University of Alberta

Soil-based Wastewater Dispersal Systems: Net Die-off and Sorption Rate Constants of *Escherichia coli* and *Enterococcus faecalis*

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Land Reclamation and Remediation

Department of Renewable Resources

Edmonton, Alberta
Fall 2008

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Your file Votre référence
ISBN: 978-0-494-47432-7
Our file Notre référence
ISBN: 978-0-494-47432-7

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ABSTRACT

Soil-based wastewater dispersal systems: Net Die-off and Sorption Rate Constants of *Escherichia coli* and *Enterococcus faecalis*

Net die off and adsorption are important soil mechanisms to remove bacterial pathogens from applied wastewater. The objectives of this study were to assess: (1) the die-off rates of two indicator organisms, *Escherichia coli and Enterococcus faecalis* at 23°C, 5°C and -18°C; (2) sorption of these organisms at 23°C and 5°C in soil samples from major horizons of a Black Chernozem and a Gray Luvisol; and (3) sorption of these organisms on soil samples from a failed at-grade, a failed mound and a failed dispersal field. Temperature significantly affected both adsorption and net die off; net die-off rate constants were higher at 23°C compared to 5°C for both organisms, and k_d values were dependent upon the organism and soil type/horizon. These constants can be used to improve the design of soil-based wastewater dispersal systems. Also, both organisms should be used in combination to detect fecal pollution in soil.

ACKNOWLEDGEMENTS

There are numerous people whom I would like to thank and acknowledge for their tremendous support. First of all, I would also like to thank my supervisor, Dr. Noorallah Juma, for his guidance and smiles throughout this research. I would also like to thank the OWTRL reseach team, Iris Bitterlich, Shandija Khan, and Baldev Chhabra for their help in the lab and setting up of the experiments. I would also like to thank the system homeowner for allowing us to trample though their backyards and collect samples.

For financial support, I wish to thank Alberta Municipal Affairs for their contributions, which made this research possible. The department of renewable resources also contributed with GRAF funding, which helped make life as a writing grad student possible.

I would like to acknowledge Guillaume Blanchet for help with statistical analysis and for turning me into an R fan.

Most of all, I would like to thank my family and friends who have been with me though many marvelous and difficult times. Thank you dad for always being there with good advice and understanding to help me though it all. Thanks Luis and family for your smiles and encouragement. Thank you Brenda, Jackie, and Mark for showing me there is a world outside of science. Also, thank you to all of CoMECIC: Adriana L., Adriana M., Catalina, Claudia, Guillaume, Jaime, Jose Pedro, Marcos, Maryam, Mathew, Kimlin, Leonardo, Po-Yuan K., Salvador, Valentin, and Virginia for the friendship I have found in each one of you.

Thank you. Thank you. Thank you.

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LIST OF ACRONYMS, ABREVEATIONS, AND UNITS

ANCOVA	Analysis of covariance
ATCC	American Type Culture Collection
BOD_5	Biological oxygen demand after 5 days
CFU	Colony forming units
E	Ellerslie
EC	Electrical conductivity
f	Porosity
K	Net die-off rate constant
K_d	Water partitioning coefficient
meq	milliequivalents
MPN	Most probable number
OWTS	Onsite wastewater treatment systems
PCA	Principal component analysis
RMH	Rocky Mountain House
TEC	Total exchangeable cations
TSS	Total suspended solids

Chapter 1

Introduction

1.1 General Overview and Objectives

The disposal of sewage effluent through centralized and onsite wastewater treatment systems (OWTS) is a global environmental problem. Centralized systems consist of a network of sewage collection pipelines, a municipal wastewater treatment system, and disposal of treated wastewater into rivers, lakes, or oceans. In contrast, OWTS treat the sewage to various degrees and disperse it onto the soil surface or subsurface. The objective of this chapter is to provide an overview of the problem, a framework of issues related to the treatment and disposal of sewage effluent, and a set of objectives for this thesis.

1.2 Introduction

Civilization has always been concerned with the removal of sewage effluent from living areas. Examples of this can be found throughout history such as the brick sewage system of the Indus Valley Civilization and the use of "night soil" or sewage in parts of China for crop fertilization (Burkes 1994).

Wastewater systems, in particular, have undergone great transformations. Continual research and investment in infrastructure has led to the development of vast sewage pipelines and sophisticated urban wastewater treatment facilities. For example, the City of Edmonton's Gold Bar wastewater treatment plant has a design capacity of 310 million litres per day. These systems are complex and carefully monitored. Liquid treatment processes consist of pre-treatment, primary, secondary, and tertiary treatment which includes biological nutrient removal followed by UV disinfection for efficient removal of organic matter, pathogens, and nutrients. The use of these systems is limited to areas with a direct physical connection to the facilities (Juma et al. 2007).

Rural homes use OWTS to treat their wastewater. The most common treatment system consists of septic tank and a standard subsoil absorption field which have been in use for more than 200 years across the world (Carroll et al. 2006). In addition to this, four other types of systems are being used in Alberta:

septic tank with a mound, septic tank with an open discharge, advanced treatment with standard septic field, and advanced treatment with an at-grade pressurized distribution system (Alberta Safety Codes Council 2007).

Although these systems are used for different applications and vary in the degree of treatment of wastewater, they do have a common denominator; soil is considered to be the final disposition point and last stage of the treatment process. Onsite wastewater disposal represents a significant volume of wastewater discharged to the subsurface in Canada (Environment Canada 2001b).

Studies have shown that, under the appropriate conditions, soil can be very effective at treating wastewater. Hegedorn et al. (1978) reported that it is possible to obtain complete bacterial removal in 30 cm to 90 cm below the base of a drainfield trench. Van Cuyk et al. (2004) reported that 99.9% of fecal coliform bacteria were removed during unsaturated flow to a depth of 60 cm. Unfortunately, this is not always the case; system failure caused by inappropriate sitting, design issues, or soil conditions such as macropores could promote long-distance transport of pathogens derived from OWTS (Smith et al. 1985; Carroll et al. 2006)

In contrast to water discharged from municipal wastewater treatment facilities, there is currently no regulation concerning the quality of the effluent which is dispersed on a soil adsorption field. Codes regulating onsite systems have focused primarily on hydraulic performance, minimizing discharge levels of organic matter, measured in terms of BOD_5 and total organic nitrogen, and nutrients measured in terms of ammonia (NH_4^+), nitrate (NO_3^-), and inorganic phosphorous (PO_4^{3-}). There are no codes for pathogens or even pathogenic indicators such as total coliforms, fecal coliforms or *E. coli* in the soil treating sewage effluent.

The lack of guidelines combined with the increased use of OWTS may dramatically increase the risk of pathogen pollution in waterways. In Canada, OWTS are already anticipated to be an important contributor of pathogen pollution in aquatic ecosystems (Environment Canada 2001a).

There is a great need for better understanding of the soil as a treatment system and how pathogens interact with it. In addition, an understanding of the environmental persistence and fate of enteric pathogens introduced into soil with sewage sludge is necessary to provide the sound scientific basis to management

practices designed to mitigate the potential microbiological health risks (Topp et al. 2003).

1.3 Onsite Wastewater Treatment Systems

In areas lacking central sewage treatment facilities, OWTS are used. It is estimated that in the province of Alberta, there are from 200,000 to 250,000 OWTS which serve over 535,000 people. That is approximately 37.1 billion litres of sewage per year. Over 4,000 new systems are installed annually. The number of systems is expected to increase as there is a rise in acreage living and in rural developments from the mining, forestry, oil and gas industries (Durnie 2002).

These systems can range in complexity and grade of treatment. The simplest version consists in a septic tank where the household wastewater is collected and dispersed in a standard adsorption field. In general terms, this system works in the following manner (Figure 1.1):

1. Wastewater from the home enters the septic tank .
2. In the tank, solids settle to the bottom, where bacteria feed on the solids and break them down. The baffle prevents solids from leaving the tank until they are decomposed.
3. The liquid then flows through a pipe into a distribution box.
4. The distribution box directs the flow out into the drain field, which consists of underground pipes in shallow beds of gravel or soil.
5. The liquid flows through orifices in the drain field pipes and seeps into the gravel or soil, which filters more waste from the water and reduces harmful pathogens.

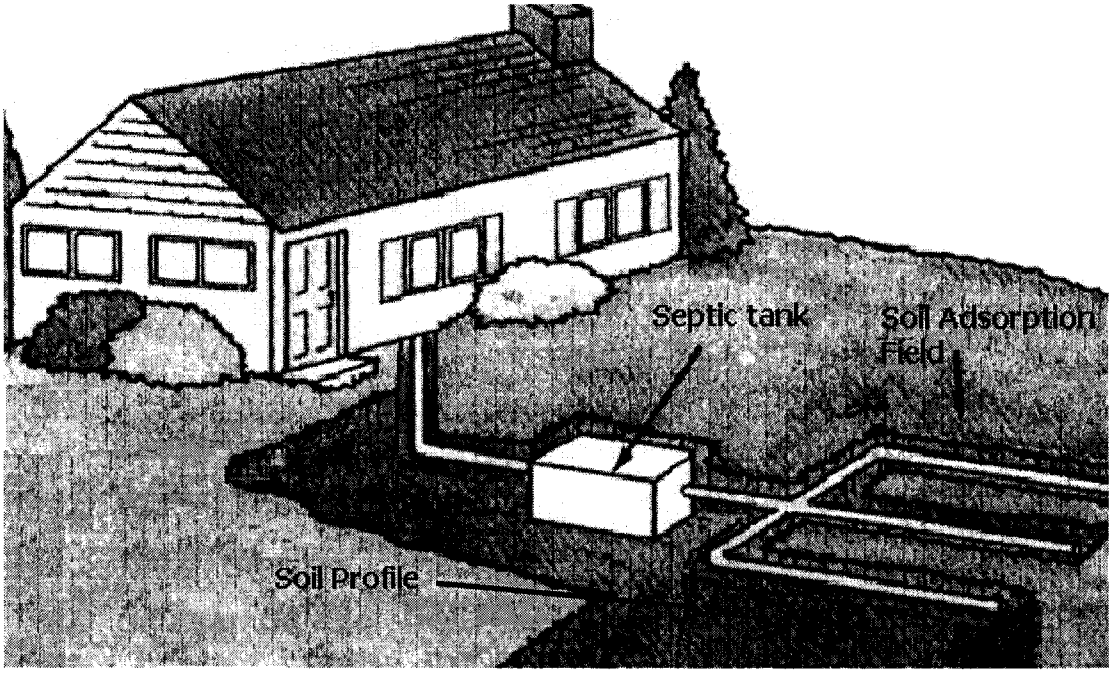

Figure 1.1: Standard septic tank gravity distribution system (adapted from: US EPA,1987)

In order to improve effluent quality, changes can be made to this system such as the addition of filters. The septic tank can also be altered by adding an aerobic treatment unit or other disinfection devices (Jantrania and Gross 2006). If the soil is deemed inappropriate or the water table is too high, alternatives to the common adsorption field include adsorption mounds, at-grade systems, intermittent sand filters, and lateral adsorption trenches among others (Jantrania and Gross 2006).

Inappropriate soil conditions occur when permeability rates are either too high or too low, the surface soil is too thin, the restrictive layer composed of clay and bedrock are too close to the soil surface, or the seasonal water tables are too high. Adsorption mounds, elevated gravel trenches, overcome these problems by separating or elevating the distribution system from the soil thereby adding more vertical space though with treatment can occur. An at-grade system is placed on the surface of the soil, taking maximum advantage of the surface organic layers and upper soil horizons. Its pressurized distribution system is designed to evenly distribute the water along the lateral.

1.3.1 Soil as a Final Disposition Point

Treatment in soil is defined as the retention, degradation, or transformation of a contaminant to some other component less harmful to the environment. There are many factors which can influence treatment efficiency of wastewater. These can be divided into site characteristics, soil physical characteristics, chemical characteristics, and soil biological activity.

Table 1.1. Site and soil characteristics which determine treatment efficiency. Compiled from Tanik and Comakoglu (1997), Jiang et al. (2005), Stevik et al. (1999)

Site Characteristics	Physical Characteristics	Chemical Characteristics	Biological Characteristics
Slope of the landscape	Bulk density	pH	Vegetation
Depth of the soil profile	Texture	Cation exchange capacity	Micro and macro fauna activity
Thickness of the subsoil/ parent material	Permeability Soil water content		Organic matter
Climate	Porosity		
Temperature	Aerobic conditions		
Proximity to ground or surface water sources	Pore size distribution		

The physical and chemical characteristics are very important for non-biological treatment. Chemical characteristics, temperature, and climate work together to determine the solubility and reactivity of the wastewater chemical contaminants. All of these can affect the appearance of micro- and macro-pores, evaporation rates, and natural predation of introduced microorganisms. Set back distances are based on the proximity of the discharge system to water sources.

All of the factors involved in treatment efficiency are highlighted when analyzing treatment mechanisms of pathogenic microorganisms. One such mechanism is filtration which can be achieved by the sedimentation and straining of

microorganisms. Another very important mechanism is the sorption or retention of any these organisms on the soil particle surfaces.

Sedimentation comes into effect when wastewater effluent is first released onto the treatment field and ponding occurs. It is the natural settling of the microorganisms in a liquid due to gravity and differences in the density of the microorganisms and soil water (Grinn et al. 2002). Straining is a mechanism similar to a filter; it is the movement constriction of a particle or microorganism when its diameter is larger than the pore opening diameter, and is inversely proportional to the soil particle size (texture).

Sorption is the attachment and detachment of the soil microorganism to the surfaces of soil particles. Jiang et al. (2005) even suggested it as the main mechanism for bacterial retention in soil because both sedimentation and straining are considered more easily reversed processes. If runoff were to occur by rainwater or saturated flow, the microorganisms could be easily washed away causing contamination of nearby areas or waterways. Adsorption, on the other hand, is supposed to be more robust, retaining the microorganisms for longer periods of time and allowing for other treatment mechanisms to take place (Hurst 1991).

1.4 Wastewater Composition

Wastewater composition from individual households is quite similar to the composition of wastewater found at large treatment facilities. The main difference is that the composition has more variability due to the individual household habits and water use rather than a homogeneous mass from many homes.

In general, wastewater contains debris, suspended solids, disease-causing pathogens (bacteria and viruses), decaying organic waste, nutrients such as nitrogen and phosphorus, and about 200 different identified chemicals which may be acutely or chronically toxic to aquatic organisms and human health (Environment Canada 1999).

The minimum mandated quality of the effluent from municipal wastewater treatment plants after tertiary treatment is: BOD_5 <20 mg/L, TSS<20 mg/L, fecal coliforms <200 CFU/100mL, total phosphorus <1 mg/L, and ammonia-nitrogen <5 mg/L. These measurements define wastewater quality and are easily measured at the

point of discharge to confirm that they meet quality standards established by the province of Alberta. (Juma et al. 2007)

1.4.1 Pathogens

Septic effluent contains a substantial number of microorganisms. The number of fecal coliforms, a group of indicator organisms, can reach 10^6-10^8 MPN/100mL. Although septic tanks remove anywhere from 25% to 75%, many organisms such as parasitic helminth eggs and protozoan cysts have a protective cover that help them pass through conventional treatment without harm. Table 1.2 shows the some pathogens found in wastewater along with their related diseases and the minimum number of pathogens necessary for infection.

Table 1.2. Pathogens found in wastewater and their infectious dose. Compiled from US FDA's Center for Food Safety and Applied Nutrition (2008).

Examples of Bacterial Pathogens found in OWTS	Related Disease	Infectious Dose (minimum)
E. coli (enteropathogenic)	Diarrhea, Cholera	10^6 - 10^{10} (MPN/100mL)
Salmonella typhi, Salmonella enteritidis	Salmonellosis, Reiter's disease	15 to 20 cells
Shigella flexneri, Shigella sonnei	Shigellosis	10 cells
Vibrio cholerae serogroup 01	Cholera	10^3 -10^7 (MPN/100mL)

1.4.2 Transmission of Illness to Humans

There are two major routes to illness by OWTS, ingestion of contaminated soil particles or ingestion of contaminated water.

Ingestion of contaminated soil particles can occur easily if there is direct contact with the onsite wastewater treatment system such as children or pets playing on the adsorption field or the ingestion of fruit and vegetables grown in the area around the system. Illness can easily be avoided if proper care is taken to isolate the system.

On the other hand, ingestion of contaminated water is a much more frequent and probable route for infection. If a soil cannot retain and treat the wastewater, pathogens could travel into underground and over ground waterways contaminating areas kilometres away from the source.

Of all the water currently withdrawn in Alberta, about 3% comes from the groundwater system. It may seem small, but this translates to approximately 500,000 domestic wells, with an annual increase of about 7,000. A high concentration of OWTS close to drinking wells has been associated with greater incidences of disease.

In the United States, it is estimated that 168,000 viral illnesses and 34,000 bacterial illnesses occur each year as a result of consumption of drinking water from systems that rely on improperly treated ground water. Malfunctioning septic systems have been identified as one potential source of ground water contamination (US EPA 2002).

