of a sterile glass fermentation vessel containing a liquid suspension of microbial media and nutrients inoculated with the experimental organism. The flow rate at which additional sterile growth media is pumped in is equal to a complete vessel volume change per time for the organism to reach the desired point of the growth curve. Effluent is usually controlled through the use of a J- or Y- tube connection at the desired level of bacteria suspension within the reactor. The effluent pump attached to this connection therefore only pumps out bacterial suspension to waste when the water level within the reactor exceeds the desired level. A series of controls with feedback mechanisms to maintain constant values for heat, pH, or other variables is sometimes also used to maintain constant growth conditions. Cultures are generally considered to have reached an equilibrium at which they can be used for

lab experiments after 5 – 6 volume changes, though some studies have shown that a longer period of time before testing allows the microbes to adapt uniformly, resulting in more consistent lab data (Senn et al., 1994). However, during adaptation, microbes may experience mutations that alter their genetic makeup and capabilities. A mutated strain may behave differently than a wild counterpart from the natural environment, and so data obtained with mutated strains may be less meaningful (Notley-McRobb et al., 2001).

There are distinct advantages and disadvantages to maintaining a chemostat culture rather than a batch culture. Chemostats provide the researcher with greater control over the culturing conditions. Cultures maintained at a constant growth stage in a chemostat may also yield more reproducible and less variable results than a culture grown under batch conditions. There is even some evidence that cultures maintained in a chemostat are more similar to populations found in natural environments. Chemostat models are frequently used to investigate the effects of environmental stresses on already nutrient-stressed populations (Senn et al., 1994). Hoover at al. (1976) found that protozoans in a chemostat culture could be maintained at a density similar to that found in the bovine rumen, though these population densities could not be maintained using other culturing techniques. However, chemostat cultures are inherently more complex, requiring more equipment, set up, and maintenance than a batch culture. Greater understanding of the kinetics and molecular changes of bacteria is required to maintain the system properly and to obtain useful results.

2.6 Conclusions: Need for Research in Bacterial Attachment and Transport

High pathogen indicator bacteria concentrations are the most common impairment responsible for 303(d) listing of streams and rivers in the United States. In order to remediate these waters, a TMDL plan must be developed for each impaired waterbody. TMDLs use

computer simulation models to quantify the current watershed pollution sources responsible for the impairment and the reductions in these sources necessary to meet water quality standards. In order to accurately describe the watershed conditions, it is necessary to accurately model the means of bacterial transport; however, because very little has been quantified regarding these transport processes, bacteria are commonly modeled as dissolved contaminates. This is a gross misrepresentation, as bacteria are discrete organisms and they also commonly attach to soil particles. This attachment may result in greater sedimentation, reduced die-off, regrowth, accumulation in bottom sediments, and resuspension of bacteria into the water column. No hydrologic simulation models, as currently used, can describe these processes due to a lack of information on the relationships between the pkanktonic and attached bacterial phases. Models, such as HSPF, have the ability to simulate partitioning between the planktonic and adsorbed phase, and transport of the two phases, however, they are not used in this manner because data on bacterial partitioning, transport, die-off, etc. in the different phases are not available to parameterize the models. Before further studies can be conducted in this area, a standard laboratory method for partitioning between soil-attached and unattached bacteria must be established. Several different methods have been used to separate and quantify these phases, but no method is widely accepted. Testing and comparison of candidate methods is a needed first step toward establishment of a standard method for bacteria partitioning. In addition, research is needed to develop equations describing the relationships between the planktonic and adsorbed bacterial phases.

CHAPTER 3: EXPERIMENTAL METHODS

3.1 Research Considerations

The ability of bacteria to attach to surfaces varies widely as a function of species, growth conditions, available nutrients, competition and predation between the species present, and soil type. Past studies using field samples in which many, if not all, of these factors are not fixed have resulted in extreme variability, making the data difficult to interpret. In order to simplify the process of testing the different methods of quantifying attachment, and to provide repeatability between experiments, this study was conducted under laboratory conditions, with as many of these variables as possible controlled.

3.1.1 Bacteria cultures

Most past experiments attempting to partition between bacteria and soil particles focused on field samples from ground water, surface water, or bottom sediments. These wild strains are influenced by surrounding conditions as well as the presence of other microbiota and so vary widely between locations. To eliminate the variability between species and strains within species, only one pure culture isolate of *E. coli* was used in the present research. *E. coli* is the current indicator organism recommended by Virginia policy makers for water quality monitoring, and it must therefore be simulated by hydrologic models in the development of TMDLs and other watershed protection activities (VASWCB, 2003). For the present research, a pure culture of *E. coli* isolate of bovine origin was obtained from the Virginia Tech Crop and Soils Environmental Sciences (CSES) Department. This stock culture was stored on soy broth agars at 4^{o}C between experiments. Every six to eight weeks the culture was aseptically transferred to new agar.

The *E. coli* cells entering a water body in the natural environment may come from a variety of animal sources, including humans, cattle, poultry, and wildlife. Land-deposited bacteria may remain on the land surface for several days prior to being washed into the stream, and so may either adapt to this new environment or experience die-off. Because it would be difficult, if not impossible, to simulate the wide variety of growth conditions cells may experience before entering a river or a stream, culturing techniques were selected to maximize attachment. Initially, *E . coli* cultures used in this research were grown under batch conditions. Growth in a nutrient broth stimulates development of cell appendages and attachment abilities more so than growth on a nutrient agar (Schillinger and Gannon, 1985), so a stock suspension of *E. coli* was grown for 24 hours in nutrient broth prior to all experiments. A flask of tryptic soy broth was inoculated using growth from the stock culture and then incubated for 2 hours at 37^{o} C followed by 22 hours at 44.5^{o}C prior to the experiments. While batch culturing was sufficient for the experiments comparing dispersion treatments, high variability in the later sorption experiments forced reassessment of this method. A chemostat reactor was ultimately used for the final sorption experiments to control bacterial growth stage and cell variability. Maintenance of the chemostat reactor is detailed in Section 3.6.3.

3.1.2 Soils

Soil particle size, organic content, nutrient availability, and pH can greatly affect the attachment of microorganisms. Consequently, a single homogenous soil type was used in all experiments. Topsoil was used to represent the type of soil bacteria attach to before being transported to water bodies via overland flow. A topsoil sample was obtained from the Virginia Tech CSES Department greenhouse. The soil was a sandy loam from the Ap soil horizon (0-20 cm depth) of soil pits in Dinwiddie County, Virginia. The soil used in this study was a mixture of

three soil pits in the area: Faceville 346B, Varina146B, and Varina 146B3. The taxonomic classification of this particular soil is: *Faceville series, Fine, kaolinitic, Thermic typic Kandiudults*. In general, the soil was mostly sand (>80%), with very little clay (3%). The silt and sand fraction were mostly composed of quartz, and the clay fraction was largely kaolinite. Table A-1 in Appendix A provides a more detailed summary of soil properties from a CSES soybean research study that used this soil in six fields near the soil pits (Schroeder, 1997). Table A-2, Table A-3, and Table A-4 in Appendix A provide the mineralogy for the sand, silt, and clay fraction of the soil (Vanwormhoudt, 1993). It is important to note that while the pHs listed in Appendix A for this soil are acidic, the soil had been limed to bring the pH up to 7.0 before storage at the CSES greenhouse. Consequently, in this study the soil pH can be considered neutral.

The soil was ground, passed through a 2 mm screen, mixed, and then autoclaved at 120^{o}C (248^{o} F) and 1.02 atm (15 psi) for 15 minutes to kill any naturally occurring soil microorganisms. Soils were handled aseptically and weighed for addition to inoculated dilution water after sterilization. During all experiments using soil, a soil control sample was also tested in addition to inoculated soil-water samples. The soil control consisted of 1.0 g of sterile soil in 100 mL of phosphate buffered dilution water and was tested using membrane filtration and mTEC agar (USEPA, 2000) to confirm that all native soil microorganisms had been killed during autoclaving. During experiments that involved mechanical mixing (Sections 3.4,3.5, and 3.6), the soil control was also mixed before testing; otherwise the sample was shaken vigorously by hand before analysis.

3.2 Selection of Experimental Partitioning Methods

A viable method for partitioning between soil-adsorbed and planktonic bacteria should be able to enumerate bacterial concentrations in a totally unknown water sample obtained in the field, and should be simple enough to integrate into current laboratory methods used to determine bacteria concentration. Possible methods identified in the literature review were multiple screen filtration, filtration with chemical or ultrasonic dispersion, centrifugation, and microscopic methods. Multiple screen filtration requires dispersion of bacteria retained on each filter to determine attached concentration. This method is considered less preferable as the bacteria retained on the filter may clump together or become attached to the filter, making the aggregates more complex and more difficult to break. Similarly, when using centrifugation, the pellet of concentrated sediment and bacteria must be dispersed to determine an attached concentration. Super concentrating the aggregates of soil and bacteria may change attachment and make the bonds more difficult to break. Also, there may be difficulties in determining the best speed to use, as planktonic bacteria are similar in size to clay particles. While microscopic methods can provide direct measures of true attachment without requiring an operational definition, they are time-consuming and require additional equipment.

Of the methods identified in the literature review, filtration and the use of dispersants appeared the most promising. Filtration is a standard method used in water quality laboratories for a number of different tests. An unknown water sample can be filtered and then the filtrate analyzed for unattached bacterial concentration. The size of the filter pores provides an operational definition for attachment, as anything retained on the filter is considered attached, while anything passing through the filter is considered unattached. Depending on the filter size, natural aggregates of bacteria containing no soil may become trapped on the filter or bacteria

attached to very fine particles may pass through the filter and be considered unattached. The filter pore size can be changed according to the researcher's needs. In this research, an 8 μm filter was used. This size filter has been used in other experiments quantifying attachment of fecal coliform (Qualls et al., 1983; Mahler et al., 2000), and is considered ideal because a single unattached *E. coli* bacterium under favorable growth conditions at its maximum size is usually between 2 μm and 6 μm in size (Bergey's Manual of Determinative Bacteriology, 1994) and should be able to pass through the pores easily. It is important to note that under more stressed conditions, individual cells may be smaller, and so aggregates of multiple cells smaller than 8 μm would be able to pass through the filter and would be considered planktonic.

In addition to using a filtration system to quantify unattached bacteria in an unknown sample, a dispersion method can be used to quantify the total amount of bacteria in the same sample. The initial unknown sample is split into two subsamples: one is filtered and the filtrate analyzed for unattached concentration, and the other is treated using a dispersion technique and analyzed for total concentration. While splitting the original sample would create additional sources of error as a result of measuring the resultant subsamples, graduated cylinders and pipettes with the lowest error available were used to minimize this issue. It is assumed that all cells detach as a result of the dispersant treatment. Attached concentration is thus measured indirectly as the difference in total and unattached concentration. Dispersion techniques disrupt the attachment of the bacteria to surfaces, either by physically breaking the bond between the bacteria and the surface or by inhibiting the bacterial metabolism and preventing the cell from making the extracellular structures needed to facilitate attachment (Paul and Jeffrey, 1985). Both dispersion using ultrasonic treatment and dispersion with a chemical surfactant have been used in

past studies quantifying attachment. In this study, the effects of these dispersion treatments will be compared to determine which is most suitable for further use in research.

3.3 Determining Die-off as a Result of Dispersion Technique

In the process of breaking bacterial attachments to soil particles or other bacteria, dispersion techniques like surfactants and ultrasonic treatment may damage the bacterial structure or metabolic capabilities, causing the bacteria to die. Consequently, before using either dispersion technique to assess bacteria-topsoil samples, it had to be determined whether die-off as a result of dispersion technique was a significant factor.

To test for significant die-off, twelve samples of 99 mL of dilution water were inoculated with 1 mL of the bacteria-broth suspension and mixed thoroughly. Nine of the samples were treated with one of three dispersion techniques, and three samples were left untreated as controls.

Table 3-1: Experimental Design for Die-off Experiments

Treatment	No. of reps.
surfactant	3
30 s ultrasonic	3
60 s ultrasonic	3
None (control)	3

The weighted test tube rack within the ultrasonic cleaning bath was designed to hold 10 mL test tubes; consequently, for each of the samples receiving either dispersant treatment, 10.0 mL of the sample was transferred to a test tube using a pipette. For those samples using a surfactant as a dispersant, two drops of Tween-85 were added to each test tube and mixed using a vortex mixer for five seconds. Those samples requiring ultrasonic treatment were placed one at a time in a weighted test tube rack within a laboratory ultrasonic cleaning bath (Fisher Scientific Solid State Ultrasonic FS-9, 50/60 Hz). The water level in the bath was adjusted to be the same level as the sample in the test tube. The bath was turned on for the appropriate amount of time

for each tube. All samples in this study were analyzed for *E. coli* concentration using serial dilutions and the membrane filtration technique with mTEC agar (USEPA, 2000). mTEC agar was selected as it is selective for coliform bacteria, and differential for *E. coli*; all coliform will produce colonies on mTEC agar, but only *E. coli* colonies will appear purple, while all other colonies will be clear or white. Appropriate plate counts were determined using the procedure outlined in Microbiological Methods for Monitoring the Environment: Water and Wastes (USEPA, 1978).

3.4 Determination of Mixing Time

Bacteria require time to develop extracellular structures necessary for attachment. Mixing water samples of soil and bacteria provides the cells with an opportunity to come into contact with soil particles suitable for attachment; however, the process of mixing may also damage cells and cause die-off. To determine the amount of time required for measurable attachment to occur, twelve samples of 99 mL of buffered dilution water were inoculated with 1 mL of *E. coli* nutrient broth suspension. Four mixing times were tested using a wrist action mechanical shaker: 15 min, 30 min, 1 hour, and 2 hours. Three samples were mixed for each time. Of these three samples, 1.0 g of sterile soil was added to two samples and one sample was left without soil to test for die-off.

Table 3-2: Experimental Design for Mixing Experiments

No. of samples	**Mixing Time**			
	15 min	**30 min**	**1 hr**	**2 hrs**
with soil	2	2	2	2
without soil	1	1	1	1

After mixing, the samples containing soil were filtered through an 8 μm filter and rinsed with 150 mL of dilution water. The filtrate was collected aseptically and analyzed for bacterial concentration using membrane filtration. The no-soil samples were analyzed for total

concentration using membrane filtration without any prior filtration or dispersion treatment. The proportion of cells considered attached was calculated using the following equation:

$$attached\ \ proportion = \frac{total\ concentration - planktonic\ concentration}{total\ concentration} \qquad (Eq.\ 2)$$

3.5 Selection of Best Dispersion Technique

In order to determine which dispersion technique served as a better means of obtaining a total concentration value in a water sample containing bacteria and soil, both the ultrasonic treatment and surfactant treatment were used to analyze subsamples of the same inoculated water-soil sample. Attached bacteria were operationally defined as those not passing through an 8 μm filter. The total concentrations obtained using each dispersant treatment were used to calculate estimates of the proportion of bacteria adhering to soil particles. Two similar experimental designs were required to answer the following experimental questions.

1. Do all non-attached bacteria pass through the 8 μm filter?
2. Do the dispersant techniques used result in significant die-off?
3. Do the dispersant methods used provide an accurate estimate of the total bacteria concentration in the sample?
4. Are the total concentration values obtained from the dispersion methods significantly different?

3.5.1 Experimental Design 1: Evaluation of filter effectiveness and die-off

A randomized complete block design (RCBD) was used to test for die-off as a result of dispersion techniques and for the ability of unattached bacteria to pass through the 8 μm filter. This design contained two controls: a bacteria suspension that contains no soil to test whether all planktonic bacteria can pass through the filter and to correct for possible die-off, and a suspension that is left untreated to obtain a true total concentration after mixing. Membrane

filtration analysis for concentration was replicated twice for each subsample. A schematic of one "block" of the experimental design is given in Figure 3-1. Ten block replications were preformed in the laboratory. Variables in this experiment are defined as:

Treatment a = immersion in ultrasonic cleaning bath for 30 s

Treatment b = addition of 2 drops Tween-85 surfactant

C_o = original total bacteria concentration, CFU/mL

$C_{a,\,s}$ = concentration of bacteria dispersed from the soil and not killed by treatment a, CFU/mL

$C_{b,\,s}$ = concentration of bacteria dispersed from the soil and not killed by treatment b, CFU/mL

$C_{f,\,s}$ = concentration of bacteria that are not attached to soil, CFU/mL

$P_{a,\,s} = \frac{C_{a,s} - C_{f,s}}{C_{a,s}}$ = proportion of bacteria attached to soil according to treatment a

$P_{b,\,s} = \frac{C_{b,s} - C_{f,s}}{C_{b,s}}$ = proportion of bacteria attached to soil according to treatment b

Ca, w = concentration of bacteria in no-soil control after treatment a, CFU/mL

Cb, w = concentration of bacteria in no-soil control after treatment b, CFU/mL

$C_{f,\,w}$ = concentration of bacteria in no-soil control that pass through the 8 μm filter, CFU/mL

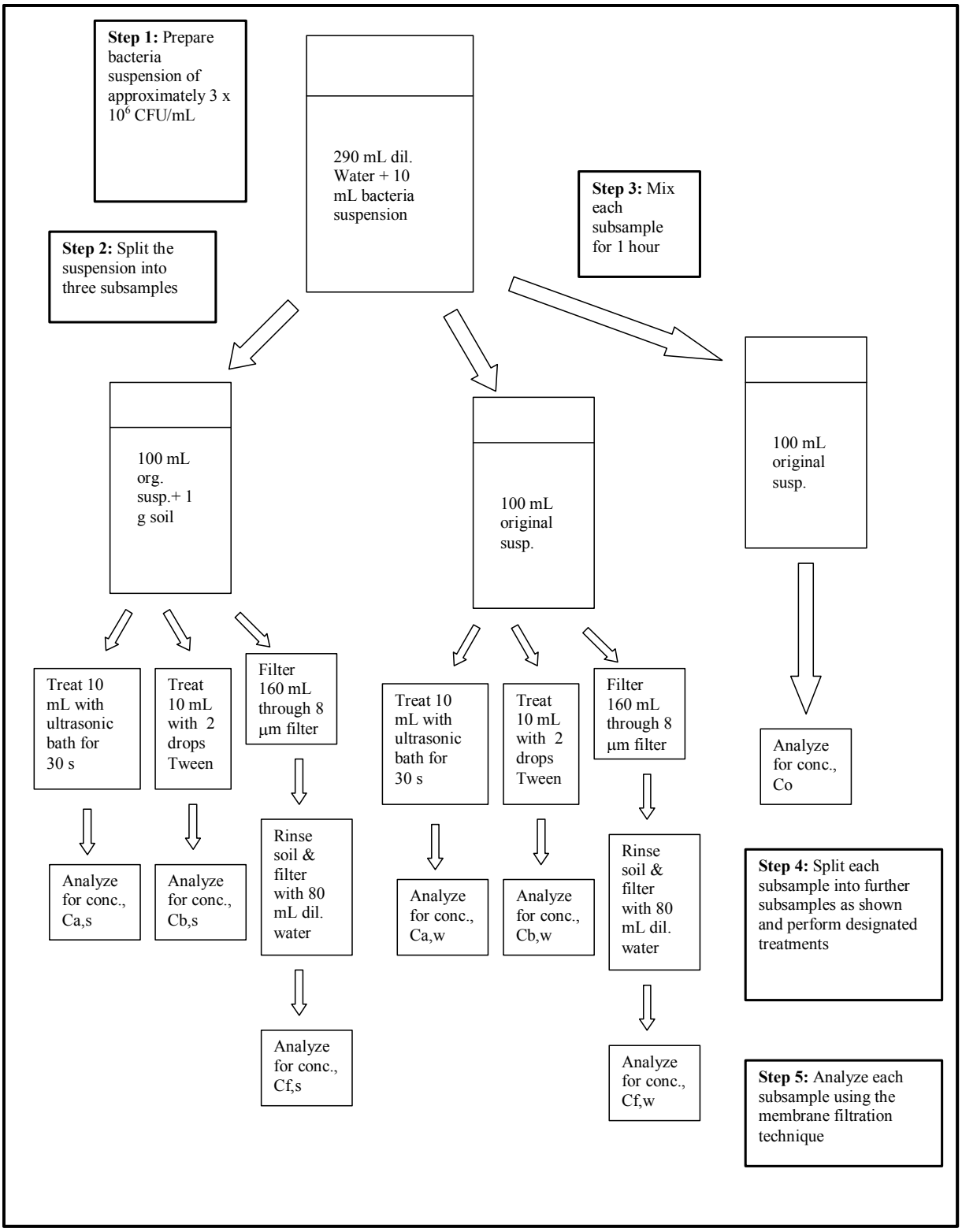

Figure 3-1: Experimental Design 1

Because the total concentration values obtained using dispersion techniques on inoculated samples without soil and the concentration value obtained after passing the unattached bacteria through the filter were expected to be roughly equal to the value obtained from the samples containing soil, only ten replications of the block design were preformed. While using more replications is always beneficial statistically, the limited time and resources available were used more heavily to compare attachment proportions obtained using the dispersion techniques (see Experimental Design 2).

