ASSESSING THE ROLE OF

PHYSICOCHEMICAL AND BIOCHEMICAL SOIL CHARACTERISTICS

ON *ESCHERICHIA COLI* ATTACHMENT.

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of GUILLAUME PATERNOSTRE find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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Food-borne diseases remain a persistent challenge to public health, causing approximately 76 million illnesses and 5,000 deaths every year in the United States (Mead, 1999). The number of incidents has more than doubled since 1987 (Tauxe et al., 1997). Consumption of produce contaminated with pathogenic bacteria, such as *Escherichia coli* O157:H7, is the primary cause of reported food-borne disease.

Agricultural animals, such as cattle and poultry, appear to be one of the most significant sources of pathogens in surface waters (Guber et al., 2005; Ling et al., 2005). Pathogens are shed in feces and can then be spread to humans either by direct consumption of the water or by consumption of crops or vegetables contaminated through irrigation.

Bacterial attachment to soil particles plays an important role in the fate and transport of pathogenic bacteria. The objective of this study was to assess the role of physicochemical and biochemical soil characteristics on attachment of a non-pathogenic *E. coli* strain. *E. coli* are commonly used as indicators of fecal contamination and can be used to simulate the fate and transport of the pathogenic *E. coli* O157:H7 strain.

The first portion of this study was to compare and contrast the physical, chemical and biochemical characteristics of six soils representative of those found across the State of Washington. The soils displayed a wide range of characteristics, including but not limited to texture, pH, organic content, cation exchange capacity (CEC) and microbial community indicators.

The second portion of the study examined *E. coli* attachment to these characterized soils using batch equilibrium experiments. *E. coli* was added to 1g of soil and shaken until attachment equilibrium was reached. Soil suspensions were then separated by centrifugation. The amount of *E. coli* sorbed was calculated as the difference between the bacterial population initially present in solution and the population present after the batch experiment. The Freundlich isotherm presented a representative model for *E. coli* attachment to each of the soils ($R^2 > 0.94$).

E. coli attachment capacity differed across the soils. Among the parameters studied, the bacterial to fungal ratio ($r^2 = 0.92$), total organic carbon content ($r^2 = 0.87$), clay percentage ($r^2 = 0.86$) and soil pH ($r^2 = 0.84$) appeared to be the characteristics with the greatest influence on attachment. These findings suggest conditions that promote *E. coli* attachment to soil particles, which has implications on development of best management practices (BMPs) to protect surface waters from contamination.

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1.1 PATHOGEN BACKGROUND

1.1.1 Food-borne illness

Food-borne illnesses are increasing worldwide. Illnesses of food-borne origin are difficult to estimate; however it is recognized that a large proportion of the 1.8 million diarrhea-related deaths in 2005 can be attributed to contaminated food and drinking water (WHO, 2007). In the United States, 76 million cases of food-borne diseases are estimated to occur each year, which result in 325,000 hospitalizations and 5,000 deaths (Mead, 1999). These diseases result from the consumption of contaminated food and are primarily caused by pathogenic bacteria, viruses, or parasites. According to WHO (World Health Organization), seven food-transmitted pathogens (*Campylobacter jejuni*, *Clostridium perfringens*, *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus, Streptococcus and Yersinia enterocolitica*) are responsible for 4 million annual infections in the United States, which account for a third of the food-borne (Table 1.1). Developed countries have food quality control systems that are well-established; however, these countries are confronted today with new dangers of contamination of their foodstuffs. The number of cases occurring yearly in developing countries is considerably higher than found in developed countries such as the United States. These differences in disease risks are due in part to differences in alimentary hygienic conditions (Figure 1.1).

Table 1.1: Annual food-borne disease estimates for the United States. Estimates are based on data provided by the Centers for Disease Control and Prevention, Atlanta, GA, USA and are typical of recent years. Adapted from Brook, 2006.

Organism	Number per year	Foods
Bacteria		
Bacillus cereus	27,000	Rice and starchy foods, high-sugar foods, meats, gravies, pudding, dry milk
Campylobacter jejeuni	1,963,000	Poultry, dairy
Clostridium perfringfens	248,000	Cooked and reheated meats
Escherichia coli O157:H7	**63,000**	Meat, especially ground meat
Other Enterpathogenic *E. coli*	**110,000**	Meat, especially ground meat
Listeria monocytogenes	2,500	Meat and dairy
Salmonella ssp.	1,340,000	Poultry, meat, dairy eggs
Staphylococcus aureus	185,000	Meat desserts
Streptococcus ssp	50,000	Dairy, meat
Yersinia enterocolitica	87,000	Pork, milk
All other bacteria	102,000	
Total bacteria	**4,177,500**	
Protozoa		
Cryptosporidium parvum	30,000	Raw and undercooked meat
Cyclospora caetanensis	16,000	Fresh produce
Giardia lamblia	200,000	Contaminated or infected meat
Toxoplasma gondii	113,000	Raw and undercooked meat
Total protozoa	**359,000**	
Viruses		
Norwalk-like viruses	9,200,000	Shellfish, many other foods
All other viruses	82,000	
Total viruses	**9,282,000**	
Total Annual Foodborne Diseases	**13,818,800**	

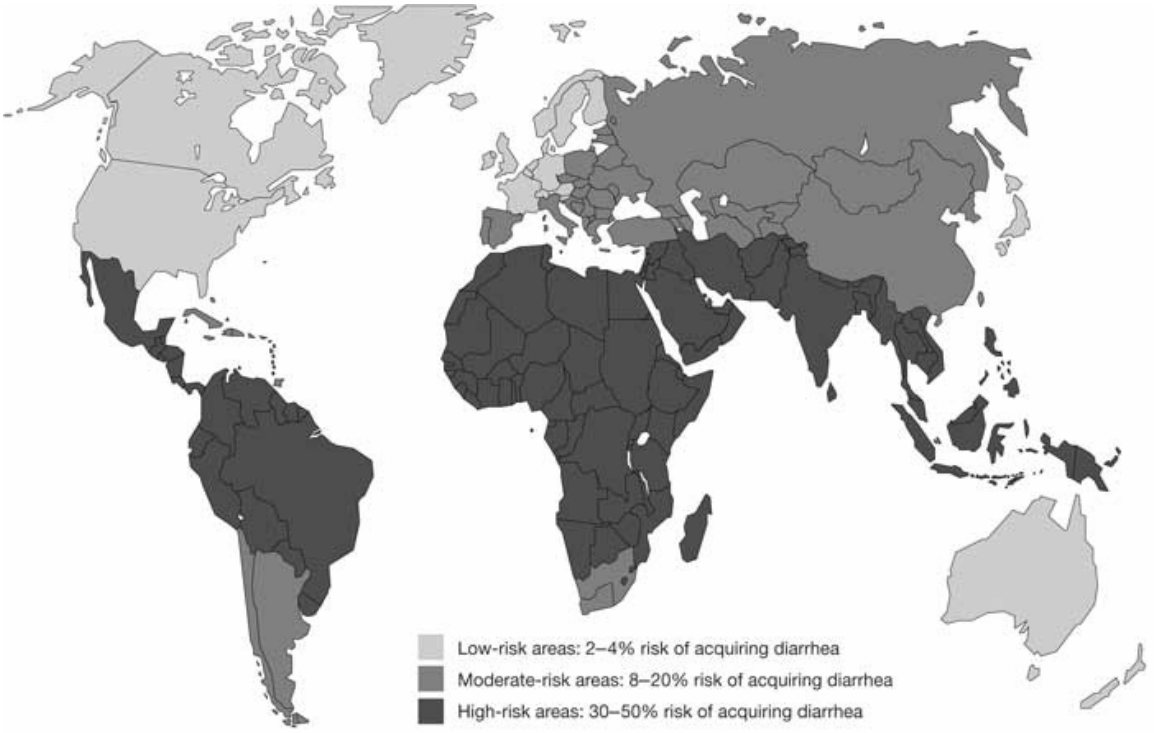

Figure 1.1: Risks of acquired food-borne illnesses in the world, people living in developing counties have a much higher probability to acquire diarrhea than the developed counties. From Herbert L DuPont, 2005.

1.1.2 Principal Sources of Contamination

Agricultural animals, such as cattle and poultry, are widely recognized as significant pathogen reservoirs (Muniesta et al., 2006). However, it is often difficult to determine if these animals carry pathogens, because they do not demonstrate virulent symptoms resulting from the bacteria. Often, toxins responsible for the infection require highly specific receptors on the host cell surface in order to attach and enter the cell. Cattle, swine, and deer do not carry these receptors and may harbor toxigenic bacteria without any ill effect. These species may shed the pathogens in their feces and spread them to humans through several different mechanisms of contamination (Figure 1.2).

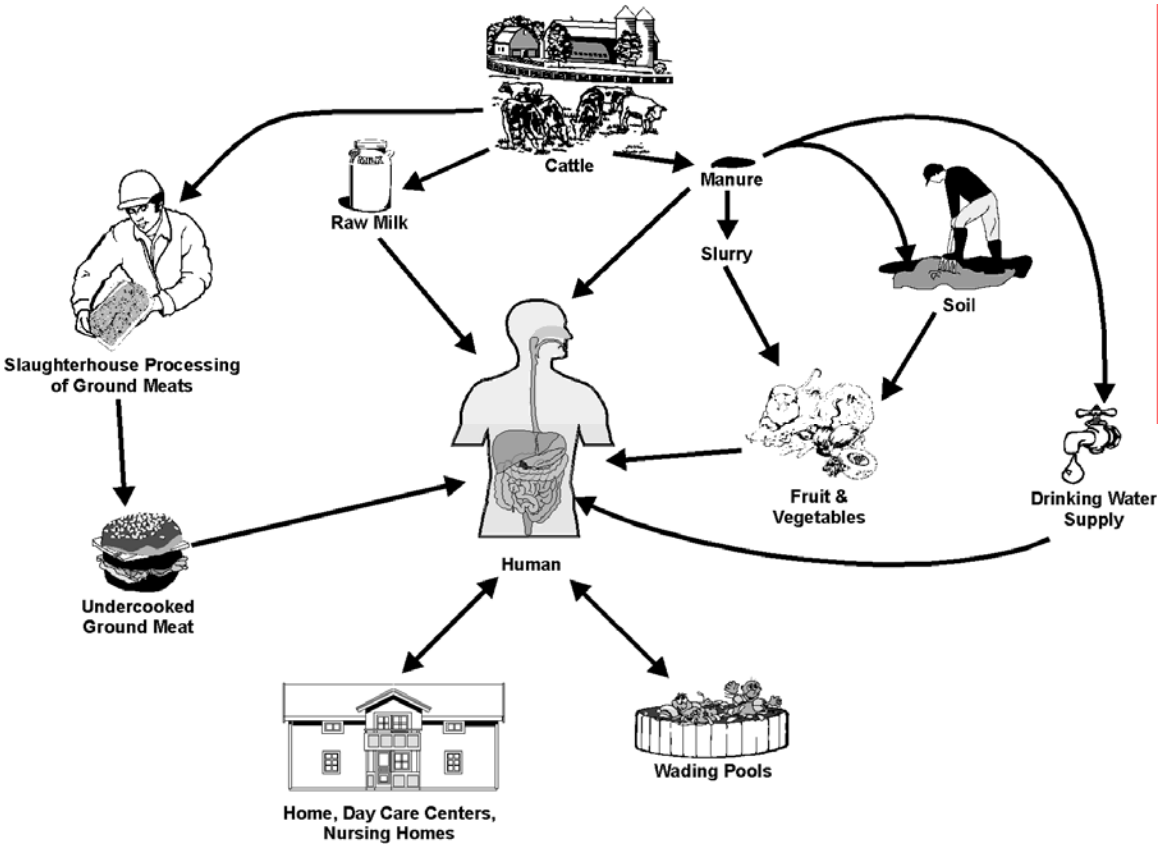

Figure 1.2: Mechanisms of pathogens contamination. From Petridis et al., 2002

The primary mechanism of human disease is through contaminated food consumption, such as undercooked ground-meat products and unpasteurized milk. Eating contaminated meat or produce can cause infection if the items have not been cooked sufficiently to kill *E. coli* O157:H7 or *Salmonella*. A second source of disease transmission is through water contamination by livestock. Freshwater plays two essential roles in the food chain because it is used for irrigation and as drinking water source. For example, approximatly 50% of the U.S. population obtains its drinking water from surface water sources, such as lakes, rivers, and reservoirs. Most of those users live in large cities or near large bodies of water (WHO, 2007).

Figure 1.3: Livestock are part of the pathogen contamination of the food chain. Contamination can be through either consumption of contaminated food or contaminated irrigation/drinking water. From www.obebeef.com

Irrigation water is increasingly implicated as a pathogen source in disease outbreaks associated with the consumption of fruits and vegetables (Table 1.3). Contamination may be due to contact with feces from domestic or wild animals during cultivation, handling or through contaminated irrigation water (Bedient et al., 1994). Fecal contamination of water and other foods, as well as cross-contamination during food preparation (with beef and other meat products, contaminated surfaces and kitchen utensils), may also lead to infection.

Table 1.2: Number of disease infections caused by *E. coli* 0157:H7 by year and location with different sources of contamination. No distinction is made between ground and surface water. From Petridis et al., 2002.

Year	Place	No of infection	Contamination source
1989	Montana, USA	243	Undercooked ground beef
1996	Sakai, Japan	5,727	Poorly washed white radish sprouts
1996	Scotland, UK	496	Undercooked ground beef
2000	Walkerton, Canada	>2,000	Contaminated drinking water
2002	Pennsylvania, USA	51	Petting infected dairy animals

Application of animal manures to agricultural lands is often an economical, practical and environmentally useful best-management option. Manure application as a fertilizer source for crops is a common historical practice (WHO, 2006); however it may introduce a potential pathogen from feces into the food system. A mature dairy cow generates about 70 kg (150 lb) of wet manure per day, which if infected would contain from 10^{10} to 10^{15} of pathogenic microorganisms (You et al., 2006). Once the pathogens are excreted from the animal, conditions are often unfavorable and their survival may be limited (Franz et al., 2005). Pathogens are able to survive manure storage, the manure applied to an agricultural field may serve as a primary source of pathogen contamination.

1.1.3 Coliform bacteria as an indicator of pathogenic contamination

Coliform bacteria are present in the environment and in the feces of all warm-blooded animals. This bacterial group consists of several genera of bacteria and belongs to the family Enterobacteriacae. Coliform is a generic name regrouping three different groups. Each of them has a different level of risk for human, from no health risk to mortal. Total coliform, fecal coliform and *E. coli* are all water quality indicators. The total coliform group is a large collection of different bacteria commonly found in the environment (e.g., soil, vegetation) that are

generally considered harmless. If only total coliform bacteria are detected, the source is probably environmental and fecal contamination is not likely (WHO, 2007).

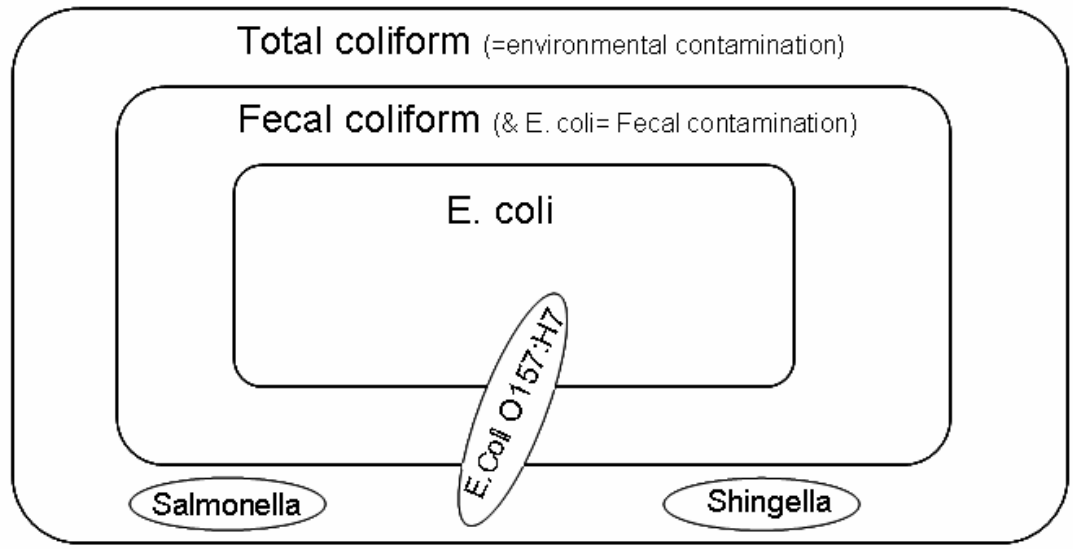

Figure 1.4: Coliform groups. Adapted form Fallon et al., 1997

Fecal coliform are sub-groups of total coliform and are characterized as being able to ferment lactose at 44.5°C. Fecal coliform bacteria are present in large numbers in feces and the intestinal tracts of humans and other warm-blooded animals (Todar, 1997). These bacteria enter water bodies from human and animal waste. If a large number of fecal-coliform bacteria (over 100 colonies/100 ml of water sample) are found in water, it is possible that pathogenic organisms are also present in water (WHO, 2007). Fecal coliform presence is usually a sign of a fecal contamination, but several fecal coliforms are not of fecal origin and originate from an environment rich in organic matter such as an industrial effluent (Barthe et al., 1998). Fecal coliforms by themselves are usually not pathogenic although they can serve as indicator organisms. Fecal coliform survival in the environment generally approximates pathogenic bacteria and their density is representative of pollution caused by feces (Brown, 1995). It is

important to note that coliform bacteria thrive in water enriched with waterfowl droppings, especially when ducks and geese use the pond year round, which can complicate analysis.