Although no such statistics exist for Canadian well water consumers, research has shown a high number of pathogens in well water. An example of this is the village of Cumberland outside of Ottawa where 1.5% of samples from 195 wells had *E. coli* and 20% of the wells had levels of total coliform high enough to cause potential health-related issues. It is suspected that the restrictive clay layer on which the village was built is the main problem. Sewage systems are also partially to blame for the poor surface water quality (City of Ottawa 2002).

The presence of fecal contamination of water sources suggests that knowledge of survival, attenuation, and survival of pathogens in soil is necessary to avoid health-related risks.

1.5 Survival of Pathogens in Soil

There have been several studies to investigate the die-off of different organisms in soil. Von Donsel et al. (1967) found that *S. typhimurium* could be found in pasture soil 9 months after acute salmonellosis had occurred in cows; *S. typhimurium* was found in garden soil at least 280 days after contamination; *Brucella abortus* was shown to survive up to 125 days in soil during winter. In a 60-week study of soil on which biosolids were applied, fecal streptococci numbers decreased

but remained detectable throughout the experiment. Fecal coliforms and *Salmonella* remained detectable after 34 and 16 weeks, respectively (Gibbs et al. 1997).

In most of these earlier studies, although survival of the organisms was studied, links to soil characteristics were often not taken into account.

1.6 Indicator Organisms

Pathogens are unfortunately few in quantity and many in species to quantify effectively in the laboratory, and therefore, indicator organisms of fecal contamination are used. In this case the coliform group is an adequate indicator species since they are not necessarily pathogenic, found in abundance, are said to have a longer life span than other pathogens; most importantly, they are exclusive to excretions of warm blooded animals. These indicator organisms can therefore be used to distinguish native soil microorganisms from microorganisms derived from sewage effluent. A better understanding of how these indicator organisms survive or behave in the soil environment could give better indications of how pathogenic bacteria might act and how effective soil treatment can be enhanced.

During the course of this project the following indicator microorganisms were used, *Escherichia coli* (ATCC 25922) and *Enterococcus faecalis* (ATCC 29212). These organisms have both been used in water and wastewater regulation codes to indicate contamination from human sources and have both been reported to have longer life spans than most pathogens.

1.6.1 *Escherichia coli*

E. coli is a Gram negative, facultative anaerobic, straight, bacillus shaped, non spore forming bacterium (2.0 to 6.0 μm in length, 1.1 to 1.5 μm in diameter) occurring singly or in pairs (Bergey et al. 1984). It can be commonly found in lower intestines of human and mammals, helping in the digestion processes, food breakdown and absorption, and vitamin K production.

Although most strains of *E. coli* are considered harmless organisms, some strains are responsible for illness. Three general clinical syndromes can result from infection with pathotypes: enteric/diarrhoeal disease, urinary tract infections, and sepsis or meningitis (Kaper et al. 2004).

According to the American Public Health Association (APHA, 2005), *E. coli* and other coliforms are one of the most widely accepted bacterial indicators of fecal

pollution. *E. coli,* in particular, is an excellent indicator since it is one of the bacteria in highest concentration within human feces. It is the fecal coliform indicator of choice when analyzing potable water and wastewater.

1.6.2 *Enterococcus faecalis*

E. faecalis is a Gram positive, coccus shaped microorganism. It has the ability to survive many environmental extreme temperatures and high salt and ionic concentrations, such as in solutions of 6.5% NaCl. Although it was generally considered to be innocuous, it has now emerged as a major cause of nosocomial infections such as urinary tract infections, wound infections, bacteremia, and endocarditis. It has also been reported to have evolved to more antibiotic resistant forms.

Mallmann and Litsky (1951) showed that *E. faecalis* was the only organism, other than coliforms, that was found in sewage and could be used as an indicator of fecal contamination. Since then, this intestinal enterococcus has been recommended by the World Health Organization for the testing of recreational water such as swimming pools, lakes, and marine waters. It is considered by some to be a more conservative indicator organism than *E. coli.*

1.7 Project Objectives

In order to address the issue of increasing soil treatment effectiveness and the implementation of better regulations in Alberta, this research was conducted to study the survival and adsorption of two indicator organisms, *Escherichia coli* and *Enterococcus faecalis,* in soil samples from major horizons of two soils commonly found in central Alberta, an Eluviated Chernozem and a Dark Gray Luvisol, and in soil samples obtained from failed OWTS.

The objectives of this research project were:

1) Determine the survival of two indicator organisms: *E. coli* (ATCC 25922) and *E. faecalis* (ATCC 29212), at three different temperatures: 20°C, 5°C, and -20°C, on samples from the major horizons of an Eluviated Black Chernozem obtained from

Ellerslie, Alberta and a Dark Gray Luvisol obtained from Rocky Mountain House, Alberta (Chapter 2).

2) Determine the adsorption rate constants of the two indicator organisms: *E. coli* (ATCC 25922) and *E. faecalis* (ATCC 29212), at two different temperatures: 20°C and 5°C, on samples from the major horizons of an Eluviated Black Chernozem obtained from Ellerslie, Alberta and Dark Gray Luvisol obtained from Rocky Mountain House, Alberta (Chapter 3).

3) Determine the adsorption rate constants of two indicator organisms: *E. coli* (ATCC 25922) and *E. faecalis* (ATCC 29212), on soil samples obtained from a failed septic field, a failed mound, and a failed at-grade dispersal site (Chapter 4).

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Chapter 2

Net Die-Off of Indicator Organisms in Soil Samples of a Dark Gray Luvisol and an Eluviated Black Chernozem

2.1 Introduction

Onsite wastewater treatment systems (OWTS) are designed to treat raw sewage and increase the quality of sewage effluent by reducing the biological oxygen demand (BOD_5), total suspended solids (TSS), and microorganism count before dispersing it onto the soil. OWTS draw on a wide range of technologies from the traditional septic tank to the use of different types of filters, aerobic treatment units, and advanced treatment plants. The use of at-grade soil-based dispersal systems is increasing because this method uses the whole soil to polish the treated effluent as it passes through the soil.

This effluent carries with it many pathogenic microorganisms. These bacteria are highly specialized to survive within the human body and can become stressed or inactivated when faced with the harsh soil environment after being dispersed. Soil represents a different variety of conditions in terms of moisture content, temperature, pH, substrate and nutrient availability, amount of dissolved oxygen, presence of toxic substances, soil texture and structure. The cation exchange capacity, organic matter, and soil clay content influence the adsorption rate of microorganisms which in turn affects their inactivation rate. If irreversible adsorption occurs, the microorganisms are considered to become non-viable and are no longer a contamination threat. Even if all these conditions prove favourable, the microbes are subject to predation or competition by other microorganisms (Van Donsel et al. 1967; Foppen and Schijven 2006).

The survival rate of any indicator organism will also depend on its properties. Many microorganisms have the ability to become encapsulated or dormant in a time of stress. Hagedorn et al. (1978) showed that both *E. coli* and *E. faecalis* survived in appreciable numbers in saturated soil throughout a 32-day sampling schedule. Gerba et al. (1975) found that coliform bacteria traveled 0.6 m in a fine sand loam and up to 830 m in a sand-gravel.

Lang et al. (2007) have suggested that there may be particular strains of *E. coli* which have adapted to thrive in the soil environment. While these strains are limited to specific area and climate conditions, it is important to better understand how each limiting factor contributes to the inactivation or survival and subsequent replication.

2.2 Factors Controlling Microbial Survival in Soils

2.2.1 Moisture Content

Cools et al. (2001) showed that higher moisture content and lower incubation temperature favoured the survival of *Enterococcus ssp.* and *E. coli.* Kibbey et al. (1978) had found similar results with the survival rates of *Streptococcus faecalis (Enterococcus faecalis).* The rates were the highest when the soil was saturated and much lower in air-dried soil.

Since the basis of onsite wastewater systems is the dispersion of wastewater, the dispersal field is expected to have high moisture content. Juma et al. (2007) determined that the average gravimetric moisture content of several at-grade septic systems for the 7.5 to 30 cm layers at the mid point position between dispersal orifices was 24%. This was significantly higher than the control soil where the moisture content was between 16% to 19%. They also found that the soil moisture content increased with depth. Therefore, moisture content is not considered a limiting factor to the pathogen survival time in a dispersal field.

2.2.2 Soil Texture and Structure

The particle size distribution can greatly influence pathogen survival. Both Marshall (1975) and Van Veen et al. (1997) found a higher survival rate of inoculated organisms in finer textured than coarse soils under the same conditions. Vargas and Hattori (1986) found the surviving inoculant bacteria to be localized in the 1 mm to 2 mm soil aggregates when co-introduced with a grazing protozoan species. Therefore, the soil texture, pore size distribution, and aggregate size greatly control the fate of introduced microorganisms.

2.2.3 Competition, Predation, and Nutrient Availability

Competition and predation are also considered to be very important factors in controlling introduced microorganisms. Predators such as protozoa are known to graze upon microorganisms such as those introduced by dispersal of sewage effluent on soil. Soils with a high clay content have more porosity, but the size of the pores is much smaller compared to sandy soils. Therefore, there is greater bacterial protection in microhabitats of a clay soil since predators cannot enter the fine pores (Rutherford and Juma 1992; England et al. 1993).

Introduced microorganisms also have to compete with native soil species for nutrients. However, this is not necessarily difficult in areas directly under the orifices of dispersal systems because the applied effluent has a high nutrient content.

2.2.4 Temperature

Temperature is one of the most important factors influencing the die-off rate coefficient (Foppen and Sheijven 2006). Therefore, it is one of the most important factors controlling the inactivation of pathogens introduced into the soil (Hurst 1991).

Generally, pathogenic bacteria will grow in the temperature range of 4°C to 60°C. However, researchers have found that the survival time of enteric bacteria tends to decline in warmer soils compared to cooler soils (Van Donsel et al. 1967, Andrews et al. 2004); the die-off rate may be doubled with every 10°C increase in temperature from 5°C to 30°C (Reddy et al. 1981). Temperatures lower than freezing even favoured survival as demonstrated in experiments with *S. typhosa* and *B. abortus*, which survived up to 125 days in soil during winter (Van Donsel et al. 1967).

Although it is possible to assume that temperature does modulate the growth rate of bacteria, Lang et al. (2007) suggest that it should not be used to directly determine an organism's ability to survive in the soil environment.

2.3 Objectives

The objective of this study was to quantify the net die-off rates of two indicator bacteria, *E. coli* and *E. faecalis*, in sterilized and non-sterilized soil samples from different horizons of an Eluviated Black Chernozem and a Dark Gray Luvisol.

2.4 Materials and Methods

2.4.1 Site Description and Soils

In order to measure net die-off (Chapter 2) and attenuation (Chapter 3) of two introduced fecal coliforms, soil samples were obtained from the Ah and Bt horizons of an Eluviated Black Chernozem from Ellerslie Research station located 10 km south of the University of Alberta Campus (53° 25'N, 113° 33'W) and from the Ah, Ae, and Bt horizons of a Dark Gray Luvisol from a site located 10 km south east of the town of Rocky Mountain House (52° 22'31"N, 114°55'18"W).

The soil types ranged from a silt loam to a clay loam. The pH of these the horizon samples was slightly acidic ranging from 4.9 to 6.9. None of the soil horizons were saline.

Table 2.1 Soil properties of selected horizons of an Eluviated Black Chernozem from Ellerslie and a Dark Gray Luvisol soil from Rocky Mountain House (RMH)

		Sand	Silt	Clay	Organic			EC
[z]Location	Horizon	Percent (%)			C	Textural Class	pH	dS/m
Ellerslie	Ah	24	58	18	6.83	Silt Loam	5.6	0.047
Ellerslie	Bt	37	33	30	0.67	Clay Loam	6.4	0.048
RMH	Ah	61	32	7	3.78	Sandy Loam	4.9	0.053
RMH	Ae	24	71	6	0.84	Silt Loam	5.1	0.032
RMH	Bt	16	50	35	1.75	Silt Clay Loam	6.9	0.187

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol

2.4.2 Preparation of Soil Samples

The soil samples from each horizon were dried and passed through a 2 mm sieve. Since the soil samples were disturbed, it was necessary to recalculate the bulk density. This was done by lightly compacting the soil samples into containers of a known volume and then weighing the container.

Total soil porosity was determined using a saturation test. Plastic rings (5.0 cm internal diameter and 2.5 cm high) were filled with soil were left to saturate in 0.5 cm of water overnight then weighed (wet weight). These samples were then dried at 105°C to constant weight (dry weight). The weight difference between the wet weight and dry weight was attributed to the water content lost from the pores and

yielded an approximation of the total pore space of the soil sample. These data were used to adjust the moisture content.

Three composite soil samples from each horizon were tested microbiologically to assess the presence of the indicator organisms using the most probable number technique. In every case, the result was negative indicating there was no evidence of prior soil contamination by these organisms.

Soil sample sterility was achieved by autoclaving a separate set of soil samples three successive times during one week. Sterility was verified microbiologically using a PDA streak plate. No glucose amendment was used in this soil.

2.4.3. Initial Inoculation and Incubation

In order to better understand the net die-off rates in the Alberta environment, the non-sterilized soil samples were incubated at 23°C (laboratory room temperature), 5°C, maintained by a fridge (Woods), and -18°C, maintained by a freezer (Woods). The sterilized soil samples were incubated at room temperature.

The indicator organisms *E. coli* (ATCC 25922) and *E. faecalis* (ATCC 29212) were left incubated at 37°C in the commercially available media (dehydrated media manufactured by DIFCO laboratories except where noted), Tryptic Soy Broth (TSB), and brain/heart infusion broth (BHIB), respectively. These cells were then suspended, washed twice in phosphate buffered solution (PBS, NaCl: 8.5 g/L, KH_2PO_4: 0.3 g/L, Na_2HPO_4: 0.6 g/L), pH 6.8, and resuspended together in the same suspension to a density of approximately 10^4 to 10^5 cells per mL, calculated by using direct count with a Petroff-Hausser chamber. These counts were verified by the using the Most Probable Number method (MPN).

From each horizon sample, 10-gram samples were weighed and transferred into 100 mL sterile Erlenmeyer flasks. Non-sterilized soils samples were spiked with a glucose solution (100 µg/g soil) in order to stimulate the natural soil microflora and fauna. Both the sterilized and non-sterilized soil samples were then inoculated with the indicator organisms. The inoculum suspension, glucose water (for non-sterilized soils), and distilled water were added to each soil sample so as to have a final moisture content of 60% of the total soil porosity.

Sampling was conducted six times during the experiment at approximately 1, 3, 6, 30, 45, and 60 days. Samples were aerated for approximately 5 minutes under the Biosafety cabinet (Canadian Cabinets BM4-2A-49) at day 14 and 40.

2.4.4 Microbiological Analysis

Three samples were taken from each incubation temperature at the dates mentioned. Each individual sample was aseptically mixed with 90 mL of phosphate buffered solution (pH 6.8) in 200 mL milk dilution bottles and mixed at 150 rpm for 10 minutes. This mixture was immediately diluted in test tubes containing 9 mL of PBS.

The indicator microorganism concentration (*E. faecalis* and *E. coli*) was measured throughout the experiment using the 3-tube Most Probable Number technique with a multiple tube fermentation described in the APHA Standard Method for Water and Wastewater (2005), section 9221B.

The measure of *E. coli* was determined using Lauryl Tryptose broth (LTB) with inverted Durham tubes in which diluted samples were incubated for 48 ± 3 hours at 35 ± 0.5°C. Positive tubes show both growth (turbidity) and presence of gas. Verification of these microorganisms was done using the GAD method. In this method, a positive confirmation of *E. coli* is indicated by a change in color of the GAD regent (L. glutamic acid, sodium chloride, bromocresol green, Triton X-100, and water) from yellow to blue after being incubated with centrifuge concentrated bacterial cells for 1 h at 35°C.

E. faecalis was determined on Azide dextrose broth. The same dilutions used with LTB would be used with this broth and then incubated at 35 ± 0.5°C for 48 ± 3 hours. Portions of growth from the positive tubes were verified by streaking on Esculin Iron (EI) agar plates then incubating for 24 ± 2 hours at 35 ±0.5°C. Brownish-black colonies with brown halos indicated the presence of fecal streptococci.

Previous tests showed that both media were selective for their specific microorganism and did not promote the growth of the other. This means, for example, that *E. faecalis* would not grow in LTB.

2.4.5 Statistical Analysis

Once the MPN data were collected for each, an MPN calculator (MPN Calculator Build 23) was used to calculate the number of organisms per mL of soil suspension.

No statistical analysis was conducted for the survival rates of the indicator organisms in sterilized soil from Ellerslie and Rocky Mountain House. The results were instead plotted and fitted to a polynomial curve representing the exponential growth (log), stationary (lag), and death phases of a microorganism growing in ideal conditions.