3.5.2 Experimental Design 2: Comparison of dispersion treatments

More repetitions were required to draw conclusions about the proportion of bacteria attached to soil as calculated using the total concentration value obtained from the dispersion treatments as greater variability was suspected. A second RCBD was designed for this purpose. This RCBD was almost identical to the first design, but eliminated the no-soil branch of subsampling to allow for greater overall block replications (Figure 3-2). Each membrane filtration analysis for concentration was replicated twice.

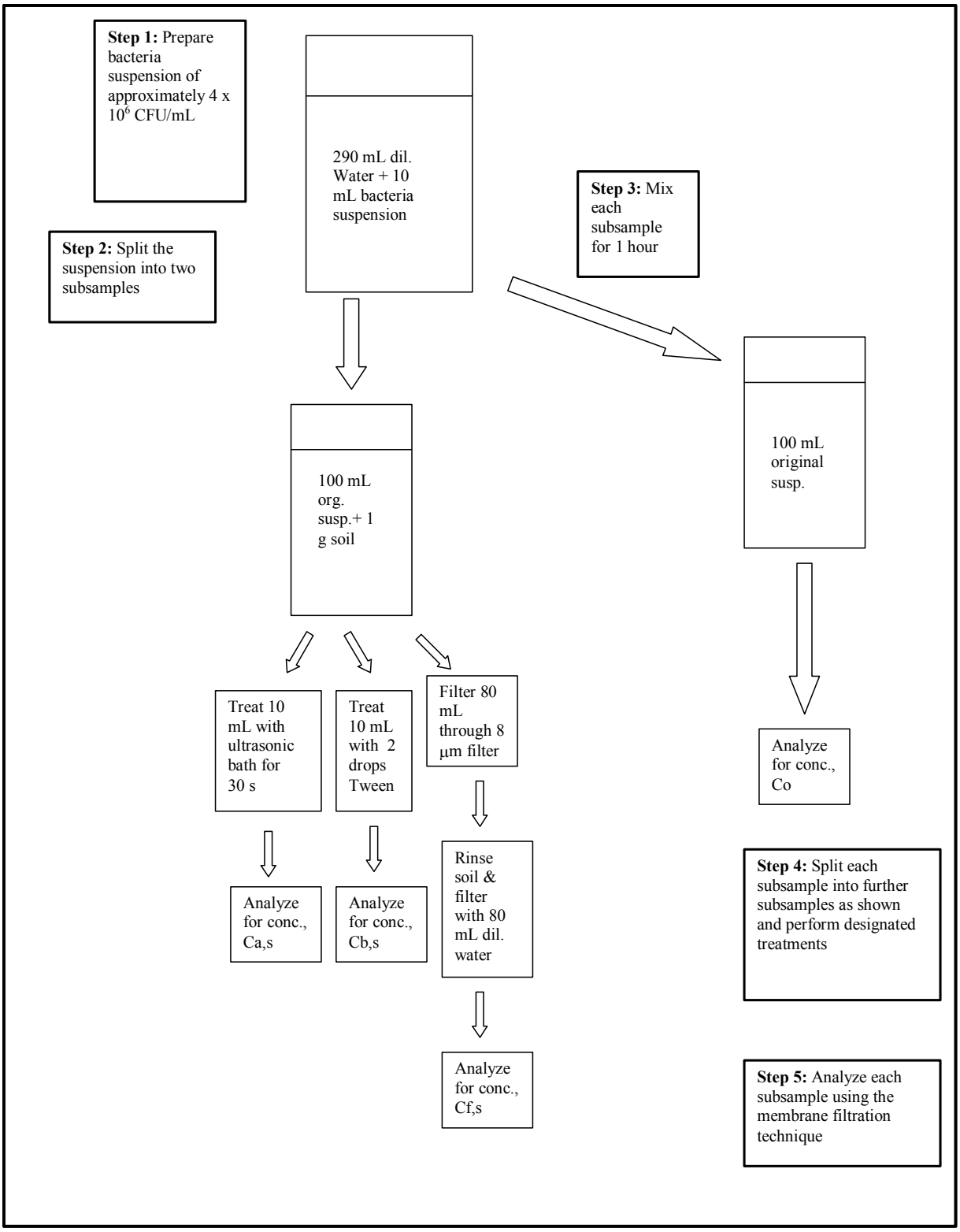

Figure 3-2: Experimental Design 2

Ten block replications of this experimental design were preformed in the laboratory. Data from the soil-water and control subsamples of the previous RCBD could also be used for analysis, bringing the total number of block replications up to twenty.

3.6 Sorption Experiments

3.6.1 Initial runs using batch cultures

E. coli cultures were grown in batch flasks for 24 hours as described in Section 3.1.1. Bottles containing phosphate buffered dilution water were inoculated with the bacteria suspension in amounts described in Table 3-3. Amounts of sterilized sandy loam topsoil were added aseptically to each sample in quantities listed in Table 3-3. These bacteria-soil combinations were designed to be a starting point for analysis, with further different bacteria-soil ratios to be added as needed after initial analysis. Two different bacterial concentrations and five total suspended solids (TSS) values were initially considered.

Table 3-3 Bacteria-soil combinations for sorption experiments with batch culture

Sample	mL of Bacteria Suspension	mL of Dilution Water	g Sterile Soil
1	1.0	99.0	0.5
2	1.0	99.0	1.0
3	1.0	99.0	2.0
4	1.0	99.0	3.0
5	1.0	99.0	5.0
6	0.1	99.9	0.5
7	0.1	99.9	1.0
8	0.1	99.9	2.0
9	0.1	99.9	3.0
10	0.1	99.9	5.0

Each sample was mixed on a wrist action shaker for one hour prior to analysis for bacterial concentration in accordance with the mixing results discussed in Section 4.2. After mixing, 10 mL of each sample was pipetted into a test tube and treated with two drops of Tween-85 surfactant. After thorough mixing on a vortex mixer, this sample was analyzed for total

bacterial concentration using serial dilutions and membrane filtration. The remaining 90 mL of each sample was filtered through an 8 μm filter and then washed with 180 mL of additional dilution water. Filtrate was collected in an aseptic reservoir and analyzed using membrane filtration for the attached concentration of *E. coli*. A schematic of this analysis procedure is given in Figure 3-3. The attached concentration of bacteria for each sample was calculated using Equation 3.

$$attached\ concentration, CFU/g = \frac{(total - planktonic, CFU/mL) * Volume, mL}{soil\ mass, g} \qquad (Eq.\ 3)$$

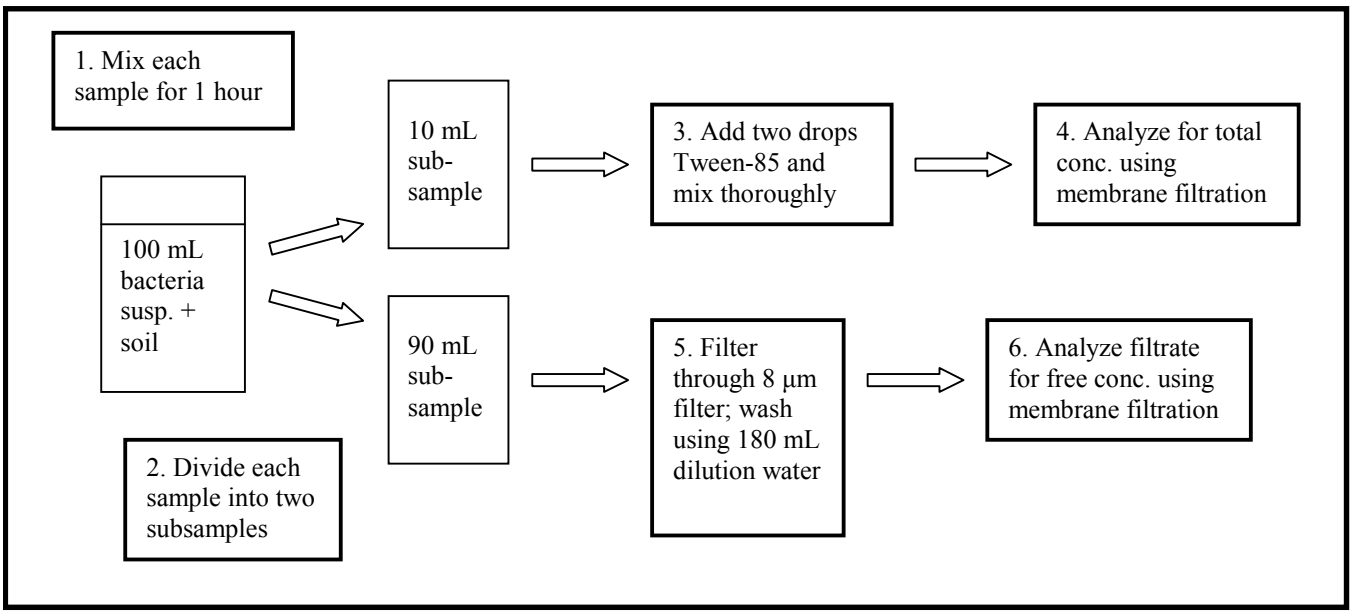

Figure 3-3: Partitioning method for sorption experiments

3.6.2 Determining the experimental growth curve

The data collected from the sorption experiments using batch cultures were highly variable and could not be reproduced. In order to control this variability, the method of culturing was reassessed. To decide how to proceed, the growth stage of *E. coli* in tryptic soy broth after 24 hours of incubation was first determined. Using a pipette, 100 mL of broth was transferred to each of four side-arm flasks. Three flasks were inoculated with bovine *E. coli* from a stock agar

culture, while the fourth flask served as a blank control. The blank was used to set a spectrophotometer to 100% transmittance, then the transmittance of each of the three inoculated flasks was determined using the spectrophotometer. All four flasks were placed in the 37° C incubator for two hours, and then transferred to the 44.5° C incubator for the remainder of the experiment. Transmittance readings for the three inoculated flasks were taken roughly every two hours for 30 hours, each time first setting the spectrophotometer to 100% using the blank. The spectrophotometer wavelength (λ) was set at 600 nm. Transmittance was used to calculated absorbance using the following equation where A is absorbance and T is transmittance as a decimal:

$$A = \log\left[\frac{1}{\frac{T}{100}}\right] \qquad \textit{(Eq. 4)}$$

Absorbance of a broth culture is directly related to cell count. The more cells in a culture, the greater the absorbance. Plotting absorbance of the culture versus the time of the experiment should give a curve similar to the typical growth curve discussed in Section 2.5.1.

3.6.3 Use of a chemostat to provide balanced growth

Data collected from the sorption experiments described in Section 3.6.1 previously were extremely variable and could not be reproduced. Results from the growth curve experiment confirmed that the *E. coli* in the batch cultures were at stationary phase, which is considered a period of extreme change and variability between cells as they attempt to adapt to a nutrient-limited environment. In an attempt to control this variability, *E. coli* were cultured in a LH Fermentation 2.0 L chemostat reactor prior to experiments rather than in batch cultures to provide a stock culture at a balanced growth state. MasterFlex peristaltic pumps were used to control the influent and effluent flows. The level of bacteria suspension was maintained at 1.5 L

through the use of a J-tube positioned at the 1.5 L level of the reactor. When the suspension level rose above the level of the J-tube, liquid was pumped out; otherwise, only air would be pumped. The reactor environment was mixed by an impeller at 500 rpm.

Aerobic conditions were maintained in the chemostat to mimic the conditions of overland flow or streamflow. An aquarium aerator bubbled in sterile air, and the influent growth media was diluted to a concentration of 1:10 to ensure that the cells remained nutrient-limited, rather than oxygen-limited. Attempts to run the chemostat using undiluted soy broth resulted in anaerobic conditions, with dissolved oxygen concentrations of effectively zero.

A heated water bath at 44.5^{o}C was pumped through tubing wrapped around the reactor to maintain a temperature of 44.5^{o}C. A LH Fermentation temperature control unit constantly monitored temperature. A schematic of the chemostat set-up used is given in Figure 3-4 and a picture of the chemostat set up is given in Figure 3-5.

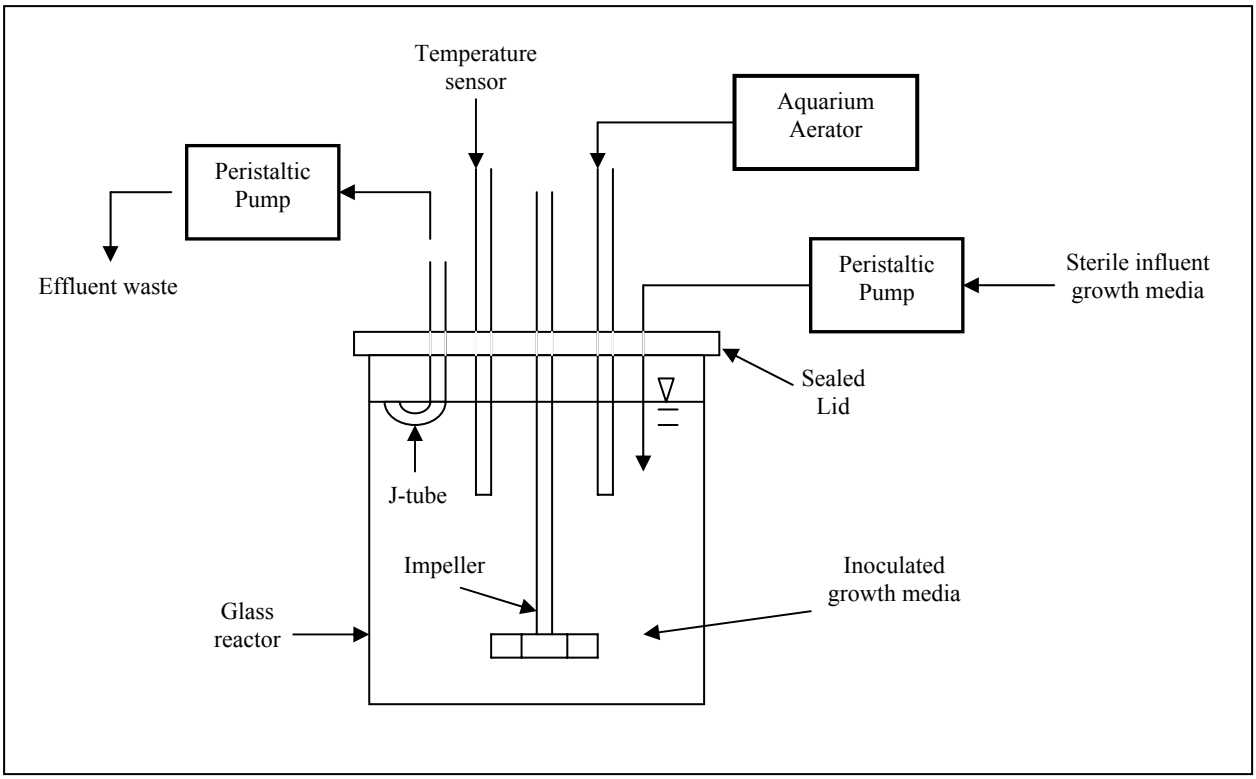

Figure 3-4 Schematic of chemostat set-up for sorption experiments

Figure 3-5: Chemostat set-up for sorption experiments

Influent and effluent flow rates are determined based on a dilution rate, D:

$$\frac{V}{Q} = \frac{1}{D} = \text{HRT} \qquad \textit{(Eq. 5)}$$

where V is the volume maintained in the reactor in liters and Q is the flow rate through the reactor in liters per hour. The hydraulic retention time (HRT) was set at 8 hours, so that the entire volume would change over every 8 hours, resulting in three total volume changes every 24-hours. The resultant dilution rate, D, for the chemostat was 0.125 hr^{-1}. Influent flow rate for a volume of 1.5 L and an HRT of 8 hours was 3.1 mL/min.

After sterilization and set-up, the growth suspension in the chemostat was inoculated with the culture and allowed to grow under batch conditions to 80% of maximum growth before beginning pumping to prevent washout of the culture. According to the experimental growth curve (Figure 4-13) 80% of maximum growth occurred around 10 hours. Generally, chemostat cultures are considered at equilibrium after five to six full volume changes (Senn et al., 1994). After turning on the pumps, the chemostat was allowed to cycle for at least 40 hours (five retention times) before samples were withdrawn for use in sorption experiments.

Samples for sorption experiments were withdrawn from the reactor using a sterile suction sampler. These samples were used as a stock suspension for use in sorption experiments set up almost identically to the initial sorption experiments described in Section 3.6.1. Bottles containing phosphate buffered dilution water were inoculated with the suspension in the amounts given in Table 3-4. Sterile soil was added aseptically in the amounts given in Table 3-4. The number of different soil amounts/TSS concentrations was reduced from five to two, as variations in TSS were suspected as an additional cause of variation in the initial sorption experiments. Each sample was mixed for an hour, and then analyzed for total concentration using Tween-85 surfactant and planktonic concentration using filtration through an 8 μm screen according to the procedure outlined in Figure 3-3. However, after filtration through the 8 μm screen, the filters were only washed with 90 mL dilution water rather than 180 mL, to avoid overfilling of the sterile reservoir used in the initial sorption experiments.

Table 3-4: Bacteria-soil combinations for sorption experiments with chemostat suspension

Sample	Vol Bacteria Susp, mL	Vol Dilution Water, mL	Sterile Soil, g
1	0.1	99.9	1.0
2	0.3	99.7	1.0
3	0.5	99.5	1.0
4	0.7	99.3	1.0
5	1.0	99.0	1.0
6	3.0	97.0	1.0
7	5.0	95.0	1.0
8	7.0	93.0	1.0
9	10.0	90.0	1.0
10	30.0	70.0	1.0
11	0.1	99.9	0.5
12	0.3	99.7	0.5
13	0.5	99.5	0.5
14	0.7	99.3	0.5
15	1	99	0.5
16	3	97	0.5
17	5	95	0.5
18	7	93	0.5
19	10	90	0.5
20	30	70	0.5

CHAPTER 4:RESULTS AND DISCUSSION

The experimental data from the laboratory experiments described in the previous chapter indicated that the partitioning method proposed was successful in enumerating total and planktonic concentrations of *E. coli*. Additionally, a large proportion of *E. coli* (78%) became attached in all experiments. Results of each individual experiment are given and their implications discussed in the following sections.

4.1 Determining Die-off

Values obtained from analysis are given in Table B-1 in Appendix B. Averaging the concentrations for each treatment over all subsamples and samples yielded the means given in Figure 4-1. Error bars indicate the 95% confidence interval of these means.

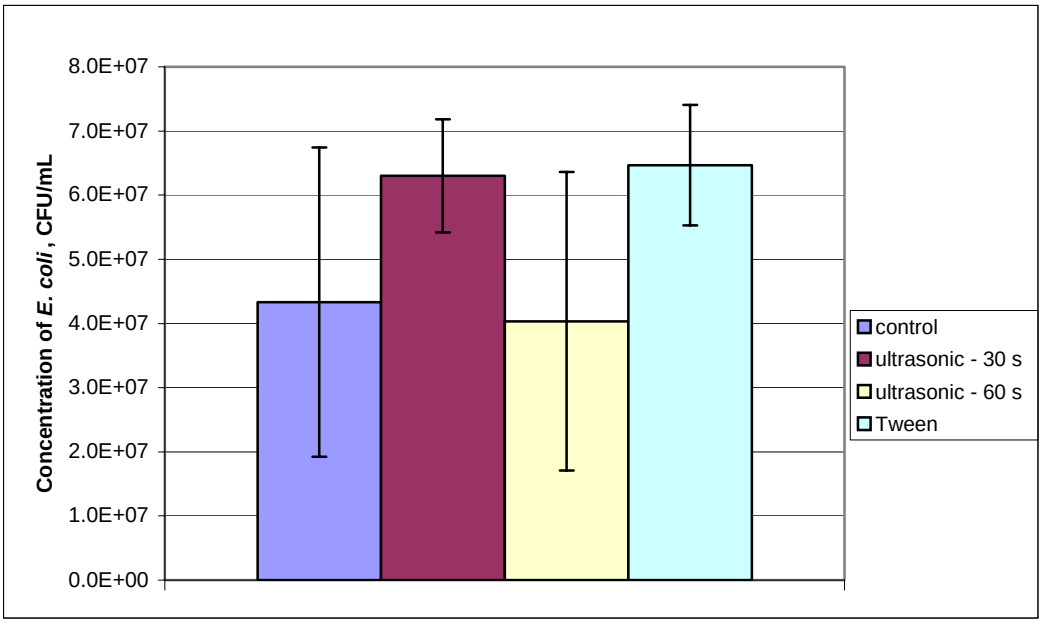

Figure 4-1 : Arithmetic Means for for Dispersion Treatments

These concentration values were analyzed using Statistical Analysis Software (SAS). All SAS codes are given in C.1 in Appendix C. A multiple comparison test, Tukey's test, was used to compare the averages. Using an alpha of 0.05, there were no statistically significant

differences, despite the differences observed visually when examining the above bar graph (D.1, Appendix D).

