EXAMINATION OF THE TRANSPORT AND RETENTION AND EXPLORATION OF THE SPATIAL DISTRIBUTION OF MICROBIAL INDICATORS IN SOIL AGGREGATES

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the Degree of

MASTER OF SCIENCE

Fisheries and Wildlife

2010

ABSTRACT

EXAMINATION OF TRANSPORT, AND RETENTION AND EXPLORATION OF THE SPATIAL DISTRIBUTION OF MICROBIAL INDICATORS IN SOIL AGGREGATES

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Runoff and infiltration of bacterial pathogens from agriculturally managed fields are of significant public health concern. The objectives of this research were to 1) examine the transport and retention of the bacterial indicators *E. coli* and *Ent. faecium* and 2) explore the spatial distribution of *E. coli* in soil aggregates. To conduct these experiments, a bacterial extraction method was developed. The aggregates used came from fields with three different soil treatments: conventionally tilled with chemical input (T1), non-tilled with chemical input (T2) and native with no chemical input (T7). The bacterial extraction method yielded 108% and 92% recoveries of *E. coli* in T1 and T7, respectively, and 97% and 119% recoveries of *Ent. faecium* in T1 and T7, respectively. In transport experiments, only T1 exhibited significantly less bacteria in the effluent from dry aggregates compared to saturated aggregates, illustrating the importance of soil treatment and moisture on bacteria transport. Similarly, soil treatment, and moisture had an effect on *E. coli* spatial distribution. At air-dry conditions, three aggregate subsections exhibited statistical differences across all soil treatments compared to one subsection at 30% moisture content. T1 exhibited the highest variability as illustrated by statistical differences in the bacterial concentration within three subsections. This study showed that aggregates are useful models in understanding factors that influence bacterial transport.

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TABLE OF CONTENTS

LIST OF TABLES

Table

Table

Table

Table

Table

Table

Table

Table

LIST OF FIGURES

Figure
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Figure
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Chapter 1.

Literature Review

1.1 Introduction

How do we keep our water safe? Although a seemingly simple and unintimidating question, for the past few decades it has been one plaguing politicians, scientists and global citizens alike. As it stands, the world population is approaching 7 billion people worldwide and within the span of 50 years, the population is predicted to increase by 2 billion, mostly in developing countries (United States Census Bureau, 2009, United Nations, 2008). This rapid increase in population will no doubt place significant strain on global resources, not the least of which is water quality and quantity. It is already estimated that 1.1 billion people worldwide have no access to improved sources of water with a further 2.6 billion having no access to improved sanitation systems (WHO, 2000). Polluted water and lack of sanitation are the main routes of human exposure to pathogenic microorganisms and has been reported to cause 9 million cases of gastrointestinal illnesses annually across the globe (Rose et al., 2001, WHO, 2009)

However, we need not look beyond the borders of the United States for assurance that this issue is worthy of attention. Although the passage of the Safe Drinking Water Act (SDWA) and the Clean Water Act (CWA) have some provided stringent guidelines, curbing point-source waterborne outbreaks in the past 30 years, the threat of non-point source waterborne outbreaks has proven to be a more daunting task (USEPA, 2004, Craun, 2006). Potable supplies and recreational waters such as rivers, lakes and coastal waters are constantly receiving human and animal fecal discharges through agricultural and storm water runoff, waste water utilities and septic tank systems (USEPA, 2009). Pathogenic agents such as *E. coli* O157:H7, *Vibrio* sp., *Giardia*, *Cryptosporidium*, Hepatitis A and Noro viruses may be transmitted through contact with

such untreated waters (Eaton, 2005, Crockett, 2007, Field, 2003, Gaffield et al., 2003, Gerba and Smith 2005).

Agricultural non-point source pollution is the leading cause of microbial impairment in rivers and lakes and a major contributor to groundwater contamination (USEPA, 2004a). Much of the blame falls on agricultural management practices at confined animal feeding and manure spreading operations (Jones, 1980, Thunegard, 1975, Goss and Richards, 2008, Thurston-Enriquez et al., 2005). Following intensive irrigation, snowmelts or excessive rainfall, the manure-borne pathogens from farm fields can percolate through the soil and into the underlying water table or may runoff into surface water (Goss and Richards, 2008, Gerba and Smith, 2005, Auld et al., 2004, Curriero et al., 2001). Tillage practices also play a significant role in the degree of pathogen percolation in the soil (Abu-Ashour et al., 1998). Aggressive grinding and pulverizing by tillage machinery causes soil erosion and disrupts soil aggregate's structural integrity (Lal et al., 1997a). During these cultivation processes, pores, which serve as microbial highways through the vadose zone are destroyed and/or restructured (Hillel, 1998, Hattori and Hattori, 1976). Water quality pollution investigations of transport mechanisms of well characterized model microorganisms (such as fecal indicator bacteria) are needed to improve the understanding of the aforementioned environmental and human factors in relation to soil-microbial interactions.

1.2 Current Knowledge on Water Quality Indicators

The use of microbial indicators may be traced back to 1880 following identification *Escherichia coli* (initially termed *Bacillus coli communis*) and *Klebsiella*

pneumonia by microbiologists Von Fritsch and Escherich respectively (Ashbolt et al., 2001). Both microorganisms, characteristically found in human feces, were thought to indicate the pollution of a water source and the possible presence of other fecal pathogens (Buchanan and Gibbons, 1974). Because of the non-specificity of the detection culture media at the time, the aforementioned bacterial genera as well as others (*Enterobacter* and *Citrobacter*) were assembled into the first indicator group, termed total coliforms (Ashbolt et al., 2001).

Total coliforms were first adapted as indictors in 1914 by the United States Public Health Service which was responsible for supervision of water safety until the formation of the USEPA (Maier et al., 2000). The coliform group was defined as being composed of Gram negative (i.e. have a thin, semi-permeable peptidoglycan layer), non-spore forming, rod-shaped bacteria that are capable of growth in the presence of bile salts and ferment lactose with the production of acid and gas at 35±2°C within 24-48 hours (Beveridge, 2000, Eaton, 2005). However, we now know that some of the bacteria associated with the coliforms have no correlation with fecal pollution. Such is the example with some *Klebsiella* which have been found in paper mill effluents, cotton mill waste waters and textile plant effluents (Caplenas and Kanarek, 1984, Campbell et al. 1976, Dufour and Cabelli, 1976).

With the introduction of modern detection techniques and the need for more specific indicators relating to fecal pollution, multiple indicators have been introduced in the following decades with varying degrees of success. Classically, these indicators' utility has been assessed based on the degree of their convergence with the "ideal indicator" concept. From the perspective of water quality monitoring, the attributes of

an ideal indicator should include: 1) presence in concurrence with pathogens 2) higher resistance to disinfection than pathogens 3) higher in numbers as compared to pathogens 4) readily and easily detection in all types of waters 5) stable in the environment, i.e. cannot multiply in the environment and 6) an indication of the degree of contamination and hazard in relation to its density (Griffin et al, 2001, Scott et al., 2002, Yates 2007). In light of such rigorous criteria, it is quite obvious that an ideal indicator has yet to be identified. In any case, the search for such a microorganism may be of secondary importance. Instead, evaluation of indicators based on their intended usage has proven to be more useful (Dufour, 1984, Ashbolt et al., 2001). *E. coli* and *Enterococcus* spp. have been used as the primary bacteriological indicators for the past three decades and their taxonomy and utility as water quality indicators are discussed in detail in the following sections.

1.2.1 *Escherichia coli* as a Fresh Water Quality Indicator

With its abundance in animal and human feces as well as its relationship with recreationally-acquired gastroenteritis, *E. coli* had been a good candidate to be an index for fecal contamination. In 1984, Dufour showed a strong correlation between *E. coli*'s density and incidence of swimming-associated gastroenteritis in fresh waters (1984). The need for such a relationship was underscored by the findings that the previously reported that fecal coliforms (a sub-group of the coliforms) lacked any significant association with gastrointestinal illnesses (USEPA, 2004). Subsequently and ever since, *E. coli* has been used as the primary indicator for fecal pollution in fresh waters by many states.

As with most indicator bacteria, detection of *E. coli* was based on a presence/absence biochemical tests or agar based culture media that required incubation

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longer than 24 hours resulting in colony forming units (CFU). From a water quality monitoring perspective, either choice was inadequate. While a presence/absence test may indicate fecal pollution, information about the density or extent of pollution cannot be ascertained without a most probable number format. And although the culture media could resolve the quantification issue, it had two disadvantages: 1) the passage of 24 hours after sampling to search for evidence may not be useful since human contact with impaired water could have already taken place and 2) selective agents in media were shown to inhibit environmentally stressed organisms (Ashbolt, et al., 2001). The development of the mTEC and then the modified mTEC agar are the most common methods for enumerating *E. coli*. Utilizing the β-D-glucuronidase enzyme reaction to catabolize glucuronic acid development, *E. coli* produces red or magenta colonies within 24 hours (USEPA, 2002). The method's two hour resuscitation period induced growth of stressed organisms while increasing the detection of *E. coli* within 90% accuracy. Furthermore, the reliability, efficiency and technical ease at which an analyst may perform the aforementioned method added another "ideal indicator" attribute to *E. coli*'s resume.

Nevertheless, as with other indicators, *E. coli* has its limitations. It has been found to replicate in tropical and subtropical soils (due to warm, humid climates that are conducive to *E. coli* growth), thereby altering the true incidence of fecal pollution (Solo-Gabrielle et al., 2000). Additionally, sensitivity to chlorination places doubts about its utility in treatment plant facilities due to potential underestimation of more chlorine-resistant pathogens (Miescier and Cabelli, 1982).

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1.2.2 *Enterococcus* spp. as Marine Water Quality Indicators

From the years of 1972-1979, Cabelli conducted a series of epidemiological studies on fecal indicators in recreational beaches and lake water in the United States and Egypt (1983). Regression analysis showed strong correlation between gastrointestinal symptom of swimmers in these water bodies and *E. coli* and *Enterococci* over the other microorganisms tested. However, *Enterococcus* spp. exhibited higher correlation coefficients for their mean densities than *E. coli* in marine water. These results implicitly illustrated the value of *Entercocci* as an indicator for pathogens that resist chlorination and can survive in highly saline environments. Persistence of *Enterococci* under conditions that are detrimental to microorganisms can be explained by *Enterococci's* cell properties. The *Enterococcus* spp. are Gram positive (i.e. have a thick, relatively impermeable peptidoglycan layer) cocci that optimally grow at 44±0.5^{0}C, 6.5% Sodium Chloride and an unusually high pH of 9 (Ashbolt et al., 2001, Eaton et al., 2005).

As a general rule, methods have not been developed to specifically identify *Enterococcus* to the species level when assessing water quality. However, the relationship of a species with specific source of fecal pollution is a great advantage from an epidemiological standpoint, effectively narrowing down the list of suspects for outbreak investigation. While there are no exclusive delineations, *E. faecalis* and *Ent. faecium* have generally been associated with human fecal pollution, while *E. bovis*, *E. equinus* and *E. avium* have been found to be indicative of presence of animal fecal matter (Eaton, 2005).

Like most bacteria, *Enterococci* have been plagued with nomenclature ambiguities and elusive detection techniques after they were first isolated in 1899.

Typical qualitative detection techniques were biochemical examinations such as the Lancefield classification scheme where the bacterium would react with a serological antiserum (Murray, 1990). During the same time period that Cabelli was conducting the study that would place *Enterococci* at the forefront of water quality indicators, Levin et al. were devising a selective method for its enumeration using membrane filtration (Levin, 1975). Since then, their method has been modified making it quicker and less technically demanding. Method 1600, using mEI media for rapid enumeration through membrane filtration is the most recently approved and recommended (USEPA, 2002a). The mEI media contains indoxyl β-D-glucoside, which is a chromogenic agent hydrolyzed by *Enterococcus*'s β-glucosidase enzyme resulting in a diffuse halo appearance around the a positive colony (Messer and Dufour, 1998). Unlike previous methods that required days, the detection for *Enterococcus* using mEI could be enumerated within 24 hours. The mEI method has made *Enterococci* a readily utilizable indicator for water quality assessment in marine and freshwater systems.

1.2.3 Use of fecal indicators in the agricultural environments

Findings in the National Water Quality report by the EPA showed that approximately 18% and 14% of assessed river and lake miles respectively were impacted by agricultural pollution, the leading contamination of which is pathogenic bacteria (USEPA, 2004a). Agricultural pollution originates from runoff associated with manure applied to fields, deposited by grazing livestock or leached from faulty septic tank systems on agricultural fields (Simpson et al., 2002, Tyrrel and Quinton, 2003, Gerba and Smith 2005). Pathogenic bacteria associated with agricultural fecal waste and manure

include *E. coli* O157:H7, *Salmonella*, *Campylobacter jejuni*, *Listeria*, *Helicobacter* and *Shigella* (Jamieson et al., 2002, Gerba and Smith, 2005).

While there are no standard guidelines for assessing fecal pollution on agricultural fields, fecal coliforms (especially *E. coli)*, *Enterococci* and *Salmonella* are frequently used as indicators (Duffy, 2003, Benham et al., 2006, Holley et al., 2008). When infected, cattle may shed 10^2-10^5 and 10^2-10^7 CFU per g of feces of *E. coli* and *Salmonella*, respectively (Himathongkhama, 1999). After applying the manure to the field, the bacterial concentrations may decline 2-4 orders of magnitude within 9 weeks (Natvig et al., 2002). However, indicator bacterial populations are known to survive in sediments for extended periods of time and may persist for months or even years (Mallmann and Litsky, 1951, Gerba and Smith, 2005, Anderson et al., 2005). After subsequent manure application, indicator bacteria may regrow causing false positives and unwarranted concern for fecal pollution (Natvig et al., 2002, Unc et al., 2006).

Best management practices (BMPs) have usually been based on research that examined the mitigation of indicator survival in soil (USEPA, 2003, Benham, 2006). For example, it is recommended to apply manure to agricultural fields during summer temperatures because at cooler temperatures manure-borne bacterial indicators have been observed to survive longer (Himathongkham et al., 1999, Mannion et al., 2007). Furthermore, storage practice and application of manure have shown to play an implicit role in indicator viability (Unc and Goss 2003). Thunegard has shown that 35% of fecal samples stored as liquid slurry contained *Salmonella* spp. as opposed to only 6% of composted solid manure (1975). The process of composting, where the temperature of

manure is raised 70^0C prior to field application, is thought to be behind this pronounced difference (Jones, 1980, Cools et al., 2001, Gerba and Smith, 2005).

Additional processes to mitigate pathogen load in agricultural soil include i) Lime stabilization, where pH of manure is raised to 12 by adding hydrated lime ($Ca(OH)_2$), quicklime (CaO), or lime containing kiln dust or fly ash ii) anaerobic digestion, which entails placing manure in an oxygen-free environment between 15-30 days at an elevated temperature, iii) aerobic digestion, where the manure is frequently agitated with oxygen or air for 20-60 days and iv) air-drying the manure for at least three months prior to land application (USEPA, 2003, Gerba and Smith, 2005).

1.3 Water Quality Monitoring Deficiencies

The diffuse nature of agricultural runoff has been troublesome for regulatory agencies to monitor (Wiebe, 2006). While regulations such as the Resource Conservation and Recovery Act and SDWA have addressed point source pollution, regulatory measures for non-point pollution due to agricultural runoff remains elusive (Nielsen, 1991, Wiebe, 2006). The passage of Section 319 of the CWA was meant to address this concern by providing state and territories grant money to establish pollution control strategies (Great Lakes Commission, 2004). Best management practices (BMPs) were put into place by these entities to introduce suitable agricultural management practices and provide barriers for water quality impairment (Michigan Department of Agriculture, 2010, Mackler and Merkle, 2000).

Although implementing BMPs was meant to curb microbial transport to drinking or contact water, microbial outbreaks being traced back to livestock is still a public health

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problem (Cooley et al., 2007). A pertinent example of this is the *E. coli* O157:H7 and *Campylobacter jejuni* outbreak in Washington County Fair in New York. Following a heavy rainfall event, runoff from a nearby farm leached into a well used by vendors to supply attendees with drinking water and ice. Sixty five persons were hospitalized, two of which died of hemolytic uremic syndrome (CDC, 2001).

Generally, there are no regulations concerning direct monitoring of agricultural soils after manure application or if there are, the monitoring and surveillance strategies are based on poor understanding of soil microbial transport (Cullen et al., 1995, Mackler and Merkel, 2000, Gagliardi and Karns, 2000). Traditional surveillance has usually taken place at sites that are suspected to be on the receiving end of the pathogenic load, i.e. recreational or ground waters (Gagliardi and Karns, 2000). Although this approach is seemingly logical because these bodies of waters are the points of human contact, it has been criticized because evidence of contamination may come after the water source has already been compromised (Cullent et al., 1995).

Even if surveillance is required, implementing guidelines for monitoring the soil necessitates thorough understanding the region's hydro-geological dynamics that effect soil-microbial interactions (Steenhuis et al., 1995, Powelson and Gerba, 1995, Jamieson et al, 2002). These dynamics can vary from region to region or even temporally, causing predictions about microbial runoff or infiltration to be dubious (Unc and Goss, 2004, Smucker et al, 2007). For example, winter manure application in areas of extreme cold may be a public health concern. The soil may become fractured due to freezing and thawing cycles, creating pathways for bacterial movement to ground water (Jamieson et al, 2002, Rosa et al., 2009). Furthermore, the ability of the soil to filter pathogens under

dry condition may be altered under extreme environmental changes such as excessive rainfall leading to ground water contamination (Mahler et al., 2000, Curriero et al., 2001, Stevik et al., 2004).

Aside from the regrettable cost of lives, there is also a hefty economic price tag due to inadequate monitoring. The tragedy of Milwaukee's *Cryptosporidium* outbreak, which not only caused 403,000 cases of illness, cost the state of Wisconsin $96.2 million in medical costs and productivity loss (Corso et al., 2003). Waterborne outbreak prevention and curbing economic loss necessitates successful monitoring strategies rooted in scientific understanding of the complex dynamics of microbial-soil interactions (Nielsen, 1991).

1.4 Microbes in Soil

The soil environment houses approximately 10^9 bacteria per gram and is one of the most microbially diverse terrestrial systems on earth (Torsvik et al., 1989). Bacteria are the most numerous microorganisms in soil and play a significant role in soil processes and plant physiologies such as carbon decomposition and mineralization, nitrogen fixation, ammonification, nitrification and denitrification (Foster, 1988, Tan, 1994). Likewise, soils in rural and agricultural areas may harbor zoonotic pathogens such as *E. coli* O157:H7, *Camplyobater jejuni*, *Cryptosporidium* that pose notable human risks (Duffy, 2003, Gerba and Smith, 2005). The specific mechanisms of survival and transport of bacteria in such a diverse environment is influenced by the complex interplay of chemical and physical properties of the soil as well as the biological processes (Hattori

and Hattori, 1976, Tisdall and Oades, 1982, Ranjard and Richaume, 2001, Jamieson et al., 2002).

1.4.1 Soil Aggregates and Microbial Interactions

Soil is defined as the heterogeneous outer layer of earth's terrestrial surface that influences the planet's climatological and hydrological cycles and serves as a growth medium for a community of living organisms (Hillel, 1998). Physically, the structure of soil is a key factor in its ability to support plant and animal life and moderate nutrient and water cycling (Lal et al., 1997a, Bronik and Lal, 2005). Soil's structure can typically be characterized based on the association of its structural units, i.e. its clay, silt and sand content (Hillel, 1998). Soils with appreciable clay content tend to associate themselves into composite structural sub-units called aggregates which may vary from several millimeters to centimeters in size (Hillel, 1998). The stability of these associations is a function of physical flocculation and biological cementing substances that holds these clusters together (Bronik and Lal, 2005). The soil aggregate's structural arrangement results in the creation of niches or compartmentalized habitats for bacteria (Mummey and Stahl, 2004, Mummey et al., 2006). Such an environment protects against intrusion of larger predators, provides a buffer between competing microorganisms and allows for stable moisture conditions (Vargas and Hattori, 1986, Ranjard and Richaume, 2001, Zhou et al., 2002).