The analysis of the net die-off curves was done using the R statistical language (R Development Core Team, 2008). The regression equation used to calculate the net die-off rates was a simple first order decay equation known as Chick's law. It is used for determining the fate of bacteria in an unfavourable environment. The equation was:

$$\left(\frac{dN}{dt}\right) = -kN$$

[Equation 2.1]

This can be integrated to:

$$\ln\left(\frac{N}{N_0}\right) = -kt$$

[Equation 2.2]

Where N_0 is the initial number, N is the residual after a given time (t), and k is the net die-off rate constant. (N/N_0) represents the proportion of indicator organisms that survive while (1- (N/N_0)) is the proportion of indicator organisms which became inactive. The half life of the bacteria, independent of the starting concentration, is given by:

$$t_{\frac{1}{2}} = \frac{\ln(2)}{k}$$

[Equation 2.3]

Once fitted, all data were confirmed to follow a normal distribution using the Shapiro-Wilks test.

The effects of temperature on the survival of *E. coli* and *E. faecalis* within the different soil types was examined using an analysis of covariance (ANCOVA) on each of the linear regressions found above. The three regressions and their respective data points were also plotted with their 95% confidence intervals to visually distinguish similarities or differences in treatment die-off effects. Although not all the regression curves were significant (p-value <0.05), they were still analyzed in the ANCOVA. This was done to in order to be able to analyze the data in a similar manner for all horizons and temperature treatments.

2.5 Results

2.5.1 Survival Rates in the Sterilized Soil Samples

The absence of competitive or predatory organisms in the soil allowed significant growth of both indicator organisms at room temperature. Figures 2.1 and 2.2 show the growth curves of *E. coli* in different horizons of the two soil profiles. Figures 2.3 and 2.4 show the growth curves of *E. faecalis*.

E. coli thrived in the Bt horizons of both soils growing to a maximum of 1.5 log of the original concentration of inoculated organisms. The least growth was found in both Ah horizons. The growth or log phase was sustained for a much longer period of time in the Black Chernozem.

E. faecalis thrived in the Chernozemic Ah horizon, but in both cases, growth was not as prolific as for *E. coli,* reaching a maximum of only 1.5 log of the original concentration in the Black Chernozem. Although the log, lag, and death curves can still be discerned in the Dark Gray Luvisolic soil from Rocky Mountain House, the net growth of *E. faecalis* on this soil was very low; its maximum growth reached only 0.008 log of the original concentration in the Bt horizon. The Bt horizon of the Black Chernozem and the Ae horizon of the Gray Luvisol seemed to have a rapid growth phase immediately after introduction to the soil, but most of its life span during the 60 days was spent in the lag (stationary) or death phases. Overall, the net growth rate of *E. faecalis* was lower than *E. coli* in the major horizons of the two soils.

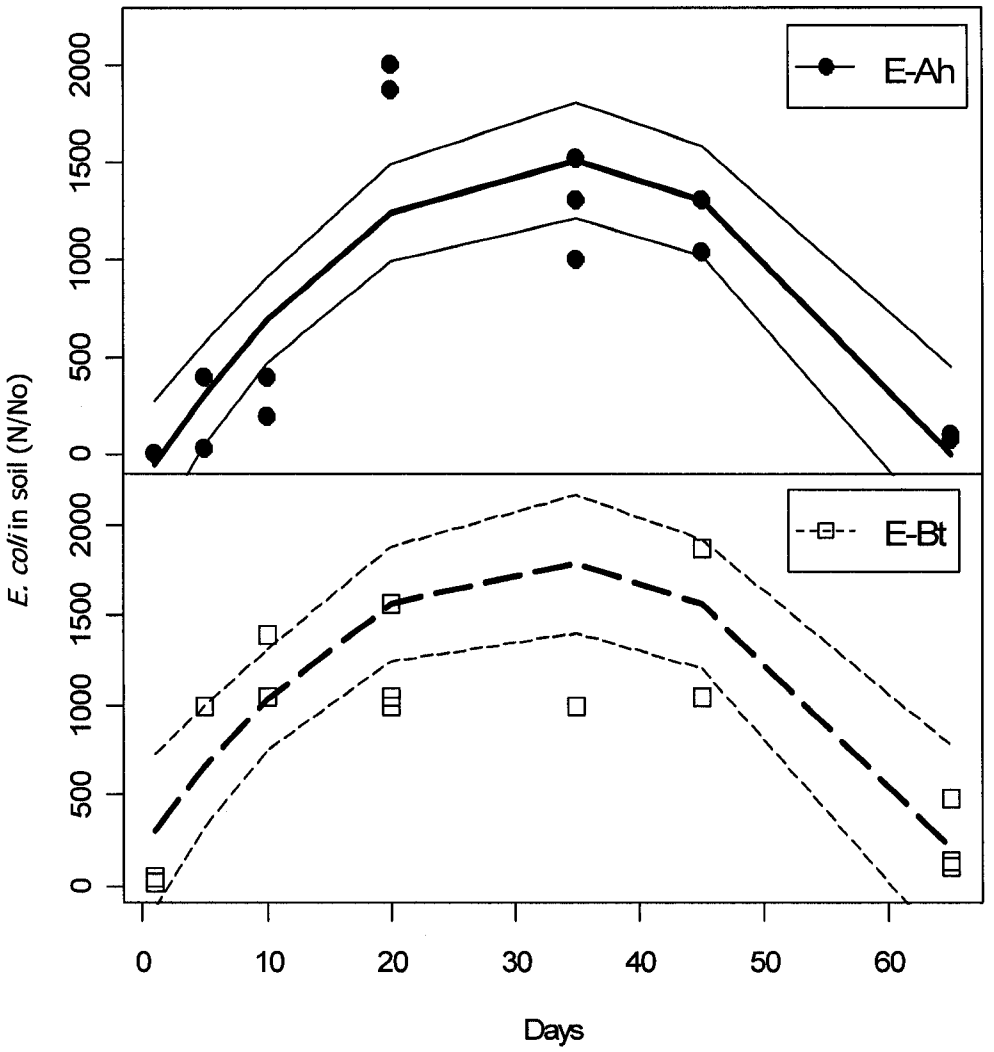

Figure 2.1: Plot of *E. coli* survival on sterilized Ah (E-Ah) and Bt (E-Bt) horizons of the Black Chernozem from Ellerslie (Bold). Lighter lines represent the 95% confidence intervals.

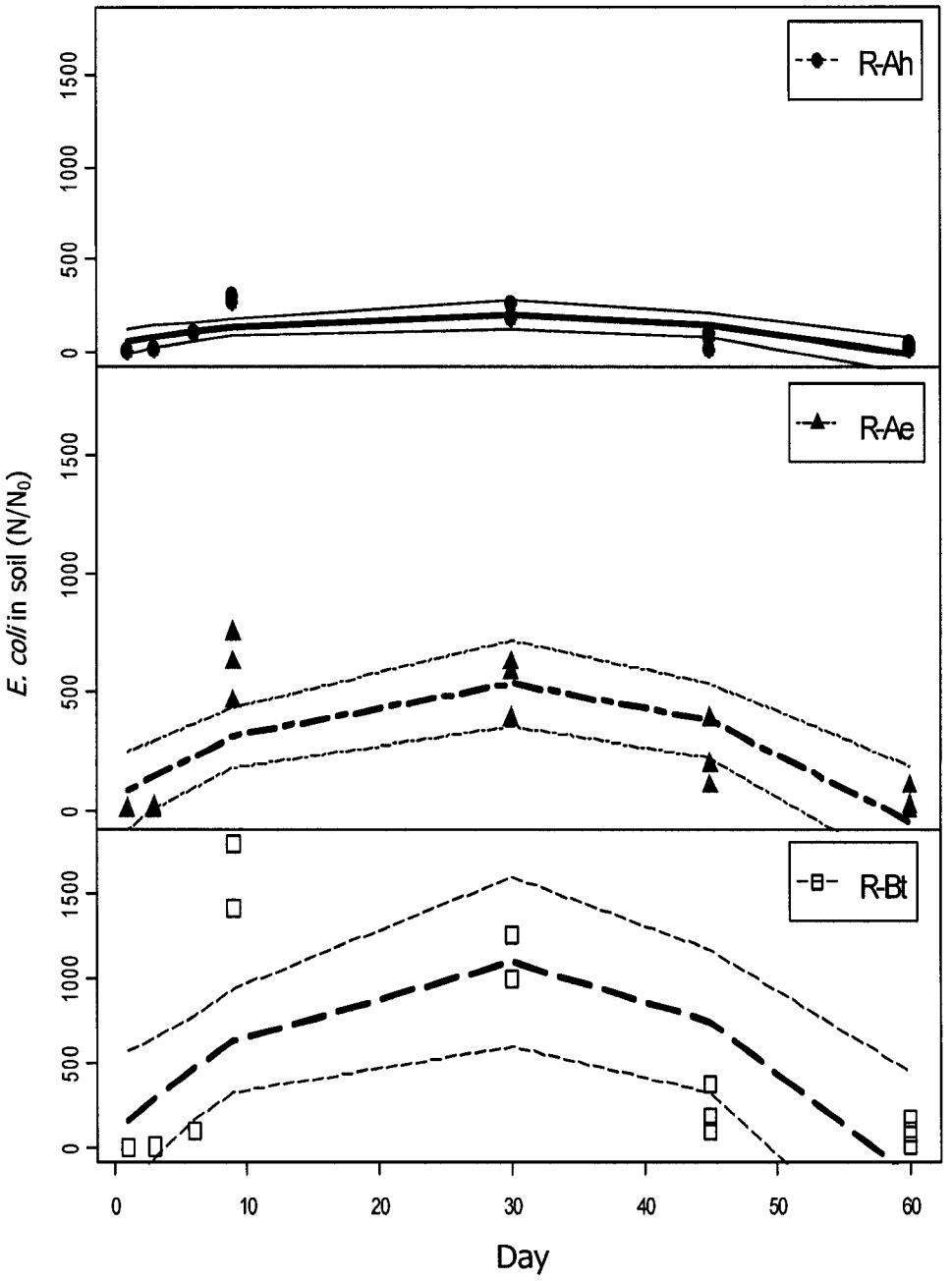

Figure 2.2: Plot of *E. coli* survival on sterilized Ah (R-Ah), Ae (R-Ae), and Bt (R-Bt) horizons of the Gray Luvisolic soil samples from Rocky Mountain House. Lighter lines represent the 95% confidence intervals.

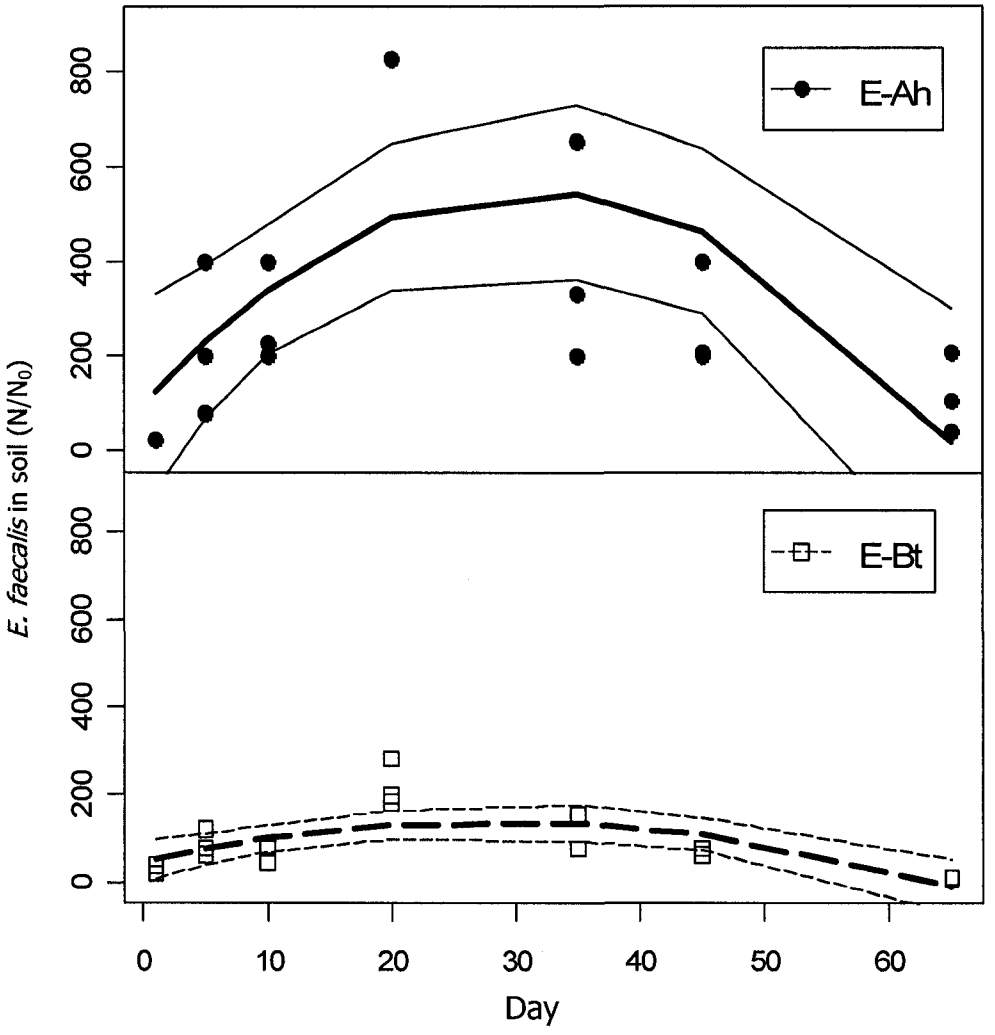

Figure 2.3: Plot of *E. faecalis* survival on sterilized Ah (E-Ah) and Bt (E-Bt) horizons of the Black Chernozemic soil samples from Ellerslie. Lighter lines represent the 95% confidence intervals.

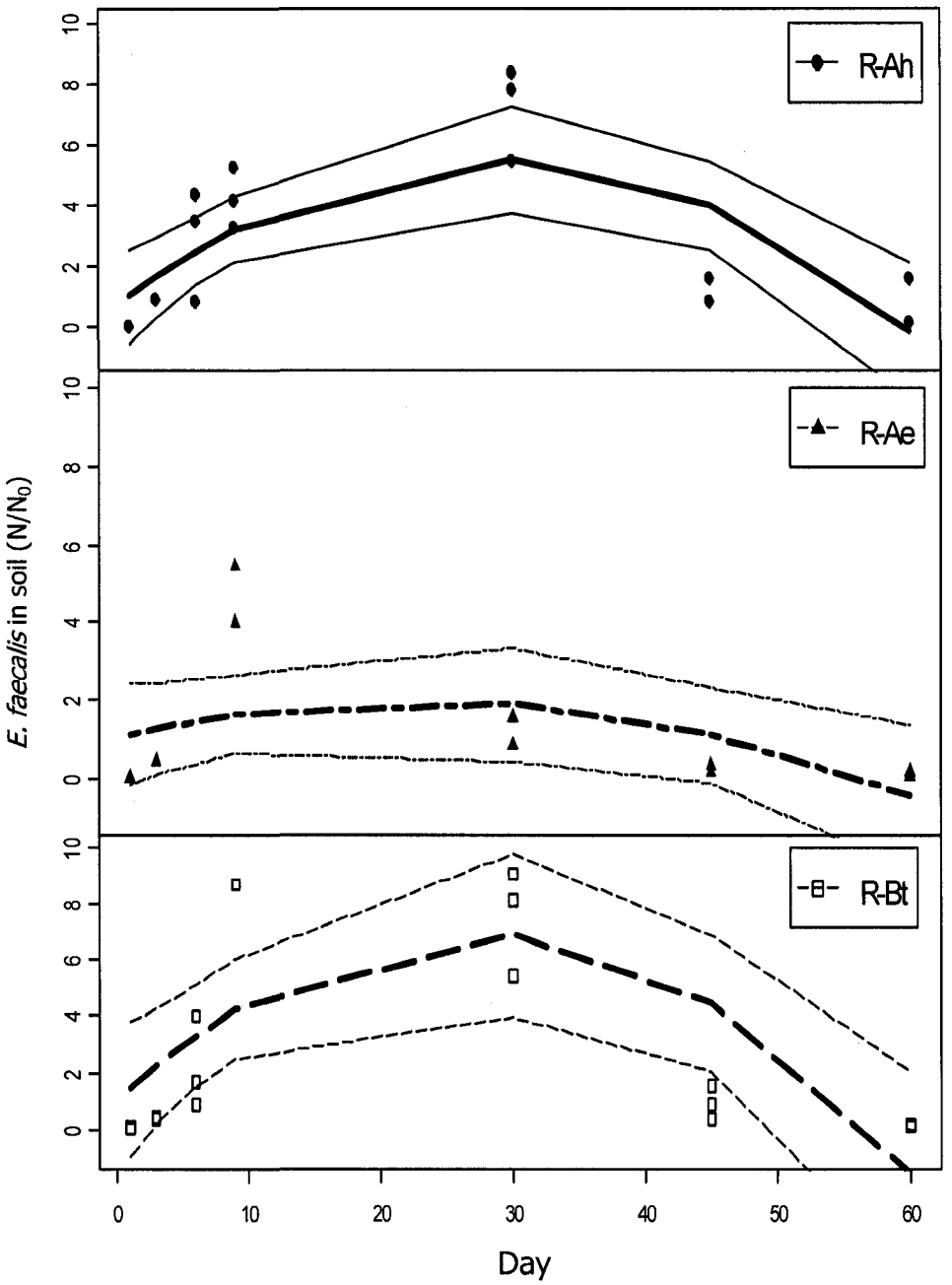

Figure 2.4: Plot of *E. faecalis* survival on sterilized Ah (R-Ah), Ae (R-Ae), and Bt (R-Bt) horizons of Gray Luvisolic soil from Rocky Mountain House. Lighter lines represent the 95% confidence intervals.