To further investigate possible treatment differences, some sample values were removed during statistical analysis because they were suspected outliers (Table B-1). As shown in Figure 4-1, the confidence intervals for the control and 60 s ultrasonic data sets were much larger than the confidence intervals for the other two datasets suggesting that some outlying values in these data sets were adding variability. Both subsample values for sample 1 (control) were removed because they were less than 50% of the value of the other control concentration values. While only one of these values was outside of the confidence interval and a technical outlier, $1x10^7$ CFU/mL, both values were removed because similar problems with these plates were observed in the laboratory. During analysis, it was noted that the colonies on these plates were extremely tiny, just barely visible. It is suspected that these plates, which were adjacent in the incubator, did not receive enough moisture or warmth to grow properly. Subsample 2 of sample 7 (ultrasonic – 60 s) was also discarded because there was no growth on the plate. Since there was growth within the optimum range of 20-80 CFU/plate on the other 5 plates in this treatment group, it seems strange that there was no growth whatsoever on this plate. The value zero is outside the confidence interval, and so can be considered an outlier, possibly the result of an unknown experimental error.

The removal of these values resulted in an unbalanced design, with unequal numbers of samples for each treatment and unequal numbers of subsamples for each sample. Rather than calculating a simple mean, the least-squares means were calculated for each value for comparison (Table 4-1). Least-squares means are used to correct for the unbalancing of a design due to the removal of data points.

Table 4-1: Comparison of Arithmetic and Least Squares Means

	***E. coli* Concentration, CFU/mL**	
Treatment	**Arithmetic Mean**	**Least Squares Means**
control (no treatment)	4.33E+07	5.75E+07
ultrasonic - 30 s	6.30E+07	6.30E+07
ultrasonic - 60 s	4.03E+07	4.82E+07
Tween-85 surfactant	6.47E+07	6.47E+07

These least squared means values were then plotted on a bar graph for visual comparison (Figure 4-2). As expected, with the outlying values removed, the confidence intervals were of comparable magnitude.

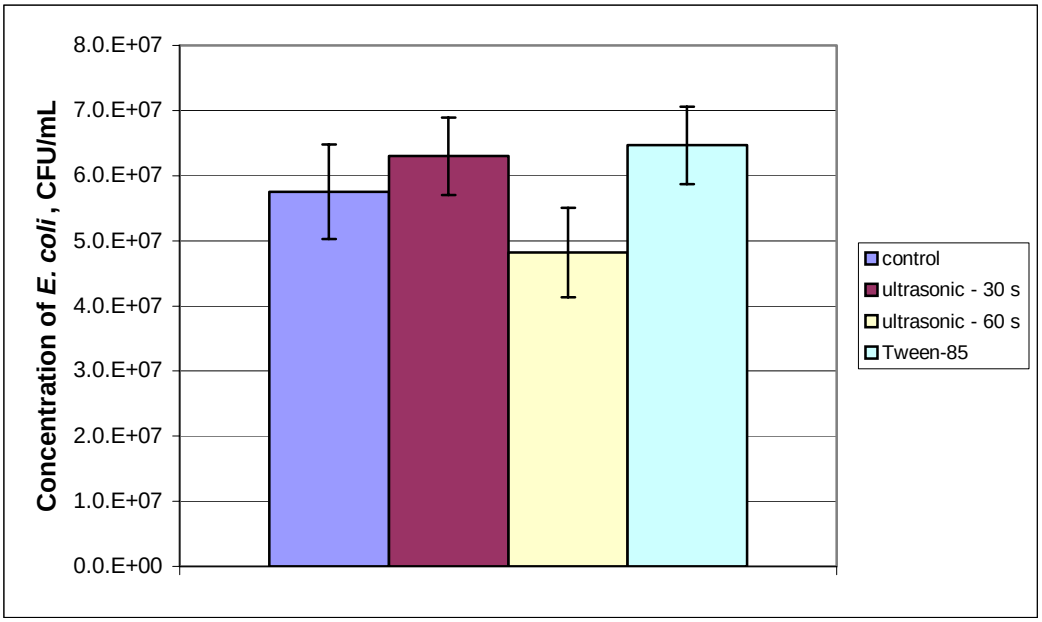

Figure 4-2 : Least Squares Means for Dispersion Treatments

A multiple comparison test using least-squares means with an alpha of 0.05 was used to test for differences between the treatments (C. 2, Appendix C). Using this test, a p-value of less than α (0.05) is considered significant. Again, though visually there appeared to be differences between treatments, no significant differences were calculated. However, the difference between

the Tween-85 concentrations and the 60 s ultrasonic concentrations resulted in an $\alpha = 0.0634$, which was almost significant (D.2, Appendix D).

While there was no statistical difference between the treatment means, treatment with 60 s of submersion in the ultrasonic bath yielded the lowest overall mean, even after outlying data values were removed. Also, bacteria exposed to this treatment resulted in much smaller colonies when grown on mTEC agar for 24 hours (Figure 4-3). Both the lower overall mean value and the tiny amounts of growth on the agar plates suggested that 60 s might be too long for the ultrasonic treatment, and caused the cells to die or reduced their viability. When cells undergo stress, they may also enter a viable but not culturable (VBNC) state to extend survival. Previous studies have found that smaller colony growth on agar plates might be the first step in a cell's switch to a VBNC state. VBNC is a metabolically inert state; because the metabolic processes have shut down, the cell will not grow to produce colonies on an agar (Barcina et a, 1990). Even if the *E. coli* cells are not killed by 60 s of sonication, if they are rendered VBNC, they cannot be detected using membrane filtration. Consequently, 60 s of ultrasonic treatment was abandoned as a possible dispersion treatment in further experiments.

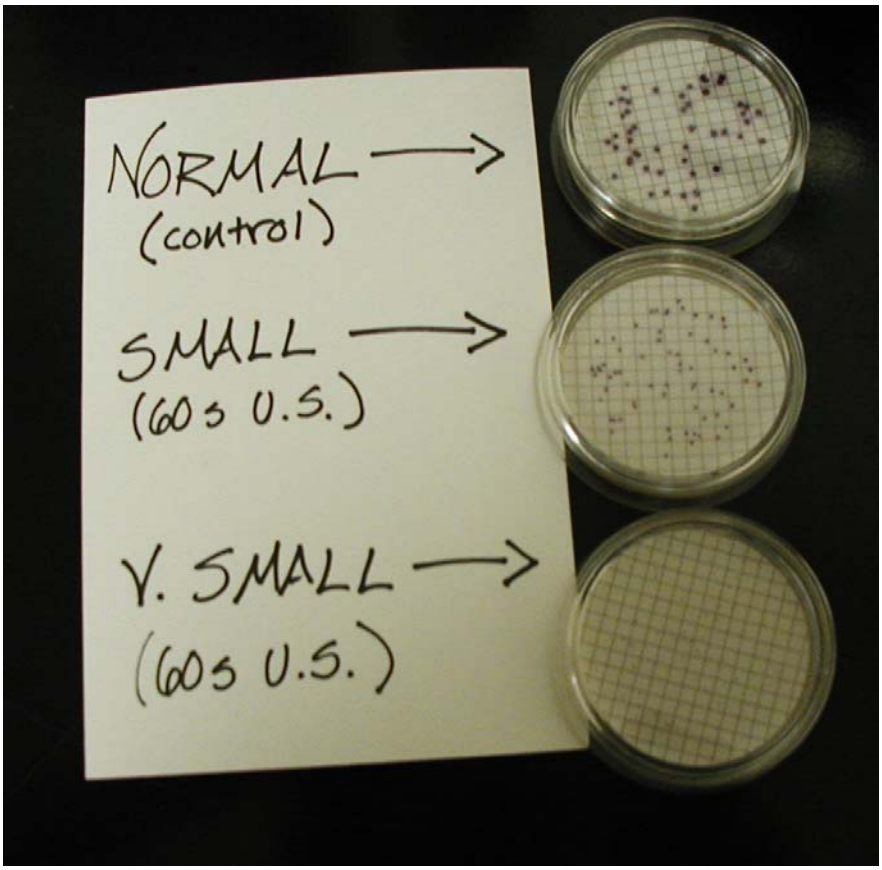

Figure 4-3: Comparison of colony size in die-off experiments

Both the 30 s ultrasonic treatment and treatment with Tween surfactant yielded values greater than the control concentration. This suggests that these treatments did not cause any die-off and perhaps disaggregated naturally occurring bacterial clumps, resulting in a higher total concentration. Further experiments were conducted to compare the effects of both treatments in order to determine which provided the best measure of total concentration.

4.2 Determination of Mixing Time

Raw data values for the mixing experiments are given in Table B-2 in Appendix B. The soil control, which tested for soil sterilization as explained in Section 3.1.2, was negative for any bacterial growth. Concentrations for the control samples were graphed in Figure 4-4. There was no noticeable trend in concentration versus mixing time. Longer mixing times neither increased

nor decreased concentration. Because only one sample was used for each time as a control, there was no statistical analysis. Differences in concentration were relatively small, considering the variability inherent in plating techniques, so it was assumed that any die-off as a result of mixing time could be ignored.

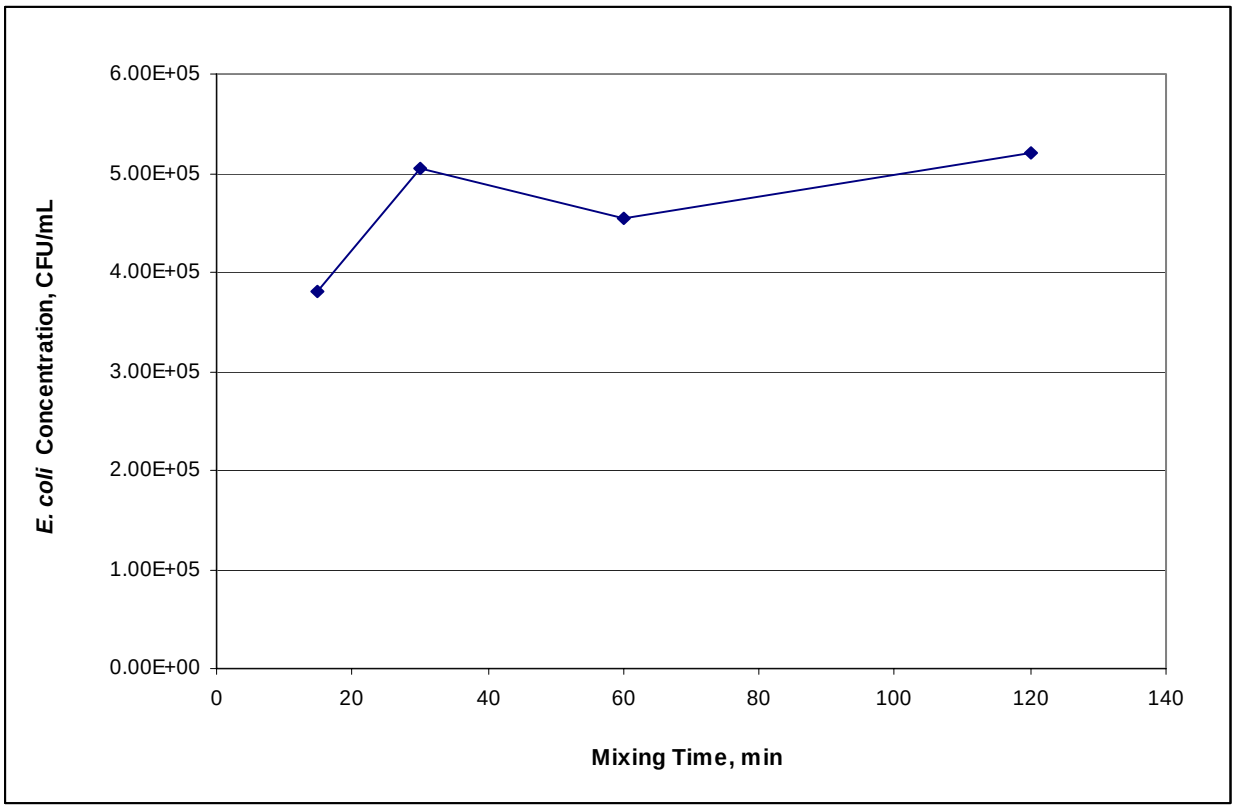

Figure 4-4: Total Concentrations for Untreated Controls

The average concentration of the filtrate from soil samples is given in Figure 4-5. This filtrate can be considered the unattached concentration of *E. coli* in the sample, as all attached bacteria should have been retained on the 8 μm filter. There appeared to be an initial decrease between 15 minutes and 30 minutes of mixing time, but then the filtrate concentration remained relatively constant. Assuming that decreases in filtrate concentration were due to increases in soil

attachment, the greatest amount of attachment appeared to occur during the first 30 minutes of mixing.

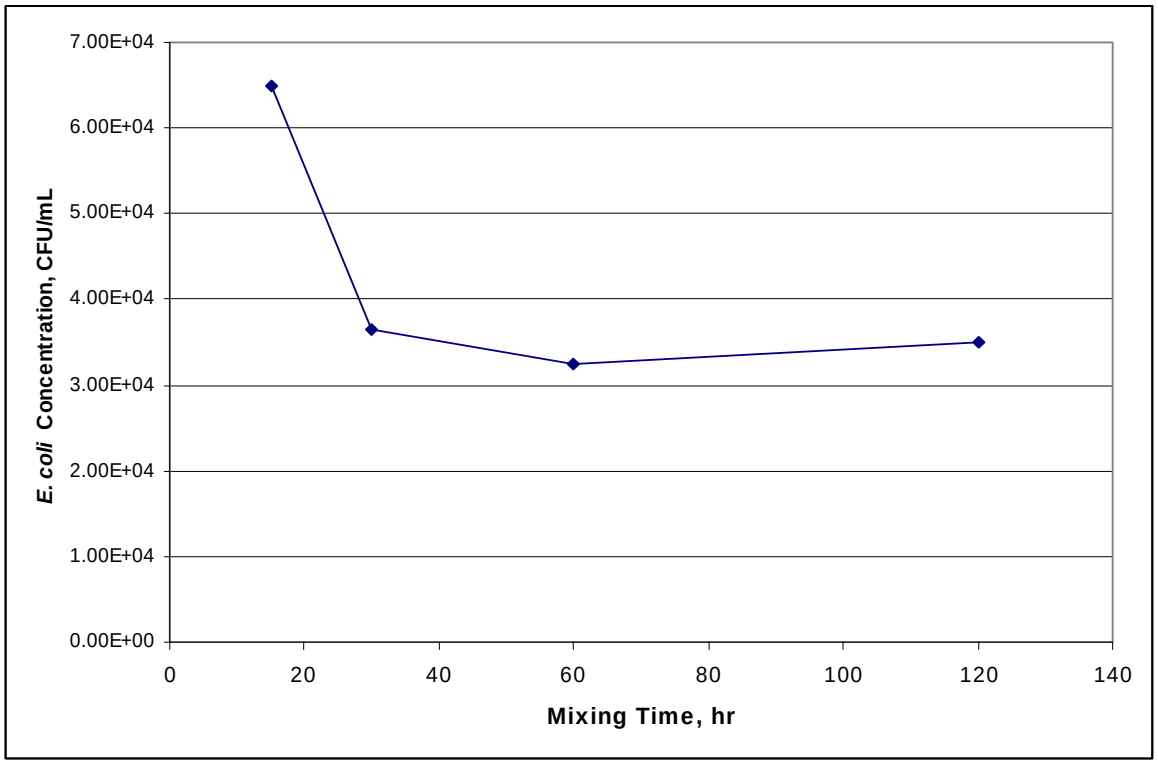

Figure 4-5. Average Concentration of Filtrate from Samples with Added Soil

A proportion of bacteria considered attached after each mixing time was calculated using Equation 2:

$$proportion\ attached = \frac{total\ concentration - planktonic\ concentration}{total\ concentration} \qquad (Eq.\ 2)$$

The concentration of the control sample containing no soil was considered a measure of total concentration for each time, and the average concentration in the filtrate was considered the planktonic concentration. Using these values, average attached proportions were calculated for each time and plotted in Figure 4-6. As expected, the attached proportion of *E. coli* increased in the first 30 minutes of mixing, and then seemed to level off and become constant.

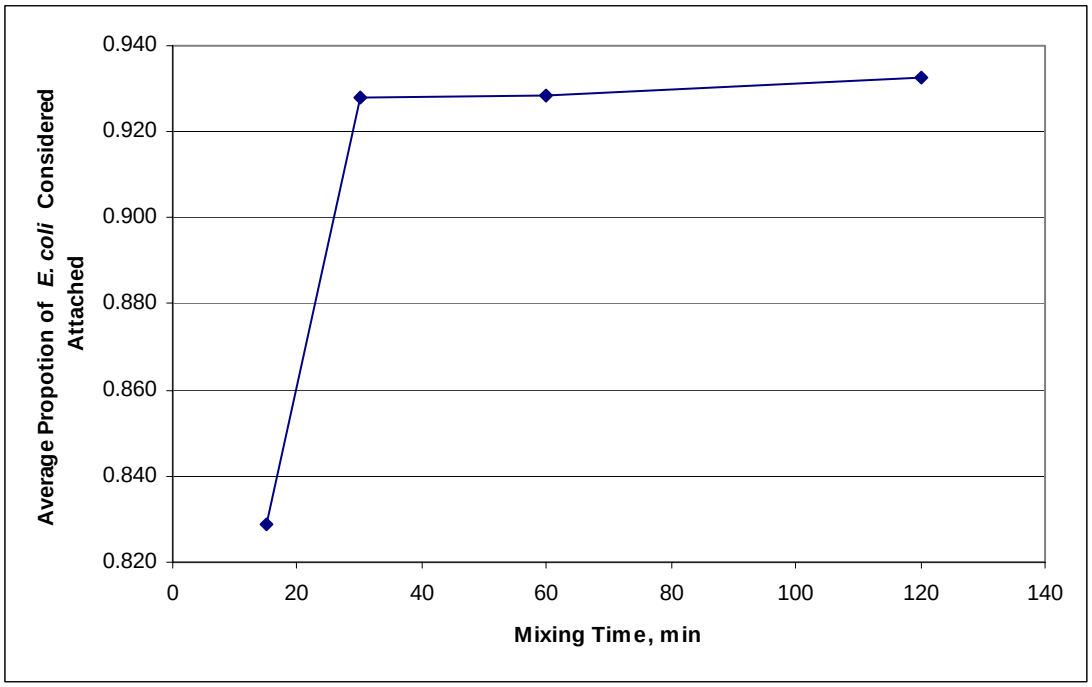

Figure 4-6 Proportion of *E. coli* Considered Attached for Different Mixing Times

The proportion data from this experiment could not be analyzed statistically because the values used are not independent. To obtain a true value of total concentration for each sample containing soil, the sample should have been split into two subsamples, with one subsample analyzed for total concentration, and one filtered and analyzed for planktonic concentration. To analyze a subsample containing soil for total concentration, a dispersant method would have to be used; however, the effects of these dispersants had not yet been determined. This experiment was used as a quick means of establishing a suitable mixing time for the samples in the experiments that would compare the dispersants.