Reaching these microhabitats is facilitated by the soil aggregate's pore structure characteristics (Hattori, 1988, Foppen, 2005). Pores may be continuous allowing two way movement or may have dead ends or become completely closed causing the bacterial

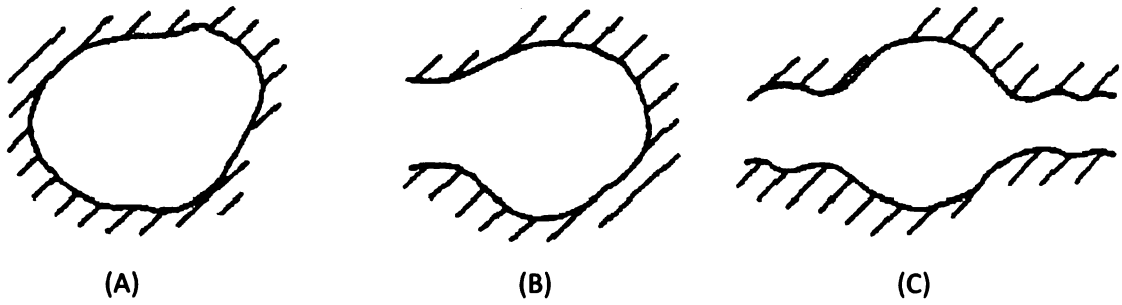

Fig 1.1. Soil pore types: A) closed pore, B) dead-end pore and C) continuous, open-ended pore. Figure from Hattori and Hattori, 1976.

entrapment (Fig 1.1). The pore size is equally as important in bacterial distribution in soil aggregates (Stevik et al., 2004). It is usually within the smaller pores of approximately 2μm (but not under 0.8μm) that bacteria may colonize (Ranjard and Richaume, 2001). In larger pores, however, sorptive forces may not be enough to retain the bacteria following a high hydraulic flow, subsequently flushing bacteria out of the aggregate (Hattori, 1988, Stevik et al., 2004).

As previously mentioned, formation of the aggregate structure is usually attributed to the spatial arrangements of the cemented subunits. Organic matter, the main biological cementing substance, is thought to be the single most important factor in maintaining aggregate structural stability which contributes to the soil's pore characteristics (Tisdall and Oades, 1982, Lal et al., 1997b). Soil organic matter is formed from sloughed plant cell components (soluble exudates, lysates, and decaying root hairs), bacterial cell components (slime layer, capsule, and degraded metabolites) fungal components (hyphae) or animal residue (Foster, 1988). Typically, the components of soil organic matter can be classified into three categories based on their resistance to environmental stress: A) transient binding agents which are rapidly decomposable organic materials such as microbial, fungal or plant polysaccharides, B) temporary

cementing agents such as plant roots, fungal hyphae and animal residues which a provide a greater surface area for the binding of clay particles thereby affecting the stability of larger groups of aggregates and C) persistent cements composed of complexes of clays, polyvalent metals and organic matter-derived resistant fragments from plant roots, fungal hyphae and degraded bacterial metabolites (Tisdall and Oades, 1982, Martens and Frankenberger, 1992).

Physical disruption due to cultivation practices such as tillage compromise the structural integrity of the aggregate by stimulating oxidation and loss of organic matter (Tisdall and Oades, 1982). This causes redistribution of soil components and changes pore size distribution and continuity (Hillel, 1998, Leij et al., 2002). However, studies show conflicting results as to whether this variability results in a decrease or increase in porosity (Lipiec et al., 2006). Nevertheless, this disturbance is thought to cause disruption of the bacterial microhabitats and affect their distribution (Peixoto et al., 2006).

1.4.2 Survival of Bacterial Pathogens in soil

Aside from reinforcing the soil aggregate, the soil organic matter is the primary nutrient cycling substrate for soil-colonizing bacteria (Foster, 1988, Ranjard and Richaume, 2001). The availability of the organic matter is contingent upon the degree of which it is decomposed and physical barriers in the soil which inhibit bacteria from reaching it (Hattori and Hattori, 1976, Tan, 1994). Under these circumstances, soil bacteria persist at a low metabolic rate to adapt to extreme nutrient limiting conditions (Rozak and Colwell, 1987).

Following manure application, however, soil is loaded with nutrients that enhance survival of introduced zoonotic bacteria in their new environment (Himathongkhama, 1999, Natvig et al., 2002, Peacock 2004). The persistence of these pathogens in soil is controlled by a myriad of factors including soil moisture content, texture, pH, temperature, nutrient availability and predatory activities within soil and the capacity of the microbe to avoid or resist these stresses (Jamieson et al., 2002, Unc and Goss, 2003, Unc and Goss, 2004, Goss and Richards, 2008). For example, viability of *E. coli* and *Enterococcus* have shown marked difference when inoculated within sandy, loamy and loamy sandy soil with 60, 80 and 100% moisture content at 5, 15 and 25^0C (Cools et al., 2001). While both microorganisms favored higher soil moisture and lower temperatures, *E. coli* survived better in sandy soil and *Enterococcus* spp. preferred loamy soil. Additionally, *Enterococcus* showed greater resistance to desiccation by surviving longer than *E. coli* at lower moisture content. This may indicate *Enterococci*'s increased production of exopolysaccharides in response to desiccation stress (Roberson and Firestone, 1992, Hartke et al., 1998).

1.4.3 Factors of Bacterial Transport in Soil

Bacteria movement within soil can occur either actively through motility or passively by being carried with water through soil pores (Lindqvist and Bengtsson, 1991). Passive transport includes an amalgam of physical processes that are termed 'preferential flow', signifying movement of bacteria as a particle across a defined pathway (Coppola et al., 2009). Pathways that mediate preferential flow are created by micro and macro-pores, fissures and cracks in the soil (Smucker and Hopmans, 2007, Coppola et al., 2009). Active microbial motility on the other hand is a physiological

process that transports bacteria in response to an environmental stimulus (Hattori and Hattori, 1976, Ford and Harvey, 2007). Bacteria may possess flagella that act as motors to promote transport in liquid media (Manson, 1990, Eisenbach, 1990). The flagella drive bacteria to preferentially swim towards chemical stimuli such as nutrients or oxygen (Adler, 1966). Because soil alternates between unsaturated and saturated conditions, the moisture content plays an integral role in bacterial motility in soil (Soby and Bergman, 1983). The ability of soil to retain moisture is usually a function of its pore size and connectivity (Ranjard and Richaume, 2001). When these pore spaces are saturated, they are thought to effectively act as microbial highways (Abu-Ashour et al., 1998, Zaval'skii and Voloshin, 2003).

While passive and active transport govern microbial movement within soil, there are two processes that retard their movement: the filtration capacity of soil and sorptive interactions between the soil and the microorganisms (Lindqvist and Bengtsson, 1991, Gannon et al., 1991, Unc and Goss, 2003). Filtration is a process by which particles are physically trapped by colliding on the surface of a porous medium (Foppen et al., 2005). This process is generally thought to be the main mechanism for mitigating microbial percolation through the soil (Jamieson et al., 2002). Microbes act as particles that collide with the eluvial particles in soil by physical impediment (Foppen et al., 2005). Physical straining or retention is influenced by the microorganisms' size and morphology and soil's pore and grain sizes (Fontes et al., 1991, Huysman and Vestraete, 1993, Stevik et al., 2004, Bolster et al., 2009).

Adsorption is an attachment process by which the chemical interactions are dictated by varying properties of the soil's substratum, the microorganisms' cell wall

characteristics, fluctuations in the soil environment and contact time between the microorganism and the soil sediment (Powelson and Gerba, 1995, Hattori and Hattori, 1976, Foppen et al., 2005). Chemical forces (Van der Waals, electrostatic and ionic bonding) are influenced by the soil particle size and texture, presence of cations (Ca^{2+}, Na^{+}, Fe^{3+}), clay and soil organic matter cation exchange capacity (Unc and Goss, 2003, Stevik et al., 2004). The variations in bacterial cell hydrophobicity, electrostatic charge and extracellular polysaccharides also influence these chemical interactions (Stenström, 1989, Fontes et al., 1991, Bolster et al., 2006). After prolonged contact time, these factors influence can change from reversible microbial adsorption to irreversible microbial adhesion to soil (Powelson and Gerba, 1995). Ultimately, environmental factors such as high hydraulic flow events (rainfall and snowmelts), pH change (lime stabilization, manure loading and rainfall) and temperature fluctuations can reduce microbial contact time with soil substratum and alter surface charges and metabolic physiology, causing desorption (Powelson and Gerba, 1995, McEldowny and Fletcher, 1988, Guber et al., 2005).

In reality the various mechanisms for transport and retardation are interdependent and even may occur simultaneously or influence each other (Smucker and Hopsmans, 2007). For example, while motility itself facilitates microbial movement, it may also increase adsorption due to the attachment of flagellar exopolysaccharides to the soil substratum (McCaulou and Bales, 1995, Van Loosdrecht and Zehnder, 2005). Similarly, the mechanism of adsorption can be a byproduct or influence filtration (Stevik, 2004). After bacteria collide with soil particles, they may be able to form chemical bonds after extended contact time (Huysman and Vestraete, 1993). Alternatively, microbes that are

sorbed to fine clay particulates (>2μm) may increase bacteria colloidal diameter thereby increasing filtration (Mahler et al., 2000).

1.5 Research Objectives

Research has indicated that transport mechanisms have typically been characterized at fields scale and laboratory bulk soil column models, which average localized heterogeneities (Gagliardi and Karns, 2000, Unc and Goss, 2003, Bolster et al, 2006). While only one study has been found to address the transport at the aggregate scale (Guber, 2009), further research is needed to address relationships of bacterial transport processes within aggregates. Examining transport at this scale using traditionally utilized indicators may elucidate specific transport mechanisms that occur due to the microscale interactions and the public health risk associated with these mechanisms.

The objectives of this research were to examine i) bacteria association with soil aggregates from different agricultural management practices, and ii) transport, retention and spatial distribution using well-characterized indicator bacteria as models. Achieving these objectives entailed the following:

1. Development of methods for assessing bacterial extraction from aggregates.
2. Evaluation of bacterial retention and survival in aggregates.
3. Examination the retention and transport behavior of *E. coli* and *Ent. faecium* in tilled and non-tilled soils at unsaturated and saturated conditions.
4. Exploration of spatial distribution of bacteria within inoculated soil aggregate subsections.

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Chapter 2.

Material and Methods Development

2.1 Introduction

Transport of bacteria through agricultural soil and into the underlying ground water or runoff to nearby surface water poses a public health risk. When humans come in contact with receiving waters, they may be infected by pathogenic microorganisms. Conventional agricultural management practices such as mechanical tillage modify the soil structure and may alter pore structure. Because these pores act as conduits for interaggregate and intraggregate microbial movement, it is hypothesized that such practices may play a role in managing microbial risk. For example, when soil is tilled microbial infiltration may be retarded due to the disruption of pore networks. Therefore, the objective of this study was to examine the transport of the bacterial indicators, *E. coli* and *Ent. faecium*, at the macroaggregate scale and investigate the correlation with structurally modified soil aggregates through conventional tillage and soil aggregates that have received no agricultural modification for 20 years, both sampled from the Kellogg Biological Station Long Term Ecological Site.

Preceding the experimental analyses to address the aforementioned objectives, a novel method was designed for bacterial extraction and obtaining optimal recovery of viable spiked bacteria in soil aggregates. Following the methods optimization, three experimental methods were designed for the following objectives

1. Evaluation of mechanisms controlling bacterial retention in aggregates.
2. Examination of the transport behavior of *E. coli* and *Ent. faecium* in tilled and non-tilled soils at unsaturated and saturated conditions.
3. Exploration of spatial distributions of bacteria within subsections of inoculated soil aggregates.

2.2 Methods Development

2.2.1 Soil aggregates. Soil samples were collected at the Long Term Ecological Research Site (LTER), Kellogg Biological Station (KBS) located in southwest Michigan (85^0 24` W longitude, $42^0$ 24` latitude) in November, 2008. Soil at the site are Typic Hapludalfs (of the Alfisol order) made up of either fine loamy, mixed mesic Kalamzoo series or coarse loamy, mixed mesic Oshtemo series. The 60 hectare site is subdivided into six replicates of 1 hectare plots exposed to eight different agricultural management treatments (Fig. 2.1).

For this study aggregates were collected from three different treatment plots: the T1 treatment plot had conventional tillage (chisel-plowed) with a corn-soybean-wheat rotation field and was conventionally fertilized (3.35±0.3 kg N ha-1 day-1). The T2 treatment plot received no tillage but had corn-soybean-wheat rotation and was conventionally fertilized. The T7 treatment was native successional plot and received no tillage after spring 1989 (Robertson et al., 2000). From each replicated plot, sample soil blocks, approximately 15 x 15 cm in size were extracted from 0-20 cm depths using a sharp flat spade. Soil was air-dried and then manually sieved by gently shaking for 30 seconds into different aggregate sizes. The aggregates of 4-6.3 mm size fraction were used for this study and stored at laboratory temperature in a plastic container for all experiments. Aggregate level texture, C and N content and bulk soil densities are described in Tables 2.1 and 2.2.

2008 KBS LTER Main Site

- T1- conventional tillage with corn-soybean-wheat rotation and fertilization
- T2- non-tilled with corn-soybean-wheat rotation and fertilization
- T7- native successional with no tillage after 1989

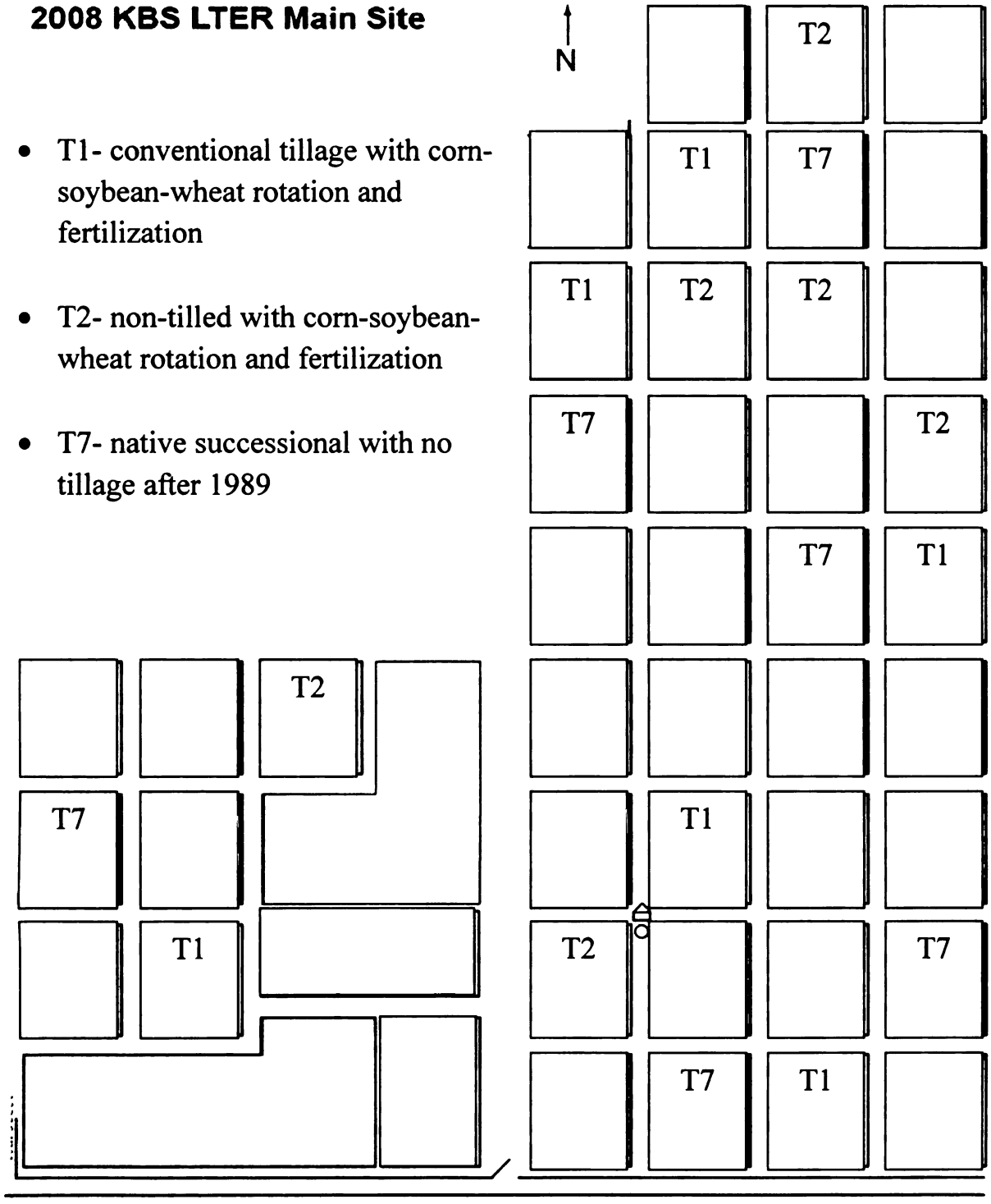

Fig 2.1. KBS LTER soil sampling plots.

Table 2.1. Average percentages of soil texture in aggregate treatments T1, T2 and T7 (Chun, 2010, Unpublished). Standard deviations are indicated in parentheses.

Soil Aggregate Treatment (0-15cm depth)	Texture (percent)		
	Sand	Silt	Clay
Tilled - T1 treatment	21 (18%)	35 (19%)	44 (2.5%)
Non-tilled - T2 treatment	35 (27%)	31 (18%)	34 (10%)
Native successional - T7 treatment	27 (17%)	34 (13%)	39 (5%)

Table 2.2. C, N and bulk soil densities of soil aggregate treatments T1, T2 and T7 (Grandy and Robertson, 2007). Standard deviations are indicated in parentheses.

Soil Aggregate Treatment (0-5cm depth)	Total Average (g/m^2)		Bulk Density (g/cm^3)
	Carbon	Nitrogen	
Tilled - T1 treatment	621 (51.1)	57.3 (5.31)	1.37 (0.01)
Non-tilled - T2 treatment	885 (55.1)	81.0 (4.66)	1.36 (0.03)
Native successional - T7 treatment	1,1001 (38.6)	86.1 (3.54)	1.21 (0.02)

2.2.2 Bacterial extraction method. A vortexing and membrane filtration (VMF) method was adapted from the technique devised by Singh to enumerate the native bacteria in the

whole, inner and outer layers of the T1, T2 and T7 soil aggregates (2007). First, each whole soil aggregate was weighed to the nearest thousandth gram and placed into a 15ml centrifuge tube (Difco, Franklin Lakes, NJ, #236940) containing 10ml of 1X Phosphate Buffered Water (PBW) (pH 7-7.2). The 1X PBW stock was made in accordance with Standard Methods for Examination of Water and Waste Water guidelines (Eaton, 2005). The centrifuge tubes containing the aggregate and PBW were votexed at full speed for 3 minutes, inverted 20 times, then vortexed again for another 3 minutes. In the initial experiments investigating native bacterial concentrations, the soil sediment was allowed to settle for 20 minutes before proceeding to membrane filtration. However, after evaluation, the settling step was eliminated from the method due to underestimating the viable bacterial counts (see section 3.1.3). After vortexing, the samples were serially diluted ten-fold in 1X PBW. Dilutions were then membrane filtered through 0.45µm pore sized membrane filters (Pall Corp., Ann Arbor, MI, #T914361) following Standard Methods, Total Colifrom (TC) bacteria were enumerated using a membrane filter procedure (Eaton, 2005). Total Heterotrophic (HPC), TC (and *E. coli*) and *Enterococcus* bacterial concentrations per gram of soil aggregate were assayed using Tryptic Soy Agar, m-Endo LES agar and mEI (BD and Co., Sparks, MD; #236920; #273620; #214881), respectively. Each sample dilution was processed in triplicates for all media, then placed bottom-side up in a 35±0.5^{0}C incubator for HPC and TC while mEI plates were incubated at 41±0.5^{0}C. All media was incubated for 24 hours prior to enumeration. Bacterial concentrations were calculated by manually counting colony forming units (CFU) from dilution plates and back calculating to original concentration. The original concentration was divided by the soil aggregate's weight and reported at CFU/g of soil.