2.5.2 Net die-off Rate Constants and Half Lives of Indicator Organisms

The results from the regression lines to fit first order decay equation for a one time addition of *E. coli* and *E. faecalis* on non-sterilized soils are presented in Tables 2.2 and 2.3, respectively. Overall, the linear regression analysis indicated highly significant effects of different temperatures on net die-off rate constants for the indicator organisms in major horizons of the two soils. All slopes were negative indicating the eventual die-off of all indicator microorganisms. The detection limit in this case was 3 CFU/mL.

Table 2.2: Net die-off rate constants and half live of *E. coli* at three temperatures in major horizons of the two soils.

[z]Location	Horizon	Temperature	[y]k	Intercept	[x]R^2	[w]p-value	Half-life (days)
Ellerslie	Ah	23°C	-0.08	-5.7	0.63	<0.001	8.7
Ellerslie	Ah	5°C	-0.13	0.01	0.74	<0.001	5.5
Ellerslie	Ah	-18°C	-0.021	-6.0	0.10	0.16	32.8
Ellerslie	Bt	23°C	-0.05	-2.1	0.72	<0.001	13.3
Ellerslie	Bt	5°C	-0.14	1.0	0.86	<0.001	4.9
Ellerslie	Bt	-18°C	-0.05	-4.6	0.43	0.001	15.1
RMH	Ah	23°C	-0.07	-3.4	0.31	0.008	10.0
RMH	Ah	5°C	-0.04	-1.5	0.44	0.003	16.3
RMH	Ah	-18°C	-0.07	-7.2	0.67	<0.001	9.8
RMH	Ae	23°C	-0.11	-3.2	0.67	<0.001	6.3
RMH	Ae	5°C	-0.07	-2.8	0.64	<0.001	9.3
RMH	Ae	-18°C	-0.04	-8.8	0.39	0.002	18.9
RMH	Bt	23°C	-0.12	-2.0	0.83	<0.001	5.8
RMH	Bt	5°C	-0.08	-1.6	0.71	<0.001	8.3
RMH	Bt	-18°C	-0.06	-6.4	0.44	0.001	12.1

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol
[y]k= slope of the regression line (first order rate constant)
[x]R^2 is the coefficient of determination
[w]p-values <0.05 indicate a significant regression fit

For both soils, the number of *E. coli* decreased exponentially at all three temperatures. The half-life of the *E. coli* population ranged from 5.8 to 13.3 days at 23°C, 4.9 to 16.3 days at 5°C, and 9.8 to 32.8 days at -18°C in both soils. The temperature responses at 23°C and 5°C were more similar compared to -18°C.

The half lives of the indicator organisms in the Ah and Bt horizons of the Chernozemic soil were at 5°C were 5.5 and 4.9 days, respectively, compared to a 8.7 and 13.3 days at 23°C, and 32.8 and 15.1 at -18°C. Therefore, at 5°C, this soil would have fewer organisms after 10 days than at 23°C or -18°C.

The half-life of *E. coli* in the Ae and Bt horizon soil samples from Rocky Mountain House showed inverse trend. As temperature dropped from 23°C to -18°C, the half life of the *E. coli* population increased. However, this was not the case in the Ah horizon as the shortest half-life, 9.8 days, was observed at -18°C, while the longest, 16.3 days, was at 5°C.

The overall shortest half-life, 4.9 days, was found in the Chernozemic Bt horizon from Ellerslie at 5°C, while the longest half-life, 32.8 days, was measured in the Chernozemic Ah horizon at -18°C. The inconsistent half-life of this indicator organism in the soil samples at the different incubation temperatures indicate there are other mechanisms that influence die-off rates.

The half-life of the *E. faecalis* population ranged from 5.0 to 11.9 days at 23°C, 12.8 to 56 days at 5°C, and 20.6 to 45.1 days at -18°C in both soils. There was an increase in half-life as temperature decreased in all samples but those from the Ae and Bt horizons from Rocky Mountain House who had longer half-lives at 5°C.

The population of *E. faecalis* on the soil from Ellerslie followed the pattern described above. The half-life of this indicator organism almost doubles from 5°C, 12.8 and 14.9 days, to -18°C, 37.8 and 26.2 days respectively.

In the Luvisolic soil from Rocky Mountain House, the influence of temperature is not so evident. However, the half-life of *E. faecalis* is at least 5 times longer at 5°C than at 23°C in all horizons of this soil.

Table 2.3: Net die-off rates constants and half life of *E. faecalis* at three temperatures in major horizons of the two soils.

[z]Location	Horizon	Temperature	[y]k	Intercept	[y]R^2	[w]p-value	Half Life (days)
Ellerslie	Ah	23°C	-0.058	-6.85	0.67	<0.001	11.9
Ellerslie	Ah	5°C	-0.054	-4.08	0.62	<0.001	12.8
Ellerslie	Ah	-18°C	-0.018	-4.41	0.27	0.02	37.8
Ellerslie	Bt	23°C	-0.109	-4.40	0.87	<0.001	6.4
Ellerslie	Bt	5°C	-0.047	-3.77	0.37	0.005	14.9
Ellerslie	Bt	-18°C	-0.027	-4.99	0.11	0.13	26.2
RMH	Ah	23°C	-0.064	-4.89	0.30	0.01	10.8
RMH	Ah	5°C	-0.012	-4.58	0.21	0.05	56.0
RMH	Ah	-18°C	-0.018	-5.04	0.10	0.16	38.5
RMH	Ae	23°C	-0.094	-4.58	0.54	<0.001	7.4
RMH	Ae	5°C	-0.018	-4.11	0.25	0.03	38.8
RMH	Ae	-18°C	-0.015	-4.73	0.11	0.15	45.1
RMH	Bt	23°C	-0.138	-3.28	0.83	<0.001	5.0
RMH	Bt	5°C	-0.020	-3.86	0.34	0.01	34.4
RMH	Bt	-18°C	-0.034	-4.15	0.34	0.006	20.6

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol;
[y]k= slope of the regression line (first order rate constant)
[x]R^2 is the coefficient of determination
[w]p-values <0.05 indicate a significant regression fit

Overall, the highest net die-off rates occurred at 23°C in the Bt horizons of both soils with half-lives ranging from as little as 5 and 6.4 days. In fact, all the net die-off rate constants at 23°C ranged from -0.138 to -0.058, as seen in table 2.2 and table 2.3.

2.5.3 Temperature Treatment Effects on Net Die-off Rate Constants Within Soil Horizon Samples

Figures 2.5 and 2.6 show that the relative number of active *E. coli* bacteria found within each of the soil sample horizons decreases through time at all the three different temperature treatments: 23°C, 5°C, and -18°C. Similar plots for *E. faecalis* are presented in Figures 2.7 and 2.8.

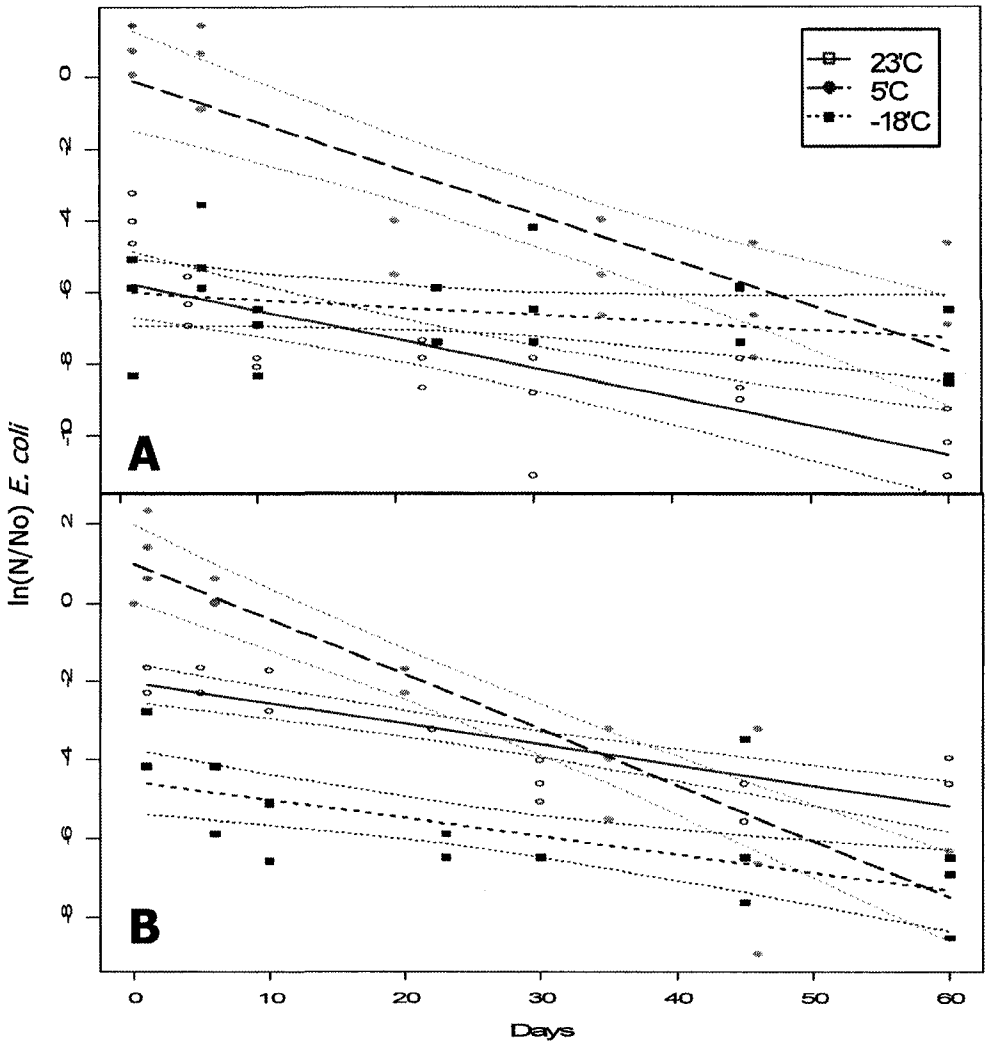

Figure 2.5: Effect of temperature on the net die-off rates (with 95% confidence intervals) for *E. coli* in the Ah (Figure 2.5A) and Bt (Figure 2.5B) horizons of the soil from Ellerslie (Black Chernozem). Lighter lines show the 95% confidence intervals.

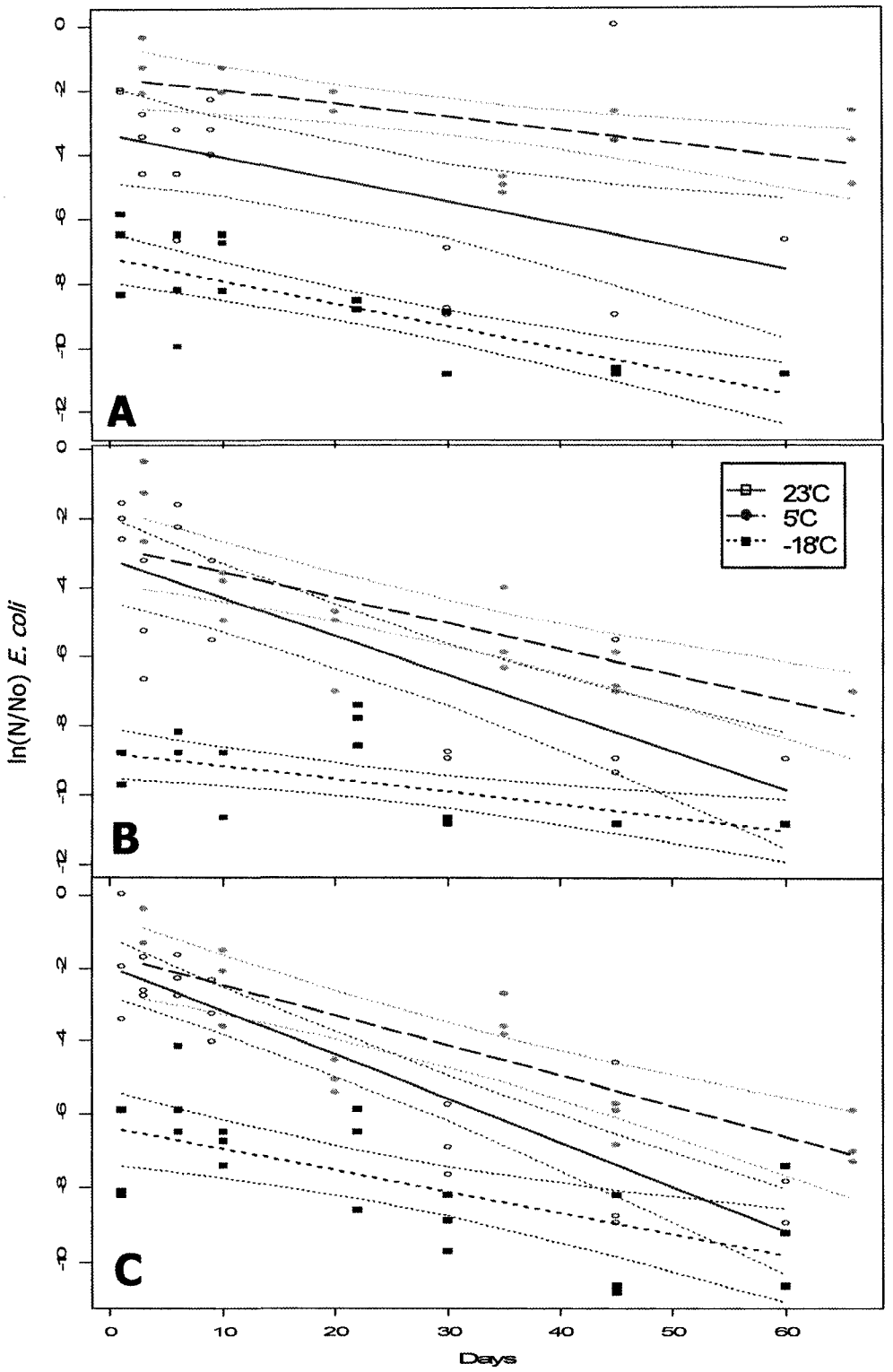

Figure 2.6: Effect of temperature on the net die-off rates (with 95% confidence intervals) for *E. coli* in the Ah (Figure 2.6A), Ah (Figure 2.6B) and Bt (Figure 2.6C) horizons of the soil from Rocky Mountain House (Gray Luvisol). Lighter lines show the 95% confidence intervals.

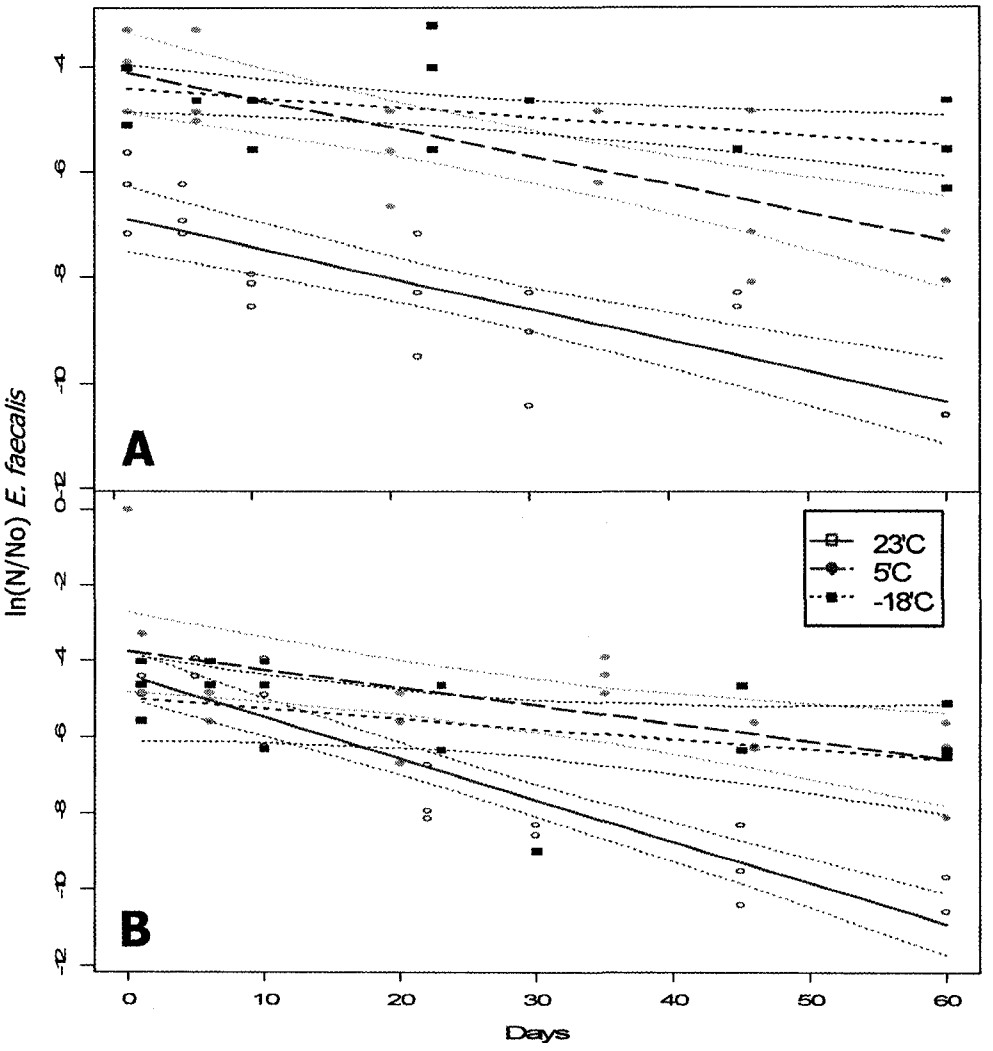

Figure 2.7: Effect of temperature on the net die-off rates (with 95% confidence intervals) for *E. faecalis* in the Ah (Figure 2.7A) and Bt (Figure 2.7B) horizons of the soil from Ellerslie (Black Chernozem). Lighter lines show the 95% confidence intervals.