As revealed by Figure 4-5 and Figure 4-6, there is measurable attachment after only 15 minutes of mixing time, and after 30 minutes the proportion of bacteria attached does not change much after further mixing. This agrees with the study by Huysman and Verstraete (1993), which found that attachment to soil particles by *E. coli* occurs mostly within the first 15-20 minutes of

mixing with the soil. Because the proportion of bacteria considered attached does not change notably after 30 minutes of mixing, a mixing time of 1 hour was selected for further experiments, as this setting was the most convenient to use with the available equipment and expected analysis time for each sample.

4.3 Selection of Best Dispersion Method

4.3.1 Experimental Design 1: Filter effectiveness and die-off

Ten block replications of the experimental design in Figure 3-1 were preformed to compare total concentration measurements obtain using different treatments. Raw data values are given in Table B-3 in Appendix B. The soil control, which tested for soil sterilization as explained in Section 3.1.2, was negative for any bacterial growth. A graph of the resultant experimental means and confidence intervals is given below in Figure 4-7. The data means were compared using SAS (C.3, Appendix C). There were no significant differences between the means using Tukey's multiple comparison test at an $\alpha = 0.05$ (D.3, Appendix D).

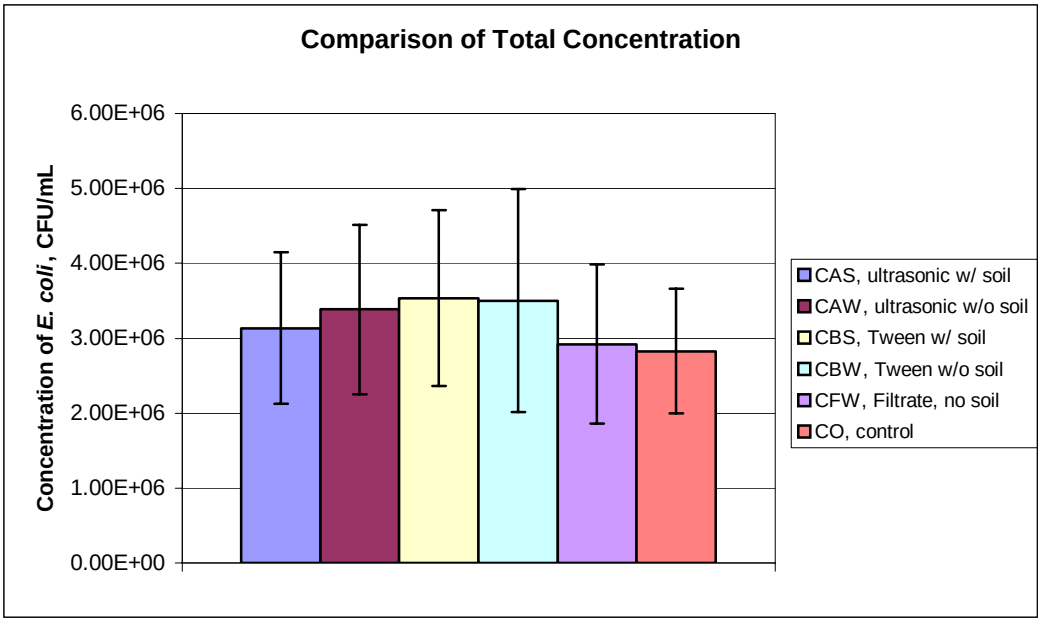

Figure 4-7: Comparison of Total Concentrations

The data obtained from this experiment could be used to answer the first two questions posed in the experimental methods section. Passing a sample of unattached (no soil present) *E. coli* through a 8 μm filter and then analyzing the filtrate for concentration yielded a concentration that was not statistically different from a value obtained by just analyzing the initial sample. The concentration of this filtrate, CFW, was actually just slightly higher on average than the concentration of the non-filtered control, CO. Therefore, it was assumed that no unattached bacteria are trapped on the filter during this process, and that analysis of the filtrate would yield an accurate unattached concentration.

Treatment with dispersion methods did not result in significant die-off, regardless of whether or not soil was present in the sample. The four total concentration values obtained after a dispersion technique was applied to the subsamples were higher on average than the total concentration obtained from the untreated control, indicating that the dispersion treatments might have dispersed clumped bacteria as well as removing them from soil particles, resulting in higher

plate counts. Concentration values obtained from samples dispersed using Tween were higher on average than concentration values obtained using ultrasonic treatment, suggesting that treatment with Tween might be more thorough in dispersing bacteria than submersion in the ultrasonic bath. Interestingly, those samples treated with a dispersant that contained soil had slightly higher concentration values than those samples treated with a dispersant that did not contain soil, though this difference was not significant. Past studies have suggested that attachment can be protective and prolong cell survival. Perhaps the dispersants did result in some cell death, but this was not measurable or was masked by the disaggregation of cell-cell aggregates. If so, attachment to soil particles in samples with soil could have protected the cells from cell death as a result of the dispersants. A greater number of block repetitions would have been required to discern any significant differences in effect between the dispersion treatments or between the samples that did or did not have added sterile topsoil.

4.3.2 Experiment 2: Comparison of attachment values

Using the data from Experiment 1 and ten block replications of Experimental Design 2 (Figure 3-2), twenty block sets of data were available for analysis. Raw data values are given in Table B-4 in Appendix B. A graph of the total and planktonic concentration mean values obtained from the analysis and the confidence intervals for each data set is given in Figure 4-8.

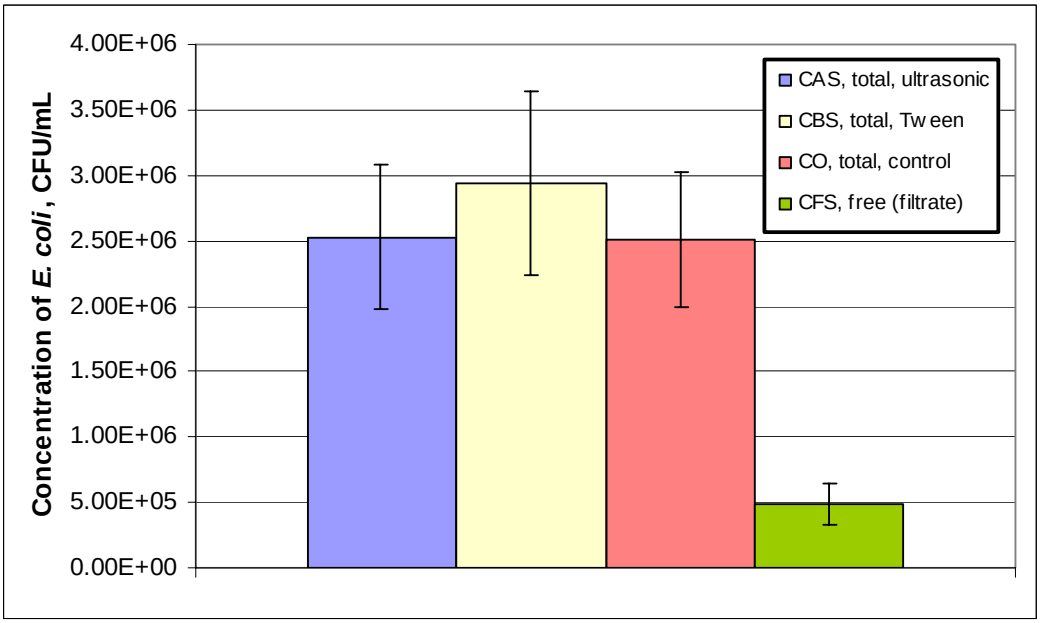

Figure 4-8: Comparison of Total and Planktonic Concentrations

SAS programs are given in C.4, Appendix C. The concentrations were compared using Tukey's multiple comparison test with an α of 0.05. The difference between the concentration of the filtrate, which was considered the planktonic concentration, was significantly different from the three measures of total concentration of bacteria in the sample, indicating that a significant portion of the bacteria were attached to soil particles. There were no statistically significant differences between the values of total concentration obtained from the control or from either dispersion technique (D.4, Appendix D). Because there were no significant differences, the values obtained using any of the dispersion methods would be reasonably accurate in obtaining values of total concentration from an unknown sample and resulted in no measurable die-off. However, concentrations were highest with Tween, suggesting that it was a more effective dispersant. It is important to note that while there was no measurable die-off, cell death or a reduction in culturability could have occurred. If cells are significantly clumped even when there are no soil particles, plate counts would be lower. Even if a dispersion treatment resulted in some

cell death, if it dispersed cell aggregates enough to result in higher plate counts than the control, no die-off would be measured. Research using more accurate measures of die-off, such as substrate uptake, would be required to definitively state the degree of die-off involved.

The values of total concentration and planktonic concentration were used to calculate proportions of the total sample concentration that were considered attached to the soil particles using Eq. 2. Proportions of attached bacteria were calculated separately using each of the total concentration values for each sample. Differences between the proportions yielded by each technique were analyzed using SAS (C.5, Appendix C). The average proportion of attached bacteria for each technique is given in

Table 4-2. Tukey's multiple comparisons test using an α of 0.05 indicated that there were no significant differences between the proportions. SAS output is given in D.5 in Appendix D.

Table 4-2 Comparison of Proportions of Attached Bacteria

Total Concentration Value	Attached Proportion
ultrasonic treatment	0.80
Tween-85 surfactant	0.78
control (no treatment)	0.77

In this experiment, between 77% and 80% of *E. coli* were attached to soil particles. It is important to note that attachment has been defined as retention on an 8 μm filter. Bacteria passing through the filter may be attached to one another or to very small clay particles, but these attachments were considered too small to be significant. Similarly, bacteria retained on the filter may be attached solely to other bacteria in large clumps and not to soil particles. Regardless, this high level of attachment indicates that current bacterial modeling techniques, which model bacteria as dissolved or planktonic chemicals, misrepresent the actual process involved in bacterial transport. It is also important to note that these experiments used only one type of soil, a

sandy loam. Numerous studies have indicated that an increase in clay content will increase bacterial attachment. Clays are very reactive. While both clays and bacteria have an overall negative charge, clays have positive edges that permit bacterial attachment, or may attract divalent cations that also attract cells (Marshall, 1975). Since the clay content of the soil used in this experiment was low, it would be expected that greater than 78% of bacteria would become attached if a soil with higher clay content, like a silty loam or clay loam, was used.

The values of the attached proportion of bacteria in the sample were roughly the same, regardless of the value used as total concentration. The slight differences between the values are difficult to interpret due to masking problems. A greater total concentration would yield a greater value for the attached concentration in the numerator, but also a greater total concentration value in the numerator. Also, there was a large amount of variability in attachment proportions between samples, as shown in Table 4-3. Measured attachment ranged between 7.3% of the total concentration to 97.2%. This wide variation in proportion of the total concentration considered attached suggests that the bacterial cells in this experiment did not behave in a homogenous manner with regard to attachment. Consequently, the main usefulness of this measurement is to illustrate the large quantity of bacteria that will preferentially attach to soil particles, rather than to provide a numeric estimate of bacteria attachment to soil particles. Experiments using a stock bacteria suspension of more homogenous cells would be required to determine an exact attached proportion.

Table 4-3. Percentage of *E. coli* Considered Attached for Each Experimental Sample

	Percent of *E. coli* Attached		
Run	**Ultrasonic**	**Tween-85**	**Control**
1	85.7%	94.0%	93.2%
2	93.8%	96.9%	95.3%
3	43.3%	10.5%	7.3%
4	70.0%	63.2%	64.4%
5	79.7%	44.1%	53.6%
6	63.3%	83.2%	84.3%
7	80.2%	90.3%	64.0%
8	68.3%	74.0%	76.3%
9	76.9%	73.8%	66.0%
10	73.1%	61.1%	74.4%
11	95.3%	95.7%	94.8%
12	95.5%	86.0%	97.2%
13	96.3%	98.5%	91.9%
14	87.6%	89.9%	85.7%
15	91.9%	89.9%	94.9%
16	86.1%	78.9%	86.8%
17	93.2%	95.8%	94.8%
18	86.6%	94.1%	92.1%
19	71.9%	71.9%	67.8%
20	69.1%	68.1%	54.5%
AVG	**80.4%**	**78.0%**	**77.0%**

Both of the dispersion techniques tested yielded higher average total concentration values than the total concentration value obtained by analyzing the untreated control, indicating that not only was there no measurable die-off, but that these dispersion methods might break up natural bacterial aggregates, resulting in higher plate counts. While there was no significant difference between the concentration values obtained using these techniques, treatment with Tween-85 was selected as the most promising method for further research. The samples treated with Tween yielded the highest total concentration values, indicating that Tween was the most effective at breaking up bacterial and soil-bacteria aggregates and provided a better estimate of the true total concentration. A method of partitioning between attached and planktonic bacteria using Tween is also more practical and inexpensive for water quality laboratories, as it does not require any new equipment, like an ultrasonic cleaning bath, but only one new reagent.

4.4 Sorption Experiments

4.4.1 Initial sorption experiments using batch cultures

The entire set of bacteria-soil combinations given in Table 3-3 was not completed due to extreme variability and an inability to reproduce results. The raw data that were collected is given in Table B-5 in Appendix B. The soil control, which tested for soil sterilization as explained in Section 3.1.2, was negative for any bacterial growth. It is important to note that the five usable data points all came from one day's work in the laboratory using the same batch culture as the stock solution. Other trials using different bacteria-soil combinations and repetitions of the combinations listed were conducted on subsequent days using different batch cultures inoculated the day before those particular experiments. All these attempts yielded plates with colonies too numerous to count. Attempts to further dilute samples to obtain concentrations failed and still yielded overgrown agar plates. A graph of the planktonic concentration versus attached concentration of cells for the five successful samples is given in Figure 4-9. The data were analyzed using the linear regression trendline function in Microsoft Excel. The y-intercept was set as zero, as it was assumed there was no background attached bacteria concentration.

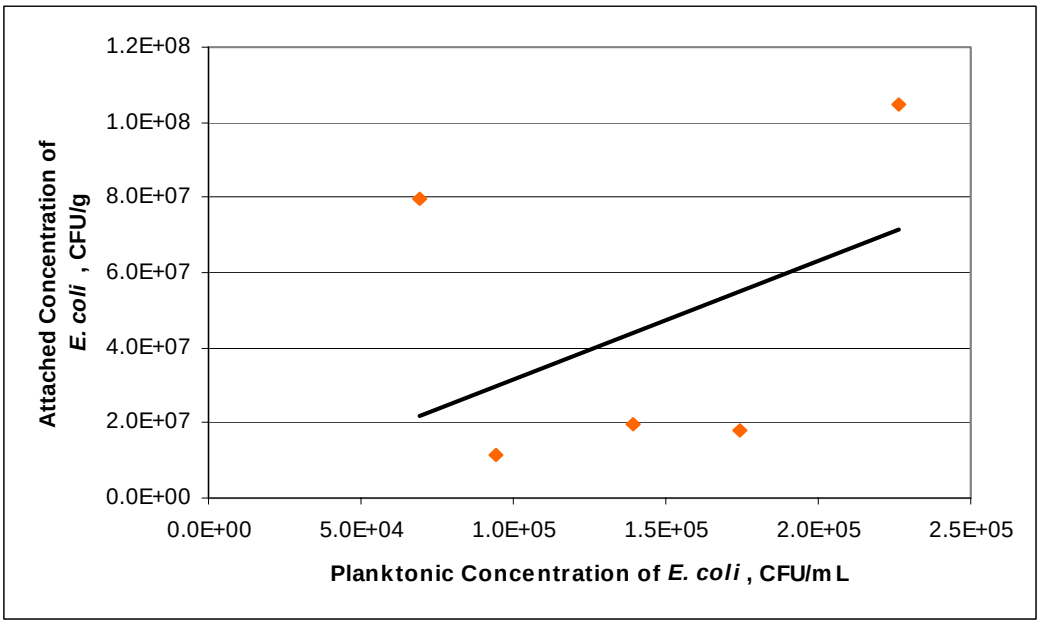

Figure 4-9. Attached versus Planktonic *E. Coli* Concentration from Batch Culture Experiments

The linear regression equation given by Excel relating planktonic and attached concentration is given below as Eq. 6. Attached concentration, A, is in CFU/g, and planktonic concentration, P, is in CFU/mL. This equation had an R^2 coefficient of 0.0686.

$$A = 320*P \qquad \textit{(Eq. 6)}$$

The extremely low R^2 value indicates that these experiments were unsuccessful in establishing a relationship between planktonic *E. coli* concentration and attached *E. coli* concentration. Langmuir and Freundlich isotherm equation models (described in more detail in 0) were also used in an attempt to model the data but resulted in even lower R^2 values of effectively zero. Two possible causes of this extreme variability were the high variation in TSS values used and the physiological state of cells grown in batch culture.

Total suspended solids values in the samples tested ranged from 1,000 mg/L (0.1 g in a 100 mL sample) to 50,000 mg/L (5.0 g in a 100 mL sample). Larger TSS values would be expected to provide more possible attachment sites for cells, and therefore result in greater

attachment. In an effort to determine the effect of the large range of TSS values on the observed bacterial attachment, the proportion of bacteria attached was calculated using Eq. 3 and graphed versus the amount of soil added to the 100 mL sample (Figure 4-10).

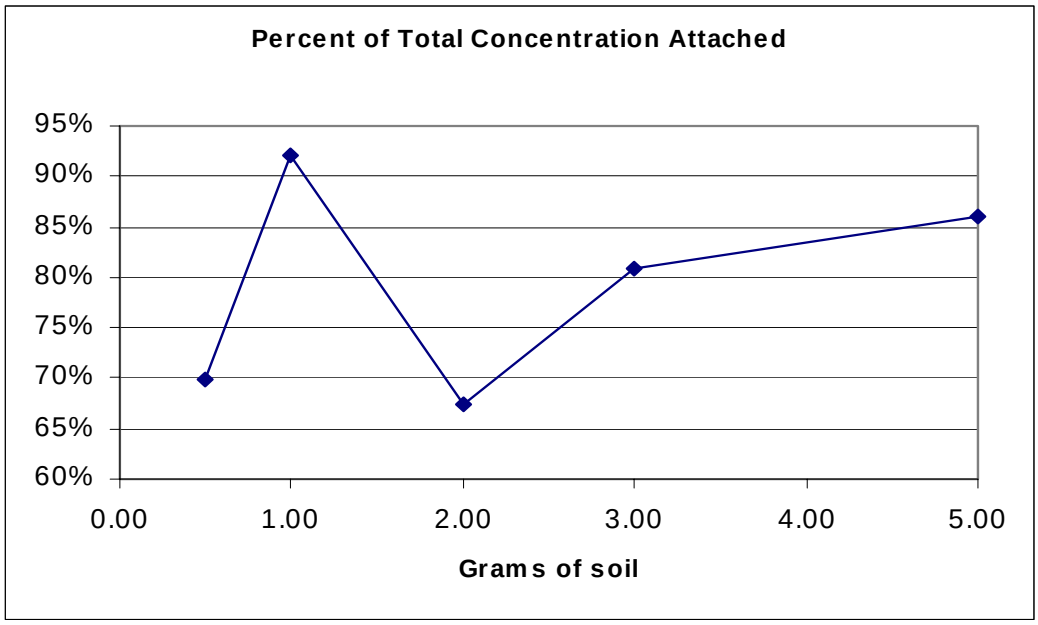

Figure 4-10. Percent of *E. coli* attached in initial sorption experiments

As revealed by Figure 4-10, no real pattern in attachment was discernable from the five data points available. Attachment did not appear to increase as soil was added, nor did it appear unaffected. Instead, the proportion of cells attached to soil seemed random. If TSS concentration had no effect on attachment, the proportion of attached cells should have remained constant. This high variability suggested that the cells themselves were behaving in a variable manner, perhaps due to physiological state.

Another indication of the high variability of the data results was the inability to repeat sample trials or obtain more than five usable data points. More than ten other samples containing different bacteria-soil combinations were tested in the same way but did not yield any usable results as all these samples' membrane filtration agar plates were totally overgrown. Attempts to

dilute the samples further proved unsuccessful. Using past information on successful dilutions for past samples did not seem to help predict the necessary dilution for a future sample. The inability to predict the necessary dilution could be due to different concentrations in the stock batch cultures; perhaps, even though each batch culture was inoculated and incubated using the same procedure, the population grew to a different concentration. Also, the bacteria in the batch cultures may have exhibited different attachment capabilities, resulting in varying proportions of free and attached bacteria.