E. coli is the most common species of the family Enterobacteriacae (Brown, 1995). Other less prevalent species include *Citrobacter*, *Enterobacter* and *Klebsiella* (Elumd et al., 1999; Edberg et al., 2000). There are many different types of *E. coli* distinguished immunologically by serotyping. The current typing system is based mainly on three types of antigens: the somatic (O) antigen which corresponds to terminal sugar on the cell surface lipopolysaccharide (LPS), the capsular (K) antigens and the flagellar (H) antigen. These Gram-negative rods represent 80 to 90% of the fecal coliforms detected (Barthe *et al.*, 1998) and are one of the most intensively studied living species. Most *E. coli* are harmless and originate from the digestive systems of animals. *E. coli* are not always confined to the intestine, and their ability to survive for periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination in soil and water. However, some strains of *E. coli* such as *E. coli* O157:H7 can cause serious health concerns (Armstrong et al., 1996).

1.1.4 Pathogenic contamination by *E. coli* O157:H7

Since 1982, *E. coli* O157:H7 has been recognized by the Centers for Disease Control (CDC) as an emerging food-borne pathogen (Amstrong et al., 1996). *E. coli* can cause illness: (i) when uropathogenic *E. coli* leave the intestinal tract and enter the urinary tract; (ii) when the bacteria leave the intestinal tract through a perforation into the abdomen and (iii) when certain pathogenic strains like *E. coli* O157:H7 transmitted by food or water are ingested (Muniesta et al., 2006). There are five classes of pathogenic *E.coli*: 1) enterotoxigenic *E. coli* (ETEC), which produces enterotoxins; 2) enteroinvasive *E. coli* (EIEC), invades the colonic epithelium; 3) enteroaggregative *E. coli* (EAggEC), auto-agglutinates bacterial cells; 4) enteropathogenic *E.*

coli (EPEC), produces a pedestal-like structure and produces attaching-and-effacing (A/E) histopathology; and 5) enterohemorhagic *E. coli* (EHEC), causes severe abdominal cramps and bloody diarrhea (Muniesta et al., 2006). Alternative O157:H7 is a mutant form of *E. coli* that acquired a powerful toxin of another bacterium: *Shigella dysenteriae* (Wang et al., 2003). An estimated 73,000 cases of infection and 61 deaths form O157:H7 occur each year in the United States alone (WHO, 2007). Symptoms indicating *E. coli* O157:H7 infection include abdominal cramps and diarrhea that may progress to bloody diarrhea and occasionally to kidney failure, especially in young children and elderly people. The incubation period can range from three to eight days, with a median of three to four days. Most patients recover within 10 days, but in a small number of patients (particularly young children and the elderly), the infection may lead to a life-threatening disease, such as haemolytic uraemic syndrome (HUS). HUS is characterized by acute renal failure, haemolytic anemia (breakdown of red blood cells) and thrombocytopenia (low level of platelets in blood). The minimum number of *E. coli* O157:H7 required to cause disease is not known, but it is suspected that only a small number of bacteria is required (Arizona Department of Health Services, 2008).

1.2 BACTERIAL FATE AND TRANSPORT

Microbial contamination of surface water is a serious concern worldwide because of the possible disease transmission. In many countries, surface water provides approximately 60% of the potable water used for human consumption (Rust et al., 2005). Pathogen transport rate and survival are two factors that govern whether a water source will become contaminated (Fallon et al., 1996).

1.2.1 Bacterial Transport

Many researchers have attempted to predict microorganism transport in surface and groundwater systems based on advection (movement of organisms carried by water in the direction of flow), dispersion (in the direction perpendicular to the flow direction) and sorption mechanisms. Hydrology, chemical properties of water, soil and microbial characteristics all have an influence on bacterial transport (Teutsch et al., 1991, Abu-Ashour et al., 1994; Jordan et al., 2003).

Bacterial transport is directly proportional to the surface and groundwater flow (Fallon et al., 1996). In 1986, a French hydraulic engineer, Henry Darcy, developed an empirical relationship for water flow through porous media (Schnoor, 1997). He found that the specific water discharge was directly proportional to the energy driving force (the hydraulic gradient) according the following relationship:

$$\boxed{\mathfrak{v}_x \alpha (\Delta h) / (\Delta x)}$$

where: $\mathfrak{v}_x$ = specific discharge in the x-direction, LT^{-1}
Δh = the change in head from point 1 to point 2, L
Δx = the distance between point 1 and point 2, L
$\Delta h/\Delta x$ = the hydraulic gradient in the x-direction, dimensionless

The actual velocity is the specific discharge divided by the fractional porosity under saturated conditions, which is greater than the specific discharge because water is forced through the narrow pore spaces, creating faster movement (Schnoor, 1997). This relation can be express as:

$$u_x = \mho_x / \eta \text{ or } u_x = \mho_x / \eta_e$$

where: u_x = actual fluid velocity, LT^{-1}
η = porosity or void volume/total volume
η_e = effective porosity,which reflects the interconnected pore volume through which water actually moves.

Even though bacterial transport is proportional to the water flow, other phenomena can retard movment. One of the most important reactions that a bacterium can undergo is sorption (Guber et al., 2005; Mankin et al., 2007). Sorption is also referred to as adhesion or attachment to soil particles (Mankin et al., 2007). Many factors affect bacterial sorption, and mechanisms of the sorption process are not fully understood despite intensive research in bacterial cell-wall chemistry and surface thermodynamics (Cunliffe et al., 2000).

Although this attachment to soil depends on the morphologic features of the bacteria, soils characteristics have a great influence. Relatively little is known about the transport rates of free cells versus attached cells, which are likely to differ substantially due to the difference in particle sizes and densities (Schnoor, 1997). Three different isotherms have been applied to describe sorption of bacterial cells to soil particles:

The linear isotherm

$$S = K_d C$$

The Freundlich isotherm

$$S = K_f C^{1/n_f}$$

And the Langmuir isotherm

$$S = \frac{S_{\max} KC}{1 + KC}$$

where S = cell number as colony forming units (cfu) sorbed per gram of sorbent (cfu.g^{-1})
K_d = partition coefficient, also called distribution coefficient (mL.g^{-1})
C = bacterial concentration in the liquid phase (cfu.mL^{-1})
S_{max} = maximum sorption capacity of the sorbent or density of bacterial cells in solid phase (cfu.g^{-1})
K = constant related to the binding strength
K_f = Freundlich adsorption coefficient, which is related to adsorption capacity
n_f = linearity coefficient, which is related to adsorption intensity (Miller et al., 2001; Wang et al., 2002).

Sorption mechanisms can also function for bacterial removal from contaminated waste water, introducing the notion of riparian buffer and vegetative filter strips as best management practices, discussed later in this paper.

1.2.2 Survival

1.2.2.1 Environmental Characteristics

Environmental characteristics that affect microbial survival include: soil composition, temperature, water content and hydrology of the area (Dorner et al., 2006). Temperature is a persistent factor in pathogen survival (Table 1.4). For instance, a pathogen such as *E. coli* O157:H7 can survive longer when the temperature of the environment is colder. Under controlled laboratory conditions, *E. coli* O157:H7 survived at least 100 days in bovine manure

frozen at -20°C and 100 days in bovine manure incubated at 4 or 10°C (Guan et al., 2006). Wang et al. (1996) reported longer survival in manure at lower temperatures. Both pathogens survived less well in liquid manure than in solid manure at 20 and 37°C while the opposite was true at 4°C (Guan et al., 2006). Temperature affects the die-off rates of the organisms and also affects the density and viscosity of water altering the water velocity (Teutsch, 1991). An increase in temperature will also cause increased molecular diffusion.

Table 1.3: Survival time (days) of two pathogens: *Salmonella* and *Escherichia coli* O157:H7 relating to the environment and temperature. Adapted from Guan et al., 2006.

Temperature		*Salmonella*	*Escherichia coli O157:H7*
WATER	**Frozen**	>182 d	>300 d
	Cold (5°C)	>182 d	>91 d
	Hot (30°C)	45-152 d	49-84 d
SOIL	**Frozen**	>84 d	>300 d
	Cold (5°C)	63 d	99 d
	Hot (30°C)	>45 d	>56 d
CATTLE MANURE	**Frozen**	>196 d	>100 d
	Cold (5°C)	84-196 d	70d
	Hot (30°C)	48 d	49-56d

Sunlight can affect bacteria through drying (Gibbs, 1995) or from the bactericidal action of ultraviolet radiation (Gerba et al., 1975). Bacteria are protected from the effects of sunlight if they are not on the soil surface or are in shade (Wray, 1975). Ultraviolet (UV) radiation affects

molecules of bound water and can kill cells through its action on the nucleic acids of microorganisms. Ultraviolet radiation, particularly that of 260 nm in wavelength, causes the formation of thymine dimers, inhibiting DNA replication and resulting in mutations (Pelczar et al., 1986). Some pathogens can repair minor damage using enzymes and death will only result if the damage caused by UV is greater than that which can be repaired. The sunlight reaching the Earth's surface contains small amounts of UV radiation in the optimal range for killing microbes (Gascón et al., 1995). Therfore it is unlikely that UV radiation will be a major component in pathogen death, especially when direct exposure to sunlight is limited.

Microorganisms thrive in moisture rich soils, because water provides substrated and a means of nutrient transport, as well as organism transport. The pH of the environment affects the solubility of compounds and the charge distribution in the system, which affects the adsorption of organisms onto the soil. The advantages of soil adsorption, similar to floc formation advantages, include protection and nutrient and substrate sharing by the organisms. In addition to pH, adsorption is also affected by soil type, texture, particle size distribution, and pore size distribution (Teutsch, 1991; Abu-Ashour, 1994).

1.2.2.2 Physical and Chemical Properties of Water and Soil

Physical and chemical characteristics strongly influence pathogen survival in soils (Nicholson et al., 2003; Franz et al., 2005; You et al., 2006). Studies with *E. coli* O157:H7 showed that concentration in soils was positively correlated to total nitrogen content ($r = 0.86$), nitrate content ($r = 0.82$) and total carbon content ($r = 0.82$) (Franz et al., 2005). The study also demonstrated that soil management affected pathogen survival. *E. coli* O157:H7 disappeared more quickly in organically managed soil than in conventionally managed soil. Sandy soil (versus clay soil) also contributed to increased rates of pathogen loss. This may have been due to

the relatively high levels of nitrate, total nitrogen and total carbon in this specific sandy soil. Pathogens such as *E. coli* O157:H7 survive longer in soils with the presence of a sufficient amount of organic matter. Nutrients given by the organic matter are required to allow the bacteria to grow and survive. However, toxic organic matter that acts as antibiotics will decrease the survival rate of the microorganisms. Pathogen survival varies greatly between different soil types. Moreover, the interactions of microorganisms in the environment affect the survival of individual bacteria through predation, competition for nutrients and as a source of nutrients.

1.3 VEGETATIVE FILTER STRIPS

Among the five leading pollutants in U.S. water bodies, pathogens and sediment are ranked second (USDA, 2006). Non-point source (NPS) pollution from agricultural lands has been identified as a significant problem that contributes to water quality degradation. Best management practices (BMPs) have been develloped to help control the movement of potential agricultural pollutants into water resources. Vegetative filter strips (VFS) are popular BMPs for removing sediments and associated contaminants, decreasing pollutant loads from manured fields and pastures (Pajaruli et al., 2008). Representing areas of planted or indigenous vegetation situated between agricultural fields and surface waters, VFS are recommended by the USDA for reducing contaminant runoff (Krutz et al., 2005) (Figure 1.5). These natural filters function by increasing sediment deposition, enhancing infiltration and providing soil and vegetative adsorptive surface. Evidence suggests that VFS can also reduce fecal coliform and pathogen transport to surface waters, particularly in cases when the bacteria are attached to soil particles that act as a transport vehicle.

Often constructed along stream, lake, pond or sinkhole boundaries, filter strips installed on cropland not only help remove pollutants from runoff, but also serve as habitat for wildlife. Filter strips generally are more effective in trapping sediment, and therefore, sediment-bound nutrients and pesticides, than soluble nutrients and pesticides.

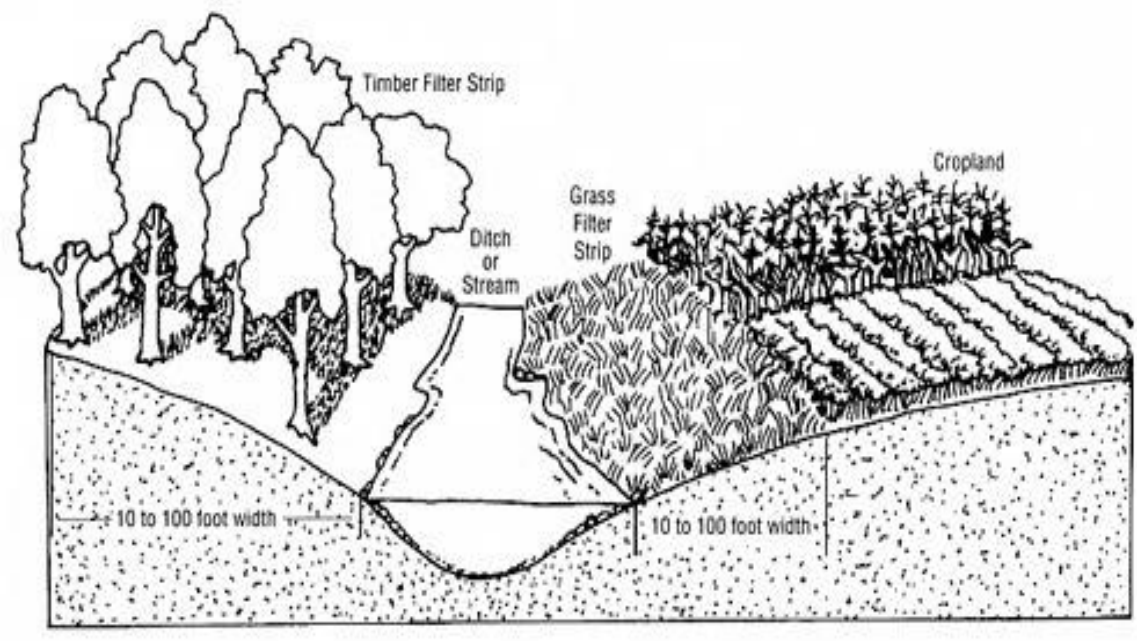

Figure 1.5: Representation of a Vegetative Filter Strip. From University of Iowa

In addition, the filter will be much more effective when the runoff passes through the vegetation in the form of shallow, uniform flow compared to conditions where the flow is concentrated in small channels or gullies. Concentrated flow channels may actually allow the runoff to bypass the vegetation in the filter strip. Shallow, uniform flow provides for maximum contact time for the removal of pollutants by several physical processes, including deposition and infiltration. Studies have to be done in order to see the impact that these VFSs can have on

pathogen and in particular *E. coli* O157:H7 trapping and removal. It is obvious that soil characteristics will have an impact on this removal.