2.2.3 Direct *E. coli* and *Ent. faecium* spiking and immediate recovery. Spiking experiments were aimed at answering two questions: 1) what was the detection limit of the assay, i.e., what was the lowest concentration of spiked bacteria recoverable and 2) what was the total percentage recovered. Stocks of *E. coli* ATCC 15597 (designation C-3000; derived from K-12 strain) and *Ent. faecium* ATCC 35667 kept at -80^{0}C, were thawed and then placed in 4ml of Tryptic Soy Broth (BD Diagnostics, Franklin Lakes, #211768) and incubated overnight at 35±0.5^{0}C in a shaking incubator. The overnight grown *E. coli* and *Ent. faecium* cultures were serially diluted 1:10 in PBW, covering a gradient of concentrations from approximately 1 CFU/soil aggregate to 1x10^7 CFU/ soil aggregate for *E. coli* and 1 CFU/soil aggregate to 1x10^4 CFU/ soil aggregate for *Enterococci*. A narrower gradient range for *Ent. faecium* was used after determination that higher concentrations would not be used in the primary flow and spatial distribution experiments so as not to over-saturate aggregates, thereby masking the effects of soil structure on bacterial transport. Subsequently, each stock dilution used for spiking was membrane filtered in triplicates onto m-Endo and mEI. After the passage of 24 hours, the CFUs were enumerated and each spiked dilution was back calculated to determine the influent spiked concentration.

The following day, the soil aggregate was placed on sterile petridish (60 x15mm) and weighed to the nearest thousandth gram. The volume of 50µl was observed to saturate the soil aggregates, hence, each spiked stock dilution used to saturate the T1 and T7 treatment aggregates with the aforementioned volume by gently touching the drops formed at the end of the micropipette tip to the aggregate. To avoid desiccation (via evaporation) and bacterial die-off in the aggregate, the spiked soil aggregates were placed

in 10ml of 1X PBW and immediately assayed using the vortex and membrane filtration method. Since only *E. coli* and *Ent. faecium* recovered concentrations were of interest, only m-Endo LES and mEI agars were used as selective growth media. CFU counts were obtained from countable dilution plates and back calculated to obtain recovered bacterial concentrations from each aggregate. Calculation of percent recovery per aggregate was obtained by dividing the recovered concentration after spiking by the total CFU (as measured per dilutions). The data were statistically analyzed as a ratio of spiked bacterial concentration to recovered bacterial concentrations.

2.2.4 Desiccation effect on *E. coli* recovery in whole and aggregate subsections. The effect of desiccation on spiked bacteria survival was critical to assess the required moisture content in the soil prior to designing the flow experiments (see next section). The percent recovery of spiked *E. coli* as a function of soil moisture content by weight was measured. *E. coli* stock was spiked into T7 aggregates at a concentration of approximately 10^4 CFU/ aggregate using the same spiking procedure mentioned earlier. The spiked concentrations were approximated based on calculations of *E. coli* stock dilution concentrations from experiments 2.2.3. To affirm the approximated concentration, the actual concentration was measured and calculated by membrane filtering the stock dilution used and enumerated following 24 hour incubation. Next, spiked aggregates were placed in petri dishes that were slightly open, allowing for evaporation while avoiding condensation and the risk of contamination. Following interval air-drying at 15 and 30 minutes, the spiked aggregates were processed using the vortex and membrane filtration method both with and without the settling step. To calculate moisture content at each time interval, aggregate weights were taken 1) prior to

spiking, 2) immediately after spiking and 3) after interval air-drying. Moisture retained in the aggregates after interval air-drying was determined by subtracting spiked aggregates' weight after interval air-drying (3) from the aggregates' weight prior to spiking (2). The obtained value was divided by the aggregates' weight prior to spiking, yielding the moisture content of the aggregate after air-drying. While the recorded weight immediately after spiking was not used in this equation, it was useful reference for the amount of moisture loss.

The desiccation analysis was further extended to spiked soil aggregates that were sliced into three subsections. The experiment aimed to ascertain the susceptibility of spiked *E. coli* in the spatial regions of soil aggregate upon exposure to desiccation stress. Preceding the soil aggregate spiking, the aggregate was cut into top, middle and bottom subsections with a stainless steel razor blade, flame-ethanol sterilizing in between each cut. The orientation of the subsections were in reference to the spatial location of the *E. coli* spike, i.e., the top section was where the *E. coli* was directly added, the middle was the section right below and the bottom was the lowest. Slices were left to dry for 0, 10, 40 and 60 minutes then processed via the vortexing and membrane filtration method without settling.

2.2.5 Soil aggregate rehydration and enrichment. Another concern was resuscitating native coliforms and *Enterococci* following prolonged hydration of soil aggregates during the flow chamber and slicing and saturation experiments. Therefore, the re-growth of both bacterial groups was assessed by suspending soil aggregates for an extended period of time. First, the soil aggregate was weighed to the nearest thousandth gram and then aseptically placed in a 15ml centrifuge tube containing either 5ml TSB or 10 ml sterilized

nano pure water. Because the brief time period that the aggregates were placed in PBW during processing was inadequate to examine re-growth of native bacteria (see results), the suspended soil aggregates were incubated for 24 hours. One set of aggregates suspensions were incubated at 35±0.5^{0}C to promote re-growth of native *E. coli* while another set was incubated at 41^{0}C for *Enterococci* resuscitation. The aggregates were processed as previously described. No growth on mEI and m-Endo indicated that there were no viable coliforms or *Enterococcus* spp. from the aggregates suspended in nanopure water and TSB. Therefore it was assumed that keeping the aggregates hydrated for less than 24 hours posed no risk of resuscitating any native fecal bacteria.

2.2.6 The effect of calcium chloride solution on *E. coli* recovery. A calcium chloride ($CaCl_2$) solution was used to prevent soil aggregates from collapsing upon application of vacuum in the flow experiments. A study by Winslow and Falk had demonstrated that with increasing $CaCl_2$ concentrations, the percent live *E. coli* (at the time named *Bacterium coli*) decreased after 24 hours even at a stable pH of 7 (Winslow and Falk, 1922). Furthermore, increasing the concentration of $CaCl_2$ resulted in higher *E. coli* die-off, ultimately reaching the maximum toxicity at 0.435M $CaCl_2$. Therefore, to ensure that the *E. coli* strain used in the experiments did not die-off, the temperature and the time that is required to keep spiked bacteria viable needed to be optimized.

Preliminary experiments were designed to examine *E. coli* recovery at 0, 2 and 4 hours at 4^{0}C and 28^{0}C suspended in a 0.5mM $CaCl_2$ solution. High recoveries after 4 hours of immersion under all the variables indicated that the 0.5mM concentration of $CaCl_2$ (used to stabilize soil aggregates) was not detrimental to *E.coli* survival. Nevertheless, it was determined that bacteria spiked in soil aggregates would not be

immersed in $CaCl_2$ solution for longer than 30 minutes while aggregate processing was completed within 3 hours in the flow chamber experiments.

2.2.7 Examination of clumping in *E. coli* and *Ent. faecium* cultures. Clumping of bacterial cells could cause errors in CFU counts (Goldman and Green, 2009). Microscopic examination of *E. coli* and *Ent. faecium* clumping were performed with a Ziess Axioskop 2 Plus model (Göttingen, Germany) using the differential interference contrast option at oil-immersion (100X) resolution. Approximately 20µl of each bacterial sample at approximately 10^7 CFU/ml were aliquoted onto glass slides and covered with a glass cover slip and sealed by nail polish. Images were captured using the peripheral camera Axiocam model MRc (München-Hallbergmoos, Germany) and stored in .jpeg and .zvi format using AxioVision software Release 4.5 (Göttingen, Germany).

Initial observation of *E. coli* cells seemed to indicate that cells clumped as readily as *Ent. faecium*. However, the aggregated appearance of the *E. coli* cells was showed that the cells were in mid-division phase. Utilizing the zoom function in AxioVision software, the images were examined more closely to observe clumping at higher magnification. Individual *Ent. faecium* cells showed distinct delineation when clumping, only few *E. coli* cells showed the same pattern (Figures 2.2 and 2.3). Most *E. coli* cells were observed to be unassociated with other cells. Conversely, *Ent. faecium* frequently clumped and sometimes exhibited formation of chains of three or more cells, a phenomenon not observed while investigating *E. coli* cells. Although this analysis is qualitative, perhaps it indicates nature of the *Ent. faecium* cell wall and its capacity to adhere to other cells more readily than *E. coli*.

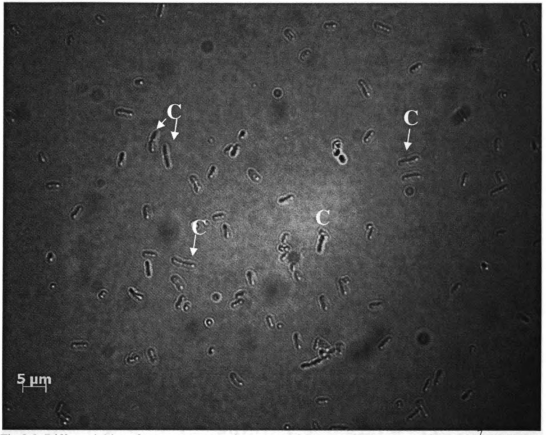

Fig 2.2. Differential interference contrast microscopy of *E. coli* cells (concentration of 10^7 CFU/ml) at 100X resolution. C indicates clumping of two cells.

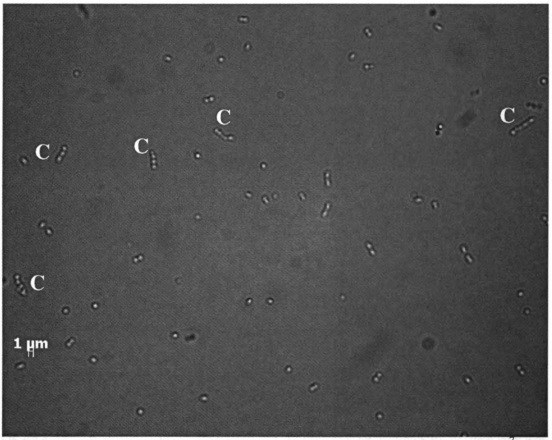

Fig 2.3. Differential interference contrast microscopy of *Ent. faecium* cells (concentration of 10^7 CFU/ml) at 100X resolution. C indicates *Ent. faecium* chains longer than two cells.

2.3 Flow chamber experiments. A glass bead matrix chamber system was designed to include a pore extraction chamber (PEC) to characterize *E. coli* and *Ent. faecium* transport through soil aggregates (Fig 2.4.) (Hyen et al., manuscript in preparation). Briefly, the system was composed of two chambers, the PEC and the collection chamber. In the PEC chamber, 2cm of sterile glass beads, 1mm in diameter, were overlaid on a single soil aggregate to avoid disruption of soil stability upon application of vacuum. The glass/soil matrix was set on a porous cindered -glass filter with 25 µm pore size to allow bacterial leaching following vacuum extraction. The collection chamber, located at the base of the PEC encased a sterile 2ml centrifuge tube designated for effluent collection and was connected to a vacuum pump (model: RPC-R, Gast, USA) and vacuum

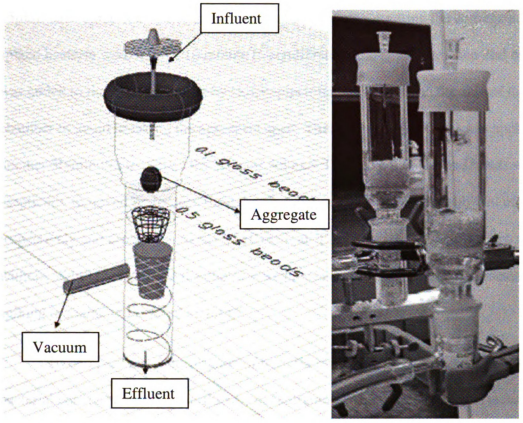

Fig 2.4 a) Glass beat matrix chamber design for investigation of bacterial transport. b) Glass bead matrix experimental setup.

controller (model: CVC2, Vacuubrand, Germany). All flow experiments were conducted at laboratory temperature to observe if motility affected transport (McCaulou and Bales, 1995).

For experiments examining bacterial transport at hydrated soil conditions, the system was saturated through capillary action by placing the PEC in 0.5mM $CaCl_2$ overnight hydrating the soil. Experiments investigating flow at non-saturated conditions did not include this pre-saturation step. Subsequently, -100cm of water vacuum was applied to the system for 30 minutes, to remove excess $CaCl_2$.

Spiking of *E. coli* and *Ent. faecium* was conducted as previously described (see direct *E. coli* and *Ent. faecium* spiking and immediate recovery) with the exception that

25µl of each stock was added to avoid potential for over saturating the aggregate. The spiked bacteria were left for 10 minute to equilibrate in the aggregate, then 2ml of $CaCl_2$ was added to the upper chamber by slowly pipetting the solution on the side of the chamber to avoid breaking the aggregate apart. Then, vacuum (-100cm) was applied for another 30 minutes to collect the effluent volume. The soil aggregate and beads were aseptically removed by forceps from the chamber and placed in 10ml and 9ml of PBW respectively and processed via the VMF method. The extracted effluent was processed without addition of PBW. All samples were processed within 2 hours after bacterial spiking to maintain bacterial viability.

To compare the concentration of bacteria retained in the aggregates against the bacterial concentration in the effluent, concentrations were calculated in the units of total CFUs. For calculating bacteria retained in soil, the CFU enumerated from plate counts were multiplied by the dilution factor and by volume of PBW the aggregate was stored in. To obtain total CFU for the effluent samples, the CFU enumerated from plate counts were multiplied by the dilution factor and by the effluent volume. Because the influent volume was different for *E. coli* and *Ent. faecium*, values had to be adjusted for statistical analysis. This was achieved by converting bacterial concentrations retained in soil and concentrations in the effluent to a ratio by dividing these concentrations by the total influent concentrations.

2.4 Aggregate peeling for native bacteria enumeration. For analysis of the spatial variation of native bacteria, the soil aggregates were peeled into three layers: exterior, transitional and inner. Only the exterior and inner layers were analyzed for native bacterial concentrations. The logic was to determine if there was a significant difference

between the two layers initially which may warrant further investigation and analysis of bacterial concentration in the transitional layer. The aggregates were peeled using soil erosion chambers (SAE) developed by Dr. Alvin Smucker and described in detail in Park and Smucker (2005) (Fig. 2.5). At the onset of soil aggregate peeling a pre-weighed, single aggregate was placed on a support screen and the top of the SAE chamber was covered with heavy duty aluminum foil. The interior wall portion of the SAE chamber was precisely machined into a uniformly knurled surface which eroded the rotating aggregate. A 350 μm-opening support screen was welded to the base of the chamber through which finely eroded soil materials dropped into the retainer of the base of the SAE. The entire SAE chamber was placed onto a spring mount onto a rotary shaker platform (Innova, Model 2300, New Brunswick Scientific Inc., Edison, NJ) and rotated at speeds ranging from 200 to 400 rpm. Rotational motion of the chamber generated frictional forces at the surface interface of each aggregate. Sequentially, 1/3 and 2/3 (exterior and transitional layers, respectively) by weight of soil aggregates were peeled and weighed. Because of the decrease of erosion rate with aggregate peeling, the peels needed to be weighed several times to assure that 1/3 and 2/3 of soil aggregates were peeled. Each concentric layer was placed in 15ml centrifuge tube containing 10ml of PBW and processed using vortexing and membrane filtration as previously described.

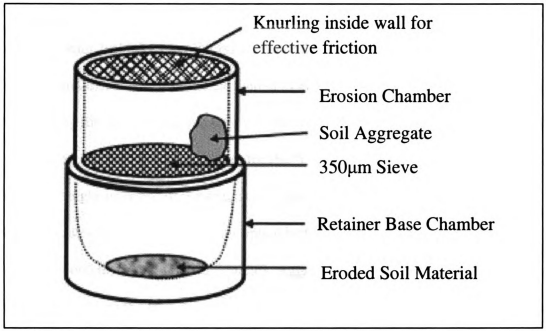

Fig 2.5. SAE chamber and retainer base used for aggregate peeling and collection (Park and Smucker, 2005).

2.5 Slicing and saturation experiments. These experiments were designed to understand the spatial distribution of spiked *E. coli* added to aggregates that had different soil moisture contents prior to added *E.coli*. Sterile DI water was applied to soil treatments T1, T2 and T7 to add 15, and 30% soil moisture contents by weight. The 15% moisture content aggregates had 16μl of DI water added and the 30% moisture content aggregates had 33μl of DI water added. The aggregates defined as 0% moisture content aggregates were air-dry aggregates that did not have any DI water added prior to spiking *E. coli*. *E. coli* is widely accepted as the primary indicator in agricultural soils and due to the fact that this was an exploratory examination of spatial distribution, *E. coli* was used for these experiments.

Calculating volumes that would be applied to the aggregates to add the specified moisture contents was achieved by preliminary weighing experiments. Five replicates of

T1, T2 and T7 air-dry aggregates were placed in petri dishes and presumed to be "fully" saturated with 1ml DI water. Excess DI water that did not saturate the aggregates was pipetted and discarded and then the weight of each aggregate was measured again. Subtracting the air-dried aggregate's weight from the saturated aggregate's weight yielded the amount of water in grams for the "fully" saturated air-dry aggregates. The average weight in grams of water to attain a presumed "full" saturation from each of the five replicates per treatment was multiplied by 0.15 and 0.30 to achieve 15% and 30% moisture contents for slicing experiments. Aggregates were allowed to equilibrate for 2 hours after adding the specified volume and kept inside closed containers within beakers containing water to avoid evaporation. In these experiments, the aggregates were hydrated to 0% (kept dry), 15% (using 16µl) and 30% (using 33µl). Subsequently, *E. coli* was added at a concentration of approximately 10^3 CFU/aggregate by seeding 50µl of the *E. coli* culture dilution and the aggregate was cut aseptically into seven pieces. Each subsection was assigned a number in reference to where the *E. coli* was added. Subsections 1, 2, 3, 4, and 5 correspond to the top, right, left, back and front of the aggregate, respectively (Figure 2.6). The middle section was divided into two subsections 6 and 7 which correspond to the center-middle and the bottom-middle respectively (Fig 2.7).

After slicing, each subsection was weighed and immediately placed in 1ml of PBW to prevent *E. coli* die-off. All samples were processed with the VMF method within four hours to avoid variability; in the meantime they were stored at 4^0C. Triplicate membrane filtered samples were placed on m-Endo agar and incubated at 35 ± 0.5^0C for

24 hours. *E. coli* CFU recovery per gram was calculated by the attained CFU value of subsection divided by the subsection weight.

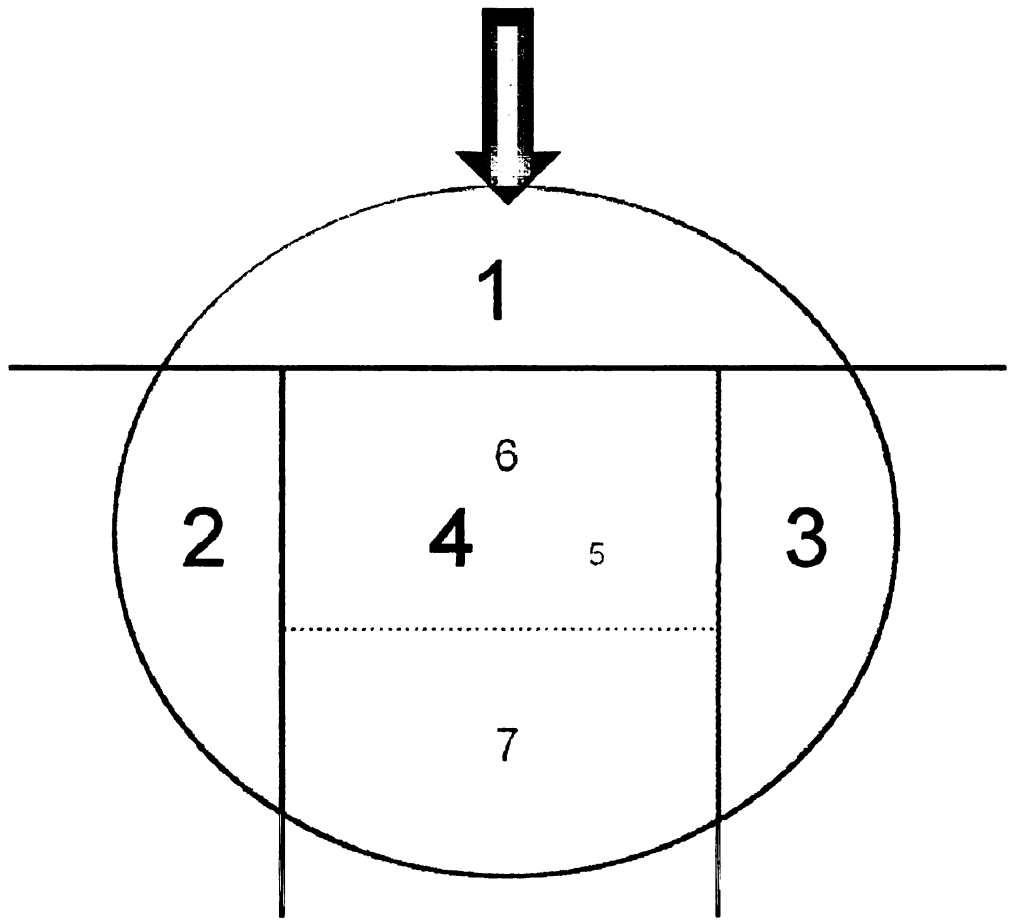

Fig. 2.6. Frontal view of the aggregate subsections. The arrow indicates the location where *E. coli* was spiked. The numerical designation of the aggregate subsections correspond to the spatial position of aggregate slicing. The larger font and darker toned numbers indicate that the sections are closer dimensional depth.