Because the suspension used to inoculate the soil-dilution water samples was a batch culture grown for 24 hours, it is likely that the *E. coli* cells within this culture had reached stationary phase. During stationary phase, the cells begin to exhibit diverse characteristics in an attempt to maximize survival in a substrate-limited environment. Because individual cells may react differently to this stress, the population becomes very heterogeneous in its characteristics. The variability between individual cells' ability to attach may have caused the high variability in the sample data. To determine the exact growth stage of these batch cultures and better diagnose the cause of the high variability in these first sorption experiments, an experimental growth curve for the culture needed to be determined. Once the growth curve was defined, the possibility of using a different method of culturing could be considered.

4.4.2 Experimental growth curve

The growth curve experiment was conducted for 30 hours to determine the growth state of *E. coli* in tryptic soy broth after 24 hours culturing in a batch flask. Graphs of absorbance vs. time and transmittance vs. time are given in Figure 4-11 and Figure 4-12. The absorbencies of the three flasks were averaged to give the representative growth curve shown in Figure 4-13. Error bars representing one standard deviation above and below each value are given in Figure

4-13. A blank control, Flask A, was used to set the spectophotomter to 100% transmittance before each reading. Raw data from the experiment are given in Table B-6 in Appendix B.

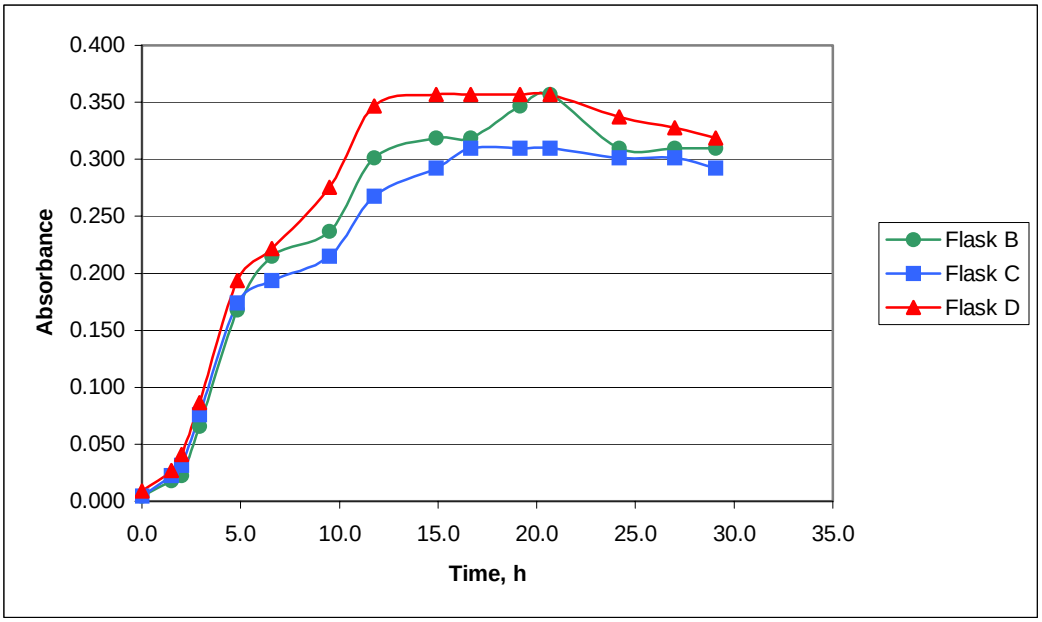

Figure 4-11. Growth Curve for each *E. coli* Tryptic Soy Broth Flask

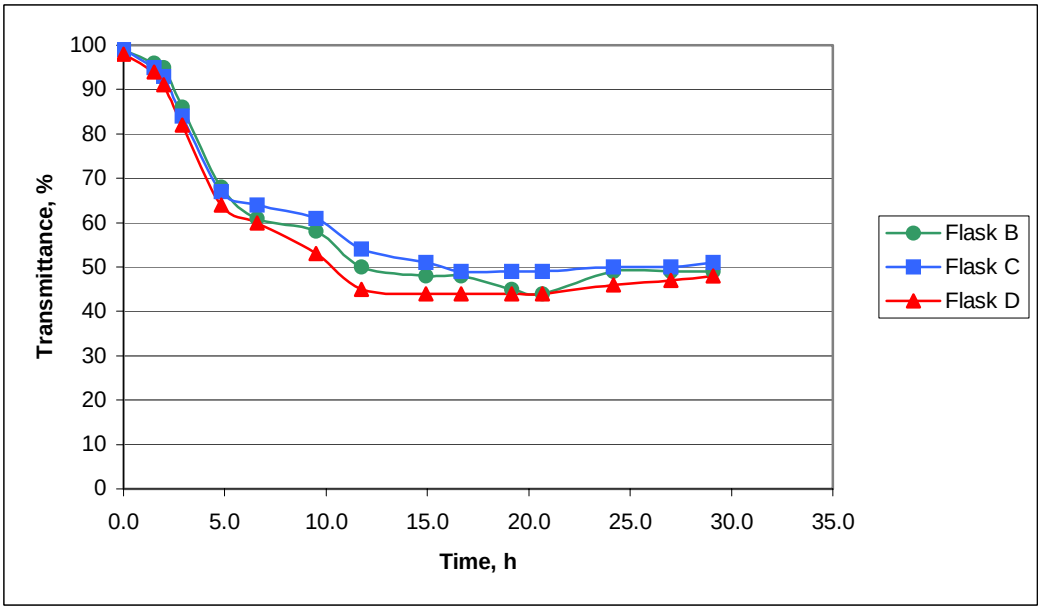

Figure 4-12. Transmittance vs. Time for *E. coli* Growth Curve Experiment

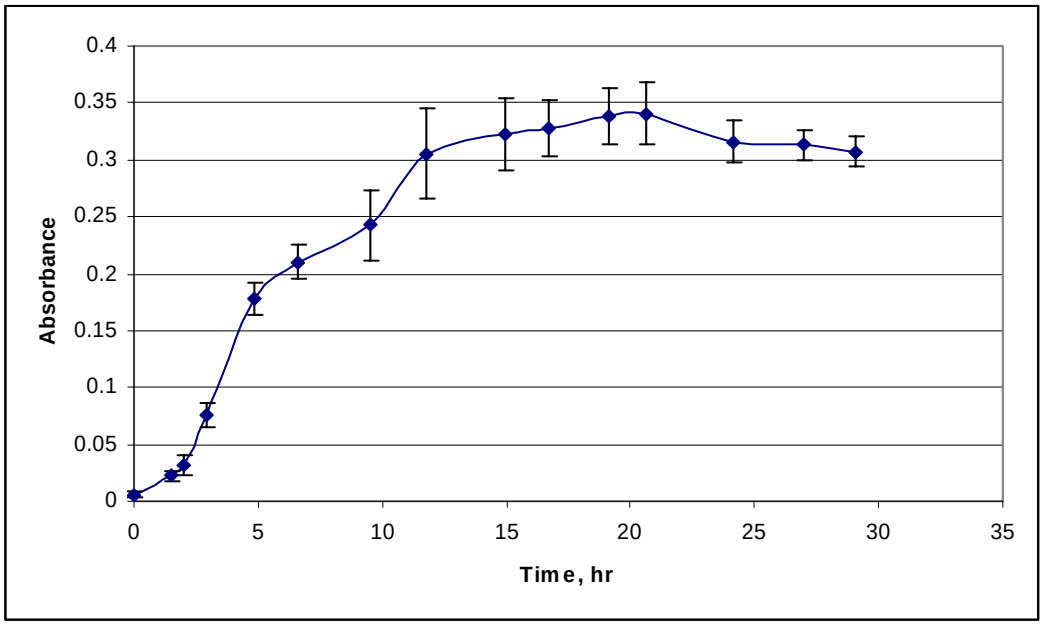

Figure 4-13. Average Growth Curve for *E. coli* in Tryptic Soy Broth

The batch cultures used in the first sorption experiments were grown for 24 hours before each experimental trial. Comparison between the experimental growth curves in Figure 4-11 and Figure 4-13 and the theoretical typical growth curve illustrated in the Jones article (1997) reveals that after 24 hours the culture was well into the stationary phase.

Once a culture reaches stationary phase in a nutrient-rich environment, the cell population begins to sense nutrient limitation and becomes stressed. In an attempt to maximize survival, individual cells express different genes and may exhibit new metabolic abilities or undergo morphological changes. Consequently, the characteristics and behavior of cells from cultures at stationary phase are extremely variable. It is likely that this high variability in cell characteristics resulted in the highly variable results in the first set of sorption experiments. Further experiments attempting to quantify attachment using batch cultures would be expected to yield similarly variable results.

In order to control this variability, it would be preferable to use a cell culture maintained in a constant nutrient concentration environment rather than in an initially nutrient-rich environment in which nutrients are eventually consumed and become scarce. Under these conditions, the culture would reach a state of balanced growth during which not only the number of cells, but the activity of the cells would be constant. A balanced growth culture population would be expected to behave in a much more homogenous manner with regard to attachment. Although the exponential phase of a batch culture is also a state of balanced growth, this is a state less representative of environmental conditions than chemostat balanced growth as the cells are not substrate-limited and are growing at the maximum growth rate. There is also experimental evidence that bacteria in the natural environment exist in a constantly nutrient-stressed state, and so a culture maintained at a similar state in a chemostat may be more representative of natural conditions (Roszak and Colwell, 1987).

Further sorption experiments were conducted using a culture from a continuous flow through reactor, or chemostat. Under these conditions, the cells could be grown continuously at a state of balanced growth under constant substrate concentration. Use of a more homogenous culture suspension was expected to give less variable results in sorption experiments.

4.4.3 Sorption Experiments using a chemostat culture

4.4.3.a Maintenance of chemostat culture

The chemostat culture described in Section 3.6.3 maintained a pure culture of *E. coli* for eight days and roughly twenty-one hydraulic retention times (HRTs). Periodic recordings of the pH, dissolved oxygen, absorbance, temperature, and *E. coli* concentration of the broth culture in the chemostat over this eight-day time period are given in Table B-7 in Appendix B. Samples withdrawn from the chemostat for use in sorption experiments are indicated. These samples were withdrawn over a period of four days and twelve HRTs.

The pH, dissolved oxygen, temperature, and absorbance of the culture remained fairly constant after pumping began; however, between day four and day six, the concentration increased by an order of magnitude, from roughly 10^8 CFU/mL to 10^9 CFU/mL. This increase in cell count was not accompanied by a measured increase in absorbance, though an increase in absorbance would be expected as the density of cells in the culture increased. Since all other factors remained relatively constant, it is difficult to determine why this increase occurred.

This increase in *E. coli* concentration could have resulted from contamination of the chemostat culture or mutation of the culture. *E. coli* colonies grown on the mTEC agar plates are purple, while other coliform colonies appear white or clear. Since all colonies observed were clearly purple, if the culture was contaminated, it was from organisms that cannot grow on mTEC agar. It is also possible that the *E. coli* bacteria within the chemostat had begun to adapt to their environment and mutated in a way that allowed the population to grow to a greater concentration. Perhaps the cells began to maintain a reduced size. Smaller cells would keep the absorbance lower even when the actual cell count increased.

It would have been necessary to monitor the chemostat more closely to determine the exact cause of this increase in concentration. Because colonies take a full 24 hours of incubation to develop on mTEC agar, this increase was not noticed until day seven, when the period of sampling for sorption experiments was almost over. By the time another plating of the chemostat concentration was counted on day eight to confirm that there was an increase in concentration despite the constant absorbance readings, the sorption experiments were complete. More frequent analysis of the *E. coli* concentration in the chemostat would have indicated this increase in concentration earlier. Also, additional platings for concentration on less selective agar may have allowed identification of a contaminant, if interaction with some contaminant was responsible for the increase.

4.4.3.b Development of isotherm equations

Ten different *E. coli* concentrations were tested for attachment at two different TSS concentrations: 5,000 mg/L (0.5 g in 100 mL) and 10,000 mg/L (1.0 g in 100 mL). Raw data for these samples are given in Table B-8 and Table B-9 in Appendix B. The soil control, which tested for soil sterilization as explained in Section 3.1.2, was negative for any bacterial growth. Nineteen of the twenty samples were considered usable data points; one sample yielded a planktonic concentration greater than total concentration, resulting in a negative attached concentration value, and was eliminated from analysis as a case of experimental error.

Isotherm equations can be used to model the equilibrium concentrations of constituents between the soil-adsorbed and free phases. Three possible isotherm equation models were considered when analyzing the *E. coli* sorption data from the experiments described in Section 3.6.3: the Linear Isotherm, the Langmuir Isotherm, and the Freundlich Isotherm. Linear regression in SAS was used to develop an equation for each model and to test for each model's fit with the experimental data.

A linear isotherm models the data according to a simple linear equation, as given in Eq. 7. The y-intercept is set at zero, as it is assumed that when the concentration of the constituent in solution, in this case the planktonic *E. coli* concentration, is zero, the attached concentration will also be equal to zero.

$$\frac{X}{M} = k * C_e \qquad \textit{(Eq. 7)}$$

Where X = mass of adsorbate

M = mass of adsorbent

k = partitioning coefficient (experimentally determined)

C_e = the non-adsorbed concentration (dissolved, or free or planktonic concentration)

The experimental data were analyzed using SAS (C.7, Appendix C) to calculate the appropriate partitioning coefficient. The adsorbed phase concentration, X/M, is equal to the attached *E. coli* concentration in CFU/g and C_e is equal to the planktonic *E. coli* concentration in CFU/mL. The resultant linear isotherm equation is given by Eq. 8. As revealed by the high R^2 correlation factor, the linear isotherm described the data very well. Additionally, statistical analysis of the data confirmed that the partitioning coefficient value was significant with an α of 0.95 (D.7, Appendix D). A graph of the data along with the regression line is shown in Figure 4-14.

$A = 595*P \qquad R^2 = 0.8804$ *(Eq. 8)*

Where A = soil-adsorbed concentration of *E. coli*, CFU/g

P = planktonic concentration of *E. coli*, CFU/mL

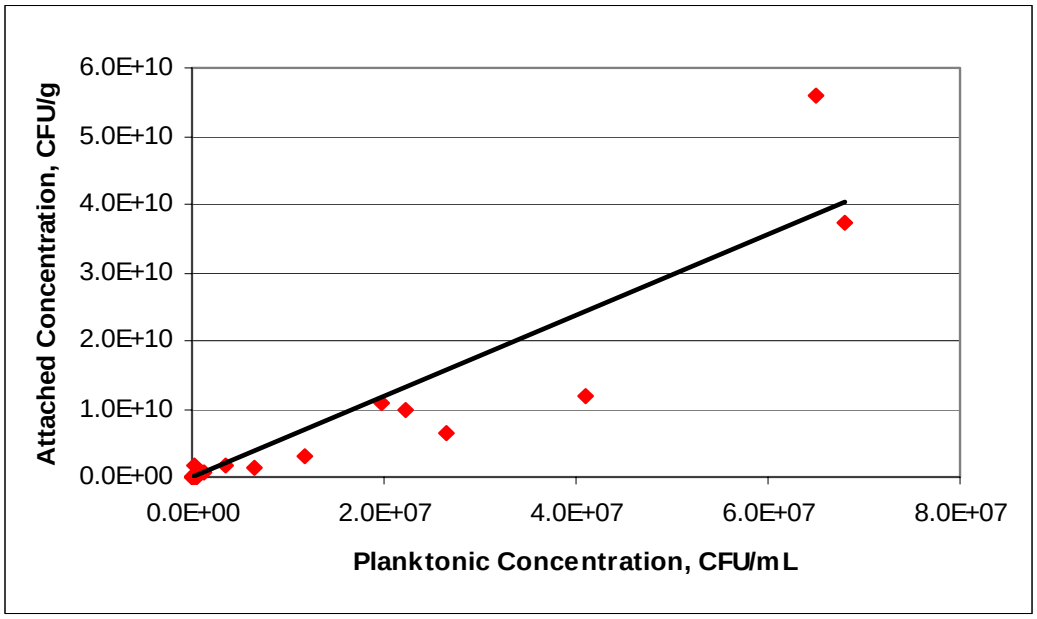

Figure 4-14 Linear Regression for Linear Isotherm Model

The Langmuir Isotherm models the experimental data using the equation given by Eq. 9. The Langmuir Isotherm assumes that the maximum adsorption is a complete monolayer; that is, there cannot be adhesion to a single site by more than one cell, and there cannot be layers of attachment or interactions between adsorbed cells. It also assumes that the energy required for attachment at different sites is homogenous.

$$\frac{X}{M} = \frac{Q^0 b C_e}{1 + b C_e} \qquad \textit{(Eq. 9)}$$

Where Q^o = adsorption maximum at a fixed temperature, or the maximum amount of adsorbate that can be adsorbed (complete monomolecular layer)

b = constant related to the binding strength of the adsorbent.

Q^0 and b are usually experimentally determined.

The Langmuir Isotherm is often rearranged to a linear form (Eq. 10) to determine if it describes the experimental data:

$$\frac{C_e}{X/M} = \frac{1}{Q^0} + \frac{1}{Q^0 b C_e} \qquad \textit{(Eq. 10)}$$

A graph of the transformed data with the regression line is given by Figure 4-15. This data was analyzed using linear regression in SAS (C.8, Appendix C). According to the SAS output (D.8, Appendix D), the slope of the line, equal to $1/Q^0b$ in Eq. 10, was significant, but the intercept ($1/Q^0$) was not significant (α=0.05). The R^2 of the linear regression line was 0.9480. The Q^0 and b values were calculated to give the Langmuir Isotherm equation in Eq. 11.

$$A = \frac{455 * P}{1 + (-4.55E - 7) * P} \qquad \textit{(Eq. 11)}$$

Where A = soil-adsorbed concentration of *E. coli*, CFU/g

P = planktonic concentration of *E. coli*, CFU/mL

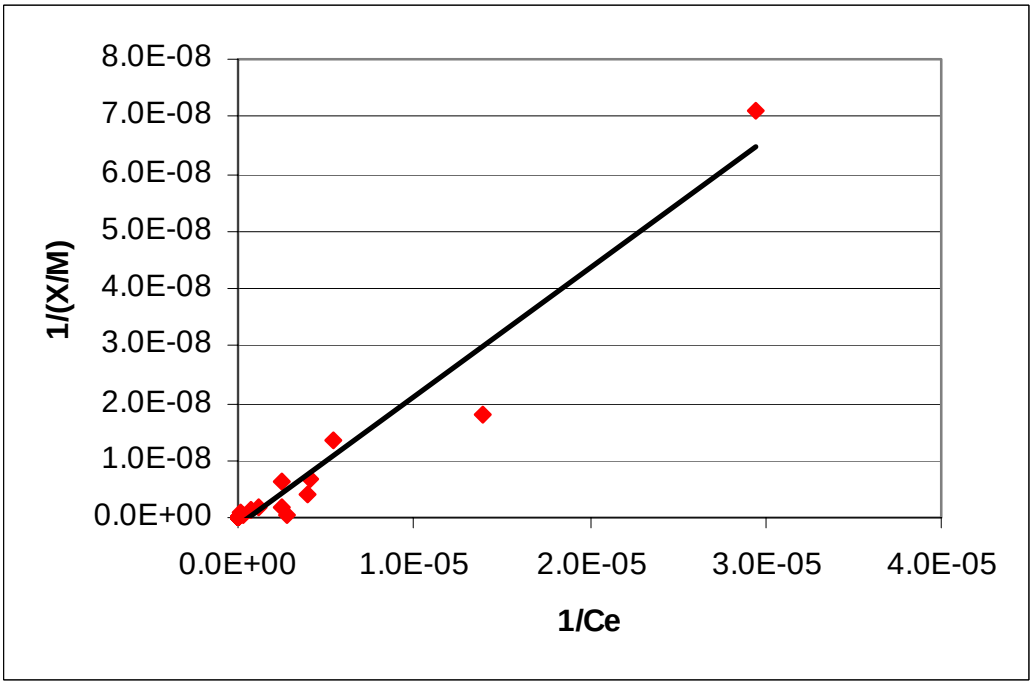

Figure 4-15. Linear Regression for Langmuir Isotherm Model

While the R^2 correlation value for the transformed data is very high, the Langmuir Isotherm does not realistically model this data. The calculated y-intercept for Eq. 10 was not significant, which is not surprising, as x=0 is far beyond the experimental data set minimum. Because the y-intercept for the transformed data is negative, the resulting value for the adsorption maximum, Q^0, is negative, which is not possible.