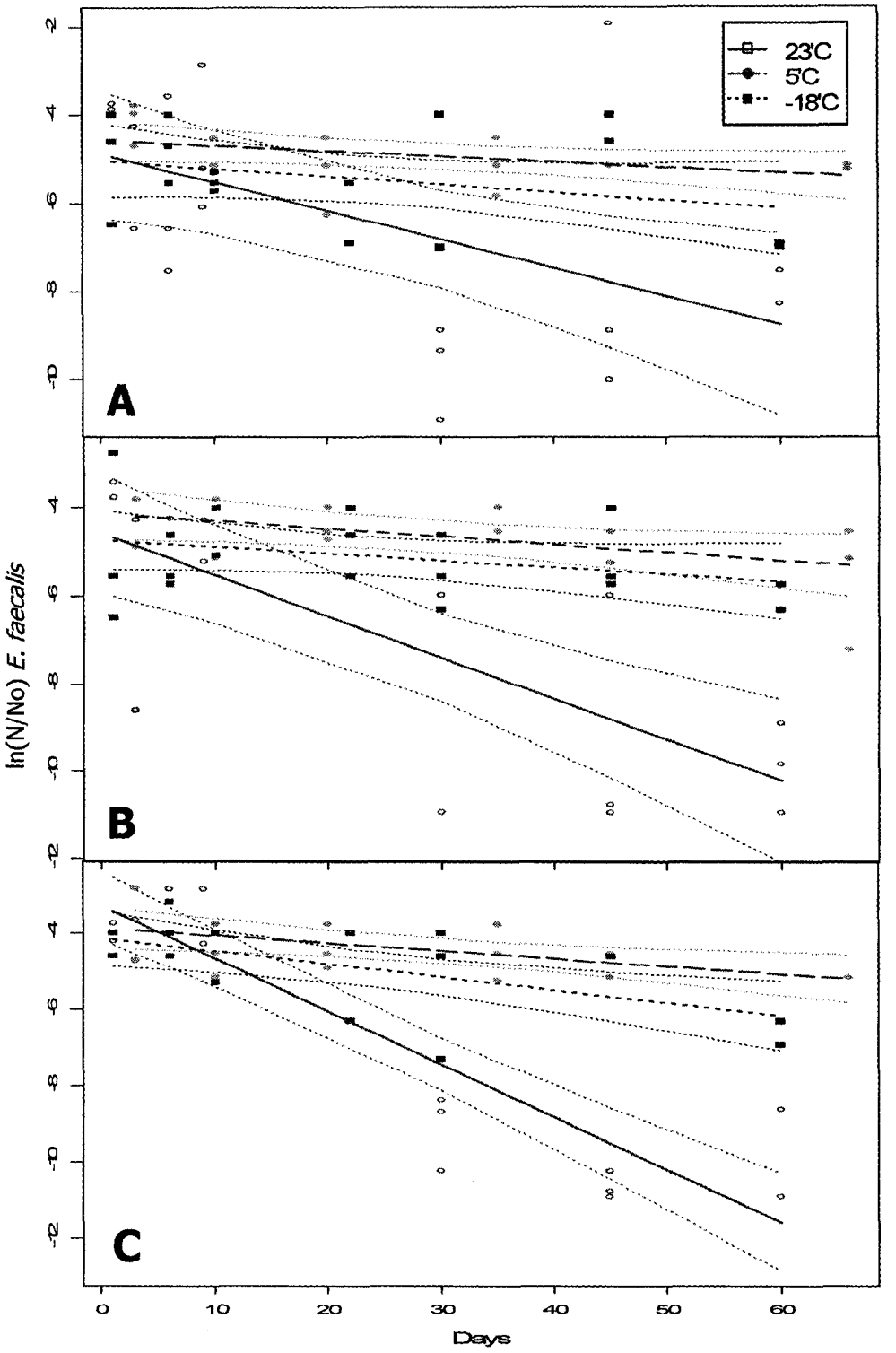

Figure 2.8: Effect of temperature on the net die-off rates (with 95% confidence intervals) for *E. faecalis* in the Ah (Figure 2.8A), Ah (Figure 2.8B) and Bt (Figure 2.8C) horizons of the soil from Rocky Mountain House (Gray Luvisol).

The ANCOVA results for the influence of different treatment temperatures on the net die-off rate constants for each of the horizon samples used for *E. coli* and *E. faecalis* are shown in Tables 2.4 and 2.5, respectively. This particular test was used to identify significant differences between intercept points and slopes thereby statistically proving that the temperature treatments influenced rate constants differently. Both the F-values and P-values are shown.

Table 2.4: ANCOVA for the temperature treatment significance of the net die-off rate constants for *E. Coli.*

Soil	Horizon	Probability of different slopes		Probability of different intercepts	
		F value	Pr(>F)	F value	Pr(>F)
Ellerslie	Ah	11.3	<0.001	47.5	<0.001
Ellerslie	Bt	22.3	<0.001	48.1	<0.001
RMH	Ah	0.8	0.45	77.4	<0.001
RMH	Ae	6.4	0.003	72.5	<0.001
RMH	Bt	5.5	0.007	54.4	<0.001

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol;
[w]p-values <0.05 indicate a significant regression fit

Table 2.5: ANCOVA for the temperature treatment significance of the net die-off rate constants for *E. faecalis.*

Soil	Horizon	Probability of different slopes		Probability of different intercepts	
		F value	Pr(>F)	F value	Pr(>F)
Ellerslie	Ah	6.0	0.005	102.9	< 0.001
Ellerslie	Bt	9.3	<0.001	14.7	<0.001
RMH	Ah	3.3	0.04	5.5	0.007
RMH	Ae	9.9	<0.001	15.7	<0.001
RMH	Bt	33.5	<0.001	24.7	<0.001

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol;
[w]p-values <0.05 indicate a significant regression fit

As seen on the table, the net die-off rate constants of both indicator organisms varied significantly with each of the different horizons in the soil profile.

In order to better understand how each of these curves differ, it is possible to analyze the 95% confidence limits of each regression line and compare with other treatments. For example, Figure 2.1A shows the net die-off rates for *E. coli* on an Ah horizon from Ellerslie. The overlapping of areas indicate that it may be difficult to separate treatment effects for temperatures -18°C and 23°C until after day 20. The same is true for all treatments after day 50. This does not mean that the rates are the same or that there are no differences depending on the treatment, but simply that all the microorganisms remaining have the same chance of survival at both temperatures after 50 days. A similar pattern after day 50 is found on Ellerslie Bt horizon. Also on the Bt horizon, the room temperature treatment does not cause net die-off as quickly as the other two before day 60.

Analyzing *E. coli* on the horizons from Rocky Mountain House, there are very similar net die-off rate constants per treatment: -18°C caused inactivation first followed by 23°C, finally, the 5°C treatment had the longest microorganism survival time. After day 50, the indicator bacteria concentration is very similar on both the Ae and Bt horizon. The same horizons have similar microorganism concentrations for the 23°C and 5°C treatments before day 10.

E. faecalis on both horizon samples from Ellerslie acted very differently than their *E. coli* counterparts. Although the 5°C and -18°C curves for Ah and the all three curves for Bt start at a similar intercept, there is no curve overlapping at the end of 60 days suggesting very different treatment effects. In both horizons, the treatment at 23°C demonstrated the highest net-die off of these microorganisms. Also in both, the 5°C and -18°C treatments were very similar suggesting a temperature threshold after which the effects would be very similar.

The Rocky Mountain House horizons Ah, Ae, and Bt at different temperatures influenced the *E. faecalis* concentration in a very similar way. In all three cases, all three treatments started at a similar concentration, but the 23°C regression line quickly fanned out indicating that this treatment induced the highest net-die off rate constants. Also in all cases, the -18°C and 5°C regression curves were very similar which indicated a similar case to the Ellerslie horizons in which the effects after a certain temperature are very similar and no longer had such a strong effect on the net die-off rates.

2.6 Discussion and Conclusions

The die-off of pathogenic microorganisms is an important part of onsite wastewater treatment in soil to aid the prevention of infectious disease and contamination of waterways. This study was set up in order to better understand the die-off rates of indicator microorganisms, *E. coli* and *E. faecalis,* within two soil profiles from central Alberta under three different temperature regimes.

2.6.1 Impact of Temperature on Net Die-off

The two organisms measured, *E. coli* and *E. faecalis*, are both important indicator organisms for wastewater. They both represent a large proportion of the bacteria in wastewater and are of longer duration than pathogenic bacteria; therefore, their presence is an indication that pathogenic bacteria could still be a potential threat.

With this study, we were able to confirm that net die-off rate constants are significantly affected by temperature and type of soil and horizon in which the organisms are introduced. Competition and predation from other microorganisms as well as other factors in the soil are important in controlling the growth of both indicator organisms; without these constraints, the indicator organisms would most likely reproduce appreciably. We also found that although both organisms are regularly used as indicators, their behaviour is not necessarily similar in all situations.

Temperature treatment significance was confirmed by using an ANCOVA analysis of the die-off plot regression. Chick's law does explain the die-off rates of both microorganisms at 23°C and 5°C. However, at -18°C, it was difficult to discern their trends. The uneven data at -18°C may have been caused by uneven freezing and thawing rates of the soil water.

Van Donsel et al. (1967) and Andrews et al. (2003) found that survival tended to decline as temperature increased. This was true in 2 out of 5 of the soil horizon samples for *E. coli* and 3 out of 5 of the soil horizon samples for *E. faecalis*. However, *E. coli* survived less time in both horizons of the Eluviated Black Chernozem at 5°C. Since this is also the soil in which this organism had the highest growth rates in sterilized soil, influences of competition by other organisms for nutrients and predation could be contributing factors to this difference.

In contrast, the net die-off rate of *E. coli* in the Ah horizon of the Dark Gray Luvisol, and *E. faecalis* in the Ah and Bt were lower at 5°C than the other two temperatures. As this soil had a lower soil organic carbon content, it could be that the amount of water soluble carbon is low. If this were true, the smaller, carbon-starved organism could enter a generalized stress resistant form in which they would be less prone to digestion from protozoa. This stressed form would be more prevalent at lower temperatures. This would not occur at -18°C because frozen water does not allow the organisms to move between pores.

Predation by protozoa and other fauna is considered to be a controlling factor in the survival of introduced bacteria. The experiments with sterilized soil samples demonstrated that without predation, growth of the indicator organisms would be quite likely. It also demonstrated that the higher nutrient availability in the Chernozemic soil allowed for significant exponential growth of both organisms. By contrast, even without predation, there was little growth of either organism in the Dark Gray Luvisol probably because of its low nutrient availability. In general, *E. coli* grew at a much quicker rate than *E. faecalis.*

Overall, both organisms reacted quite differently to the different temperature treatments. *E. faecalis* tended to live longer in soil than *E. coli* making it a longer lasting indicator, but given the right conditions, the latter organism is more prone to regrowth in soil. Even so, given the limited nutrient conditions in soil and the existence of predators, in this case, *E. faecalis* could be considered the more conservative indicator organism. One of the most important findings of this study was the confirmation of the extended survival of these types of bacteria in colder conditions.

2.6.2 Experimental Die-Off Rates and OWTS

These data were obtained with the intention of providing input into the improvement of design and regulation of OWTS. However, two important considerations must be accounted for before this can occur: The seasonal variation of dispersal field soil temperatures and the amount of microorganisms which will be dosed over time into these soils must be known.

Canadian soils in winter are affected by freezing soil conditions. This study has shown that the both organisms survived extended amounts of time under freezing conditions. Under a one-time application of organisms to the soil, between 13 and 56 days would have to pass for the organism concentration to be reduced by half. However, this will not be the case for OWTS.

An at-grade wastewater treatment system is being studied in an area close to the town of Leduc by Juma and associates (personal communication). By using temperature sensors in the soil, they have identified that dispersal of wastewater into the soil also introduces a heat influx which prevents the soil from freezing. This warmth is insulated by PVC pipe, a chamber, and mulch layer which protect the system from outside disturbances. This microenvironment maintains enough heat to prevent the soil under the system from dipping under 2°C under normal winter conditions. It also protects the system from summer heat.

Therefore, the data from 5°C may be the most important when describing the net die-off of pathogens in soil under a dispersal system. More research should be done to study die-off of other pathogens or groups of pathogens found in effluent to determine their survival rates.

The second major consideration is that the dispersal field of an OWTS is used on a continuous basis. On a weekly basis, multiple doses of effluent can be added to the soil. As an example, if 1×10^3 *E. coli* per gram of soil is introduced into a Bt horizon of a Dark Gray Luvisol at 5°C, we expect the population to be reduced by half in 8.3 days. If instead of a one-time dose, this same dose were to be continued over a period of days, there would be an accumulation of organisms in soil. After a certain amount of time, this accumulation may stabilize after a certain point. This concept is illustrated in Figure 2.9.

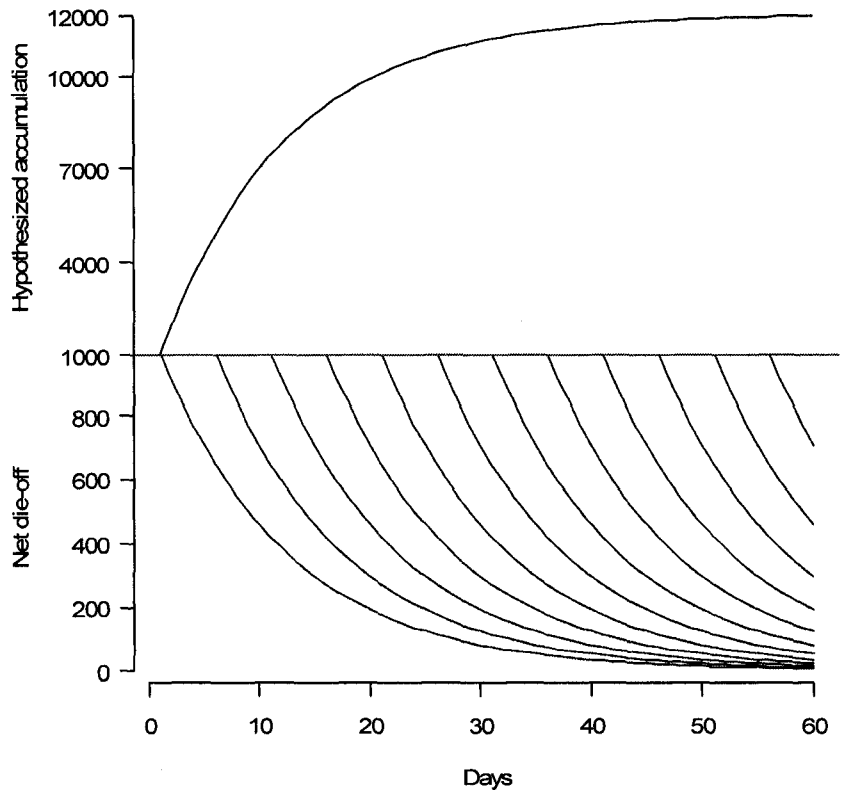

Figure 2.9: Hypothesized accumulation of microorganisms after 60 days when doses of 10^3 CFU/g are added each day. The graph is based on the net die-off rate of *E. coli* in the Bt horizon of a Dark Gray Luvisol. The lines under 1000, shown for every 5 days, represent the net die-off of organisms after daily doses of 10^3 CFU per gram of soil. The upper line represents the accumulation of organisms over time. Note change in scale under 1000 CFU/g.