2.6 Statistical analysis. Average and standard deviation values for all experimental analyses were calculated using Microsoft Excel 2007. Two-way analysis of variance (ANOVA) was conducted to evaluate statistical significance between native bacterial concentrations in T1, T2 and T7 aggregate treatments (native bacterial extraction; section 2.2.2), and for *E. coli* and *Ent. faecium* recovery ratios in T1 and T7 aggregates (*E. coli* and *Ent. faecium* spiking and immediate recovery experiments; section 2.2.3). Three-way

ANOVA was conducted on *E. coli* and *Ent. faecium* aggregate and effluent ratios in T1, T2 and T7 aggregates (flow-chamber experiments; section 2.3) and log-transformed *E. coli* recoveries from aggregate slices at 0, 15, 30% moisture contents in T1, T2 and T7 aggregates (slicing and saturation experiments; section 2.5). Because the data set contained values of zero, a value of 1 was added to the slicing and saturation data to perform log-transformations. Levene's test was used in all ANOVA to check unequal variances. Tukey's test was used for pair-wise comparisons between variables in slicing and saturation experiments and flow chamber experiments. The Akaike and Bayesion criteria were used to determine the goodness of fit model for grouped data in slicing and saturation experiments and flow chamber experiments. All statistical analyses were performed with assistance from the College of Agriculture and Natural Resource (CANR) Statistical Consulting Center using SAS Version 9.2 (SAS Institute, NC).

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Chapter 3.

Results and Discussion

3.1 Native Bacterial Concentrations

3.1.1 Native bacterial concentrations in dry soil aggregates. Initial experiments were designed to determine the levels of native bacterial populations and background levels of *E. coli* and *Ent. faecium* prior to running the spiking and immediate recovery experiments (section 3.2). Whole aggregates were evaluated for the enteric bacteria. No growth on m-Endo media (n=5) and mEI media (n=3) with any aggregate from all soil treatments indicated there were no viable coliforms or *Enterococcus* spp. in air-dried aggregates. This may be attributed to the extended storage time of the soil aggregates at laboratory conditions where the bacteria either died due to desiccation stress or underwent a metabolic shift to a viable but non-culturable state (Rozak and Colwell, 1987). There was, however, growth on TSA plates indicating presence of heterotrophic bacteria (HPC). Treatments T1, T2 and T7 contained an average of $3.02x10^5$, $3.05x10^5$ and $3.76x10^5$ CFU/ g (Fig. 3.1) with standard deviations of $8.67\ x10^4$, $1.22\ x10^5$ and $1.60x10^5$ respectively. These differences were not significant ($p>0.05$).

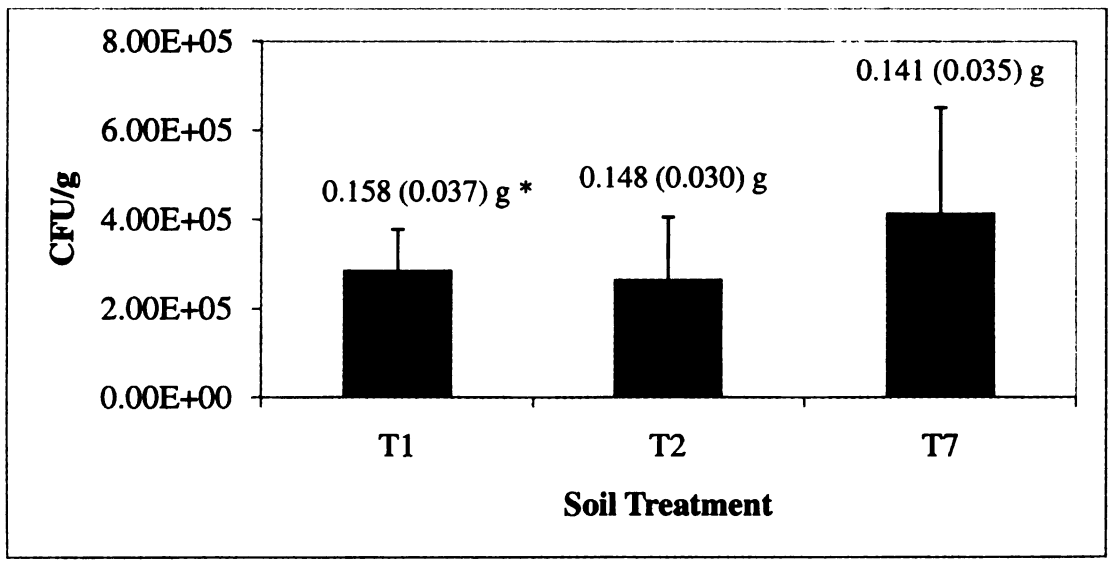

Fig 3.1. Native bacterial concentrations in aggregate treatments from T1, T2 and T7 (n=5) treatments.
* The values above each bar indicate the average weight (g) of each aggregate for each soil treatment. The values in parentheses indicate the standard deviations.

3.1.2 Effect of settling on bacterial extraction. Compiling native bacterial extraction data from whole soil aggregate treatments T1, T2 and T7 indicated a negative correlation between aggregate weight and bacterial extraction (n=15) (Fig. 3.2). It can be observed that as the soil aggregate's weight increased, the HPC/ g decreased. Arriving at such a relationship may be explained by the sorption of native bacteria to the soil particulates, the heavier of which settles much quicker in solution, thereby leading to a decreased detection and underestimation of actual viable bacteria (Richaume et al., 1993, Mahler et al., 2000). Thus an evaluation of the effect of settling on bacterial extraction and enumeration was undertaken. The T7 soil treatment was used as a proxy for all the treatments since it was shown that their bacterial concentrations were not significantly different (see section 3.1.1). Processing of two replicates of soil aggregates yielded an average of 2.83×10^6 CFU/g of native heterotrophic bacteria when the aggregate was dissolved in PBW, vortexed and assayed without settling; a 1-log increase as compared to extraction with a 20-minute settling step. This illustrated that viable bacteria adhered to soil particulates very strongly after being stored at dry conditions, and even after extensive vortexing, this attachment was not disrupted.

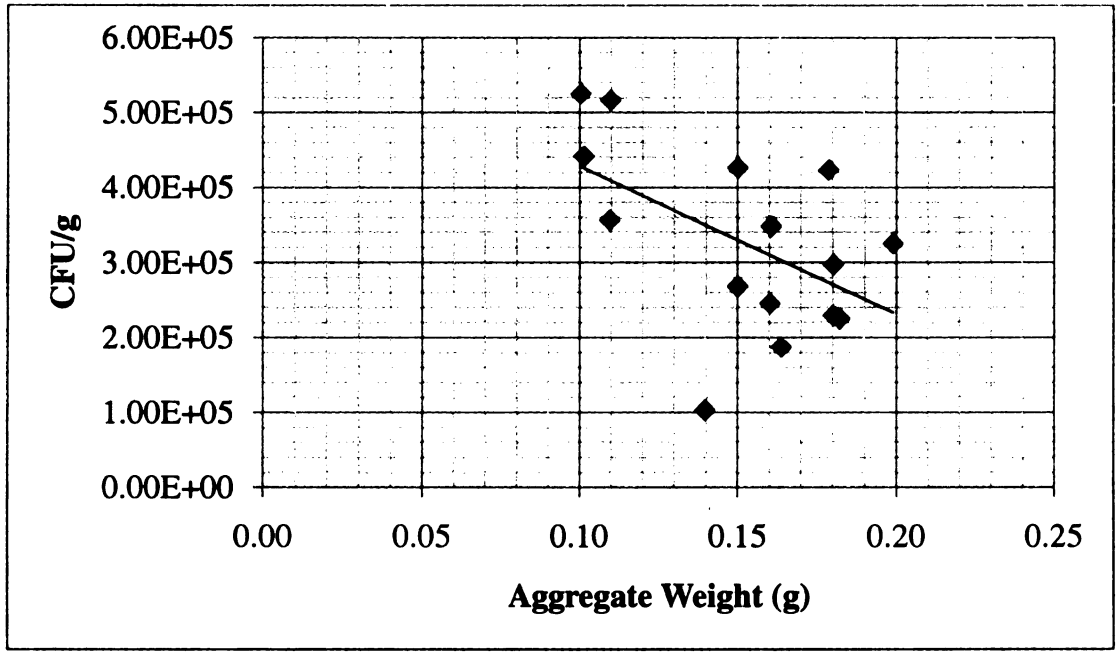

Fig 3.2. Comparison of aggregate weight from treatments T1, T2 and T7 to native bacterial colony forming units per gram of soil with a trend line (n=15).

3.2 Spiking and Immediate Recovery

3.2.1 *E. coli* and *Ent. faecium* spiking and immediate recovery. A gradient of concentrations was used to determine the detection limit for the VMF method after fully saturating the aggregate with both *E. coli* and *Ent. faecium*. T1 and T7 aggregates were spiked in triplicates and recovery rates and standard deviations were calculated after processing. The combined average of all concentrations for *E. coli* in the T1 treatment for was 108% (standard deviation= 37%) (Table 3.1). This indicated very high recovery rates could be achieved with concentrations as low as 6 CFU/ soil aggregate. Similarly, seeded T7 soil aggregates had very high recovery rates. The total percent recovery for the combined average of all concentrations was 92% (standard deviation= 31%) (Table 3.2).

Using the same procedure of spiking and processing for *Ent. faecium*, recovery rates for T1 and T7 were 97% (standard deviation=41%) and 119% (standard deviation=57%) (Tables 3.3 and 3.4). Unlike *E. coli*, average *Ent. faecium* recoveries at the lowest concentrations for T1 and T7 were below 100%. In fact, two of the replicates using T1 did not yield detectable CFUs at the low spike. This is possibly due to a lower spike of *Ent. faecium* as compared to *E. coli* or a reflection of the harsher, selective media conditions of mEI as compared to m-Endo (m-Endo being a more general growth media for coliforms).

Furthermore, *Ent. faecium* yielded higher total standard deviation values than *E. coli*. At lower concentrations this high variability may have been because *Ent. faecium* was added at approximately 3 CFU/ aggregate as compared to *E. coli* which was seeded at approximately 8 CFU/ aggregate. This is supported by recovery rates of *Ent. faecium* in T1 soil, where the detection limit was reached at the lowest concentration (Table 3.3). On the other hand, clumping and formation of chains by *Ent. faecium* cells may have caused error in CFU counts (Jennison and Wadsworth, 1940). Clumping is caused by cells attaching to each other through interactions of the extracellular components of their cell wall (Wildy and Anderson, 1964, Singh and Vincent, 1987, Hogt et al., 1986). Microscopic observations (section 2.2.7) support this assumption, indicating that *Ent. faecium* did indeed clump more readily than *E. coli*.

Table 3.1. Spiked CFU/ aggregate counts and percentage of *E. coli* recovery in T1 soil aggregates.

Replicate	Bacteria Seeded CFU/ soil Aggregate[a]	Bacteria Recovered CFU/ soil Aggregate	Percent Recovery
1	9.50	$1.33x10^1$	140%
2	7.17	$1.33x10^1$	186%
3	7.67	6.67	87%
$\bar{x}$[b]	8.11		138%
S[c]	1.23		50%
1	$7.33x10^1$	$5.33 x10^1$	73%
2	$7.67 x10^1$	$5.33 x10^1$	70%
3	$7.67E x10^1$	$1.07 x10^2$	139%
$\bar{x}$	$7.56E x10^1$		94%
S	1.92		39%
1	$7.00x10^2$	$6.17x10^2$	88%
2	$6.17x10^2$	$6.77x10^2$	110%
3	$7.00x10^2$	$5.43x10^2$	78%
$\bar{x}$	$6.72x10^2$		92%
S	$4.81x10^1$		16%
1	$1.08x10^3$	$1.75x10^3$	162%
2	$6.67x10^3$	$5.83x10^3$	88%
3	$6.83x10^3$	$9.53x10^3$	140%
$\bar{x}$	$4.86x10^3$		130%
S	$3.27x10^3$		38%
1	$9.83x10^4$	$6.77x10^4$	69%
2	$5.00x10^4$	$5.23x10^4$	105%
3	$5.00x10^4$	$4.47x10^4$	89%
$\bar{x}$	$6.61x10^4$		88%
S	$2.79x10^4$		18%
Total $\bar{x}$			108%
Total s			37%

a. All aggregates were processed immediately at laboratory temperature, using PBW as a diluent. Averages and standard deviation for each concentration and the recovery are calculated for all aggregates.
b. '$\bar{x}$' denotes the calculated mean for CFU/ aggregate and recovery percentages.
c. 's' denotes the calculated standard deviation for CFU/ aggregate and recovery percentages.

Table 3.2. Spiked CFU/ aggregate counts percentage of *E. coli* recovery in T7 soil aggregates.

Replicate	Bacteria Seeded CFU/ soil Aggregate	Bacteria Recovered CFU/ soil Aggregate	Percent Recovery
1	4.40	3.33	76%
2	5.50	3.33	61%
3	9.25	1.50×10^{1}	162%
$\bar{x}$	6.38		100%
s	2.54		55%
1	5.70×10^{1}	5.67×10^{1}	99%
2	9.25×10^{1}	5.67×10^{1}	61%
3	9.50×10^{1}	4.00×10^{1}	42%
$\bar{x}$	8.15×10^{1}		68%
s	2.13×10^{1}		29%
1	5.70×10^{2}	4.83×10^{2}	85%
2	8.09×10^{2}	8.67×10^{2}	107%
3	7.83×10^{2}	8.00×10^{2}	102%
4	6.67×10^{2}	9.33×10^{2}	140%
$\bar{x}$	7.07×10^{2}		109%
s	1.11×10^{2}		23%
1	3.73×10^{3}	4.57×10^{3}	123%
2	3.73×10^{3}	4.53×10^{3}	122%
3	9.50×10^{3}	7.33×10^{3}	77%
$\bar{x}$	5.65×10^{3}		107%
s	3.33×10^{3}		26%
1	5.17×10^{4}	4.60×10^{4}	89%
2	6.75×10^{4}	6.20×10^{4}	92%
3	9.33×10^{4}	6.87×10^{4}	74%
4	9.33×10^{4}	5.43×10^{4}	58%
$\bar{x}$	7.65×10^{4}		78%
s	2.05×10^{4}		16%
Total $\bar{x}$			92%
Total s			31%

Table 3.3. Spiked CFU/ aggregate counts percentage of *Ent. faecium* recovery in T1 soil aggregates.

Replicate	Bacteria Seeded CFU/ soil Aggregate	Bacteria Recovered CFU/ soil Aggregate	Percent Recovery
1	1.00	1.00	100%
2	1.67	N.D.*	-
3	5.67	N.D.	-
$\bar{x}$	2.78		-
S	2.52		-
1	2.38×10^1	2.00×10^1	84%
2	3.03×10^1	4.33×10^1	143%
3	4.87×10^1	4.33×10^1	89%
$\bar{x}$	3.43×10^1		105%
S	1.29×10^1		33%
1	2.70×10^2	1.98×10^2	73%
2	4.25×10^2	4.00×10^2	94%
3	3.27×10^2	3.63×10^2	111%
$\bar{x}$	3.41×10^2		93%
S	7.84×10^1		19%
1	2.67×10^3	2.83×10^3	106%
2	3.15×10^3	5.23×10^3	166%
3	3.42×10^3	3.88×10^3	114%
$\bar{x}$	3.08×10^3		129%
S	3.80×10^2		33%
1	2.58×10^4	3.67×10^4	142%
2	3.47×10^4	4.00×10^4	115%
3	3.85×10^4	4.50×10^4	117%
$\bar{x}$	3.30×10^4		125%
S	6.50×10^3		15%
Total $\bar{x}$			97%
Total s			41%

* N.D. indicates that bacterial concentrations could not be detected.

Table 3.4. Spiked CFU/ aggregate counts percentage of *Ent. faecium* recovery in T7 aggregates.

Replicate	Bacteria Seeded CFU/ soil Aggregate	Bacteria Recovered CFU/ soil Aggregate	Percent Recovery
1	1.67	1.00	60%
2	1.00	1.00	100%
3	5.67	2.00	35%
$\bar{x}$	2.78		65%
S	2.52		33%
1	$2.38Ex10^{1}$	$4.67x10^{1}$	196%
2	$3.03x10^{1}$	$5.67x10^{1}$	187%
3	$4.87x10^{1}$	$3.00x10^{1}$	62%
$\bar{x}$	$3.43x10^{1}$		148%
S	$1.29x10^{1}$		75%
1	$2.70x10^{2}$	$3.55x10^{2}$	131%
2	$4.25x10^{2}$	$3.93x10^{2}$	93%
3	$3.27x10^{2}$	$4.67x10^{2}$	143%
$\bar{x}$	$3.41x10^{2}$		122%
S	$7.84x10^{1}$		26%
1	$2.67x10^{3}$	$4.90x10^{3}$	184%
2	$3.15x10^{3}$	$4.35x10^{3}$	138%
3	$3.42x10^{3}$	$4.05x10^{3}$	119%
$\bar{x}$	$3.08x10^{3}$		147%
S	$3.80\ x10^{2}$		33%
1	$2.58x10^{4}$	$4.33x10^{4}$	168%
2	$5.00x10^{4}$	$3.47x10^{4}$	69%
3	$3.85x10^{4}$	$3.67x10^{4}$	95%
$\bar{x}$	$3.81x10^{4}$		111%
S	$1.21x10^{4}$		51%
Total $\bar{x}$			119%
Total s			57%

At lower concentrations both bacterial species recovered from T1 and T7 treated aggregates exhibited higher standard deviations (Fig. 3.3 and 3.4). This trend may be explained from a methodological perspective. It has been established that in microbiological plate counts, decreasing bacterial concentrations result in higher counting error (Breed and Dotterrer, 1916). Additionally, at lower concentrations there was more sediment present on the media. Olsen and Bakken have observed a similar decrease of CFU/g counts as amounts of soil per plate increased (1987). From experimental observations, the sediment altered the typical spherical morphology to an irregular shape that, when colonies were in close proximity made it difficult to discern delineations between them. It also may be possible that the high density of soil on the membrane filter inhibited bacteria nutrient acquisition and growth.

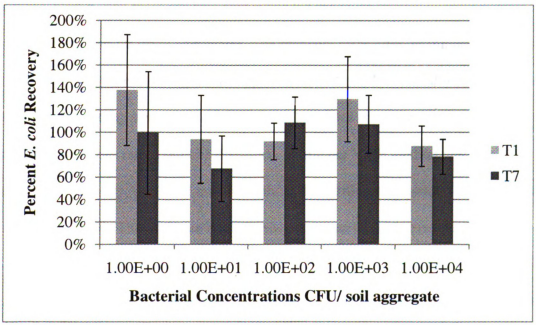

Fig 3.3. The averaged percent recoveries and standard deviations of *E. coli* in T1 (n=3) and T7 (n=3; n=4 at $1x10^2$ and $1x10^4$ concentrations).