It is vital to understand the influence soil characteristics have on bacterial sorption to particles in order to design appropriate VFS systems to protect surface water from fecal contamination. Thus the goal of this study is to examine the role that physicochemical and biochemical soil characteristics have on *E. coli* attachment.

CHAPTER 2 - SOIL ANALYSIS

2.1 INTRODUCTION

Soil characteristics are critical factors influencing natural processes. For example, bacterial attachment to soil particles can be influenced by clay content (Ling et al., 2002; Guber et al., 2005). A more robust characterization of soil properties should be examined to better understand bacterial fate and transport in the environment. This knowledge can help develop new strategies for mitigating pathogen movement from land into surface waters.

Physical characteristics such as texture class are important soil parameters. Weaver et al., (1978) studied *E. coli* adsorption onto soils having 10% and more than 50% clay content. They found that *E. coli* attachment varied from 7% in the low clay to 90% in the high clay for an initial given concentration.

Chemical characteristics of a soil should be studied as well. Parameters such as pH and specific ion content are critical for determining the type of microbial communities that can exist in a particular soil.

Microorganisms in surface soils are crucial to any natural processes. Microbial communities can be examined on the basis of some parameters that reflect the behavior of soil microorganisms, such as enzyme activity or grouping of fatty acids (Ibekwe and Kennedy, 1998; Kirk et al., 2004)**.** Dehydrogenase and β-Glucosidase are two intracellular enzymes involved in microbial respiratory metabolism (von Mersi and Schinner, 1991) and are good indicators of microbial activity in soils. Fatty acid methyl ester (FAME) and phospholipid fatty acids (PLFA) are biochemical methods that both use fatty acid biomarkers to estimate the diversity of microorganisms present in soil. The FAME technique directly extracts fatty acid acids from soil,

methylated and analyzed them by gas chromatography to detect changes in the composition of the bacterial and/or fungal community. At the same time, PLFA profiles provide a "fingerprint" of the microbial community composition within a given sample. PLFAs are the fatty acids present in the lipid bilayer of the cell membrane of bacteria (Dey et al., 2006).

To evaluate *E. coli* sorption onto soil particles in this study, we utilized six various soils from Washington State with different characteristics. The objective of this portion of the research was to characterize physicochemical and biochemical parameters in the six soils to help determine those that influence *E. coli* attachment in subsequent sections.

2.2 MATERIAL AND METHODS

2.2.1 Soil samples

Six different soils that are representative from the Pacific Northwest were collected from various sites in Washington State (Figure 2.1). Soil samples were collected from the top 10 cm at each location, passed through a 2 mm-pore size sieve (USA Standard n^{o}10), and kept in the dark in the laboratory before use. They were collected between March 2008 and July 2008.

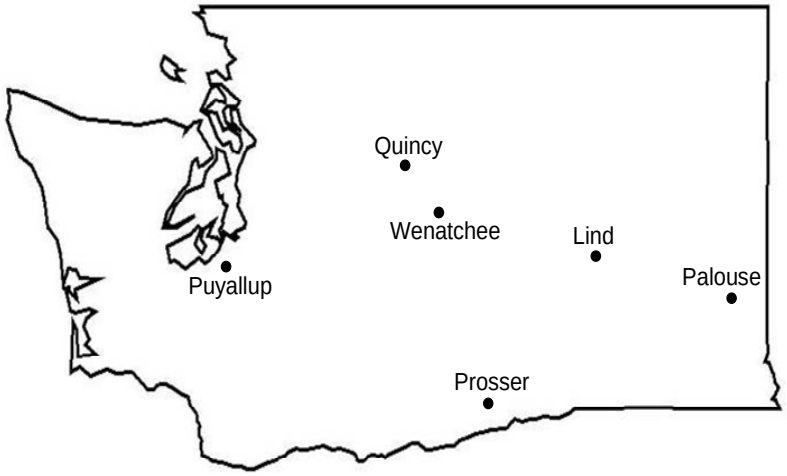

Figure 2.1: Soil sampling location in Washington State

2.2.2 Physical characteristics

The principal physical characteristics of the soil samples were studied using air-dried soil samples (Appendix A). The percent moisture was calculated as the difference in weight of a certain amount of soil before and after 16 hours of drying at 40.5^{o}C. Analysis of clay, silt and sand content of the soils were measured with the hydrometer method of Gee and Bauder (1986) after dispersion in sodium hexametaphosphate (50 g L^{-1}) and using a standard hydrometer,

ASTM no. 152 H, with Bouyoucos scale (g L^{-1}). The U.S Department of Agriculture (USDA) system for distinguishing particle size was used and fractions were classified as sand, silt and clay (Appendix A).

2.2.3 Chemical analysis

Air dried soil samples, in triplicate, were used to estimate the pertinent soil chemical properties using standard procedures (Appendix A). The pH and electrical conductivity (EC) were determined by preparing a 1:1 soil to water slurry and allowing samples to reach equilibrium at room temperature (Smith and Doran, 1996). The pH was determined with an Orion Research 811 (Boston, MA) pH meter. Electrical conductivity was measured using a digital conductivity meter (VWR International, Bristol, CT). The total carbon (TC) content was determined with a LECO CNS analyzer (LECO, St. Joseph, MI) using air dried and ground (to pass a 1-mm sieve) soil. No carbonates where detected, which allowed us to consider the TC as being total organic carbon content (TOC). Cation exchange capacity (CEC) and extractable cations (Na, K, Ca, Mg) were determined by the Analytical Science Laboratory of the University of Idaho, ID. CEC was determined using the Flow Injection Analysis described by Ruzicka et al. (1981) while the extractable cations was analyzed using Field et al. (1999) method.

2.2.4 Microbial activity

Dehydrogenase enzyme activity (DHA) and β-Glucosidase activity were determined as described by Tabatabai (1994). Soil DHA was determined based on the dehydrogenation of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenyl formazan (TPF) by microbial dehydrogenases.

Soil fatty acid methyl esters (FAME) and phospholipid fatty acids (PLFA) procedures generally follow Bligh and Dyer (1954) as described by Ibekwe and Kennedy (1999) for FAME and Petersen and Klug (1994) for PLFA. All reagents were HLPC Grade and purchased from Sigma (St. Louis, MO) except where noted. Soil samples (2 g) were placed in Teflon-lined screw cap culture tubes (16 x 100 mm) and processed according to Sherlock Microbial Identification System (Sherlock Microbial Identification System, 1996) standard protocol. Fatty acid methyl esters analysis was conducted based on saponification of soil at 100^{o}C, acid methylation at 80^{o}C, an alkaline wash, and an extraction of methyl esters of long-chain fatty acids and similar lipid compounds into hexane. Nonadecanoic acid methyl ester was included after the methylation step for both FAME and PLFA to enable quantification of identified lipids on a molar basis. Samples for phospholipid analysis were extracted as described above and further separated by solid phase extraction using 100 mg silica columns (Varian, Palo Alto, CA). Columns were conditioned with 3 mL hexane, 1.5 mL hexane/chloroform (1:1) and 100 µL chloroform and a slight vacuum (1-2 in Hg) was applied to the columns after the addition of each solvent. The columns were rinsed through the sequential addition of 1.5 mL chloroform/2-propanol (1:1) and 1.5 mL 2% acetic acid in diethyl ether with vacuum. Finally, phospholipids were eluted from the columns with 2 mL methanol, and evaporated under nitrogen in preparation of extraction of the PLFAs. The combined organic phase was evaporated to dryness under nitrogen and then redissolved in 75 µL hexane: methyl tertiary butyl ether (1:1).

Fatty acid methyl esters from FAME and PLFA extractions were analyzed on a gas chromatograph (Agilent Technologies GC 6890, Palo Alto, CA) with a fused silica column and equipped with a flameionizer detector and integrator. ChemStation (Agilent Technologies GC 6890, Palo Alto, CA) operated the sampling, analysis, and integration of the samples. Peak

identification and integration of areas were performed under the Eukary method parameters by software supplied by Microbial Identification Systems, Inc. (Newark, DE). Raw percentages of each fatty acid in each sample covered a wide range of values and were log transformed before using the covariance matrix of principal component analysis (PCA) in SAS (2002). Principal component analysis was used to reduce the dimensionality in the data and to examine associations among the soils (Tabachnick and Fidell, 2001). Kaiser's rule (Joliffe, 1986) was employed, wherein only variables with an eigenvalue greater than one are to be used for further analysis when the correlation matrix is used in PCA. In the initial analyses, correlation between principal components and fatty acids for PC1, PC2, PC3, and PC4 was computed. In PCA, the eigenvectors determine the directions of maximum variability and the eigenvalues specify the variances.

Many lipids are associated with taxonomic or functional groups of microorganisms and can provide insight into the types of organisms present in an environmental sample. Analysis of these constituents provides a biochemical fingerprint of microbial communities and yields information such as biomass, physiology, taxonomic and functional identity, and overall community structures. By comparing the biomarkers found in the six different soils with established databases, we were able to estimate the microbial biomass present in the samples. Biomarkers allowed estimating a percentage of bacteria requiring either oxygen to live (aerobe) or bacteria which require another terminal election receptor (anaerobe). The presence of a cell wall composed of a thick layer of a particular substance (peptidologlycan).was studied in order to know the percentage of Gram-positive bacteria versus Gram-negative (lack of peptoglycan) among the microbial community. Fungal-specific biochemical markers were used to estimate the biomass of mycorrhizal fungi. These fungi form symbiotic relationships in and on the roots of

host plants. Finally, under adverse cultural or environmental conditions, for example depletion of nutrients, changes in pH, temperature etc., bacteria launch “stress responses” which significantly improve their chances of survival under, or successful adaptation to, the challenges posed by such unfavourable environments.

Each sample peak from the PLFA analysis was compared against a database of known microbial fingerprints. Peaks that correspond to 12 to 20 carbon chain lengths are generally associated with microorganisms. Bacterial:fungal ratios were calculated for each sample. Peaks used as marker for bacteria were 12:0b, C12 Primary Alcohol, 14:0 iso, 15:0, 15:0 anteiso, 15:0 iso, 15:0 iso g, 15:1 cyclo, 16:0 me 10, 16:1 ω 7, 16:1 ω 7c, 16:1 ω7t, c16 alcohol, 17:0 anteiso, 17:0 cyc, 17:0 iso, 17:0me10, 17:1 ω6, 17:1 ω7i, 18:1 ω7, 18:1 ω7c, 18:1 ω9c, 19:0 cyc, 19:0 cyc c11-12, 19:0 cyc ω9c/19:1 and for fungi were. 16:1 ω5, 16:1 ω5c, 18:1 ω9, 18:2 ω6, 18:2 ω6c, 18:2 ω9, 18:3 ω3, 18:3 ω6, 18:3 ω6c. Stress indicators were calculated based on the ratios of the cyclopropyl fatty acids to monoenoic precursors and the total saturated to total monounsaturated fatty acids (Kieft et al., 1997; Bossio and Scow, 1998; Fierer et al., 2003). Specific peaks used to calculate the cyclopropyl fatty acids to monoenoic precursor ratios were cy17:0 to 16:1ω7c and cy19:0 to 18:1ω7c. The ratio of total saturated to total monounsaturated fatty acids used the ratio of the sum of 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0 to sum of 16:1ω11c, 16:1ω9c, 16:1ω7c, 16:1ω5c, 17:1ω9c, 17:1ω8c, 17:1ω7c, and 17:1ω5c. For Gram positive bacteria, markers were 15:0, 15:0 anteiso, 15:0 isoG, 16:0 Me 10, C16 alcohol, 17:0 iso, 18:1 ω9c. The markers for gram negative bacteria were 12:0b; 17:0 cyc, 18:1ω7c; 19:0 cyc, 19:0 cyc c 11-12, 19:0 cyc ω9c/19:1. Mycorrhizal markers were 16:1 ω5, 16:1 ω5c, 18:2 ω6, 18:2 ω6c, 18:2 ω9. Monounsaturated fatty acids from 14:0 to 19:0 were also evaluated (Bossio and Scow, 1998). Microbial community composition was also evaluated using monounsaturated fatty acids

from 14:0 to 19:0 (Bossio and Scow 1998). Biomass was calculated from mol response readings using the relationship determined by Bailey et al. (2002). The mol response for each sample was summed and then multiplied by an extraction efficiency factor (based on internal standards added to each run); the resultant response was then entered into the following equation: biomass = 2.4 (response) + 46.2. .

2.3 RESULTS AND DISCUSSION

2.3.1 Physical characteristics

Particle size analysis allowed classifying the six soil samples into three different classes: silt loam, sandy loam and sandsandy loam (Figure 2.2). Pullman and Lind samples were classified as silt Loam, with a percentage of 79% silt for Pullman and 66% silt for Lind. Of all the soils studied, the soil from Pullman had the highest percentage of clay of at 17% while Lind had only 4% clay. Three soil samples were classified as sandy loam: Wenatchee, Puyallup and Prosser. Their particle size distribution is very similar with around 50% sand, 45 % silt and 5% clay. The Quincy sample has been classified as sandsandy loam with 98% Sand, 2% Silt and no Clay particles.

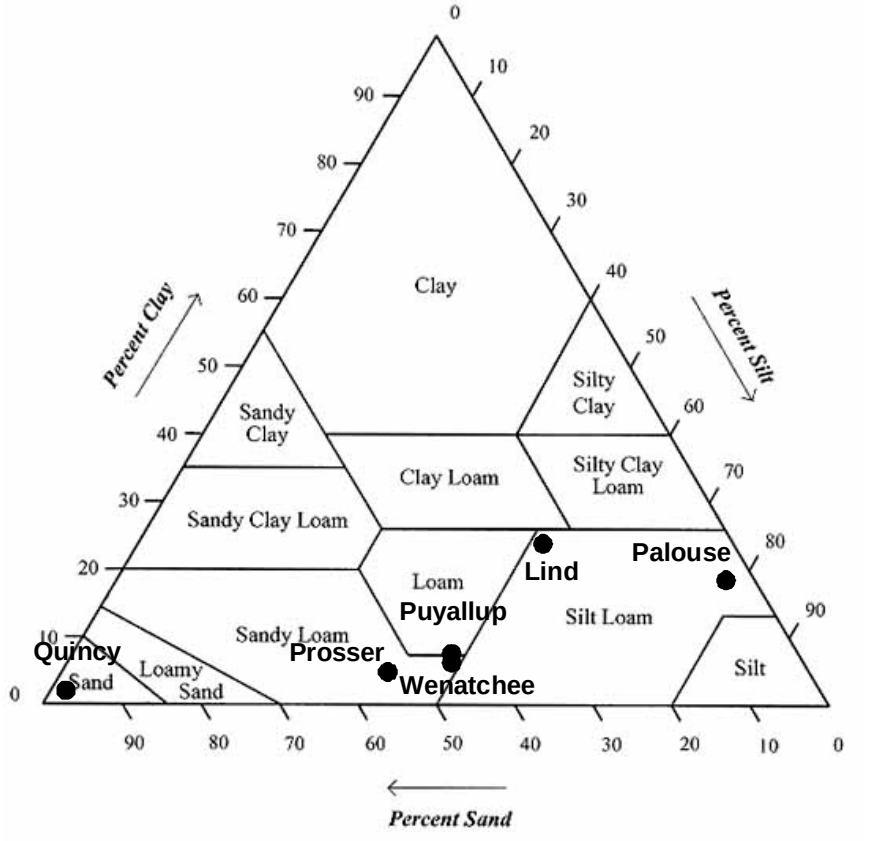

Figure 2.2: Soil repartition within the USDA classification

Percent moisture analysis showed similarities in results with the particle size distribution. The two soils with the highest percent moisture were the two silt Loam (14.60 ± 0.07% for Pullman and 8.95 ± 0.09% for Lind), followed by the three sandy Loams (ranging from 1.15 ± 0.02% to 3.69 ± 0.10%). Finally, the Quincy soil sample had the least soil moisture content, with 0.97 ± 0.02%.

2.3.2 Chemical analysis

The pH levels of the soil samples ranged from 4.71 ± 0.01 to 7.72 ± 0.10. Pullman and Puyallup samples were acidic with pH values of 4.71 ± 0.01 and 5.54 ± 0.01 respectively. All the other soils had pH values in the neutral range: between 6 and 8. Studies have shown that among various environmental factors, pH is important in affecting the surface charge of soils and the availability of plant nutrient and microorganisms (Escobar and Hue, 2008).