If soil is to be tested for regulatory purposes, the concentration and dosing rate of the microorganisms must be considered. If we only consider temperature effects, the soil under the chambers will most likely have an accumulation of microorganisms over a period of time. Further studies are needed to study the die-off rates after multiple doses and with higher concentrations of microorganisms. However, this study can potentially give guidelines to improved designs of soil-based wastewater dispersal systems in colder regions.

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Chapter 3

Adsorption of Indicator Organisms in Soil Samples from Different Horizons of an Eluviated Black Chernozem and a Dark Gray Luvisol

3.1 Introduction

Often called sticking efficiency, sorption, attachment, or retention capacity of the soil, adsorption is one of the important retardation processes referring to the attachment of microorganisms to soil surfaces. This process involves both the physical and chemical adsorptive properties of the soil environment and the adhesion properties of each microorganism involved. If it is successful, adsorption can control the availability of pathogenic bacteria in the wastewater flow and thereby hinder their contamination potential.

This interaction of bacterial transport and adsorption is not easy to understand. The adsorption of bacteria in soil is a complex phenomena controlled by soil properties, the composition of the microbial population, and in the special case of onsite wastewater treatment systems, the method of application.

Soil adsorption rates are controlled by factors such as its cation exchange capacity, organic matter and clay content, mineral composition, reaction time, adsorption site competition from other microorganisms or molecules such as calcium, magnesium, and sodium, and the temperature at which the reaction occurs (Stevik et al. 1999).

Microorganisms adsorb differently depending on factors such as their individual cell properties and charge, wastewater flow rates in soil, wastewater ionic strength, and the temperature at which the reaction would occur. If bacteria are much smaller than pore spaces, sorption is the dominant process affecting bacteria and surface interactions (Stevik et al. 2004).

Factors that affect adsorption rates that are directly influenced by the wastewater systems are the wastewater ionic strength, pH, and water flow rates.

3.1.1 Adsorption

In very general terms, adsorption is a phenomenon in which there is a change in concentration of a given substance at the interface with respect to its concentration in the bulk solution (Toth and Boowko 2002; Yaron et al. 1996). Hermannson (1999) described bacterial adsorption as the process of transfer of a cell from an unbound state in the bulk phase to a more or less firm attached state at an interface.

Adsorption may occur by hydrogen bonding, hydrophobic bonding (especially clay-organic complexes), the balance of electrical charges on the surface of the adsorbate and the adsorbent, and by acid-base reactions (Yaron 1999; Bengston and Ekere 2001)

One of the most important adsorption theories is the DLVO theory named after Derjaguin, Landau, Verwey and Overbeek who developed the theory in the 1940s. This classical theory explains that the stability of the 'colloidal system' is based on the van der Waals attractive force (V_A) and the electrical double layer repulsive force (V_R). The latter is the energy barrier which repels two particles preventing them from meeting and adhering. If this barrier is overcome by, for example, a high speed collision between particles, an attractive force will pull them into contact strongly and irreversibly.

Hermannson (1999) extended this theory into the microbiological world. With the assumption that a cell is interacting with a flat surface, the adsorption potential is defined by the attractive forces, sum of the van der Waals forces, and the repulsive forces, the sum of the electrical double layer overlap between cell and the substratum. Although treating bacteria as colloids does explain some behavior, adhesion can only be partially explained in this manner. Another extended DLVO theory was created which accounts for hydrophobic and hydrophilic interactions but still lacks a complete understanding of the bacteria interface. It also still does not take into account many soil and biological factors.

3.1.2 Bacterial Adsorption: Process

Different from chemical adsorption, which is the binding of a contaminant to the surface of a solid medium, the adsorption of microorganisms is not based only on the electrostatic properties and cationic properties the adsorbent and adsorbate. Adsorption of bacteria onto the soil surface provides a safer, nutrient rich environment and protection from predators. Bacteria, especially pathogenic bacteria, generally prefer a more sedentary, surface bound lifestyle (Dunne 2002). An example of bacterial protection can be seen in work presented by Gantzer et al. (2001) where they found that temperature had less of an effect on the survival of microorganisms if they were adsorbed to soil.

The adsorption of bacteria to surfaces can be reversible or irreversible. The degree of adsorption depends on the repulsive or attractive forces at the adsorption site and whether cells are attached by cell surface structures such as polysaccharide excretions. Other bacterial properties which determine adsorption rates are cell surface hydrophobicity, bacterium-substratum charge interactions, surface roughness, surface free energy, and bacterial motility by flagella and pili (van Loosdrecht et al. 1987b; Camper et al. 1993; Huysman and Verstraete 1993).

The attachment of bacteria to a soil surface usually occurs in three different steps: contact, primary connection, and irreversible latching onto the substratum. Contact, the bringing together of the microorganism and the soil particles to a distance less than 1nm is achieved by the flow of wastewater over the soil particles and an individual microorganism's movement caused by chemotaxis or motility by external appendages. This first contact is very important. If the wastewater flow is too fast, it will not allow sufficient time between the microorganism and the soil (Dunne 2002).

Once the organisms are close enough, primary connection is controlled by non-specific hydrophobic interactions such as the van Der Waals force explained in the DVLO theory and other electrostatic and hydrophobic interactions. The first step to adhesion is the reversibly adsorbed step. If they are to stay in this state, these cells must be kept away from the surface by repulsive electrostatic forces. They can easily be washed away with changes in flow, in the wastewater composition, or cellular motility.

The second step is the irreversible adsorption when cells are in direct contact with the surface (Marshall 1971). This type of adsorption is much more difficult to overcome (Mills 1994). This can also occur when cells excrete exopolysaccharides. These are known to form with biofilms, but in many OWTS systems, due to the low carbon content of the effluent, these will not be a great factor (Dunne 2002).

3.1.3 Bacterial adsorption: Bacterial characteristics that influence adsorption

Of all the various properties that influence bacterial adhesion to soil particles, one of the most important one is the character of the individual microorganism. Examples of this are cell size, motility, hydrophobicity, surface charge, cell membrane proteins, and the excretion of extracellular polysaccharides.

As mentioned earlier, sorption is the dominant process affecting smaller sized cells since they are not as subjected to the forces of filtration though the pores (Stevik et al. 2004). The motility provided by external appendages such as flagella can both help the cell attach to surfaces by allowing for closer, deliberate contact with surfaces, overcoming electrostatic repulsive barriers (Stevik et al. 2002; van Loosdrecht et al. 1987a) and can also be involved in detachment when the environment is no longer suitable (Dunne 2002).

Although hydrophobicity and surface charge are often studied to determine overall adsorptive potential, there is disagreement on the importance of cell properties in the bigger picture. Gannon et al. (1991) found that the transport of bacteria was not related to its surface charge. Li and McLandsborough (1999) also found no correlation between *E. coli* surface charge, hydrophobicity, and adhesion to beef muscle.

The reason the cellular characteristics are often overlooked in the onsite system is that they are different for every type of microorganism strain and species. *E. coli* for example has electrophorectic mobility values ranging from $-0.42 \times 10^{-8} mV^{-1}s^{-1}$ to $-1.84 \times 10^{-8} mV^{-1}s^{-1}$ (van Loosdrecht et al. 1987b) depending on species and growth conditions. There are hundreds of different microorganisms in sewage effluent; therefore, it is more interesting to focus on indicator organism

characteristics or the characteristics of a mass of bacteria rather than their individual characteristics.

3.1.4 Soil Adsorption: Organic matter and Clay Content, Cation Exchange Capacity

Soil systems are very complex. Since they are very heterogeneous in nature, different soils and even different soil horizons will adsorb differently. This process will also greatly depend on whatever is being adsorbed into the soil. Soil can act as an excellent filter adsorbing a wide variety of materials ranging from inorganic essential nutrients and cations such as calcium, magnesium and sodium, complex organic molecules such as pesticides to organic constituents such as pathogenic bacteria.

Soil adsorption is mediated by both positive and negative electrical charges and non-ionized functional groups on the mineral and organic constituents of soil. These colloidal particles of large surface area are measured in terms of clay and humus (Dawes and Goonetilleke 2004).

Although clay is negatively charged, some parts of the humus may be both positively and negatively charged, especially by charged groups exposed at the edges of the crystal lattice of clay particles (Huysman and Verstraete 1993). The negative charges are usually neutralized by cations absorbed to these particles and can be replaced and exchanged by other particles such as other cations or microorganisms. Another reason organic matter is very important is that its carbon content may be exploited by starved bacteria thereby affecting the physiological status of the cells (van Loosdrecht et al. 1987a).

The clay content is especially important since its high surface area and charges make it the dominant element in determining a soil's cation exchange capacity (Hagedorn et al 1978; Bengtsson and Ekere 2001). A measure of the soil's ability to adsorb and exchange cations is the Cation Exchange Capacity or CEC. A higher CEC means that the soil will have a higher rate of adsorption of positive colloids. CEC is expressed as the sum of exchangeable cations per mass of soil (mEq/100g or cmol(+)/Kg soil).

3.1.5 Other factors to Consider: Sodium

Sodium is a molecule of particular interest in this adsorption process. It is usually expressed as a ratio of sodium ions to calcium and magnesium molecules (SAR). Higher SAR valves can be very detrimental to a healthy soil system because the larger hydrated radius of sodium ions tend to exclude other ions from the same sites, disperses soil aggregates, causing clogging of soil infiltration systems with clay particles (Mills et al 1994).

Electrical conductivity is the measure of the concentration of ions in the soil solution. A soil with >4 dS/m is considered to be saline. As salinity increases, the ionic strength of the soil solution increases, bacterial retention increases and the transport of bacteria decreases. A specific example is the increase of attachment of Gram-negative bacteria to sand, quartz grains, and silica beads when there is an increase in solution salinity. It is suspected that this increase could be due to a decrease in the electrical double layer allowing for an increase in primary contact of the bacteria with the adhering surface (Camper et al. 1993; Marshall et al. 1971; Guber et al. 2005).

3.1.6 Other Factors to Consider: Temperature

Temperature is known to greatly affect adsorption rates. For inorganic colloids, adsorption is considered as an exothermic reaction therefore it tends to increase as the temperature decreases. This increased adsorption could also be due to an increase in the solubility of the adsorbate. This effect has been shown with pesticides such as atrazine and ametryne in soil (Yaron et al. 1996).

In the field of bacterial adsorption studies, there is a lack of information on the effect of temperature on bacterial adsorption in soil. Jiang et al. (2007) conducted an experiment using goethite, kaolinite and montmorillonite and found an increase in the adsorption of *P. putida* on these minerals at 15°C, 25°C, and 35°C, respectively. The temperatures with the least adsorption were 5°C and 45°C. It is thought to be more related to the physiological state of *P. putida* since its activity is optimum in the 15°C to 35°C temperature range. They stated that higher metabolism facilitated adsorption.

3.2 Objectives

The objective of this study was to quantify the adsorption of two indicator organisms, *Escherichia coli* (ATCC 25922) and *Enterococcus faecalis* (ATCC 29212), to soil samples obtained from major horizons of an Eluviated Black Chernozem and a Dark Gray Luvisol.

3.3 Materials and Methods

3.3.1 Site description and Soils

In order to measure net die-off (Chapter 2) and attenuation (Chapter 3) of two introduced fecal coliforms, soil samples were obtained from the Ah and Bt horizons of an Eluviated Black Chernozem from the Ellerslie Research station, located 10 km south of the University of Alberta Campus (53° 25'N, 113° 33'W), and from the Ah, Ae, and Bt horizons of a Dark Gray Luvisol, located 10 km south east of the town of Rocky Mountain House (52° 22'31"N, 114°55'18"W).

3.3.2 Preparation of Soil Samples

Three replicates of previously sieved and dried soil samples from each horizon were placed into 15 mL centrifuge tubes. In order to obtain adsorption curves, the mass of soil in the centrifuge tubes was increased incrementally and a fixed amount of indicator organisms were added to the soil samples. The quantity of soil mass in the centrifuge tubes was 0.2, 0.6, 1.1, 1.6, and 2 g. The adsorption experiments were conducted at 23°C or 5°C. Soil samples were incubated at their treatment temperature for 24 h prior to inoculation.

3.3.3. Initial Inoculation and Incubation

The indicator organisms *E. coli* and *E. faecalis* were incubated overnight in commercially available media (dehydrated media manufactured by DIFCO laboratories except were noted), Tryptic Soy Broth (TSB), and brain/heart infusion broth (BHIB), respectively. These cells were then suspended, washed twice in phosphate buffered solution (PBS), pH 6.8, and resuspended together in the same suspension to a density of approximately 10^4 to 10^5 cells per mL of each, calculated

by using direct count with a Petroff-Hausser chamber. These counts were verified by the using the Most Probable Number method (MPN).

Five mL of a combined suspension of *E. coli* and *E. faecalis* were added to the 15 mL centrifuge tubes and shaken with a vortex to ensure the contact of the suspension with all the soil particles. This mixture was left standing for one minute and then shaken gently at 140 rpm for 5 minutes. This contact time was chosen as it represents the minimum contact time between bacteria and soil particles during effluent flow though the soil horizons.

Separation of the soil particles was achieved using slow differential centrifugation, 48 x g for 3 minutes. The centrifugal speed was determined using $G=n^2\Phi/1800$ where n^2 is the bowl speed (RPM) and Φ is the bowl maximum inner diameter (m).

3.3.4 Microbiological Analysis

In order to obtain more precise results, the MPN method was replaced by the membrane filtration method to analyze the concentration of microorganisms in the supernatant following the APHA standard methods for water and wastewater (2005). This method only works when there is a low concentration of soil particles in suspension as they easily clog the filter and will give more a direct count of the viable colony forming units (CFU) of each microorganism.

After centrifugation was completed, 1 mL of the supernatant was diluted in 10 mL of phosphate buffered solution. Various dilutions were used with the objective of obtaining an ideal number of 20 to 200 CFU of the microorganisms when vacuum filtered though at 0.45 µm, gridded, sterile, Fisherbrand membrane, catalogue number: 09-719-555.

Two membranes per each dilution were obtained and placed in 9 mm Petri dishes containing mFC (Difco) agar for counting *E. coli* and mEnterococcus (Difco) agar for counting *E. faecalis.*

Colonies were counted with the help of a dissecting microscope. Positive *E. coli* colonies appeared blue on the mFC agar after being incubated inverted at 44.5 ± 0.2°C for 24 h. Positive *E. faecalis* colonies appeared light or dark red after incubation at 35 ± 0.5°C for 48 h.

The confirmation tests for *E. coli* consisted of inoculation of well isolated colonies into test tubes with EC medium. These were incubated at 44.5 ± 0.2°C for 24 h. Any abnormal colonies could later be tested using the GAD procedure described in the previous section.

E. faecalis was confirmed on Brain-Heart Infusion agar plate incubated at 35 ± 0.5°C for 48 h. Samples from well isolated colonies were subjected to a few drops of freshly prepared 3% hydrogen peroxide. The absence of bubbles proves a catalase negative test. Further confirmation of abnormal colonies was proven by inoculation onto Azide Dextrose Broth (incubated at 35 ± 0.5°C for 48 hours) and brain-heart infusion broth with 6.5% NaCl (incubated at 35 ± 0.5°C for 48 hours).

Previous tests showed that both media were selective for their specific microorganism and did not promote the growth of the other. This means, for example, that *E. faecalis* would not grow on mFC.

All results were tabulated and analyzed according to the APHA method with the slight modification that the results were calculated on a 1 mL basis instead of 100mL.

3.3.5 Statistical Analysis

The adsorption process can be simulated by a simple linear isotherm (Travis and Etnier, 1991;Huysman and Verstraete 1993; Ling et al 2002; Pachepsky et al. 2006):

$$S = K_d \ C \qquad \text{[Equation 3.1]}$$

where S is the concentration of bacteria adhering to soil (CFU/g soil), C is the concentration in the suspension (CFU/mL), and K_d is the partitioning coefficient. This equation assumes instantaneous equilibrium. Therefore, the k_d was found by calculating the slope of the regression line when the colony forming units of each microorganism in suspension (x-axis) versus the concentration in soil (y-axis) were plotted. The statistical analysis of these curves was done using the R statistical language (R Development Core Team, 2008). After this analysis, all data was tested to confirm a normal distribution using the Shapiro-Wilks test. If this test was negative, a permutation test of 999 permutations was used to calculate the correct probability value and regression coefficients.

An analysis of covariance (ANCOVA) (Ramette 2007) was used to relate the different adsorption isotherms of each horizon at the two different temperatures. By comparing the two regression slopes, this statistical test shows if the slopes and intercepts of the regressions are significantly different. When true, it is confirmed that temperature does influence the adsorption of these microorganisms and is therefore an important parameter to take into account when considering Alberta's cooler climate.

A principal components analysis (PCA) (Legendre and Legendre 1998) was used to create an ordination biplot to uncover certain tendencies. Since there were different tendencies at the two temperatures, two analyses were carried out at 23°C and 5°C. Type 2 scaling allows a correlation of variables based on their angles of separation.