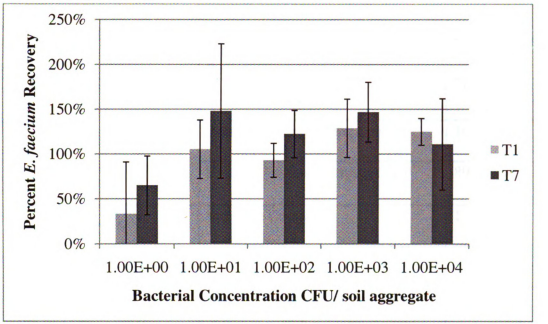

Fig 3.4. The averaged percent recoveries and standard deviations of *Ent. faecium* in T1 (n=3) and T7 (n=3) soil treatment.

3.2.2 Desiccation and *E. coli* recovery in whole aggregates. After 30 minutes of air-drying, high *E. coli* recovery persisted while the moisture content slightly decreased as compared to non-air dried aggregates. The recovery rates after 30 minutes were not significantly different for experiments processed with VMF method with settling and without the settling steps (Table 3.5). After 60 minutes of air-drying, however, very different recovery rates could be discerned. While the VMF method with settling yielded no *E. coli* recovery, the VMF method without the settling step yielded an average recovery of 49%. The moisture content was compared to the recovery rates, and a steep decline of *E. coli* recovery could be ascertained from air-dried aggregates processed with the VMF method with settling compared to the aggregates that were processed without the settling step (Fig. 3.5 and 3.6).

Table 3.5. Comparison of the VMF method with the settling step and without the settling step.

Air-drying Time	Effect of Desiccation and Attachment on *E. coli* Recovery	
30 minutes	Moisture Content	15% (n=4)
	Recovery with Settling Step	103% (n=2)
	Recovery without Settling Step	89% (n=2)
60 minutes	Moisture Content	1.5 % (n=4)
	Recovery with Settling Step	0% (n=2)
	Recovery without Settling Step	49% (n=2)

* 50µl *E. coli* was spiked in all aggregates at a concentration of approximately 10^4 CFU/ aggregate. All aggregates used for these experiments were T7.

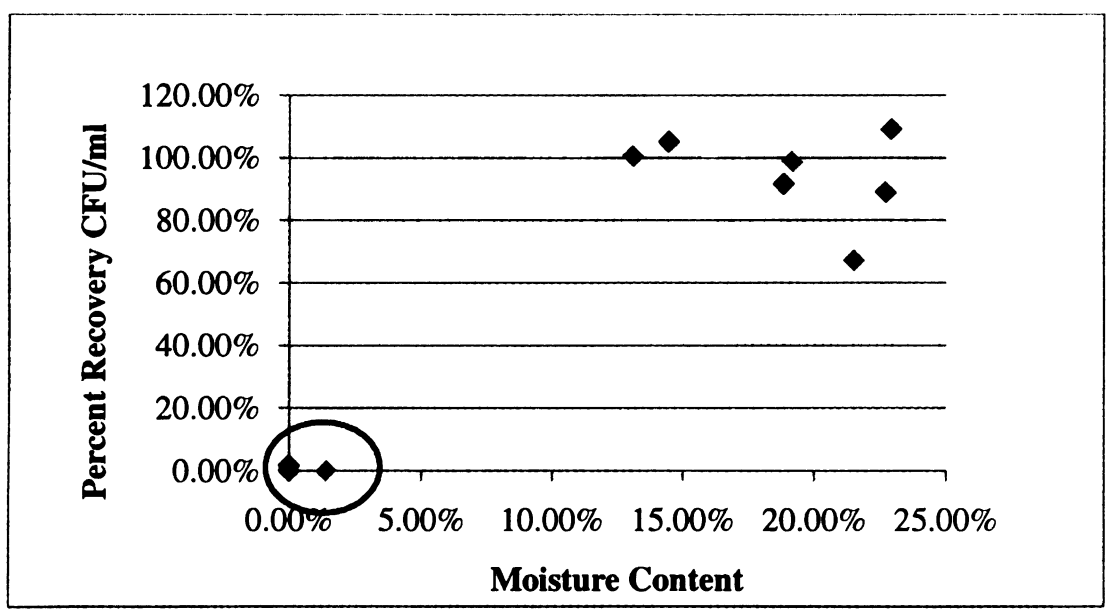

Fig 3.5. Recovery of spiked *E. coli* as a function soil moisture content when processing the whole aggregate using the VMF method with a settling step (n=13).
*The circled area highlights the steep decline of *E. coli* recovery at the lower moisture content while using the VMF method with a settling step.

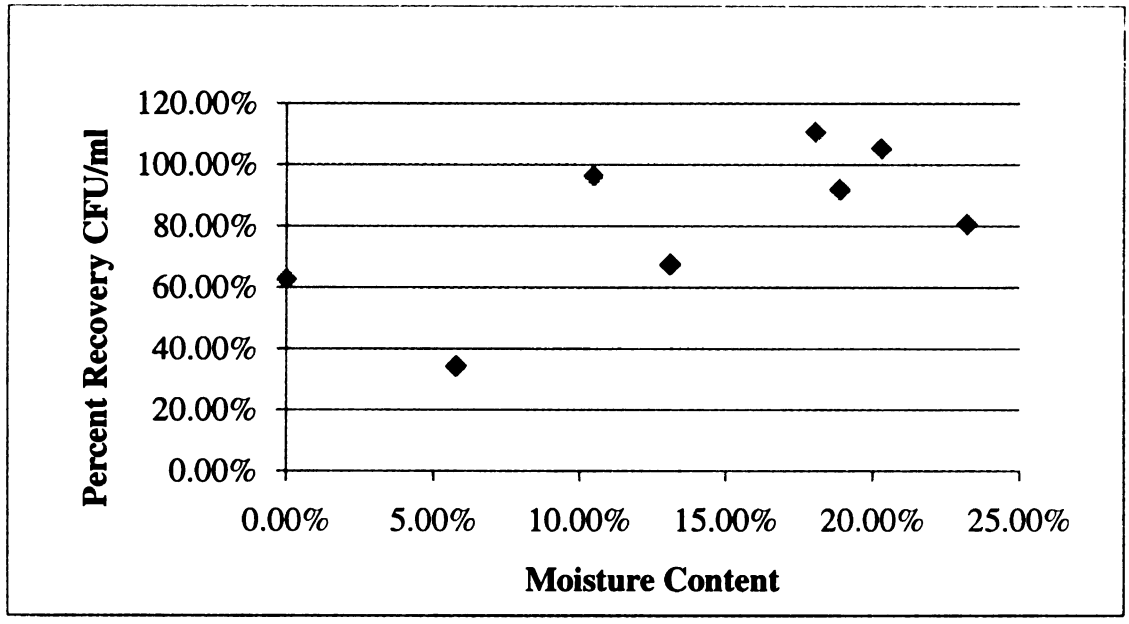

Fig 3.6. Recovery of spiked *E. coli* as a function soil moisture content when processing the whole aggregate using the VMF method without settling step (n=8).

This converges with observed adsorption of native bacterial in air-dry soil aggregates stored at room temperature which caused the underestimation of the actual viable bacterial counts (see section 3.1.3). It may be that after extended air-drying (desiccation stress) and extended contact with the soil, the spiked *E. coli* adsorbed to the soil, thus making it difficult to extract them, even with vigorous shaking (Huysman and Vestraete, 1993a). To avoid underestimation in further experiments, the settling step was excluded from the VMF aggregate processing.

The experimental evaluations of native heterotrophic bacteria (section 3.1) and spiking experiments with *E. coli* and *E. faecium* (section 3.2), indicated that the bacterial extraction method developed did provide high and consistent recoveries of bacteria from aggregates. The effects of desiccation, clumping and bacterial adhesion to soil particles were addressed in order to explain the inherent methodological errors that may occur while employing this technique. Other factors such as native bacterial resuscitation

and the effect of $CaCl_2$ on *E. coli* viability have also been addressed (sections 2.2.5 and 2.2.6) and were shown not to have an impact on the method using dried soil aggregates.

3.3. Flow Chamber Experiments

Flow experiments were designed to examine retention and transport of *E. coli* and *Ent. faecium* within T1, T2 and T7 aggregates. The investigation was conducted using air-dry (unsaturated) aggregates and saturated aggregates to observe if moisture affected the transport of either bacterial species. Transport of bacteria was evidenced by the enumeration of bacterial species in the effluent. Conversely, retention was illustrated by determining the bacterial concentrations remaining in the soil aggregate. Bacterial concentrations in effluent and bacterial concentrations retained in the aggregate were computed as log of total CFU to be able to conduct comparisons between samples.

The most apparent influence on bacterial retention and transport appeared to be due to soil saturation. At air-dry conditions, retention was high for all soil treatments and bacteria types (Figure 3.7). T1 had the highest bacterial retention capacity and no *E. coli* or *Ent. faecium* were detected in the effluent. However, the difference in retention between soil treatments was not shown to be statistically different ($p<0.05$). T2 and T7 aggregates did not retain *E. coli* and *E. faecium* as readily as T1, however the bacterial concentrations in soil aggregates were approximately 3-log higher than the concentrations in the effluent (Table 3.6). It is thought that at dry soil conditions, preferential flow occurs within smaller pores carrying the bacteria with the solution, causing them to be filtered and thus, more readily retained in the soil (Hattori, 1988, Stevik et al., 2004). Furthermore, at lower soil moisture content the bacteria may adhere to the soil particles due to reduced water-microbe interaction and thus increased contact with the solid soil

substratum (Huysman and Vestraete, 1993, Guber et al., 2009). These hypotheses are supported when comparing bacterial effluents for all soil treatments using saturated aggregates (Figure 3.8). For example, T1 treatment exhibited the complete opposite effect when compared to air-dry conditions. Effluent concentrations for *E. coli* and *Ent. faecium* were 2.7 and 1.6 log CFU/aggregate, respectively. Therefore, at higher moisture content, bacteria may have had less contact with the solid-phase soil and transported more readily when the soil was saturated.

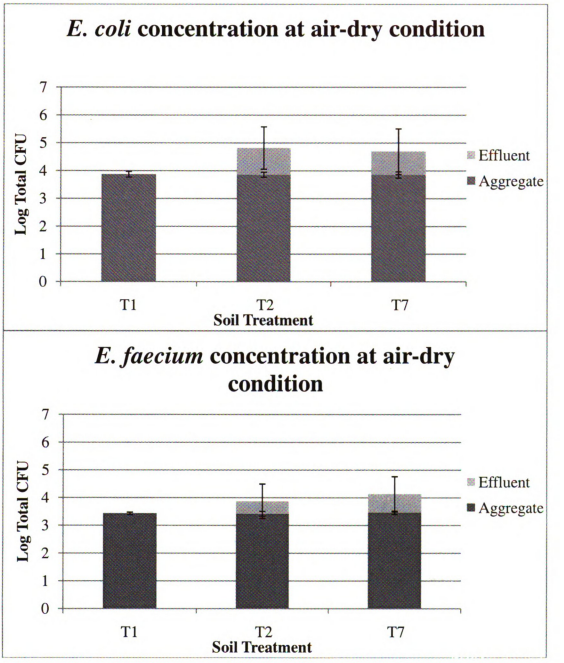

Fig 3.7. Recovery of *E. coli* and *Ent. faecium* from T1, T2, T7 aggregates and effluents at air-dry conditions (n=5).

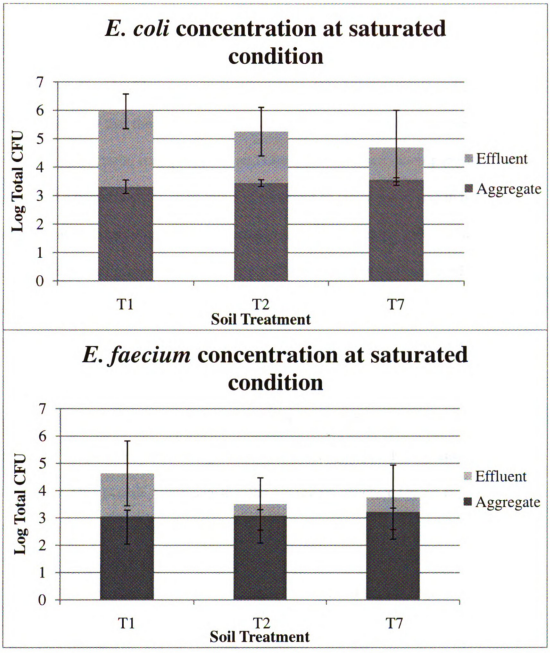

Fig 3.8. Recovery of *E. coli* and *Ent. faecium* from T1, T2, and T7 aggregates and effluents at saturated conditions (n=5).

The average effluent concentrations indicate that soil treatment was also another variable that influenced the retention and transport of bacteria. While at air-dry conditions there was no detection of bacterial concentrations in the T1 effluent, in T2 and

T7 soils, the concentrations in the effluents for *E. coli* were 0.9 and 0.8 log CFU/ aggregate (Table 3.6). On the other hand, under saturated aggregate conditions for T1 soil, higher *E. coli* effluent concentrations were detected compared to T2 and T7 (Table 3.7). It is likely that the difference in soil macroporosity may have caused the observed variation in bacterial transport (Unc and Goss, 2003). When macroporosity of soil is higher, the bacteria are filtered less and observed to leach out of soil in higher concentrations then when soil is saturated (Abu-Ashour et al., 1998). This is supported by X-ray microtomography analysis of the soil's macropores that indicated T1 had significantly more macropores than T7 (Wang et al., 2010). However, it should be noted that when examining the individual effluent replicate results, T1 had considerable variation in effluent concentrations for both bacteria types (Tables 3.8 and 3.9, first columns). While T7 was also observed to have high variability, this was due to one replicate which was considerably different than the rest (Tables 3.8 and 3.9, fifth columns). This possibly indicates that the aggregate structure for T7 is more uniform than T1's aggregate structure.

Table 3.6. Concentration of *E. coli* and *Ent. faecium* in the influent and T1, T2 and T7 aggregates and effluents (n=5).

E. coli Recoveries in Air-dry Soil			
Sample \ Soil Treatment	T1	T2	T7
Aggregate[a]	3.9	3.9	3.9
Effluent	N.D[b]	0.9	0.8
Influent	3.6	3.6	3.6
Ent. faecium Recoveries in Air-dry Soil			
Sample\Soil Treatment	T1	T2	T7
Aggregate	3.4	3.4	3.5
Effluent	N.D.	0.5	0.7
Influent	3.1	3.1	3.1

a. Aggregate, effluent, and influent concentrations were calculated as log total CFU.
b. N.D. indicates that bacterial concentrations could not be detected.

Table 3.7. Concentration of *E. coli* and *Ent. faecium* in the influent and T1, T2 and T7 aggregates and effluents (n=5).

E. coli Recoveries in Saturated Soil			
Sample\Soil Treatment	T1	T2	T7
Aggregate	3.3	3.4	3.5
Effluent	2.7	1.8	1.1
Influent	3.6	3.6	3.6
Ent. faecium Recoveries in Saturated Soil			
Sample\Soil Treatment	T1	T2	T7
Aggregate	3.0	3.1	3.2
Effluent	1.6	0.4	0.5
Influent	3.1	3.1	3.1

Table 3.8. The percent recovery of *E. coli* in effluents for T1, T2 and T7 aggregates replicates at saturated and non-saturated conditions (n=5).

	T1		T2		T7	
Replicate	Saturated	Non-Saturated	Saturated	Non-Saturated	Saturated	Non-Saturated
1	23%	N.D*	N.D	N.D	N.D	N.D
2	41%	N.D	N.D	N.D	N.D	N.D
3	10%	N.D	4%	N.D	N.D	N.D
4	2%	N.D	16%	N.D	2%	1%
5	50%	N.D	16%	1%	26%	N.D
x□	25%	-	7.2%	0.2%	5.6%	0.2%
s	20%	-	8.2%	0.5%	11%	0.5%

*N.D. indicates that there were no bacteria detected. N.D. values were treated as zeros to calculate averages (x□) and standard deviations (s).

Table 3.9. The percent recovery of *Ent. faecium* in effluents for T1, T2 and T7 aggregates at saturated and non-saturated conditions (n=5).

	T1		T2		T7	
Replicate	Saturated	Non-Saturated	Saturated	Non-Saturated	Saturated	Non-Saturated
1	1%	N.D	N.D	N.D	N.D	1%
2	47%	N.D	N.D	N.D	N.D	1%
3	1%	N.D	N.D	N.D	N.D	N.D
4	0%	N.D	N.D	N.D	N.D	N.D
5	41%	N.D	21%	1%	28%	N.D
x□	18%	-	4.2%	0.2%	5.6%	0.4%
s	24%	-	9.4%	0.5%	13%	0.5%

Three-way ANOVA - using the variables soil saturation, soil treatment, and bacterial species – indicated that there was a two-way interaction between soil saturation and soil treatment but not bacterial species. The response variable used was the ratio of bacterial effluent concentration to total influent concentration to correct for the difference between *E. coli* and *Ent. faecium* influent concentrations for the various experiments. Grouping by soil saturation indicated the best goodness of fit criteria (using the Akaike and Byesian criteria). Statistical significance was only observed in the T1 treatment between the saturated and dry aggregate (Figure 3.9). This conforms to the graphically illustrated results indicating that higher aggregate saturation and tillage increased the average bacterial effluents.

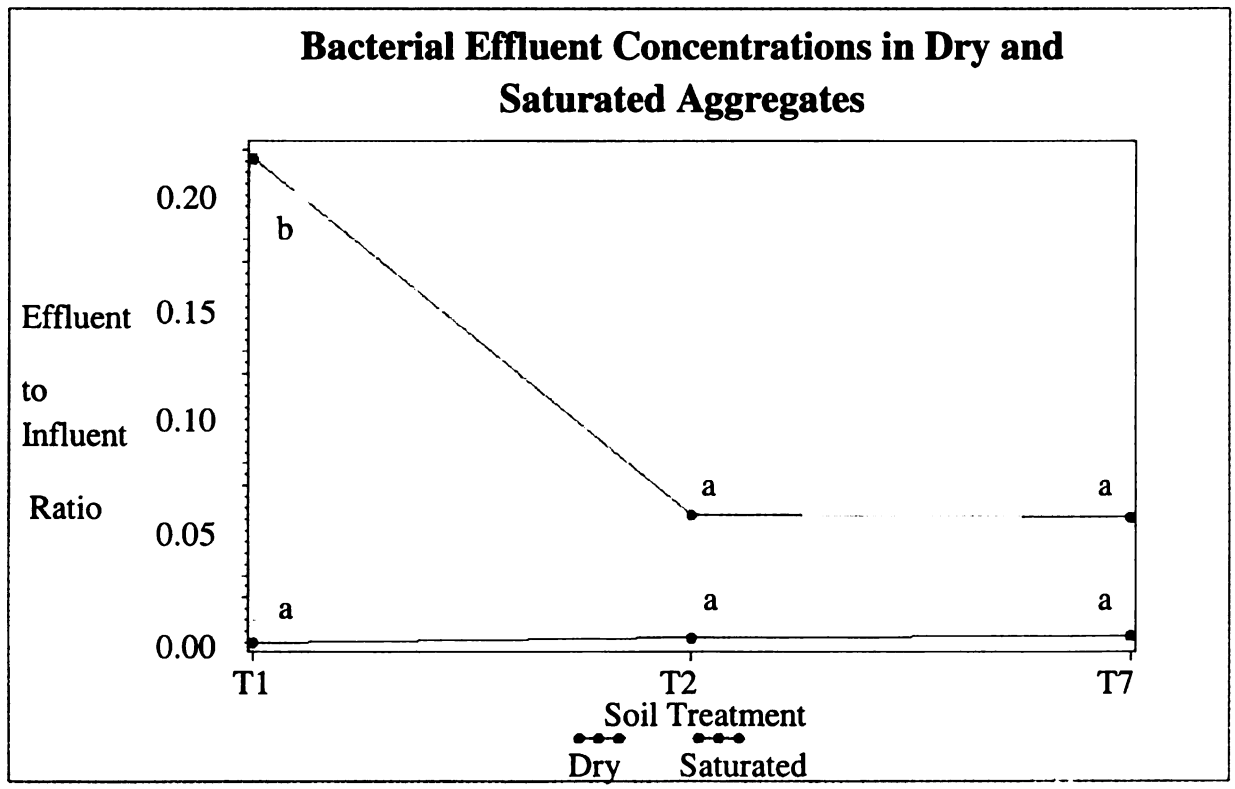

Fig 3.9. Statistical analysis of the interaction between soil treatment and soil saturation combining both bacterial species. Different letters in the effluent to influent ratio at different soil saturation indicate that they are significantly different ($p<0.05$).