The attachment of bacteria cells to soil particles likely violates the assumptions included in the Langmuir Isotherm discussed previously. Bacteria cells probably do interact with one another, collecting in cell clumps that then attach to soil particles, and form layers of attachment more than one cell thick. Also, most soil particles contain several different sites of attachment of different energy due to the shape and clay content of each particle. Since the assumptions of the physical nature of attachment for the Langmuir Isotherm are probably not valid in this case, it is not reasonable to use the Langmuir model in this case.

The general Freundlich Isotherm model is given by Eq. 12. Unlike the Langmuir Isotherm, the Freundlich Isotherm is empirical. Like the Linear Isotherm, it does not reach a maximum value and in fact, the Linear Isotherm is just a simplified case of the Freundlich Isotherm with 1/n = 0.

$$\frac{X}{M} = k * C_e^{1/n} \qquad \textit{(Eq. 12)}$$

Where n = experimentally determined coefficient.

The Freundlich Isotherm can be linearized by taking the log of Eq. 12, resulting in Eq. 13. A regression line is then fit to the logs of X/M and C_e to determine if the data conform to the Freundlich Isotherm.

$$\log(\frac{X}{M}) = \log(k) + \frac{1}{n} * \log(C_e) \qquad \textit{(Eq. 13)}$$

A graph of the transformed data with the regression line is given by Figure 4-16. These data were analyzed using linear regression in SAS (C.8, Appendix C). The R^2 factor for the linear regression line was 0.9163. According to the SAS output (D.9, Appendix D), the slope of the line, 1/n in Eq. 13, was significant; however, the intercept, log(K), was not significant (α=0.05). The resulting Freundlich Isotherm is:

$$A = 2508 * P^{0.90} \qquad \textit{(Eq. 14)}$$

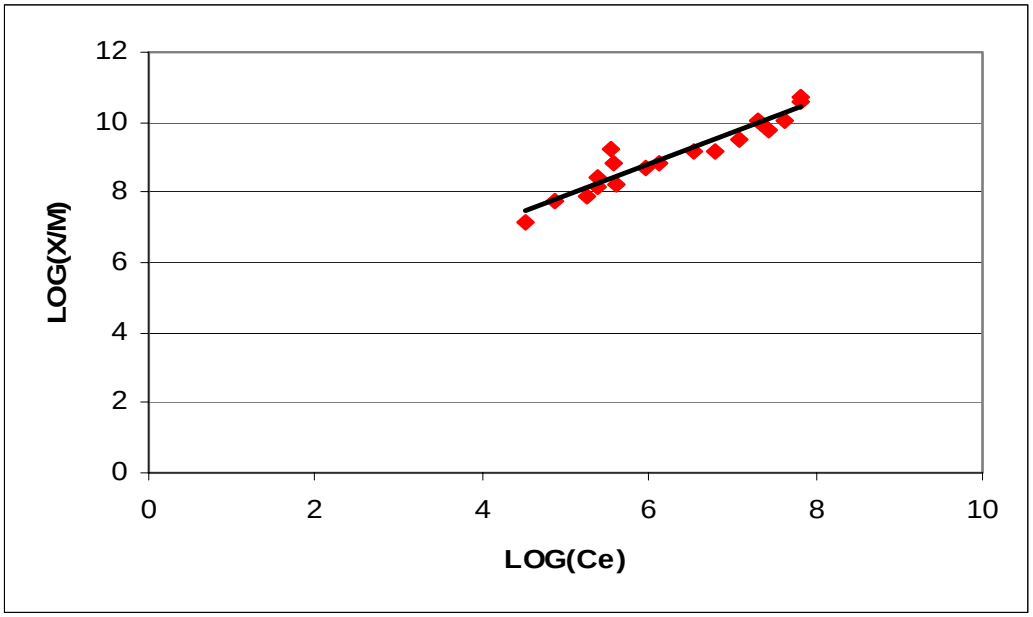

Figure 4-16. Linear Regression for Freundlich Isotherm Model

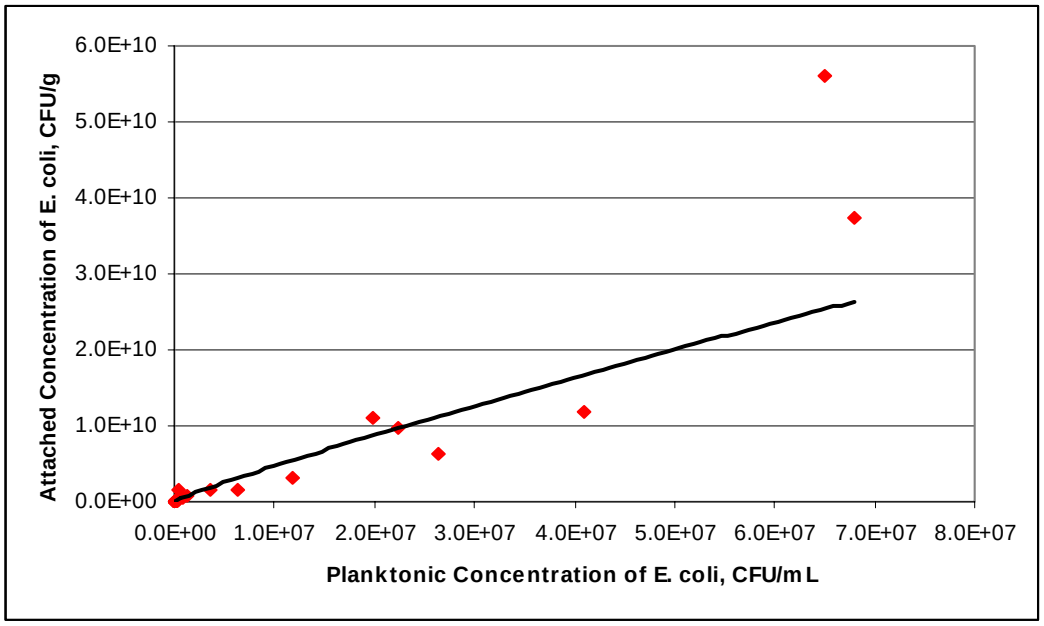

Figure 4-17. Freundlich Isotherm describing Sorption Data

The R^2 correlation factor for the Freundlich Isotherm (0.92) was slightly higher than that of the Linear Isotherm (0.88). However, the Linear Isotherm still may be the better model for use in describing the equilibrium between attached and planktonic *E. coli.* Figure 4-17, a graph of the data plotted with a line representing Eq. 14, reveals that the model is nearly linear. The 95% confidence interval for the 1/n value for the Freundlich for the experimental data was (0.76, 1.04), which includes 1.0, so the value of 1/n is not statistically different from 1.0. Also, the difference in R^2 values between the two models is very small. Because there is no clear advantage to using the Freundlich Isotherm, and because the value of 1/n is not statistically different from 1.0, until further research is conducted, it would be best to stick with the simplest model that accurately describes the data – the Linear Isotherm.

As revealed by the high R^2 correlation factor of the Linear Isotherm (0.88), the chemostat method of culturing was extremely successful in resolving the variability encountered using batch cultures at stationary phase. The corresponding equation relating attached and planktonic

concentration for the batch cultures had an R^2 of less than 0.1, illustrating little or no relationship between the two values, yet an R^2 of over 0.8 indicates that a linear equation models the data very well, as first proposed by Reddy et al. (1981).

4.4.3.c Effect of suspended solids concentration

The proportion of bacterial cells considered attached was calculated using Eq. 2 for each of the nineteen usable samples. The proportions of attached cells for each sample are given in Table B-8 and Table B-9, Appendix B. The average proportions of attached bacteria for the different TSS values and for all samples are given in Table 4-4. Interestingly, the average attached proportion of bacteria was 78%, which was the same as the average proportion considered attached during the earlier experiments using batch cultures to test dispersion methods. However, a comparison of the proportions calculated for individual samples for these experiments using batch cultures (Table 4-3) with the individual sample values in Table B-8 and Table B-9 reveals a much smaller range of attachment values for the chemostat samples. When using batch cultures, measured attachment varied between less than 10% and 97% but when using chemostat cultures, measured attachment varied only between 55% and 95%. This reduced range in measured attachment values illustrates the more homogenous attachment behavior of the balanced growth cells cultured in the chemostat as compared to the stationary phase cells cultured in a batch. Regardless, the consistency of average attachment proportions suggests that the earlier experimental results focused on selection of a dispersion method are valid for the chemostat cultures.

As shown in Table 4-4, attachment was greater for samples with a TSS concentration of 10,000 mg/L than for samples with a TSS concentration of 5,000 mg/L and this difference was statistically significant at an α of 0.05 based on the F-test (C.6, Appendix C, D.6, Appendix D).

This was expected, as a greater amount of suspended solids would also provide a greater number of potential attachment sites for the cells.

Table 4-4. Attached Proportion of *E. coli* for Chemostat Sorption Experiments

Sample TSS, mg/L	**Attached Proportion of *E. coli***
5,000	0.74
10,000	0.82
average	0.78

Though the difference between attachment for a TSS of 5,000 mg/L (74%) and a TSS of 10,000 mg/L (82%) was significant, this difference seemed small, since the amount of soil, and therefore potential attachment sites in the sample, had doubled. To further explore this difference, the experimental data used to develop the sorption equations in Section 0 was graphed and analyzed separately according to TSS value. A graph for the data from samples with a TSS of 5,000 mg/L is given in Figure 4-18 and a graph for the data from samples with a TSS of 10,000 mg/L is given in Figure 4-19. The equations provided by SAS linear regression (C.7, Appendix C) are given below. Eq. 14 relates planktonic and attached concentration at a TSS of 5,000 mg/L and Eq. 15 relates planktonic and attached concentration at a TSS of 10,000 mg/L. Attached concentration, A, is in CFU/g, and planktonic concentration, P, is in CFU/mL.

$$A = 600*P \qquad R^2 = 0.8844 \qquad \textit{(Eq. 14)}$$

$$A = 270*P \qquad R^2 = 0.9553 \qquad \textit{(Eq. 15)}$$

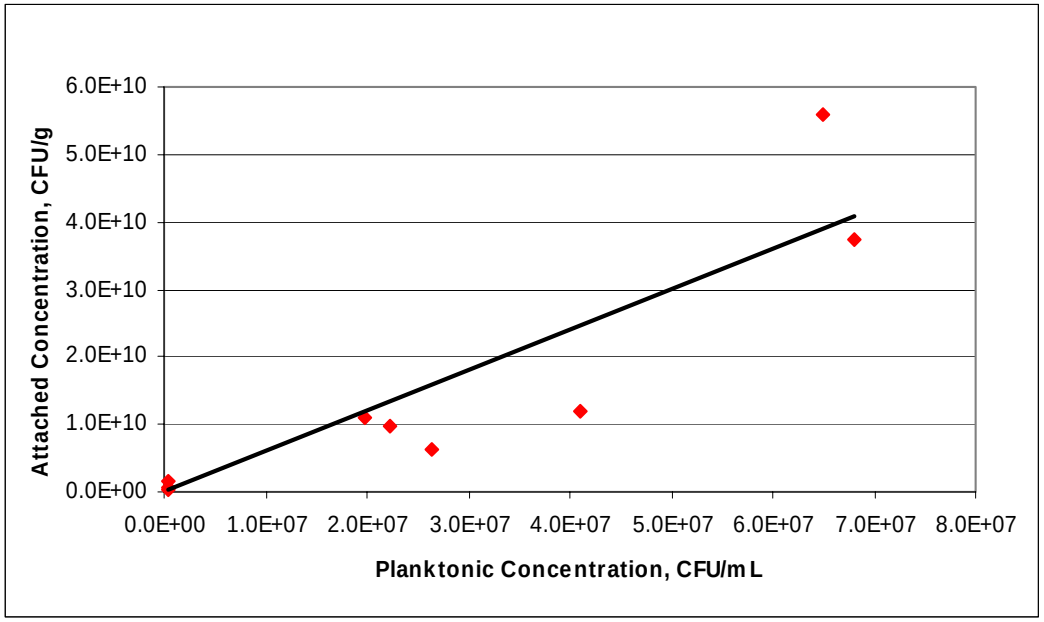

Figure 4-18. Attached Concentration vs. Planktonic Concentration for Chemostat *E. coli* Cultures for a TSS of 5,000 mg/L

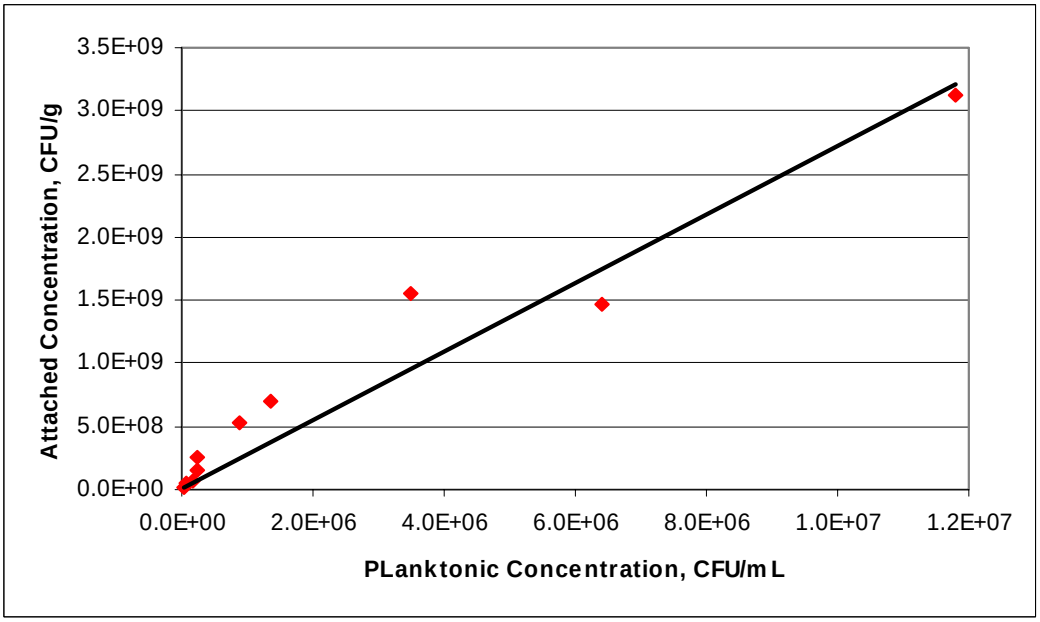

Figure 4-19. Attached Concentration vs. Planktonic Concentration for Chemostat *E. coli* Cultures for a TSS of 10,000 mg/L

Both Eq. 14 and Eq. 15 have very high R^2 values, indicating that these equations model the data very well. Both slopes are also considered statistically significant in modeling the

structure of the data ($p = <0.0001$, D.10 and D.11, Appendix D). However, the equations are very different, with the slope of Eq. 14 over twice that of Eq. 15. As revealed by the confidence intervals listed in D.10 and D.11 in Appendix D, the confidence intervals of these slopes do not overlap, indicating that they are statistically different. These differences in resultant linear equation models between TSS values may indicate that the amount of suspended solids in the sample influenced attachment. As expected, the average proportion of bacteria attached was slightly less for samples of 5,000 mg/L TSS than for samples of 10,000 mg/L TSS, which contained more soil and therefore more potential sites for attachment.

It is possible that the bacteria in this experiment were not limited by attachment sites. While there was a significant difference between the average proportion of attached cells in samples with a TSS of 5,000 mg/L and samples with a TSS of 10,000 mg/L, this difference seemed small for a doubling in available soil particles for attachment. Perhaps at these relatively large TSS concentrations, the cells are not limited by potential attachment sites; consequently, all cells that have the ability to attach do so. For this particular culture, roughly 78% of cells were able to form attachments to soil particles. However, this hypothesis assumes that all attachment is the result of the cells' physical ability to attach to the particles and is not due to chemical attraction, which is likely not true. Further research would be required to test this hypothesis and further explore the relationship between cellular attachment and total suspended solids.

4.4.3.d Limitations of isotherm equations

In modeling the attachment of contaminates to soils, isotherms relate the free concentration of the contaminant in solution to the adsorbed concentration of the contaminant per unit of soil. The linear isotherm, Eq. 8, was selected as the best representation of the experimental data. However, the practical usefulness of Eq. 8 in bacterial modeling is limited by

the understanding of the interaction between attachment and TSS concentration discussed in the previous section and also limited by the laboratory conditions of the experiments. The conditions used in this study are not representative of the very complex and variable conditions encountered in the field; however, it was necessary to simplify the variables involved in order to obtain the repeatability necessary to develop a sorption equation with high correlation and to confirm the usefulness of the partitioning method developed earlier. Because these conditions are so controlled, the resulting equations should not be considered applicable to every situation involving bacterial attachment in overland flow or in the water column. As discussed in the literature review and again revealed by the data of this study, the amount and type of soil particles available greatly influences attachment. In this study, only one type of soil, a sandy loam, was considered. A great deal of previous research has indicated that attachment increases dramatically with clay content. The sterile soil used in this study had an average clay content of only about 3%, and so higher proportions of attachment would be expected for soils with greater clay content.

Attachment is also influenced by species isolate, competition with native species, contact time with soil particles, temperature, weather, and water chemistry. In many ways the conditions used in this study can be considered ideal for cell survival and so maximized attachment. Only one species was tested under sterile condition, so there was no competition for nutrients or attachment sites and no threat of predation by other species. The water samples consisted of phosphate buffered dilution water to maintain a neutral pH. Mixing time was designed to provide an ideal amount of time for attachment without risking die-off. In the field, many of these factors would be less than ideal for bacterial survival and attachment would certainly be affected. Further experiments would be necessary to investigate the effects of these environmental factors

on bacterial attachment and to refine the linear relationships presented in this study for use in bacterial modeling.

The partitioning method used to obtain the data in this study also affects the use of the above equations for bacterial modeling. Attachment was operationally defined as the inability to pass through the pores of an 8 μm filter. Cell aggregates attached to very small clays would pass through these pores and be considered planktonic, and aggregates of bacterial cells not attached to soil particles would be considered attached. The primary purpose of modeling planktonic and attached bacteria phases separately would be to incorporate sedimentation, resuspension, different rates of die-off, and other known effects of attachment within the model. It is not known whether sedimentation and accumulation in nutrient-rich sediments or the act of attachment itself is primarily responsible for the reduced die-off of attached bacteria. Further research in this area would indicate whether it was necessary to differentiate between truly attached and truly planktonic cells for modeling purposes. However, using an 8 μm screen to separate between attached and planktonic phases does achieve the objective of separating between cells expected to settle out of the water column and those expected to remain suspended. Cells attached to aggregates smaller than 8 μm would probably not settle out of the water column at a rate significantly higher than that of truly planktonic cells. Similarly, large clumps of solely bacterial cells containing no soil might settle out faster and contribute to accumulated populations of bacteria on streambeds available for resuspension. The screen size could be changed as needed in future studies to operationally define attached bacteria as those attached to larger or smaller soil particles.

CHAPTER 5: Summary and Conclusions

5.1 Selected Partitioning Method

The first objective of this research study as stated in Section 1.1 was to identify, test, and evaluate available methods for partitioning between soil-adsorbed and planktonic *E. coli* bacteria in the laboratory. Several candidate methods were identified through a review of past research on bacterial attachment and are summarized in Chapter 2. The method selected as most promising for this research used filtration to separate planktonic bacteria for enumeration, and used a dispersion treatment to disperse attached bacteria cells from soil particles for enumeration. Two different dispersion treatments were considered: the chemical surfactant Tween-85 and immersion in an ultrasonic cleaning bath for 30 seconds. These dispersion treatments were compared and the entire method evaluated using a pure culture of *E. coli* and sterilized topsoil from Dinwiddie, Virginia in a series of randomized complete block design experiments.

Analysis of the data from these experiments confirmed both hypothesis 1 and hypothesis 2 from Section 1.2. Research hypothesis 1 stated that using the research method outlined above, a significant proportion of *E. coli* is retained on the 8 μm screen used in filtration and is so considered attached. Depending on the type of dispersion method used to determine total bacterial concentration, on average between 77% and 80% of the total bacterial concentration was considered attached. The only statistically significant differences between concentration values obtained in these experiments (Tukey's Multiple Comparison Test; α=0.05) were between planktonic (filtrate) and total concentration. Since attachment was calculated as the difference between these two concentrations, the proportion of bacteria attached is considered significant.