Electrical conductivity of most of the soils in this study ranged from 1 to 6.2 $dS.gsoil^{-1}$, except for the Lind soil, which had a high value of 15.78 ± 5.83 $dS.gsoil^{-1}$.

Table 2.1: Physical and chemical properties of the soils

			Pullman	Wenatchee	Puyallup	Quincy	Lind	Prosser
n[b] = 3	**pH**		4.71 (0.01)	6.39 (0.05)	5.54 (0.01)	7.75 (0.13)	6.31 (0.07)	7.72 (0.10)
	EC	dS/gsoil	5.78 (0.18)	1.06 (0.04)	6.22 (0.07)	1.53 (0.05)	15.78 (0.58)	2.96 (0.15)
	Percent moisture	%	14.60 (0.07)	3.69 (0.10)	1.15 (0.02)	0.97 (0.02)	8.95 (0.09)	1.99 (0)
n = 3	**TOC[c]**	mg Cg^{-1}soil	22.7 (0.9)	9.6 (0.3)	20.6 (0.3)	2.4 (0.07)	7.5 (0.1)	5.6 (0.4)
	TOC[c]	% $C.g^{-1}$soil	2.27 (0.09)	0.96 (0.03)	2.06 (0.03)	0.24 (0.007)	0.75 (0.01)	0.56 (0.04)
	Sulfur	% S.g-1soil	0.03 (0.002)	0.09 (0.0003)	0.05 (0.004)	0.0001 (0.0005)	0.008 (0.002)	0.0007 (0.0006)
	Nitrogen	% N.g-1soil	0.17 (0.006)	0.06 (0.002)	0.17 (0.003)	0.0002 (0.0007)	0.08 (0.01)	0.01 (0.002)
n = 1	**Calcium**	cmol(+)/kg	5.70	6.40	5.20	0.02	5.70	31.00
	Magnesium	cmol(+)/kg	1.20	2.00	1.00	0.02	2.30	2.70
	Potassium	cmol(+)/kg	1.70	1.40	0.68	0.08	2.40	0.35
	Sodium	cmol(+)/kg	0.09	0.09	0.17	0.05	0.25	0.19
	CEC[d]	cmol(+)/kg	22.00	16.00	15.00	7.30	13.00	18.00
n = 2	**Sand**	% (wt)	4	50	50	98	30	54
	Silt	% (wt)	79	44	45	2	66	43
	Clay	% (wt)	17	6	5	0	4	3
	Textural Class		Silt Loam	Sandy Loam	Sandy Loam	Sandsandy Loam	Silt Loam	Sandy Loam

[a] The values in parentheses are standard deviation

[b] n-Number of replicates for each analysis

[c] TOC-Total Organic Carbon (calculated from the Total Carbon and carbonates content)

[d] CEC- Cation Exchange capacity

Analysis of the total organic carbon (TOC) showed that the two soils with the lower pH had the highest TOC values with 22.7 mg-C.g^{-1}soil for Pullman and 20.6 mg-C.g^{-1}soil for Puyallup respectively. These two soils were followed by Wenatchee (9.6 mg-C.g^{-1}soil), Lind (7.5 mg-C.g^{-1}soil) and Prosser (5.6 mg-C.g^{-1}soil). Finally, Quincy had a very low TOC content with only 2.4 mg-C.g^{-1}soil.

The sulfur and nitrogen in the soil samples were very low with levels approaching zero. Ion content analysis revealed several differences and similarities among soil samples. The calcium content for each soil ranged from 5.20 cmol.kg^{-1} to 6.40 cmol.kg^{-1}, except for the low Quincy value (0.02 cmol.kg^{-1}) and a high Prosser value (31 cmol.kg^{-1}). Magnesium and potassium levels were similar for the soil samples and varied between 0.02 cmol.kg^{-1} and 2.70 cmol.kg^{-1}. The Quincy soil had the lowest values for both with 0.02 cmol.kg^{-1} for magnesium and 0.08 cmol.kg^{-1} for potassium. The highest value for sodium was 0.19 cmol.kg^{-1} for Prosser, indicating the low sodium content of all our samples. Finally, the cation exchange capacity (CEC) ranked from highest to lowest were: Pullman, Prosser, Wenatchee, Puyallup, Lind, Quincy. Quincy's CEC value (7.30 cmol.kg^{-1}) was three times less than the Pullman (22.00 cmol.kg^{-1}).

2.3.3 Microbial activity

Dehydrogenase activity (DHA) and β-Glucosidase activity were different for every soil. For both DHA and β-Glucosidase, the soil samples can be ranked from high soil activity to the lowest (Figure 2.3). The ranking from the highest microbial activity to the lowest based on the dehydrogenase (Figure 2.3 A) is the same than the one base on the β-Glucosidase activity. This ranking is from the highest to the lowest: Wenatchee, Pullman, Puyallup, Lind, Quincy, Prosser. Wenatchee soil had the greater microbial activity 5.51 ±

0.34 µg.gsoil^{-1}.hr^{-1} while Prosser had only 0.28 ± 0.07 µg.gsoil^{-1}.hr^{-1}. These values are similar to published values of soils of Washington State (Kennedy and Schillinger, 2006; Schillinger et al., 2007; Cochran et al., 2007).

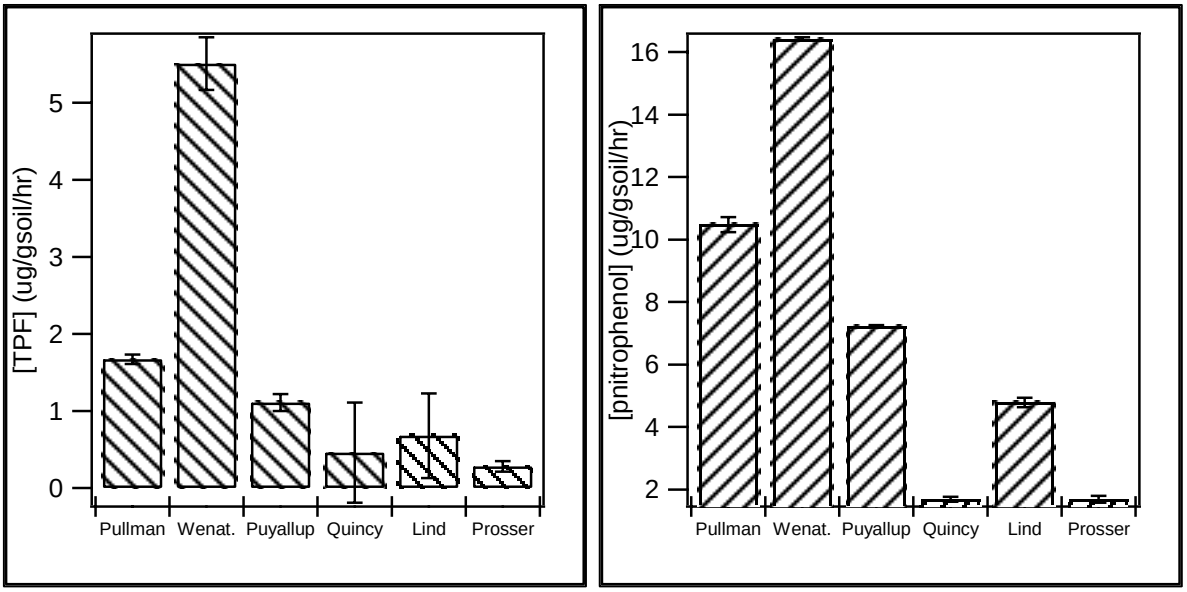

Figure 2.3: Microbial Activity comparison in the six soil samples using DHA (A) and β-glucosidase Activity (B)

FAME and PLFA are biochemical methods that can provide detailed information about the structure of the active microbial community. PCA was used to compare FAME and PLFA profiles between the six soil samples (Figure 2.4 and 2.5). The use of PCA requires that the number of samples is greater than the number of variables (Joliffe, 1986). While we did not meet these criteria here, the visual representation of the diversity of soil fingerprints showed us that we were working with a diverse group of soils.

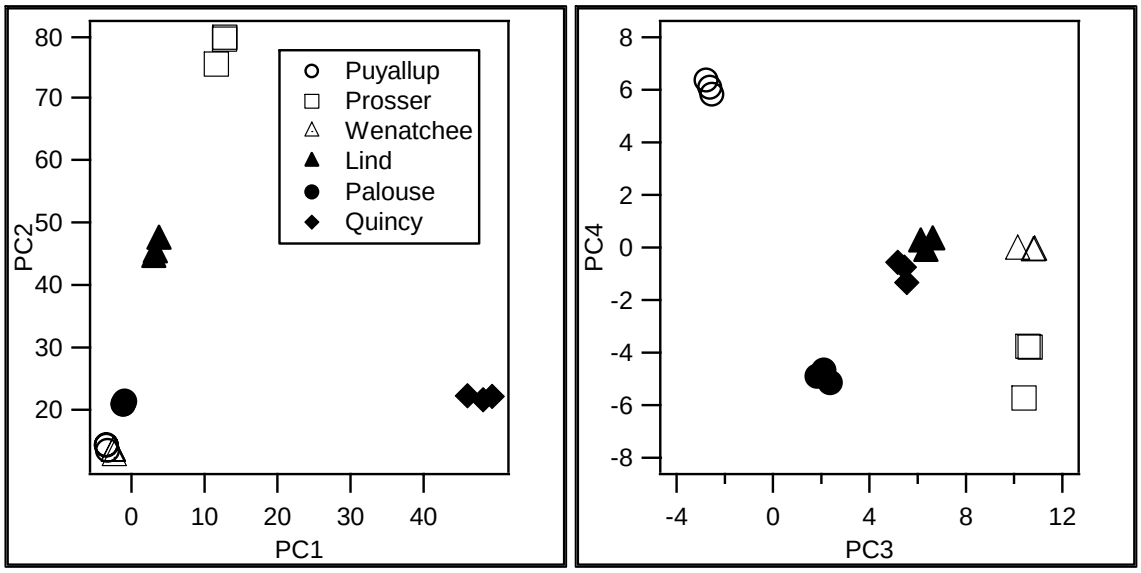

Figure 2.4: Principal component analyses (PCA) of fatty acid methyl ester (FAME) in the six different soils from Washington State, USA.

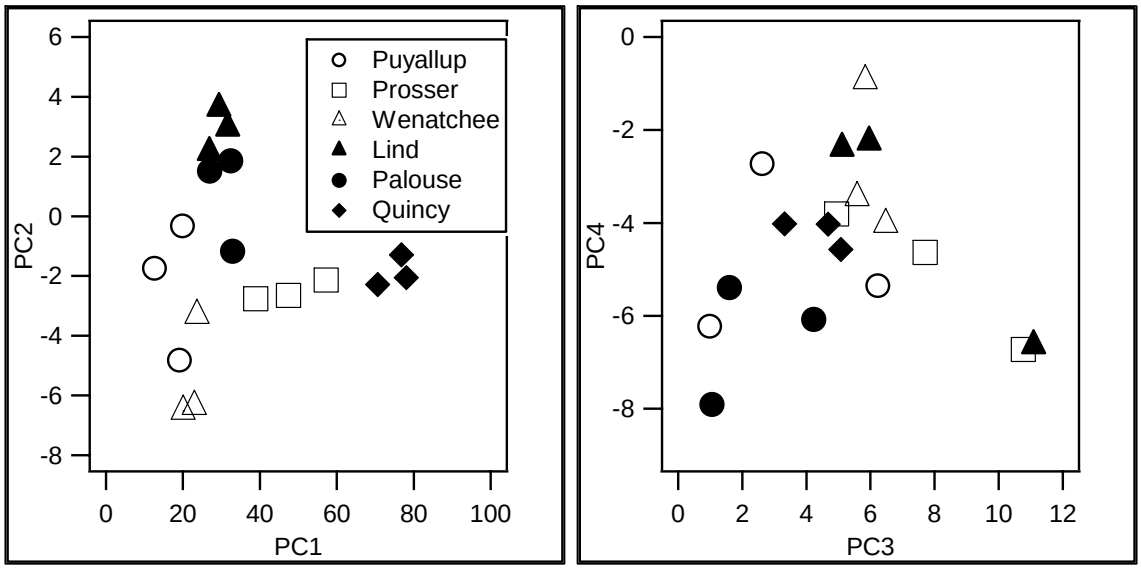

Figure 2.5: Principal component analyses (PCA) of phospholipids fatty acid methyl ester (PLFA) in the six different soils from Washington State, USA

Every soil has a very specific microbial community based on groupings of their fatty acids (Figure 2.4). This study by fatty acids extraction directly from soil, allowed us to compare differences in the composition of the bacterial and fungal community. Regarding PC1 and PC2, Pullman, Wenatchee and Puyallup had some similarities in their microbial activity (closely related), but presented some major differences regarding to PC3 and PC4. Every soil had then some differences, consequence of a different location, but some of them (Pullman, Wenatchee and Puyallup) presented some similarities in their microbial activity.

Phospholipid fatty acids analysis provides us detailed information about the structure of the active microbial activity (Figure 2.5). This fingerprint of soil microbial communities is more precise and describes the more active fraction of a soil biota than FAME. The six soil samples represented a great diversity in terms of microbial activity with a few similarities for three of them. PLFA profiles as compared in PC1 vs PC2 showed that every soil sample contained their own unique characteristics. The soils were compared as to biomass, bacterial to fungal ratio and biomarkers (Table 2.2).

Puyallup and Wenatchee soils were the two soils having the highest microbial biomass with values of 409 and 495 mg-Cg^{-1}soil, respectively. The Lind and Pullman soils were next with relatively high values of 323 and 344 mg-Cg^{-1}soil. Finally, the two soils with the lowest microbial biomass were Prosser and Quincy with values of 202 mg-Cg^{-1}soil and 134 mg-Cg^{-1}soil, respectively.

Table 2.2: Microbial biomass and response of soil microbial markers determined from phospholipid fatty acid analysis of the six soils from Washington State, U.S.A. [a]

	Microbial Biomass	pnitrophenol[c]	TPF[d]	Gram− Negative Bacteria	Gram− Positive Bacteria	Aerobic Bacteria	Anaerobic Bacteria	Mycorrhizal Fungi	Monounsatured Fatty Acids	B:F Ratio
	$mgCg^{-1}soil$	µg/gsoil/hr		% of total microbial population						
Lind	323 (34)	4.79 (0.15)	0.68 (0.55)	6.80 (0.01)	10.65 (0.01)	6.80 (0.001)	5.79 (0.01)	5.79 (0.02)	0.28 (0.008)	1.77 (0.51)
Pullman	344 (24)	10.48 (0.24)	1.67 (0.06)	9.08 (0.001)	12.47 (0.001)	9.08 (0.001)	3.90 (0.0004)	3.90 (0.007)	0.33 (0.04)	2.62 (0.34)
Prosser	202 (37)	1.69 (0.11)	0.28 (0.07)	6.23 (0.006)	7.15 (0.008)	5.46 (0.001)	4.39 (0.003)	4.39 (0.02)	0.37 (0.08)	1.61 (0.32)
Puyallup	409 (109)	7.23 (0.03)	1.11 (0.11)	5.95 (0.005)	10.43 (0.006)	5.82 (0.003)	5.21 (0.004)	5.21 (0.01)	0.2 (0.03)	2.09 (0.40)
Quincy	134 (22)	1.69 (0.08)	0.46 (0.65)	2.89 (0.005)	2.67 (0.008)	2.89 (0.008)	2.00 (0.005)	2.00 (0.008)	0.63 (0.05)	1.3 (0.08)
Wennatchee	495 (32)	16.42(0.05)	5.51 (0.34)	4.29 (0.003)	9.82 (0.003)	4.29 (0.002)	3.74 (0.001)	3.74 (0.003)	0.19 (0.008)	1.81 (0.09)

$n^b = 3$

[a] values in parenthesis are the standard deviations

[b] n-Number of replicates for each analysis

[c] pnitrophenol-Measure of the beta glucosidase activity

[d] TPF-Measure of the dehydrogenase activity (DHA)

A detailed description of the results from the PLFA analysis from all the soil samples in order of the soils from the most microbial biomass to the least follows. The Wenatchee soil contained the highest level of microbial biomass per gram of soil. This soil also contained an average of 1.81 times more bacteria fungi. Within the bacterial markers, 4.3% were Gram-negative bacterial markers while 9.8% were positive. This soil contained more aerobic bacterial markers than anaerobic, and had a low level of stress-type biomarkers.