3.4 Results

The primary properties of soil are presented in Table 3.1. This table is identical to the one presented as Table 2.1 on page 18 and is reproduced here as the data were used for the principal component analysis. The primary properties of soil The Ah horizon of the soil at Ellerslie had almost double the amount of organic C compared to that at Rocky Mountain. In contrast, the Bt soil horizons had similar amounts of clay but lower amounts of organic matter. The pH of upper horizons in the Gray Luvisol was more acidic than the Ah horizon of the Black Chernozem. Both soils were non-saline.

Table 3.1: Soil properties of selected horizons of an Eluviated Black Chernozem from Ellerslie and a Dark Gray Luvisol soil from Rocky Mountain House

[z]Location	Horizon	Sand	Silt	Clay	Organic C	Textural Class	pH	EC dS/m
		Percent (%)						
Ellerslie	Ah	24	58	18	6.83	Silt Loam	5.6	0.047
Ellerslie	Bt	37	33	30	0.67	Clay Loam	6.4	0.048
RMH	Ah	61	32	7	3.78	Sandy Loam	4.9	0.053
RMH	Ae	24	71	6	0.84	Silt Loam	5.1	0.032
RMH	Bt	16	50	35	1.75	Silt Clay Loam	6.9	0.187

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol

The cations and cation exchange capacity of the soil horizons used for this experiment are shown in Table 3.2. The dominant parent geological material of these soils was calcium carbonate; therefore, the Bt horizons are high in calcium. The second most common cation was magnesium. These soils have a high cation exchange capacity.

Table 3.2: Cations and Cation Exchange Capacity

		Na	K	Mg	Ca	sum of cations
[z]Location	Horizon			[y]meq/100g soil		
Ellerslie	Ah	0.09	0.76	2.28	23.33	26.46
Ellerslie	Bt	0.08	0.84	3.21	14.10	18.24
RMH	Ah	0.04	0.71	1.92	15.79	18.45
RMH	Ae	0.06	0.27	1.95	16.06	18.34
RMH	Bt	0.04	0.50	2.73	47.43	50.70

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol
[y]meq= milliequivalents

3.4.1 Adsorption Percentage

The adsorption efficiency in terms of percentage of microorganisms adsorbed per gram of soil are shown in Figures 3.1 and 3.2. The adsorption experiment was carried out by varying the amount of soil used, not the concentration of microorganisms in the inoculant suspension. As soil samples from the major horizons of two soils have different adsorbing surfaces, there were differences in the percentage of indicator organism that were adsorbed.

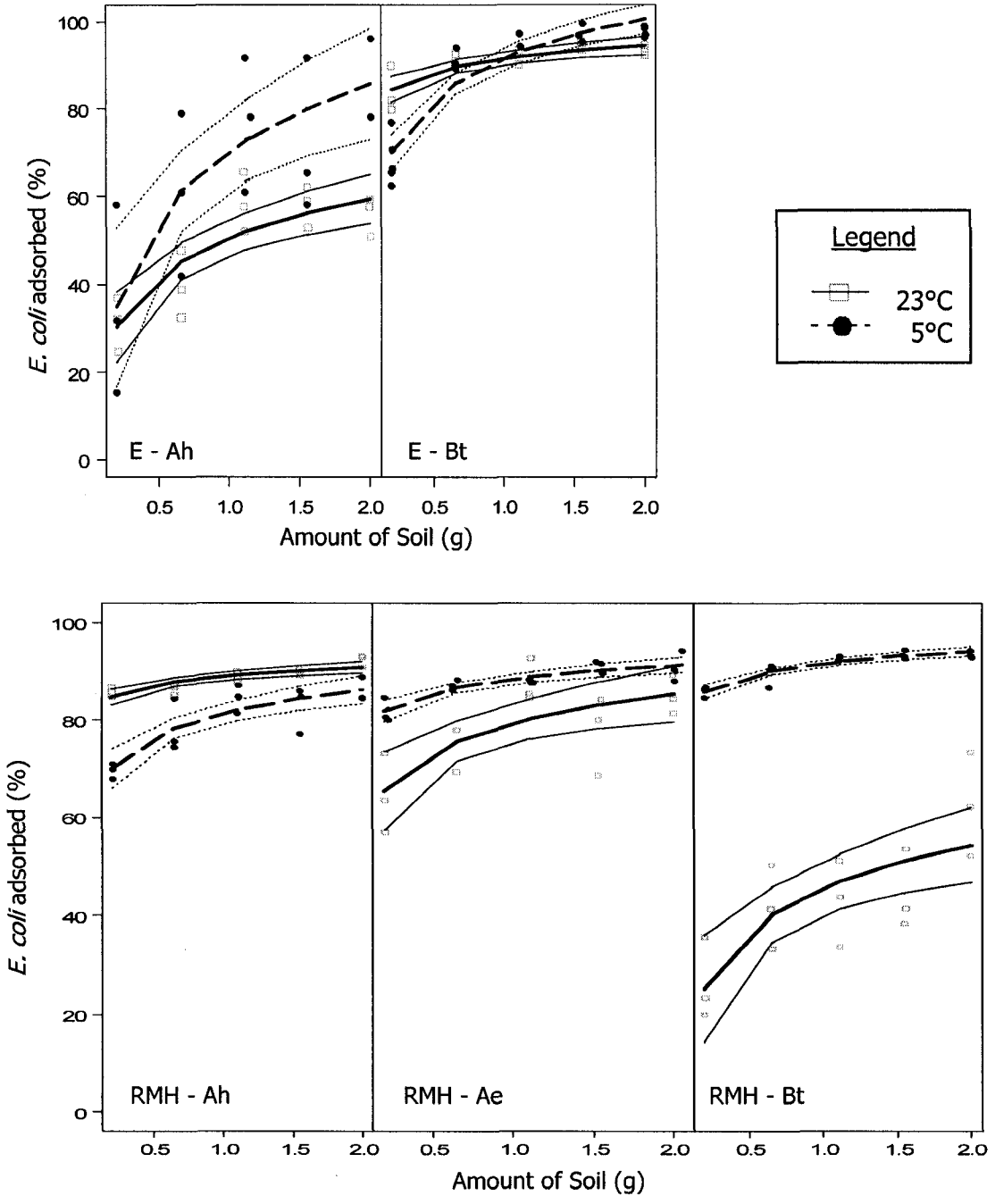

Figure 3.1: Adsorption of *E. coli* on soil horizons of: an Eluviated Black Chernozem from Ellerslie (E-Ah, E-Bt), and a Dark Gray Luvisol from Rocky Mountain House (R-Ah, R-Ae, R-Bt). Light lines indicate 95% confidence intervals.

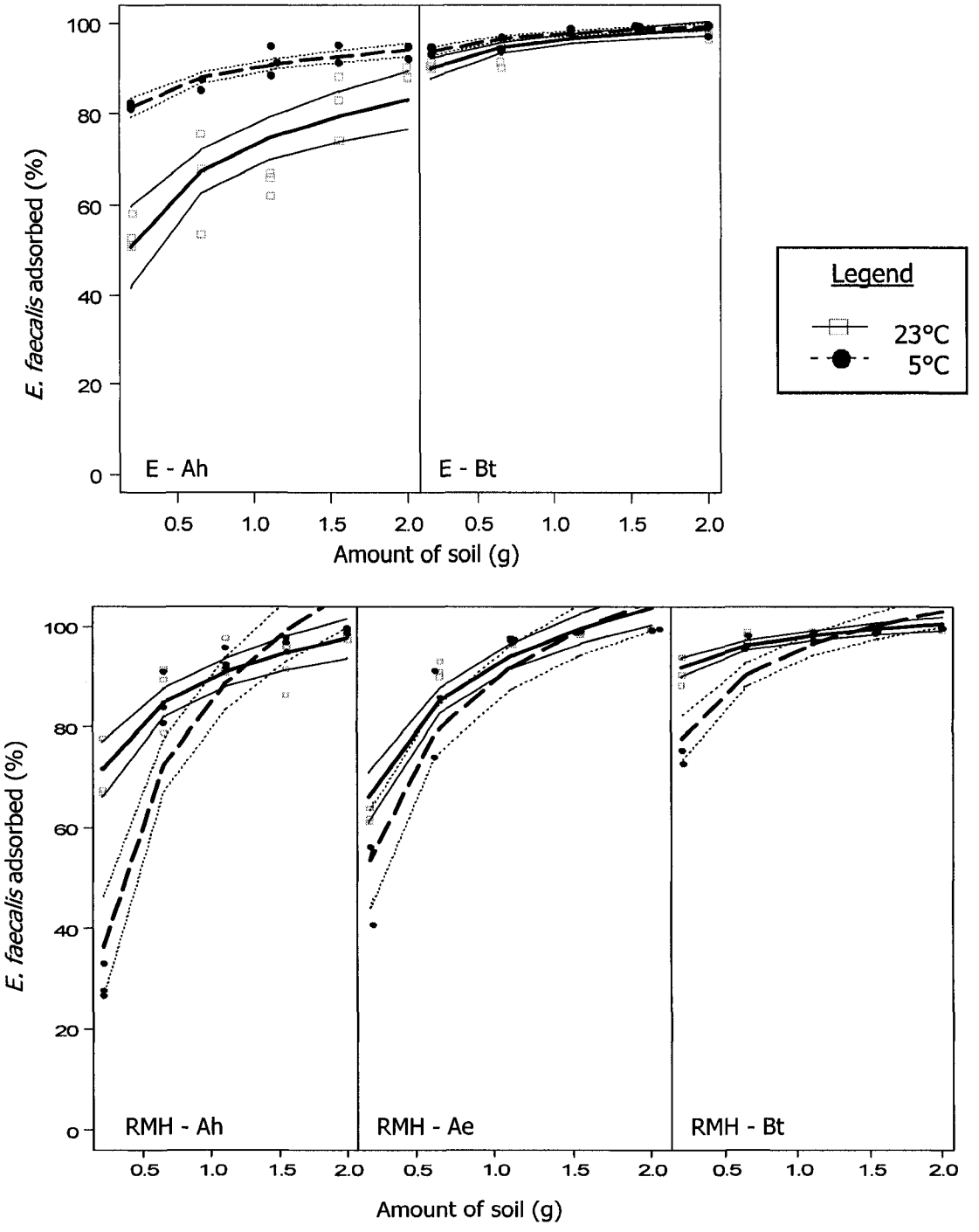

Figure 3.2: Adsorption of *E. faecalis* on soil horizons of an Eluviated Black Chernozem from Ellerslie (E-Ah, E-Bt), and a Dark Gray Luvisol from Rocky Mountain House (R-Ah, R-Ae, R-Bt). Lighter lines show the 95% confidence intervals.

3.4.2 Adsorption Partitioning Coefficients for *E. coli*

The data obtained from each adsorption experiment was used to construct the plot shown in Figure 3.3. The concentration of *E. coli* in suspension is represented on the x axis as colony forming units (CFU) per mL of supernatant, and the number of organisms adsorbed to the soil is represented on the y axis as CFU per gram of soil.

A linear regression was then calculated using the data from this graph. The slope of the line represents the K_d water partitioning coefficient. The results of these regression equations for the adsorption rates of *E. coli* on all 5 horizons sampled are shown in Table 3.3.

The results for the Shapiro-Wilks normality test results are also shown. When the Sharpiro-Wilks p-Value is significant (p-value<0.05) this means that the data do not follow a normal distribution. If this occurs, a data permutation is used to analyze the probability that the regression is significant. These data along with the slope (K_d), intercept, and the adjusted coefficient of determination (R^2) are also shown on Table 3.3

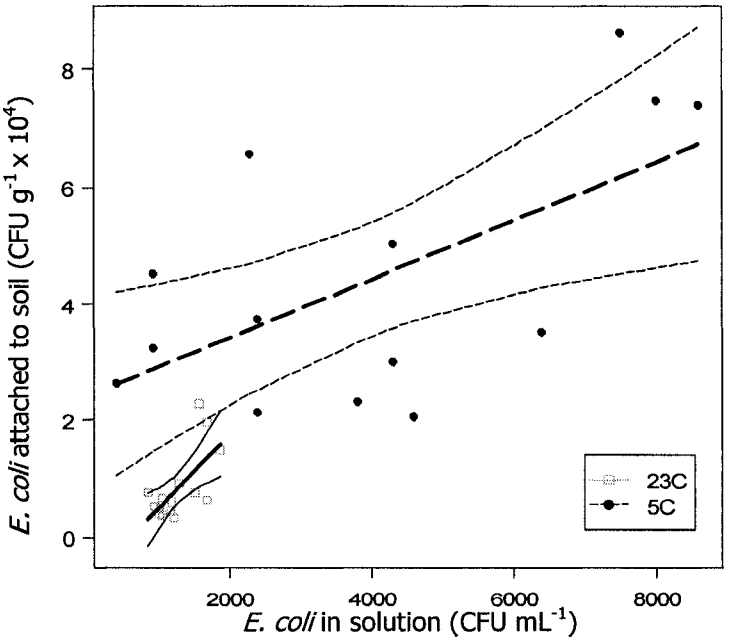

Figure 3.3: Adsorption partitioning coefficient for *E. coli* on the Ellerslie (Black Chernozem) Ah horizon at two temperatures. Dotted lines indicate 95% confidence intervals.

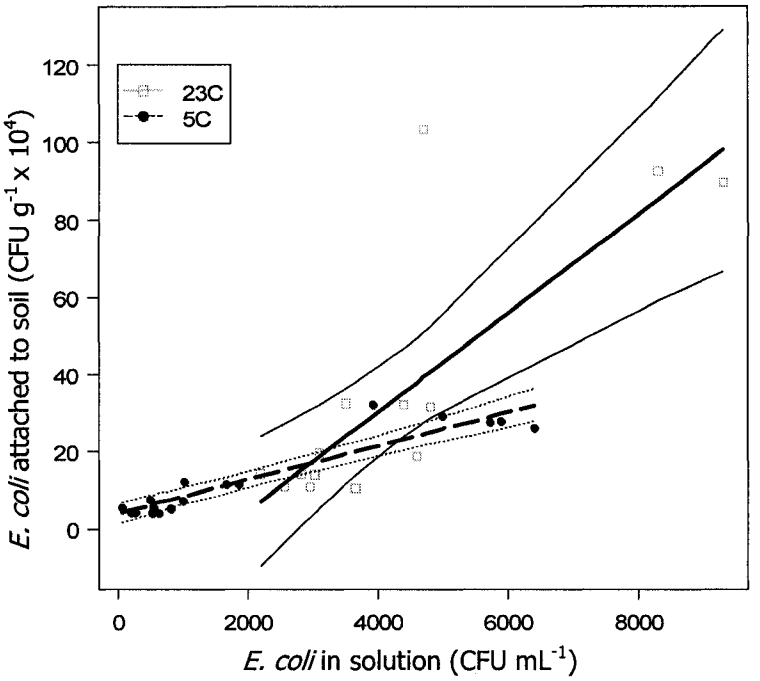

Figure 3.4: Adsorption partitioning coefficient for *E. coli* on the Ellerslie (Black Chernozem) Bt horizon at two temperatures. Lighter lines indicate 95% confidence intervals.

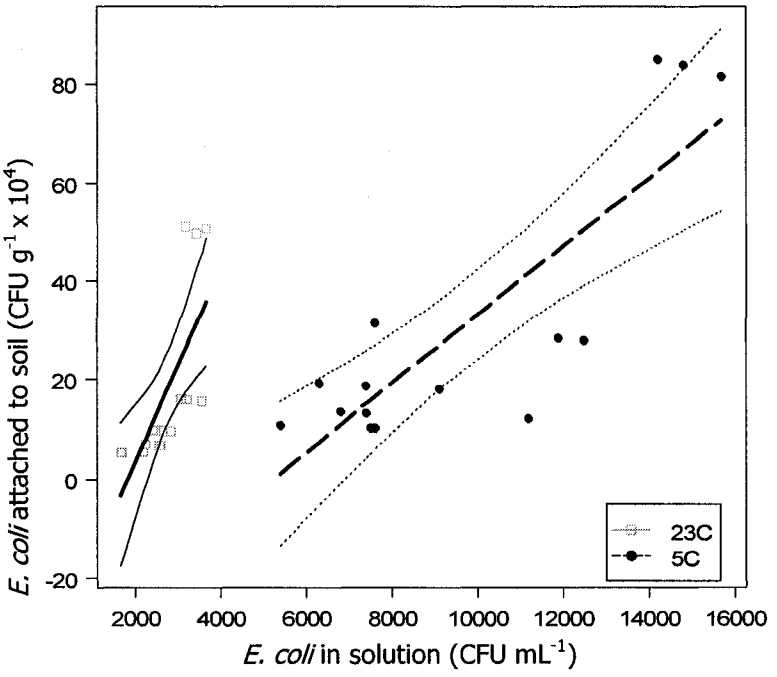

Figure 3.5: Adsorption partitioning coefficient for *E. coli* on the Rocky Mountain House (Gray Luvisol) Ah horizon at two temperatures. Lighter lines indicate 95% confidence intervals.