The second research hypothesis in Section 1.2 states that there is no significant difference between the total concentration of a sample determined using a dispersion treatment and the total

concentration obtained from an untreated sample. In the randomized complete block design experiments, the differences between the total concentration of samples treated with Tween-85, treated with 30 s of immersion in an ultrasonic cleaning bath, and left untreated were not statistically significant (Tukey's Multiple Comparison Test, $\alpha = 0.05$); however, some interesting trends were observed. Testing between these values of total concentration was included largely to correct for possible die-off as a result of dispersion treatments. On average, total concentrations obtained using a dispersion treatment before membrane filtration were actually higher than that of an untreated control, though this difference was not statistically significant. This increase in concentration after dispersion suggests that *E. coli* naturally exist in multiple cell aggregates even in the absence of soil particles. These findings suggest that current methods of bacterial enumeration using plate counts underestimate the true concentration as clumps of cells form only one visible colony that is indistinguishable from a colony formed by a single parent cell. Tween-85 was selected as the best dispersion method for use in further research as it provided the highest average total *E. coli* concentration.

The method presented in this study was successful in partitioning between soil-adsorbed and planktonic *E. coli* in laboratory-composed samples and is recommended for use in future studies of bacterial attachment. The total concentration is accurately predicted through the use of Tween-85 surfactant, and over 99% of planktonic bacteria in a sample containing no soil passed through the 8 μm filter. Additionally, the filter size used to separate the planktonic bacteria from the attached bacteria can be changed according to the objectives of the researcher. The method is inexpensive, uses readily available laboratory equipment, and could be used to analyze a field sample of completely unknown concentration in addition to laboratory samples of known concentration since it provides measures of planktonic, total, and attached concentrations.

5.2 Isotherm Equation

The second research objective listed in Section 1.1, to develop an equation relating the planktonic and attached concentration of *E. coli* was achieved through the testing of laboratory-composed samples of different soil-bacteria ratios. While cultures of *E. coli* grown under batch conditions were sufficient to evaluate the proposed method of partitioning in the laboratory as described in the previous section, batch cultures were too heterogeneous in attachment behavior to provide usable data for linear regression. Examination of cell growth stage in batch cultures revealed that the cells were at stationary phase, a highly variable physiological state. This variability was controlled through the use of a chemostat reactor, which maintained cells at the more homogenous state of balanced growth.

Equations relating attached concentration to planktonic concentration and relating attached concentration to total concentration were developed using data from samples inoculated with samples from the chemostat culture. The data were analyzed using linear regression in SAS. Linear, Langmuir, and Freundlich isotherm models were considered, and the linear isotherm was selected as the best description of the experimental data. Attached concentration was strongly linearly correlated with planktonic concentration, as revealed by the high R^2 value (0.8804) of the linear regression equation describing the data (Eq. 8). The slope of this equation, which could be used as a linear isotherm, was statistically significant for an α of 0.05. This confirms hypothesis 3 from Section 1.2, which states that the adsorption of *E. coli* to soil particles can be mathematically described.

While the high R^2-correlation factor and significant slope indicate that Eq. 8 is an excellent representation of the structure of the data obtained in these experiments, this equation was developed under extremely controlled conditions not representative of the complexity and

variation of environmental conditions. Under natural conditions, changes in water chemistry, soil type, and competition with native species would greatly affect measured attachment. Consequently, despite the high correlation factors associated with the generated equations, the immediate application of these equations into water quality models is questionable and not recommended. The effect of changing environmental factors was illustrated by the differences when linear regression was performed on data sets with different initial TSS concentrations. Resultant equations differed widely, and would predict different attached bacterial concentrations. The difference between these equations is suspected to be the result of an environment in which the available soil particles are not saturated with bacteria, and so the cells are not limited by available attachment sites. Further research would be required to better understand and characterize the interactions between *E. coli* and soil particles.

The average proportion of *E. coli* considered attached in these sorption experiments using a chemostat culture was 78%, the same exact value as the average proportion of attached *E. coli* in the batch experiments testing dispersion treatments. This is important, as it indicates that the results from the batch experiments, which were used to select a dispersant, were valid for use on the chemostat culture. It also suggests that on average, regardless of physiological state, about 78% of bovine *E. coli* will attach to a sandy loam soil, though variability in attachment will be much higher for cultures not maintained at a state of balanced growth.

5.3 Suggestions for future research

There is a definite need for more information on bacteria attachment to soil particles to improve water quality modeling. The partitioning method discussed in this study was successful in partitioning between soil-adsorbed and planktonic bacteria and could be used in future experiments examining different aspects of bacteria-sediment interactions. It is important to

recognize that while the sorption equations given in 0 illustrate the successful use of this partitioning method, this particular equation is only relevant for this one bacterial strain, one soil type, and controlled laboratory conditions. Before a method of modeling soil interactions can be established, more research must be conducted, particularly under field conditions.

Bacteria vary widely between strains, sources, and environmental conditions. Before conducting large-scale field experiments, it would be best to first establish behavior patterns under more specific controlled laboratory experiments. An understanding of behavior under lab conditions will aid in the design of field experiments and analysis of field data, which is usually high in unexplained variability. Field studies must follow lab studies before accurate modeling methods are established to ensure that water quality models reflect the behavior of bacteria under natural conditions.

5.3.1 Lab-scale research suggestions

- There is research suggesting that bacteria grown under laboratory conditions may undergo mutations that result in the loss of some stress responses. It is necessary to identify those genes affected by these mutations to determine if these are also genes responsible for attachment structures and behavior. If relevant genes are being loss through laboratory cultures, a new method of culturing must be established.
- In this study, all *E. coli* retained on the 8 μm filter were considered attached. In reality, some of these bacteria may have been only loosely associated with the soil particles, or bacteria attached to very small particles may have passed through the filter pores. While only those cells attached to relatively large particles will undergo significantly faster sedimentation, there is evidence that attachment alone results in reduced die-off. Therefore, it would be

useful to confirm proportions of attached bacteria microscopically to determine a "true" attached proportion.

- Die-off was not investigated in this study. There has been research suggesting that attachment or association with particles results in extended survival; however, this has not been quantified under laboratory or field conditions. Quantification of the relative rates of soil-adsorbed and planktonic bacterial die-off is necessary to model these phases of bacteria separately.
- Only one specific type of topsoil was used as an attachment medium in this study. The particle size, pH, organic matter, and amount of soil present can affect attachment. A study investigating and quantifying the effects of these factors on a specific species' attachment behavior would be useful for modeling.
- There have been numerous observations of the release of indicator bacteria from sediments. Attempts have been made to quantify the amount of bacteria that can be released by polluted sediments in the field. A laboratory-scale study quantifying the amount of bacteria accumulation and resuspension from a sediment bed would aid in the prediction of bacterial concentrations in streams by hydrologic models.
- Research into the behavior of pathogen indicators assumes that these behaviors will be similar to that of actual pathogens; however, this is not always the case. Recent studies have found that current water quality indicators and standards are not always as sensitive or predictive as hoped. Before an attempt is made to consolidate all data from die-off, attachment and resuspension studies into a water quality simulation model, studies need to be conducted exploring the behavior of actual waterborne pathogens of concern, including non-bacterial pathogens such as protozoans and viruses. The fate and transport, including

attachment and resuspension, of pathogens must be studied both in the lab and in the field to determine whether studies on the behaviors of pathogen indicators can be used to model and predict pathogen behavior.

5.3.2 Field-scale research suggestions

- There is currently no available field data characterizing bacterial attachment during overland flow events. Land-applied bacteria likely undergo attachment during this process, and so the concentrations of attached and planktonic bacteria entering a water body would be largely determined during overland flow events. Data is necessary to develop, calibrate, and validate inclusion of bacteria attachment effects in water quality models that simulate overland flow. Rainfall simulator studies in which the runoff was analyzed using the laboratory methods described in this study are recommended.
- While there is a considerable amount of data from past studies on the concentrations of bacteria in stream sediments, there is little if any field data on the amount of attached bacteria within the water column. Attachment may also occur as bacteria deposited in the stream come into contact with the suspended solids within that stream. A field study collecting samples during storm events and ambient conditions that tested the samples using the method outlined in this study would provide useful data for future modeling efforts.

5.3.3 Modeling research suggestions

- All research on die-off and attachment collected needs to be consolidated into a water quality hydrologic model that could be used in water quality management. The model needs to be calibrated and validated with existing observed data from streams and rainfall simulator studies to confirm the accuracy of modeling attachment both in-stream and during overland flow events.

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Appendix A Background Soil Data

Table A-1 Soil Characteristics (adapted from Schroeder, 1997)

	Particle Size, %							Exchangeable Ions, ppm				
Soil Control	**Sand**	**Silt**	**Clay**	**% Carbon**	**Bulk Density, g/cm^3**	**pH[a]**	**Effective Cation Exchange Capacity, cmol/kg**	**Ca**	**Mg**	**K**	**H**	**Al**
1	84.9	13.5	1.6	0.48	1.93	5.9	1.37	1.0	0.26	0.01	2.8	0.10
2	84.8	13.7	1.5	0.36	1.93	5.6	1.13	0.8	0.14	0.09	1.4	0.10
3	80.9	14.4	4.7	0.43	1.90	5.7	1.41	0.9	0.28	0.13	1.2	0.10
4	78.2	14	7.8	0.69	1.88	5.7	2.08	1.2	0.49	0.24	6.6	0.15
5	83.1	14.5	2.4	0.64	1.84	5.3	2.09	1.4	0.49	0.15	0.0	0.05
6	83.5	15.3	1.2	0.57	2.03	5.4	1.79	1.2	0.41	0.13	0.2	0.05
AVG	**82.6**	**14.2**	**3.2**	**0.53**	**1.92**	**5.6**	**1.65**	**1.1**	**0.35**	**0.13**	**2.0**	**0.09**

[a]the soil was limed prior to storage to bring the pH to neutral

Table A-2 Sand Mineralogy (adapted from Vanwormhoudt, 1993)

	Mineralogy by mass, % (g/100 g soil)								
Soil Pit	**Kaolinite**	**Chloritized Vermiculite**	**Vermiculite**	**Smectite**	**Mica**	**Quartz**	**Gibbsite**	**Feldspars**	**Interstratified 2:1 Phyllosilicates**
Faceville 346B	1.38	1.71	trace	0	0.42	0.29	0.38	trace	trace
Varina 146B	5.64	2.98	0	trace	0.74	0.64	0.64	0	trace
Varina 146B3	10.98	7.99	0	0	0.2	0	0.8	0	0
AVG	**6.00**	**4.23**	**trace**	**trace**	**0.45**	**0.31**	**0.61**	**trace**	**trace**

Table A-3 Silt Mineralogy (adapted from Vanwormhoudt, 1993)

	Mineralogy by mass, % (g/100 g soil)					
Soil Pit	**Kaolinite**	**Chloritized Vermiculite**	**Mica**	**Quartz**	**Feldspars**	**Interstratified 2:1 Phyllosilicates**
Faceville 346B	0.79	0.52	1.7	9.56	0.13	0.39
Varina 146B	2.54	0.56	2.54	8.18	0.28	0
Varina 146B3	13.02	0	0	15.28	trace	0
AVG	**5.45**	**0.36**	**1.41**	**11.01**	**0.21**	**0.13**

Table A-4 Clay Mineralogy (adapted from Vanwormhoudt, 1993)

	Mineralogy by mass, % (g/100 g soil)	
Soil Pit	**Qz**	**Heavy Metals**
Faceville 346B	69.05	13.14
Varina 146B	59.45	13.94
Varina 146B3	37.84	9.46
AVG	**55.45**	**12.18**

Appendix B Raw Data

Table B-1 Die-off Experiment Raw Data

Sample	Plate	Treatment	CFU/plate	Dilution	Concentration (CFU/mL)
1	1	control	20	1.00E-06	2.00E+07
	2	control	10	1.00E-06	1.00E+07
2	1	control	49	1.00E-06	4.90E+07
	2	control	53	1.00E-06	5.30E+07
3	1	control	65	1.00E-06	6.50E+07
	2	control	63	1.00E-06	6.30E+07
4	1	ultrasonic - 30 s	61	1.00E-06	6.10E+07
	2	ultrasonic - 30 s	55	1.00E-06	5.50E+07
5	1	ultrasonic - 30 s	60	1.00E-06	6.00E+07
	2	ultrasonic - 30 s	57	1.00E-06	5.70E+07
6	1	ultrasonic - 30 s	78	1.00E-06	7.80E+07
	2	ultrasonic - 30 s	67	1.00E-06	6.70E+07
7	1	ultrasonic - 60 s	47	1.00E-06	4.70E+07
	2	ultrasonic - 60 s	0	1.00E-06	0.00E+00
8	1	ultrasonic - 60 s	45	1.00E-06	4.50E+07
	2	ultrasonic - 60 s	34	1.00E-06	3.40E+07
9	1	ultrasonic - 60 s	65	1.00E-06	6.50E+07
	2	ultrasonic - 60 s	51	1.00E-06	5.10E+07
10	1	Tween (2 drops)	54	1.00E-06	5.40E+07
	2	Tween (2 drops)	63	1.00E-06	6.30E+07
11	1	Tween (2 drops)	78	1.00E-06	7.80E+07
	2	Tween (2 drops)	68	1.00E-06	6.80E+07
12	1	Tween (2 drops)	69	1.00E-06	6.90E+07
	2	Tween (2 drops)	56	1.00E-06	5.60E+07

Table B-2 Mixing Time Experiment Data

Time (min)	Average Control (no soil) Concentration	Average Filtrate (Unattached) Concentration
15	3.80E+05	6.50E+04
30	5.05E+05	3.65E+04
60	4.55E+05	3.25E+04
120	5.20E+05	3.50E+04

Table B-3 Best Dispersant Experiment 1 Raw Data

rep	CFS[a]	CAS[b]	CBS[c]	CFW[d]	CAW[e]	CBW[f]	COS[g]
1	1.02E+05	7.15E+05	1.70E+06	1.35E+06	1.35E+06	1.15E+06	1.50E+06
2	1.28E+05	2.05E+06	4.15E+06	8.40E+05	1.95E+06	7.20E+05	2.75E+06
3	7.65E+05	1.35E+06	8.55E+05	1.02E+06	1.11E+06	9.95E+05	8.25E+05
4	5.70E+05	1.90E+06	1.55E+06	1.41E+06	1.30E+06	1.75E+06	1.60E+06
5	1.23E+06	6.05E+06	2.20E+06	3.38E+06	3.55E+06	2.85E+06	2.65E+06
6	4.95E+05	1.35E+06	2.95E+06	1.46E+06	3.70E+06	1.50E+06	3.15E+06
7	8.10E+05	4.10E+06	8.35E+06	4.31E+06	4.45E+06	7.20E+06	2.25E+06
8	1.65E+06	5.20E+06	6.35E+06	4.20E+06	5.85E+06	8.05E+06	6.95E+06
9	1.55E+06	6.70E+06	5.90E+06	8.10E+06	8.75E+06	9.05E+06	4.55E+06
10	5.25E+05	1.95E+06	1.35E+06	3.15E+06	1.85E+06	1.75E+06	2.05E+06
AVG	**7.82E+05**	**3.14E+06**	**3.54E+06**	**2.92E+06**	**3.39E+06**	**3.50E+06**	**2.83E+06**

Table B-4 Best Dispersant Experiment 2 Raw Data

Run	CFS[a]	CAS[b]	CBS[c]	COS[g]
1	1.02E+05	7.15E+05	1.70E+06	1.50E+06
2	1.28E+05	2.05E+06	4.15E+06	2.75E+06
3	7.65E+05	1.35E+06	8.55E+05	8.25E+05
4	5.70E+05	1.90E+06	1.55E+06	1.60E+06
5	1.23E+06	6.05E+06	2.20E+06	2.65E+06
6	4.95E+05	1.35E+06	2.95E+06	3.15E+06
7	8.10E+05	4.10E+06	8.35E+06	2.25E+06
8	1.65E+06	5.20E+06	6.35E+06	6.95E+06
9	1.55E+06	6.70E+06	5.90E+06	4.55E+06
10	5.25E+05	1.95E+06	1.35E+06	2.05E+06
11	1.04E+05	2.20E+06	2.40E+06	2.00E+06
12	1.11E+05	2.45E+06	7.95E+05	3.90E+06
13	7.50E+04	2.05E+06	4.85E+06	9.25E+05
14	1.56E+05	1.26E+06	1.55E+06	1.09E+06
15	1.86E+05	2.30E+06	1.85E+06	3.65E+06
16	2.64E+05	1.90E+06	1.25E+06	2.00E+06
17	2.42E+05	3.55E+06	5.80E+06	4.65E+06
18	1.47E+05	1.10E+06	2.50E+06	1.85E+06
19	2.39E+05	8.50E+05	8.50E+05	7.40E+05
20	4.89E+05	1.58E+06	1.54E+06	1.08E+06
AVG	**4.92E+05**	**2.53E+06**	**2.94E+06**	**2.51E+06**

[a] Concentration of 8 μm filtrate, sample contained soil
[b] Total concentration after immersion in ultrasonic bath, sample contained soil
[c] Total concentration after addition of Tween-85 surfactant, sample contained soil
[d] Concentration of 8 μm filtrate, sample did not contain soil
[e] Total concentration after immersion in ultrasonic bath, sample did not contain soil
[f] Total concentration after addition of Tween-85 surfactant, sample did not contain soil
[g] Total concentration, no treatment (control)

Table B-5 Raw Data from Initial Sorption Experiment Using Batch Cultures

		Total (Tween)			Free (Filtrate)				Attached	
mL bac. susp.	**g Soil**	**CFU**	**Dilution Level**	**Conc, CFU/mL**	**CFU**	**Dilution Level**	**Conc, CFU/mL**	**Conc, CFU/mL**	**Conc, CFU/g**	**% Attached**
1.0	0.5	73	1.00E-04	7.30E+05	84	1.00E-03	2.52E+05	4.78E+05	9.56E+07	65%
		77	1.00E-04	7.70E+05	67	1.00E-03	2.01E+05	5.69E+05	1.14E+08	74%
1.0	1.0	98	1.00E-04	9.80E+05	19	1.00E-03	5.70E+04	9.23E+05	9.23E+07	94%
		75	1.00E-04	7.50E+05	27	1.00E-03	8.10E+04	6.69E+05	6.69E+07	89%
1.0	2.0	39	1.00E-04	3.90E+05	47	1.00E-03	1.41E+05	2.49E+05	1.25E+07	64%
		68	1.00E-04	6.80E+05	69	1.00E-03	2.07E+05	4.73E+05	2.37E+07	70%
1.0	3.0	75	1.00E-04	7.50E+05	51	1.00E-03	1.53E+05	5.97E+05	1.99E+07	80%
		70	1.00E-04	7.00E+05	42	1.00E-03	1.26E+05	5.74E+05	1.91E+07	82%
1.0	5.0	61	1.00E-04	6.10E+05	26	1.00E-03	7.80E+04	5.32E+05	1.06E+07	87%
		75	1.00E-04	7.50E+05	37	1.00E-03	1.11E+05	6.39E+05	1.28E+07	85%

Table B-6 Raw Data from Growth Curve Experiment

	Flask B		Flask C		Flask D		
Time, h	**Trans, %**	**Absorbance**	**Trans, %**	**Absorbance**	**Trans, %**	**Absorbance**	**Avg Absorbance**
0.0	99	0.004	99	0.004	98	0.009	0.006
1.5	96	0.018	95	0.022	94	0.027	0.022
2.0	95	0.022	93	0.032	91	0.041	0.032
2.9	86	0.066	84	0.076	82	0.086	0.076
4.8	68	0.167	67	0.174	64	0.194	0.178
6.6	61	0.215	64	0.194	60	0.222	0.210
9.5	58	0.237	61	0.215	53	0.276	0.242
11.8	50	0.301	54	0.268	45	0.347	0.305
14.9	48	0.319	51	0.292	44	0.357	0.323
16.7	48	0.319	49	0.310	44	0.357	0.328
19.2	45	0.347	49	0.310	44	0.357	0.338
20.7	44	0.357	49	0.310	44	0.357	0.341
24.2	49	0.310	50	0.301	46	0.337	0.316
27.0	49	0.310	50	0.301	47	0.328	0.313
29.1	49	0.310	51	0.292	48	0.319	0.307