The Puyallup soil contained the second highest level of microbial biomass per gram of soil. This soil contained 2.09 times more bacteria than it contains fungi, making this soil the second highest bacteria to fungi ratio after Pullman. Within these bacterial markers, 6% were Gram-negative while 10% were Gram-positive. This soil contained more aerobic bacterial markers than anaerobic, and had a low level of stress as did the Wenatchee soil.

The Pullman soil contained 344 mg-C per gram of soil. This soil had in average 2.62 times more bacteria than fungi, making this soil the one with the highest bacterial to fungial ratio. Within these bacteria, 9.1% were Gram-negative while 12.5% were Gram-positive. This soil contained more aerobic bacteria markers than anaerobic, and had a low stress level.

The Pullman and the Lind soil were similar to each other when the characteristics studied from the PLFA analysis were compared. The microbial biomass for this soil was 323 mg-C per gram of soil. This soil contained 1.77 times more bacteria than it contained fungi. Within these bacteria, 6.8% were Gram-negative bacterial markers while 10.7% were Gram-positive. This soil contained more aerobic bacterial markers than anaerobic.

The Prosser soil had a low microbial biomass of 202 mg-C.g^{-1}soil. The bacterial:fungal ratio was 1.6 with 6.8% of Gram-negative bacterial markers while 10.2%

were Gram-positive, This soil contained more aerobic bacterial markers than anaerobic. The stress level is the highest with an indicator of 14.9.

The Quincy soil contained the lowest amount of microbial biomass per gram of soil (102). For this soil, all the microbial PLFA values were low. In fact, the Quincy soil was consistently lowest in all the microbial markers calculated. Nevertheless, it still contained more bacteria than fungi and more Gram-negative than Gram-positive. Finally, the stress level of this soil was one of the highest with a value of 14.6%.

The six soils studied here were diverse in their microbiology. The two soils with the lowest microbial biomass also had low values in the other microbial measurements except that they both had a high level of stress markers. These stress markers could in fact show that the microbial populations were faced with difficult survival. Moreover, soils with a high microbial activity had a lower stress indicator.

2.4 CONCLUSION

In this study, we contrasted and compared the physical, chemical, biochemical characteristics of six soils from Washington State. Information from these experiments will allow interpretation of *E. coli* attachment onto different soil types. To date, very few studies have examined the impact of factors such as soil texture, electric conductivity, pH, carbon content, extractable cations, cation exchange capacity or microbial activity when evaluating sorption of *E. coli* to soil particles.

CHAPTER 3 - SORPTION STUDIES

3.1 INTRODUCTION

Pathogenic pollution of surface water is a great concern to the public due to the potential for disease transmission (Ling et al., 2002). Freshwater plays two essential roles in the food chain: it is used as both an irrigation and drinking water source. In the United States, 76 million cases of food-borne diseases are estimated to occur each year, which results in 325,000 hospitalizations and 5,000 deaths (Mead, 1999). These illnesses result from the consumption of contaminated food and are primarily caused by a variety of pathogenic bacteria, viruses, or parasites. Agricultural animals such as cattle and poultry are widely recognized as the most important pathogen reservoirs in the environment and can contaminated food via fecal material (Muniesta et al., 2006; Echeverry, 2006). Although considered a beneficial fertilizer and soil amendment, the 70 kg (150 lb) of wet manure that a mature dairy cow generates per day can serve as a reservoir for 10^{10} to 10^{15} bacterial human pathogens (Guber et al., 2005; Guan et al., 2006).

Bacterial sorption to soil particles is an important mechanism in understanding how to best prevent contamination of surface waters (Mankin et al., 2007; Nola et al., 2002; Guber et al., 2005). Many factors that affect bacterial sorption are not fully understood despite intensive research on bacterial cell-wall chemistry and surface thermodynamics (Cunliffe et al., 2000). Although soil attachment depends on bacterial morphologic features, soil characteristics contribute a significant influence (Nola et al., 2002). Few studies have examined the impact of such factors as soil texture, electric conductivity, pH, carbon content,

extractable cations, cation exchange capacity or microbial activity when evaluating sorption of *E. coli* to soil particles.

To better understand pathogen adsorption onto soil particles, we examined the attachment of an *Escherichia coli* strain onto six soils with different characteristics representative of the Pacific Northwest to demonstrate the role soil characteristics have on bacterial attachment.

3.2 MATERIAL AND METHODS

3.2.1 Soils

Six different soils were collected from various sites in Washington State for use in this study (Figure 2.1). Methods used to characterize the physical, chemical and biochemical characteristics are shown in Chapter 2.

3.2.2 *E. coli*

This study used *E. coli* H4H, originally isolated and maintained by the College of Veterinary Medicine at Washington State University, Pullman WA. The cells of this strain are motile and rod-shaped. The original *E.coli* H4H inoculum was prepared by growing a culture in Tryptic sulfate broth (Difco, Detroit, Mich.) overnight at the laboratory temperature (22 ± 1^{o}C). Inoculation of 1 mL of this culture onto 24 mL of fresh Tryptic sulfate broth (Difco, Detroit, Mich.) was placed on an orbital shaker at 110 ± 10 rpm during a sufficient time to adjust to a concentration ranged from 10^7 to 10^8 colonies.mL^{-1}. This estimation of the *E.coli* population was done by using optical density measurement at 600 nm (Spectronic 20 Genesys, Thermo Electron Scientific Instruments, Madison, WI; Miller et al., 1972).

3.2.3 Bacterial Enumeration

The membrane-filtration method was used for *E. coli* enumeration as described in the APHA Standard Methods (Part 9222G; APHA, 1998). 50mL sterile polypropene centrifuge tubes containing the *E.coli* solution were vortexed before samples of 50 µl to 1 mL were taken for serial dilution into 0.1 NaCl and plated to determine the concentration range of *E.*

coli in each sample. Samples were filtrated onto a 0.45 µm gridded sterile filter membrane and then placed onto m-FC media (Difco, Detroit, Mich.) that was re-hydrated in 1 L water containing 10 mL of 1% rosolic acid in 0.2 N NaOH. The mFC plates were incubated at 44.5 ± 0.2°C for 24 ± 2h in a water bath in order to avoid any temperature variation. Colonies produced by fecal coliform bacteria and then by the *E. coli* strain on m-FC medium were blue. Bacterial counting was made using the calculation recommended in the Standard Method (Part 9222B):

$$\textit{E. coli} \text{ colonies } .mL^{-1} = \textit{E. coli} \text{ counted / mL sample filtered}$$

If the colonies were in the countable range (20 to 60 cfu) otherwise using the estimation showed in the same Standard Method.

$$\textit{E. coli} \text{ colonies } .mL^{-1} = \text{Sum of E. coli counted in all samples / Sum of mL sample filtered}$$

Data from each sample were obtained in triplicate and the mean was recorded for the analysis. Results were recorded as colony forming units per millimeter ($CFU.mL^{-1}$)

3.2.4 Adsorption experiments

3.2.4.1 Temperature

In previous studies, Guber et al. (2007) used a batch experiment at 8°C while Mankin et al. (2007) used a different laboratory temperature (22°C). In order to determine the temperature to use in these experiments, a comparison of the *E. coli* H4H growth at different temperature was made. One mL of *E. coli* H4H culture was inoculated onto two 50 mL Erlenmeyer flasks containing 24 mL of fresh Tryptic sulfate broth (Difco, Detroit, Mich.).

Both Erlenmeyers were placed on an orbital shaker at 110 ± 10 rpm at 8°C and 22°C. Estimation of the *E.coli* population was done by using optical density measurement at 600 nm (Spectronic 20 Genesys, Thermo Electron Scientific Instruments, Madison, WI; Miller et al., 1972). Microbial growth was minimal at 8°C (Figure 3.2).

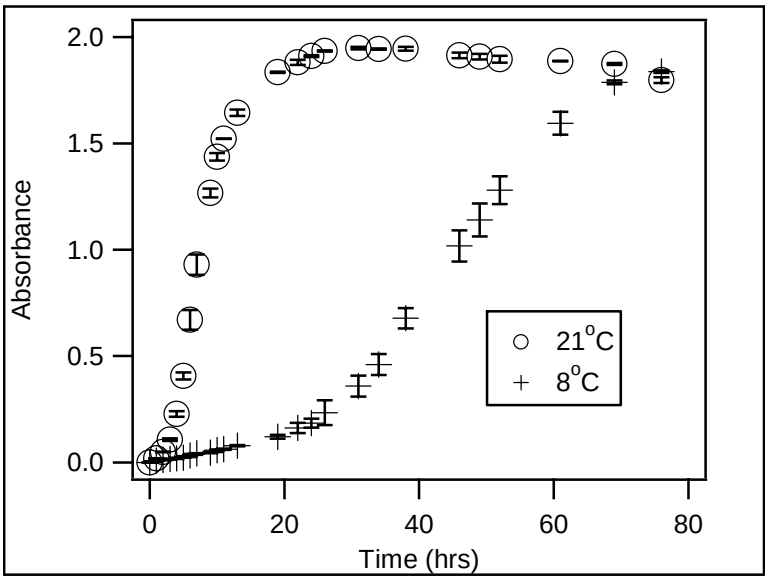

Figure 3.1: *E. coli* H4H growth curve at 8°C and 24°C

3.2.4.2 Batch equilibrium

A batch equilibrium technique was used to determine bacterial sorption to the different soils. All treatments were performed in triplicate. A mass of 1 g of dry weight soil was added to 50 mL sterile polypropene centrifuge tubes. To facilitate the mixing of soil and bacteria, centrifuges tubes were filled with 9 mL of 0.1 M NaCl. Centrifuges tubes were placed horizontally on an orbital shaker at 110 ± 10 rpm overnight in a VWR Model 2020 incubator set at 8 ± 1°C. No background counts of *E. coli* and fecal coliforms were detected on the soils. The soil suspensions where then inoculated with 1mL of individual *E. coli* concentrations (from 10^6 to 10^8) and shaken for 2 hours as described above. The *E. coli* populations in suspensions were obtained by serial dilution of an initial *E. coli* inoculum in

TSB onto specific quantities of 0.1 NaCl. The time required for sorption to reach equilibration was determined experimentally (internal data not shown), which corresponded with the equilibrium time described by Olivier et al. (2007). A refrigerated Sorval centrifuge (model RC 50+, Dupont) was used to separate sorbed *E. coli* from *E. coli* still in suspension. Stokes' law was used in order to determine the centrifugation time and speed because of the difference of the difference between bacterial (1.0 to 1.1 $g.cm^{-3}$) and clay (2.6 $g.cm^{-3}$) densities (Ling et al., 2002). The assumption of the fact that all clay particles were spherical and have the same density was made in order to apply Strokes' law. Finally after a 5 min centrifugation time at 1490±10rpm, the supernatant was removed and plated for *E. coli* enumeration following the method previously detailed. Controls containing only soil and 0.1 M NaCl were made in order to confirm the fact that no background of any kind of fecal coliform was present on any soil studied. The amount of attached *E. coli* was calculated from the difference between the amount applied and the amount recovered in the supernatant.

The Freundlich isotherm equation:

$$S = K_f C^{1/n_f}$$

was fitted to the data, where S is the equilibrium concentration of colony-forming units attached to gram of soil (CFU g-1), C is the solution concentration of bacteria (CFU L-1), Kf is the Freundlich adsorption coefficient related to adsorption capacity and n_f the linearity coefficient related to adsorption intensity.

3.3 RESULTS AND DISCUSSION

3.3.1 Principal soil characteristics

Soils characteristics have been studied in detail and results are expressed in Chapter 2. Briefly, two soils were classified as silt loams (Pullman and Lind), three as sandy loams (Wenatchee, Puyallup and Prosser) and one as sandsandy loam (Quincy). pH levels of the soil samples ranged from 4.71 ± 0.01 to 7.72 ± 0.10. Pullman and Puyallup samples were acidic with pH values of 4.71 ± 0.01 and 5.54 ± 0.01 respectively. Analysis of the total organic carbon (TOC) showed that the two soils with the lower pH had the highest TOC values with 22.7 mg-C.g^{-1}soil for Pullman and 20.6 mg-C.g^{-1}soil for Puyallup. The ranking from the highest microbial activity to the lowest based on the enzyme dehydrogenase is identical to that ranking from β-Glucosidase activity. This ranking is from the highest to the lowest: Wenatchee, Pullman, Puyallup, Lind, Quincy, Prosser.

3.3.2 *E.coli* attachment

The experimental data on *E. coli* attachment to the six soils from different regions in Washington State are shown in Figure 3.3. Biochemical and physicochemical soil characteristics had a strong influence on *E. coli* attachment. The number of *E. coli* cells sorbed for a same initial concentration varied with the type of soil used.

The applicability of the Freundlich isotherm had been shown in other studies (Mankin et al., 2007) and the values of the coefficient of determination (R^2) for the fits of *E. coli* H4H data to the Freundlich equation were high enough to confirm this statement. The effect of soil characteristics on *E. coli* attachment are reflected by the isotherm parameters (Table 3.3).

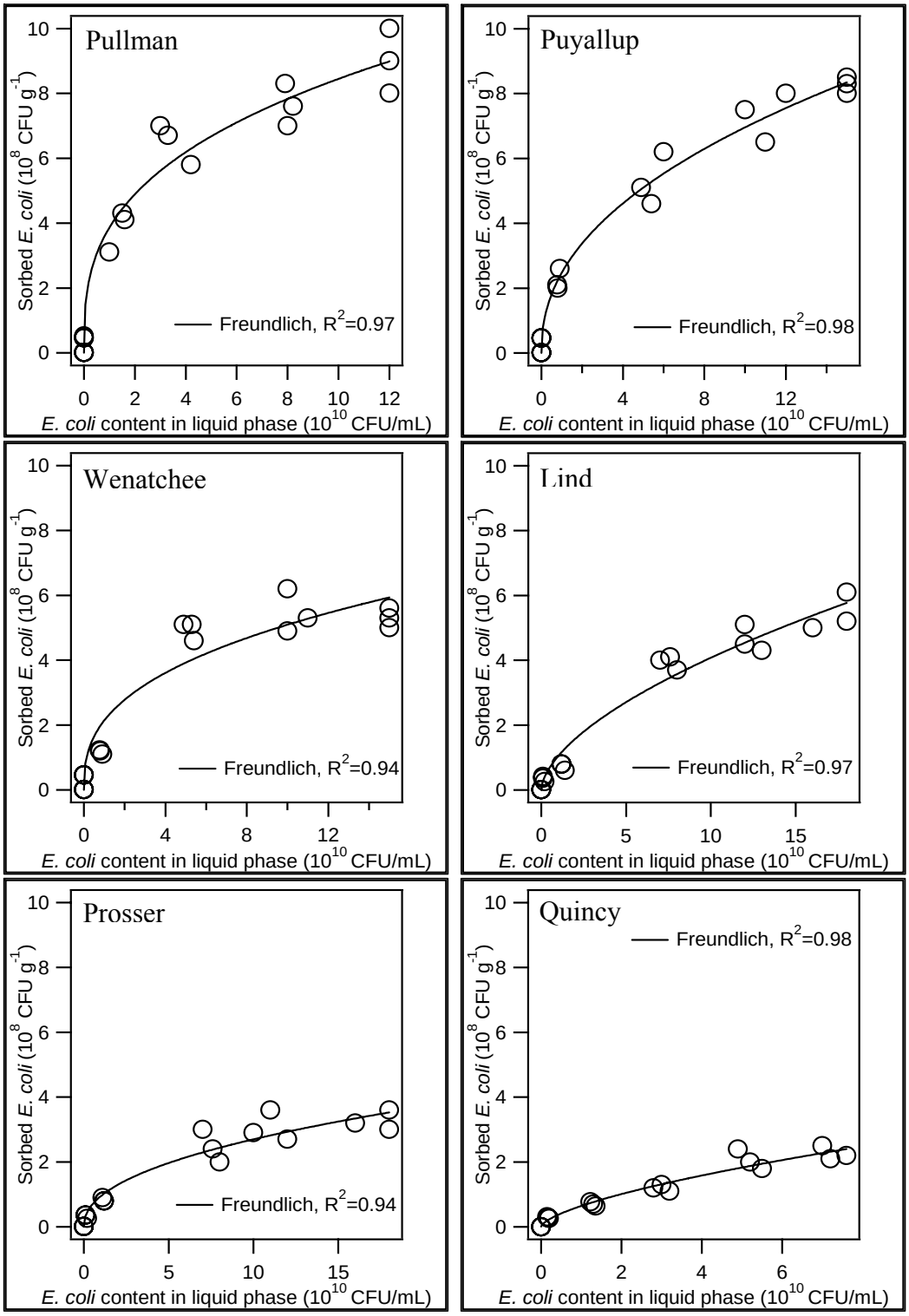

Figure 3.2: Attachment isotherms of Escherichia coli for Pullman, Puyallup, Wenatchee, Lind, Prosser and Quincy.