3.4 Bacterial Spatial Distribution

3.4.1 Native bacterial concentrations in soil aggregate interior and exterior layers.

Differences between native bacterial counts from the exterior and interior soil layers of T1, T2 and T7 were minute. T1, T2 and T7 contained an average of $1.02x10^6$, $3.78x10^5$ and $9.45x10^5$ CFU/g in the exterior layer, while the interior soil layers contained $8.28x10^5$, $3.26x10^5$ and $8.43x10^5$ CFU/g respectively. Although it is generally accepted that the interior region of the soil harbors more bacteria than the exterior, our experimental results indicated otherwise (Hattori and Hattori, 1976, Ranjard and Richaume, 2001) (Figure 3.10). It could be that the after such an extended storage period, moisture content even in the interior of the soil was too low to sustain the larger concentration of viable bacteria (Stevik et al., 2004). It also possible that this is the actual representation of natural bacterial distribution of aggregates collected. The soil used for these experiments was sieved after sampling to separate different sized macroaggregates, therefore, we may have processed the macroaggregate fractions that naturally contained a homogenous distribution of native bacteria. The statistical difference of HPC in between soil treatments could not be determined because only two replicates of each layer were analyzed.

When comparing the concentrations of extracted native bacteria from whole T1 and T7 aggregates (see section 3.1.1) to the total native bacterial concentrations of the inner and exterior concentric layers of same soil treatments, the separated aggregates yield approximately 1-log higher concentration. The process of separating the aggregate is thought to be behind the observed increase where bacterial extraction improves

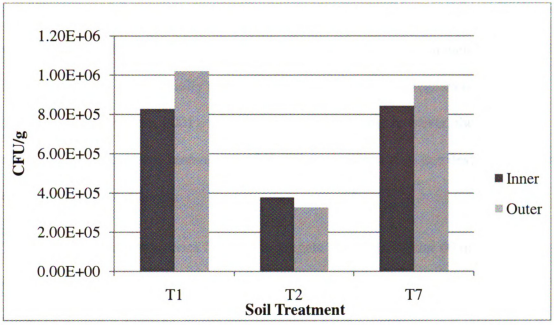

Fig 3.10. Heterotrophic bacterial concentrations in interior and exterior layers of soil treatments T1, T2 and T7 (n=2).

homogeneous distribution of bacteria that were clumped in the aggregate (Richaume et al., 1993). This may also be due to bacterial adsorption and particle interference (discussed in section 3.1.2) that was overcome after the physical disruption of the soil aggregate by separating it into layers.

3.4.2 Desiccation and *E. coli* recovery in aggregates subsections. To examine the drying affect on spatially distributed bacteria in the aggregate, aggregates were separated into three subsections in relation to where the *E. coli* was seeded (the SAE chamber was not used for these experiments because the aggregates were moist after spiking and thus it would be difficult to erode the aggregate accurately). The subsections were then allowed to air-dry for 0, 10, 40 and 60 minutes. Aggregate subsections after desiccation showed a decline in *E. coli* recovery (Table 3.10). It is noteworthy to point out that at all time intervals, higher recoveries were always observed within the middle subsections. This is especially true at 40 minutes of air drying, where the middle section showed a recovery

of 11.5% as compared to the 0.7% and 4.3% recoveries in the top and bottom subsections. The observed results coincide with what might occur in nature, as bacteria have been shown preferentially relocate to the center of soil aggregates for protection (Ranjard and Richaume, 2001). At 60 minutes of air-drying, however, there was not much difference between recovery yields in all three subsections due desiccation stress at the lower moisture content.

Total *E. coli* recoveries from the aggregate subsections after 60 minutes of air-drying were lower (7.9%) as compared to *E. coli* recoveries from whole aggregates (49%) (Tables 3.5 and 3.8). While the aggregates were stable when wet (at times 0 and 10 minutes), once dried the aggregates were flaking (times 40 and 60 minutes) during the slicing procedure (flaking from the subsections). These pieces may have had *E. coli* cells adsorbed to them, leading to the underestimations of actual *E. coli* recovery. To ensure there was no underestimation due to flaking and to avoid desiccation stress due to low moisture content, sliced aggregates were then processed immediately (results described in below in section 3.4.3)

Table 3.10. Averaged recovery of *E. coli* from sliced soil aggregate subsections (n=2).

Time (minutes)	Subsection			Total Recovery
	Top	Middle	Bottom	
0	23.4%	30.2%	23.7%	77.3%
10	17.2%	29.5%	17.8%	64.5%
40	0.7%	11.5%	4.3%	16.5%
60	2.7%	3.2%	2.0%	7.9%

3.4.3 Slicing and saturation experiments. These experiments were designed to explore the effect of soil saturation and soil treatment on bacterial distribution within the

aggregate. Aggregates that were air-dry and pre-saturated to 15% and 30% moisture content were seeded with *E. coli* and then sliced into seven subsections to examine bacterial translocation. Subsections 1, 2, 3, 4, and 5 corresponded to the top, right, left, back and front of the aggregate respectively and the middle section was divided into two subsections 6 and 7 which corresponded to the center-middle and the bottom-middle respectively (see section 2.5). Calculation of *E. coli* concentrations were based on log CFU/ g.

At air-dry condition (i.e. 0% moisture content), T1 had high variability in *E. coli* concentration in five of the seven slices indicating a non-uniform distribution of *E. coli* in the three replicates (Figure 3.11A). T2 also showed variability at 0% moisture content, but variability was confined to three subsections (Figure 3.12A). T7 showed the most even distribution of *E. coli* as compared to T1 and T2, by exhibiting the least variability in aggregate subsections at 0% moisture content (Figure 3.13A). These observations can be explained by the variability in *E. coli* recovery replicates per subsection. While *E. coli* could not be detected in at least one replicate per slice, T2 had only two replicates at which *E. coli* could not be detected. *E. coli* in T7 subsections on the other hand, could be detected in all replicates (Figures 3.13A, 3.13B, and 3.13 C).

When the aggregates were saturated, the dynamics of *E. coli* spatial distribution were different in T1 and T2 treatments. At moisture contents of 15% only one slice in T2 treatment exhibited high variability while across all treatments at 30% moisture content very minute variability was observed (Figures 3.11B, 3.11C, 3.12B and 3.12C). It's apparent that at increasing moisture content, *E. coli* spreads more evenly within the aggregates T1.

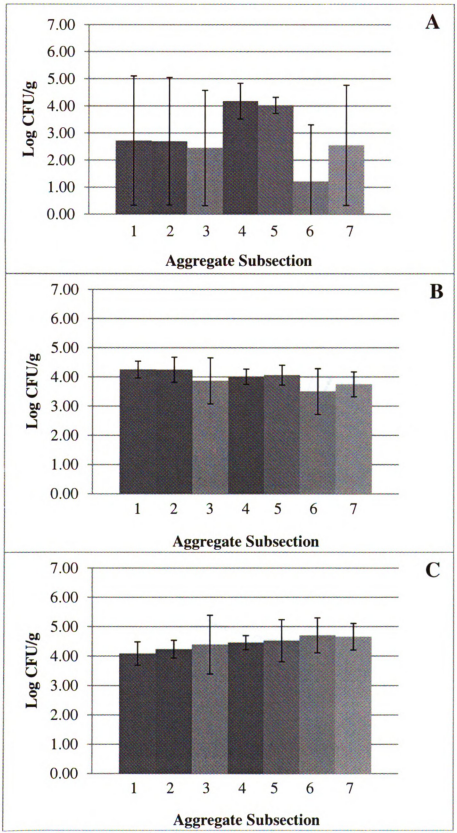

Fig 3.11. *E. coli* concentrations in T1 aggregate slices at A) 0% moisture content, B) 15% moisture content and C) 30% moisture content (n=3).

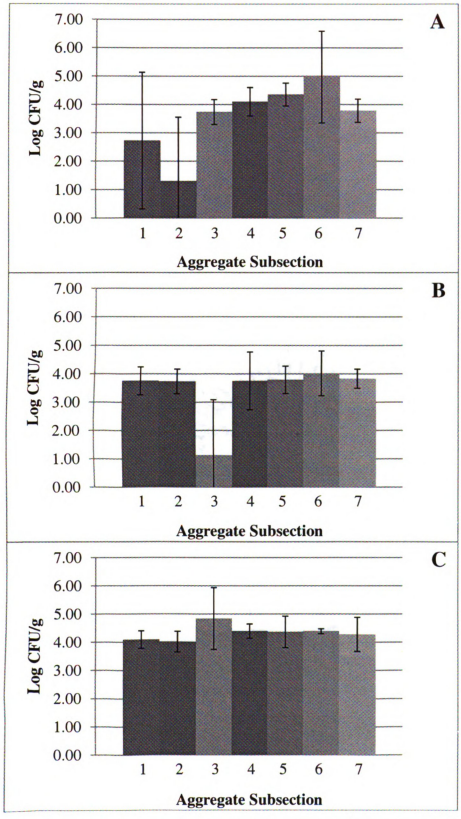

Fig 3.12. *E. coli* concentrations in T2 aggregate slices at A) 0% moisture content, B) 15% moisture content and C) 30% moisture content (n=3).

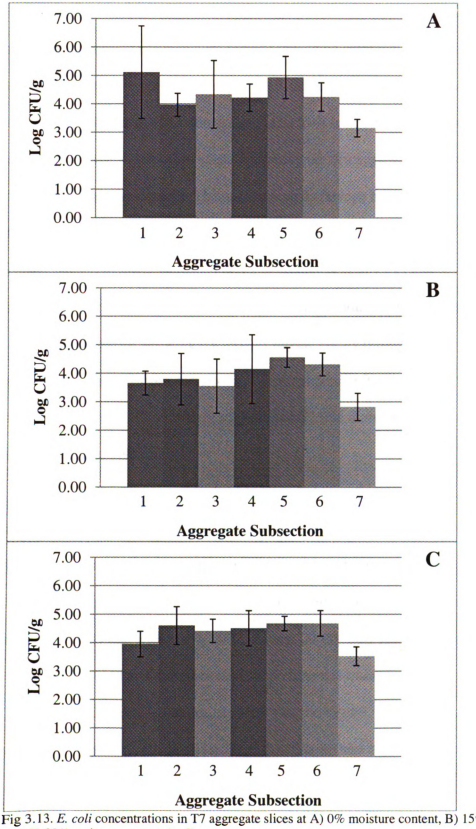

Fig 3.13. *E. coli* concentrations in T7 aggregate slices at A) 0% moisture content, B) 15% moisture content and C) 30% moisture content (n=3).

To understand the difference in *E. coli* distribution, both the effect of the soil aggregate treatment and moisture content should be assessed. It is of interest that *E. coli* distribution in T7 treatment did not seem to change between any of the moisture contents and was evenly distributed among the slices. This is in contrast to T1 soil which exhibited entirely opposite distribution when comparing air-dry aggregates and aggregates with increasing moisture content. The only known difference between treatments is tillage and fertilization. Tillage could have altered the soil's pores structure as reflected in its higher bulk density (i.e. lower porosity) of T1 in comparison to T7 (Table 2.3). As a consequence it is possible that that the pores that conduct flow are more uniform in T7 aggregate, therefore allowing bacteria to spread evenly throughout the aggregate when it was air-dry. Results from section 3.4, where bacterial effluent concentrations in T1 were more variable than T7, similarly suggest that T7 had more uniform pore characteristics (Tables 3.8 and 3.9).

T2 on the other hand seemed to lie in between the extremes of *E. coli* distribution at 0% moisture content. T2 aggregates did not receive any tillage, but did receive N-fertilization and had agricultural corn-wheat and soybean crop growth. The addition of fertilizer and growth of non-native agricultural plants are known to alter the soil organic matter (Grandy and Robertson, 2007, Liebig, 2002). This alteration can impact the soil's structural stability and therefore alter its preferential transport pathways (Blazier et al., 2008). From these observations, it seems that the impact of fertilization has less of an effect as opposed to tillage on bacterial distribution and thus the aggregate pore continuity.

Statistical analyses confirmed the graphically illustrated results. Three-way ANOVA using the variables of soil saturation, soil treatment and subsection number was used. The response variable in the experiment was the natural log of CFU/g. Levene's test for unequal variance was significant indicating unequal variances in all variables. Variances were highest in the aggregate saturation and subsection variables. Grouping by aggregate saturation indicated the best goodness of fit criteria (using the Akaike and Byesian criteria). Interestingly, by grouping the data using the aggregate saturation, the 0% moisture content indicated the highest variability estimate (6.08) as compared to T2 (1.81) and T7 (1.14). Next, a repeated measure was run to reduce individual differences between aggregate subsections using a compound symmetry model structure. The ANOVA was found to have a three-way interaction between all variables.

Using two combinations of two-way interactions allowed the investigation of the influencing factors in *E. coli* recoveries as affected by the subsection location. The first two-way interaction examined was between the soil saturation and the subsection number. Similar to Figure 3.11, the statistical analysis showed that three subsections, 3, 6 and 7 (i.e. the left, center-middle and the bottom-middle section) exhibited statistical significance in between aggregate saturations of 0, 15 and 30% in T1 soils (Figure 3.14). Only two subsections in T2 exhibited statistical differences and none in T7 soil (Figures 3.15 and 3.16). It is noteworthy, that at all moisture contents, the center-bottom subsection (7) in T7 exhibited the least concentration of *E. coli*.

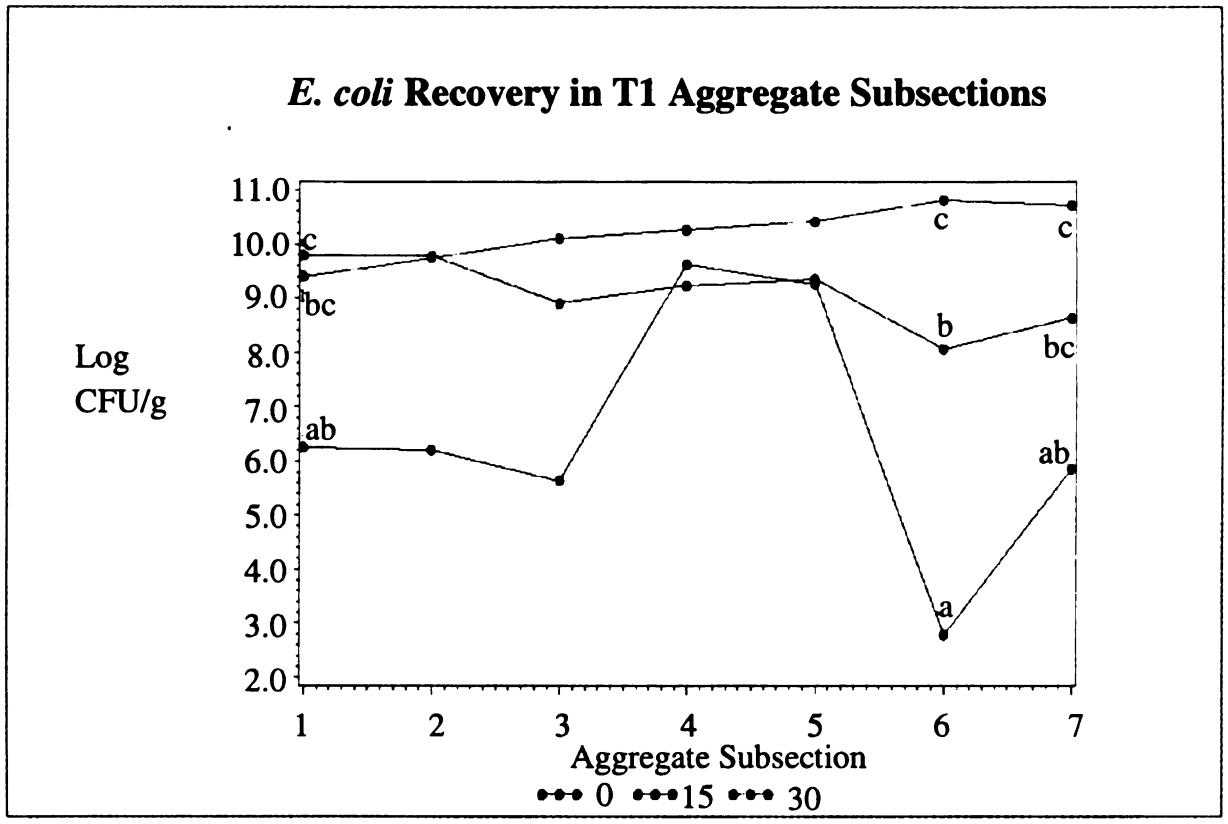

Fig. 3.14. Statistical comparison of *E. coli* recoveries at 0, 15, and 30% moisture content in T1 aggregate subsections. Moisture contents at specific subsections with different alphabets indicate that *E. coli* concentrations are significantly different.

The second two-way interaction examined was between the soil treatment and the subsection number. At the 0% moisture content, the greatest numbers of subsections with statistical differences between all soil treatments were observed (Figure 3.17). It's noteworthy to point out the statistical difference in subsection 6 (center-middle) between T1 and T2 as well as T1 and T7. At the 15% moisture content, there were only two subsections exhibiting statistical significant results (Figure 3.18). Finally, at the 30% moisture content, there was only one subsection (7; the center-bottom) that exhibited statistical difference between T1 and T2 as well as T1 and T7 aggregates (3.19).

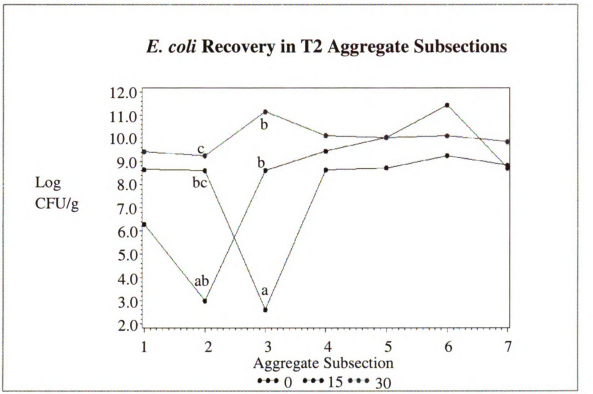

Fig. 3.15. Statistical comparison of *E. coli* recoveries at 0, 15, and 30% moisture content in T2 subsections.

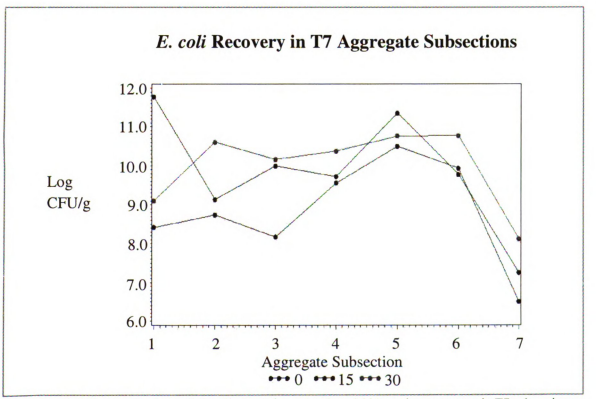

Fig. 3.16. Statistical comparison of *E. coli* recoveries at 0, 15, and 30% moisture content in T7 subsections.