Table B-7 Chemostat Monitoring Data

Sample ID	Day	Time, hr	Trans., %	Abs.	DO, mg/L	Chemostat Temp., C	pH	Dilution	CFU	CFU	Avg Cell Conc.	Comments
A	1	0	90	0.046	ND	43.9	6.2	1.00E-06	21	26	2.35E+07	starter culture used to inoculate chemostat
	1	3.5	96	0.018	5.13	42.7	6.2	ND	ND	ND	ND	turned water bath to 50 C
	1	7.5	79	0.102	5.34	44.0	6.3	ND	ND	ND	ND	stopper on chemostat leaked 300 mL; pumped at max 30 min to 1.5 L
	1	11	70	0.155	5.43	43.6	7.2	ND	ND	ND	ND	begin pumping; turn water bath to 52 C
	1	13	73	0.137	5.16	43.9	7.0	ND	ND	ND	ND	
	1	15.25	76	0.119	4.83	44.0	7.0	ND	ND	ND	ND	
	2	26	77	0.114	5.04	44.2	7.2	ND	ND	ND	ND	
B	2	27.5	80	0.097	5.08	44.3	7.0	1.00E-07	6	17	1.15E+08	adjusted thermometer depth
	2	32	79	0.102	5.12	44.1	7.2	ND	ND	ND	ND	
	2	37.5	78	0.108	5.3	44.6	7.1	ND	ND	ND	ND	water bath turned to 51 C
	3	48.5	79	0.102	5.23	43.6	7.2	ND	ND	ND	ND	water bath turned to 51.5 C
C	3	52.5	79	0.102	5.13	43.9	7.0	1.00E-07	7	12	9.50E+07	use for samples #1-3
D	3	55	81	0.092	5.26	43.8	7.1	ND	ND	ND	ND	use for samples #4-5
	3	62	79	0.102	4.75	43.5	7.0	ND	ND	ND	ND	
E	4	73	79	0.102	4.65	43.5	7.0	ND	ND	ND	ND	use for samples #6-8
F	4	75.5	80	0.097	5.64	43.9	6.9	ND	ND	ND	ND	use for samples #9-10; add water to water bath
	4	88	84	0.076	4.53	44.2	7.1	ND	ND	ND	ND	turned water bath to 51 C
G	4	96	81	0.092	4.71	43.6	6.9	1.00E-07	8	19	1.35E+08	use for samples #11-13
	5	106.5	78	0.108	4.75	43.7	7.3	ND	ND	ND	ND	
	6	121	81	0.092	4.87	43.5	7.3	ND	ND	ND	ND	
H	6	124	80	0.097	4.88	43.3	7.1	1.00E-07	138	125	1.32E+09	use for samples #14-16; turn water bath to 52 C
	6	127.25	80	0.097	ND	44.1	7.1	ND	ND	ND	ND	DO probe at CEE 5104

B. 7 Chemostat Monitoring Data (cont'd)

Sample ID	Day	Time, hr	Trans., %	Abs.	DO, mg/L	Chemostat Temp., C	pH	Dilution	CFU	CFU	Avg Cell Conc.	Comments
	6	132	80	0.097	4.82	43.7	7.0	ND	ND	ND	ND	
I	7	146.25	79	0.102	4.88	44.1	7.0	1.00E-08	9	15	1.20E+09	use for samples #17-19
J	7	149	79	0.102	4.36	43.5	7.0	ND	ND	ND	ND	use for samples #11 & #20
	7	156.75	80	0.097	4.85	44.6	7.3	ND	ND	ND	ND	turn water bath to 51 C
	8	169.25	78	0.108	ND	43.5	7.2	ND	ND	ND	ND	DO probe at CEE 5104 lab
	8	175	80	0.097	5.09	43.6	7.3	ND	ND	ND	ND	kill reactor

Table B-8 Sorption Experiments using Chemostat Culture, TSS = 10000 mg/L

	Total				Free				Attached		
Sample	CFU	CFU	Dilution	Concentration, CFU/mL	CFU	CFU	Dilution	Concentration, CFU/mL	Concentration, CFU/mL	Concentration, CFU/g	% (CFU/mL basis)
1	15	20	1.00E-04	1.75E+05	19	15	1.00E-03	3.40E+04	1.41E+05	1.41E+07	80.57%
2	56	69	1.00E-04	6.25E+05	30	42	1.00E-03	7.20E+04	5.53E+05	5.53E+07	88.48%
3	100	84	1.00E-04	9.20E+05	88	97	1.00E-03	1.85E+05	7.35E+05	7.35E+07	79.89%
4	26	29	1.00E-05	2.75E+06	13	12	1.00E-04	2.50E+05	2.50E+06	2.50E+08	90.91%
5	16	19	1.00E-05	1.75E+06	12	12	1.00E-04	2.40E+05	1.51E+06	1.51E+08	86.29%
6	53	72	1.00E-05	6.25E+06	45	44	1.00E-04	8.90E+05	5.36E+06	5.36E+08	85.76%
7	80	86	1.00E-05	8.30E+06	68	69	1.00E-04	1.37E+06	6.93E+06	6.93E+08	83.49%
8	18	20	1.00E-06	1.90E+07	18	17	1.00E-05	3.50E+06	1.55E+07	1.55E+09	81.58%
9	24	18	1.00E-06	2.10E+07	25	39	1.00E-05	6.40E+06	1.46E+07	1.46E+09	69.52%
10	47	39	1.00E-06	4.30E+07	64	54	1.00E-05	1.18E+07	3.12E+07	3.12E+09	72.56%

Table B-9 Sorption Experiments using Chemostat Culture, TSS = 5000 mg/L

	Total				Free				Attached		
Sample	**CFU**	**CFU**	**Dilution**	**Concentration, CFU/mL**	**CFU**	**CFU**	**Dilution**	**Concentration, CFU/mL**	**Concentration, CFU/mL**	**Concentration, CFU/g**	**% (CFU/mL basis)**
11	14	10	1.00E-05	1.20E+06	12	28	1.00E-04	4.00E+05	8.00E+05	1.60E+08	66.67%
12	77	90	1.00E-05	8.35E+06	16	20	1.00E-04	3.60E+05	7.99E+06	1.60E+09	95.69%
13	39	32	1.00E-05	3.55E+06	14	25	1.00E-04	3.90E+05	3.16E+06	6.32E+08	89.01%
14[a]	179	185	1.00E-06	1.82E+08	106	125	1.00E-06	2.31E+08	-4.90E+07[a]	-9.80E+09[a]	-26.92%[a]
15	69	80	1.00E-06	7.45E+07	81	117	1.00E-05	1.98E+07	5.47E+07	1.09E+10	73.42%
16	53	90	1.00E-06	7.15E+07	128	95	1.00E-05	2.23E+07	4.92E+07	9.84E+09	68.81%
17	62	54	1.00E-06	5.80E+07	135	129	1.00E-05	2.64E+07	3.16E+07	6.32E+09	54.48%
18	32	19	1.00E-07	2.55E+08	35	33	1.00E-06	6.80E+07	1.87E+08	3.74E+10	73.33%
19	37	32	1.00E-07	3.45E+08	36	29	1.00E-06	6.50E+07	2.80E+08	5.60E+10	81.16%
20	13	7	1.00E-07	1.00E+08	15	26	1.00E-06	4.10E+07	5.90E+07	1.18E+10	59.00%

[a]This sample was not used in sorption equation calculations due to the negative attachment value (probable error/plate mix-up)

Appendix C SAS Code

C. 1 SAS Code for Die-off Experiment, All Data

```
data dieoff;
input trt $ rep cfu;
lines;
[DATA]
;
run;

proc glm data=dieoff;
class trt rep;
model cfu = trt rep(trt);
test h = trt e = rep(trt);
means trt / lsd e=rep(trt);
proc means data = dieoff alpha=.05 clm mean std;
   class trt;
run; quit;
```

C. 2 SAS Code for Die-off Experiment, Outliers Excluded

```
data dieoff;
input trt $ rep cfu;
lines;
[DATA]
;
run;

proc glm data=dieoff;
class trt rep;
model cfu = trt rep(trt);
test h = trt e = rep(trt);
*means trt / lsd e=rep(trt);
lsmeans trt / pdiff adjust = T e = rep(trt);
lsmeans trt / CL;
run;
```

C. 3 SAS Code for Best Dispersant Method, Experiment 1

```
data rcbd1;
input trt $ rep conc;
cards;
[DATA]
;
proc glm data=rcbd1;
class trt rep;
model conc=rep trt trt*rep;
test h=trt e=rep*trt;
means trt /tukey e=rep*trt;
run;
proc means data = rcbd1 alpha=.05 clm mean std;
   class trt;
run;
quit;
```

C. 4 SAS Code for Best Dispersant Method, Experiment 2

```
data rcbd2;
input trt $ rep conc;
cards;
[DATA]
;
proc glm data=rcbd2;
class trt rep;
model conc=rep trt trt*rep;
test h=trt e=rep*trt;
means trt /tukey e=rep*trt;
run;
proc means data = rcbd2 alpha=.05 clm mean std;
   class trt;
run;
quit;
```

C. 5 SAS Code for Comparison of Proportion Attached/Batch Experiments (Randomized Complete Block Design)

```
data prop;
input trt $ block conc;
lines;
[DATA]
;
run;

proc glm data=prop;
class trt block;
model conc = trt block;
means trt/tukey;
means block;
run; quit;
```

C. 6 SAS Code for Comparison of Proportion Attached/Chemostat Sorption Experiments (Completely Randomized Design with no subsampling)

```
Data chemo;
input TSS prop;
lines;
[DATA]
;
run;

proc glm data=chemo;
class TSS;
model prop=TSS;
run; quit;
```

C. 7 SAS Code for Linear Regression, Intercept = Zero:

```
data sorption;
input x y;
lines;
[DATA]
;
run;

proc reg data=sorption;
model y = x /clb clm cli NOINT;
run; quit;
```

C. 8 SAS Code for Linear Regression, Intercept ≠ Zero:

```
data sorption;
input x y;
lines;
[DATA]
;
run;

proc reg data=sorption;
model y = x /clb clm cli;
run; quit;
```

Appendix D SAS Outputs

D.1 Tukey's Multiple Comparison Test for Die-off, All Data

Treatment ID Key:

A	control (no dispersion)
B	30 s ultrasonic
C	60 s ultrasonic
D	Tween-85 surfactant

Tukey's Multiple Comparison Test:

Alpha	0.05
Error Degrees of Planktonicdom	8
Error Mean Square	5.331E+14
Critical Value of t	2.306
Least Significant Difference	3.07E+07

Means with the same t-grouping letter are not significantly different:

t-Grouping	Mean	trt
A	6.47E+07	D
A	6.30E+07	B
A	4.33E+07	A
A	4.03E+07	C

D. 2 Tukey's Multiple Comparison Test for Die-off, Outliers Excluded

Treatment ID Key:

A	control (no dispersion)
B	30 s ultrasonic
C	60 s ultrasonic
D	Tween-85 surfactant

Standard errors and probabilities were calculated using the Type II Mean-Square for rep(trt) as an error term. Values less than α (0.05) indicate a significant difference between the compared treatment concentrations.

i/j	A	B	C	D
A		0.5009	0.2952	0.3859
B	0.5009		0.088	0.8169
C	0.2952	0.088		0.0634
D	0.3859	0.8169	0.0634	

D. 3 SAS Output for Best Dispersant Method, Experiment 1

Treatment ID's are given below. These designations are the same as those in the schematic of the experimental procedure given in Figure 3-1.

ID	subsample contained soil?	Description
CAS	Y	total concentration after 30 s ultrasonic
CAW	N	total concentration after 30 s ultrasonic
CBS	Y	total concentration after 2 drops Tween-85
CBW	N	total concentration after 2 drops Tween-86
CFS	Y	concentration that passed through 8 mm filter
CFW	N	concentration that passed through 8 mm filter
COS	N	original suspension control; no treatment

Tukey's Multiple Comparison Test:

Alpha	0.05
Error Degrees of Freedom	54
Error Mean Square	3.488E+12
Critical Value of Studentized Range	4.33055
Minimum Significant Difference	1.81E+06

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ. Means with the same t-grouping letter are not significantly different.

t-grouping	Mean	trt
A	3.54E+06	CBS
A	3.50E+06	CBW
A	3.39E+06	CAW
A	3.14E+06	CAS
A	2.92E+06	CFW
A	2.83E+06	COS
B	7.82E+05	CFS

ANOVA Table:
Dependent variable = conc (Concentration)

Source	DF	Sum of Squares	Mean-Square	F-Value	Pr>F
Model	69	7.65086E+14	1.10882E+13	36.92	<0.0001
Error	70	2.10252E+13	3.0036E+11		
Corrected Total	139	7.86111E+14			

R-Square	Coeff Var	Root MSE	conc Mean
0.973254	19.09686	548051.1	2.87E+06

Tests of Hypotheses Using the Type III MS for trt*rep as an Error Term:

Source	DF	Type III SS	Mean-Square	F-Value	Pr>F
trt	6	1.10855E+14	1.84758E+13	5.3	0.0002

D. 4 SAS Output for Best Dispersant Method, Experiment 2

Treatment ID's are given below. These designations are the same as those in the schematic of the experimental procedure given in Figure 3-2.

ID	subsample contained soil?	Description
CAS	Y	total concentration after 30 s ultrasonic
CBS	Y	total concentration after 2 drops Tween-85
CFS	Y	concentration that passed through 8 mm filter
COS	N	original suspension control; no treatment

Tukey's Multiple Comparison Test:

Alpha	0.05
Error Degrees of Freedom	57
Error Mean Square	2.737E+12
Critical Value of Studentized Range	3.74268
Minimum Significant Difference	9.79E+05

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ. Means with the same letter are not significantly different.

t-grouping	Mean	trt
A	2.94E+06	CBS
A	2.53E+06	CAS
A	2.51E+06	COS
B	4.92E+05	CFS

ANOVA Table:
Dependent Variable: conc (concentration)

Source	DF	Sum of Squares	Mean-Square	F-Value	Pr>F
Model	79	5.43787E+14	6.88338E+12	32.36	<0.0001
Error	80	1.70164E+13	2.12706E+11		
Corrected Total	159	5.60804E+14			

R-Square	Coeff Var	Root MSE	conc Mean
0.969657	21.79057	461200.1	2.12E+06

Tests of Hypotheses Using the Type III MS for trt*rep as an Error Term:

Source	DF	Type III SS	Mean-Square	F-Value	Pr>F
trt	3	1.45493E+14	4.84978E+13	17.72	<0.0001

D.5 SAS Output for Comparison of Proportions, Best Dispersant Method Experiment 2

Treatment ID Key:

trt	measure of total concentration[a]
pas	30 s immersion in ultrasonic cleaning bath
pbs	2 drops Tween-85
pos	no treatment

[a]all proportions used the 8 μm screen filtrate concentration as a measure of the planktonic concentration

Tukey's Multiple Comparison Test:

Alpha	0.05
Error Degrees of Freedom	38
Error Mean Square	0.006823
Critical Value of Studentized Range	3.44902
Minimum Significant Difference	0.0637

Means with the same letter are not significantly different:

t-grouping	Mean	trt
A	0.80390	pas
A	0.77995	pbs
A	0.76965	pos

D. 6 Comparison of Proportion of E. coli Attached for 5,000 mg/L and 10,000 mg/L, Sorption Experiments, Chemostat cultures

ANOVA Table:

Source	DF	Sum of Squares	Mean-Square	F-Value	Pr>F
Model	1	0.03339511	0.03339511	3.09	0.097
Error	17	0.18397846	0.01082226		
Corrected Total	18	0.21737357			

R-Square	Coeff Var	Root MSE	prop Mean
0.15363	13.34952	0.10403	0.77928

D.7 SAS Linear Regression Output, Chemostat Cultures, Attached (CFU per g) vs. Planktonic, All TSS Values → Linear Isotherm

ANOVA Table:

Source	DF	Sum of Squares	Mean Square	F-value	Pr>F
Model	1	4.35669E+21	4.35669E+21	132.5	<0.0001
Error	18	5.91872E+20	3.28818E+19		
Uncorrected Total	19	4.94856E+21			

Root MSE	5734261845
Dependent Mean	7504889474
Coeff Var	76.40701
R-Square	0.8804
Adj R-Square	0.8738

Parameter Estimates:

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr>t
planktonic	1	594.85861	51.67887	11.51	<0.0001

		95% Confidence Limits	
Variable	**DF**	**Upper**	**Lower**
planktonic	1	486.28533	703.43189

D.8 SAS Linear Regression Output, Chemostat Cultures, Attached (CFU per g) vs. Planktonic, All TSS Values → Langmuir Isotherm

ANOVA Table:

Source	DF	Sum of Squares	Mean Square	F-value	Pr>F
Model	1	4.5011E-15	4.55011E-15	309.78	<0.0001
Error	17	2.49699E-16	1.46882E-17		
Corrected Total	18	4.79981E-15			

Root MSE	3.83251E-09
Dependent Mean	6.69111E-09
Coeff Var	57.2777
R-Square	0.948
Ad R-Square	0.9449

Parameter Estimates:

Variable	DF	Parameter Estimate	Standard Error	t-Value	Pr>t
Intercept	1	-1.25035E-09	9.88254E-10	-1.27	0.2229
Invplank	1	0.00224	0.00012742	17.6	<0.0001

Variable	DF	95% Confidence Interval	
Intercept	1	-3.33539E-09	8.3468E-10
Invplank	1	0.00197	0.00251

D.9 SAS Linear Regression Output, Chemostat Cultures, Attached (CFU per g) vs. Planktonic, All TSS Values → Freundlich Isotherm

ANOVA Table:

Source	DF	Sum of Squares	Mean Square	F-value	Pr>F
Model	1	16.27303	16.27303	186.02	<0.0001
Error	17	1.48717	0.08748		
Corrected Total	18	17.7602			

Root MSE	0.29577
Dependent Mean	9.06252
Coeff Var	3.26368
R-Square	0.9163
Ad R-Square	0.9113

Parameter Estimates:

Variable	DF	Parameter Estimate	Standard Error	t-Value	Pr>t
Intercept	1	3.39935	0.42073	8.08	<0.0001
Logplank	1	0.89655	0.06573	13.64	<0.0001

Variable	DF	95% Confidence Interval	
Intercept	1	2.51169	4.28702
Logplank	1	0.75786	1.03523

D.10 SAS Linear Regression Output, Chemostat Cultures, Attached (CFU per g) vs. Planktonic, TSS = 5,000 mg/L

ANOVA Table:

Source	DF	Sum of Squares	Mean Square	F-value	Pr>F
Model	1	4.36291E+21	4.36291E+21	61.18	<0.0001
Error	8	5.7052E+20	7.1315E+19		
Uncorrected Total	9	4.93343E+21			

Root MSE	8444822992
Dependent Mean	14965555556
Coeff Var	56.4284
R-Square	0.8844
Adj R-Square	0.8699

Parameter Estimates:

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr>t
planktonic	1	600.06097	76.71809	7.82	<0.0001

		95% Confidence Limits	
Variable	**DF**	**Upper**	**Lower**
planktonic	1	423.14873	776.97321

D.11 SAS Linear Regression Output, Chemostat Cultures, Attached (CFU per g) vs. Planktonic, TSS = 10,000 mg/L

ANOVA Table:

Source	DF	Sum of Squares	Mean Square	F-value	Pr>F
Model	1	1.44542E+19	1.44542E+19	192.49	<0.0001
Error	9	6.75808E+17	7.50898E+16		
Uncorrected Total	10	1.513E+19			

Root MSE	274025129
Dependent Mean	790290000
Coeff Var	34.674
R-Square	0.9553
Adj R-Square	0.9504

Parameter Estimates:

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr>t
planktonic	1	272.06237	19.60929	13.87	<0.0001

		95% Confidence Limits	
Variable	**DF**	**Upper**	**Lower**
planktonic	1	227.70307	316.42167

VITA

The author, Leigh-Anne Henry, was born in Dallas, Texas in 1980. Only a true Texan for the first two years of her life, she moved to Maryland and grew up outside Baltimore. She graduated from Mount de Sales Academy in Catonsville, MD in 1998 and began her undergraduate career as a general engineering major the following fall at Virginia Tech. Throughout the author's undergraduate years at Virginia Tech, she remained an extremely active member of the honors program and was a founding member of the Hillcrest Honors Community. After two years spent as a successful but unhappy aerospace engineering major, the author decided to switch directions to pursue a career in a field she actually enjoyed. She graduated magna cum laude with a B.S. in Biological Systems Engineering in the summer of 1998 and promptly began her graduate work in the same department.