Table 3.1: Parameters of the Freundlich isotherms.

	K_f [b]	n_f [c]	R^2
Pullman	3.86(0.33)	0.34 (0.04)	0.97
Puyallup	2.47 (0.19	0.45 (0.03)	0.98
Wenatchee	2.14 (0.34	0.38 (0.06)	0.94
Lind	1.05 (0.16	0.59 (0.06)	0.97
Prosser	0.94 (0.14	0.46 (0.06)	0.94
Quincy	0.64 (0.08	0.65 (0.07)	0.98

[a] Values in parenthesis are the standard deviations

[b] Freundlich adsorption coefficient

[c] Lineary coeficient

All soils presented sorption capacity (Figure 3.3). Pullman soil with the highest *E. coli* sorption had a Freundlich coefficient 16.6% higher than the Quincy soil. Sorption capacity of every soil is reflected by the Freundlich coefficient (K_f). In this study the soils can be classified from the most sorbent to the less as Pullman, Puyallup, Wenatchee, Lind, Prosser and Quincy. Very few studies have examined the impact of factors such as soil texture, pH, carbon content, cation exchange capacity or even microbial activity when evaluating sorption of *E. coli* to soil particles.

To analyze the role theses physicochemical and biochemical soil characteristics on *E. coli* attachment, Pearson Product Moment Correlation (r) of each parameter was calculated in order to determinate the coefficient of determination (r^2) and p-value (Table 3.2 and Table 3.3).

Concerning the physicochemical properties, the percentage of clay particles played an important role on *E. coli* sorption ($r^2 = 0.86$). This result is not unusual, as a higher sorption capacity for clay has been reported in several studies for E. coli and other bacteria. Huysman

and Verstrate (1993) showed that 93.6% of *E. coli* were attached to a clay loam (28% clay), whereas 20 % were attached to a sandy soil (3%). Our results corresponded with these findings. Pullman soil had the most clay content (17%) and the larger Freundlich coefficient while no clay particles were detected in the Quincy soil which exhibited the lowest Freundlich coefficient ($K_f = 0.64$).

Table 3.2: Correlations of the physical and chemical parameters of the soils with the Freundlich adsorption coefficient.

	r	r^2	p-value
pH	**-0.92**	**0.84**	**0.0001**
EC	-0.04	0.00	0.1349
Percent moisture	0.63	0.40	0.1706
TOC	**0.93**	**0.87**	**0.0193**
Sulfur	0.51	0.26	0.0044
Nitrogen	0.86	0.74	0.0053
Calcium	-0.24	0.06	0.1452
Magnesium	-0.12	0.01	0.6351
Potassium	0.35	0.13	0.2519
Sodium	-0.26	0.07	0.0065
CEC	0.75	0.56	0.0001
Sand	-0.73	0.53	0.0047
Silt	0.66	0.43	0.0019
Clay	**0.93**	**0.86**	**0.1333**

r - Pearson coefficient

r^2 - coefficient of determination

The total organic content of the soils had the same Pearson coefficient than the clay content (0.86) showing the importance of this parameter on *E.coli* sorption.

Pearson correlation showed a negative correlation between the sand content and the *E. coli* attachment (-0.73). The effect of soil pH on *E. coli* attachment showed a negative Pearson correlation close from 1 (-0.92) and a coefficient of determination of 0.84. There is then a negative relation between soil pH and *E. coli* attachment. In other words, an increase in pH resulted in a decrease of *E. coli* attachment. School and Harvey (1992) observed that a greater number of *Arthrobacter* were attached to quartz at pH 5.04 than at pH 7.52.The authors hypothesized that an increased in pH resulted in an increase of the negative charge of the quartz surface, such that less negatively charged bacteria cells encountered favorable attachment sites on the quartz surface. Our study confirmed this finding, the two soils with the lower pH: Pullman with pH 4.71 and Puyallup with pH 5.51.

Finally, we studied the effects of microbial activity on *E. coli* attachment using correlations (Table 3.3). Bacterial to fungal ratios were correlated with *E. coli* attachment to the six different soils studied ($r^2 = 0.92$). The postive relation between *E. coli* attachment and the ratio between bacteri and fungi follows the theoretical expectations in that fungi typically require more carbon as an energy source than bacteria. This may indicate that when less fungi than bacteria are present in a soil, more carbon will be available and greater *E. coli* attachment would result. The greater sorption in soils with high bacteria fungal ratios may also indicate that other bacteria consortia are beneficial to this sorption. Further investigation into this phenomenon is necessary to understand this correlation.

Table 3.3: Correlations of the physical and chemical parameters of the soils with the Freundlich adsoption coefficient.

	r	r^2	p-value
Microbial Biomass	0.60	0.36	0.0002
pnitrophenol	0.66	0.43	0.0536
TPF	0.36	0.13	0.8115
Gram-Positive Bacteria	0.66	0.44	0.0025
Gram-Negative Bacteria	0.76	0.57	0.0009
Aerobic Bacteria	0.71	0.50	0.0031
Anaerobic Bacteria	0.14	0.02	0.0101
Mycorrhizal Fungi	0.14	0.02	0.0101
Monounsatured Fatty Acids	-0.49	0.24	0.0129
B:F Ratio	**0.96**	**0.92**	**0.9756**
Stress Indicators	-0.54	0.29	0.0078

r - Pearson coefficient
r^2 - coefficient of determination
pnitrophenol-measure of the beta glucosidase activity
TPF-measure of the dehydrogenase activity

Linear regression shows that the correlation between *E. coli* attachment and the soil characteristics is greater than 0.84 (Figure 3.4). These characteristics are percentage of clay, TOC, pH and the ratio between bacteria and fungi. Within the six different soils studied Pullman had the most favorable clay percentage, TOC, pH and bacterial to fungial ratio while Quincy had the least favorable characteristics. Linear trend lines where plotted to observe the relationship between these parameters and the *E. coli* attachment.

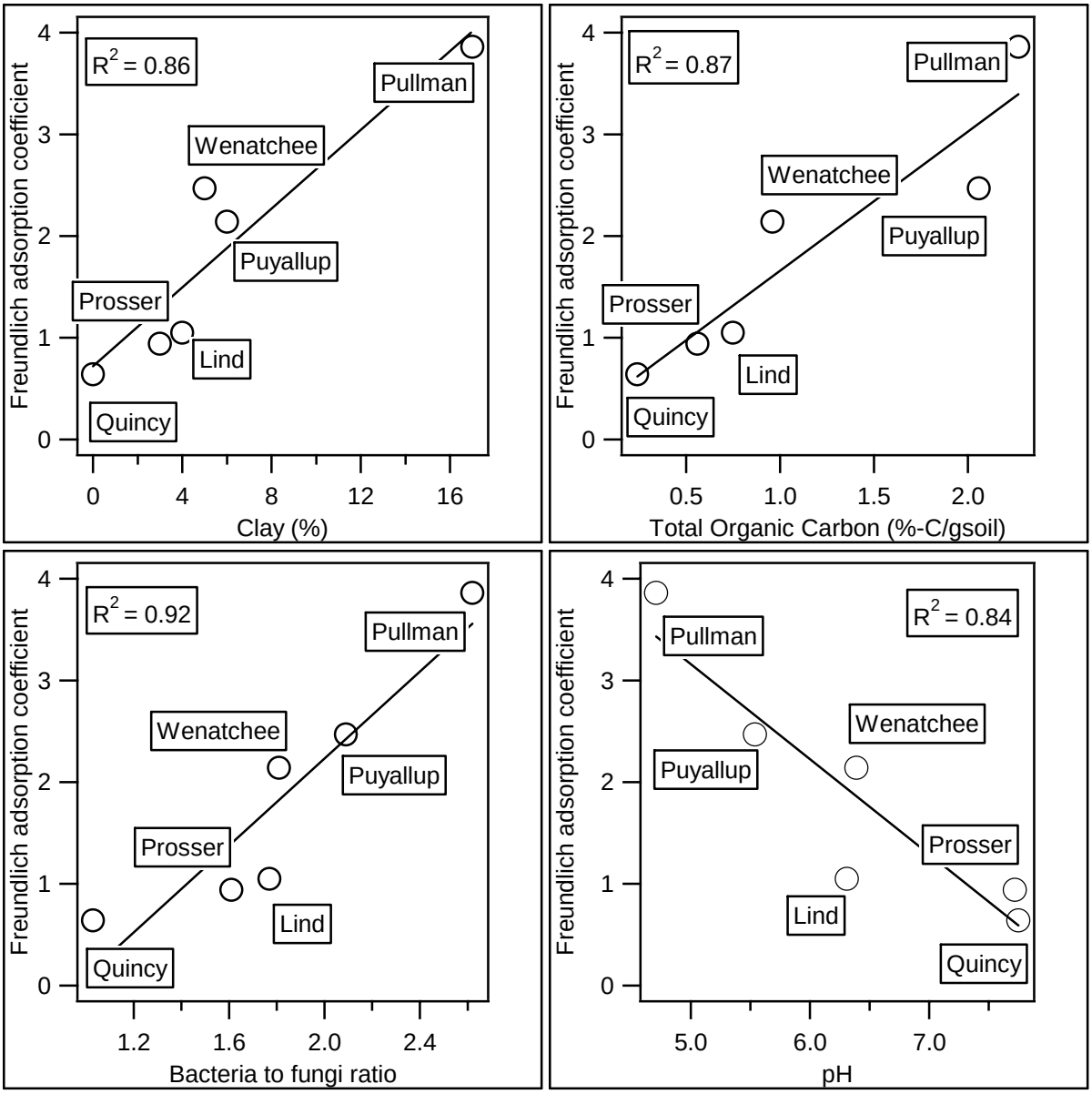

Figure 3.3: Regression of selected soil characteristics on *E. coli* attachment to soil particles.

CHAPTER 4 - CONCLUSIONS AND RECOMMENDATIONS

4.1 CONCLUSIONS

Results of this study showed that physicochemical and biochemical soil characteristics had an important impact on *E. coli* attachment. A Pearson correlation between the different soil characteristics and the Freundlich coefficients was executed in order to evaluate the impact that soil characteristics can have on bacterial attachment.

We observed that:

- The values of the coefficient of determination (R^2) for the fits of *E. coli* H4H data to the Freundlich equation were high enough (>0.94) to confirm that *E. coli* attachment to soil particles followed the Freundlich isotherm.
- Every studied soil from Washington State presented various differences in their physicochemical and biochemical characteristics.
- Among the physical and chemical characteristics, the percentage of clay particles played an important role on *E. coli* sorption (r = +0.93). Combined to the clay content, the total organic content had a strong influence on E. coli sorption (r = 0.93). Moreover, there is then a negative relation between soil pH and *E. coli* attachment. An increase in pH will result here in a decrease of *E. coli* attachment.
- Concerning the biochemical characteristics, the bacterial to fungal ratio in a soil was the parameter having the most importance with a Pearson coefficient of 0.96.

4.2 APPLICABILITY

As described in Chapter 1, surface water is increasingly implicated as a pathogen source in disease outbreaks associated with the consumption of fruits and vegetables. The goal of this study was to examine the role that physicochemical and biochemical soil characteristics have on *E. coli* attachment. Knowing this impact, it possible to understand the influence soil characteristics have on bacterial sorption to particles in order to design best management practices (BMPs) to prevent transport of pathogens to surface water.

Vegetative filter strips (VFS) have been developed to help control the movement of potential agricultural pollutants into water resources. They are a popular BMP for removing sediments and associated contaminants, decreasing pollutant loads from manured fields and pastures. Evidence suggests that VFS can also reduce fecal coliform and pathogen transport to surface waters, particularly in cases when the bacteria are attached to soil particles that act as a transport vehicle. Impact of soil characteristics on bacterial sorption can help to optimize VFS design. This study suggests that altering soil characteristics to increase soil sorption capacity may present a natural way to increase bacterial attachment, which could correspondingly mitigate pathogen transport to surface waters.

Soil characteristics should be considered in VFS design from another perspective as well. A primary goal of VFSs is to reduce runoff velocity and increase infiltration to promote depositional settling of soil particles and associated contaminants. Soil parameters influence particle settling rates under these reduced flows and are thus an important consideration. Furthermore, knowledge of bacterial sorption to a soil under different scenarios allows for the calculation of removal efficiency of pathogens attached to soil particles. This information can then be used to determine the needed VFS width for effective pathogen removal.

Our study showed that parameters like TOC, clay percentage and pH greatly influence *E. coli* attachment. The addition of soil amendments may improve the physical, chemical and microbial characteristics of a soil like Quincy, Prosser or Lind, soils that demonstrated a low sorption capacity. A soil amendment is any material added to a soil to improve properties, such as water retention, plant growth or water infiltration. Mixing these soils with manure can be a way to improve their sorption capacity and VFS performance. However, fresh manure may contain a high ammonia levels and have a high risk of containing pathogens (Chapter 1). Studies on pathogen survival in a slurry showed *E. coli* persisted for 7 days and *Crypstosporidium* survived for 4 weeks (Guan et al., 2002). It is imperative, therefore, that aged manure (at least six months old) be used to reduce these risks. Soil parameters, as well as the characteristics of any applied amendment, are critical to successful VFS implementation.

4.3 RECOMMENDATIONS FOR FUTURE RESEARCH

The following are recommendations for future research assessing the role of physicochemical and biochemical soil characteristics on *E. coli* attachment.

- Increasing the amount of bacteria added to the soil solution or decreasing the amount of soil should allow observing sorption saturation. Knowing this saturation limit will allow for better analysis using different isotherms, which will facilitate comparison with other studies.
- Total organic carbon content and clay percentage were the two physicochemical characteristics having the largest role on *E. coli* sorption in this study. Removing the total organic carbon content from the soil can allow determining the extent clay content plays and correspondingly the role of TOC on *E. coli* sorption.
- An attachment study using different *E. coli* strains would provide a more robust understanding of bacterial fate and transport phenomenon associated with soils. Ideally this will investigate the comparative role soil characteristics have on different *E. coli* strains, including the pathogen strain *E. coli* O157:H7.

APPENDIX A: Soil characterization

Standard Operating Procedures (SOP)

WASHINGTON STATE UNIVERSITY CIVIL AND ENVIRONMENTAL ENGINEERING

Soil Characterization

Standard Operating Procedures (SOP)

Washington State University

Center for Environmental, Sediment and Aquatic Research (CESAR)

c/o Jeffrey L. Ullman

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Pullman, WA, 99164

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Compiled from the protocols used in Dr A. Kennedy Laboratory, USDA, Pullman, WA

Date: July 1st, 2008

Table of Contents

PART I: Introductions & Field Data Collection

A. Scope and Application:

The procedures used here apply to soil field collection and laboratory activities for the CESAR group of Washington State University. Procedures outlined include field sample collection, transportation, and a complete soil analysis.

The objective of this SOP is to provide consistent, well defined methods for use in multiple research applications.

B. Field Sampling:

i. ***Site Location:*** Every well open area constituted of soil can be taken as a site. The first 10 cm layer of soil is known as the surface layer and is considered as the most favorable location regarding to the microbial activity.

ii. ***Equipment*:** The following equipment is to be considered for each sampling trip although each item may not be necessary depending on the situation.