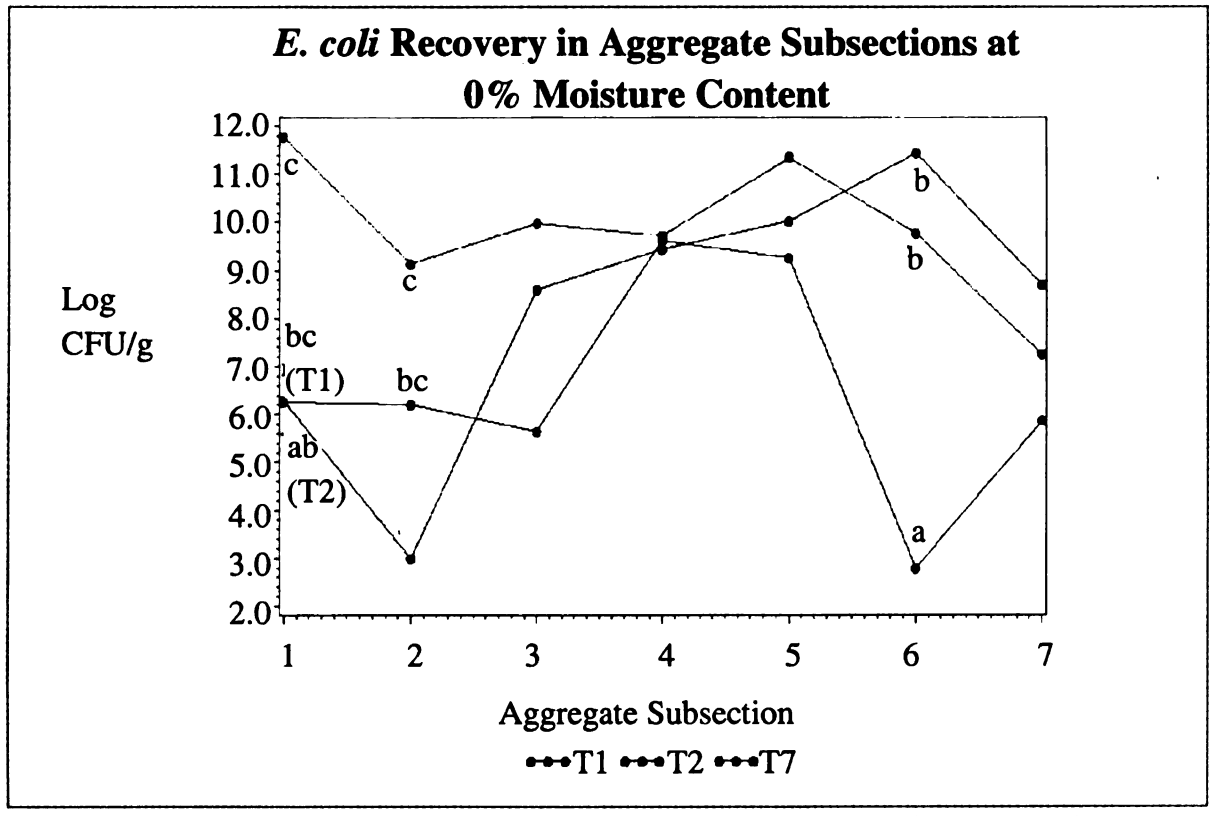

Fig. 3.17. Comparison of *E. coli* recoveries in T1, T2, and T7 aggregates subsections at 0% moisture content. Moisture contents at specific subsections with different alphabets indicate that *E. coli* concentrations are significantly different.

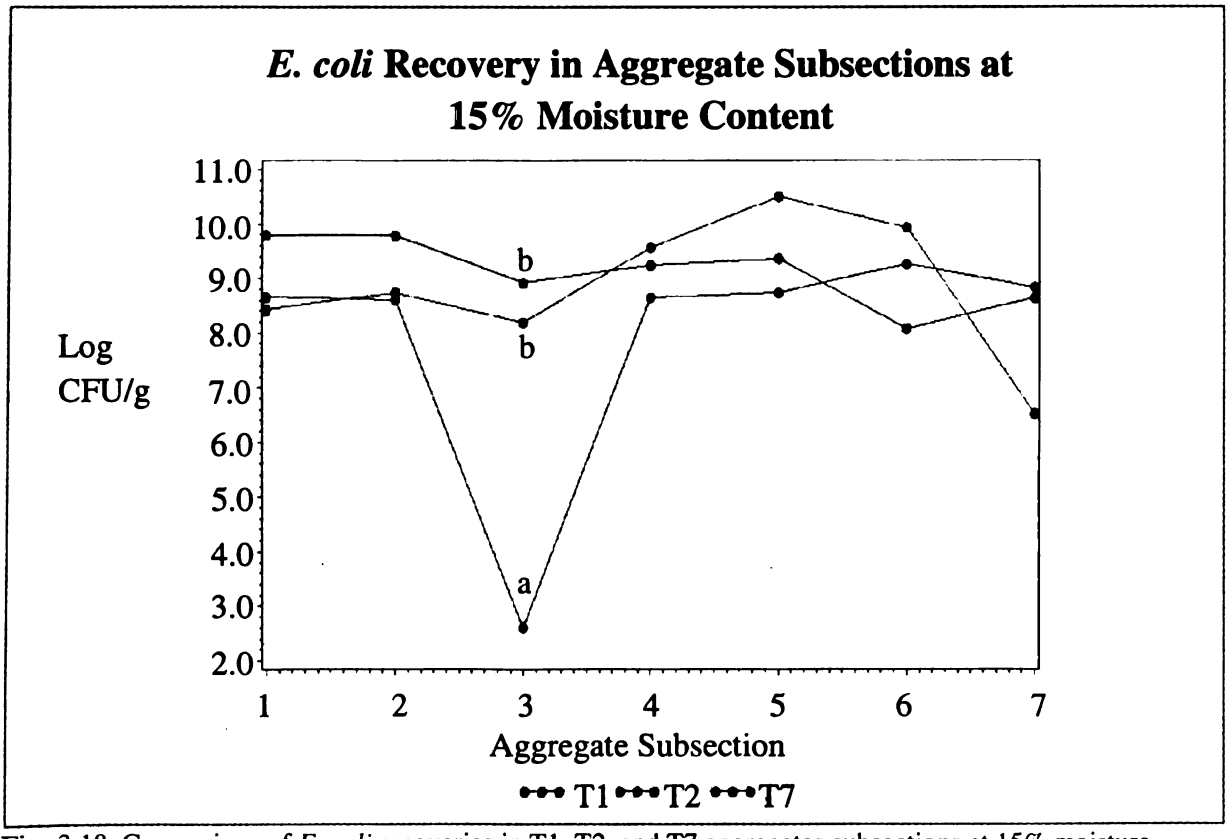

Fig. 3.18. Comparison of *E. coli* recoveries in T1, T2, and T7 aggregates subsections at 15% moisture content.

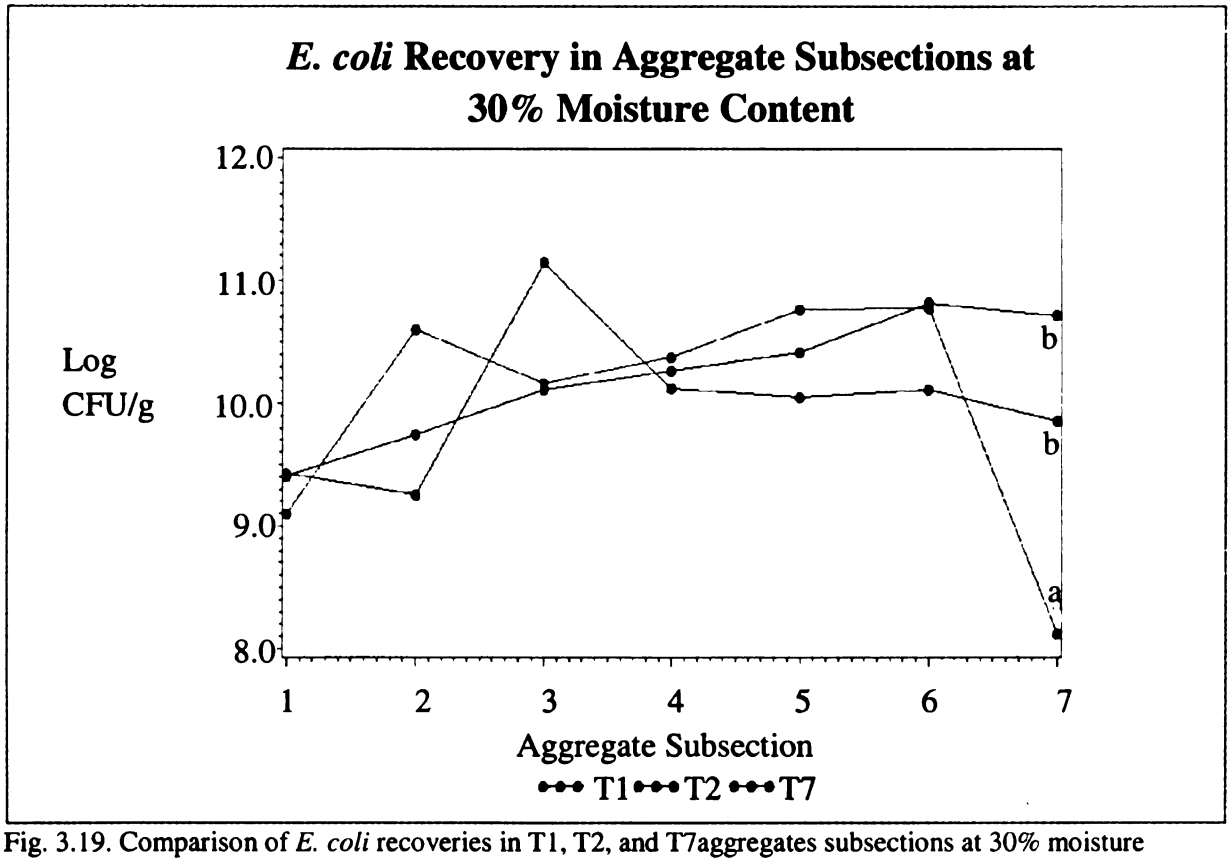

Fig. 3.19. Comparison of *E. coli* recoveries in T1, T2, and T7aggregates subsections at 30% moisture content.

Statistical analyses indicated that all three variables (soil treatment, moisture content and subsection location) had an effect on the bacterial spatial distribution of *E. coli* in the aggregates. It was observed that T1 had the highest variability between subsections and most often exhibited significant differences when compared to other soil treatments at 0% moisture content (Figure 3.14). It also appeared that when the moisture content is high, the soil treatment does not seem to impact *E. coli* distribution as much. At 0% moisture content there were three subsections that exhibited significant differences between the soil treatments as compared to only one subsection at 30% moisture content. This is probably because as more water was added to the aggregates at increasing moisture contents, more pores were filled creating a continuous pathway for *E. coli*

movement (Hillel, 1998). *E. coli*'s flagellar motility could have utilized the water continuity to disperse within the aggregate (Soby and Bergman, 1983).

Of interest was the impact the aggregate subsection had across all treatments. As mentioned earlier, T1 had the least *E. coli* concentration in subsection 6 across all moisture contents (Figure 3.14) and this subsection had significantly lower *E. coli* concentrations in T1 compared to T2 or T7 (Figure 3.17). T2 and T7 did not exhibit any significant differences in subsection 6 at any moisture content. This is of importance because subsection 6 is the center-middle, possibly indicating inaccessibility of that area in T1 soil. Also interesting is that subsection 7 (center-bottom) in the T7 aggregates always had less *E. coli* concentrations (Figures 3.16 and 3.19). The method of *E. coli* addition to the aggregates could have influenced this. *E. coli* was added to the top subsection, therefore it may not have reached the bottom subsection before slicing the aggregate.

It is important to note that the second replicate for T1 aggregate at 0% moisture content had no recovery in five of the seven subsections and very low bacterial concentrations in the other two subsections (See Appendix, Table A.6). This may indicate that the *E. coli* died-off during the slicing process whereby the aggregates may have been left to air-dry for an extended period of time. This puts into question the results of the T1 aggregate at 0% moisture content, because the statistical analysis may have been skewed due to the lower concentration in the second replicate. Nonetheless, the slicing and hydration experiments will be performed again in future work and statistical analysis will be subsequently conducted to compare with these results.

3.5 Discussion

3.5.1 Bacterial extraction method from soil. Over the last 60 years, many approaches to extract and detect bacteria in soil have been developed, each with their respective advantages and limitations (Ranjard and Richaume, 2001). Studies that have addressed bacterial extraction have usually utilized a variation of an agitation (i.e. vortexing or blending), sonication or fractionation method (Hattori, 1988, Mahler et al., 2000, Holdaway, 2003, Singh, 2007, Boehm et al., 2009). To detect bacteria, culturing methods using agar have been the most prevalent (Fontes et al., 1991, Gannon et al., 1991, Mahler et al., 2000, Guber et al., 2005, Bolster et al. 2006). However, other studies have used most probable numbers (MPN) liquid based assays, immunogenic assays, microscopic evaluations and genetic analyses (Bakken and Olsen, 1987, Richaume et al., 1993, Mummey et al., 2006, Zimmerman et al., 2009).

In this study, the vortexing extraction and membrane filtration culture method seemed to underestimate native bacterial population but proved to be suitable for detecting spiked bacteria. Underestimation was most likely due strong adhesion of native bacteria to the soil particles because of the very dry soil conditions. The vortexing agitation was not enough to detach the bacteria from the soil particles. But in combination with peeling or fractioning the aggregate, more native bacteria appeared to become detached from soil. Thus it is recommended to keep soils moist after sampling to avoid bacterial desiccation and strong adhesion to soil particles. It also may be useful to employ a combination of methods to increase the accuracy of native bacterial extraction.

Culture media and growth conditions were most likely limiting factors in detecting native bacteria as well. Many species of native soil bacteria have slower growth rates, so the 24 hour incubation period may have not been enough for most of the bacteria to grow (Rozak and Colwell, 1989). Therefore, a longer incubation period on low nutrient media has been recommended for enumerating native soil bacteria (Olsen and Bakken, 1987, Davis et al., 2005).

The very high recovery rates (section 3.2.1) of *E. coli* and *Ent. faecium* indicate that the vortexing and membrane filtration method was optimal for aggregate spiked bacteria. This high recovery was evident even at low bacterial concentrations. However, similar to native bacteria, spiked bacteria may succumb to desiccation stress, so it is important to ensure that the soil does not dry out. It is recommended to process the spiked aggregate within 2 hours to avoid bacterial growth or die-off.

In regards to detection of spiked bacteria, the method had its limitations. *Ent. faecium* seemed to clump and attach to soil particles more readily than *E. coli*, causing high variability and underestimation of bacterial influent concentrations. A possible improvement to this method would be to introduce a series of washing and centrifugation steps when the adding stock dilutions of bacteria to soil aggregates (Bolster et al., 2006). In preparing the stock one could minimize clumping by removing nutrients in the stock culture. It also may be beneficial to add detergents such as Tween 80 to the washed stock culture and/or during the extraction with vigorous vortexing to detach the bacteria and to decrease the appearance of clumps (McConville et al., 1974).

3.5.2 Bacterial retention and transport in soil. When bacteria are applied to soil they may be retained by adsorbing to soil surfaces or become trapped in soil micropores (Stevik et al, 2004). Our experiments illustrate that this high capacity to retain bacteria can be discerned even at the macroaggregate scale. Perhaps the most important factor in bacterial transport was the soil moisture content. This is of interest because after rainfall, soil is saturated and thus pathogenic bacteria in soil may runoff into adjacent water bodies or be carried into ground water, posing a public health risk (Unc and Goss, 2004, Muirhead et al., 2006).

When the soil was dry, both indicator bacteria were found to be retained in aggregates in high concentrations even after flushing with solution (Figure 3.7). It is thought that when the soil is at low moisture content, capacity of bacteria to adsorb to soil increases (Jamieson et al., 2002). This is supported by our experiments that have indicated that native bacteria and spiked bacteria can strongly adhere to soil particles when soil is dry (sections 3.1.2, 3.2.2 and 3.4.2). Furthermore, Guber et al. have shown that fecal coliforms adhered to dry soil 2.5 times more readily than water saturated aggregates (2009). It is also possible that the addition of $CaCl_2$ enhanced bacterial retentions in soil aggregates. Ca^{2+} ions in the $CaCl_2$ solution may form ionic bridges between the bacteria and the soil, thereby increasing attachment (Stevik et al., 2004). Although the micropores (sizes <2um) in our soil aggregates have not been characterized, filtration could have also increased retention. Hattori has shown that when soil is dry, bacteria is passively carried with the solution in small pores due to capillary force (1988). There they could get stuck and may not be able to exit the soil aggregate even when flushed (Powelson and Gerba, 1995).

Soil structure seems to be important in bacteria retention, however, it is not well understood. Some studies indicate that physical re-structuring of soil can retard bacterial movement from soil surface to groundwater, thereby suggesting tillage as a suitable agricultural management practice (Abu-Ashour et al., 1998, McMurry et al., 1998). However, other studies have indicated tillage can have a variable effect on bacterial transport into groundwater and may even enhance runoff (Stoddard et al., 1998, Gagliardi and Karns, 2000, Jenkins et al., 2008). Results from the flow chamber experiments described in Chapter 3, corresponded to the latter studies. Although the average leached bacterial concentration for T1 (tilled) aggregates was higher as compared to non-tilled aggregates, the effluent concentrations were highly variable (Figure 3.8). This inconsistency most likely suggests that the tillage causes the aggregates to have non-uniform structures, making measurement of bacterial transport even at the macroaggregate scale more uncertain.

The type of bacterial species seemed to contribute less to the retention than soil moisture or soil management practice. This was not anticipated because *Enterococci* are known to exhibit higher retention in soil when compared to *E. coli* (Mahler et al., 2000). This is because they can adsorb to clay particles and clump together, making extracting them from soil more difficult (Stenström, 1989, Huysman and Vestraete, 1993). Microscopic evaluations (section 2.2.7) support this, because unlike *E. coli*, *Ent. faecium* were observed to clump more readily and form chains of three or more cells as it grows. Therefore, it is tempting to conclude that *Ent. faecium* was retained more readily than *E. coli* as evidenced by higher recovery of the latter in effluent of T1 treatment at saturated conditions (Table 3.7). However, these results are not statistically significant. Therefore,

at the aggregate-scale, the different bacterial species, cell properties and motility did not have a substantial affect on retention. At larger scales, such as in field conditions, this affect could be magnified due to increased contact with soil increasing chances of filtration and/or adsorption. More work is needed to address the observed trends to determine significance.

Finally, more than one flushing regime could be done to simulate multiple rainfall events. Other experiments have illustrated that the bulk of bacteria concentrations are drained from the soil after more solution has passed through the soil column (Fontes et al., 1991, Foppen et al., 2005). Therefore, it is recommended to conduct aggregate-scale flow experiments with multiple or continuous flushes to better understand the transport of bacteria under extensive or continuous rainfall.

3.5.3 Spatial distribution of *E. coli* in soil. The importance of understanding spatial distribution of spiked bacteria may help us ascertain the movement of pathogens loaded in soil after addition of manure slurry (Unc and Goss, 2004). Pathogens carried in liquid manure can percolate and distribute through the soil (Cools et al., 2001). To our knowledge, there have been no studies investigating spiked bacterial spatial distribution by subsectioning soil aggregates.

Similar to bacterial retention and transport, bacterial spatial distribution was largely influenced by saturation of the aggregate. We observed that soil structure plays a vital, yet secondary role. When the soil was saturated (at 30% moisture content by weight), the *E. coli* distributed throughout the aggregates regardless of soil treatment (i.e. tilled or non-tilled soil). This is possibly because most of the pore spaces where the

motile *E. coli* could disperse were filled with water allowing it to move freely within the aggregate.

Tillage is known to affect the aggregate's structure by compressing the soil and destroying the organic binding agents that the soil keep the soil aggregate stable (Tisdall and Oades, 1982, Hillel, 1998). The variable distribution of *E. coli* in tilled aggregates confirms the unpredictable effect tillage aggregate structure. This is further supported by the results from the flow chamber experiments where the effluent concentrations in saturated T1 aggregates were highly variable, suggesting the non-uniformity of flow conducting pores. Furthermore, the lower recoveries in the center-most subsection as compared to the non-tilled aggregates indicate that the tillage may alter the natural diffusion of bacteria to the inner part of the aggregate where they could colonize.

Due to the fact that these experiments were exploratory, only *E. coli* was used to simulate spatial distribution in soil aggregates. It would be of interest to examine the translocation of other bacterial species that are non-motile such as *Enterococci*. This would confine the spatial distribution due only to preferential flow, and thus help understand if motility of the bacteria has an affect or alters distribution.