- Copy of SOP
- Labeling tape & Marker
- Pencil/Pen
- 5 gallon buckets with lids (As many a your need)
- 4 mm sieves USA standard
- Shelve

iii. ***Field Site Characterization*:** Specific data needs to be collected at the sampling site in order to prevent any mistake and to be able to track every sample.

- Sample name, date, & time
- Site description including location & weather.

iv. ***Field Sampling Procedures*:** The first 10 cm of soil can be collected using a shelve on different locations of the site in order to have a representative sample.

PART II: Laboratory Data Collection

A. pH and Electrical Conductivity Protocol

i. Introduction

Soil pH is critical in determining the type of microbial and plant communities that can exist in a particular soil, partially because of the effect of pH on nutrient availability. For example, at pH values of 5 and more acidic, N, Ca, Mg, P, K, S, and Mo become limiting, while Fe, Mn, Zn, Cu, and Co become toxic. Microorganisms respond to pH as well. Acidic soils are difficult for bacteria to tolerate, while fungi do well under a wide range of pH values. Generally speaking, a pH between 6 and 7 is ideal.

Electrical conductivity is a measure of the salt concentration in soil. The salt concentration is of importance to plant growth, because a high concentration of soluble salts can interfere with plant growth. Also, microorganisms differ in their ability to tolerate high salt concentrations, which can shift microbial community functions (nutrient cycling, etc.). Generally, less than 4 $dS.m^{-1}$ (decisiemens per meter) is healthy.

ii. Equipment:

- 1 scoop/10 g of soil per sample.
- 1 scoop of soil weighs approx. 10 grams.
- Soil:water 1:1
- 10 mL deionized water per sample
- 1 50 mL Fisher brand plastic wide mouth conical centrifuge tube per sample
- pH meter and probe.
- Electrical conductivity meter and probe

iii. Procedure:

• Weigh 1 scoop (approx. 10 grams) of soil into the 50 mL centrifuge tube and add 10 mL of deionized water the automatic pipettor.

• Place cap on the tube tightly.

• Place the rack of sample on the end-over-end shaker and allow to shake over night.

• The next day remove samples from the shaker and allow to settle to separate soil from solution. Sample can be centrifuged at 3000 rpm for 5 min. to aid in separating.

• Calibrate the pH (7.0) and conductivity meters. See below for instructions to calibrate meters.

• Read conductivity and pH, recording data to the computer using winwedge. Measure conductivity first and then pH because conductivity readings are more stable if the soil is not disturbed.

iv. Calibration of the meters

pH Meter

• Press CAL key to initiate calibration sequence. The last calibration buffer range will be displayed i.e. 7.

• Press the ▲▼ keys to select the correct buffer range (either 7, 4-7, or 7-10). Select the correct buffer or buffer range and press yes.

• Place electrode in the calibration solution. When READY light is displayed press yes to accept buffer value. SLP will be displayed while the calculated slope is displayed.

• Press yes to accept calibration. A 7 (or what ever buffer was selected) will be displayed to the left side of the screen. This indicates that the meter has been calibrated.

• Rinse the electrode and proceed to measure pH.

EC Meter

• Before the EC meter can be calibrated the existing calibration must be cleared. Do so by pressing the CHECK key. "CHK" will appear in the top right corner of the display.

• Press and hold ENTER for 10 seconds, "0" will be displayed in the far left corner of the display to indicate that the calibration data has been cleared.

• Press ENTER to return to measurement mode.

• Insert the probe into the EC calibration solution. Press the mode key until you come to temperature in ºC.

• Refer to the EC standard solution bottle for the correct concentration at the current temperature of your solution. For example @ 25º C the EC should be 1,413 $uS.cm^{-1}$.

• Press the ▼▲ keys until the correct concentration is displayed.

• With the correct value on the display, press the ENTER key to enter the value as a calibration point.

• The meter is now calibrated and CAL will be displayed in the upper left corner of the display.

B. Percent Moisture Protocol

i. Introduction

Percent moisture is important for two reasons. First, the amount of water in the soil affects the amount of soil that you weigh out for analysis. For example, if you have a soil the has 50% moisture, and a soil the has 3% moisture and you need to weigh out 5g to run dehydrogenase. Unless you correct for the water in the sample, the samples will have different amount of soil even though both weigh 5g. Secondly, knowing the percent moisture of your samples is handy when trying to understand other data, such as microbial activity. A soil that has 15% moisture will probably have more microbial activity compared to a very dry soil.

ii. Equipment:

- Approximately 5g (or 1 scoop Aprox. 10 G) of soil per sample
- 1 soil can per sample
- Oven located in room 218 heated to aprox. 105ºC
- Computer connected to a scale through WINWEDGE
- An excel spread sheet listing sample ID's with associated can #

iii. Procedure:

• Place an empty soil can on a tarred scale and record the empty can weight to the excel spread sheet through winwedge. Ensure the number on the can corresponds to the can # in the spreadsheet.

• With the soil can on the scale, tare the scale and weigh approximately 5g (or 1 scoop Aprox. 10 G) of soil into the can. Record the exact weight of soil to the excel sheet.

Note: If there is a limited amount of sample use 5g or less of soil to ensure there is enough sample for remaining analyses.

• After all wet soil weights have been recorded place cans on a tray in the drying oven with can lids on the bottom of the can to allow the soil to dry completely. Close the drying oven door completely and allow to dry for 24 hours.

• After 24 hours retrieve cans from the oven. Be sure to use oven gloves. Cap the cans as soon as they come out of the oven to avoid the soil from absorbing atmospheric moisture.

• Place the can on a tarred scale and record the weight.

• Proceed with calculations as outlined below.

• To clean soil cans wipe with a dry Kimwipe. Do not use water as this will cause cans to rust.

iv. Calculations:

Formula to calculate % moisture: ((Wet weight – Dry weight) / Dry weight)

C. Particle Size Analysis by Hydrometer

i. Introduction

The Particle Size Analysis is a method used in order to determinate the soil texture. It is a property used to describe the relative proportion of different grain size of mineral particles in a soil. They are grouped according to their size into what are called soil separates. These separates are typically named clay, silt, and sand. The soil texture will have a very important influence on the microbial sorption.

ii. Equipment:

- Laboratory Balance accurate to 0.01 g.
- Oven capable of drying samples at 105°C.
- Metal containers to dry soil samples.
- 600 ml beaker.
- 100 ml graduated cylinder.
- reciprocating shaker and one liter bottles for dispersing soil samples.
- Standard Bouyoucos hydrometer (ASTM 152H) with scale in $g.L^{-1}$.
- Sedimentation cylinders. 1,000 ml sedimentation cylinder
- A plunger to shake the soil suspension in the cylinders.

iii. Reagents

Sodium hexametaphosphate (HMP) stock solution at 50 $g.L^{-1}$.

iv. Procedure

1. Soil preparation.

Air dry soil sample. Place around 100g of soil to air dry during 48 hours

2. Oven dry mass determination.

• Weigh empty drying tin. Record as Tare.

• Weigh approximately 10 g of air-dry soil into drying tin and record as Wet gross weight .

• Dry sample for 16 hours or overnight in oven at 105°

• Weigh dried sample in tin and record as Dry gross weight.

3. Soil dispersion.

• Weigh 40-50 g of air dry soil sample into 600 ml beaker or cup. Record as Sample weight. (If soil is very sandy, increase mass to 80-100 g.)

• Add 100 ml of HMP stock solution to beaker.

• Add about 250 ml distilled water to beaker, place on an orbital shaker overnight.

4. Hydrometer measurements

• Transfer suspension to sedimentation cylinder using a wash bottle to remove all soil from the dispersing cup or bottle. Fill cylinder with distilled water to 1,000 ml mark.

• Prepare a blank solution by placing 100 ml HMP stock into another cylinder, filling to the mark with distilled water, and mixing as described in step 4-c.

• Mix contents of cylinder thoroughly with mixing plunger using strong upward strokes and gentle downward strokes.

• Begin recording time immediately after removing plunger or setting cylinder down.

• Insert soil hydrometer into suspension and read to nearest 0.5 unit after exactly 30 and 60 seconds of settling time. Gently remove hydrometer. Record 30 second reading as RL1 and

60 second reading as RL2 (take several RL1 and RL2 readings to check for reproducibility). Insert hydrometer into blank solution and record reading as R1 and R2. Measure temperature of blank solution to nearest 1°C and record as Temp 1 and Temp 2.

• Repeat hydrometer measurements at approximately 90 minutes and 24 hours. Record time (in minutes) as T3 and T4, respectively. Record hydrometer readings in soil suspension as RL3 and RL4. Record blank readings as R3 and R4.

• Repeat temperature measurements in blank solution and record as Temp 3 and Temp 4.

D. β-Glucosidase Activity

i. Introduction

β-glucosidase is a member of a group of enzymes that catalyze the following general reaction:

glycosides + H_20 -> sugar + aglycons

Specifically, β-glucosidase is an extracellular enzyme which catalyzes the breakdown (hydrolysis) of maltose and cellobiose. These breakdown products are believed to be important sources of energy for soil microorganisms. Fungi, yeast, and plants commonly produce β-glucosidase. β-glucosidase activity has been correlated with organic C content in the surface soils.

ii. Equipment:

- Clean glass scintillation vials (1 per sample) w/ caps
- Whatman #2 filter paper (1 per sample)
- Plastic funnels (20) in wooden stands above sink

- 1 g soil per sample
- 1-50ml Erlenmeyer flask per sample
- Spectrophotometer (in back left corner of lab as you walk in)

iii. Reagents

1. Modified Universal Buffer (MUB) pH = 6:

- Modified Universal Buffer (MUB) Stock Solution. Dissolve 12.1 g of tris(hydroxymethyl)aminomethane (THAM), 11.6 g of maleic acid, 14.0 g of citric acid, and 6.3 g of boric acid in 488 ml of 1 N sodium hydroxide (NaOH) and dilute the solution to 1 liter with deionized water. Store in refrigerator.

- Modified Universal Buffer (MUB) pH = 6: Place 200mL of MUB stock solution in a 1 L beaker with a magnetic stir bar, and place on a magnetic stirrer. Adjust the pH of the solution to pH 6.0 with 0.1 *M* hydrochloric acid (HCL), and adjust the solution to 1L with DI water.

2. PNG (*p*-Nitrophenyl-β-D-glucoside) Solution, 0.05 *M*: Dissolve 0.654 g of PNG in about 40 mL of MUB pH 6.0, and adjust the volume to 50 mL with the same buffer. Store the solution in the refrigerator.

3. Calcium Chloride ($CaCl_2$) 0.5 *M:* Dissolve 7.35 g of $CaCl_2$ ($2H_2O$) 75 mL of water and, dilute the volume to 100mL.

4. Tris(hydroxymethyl)aminomethane (THAM-NaOH) extractant solution, 0.1 *M*, pH=12: Dissolve 6.1 g of THAM in about 400 ml of water, adjust the pH of the solution to 12 by titration with 0.5 *M* NaOH, and dilute the volume to 500 ml with deionized water.

5. Tris(hydroxymethyl)aminomethane (THAM) diluent, 0.1 *M*, pH ~ 10: Dissolve 6.1 g of THAM in 400 ml of water, and adjust the volume to 500 mL with water.

6. Standard *p*-nitrophenol stock solution (for the spectrophotometer)**:** Dissolve 0.5 g of the p-nitrophenol in about 350 mL of water, and dilute the solution to 500 mL with water. Store the solution in a refrigerator.Dilute 1 ml of the standard *p*-nitrophenol stock solution to 100 mL in a volumetric flask and mix thoroughly.

Pipette 0-,1-,2-,3-,4-, and 5-mL aliquots of this diluted standard solution into 50-mL Erlenmeyer flasks, adjust the volume to 5 mL by addition of water.

Add 1 mL of 0.5 *M* $CaCl_2$ and 4 mL of 0.5 *M* NaOH; mix and filter the resultant suspension.

Record readings on spec (410 nm), rinsing between each standard with 0.1 *M* THAM pH~10.

To read the samples first start up the WinWedge program from the start menu. Select the spec 401 from the file menu and then select Activate and test mode. Highlight the cell in the excel worksheet data is to recorded. Place sample in the cuvete (approx. 2mL) place the cuvete into the spec and press “second function then print” this will record the data on the display to the spreadsheet.

iv. Procedure

• The day before you plan to run this assay, make sure that you have all the reagents you need made.

• Weigh out 1 g of soil (dry weight) in a 50-ml Erlenmeyer flask.

• Add 0.25 ml of toluene (**optional**), 4 ml of MUB pH=6, and 1 ml of PNG solution. Stopper the flask, and swirl it for a few seconds to mix the contents.

• Incubate the flasks for 1 HOUR at 37^{o} C. TURN ON SPECTROPHOTOMETER and set to a wave length of 410 nm.

• After 1 hour, remove the flasks from the incubator and perform the following steps.

• Add 1 ml of 0.5 *M* $CaCl_2$ and 4 ml of 0.1 *M* THAM buffer pH=12 and swirl the flask.

• Filter the soil suspension through a Whatman #2v filter in a funnel (set up in the wooden funnel racks above the stainless steel sink) into labeled glass scintillation vials.

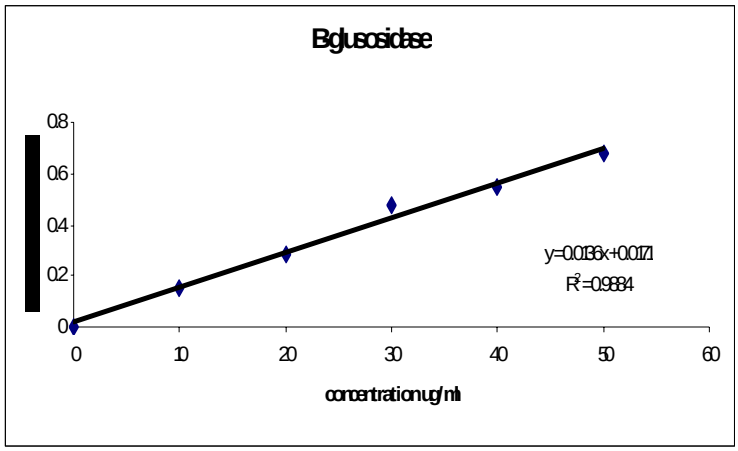
• Samples can now be read on the Spectrophotometer. Samples may need to be diluted to fall within the calibration standards. Any absorbance that is greater than the absorbance of the highest standard will need to be diluted. A 1:5 dilution is usually sufficient. Dilute samples with THAM pH 10.

v. Calculations

• From the absorbance data collected on your standards, you will be able to calculate the *p*-nitrophenol concentration in $ug.gsoil^{-1}.hr^{-1}$ of your soils.

• Enter your *p*-nitrophenol standard concentrations in one column and the corresponding absorbance values in the column just to the right of it, and run a regression with the concentrations as the independent variable and the absorbance values as the dependent. You should get an r^2 value close to .99; no lower than .95 for a good set of standards.

Concentration (ug/mL)	**Absorbance (nm)**
0	0.003
10	0.154
20	0.281
30	0.481
40	0.548
50	0.681



• From the regression output you will get a **x coefficient** and **constant** a value.

• Solve the equation for Concentration (X).

• Now that you have the *p*-nitrophenol concentration, you can calculate the rate of production per gram of soil per hour:

***p*-nitrophenol conc. ug/ul x 10.0 ml assay / 1 g soil**

E. Dehydrogenase

i. Introduction

Dehydrogenase enzymes are important catalysts in the transfer of H^+ ions during the oxidation of soil organic matter. Since dehydrogenase is a common enzyme in microorganisms, it is thought to be a good indicator of microbial activity in soils.

What happens in the assay is that triphenyl tetrazolium chloride (TTC) acts as a H^+ acceptor. While microbes are oxidizing soil organic matter, TTC accepts H^+ ions, and in doing so, forms triphenyl formazan (TPF), which turns a reddish color when extracted with methanol. The redder the extract, the greater the microbial activity. We then measure the red extract on a spectrophotometer.

ii. Equipement

- large test tubes with #2 rubber stoppers
- glass scintillation vials
- Whatman #2 filter paper
- funnels
- 1 ml 3% triphenyl tetrazolium chloride (TTC) per sample made in volumetric flask
- 2 ml 2% $CaCO_3$ per sample
- Either one 4.5mL cuvet per sample, or a microtiter plate for every 15 or so samples

iii. Procedure

Day 1:

• Weigh 5g of soil into large test tubes. Label tubes with sample ID code, rep., and treatment.

• Add 1.0ml of 3% TTC (3g TTC in volumetric flask, add deionized water to bring to100ml total). There might already be some in the almond frig, but make sure that it is no more than 1 month old.

• Add 2.0ml of 2% $CaCO_3$ stock (2g $CaCO_3$/100ml dH_2O). This can be done in a beaker by adding 100ml deionized water (measured with a graduated cylinder) to the $CaCO_3$. Place beaker with solution on stir plate and stir while you add to soil.

d. Mix tube by vortexing and then plug tube with #2 stopper. Try to avoid splattering soil up the sides of the tube by holding fingers low on the test tube during vortexing.

• Incubate for 24 hours at 37^0C in the incubator in rm 212. Be sure to note the exact number of hours plus minutes of incubation. Be sure to turn on the incubator at least 1 hour before using to incubate samples to allow the temperature to equilibrate @ 37°C.

Day 2:

• Remove tubes from incubator, add 10ml of Methanol (MeOH Reagent Grade), then stopper and vortex for 1 minute. Let tube settle for a few minutes.

• Decant solution through funnel with Whatman #2 filter paper, into glass scintillation vials.

• Add another 10ml MeOH to each tube, vortex and let tube settle again. Decant through same filter into same vial.

d. Samples can now be read on the Spectrophotometer at 492 nm. Samples may need to be diluted to fall within the calibration standards. Any absorbance that is greater than the

absorbance of the highest standard will need to be diluted. A 1:2 dilution is usually sufficient. Dilute with Methanol.

F. Total Organic Carbon

• Start by setting up the computer by attaching the RS-232 cable from the computer to the balance that you will use to weigh samples.

• Open the "Leco Weight Template" on the shared folder and add your sample ID's to this sheet. This is the format that weight need to be in when delivered to Ron for analysis. All sample ID's from our lab need to start with a 1 (ie. 1IRRBurn06). This helps us to track Leco charges and locate our samples.

• Open Winwedge from the start menu and activate the file that corresponds to the Balance you are using. Once you have activated the file, test communication by putting the cursor in cell of the excel spreadsheet and press the send button (print, →, send, read etc..). Whatever is displayed on the scale should have been sent to the excel spreadsheet.

• After establishing communication then you can proceed with weighing individual samples. Weight each sample into the Leco combustion boats following the instructions below for Soil vs Residue.

• As you proceed to weigh samples stop periodically and check to make sure the sample being weighed correlates to the sample ID in the spreadsheet and that it also correlates to the correct rack position. It is also a good idea to save the file at this time as well.

• Be sure to wipe the spatula between each sample as to avoid carry-over into the next sample.

i. Grain / Residue

• Residue samples are normally ground to a fine texture before Leco analysis.

• The last three samples of each rack will need to be a standard, which is usually orchard leaves. Each standard needs to weigh about .2000 grams, and no com-cat needs to be added.

• For grain and residue samples weigh out about .200 grams and add about .7500 of a gram of com-cat. This is done by weighing the residue and sending that weight to the spreadsheet, then tarring the scale and then weighing the com-cat. Com-cat should be evenly sprinkled over the residue to allow for good combustion.

• Boats that have not been used for soil and are relatively free of debris are the best to use when weighing residue.

ii. Soil

• Soil that is to be weighed for Leco should be dried in tins in the oven for at least 24 hours to ensure they are dry.

• Each soil sample will need to weigh about .5000 grams. Soil should be spread out evenly over the length of the boat.

• Com-cat does not need to be added to soil standards or soil samples unless you are looking for low levels of sulfur.

• After you are done weighing a set of samples save the file to both the hard drive and a floppy disk. The floppy disk will go down to Ron with the samples and be used to load the weights into the machine. The file needs to be saved to the hard drive because often floppy discs fail or cannot be read on other computers.

• After samples have been run results can be viewed and downloaded for the Leco website. Go to http://jsn225.cahe.wsu.edu/netleco. You will then be prompted to enter a user name and password. This is the same you WSU network username and password.

APPENDIX B: E. coli Sorption to Soil Particles

Standard Operating Procedures (SOP)

WASHINGTON STATE UNIVERSITY CIVIL AND ENVIRONMENTAL ENGINEERING

E. coli Sorption to Soil Particles

Standard Operating Procedures (SOP)

Washington State University

Center for Environmental, Sediment and Aquatic Research (CESAR)

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Created by: Guillaume Paternostre, Graduate Research Assistant

Date: September 1st, 2008

Reviewed by:

Date:

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Laboratory Data Collection

A. Media and Reagent Preparation

i. Tryptic Soy Agar

Follow the directions given in the *Difco & BBL Manual* for Bacto™ Tryptic Soy Agar (or BBL™ Trypticase™ Soy Agar) with the following modifications:

• The "Directions for Preparation" gives the amount for making up 1 L so adjust accordingly to the volume you need. Note that 1l of media will give you around 50 plates.

• Make up the TSB in a clean 1000 ml erlenmeyer flask.

• I recommend that you add the powder in the flask first, and then put in half the water, swirl to dissolve, then the rest of the water, swirl to completely dissolve and mix. This minimizes the chance that any stray powder will get stuck at the top of your flask.

• Cover the opening of the flask with a layer of aluminum foil.

• Heat until boiling, promptly remove from heat. Do not sterilize by autoclaving.

• Allow it to cool to approximately 60°C.

• Pour then molten agar into standard-sized petri dishes until they are about half full

• Allow the plates to solidify, then invert and stack them for storage.

• Fresh plates should be stored for at least two days at room temperature before use to ensure against contaminants or two weeks in the fridge at 4°C.

ii. Tryptic Soy Broth

Follow the directions given in the Bacto™ Tryptic Soy Broth (TSB) media container with the following modifications:

• The “Directions for Preparation” gives the amount for making up 1 L so adjust accordingly to the volume you need.

• You can omit the second step in the directions “Warm gently until solution is completely dissolved.” TSB usually goes into solution quite easily at room temperature.

• I recommend that you put the powder in the flask first, and then put in half the water, swirl to dissolve, then the rest of the water, swirl to completely dissolve and mix. This minimizes the chance that any stray powder will get stuck at the top of your flask.

• Make up the TSB in a clean 1 L bottle.

• Cover the bottle with a lid but not tight that the pressure can’t escape from the flask. I recommend completely tightening the cap and then loosening it about a quarter of a turn.

• Autoclave the fresh media (125°C during 15 minutes, program P09 in Dana 117). When autoclaving, it is important to fill up the bottle no more than 700 ml, this helps to prevent the liquid from becoming superheated and boiling over.

• After autoclaving, label the bottle with the type of media, the date and your initials (ex: TSB, 08/08/08, GP). Finally, store the fresh medium at room temperature.

iii. m-FC Media

• Suspend 52 g of the powder in 1 L of purified water. Mix thoroughly. I recommend that you put the powder in the flask first, and then put in half the water, swirl to dissolve, then the rest of the water, swirl to completely dissolve and mix. This minimizes the chance that any stray powder will get stuck at the top of your flask.

• Heat with frequent agitation until boiling, promptly remove from heat, and cool to below 50°C. Do not sterilize by autoclaving.

• Add 10 mL of a 1% solution of Rosolic Acid in 0.2M NaOH (for the 1% Rosolic acid, dissolve 0.25 g of Rosolic acid in 25 mL of 0.2M NaOH in a 25mL flask).

• Continue heating for 1 minute. DO NOT AUTOCLAVE.

• Dispense 5- to 7-mL quantities to 50- × 12-mm petri plates and let solidify. Refrigerate finished medium, preferably in sealed plastic bags or other containers to reduce moisture loss, and discard unused agar after 2 weeks.

iv. Rinse Water

• Add 1.25 L Stock Phosphate Buffer solution and 5.0mL Magnesium Chloride solution to 1 L reagent grade-water in a clean 1 L dilution bottle.

Stock Phosphate Buffer:

- Dissolve 34.0g of potassium dihydrogen phosphate (KH_2PO_4), in 500 mL reagent-grade water.
- Adjust to pH 7.2 ± 0.5 with 1*N* sodium hydroxide (NaOH).
- Dilute to 1 L with reagent grade water.
- Discard turbid solution.

Magnesium Chloride:

- Dissolve 81.1 g of Magnesium Chloride ($MgCl_2.6H_2O$) in 1L reagent-grade water

• Mix thoroughly and dispense in 2 autoclavable squiz bottle.

• Autoclave the bottles (125°C during 15 minutes, program P09 in Dana 117).

v. 0.1M NaCl

• Dissolve 5.844 g of Sodium Chloride (NaCl) in 1 L of reagent-grade water.

• Cover the bottle with a lid but not tight that the pressure can't escape from the flask. I recommend completely tightening the cap and then loosening it about a quarter of a turn.

• Autoclave the fresh solution (125°C during 15 minutes, program P09 in Dana 117). When autoclaving, it is important to fill up the bottle no more than 700 ml, this helps to prevent the liquid from becoming superheated and boiling over.

• After autoclaving, label the bottle with the type of media, the date and your initials (ex: 0.1M NaCl, 08/08/08, GP).

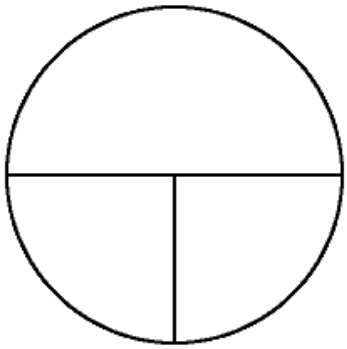
• Store at room temperature.

B. Maintain the E. coli culture

The method described here is called the "T" streak and is one of the easiest (adapted from the center for polymer education website).

• Light a Bunsen burner in your bench space. To maintain sterile conditions, inoculation should occur within 20 cm of the flame. Wait 20 seconds before opening the TSA petri dish and inoculating. This gives the flame time to sterilize the local air. Remember that you want to achieve sterile conditions. Do not work with the plate close to your face. This will violate the sterile environment.

• Use a marker or wax pencil to draw a T on the bottom of a TSA plate of nutrient agar. This divides the plate into three sections. Label the side of your plate with the bacteria, date and your initials.

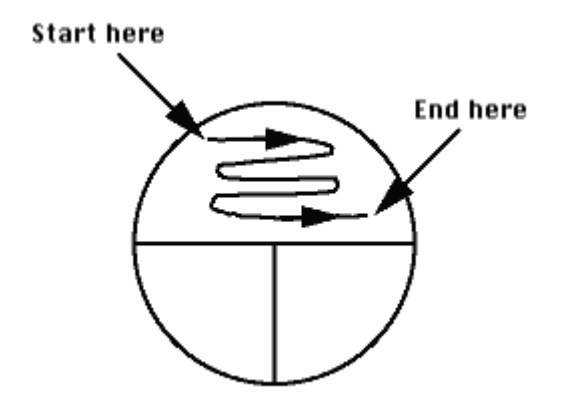


• Sterilize the inoculating loop by holding its tip in the flame until it turns red.

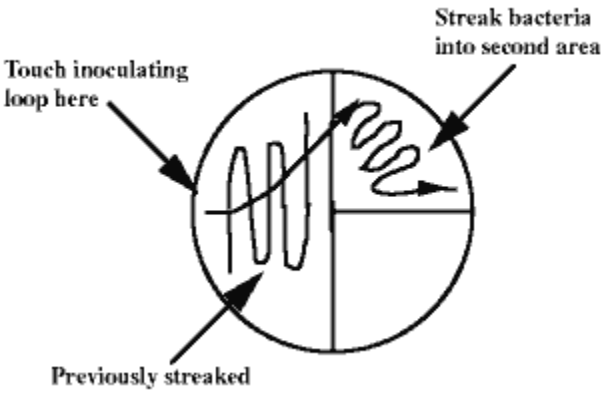
• Lift up the lid of the plate you will be inoculating and poke the inoculating loop through the agar close to the side of the petri dish to cool it. This prevents the heat from killing the bacteria sample you want to use. The heat will not harm the agar. Try to lift the lid of the plate up only as much as is necessary to put the loop inside. If you completely remove the lid, it can become contaminated with bacteria from the environment.

• Touch the loop to the edge of the colony growing on the "old plate". Then take the loop and place the lid securely back on the plate.

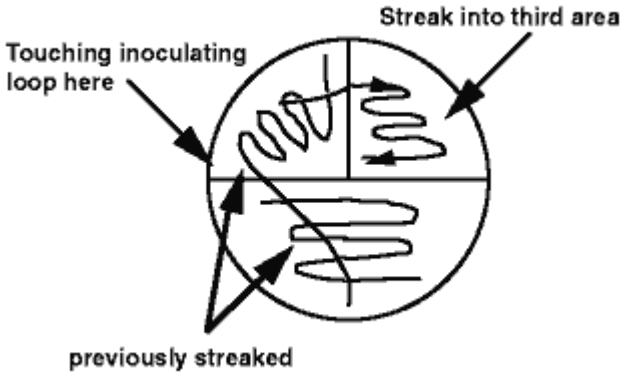
• Set the plate you will be streaking so that its bottom is sitting on the bench top and you can see the T clearly. The largest section should be at the top. Carefully lift up the lid and touch the inoculating loop to the upper left hand corner of the largest section of the plate. Move the loop from left to right, back and forth, across the surface of the agar. Since nutrient agar is a gel with properties similar to Jell-O, do not push down with the loop or you will gouge the agar.



• Replace the lid of the petri dish and flame the loop again to kill any remaining bacteria on it. Rotate the plate 90 degrees counterclockwise. Carefully lift the lid slightly and touch the loop into the left side of the plate which contains the area you streaked in the previous step. Move the loop across the surface of the agar until it is in the smaller section in the upper right of the plate. Within that quarter of the plate, move the loop back and forth across the agar surface



• Repeat the last step



• Replace the lid of the petri dish and flame the loop again to kill any remaining bacteria on it.

• Seal the petri dish with a layer of parafilm around the edge. This keeps the agar from drying out while it is in the incubator. Incubate the streak plate at 35C until you can see individual colonies.

• After 24 hours incubation, store you plate in the fridge at 4C during a month.

C. E. coli inoculation onto TSB.

• In a 50 mL sterile plastic centrifuge tube, insert 25 mL of fresh TSB avoiding any contamination by using a sterile pipette.

• Close the centrifuge tube.

• Light your Bunsen burner.

• Flame the inoculating loop to redness by holding it pointed down into the flame, starting near the handle and then moving the loop into the flame. This technique sterilizes the loop and, if wet with a culture, heats up the loop without spattering bacteria into the air and onto the surrounding area.

• Let the loop cool a minute. A hot loop will damage the bacteria cells.

• Remove the top of the E.coli plate and keep it in your hand avoiding any contamination.

• Insert the cooled sterilized loop into the plate and carefully touch an isolated colony with the extremity of your loop.

• Remove the loop being careful again to not touch anything.

• Open the plastic tube containing the 25 mL TSB.

• Insert the loop into the broth and shake to remove the bacteria.

• Resterilize the inoculating loop and place it on the table. NEVER place a contaminated loop on the table.

• Gently shake your broth culture. Label it with: today's date, microbe's name, your initials

• Let your inoculated tube sit overnight at room temperature.

D. Growth Curve

In order to be able to show a growth curve for a certain kind of bacteria, you have to answer the question how many bacteria are present in this sample?

Measuring the optical density (O.D.) of a sample is an indirect method of determining the number of cells present. The amount of light of a specific wavelength that is absorbed by a culture is related to the number of cells. This is a very fast and easy way to count cells when possible.

Day 1:

• *E. coli* inoculation onto TSB: See section C of this SOP.

Day 2:

• Turn on the spectrophotometer and set it to 600 nm.

• Set the absorbance to 0 using un-inoculated TSB media as a blank.

• Add 24 mL of TSB in a sterile 50 mL Erlenmeyer.

• Vortex the inoculated TSB from day 1 during 7 seconds.

• With a micro-pipette, take 1 mL of the old inoculum and inoculate it in the Erlenmeyer.

• Place the inoculated Erlenmeyer in the orbital shaker in Dana 117 at 30C and 110 rpm.

• Measure the absorbance of your inoculated sample after: 1, 2, 3, 4, 6, 8, 12, 16, 24 hours.

• Plot the absorbance against time.

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