3.6 Conclusion

Perhaps the most interesting aspect of the research is the high attachment rate of bacteria to soil. As a survival mechanism against stress, bacteria are thought to increase exopolysaccharide production for protection which increases adsorption to soil due to attachment to soil particles (Gerba and Mcleod, 1978, Wilkinson, 1958, Roberson and Firestone, 1992, Abu Lail et al., 2007). From a water quality perspective this ideal,

because it suggests that bacteria would be retained more readily in soil and would not runoff in surface or transport into groundwater. However, as we have observed, there are factors can reduce or alter the soil capacity. The increasing of soil saturation was the most important variable in enhancing bacterial transport and spatial distribution. This highlights the concern when applying manure-fertilizers followed by extensive irrigation or prior to excessive rainfall where the bacteria may percolate through the soil and contaminate the ground water (Stoddard et al., 1998). Studies that have examined the soil saturation's affect on bacterial transport on field-scale and column studies have reported similar results (Jamieson et al., 2002). Therefore, as an agricultural best management practice, application of manure followed by intensive irrigation or during seasons with increased precipitation is discouraged.

While disturbing the soil has been reported to increase the soil's filtration capacity and inhibit bacterial transport, the aggregate experiments indicated otherwise (Abu-Ashour et al., 1998). Tillage increased bacterial transport in flow experiments and altered *E. coli* distribution within aggregates. This illustrates negative and often confounding effect tillage has on filtration because of its impact on the aggregate's pore structure. Studies that observe bacterial transport in large scale experiments to provide information on effect of tillage may not discern differences due to the averaging of the soil heterogeneities (Guber et al., 2009).

The elucidation of these interactions at such a small scale indicates the usefulness of utilizing aggregates for modeling soil-microbial interactions. It even suggests that the major factors, such as tillage and soil moisture, that influence bacterial retention, transport and spatial distribution can be explained. Because of this, it is proposed that

more research for characterizing bacterial transport in soil on the aggregate-scale would be of great importance.

In addition to follow up experiments suggested in the discussion section, it would be of interest to observe the retention and transport of the bacterial indicator *Clostridium prefringens*. This is because it is utilized in warmer tropical climates and has the advantage over *E. coli* and *Enterococci* in that it does not replicate in the soil environment (Fujioka and Byappanahalli, 2001, Desmarais et al., 2002). It also may be of interest to compare bacterial transport with an indicator virus such as coliphage. Viruses do not replicate, have entirely different properties and are a fraction of the size of bacteria (Powelson and Gerba, 1995).

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Appendix Raw Data for Analysis

Table A.1. Data for heterotrophic plate counts for bacterial extraction from whole aggregate experiments.

Sample/Dilution	10^{-2} Dilution (CFU)	10^{-3} Dilution (CFU)	10^{-4} Dilution (CFU)	Aggregate weight (g)	Aggregate treatment
V4	49, 37, 38	5, 3, 9	0, 1, 0	0.1801	T1
V5	17, 13, 13	1, 1, 0	0, 0, 1	0.1397	T2
V6	69, 58, 65	7, 7, 10	0, 0, 1	0.1501	T7
V10	46, 31, 44	4, 2, 1	-	0.15	T1
V11	47, 41, 40	0, 3, 2	-	0.1802	T2
V12	43, 49, 31	0, 4, 2	-	0.1821	T7
V13	48, 35, 51	4, 5, -	-	0.1013	T1
V14	57, 58, 52	10, 5, 6	-	0.1606	T2
V15	56, 50, 52	4, 8, 9	-	0.1004	T7
V16	43, 34, 41	4, 0, 7	-	0.1602	T1
V17	66, 79, 82	5, 3, 6	-	0.1787	T2
V18	24, 36, 32	3, 1, 3	-	0.1637	T7
V19	57, 60, 77	5, 6, 2	-	0.1989	T1
V20	40, 38, 39	8, 1, 3	-	0.1095	T2
V21	56, 63, 51	10, 12, 4	-	0.1098	T7

*Note: CFU values for dilution plates are in triplicates for each sample.

Table A.2. Data for whole aggregate desiccation and *E. coli* recovery experiments.

Date of Experiment	Air-drying time (minutes)	10^{-2} Dilution (CFU)	10^{-3} Dilution (CFU)	Aggregate dry weight (g)	*E.coli* solution weight(g)	Aggregate weight after air-drying (g)
3/12/2009	None	53, 53, 57	4, 7, 5	0.2531	0.0485	-
	120	0, 0, 0	-	0.3117	0.0487	0.3160
3/13/2009	None	28, 42, 24	6, 2, 2	0.2238	0.0481	-
	120	0, 0, 0	-	0.2657	0.0498	0.2656
3/14/2009	None	39, 43, 49	-	0.2160	0.0495	-
	120	0, 0, 0	-	0.2461	0.0462	0.2412
4/15/2009	None	79, 61, 57	7, 5, 4	0.2585	0.0487	-
	30	74, 70	-	0.2450	0.0491	0.2771
	60	2, 2	-	0.2825	0.0500	0.2936
4/16/2009	None	45, 44, 49	1, 4, 2	0.2062	0.0468	-
	30	57, 49, 57	2, 4, 4	0.1997	0.0473	0.2286
	60	7, 13, 7	-	0.2510	0.0489	0.2631
	90	0, 0, 0	-	0.2548	0.0483	0.2562
5/11/2009	None	68, 62, 56	5, 7, 8	0.2358	0.0445	-
	15	67, 74, 72	4, 11, 7	0.1884	0.0492	0.2266
	30	65, 83, 76	6, 9, 7	0.1630	0.0495	0.1924
	45	64, 61, 70	8, 7, 7	0.2035	0.0493	0.2248
	60	41, 41, 45	-	0.1562	0.0480	0.1717
11/17/2009	None	51,33,45	0,4,3	0.2070	0.0496	-
	30	39,33,36	3,3,6	0.2055	0.0492	0.2324
	60	14,28,13	4,2,2	0.2324	0.0508	0.2458

Table A.3. Data for heterotrophic plate counts for bacterial extraction from aggregate interior and exterior layers.

Sample	10^{-2} Dilution (CFU)	10^{-3} Dilution (CFU)	Weight (g)	Aggregate Treatment	Diluent
T7 107 A	41, 36, 33	3, 2, 2	0.0375	T1	10ml
T7 107 C	35, 36, 40	3, 3, 5	0.0387	T2	10ml
T1 105 A	63, 53, 51	4, 0, 5	0.0741	T7	10ml
T1 105 C	26, 16, 30	0, 0, 1	0.0686	T1	15ml
T2 154 A	36, 41, 37	3, 4, 1	0.083	T2	10ml
T2 154 C	18, 38, 37	3, 2, 5	0.0851	T7	10ml
T7 111 A	27, 19, 17	2, 1, 1	0.0296	T1	10ml
T7 111 C	28, 28, 31	4, 6, 1	0.031	T2	10ml
T2 144 A	13, 15, 19	2, 0, 0	0.0527	T7	10ml
T2 144 C	12, 19, 10	5, 2, 1	0.0475	T1	10ml
T1 136 A	64, 64, 63	16, 8, 9	0.0704	T2	10ml
T1 136 C	94, 94, 103	11, 12, 8	0.064	T7	10ml

*Note: The letter "A" after the sample ID indicates the sample is the outer aggregate layer. The letter "C" after the sample ID indicates the sample is the inner aggregate layer.

Table A.4. Data for flow chamber experiments using saturated aggregates.

Replicate	Aggregate concentration (CFU)	Effluent concentration (CFU)	Spiked bacteria concentration (CFU)	Soil treatment	Bacterial Species
1	1.70E+03	4.98E+02	2.75E+03	T1	*E. coli*
2	9.00E+02	6.30E+02	6.50E+03	T1	*E. coli*
3	2.10E+03	2.34E+02	4.08E+03	T1	*E. coli*
4	3.27E+03	7.28E+01	4.75E+03	T1	*E. coli*
5	3.44E+03	3.44E+03	3.25E+03	T1	*E. coli*
1	3.70E+03	4.17E+00	2.75E+03	T2	*E. coli*
2	3.53E+03	1.25E+01	6.50E+03	T2	*E. coli*
3	2.83E+03	1.04E+02	4.75E+03	T2	*E. coli*
4	2.10E+03	3.92E+02	3.67E+03	T2	*E. coli*
5	2.10E+03	3.92E+02	3.67E+03	T2	*E. coli*
1	3.27E+03	N.D.	2.75E+03	T7	*E. coli*
2	4.77E+03	N.D.	6.50E+03	T7	*E. coli*
3	3.33E+03	4.53E+00	4.08E+03	T7	*E. coli*
4	3.57E+03	6.23E+01	4.75E+03	T7	*E. coli*
5	3.40E+03	1.22E+03	3.25E+03	T7	*E. coli*
1	1.30E+03	1.20E+01	1.17E+03	T1	*Ent. Faecium*
2	4.33E+02	3.78E+02	1.15E+03	T1	*Ent. Faecium*
3	1.40E+03	1.85E+01	1.38E+03	T1	*Ent. Faecium*
4	1.70E+03	0.00E+00	1.38E+03	T1	*Ent. Faecium*
5	1.27E+03	8.74E+02	1.20E+03	T1	*Ent. Faecium*
1	1.27E+03	N.D.	1.17E+03	T2	*Ent. Faecium*
2	1.77E+03	N.D.	1.15E+03	T2	*Ent. Faecium*
3	1.57E+03	N.D.	1.38E+03	T2	*Ent. Faecium*
4	1.53E+03	N.D.	1.38E+03	T2	*Ent. Faecium*
5	5.00E+02	1.36E+02	8.67E+02	T2	*Ent. Faecium*
1	1.27E+03	N.D.	1.17E+03	T7	*Ent. Faecium*
2	1.77E+03	N.D.	1.15E+03	T7	*Ent. Faecium*
3	1.57E+03	N.D.	1.38E+03	T7	*Ent. Faecium*
4	1.53E+03	N.D.	1.38E+03	T7	*Ent. Faecium*
5	5.00E+02	4.36E+02	1.20E+03	T7	*Ent. Faecium*

*Note: N.D. indicates that there were no bacteria detected.

Table A.5.Data for flow chamber experiments using air-dry aggregates.

Replicate	Aggregate Concentration (CFU)	Effluent concentration (CFU)	Spiked bacteria concentration (CFU)	Soil treatment	Bacterial Species
1	6.10E+03	N.D.	4.42E+03	T1	*E. coli*
2	6.97E+03	N.D.	3.83E+03	T1	*E. coli*
3	6.10E+03	N.D.	4.42E+03	T1	*E. coli*
4	8.97E+03	N.D.	4.83E+03	T1	*E. coli*
5	1.02E+04	N.D.	5.50E+03	T1	*E. coli*
1	6.13E+03	4.30E+00	4.42E+03	T2	*E. coli*
2	6.57E+03	N.D.	3.83E+03	T2	*E. coli*
3	6.13E+03	4.30E+00	4.42E+03	T2	*E. coli*
4	9.17E+03	1.95E+01	4.83E+03	T2	*E. coli*
5	9.10E+03	1.05E+02	5.50E+03	T2	*E. coli*
1	5.67E+03	1.31E+01	4.42E+03	T7	*E. coli*
2	6.97E+03	N.D.	3.83E+03	T7	*E. coli*
3	5.67E+03	1.31E+01	4.42E+03	T7	*E. coli*
4	8.83E+03	7.42E+01	4.83E+03	T7	*E. coli*
5	1.00E+04	N.D.	5.50E+03	T7	*E. coli*
1	2.47E+03	N.D.	1.22E+03	T1	*Ent. Faecium*
2	2.93E+03	N.D.	1.16E+03	T1	*Ent. Faecium*
3	2.47E+03	N.D.	1.22E+03	T1	*Ent. Faecium*
4	2.73E+03	N.D.	1.25E+03	T1	*Ent. Faecium*
5	3.03E+03	N.D.	1.28E+03	T1	*Ent. Faecium*
1	2.17E+03	N.D.	1.22E+03	T2	*Ent. Faecium*
2	3.20E+03	N.D.	1.16E+03	T2	*Ent. Faecium*
3	2.17E+03	N.D.	1.22E+03	T2	*Ent. Faecium*
4	3.17E+03	9.73E+00	1.25E+03	T2	*Ent. Faecium*
5	2.80E+03	1.58E+01	1.28E+03	T2	*Ent. Faecium*
1	2.63E+03	1.75E+01	1.22E+03	T7	*Ent. Faecium*
2	3.47E+03	N.D.	1.16E+03	T7	*Ent. Faecium*
3	2.63E+03	1.75E+01	1.22E+03	T7	*Ent. Faecium*
4	2.63E+03	5.30E+00	1.25E+03	T7	*Ent. Faecium*
5	3.33E+03	N.D.	1.28E+03	T7	*Ent. Faecium*

Table A.6. Data for slicing experiments using T1 aggregates.

Moisture content	0%					
Subsection	Replicate 1	Weight (g)	Replicate 2	Weight (g)	Replicate 3	Weight (g)
1	2.00E+02*	0.042	0.00E+00	0.019	8.67E+02	0.0289
2	4.00E+02	0.073	0.00E+00	0.023	1.03E+03	0.0465
3	1.33E+02	0.043	0.00E+00	0.015	8.67E+02	0.121
4	7.33E+02	0.031	6.67E+01	0.024	7.33E+02	0.0141
5	2.67E+02	0.012	2.67E+02	0.047	8.67E+02	0.0939
6	0.00E+00	0.024	0.00E+00	0.074	9.00E+02	0.2109
7	4.67E+02	0.04	0.00E+00	0.02	6.00E+02	0.1617
Influent	7.50E+03	-	7.50E+03	-	7.50E+03	-
Moisture content	15%					
Subsection	Replicate 1	Weight (g)	Replicate 2	Weight (g)	Replicate 3	Weight (g)
1	6.67E+01	0.0072	6.33E+02	0.0181	5.00E+02	0.0282
2	5.67E+02	0.0997	4.33E+02	0.0155	1.33E+02	0.0038
3	6.67E+01	0.0607	2.67E+02	0.0303	3.00E+02	0.0073
4	2.33E+02	0.0261	7.00E+02	0.0354	3.00E+02	0.0505
5	7.00E+02	0.0277	1.00E+02	0.0191	3.00E+02	0.0258
6	1.00E+02	0.2442	2.67E+02	0.0214	1.67E+02	0.0267
7	4.00E+02	0.1505	1.33E+02	0.0344	3.67E+02	0.0216
Influent	2.35E+03	-	4.48E+03	-	5.92E+03	-
Moisture content	30%					
Subsection	Replicate 1	Weight (g)	Replicate 2	Weight (g)	Replicate 3	Weight (g)
1	3.50E+02	0.0548	1.00E+02	0.0124	4.67E+02	0.0134
2	2.50E+02	0.0304	2.33E+02	0.0071	1.33E+02	0.0072
3	2.50E+02	0.0251	3.33E+01	0.0075	3.33E+02	0.001
4	6.00E+02	0.026	2.67E+02	0.014	2.67E+02	0.005
5	8.50E+02	0.061	3.33E+02	0.0277	2.67E+02	0.0012
6	4.50E+02	0.0037	3.33E+02	0.0323	3.00E+02	0.003
7	4.00E+02	0.0246	3.00E+02	0.0023	5.67E+02	0.013
Influent	4.51E+03	-	4.51E+03	-	8.47E+03	-

*Note: these *E. coli* concentrations are in CFU/ aggregate.

Table A.7. Data for slicing experiments using T2 aggregates.

Moisture content	0%					
Subsection	Replicate 1	Weight (g)	Replicate 2	Weight (g)	Replicate 3	Weight (g)
1	8.00E+02	0.022	0.00E+00	0.015	6.00E+02	0.1412
2	2.00E+02	0.025	0.00E+00	0.046	0.00E+00	0.0045
3	2.67E+02	0.036	2.00E+02	0.016	3.00E+02	0.1694
4	1.53E+03	0.037	4.00E+02	0.034	6.33E+02	0.1527
5	9.33E+02	0.015	3.33E+02	0.033	1.20E+03	0.0651
6	6.67E+02	0.0001	2.00E+02	0.03	1.03E+03	0.0564
7	6.67E+01	0.004	6.67E+01	0.026	6.67E+02	0.1274
Influent	7.50E+03	-	7.50E+03	-	7.50E+03	-
Moisture content	15%					
Subsection	Replicate 1	Weight (g)	Replicate 2	Weight (g)	Replicate 3	Weight (g)
1	2.67E+02	0.1707	3.33E+02	0.0265	3.33E+02	0.0354
2	3.33E+02	0.0407	6.67E+01	0.0385	2.00E+02	0.0178
3	0.00E+00	0.1093	1.00E+02	0.0405	0.00E+00	0.0002
4	6.67E+01	0.1665	7.67E+02	0.0588	5.00E+02	0.0145
5	1.67E+02	0.0987	4.00E+02	0.0319	5.00E+02	0.0457
6	1.33E+02	0.1006	8.33E+02	0.02	4.00E+02	0.0195
7	2.33E+02	0.0836	1.67E+02	0.0151	4.33E+02	0.0424
Influent	2.35E+03	-	4.48E+03	-	5.92E+03	-
Moisture content	30%					
Subsection	Replicate 1	Weight (g)	Replicate 2	Weight (g)	Replicate 3	Weight (g)
1	6.67E+01	0.0118	2.67E+02	0.0183	3.00E+02	0.013
2	6.67E+01	0.003	6.67E+01	0.0159	1.33E+02	0.011
3	1.33E+02	0.0076	5.00E+02	0.0336	1.67E+02	0.00013
4	3.00E+02	0.0234	1.33E+02	0.0035	3.33E+02	0.0105
5	3.00E+02	0.0236	4.00E+02	0.0418	2.33E+02	0.0023
6	4.33E+02	0.0218	6.33E+02	0.0219	3.33E+02	0.0128
7	1.00E+02	0.0176	3.00E+02	0.0217	1.67E+02	0.0019
Influent	4.51E+03	-	4.51E+03	-	8.47E+03	-

Table A.8. Data for slicing experiments using T7 aggregates.

Moisture content	0%					
Subsection	Replicate 1	Weight (g)	Replicate 2	Weight (g)	Replicate 3	Weight (g)
1	1.40E+03	0.087	8.00E+02	0.028	9.67E+02	0.1287
2	7.33E+02	0.026	9.33E+02	0.0001	6.00E+02	0.1899
3	1.40E+03	0.046	2.13E+03	0.059	6.67E+02	0.1909
4	4.67E+02	0.03	3.33E+02	0.01	1.97E+03	0.637
5	7.33E+02	0.009	1.47E+03	0.059	9.33E+02	0.1241
6	6.67E+02	0.034	1.07E+03	0.03	7.00E+02	0.0016
7	8.00E+02	0.035	2.67E+02	0.02	1.03E+03	0.1311
Influent	7.50E+03		7.50E+03	-	7.50E+03	-
Moisture content	15%					
Subsection	Replicate 1	Weight (g)	Replicate 2	Weight (g)	Replicate 3	Weight (g)
1	3.33E+02	0.0985	5.67E+02	0.0938	6.67E+02	0.1302
2	1.33E+02	0.2204	5.67E+02	0.0805	7.67E+02	0.0754
3	6.67E+01	0.2298	1.33E+03	0.0365	8.00E+02	0.0284
4	4.67E+02	0.2345	5.33E+02	0.1154	9.67E+02	0.0583
5	6.67E+02	0.3126	1.23E+03	0.0017	8.00E+02	0.1596
6	3.67E+02	0.0391	7.00E+02	0.015	1.07E+03	0.0234
7	2.67E+02	0.0516	7.33E+02	0.0197	3.33E+02	0.0311
Influent	2.35E+03	-	4.48E+03	-	5.92E+03	-
Moisture content	30%					
Subsection	Replicate 1	Weight (g)	Replicate 2	Weight (g)	Replicate 3	Weight (g)
1	4.33E+02	0.0153	3.33E+01	0.0053	6.67E+02	0.0211
2	4.67E+02	0.0396	3.67E+02	0.0114	6.00E+02	0.0297
3	5.00E+02	0.0435	1.33E+02	0.013	3.67E+02	0.0028
4	5.33E+02	0.0176	3.00E+02	0.0021	5.67E+02	0.0382
5	1.43E+03	0.0853	4.33E+02	0.0033	8.67E+02	0.0098
6	6.33E+02	0.0317	6.67E+01	0.005	1.03E+03	0.0319
7	3.67E+02	0.0109	3.00E+02	0.0043	5.33E+02	0.0086
Influent	4.51E+03	-	4.51E+03	-	8.47E+03	-