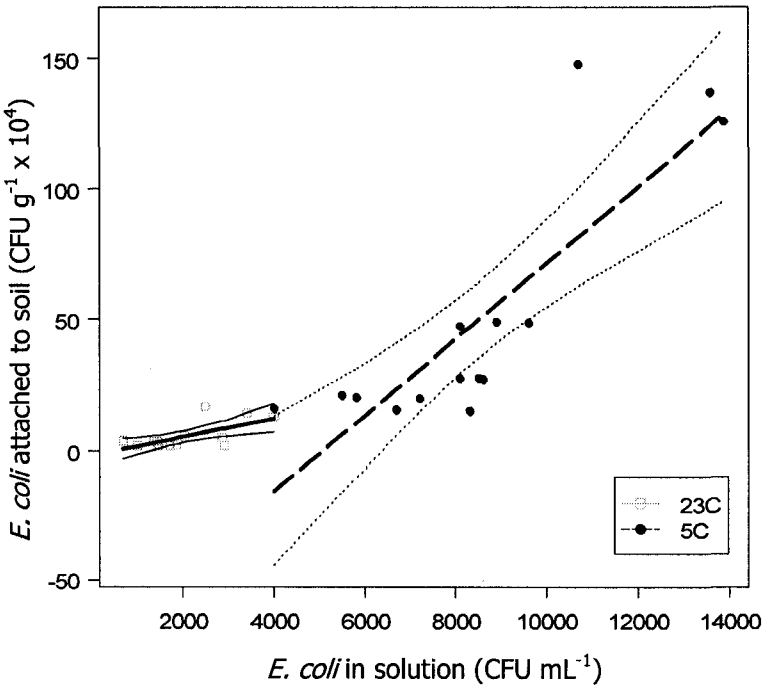

Figure 3.6: Adsorption partitioning coefficient for *E. coli* on the Rocky Mountain House Ae at two temperatures. Lighter lines indicate 95% confidence intervals.

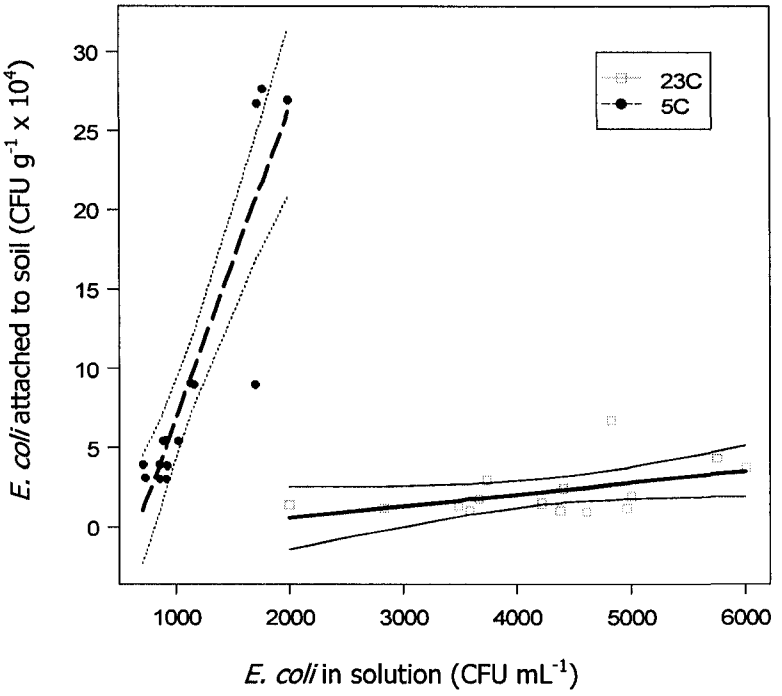

Figure 3.7: Adsorption partitioning coefficient for *E. coli* on the Rocky Mountain House Bt at two temperatures. Lighter lines indicate 95% confidence intervals.

Table 3.3: Adsorption rates of *Escherichia coli* on different soil samples from the major horizons of two soils

[z]Soil	Horizon	Temperature	[y]SW p-value	Intercept	Slope ([x]K_d)	[w]R^2	[v]p-value	Permuted [v]p-value
Ellerslie	Ah	23°C	0.37	-8217	12.8	0.45	0.006	
Ellerslie	Bt	23°C	<0.001	-208835	127.8	0.63		0.002
RMH	Ah	23°C	0.28	-353286	194.1	0.49	0.003	
RMH	Ae	23°C	0.16	-16681	35.7	0.44	<0.001	
RMH	Bt	23°C	0.02	-9623	7.5	0.24		0.027
Ellerslie	Ah	5°C	0.63	24123	5.0	0.40	0.011	
Ellerslie	Bt	5°C	0.008	40094	43.5	0.88		0.001
RMH	Ah	5°C	0.78	-364064	69.5	0.71	<0.001	
RMH	Ae	5°C	0.10	-736236	145.3	0.71	<0.001	
RMH	Bt	5°C	0.01	-129667	197.4	0.81		0.001

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol
[y]SW= Shapiro-Wilks test of normality; data considered not normal if p-value<0.05
[x]K_d=water partition coefficient
[w]R^2 is the coefficient of multiple determination
[v]p-values <0.05 indicate a significant regression fit

In every case, the data collected from the Bt horizon did not follow a normal distribution for which a data permutation was employed, as previously explained. All the regressions have a significant probability or permutated probability value.

The Ellerslie Bt and the Rocky Mountain House Ah horizons showed the highest slopes or K_d partitioning coefficients of their groups at 23°C. At 5°C the Bt horizons had higher k_d values for both soil types.

The adsorption rates in the Ah and Bt horizons of the Chernozemic soil were almost double at 23°C than at 5°C. The highest k_d of 127.8 was measured in the Bt horizon of this soil.

The two highest k_d values in the Luvisolic soil were in the Bt horizon at 5°C, 197.4, and in the Ah horizon at 23°C, 194.1. Contrary to the Chernozemic soil, the horizon of higher K_d values inverted as temperature decreased; at 23°C, the Ah horizon had higher K_d values, 194.1, for *E. coli* than at 5°C, 69.5, while at 5°C, the Bt horizon had a K_d value, 197.4, which was higher than the value of 69.5 for the Ah horizon.

In order to better understand the temperature treatment effect, an ANCOVA was conducted; results for *E. coli* are shown in Table 3.4. The regression models of adsorption of *E. coli* on all five horizons at the two temperatures, 5°C and 23°C, were significantly different, confirming that the temperature treatment was significant.

Table 3.4: ANCOVA temperature treatment significance on the adsorption of *E. coli*

		Probability of different slopes		Probability of parallel slopes		[X]Probability of different intercepts	
[Z]Soil	Horizon	F-value	[Y]Pr(>F)	F-value	[Y]Pr(>F)	F-value	[Y]Pr(>F)
Ellerslie	Ah	58.3	<0.001	0.5	0.480	15.5	0.001
Ellerslie	Bt	58.6	<0.001	11.8	0.002	-	-
RMH	Ah	33.9	<0.001	4.1	0.017	-	-
RMH	Ae	92.3	<0.001	3.7	0.064	10.4	0.003
RMH	Bt	14.5	<0.001	79.6	<0.001	-	-

[Z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol
[Y]p-values <0.05 indicate a significant regression fit
[X]If probability of parallel slopes is significant, it is not necessary to continue to test the probability of different intercepts are the regression models have proven to be significantly different.

3.4.3 Adsorption partitioning coefficients for *E. faecalis*

Table 3.5 shows that all the regressions for the K_d values of *E. faecalis* were significant and that all of these data but the Bt horizon from Rocky Mountain House were normally distributed.

The highest K_d values at both temperatures can be seen in the Bt horizons of both soils. Both soils tended towards higher K_d values with increasing depth of the horizon location.

The Chernozemic horizons had higher K_d values at 5°C, reaching at K_d of 289.5 in the Bt horizon. The lowest k_d value is from the Ah horizon at 23°C of only 21.0.

Contrary to the Chernozemic soil, the Luvisolic soil had higher K_d values at 23°C. Its highest K_d value, 202.8, is from the Bt horizon at 23°C. At 5°C, the highest K_d value is only 59.1 from the same horizon.

The regression models used to obtain K_d water partitioning coefficient for *E. faecalis* are shown in Figure 3.2 to 3.12.

Table 3.5: Adsorption partitioning coefficients of *E. faecalis* on different soil samples from the major horizons of two soils

[z]Soil	Horizon	Temperature	[y]SW p-value	Intercept	Slope ([x]K_d)	[w]R^2	[v]p-value	Permuted [v]p-value
Ellerslie	Ah	23°C	0.86	-2884	21.0	0.48	0.004	
Ellerslie	Bt	23°C	0.35	11124	161.3	0.62	<0.001	
RMH	Ah	23°C	0.10	8824	46.6	0.74	<0.001	
RMH	Ae	23°C	0.10	40016	33.0	0.98	<0.001	
RMH	Bt	23°C	0.002	20295	202.8	0.88		0.001
Ellerslie	Ah	5°C	0.34	-27817	121.9	0.77	<0.001	
Ellerslie	Bt	5°C	0.16	14664	289.5	0.66	<0.001	
RMH	Ah	5°C	0.09	123100	5.7	0.68	<0.001	
RMH	Ae	5°C	0.80	381800	15.6	0.78	<0.001	
RMH	Bt	5°C	0.37	35208	59.1	0.97	<0.001	

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol
[y]SW= Shapiro-Wilks test of normality; data considered not normal if p-value<0.05
[x]K_d=water partition coefficient
[w]R^2 is the coefficient of determination
[v]p-values <0.05 indicate a significant regression fit

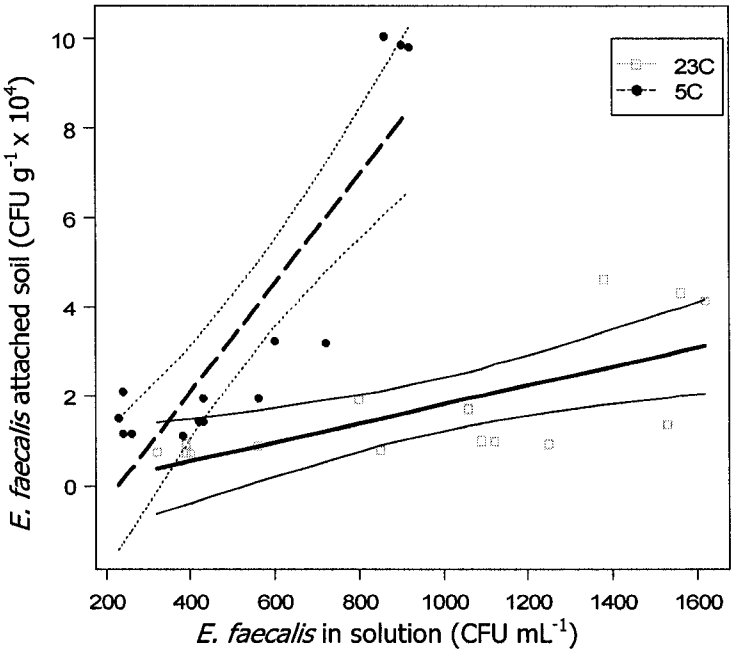

Figure 3.8: Adsorption regression of *E. faecalis* on the Ellerslie Ah horizon at two temperatures. Lighter lines indicate 95% confidence intervals.

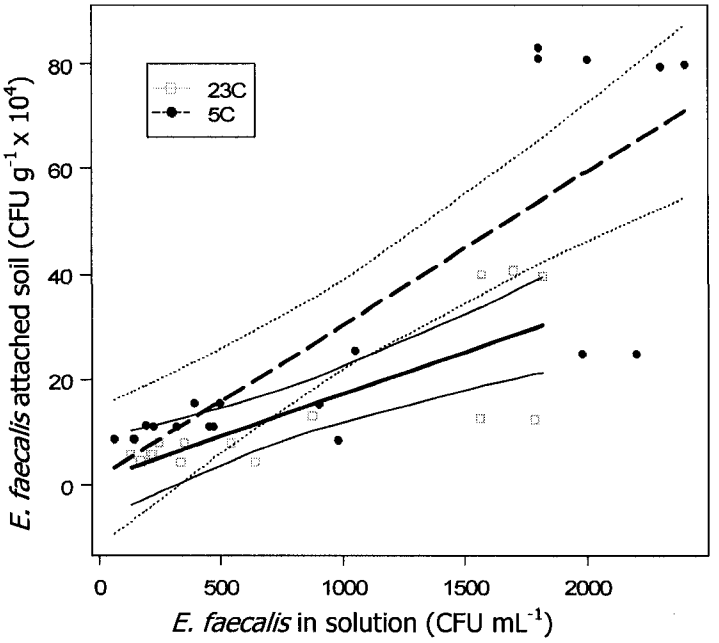

Figure 3.9: Adsorption regression of *E. faecalis* on the Ellerslie Bt horizon at two temperatures. Lighter lines indicate 95% confidence intervals.

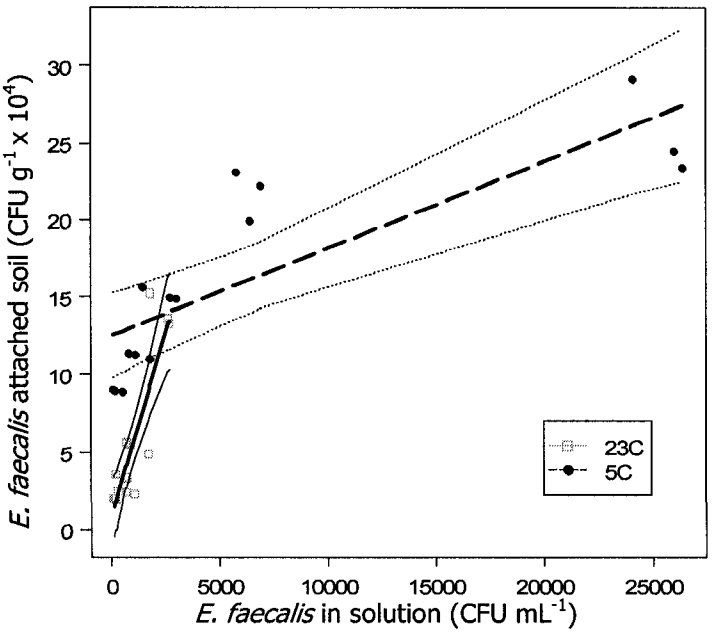

Figure 3.10: Adsorption regression of *E. faecalis* on the Rocky Mountain House Ah horizon at two temperatures. Lighter lines indicate 95% confidence intervals.

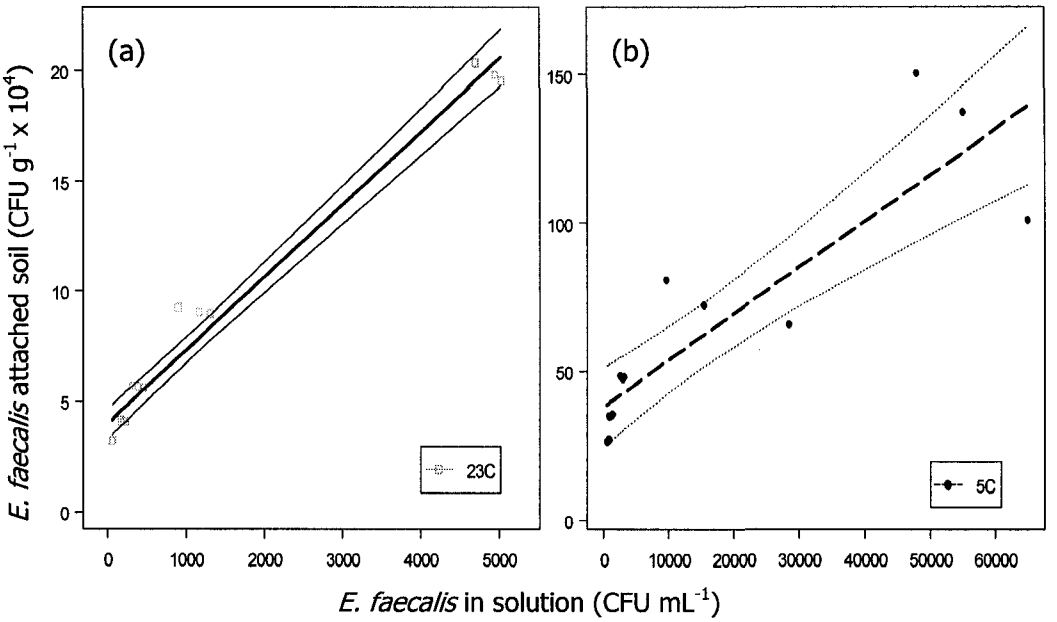

Figure 3.11: Adsorption regression of *E. faecalis* on the Rocky Mountain House Ae horizon at two temperatures, 23°C (a), and 5°C (b); The regression lines are plotted on different graphs due to the difference in scale. Lighter lines indicate 95% confidence intervals.

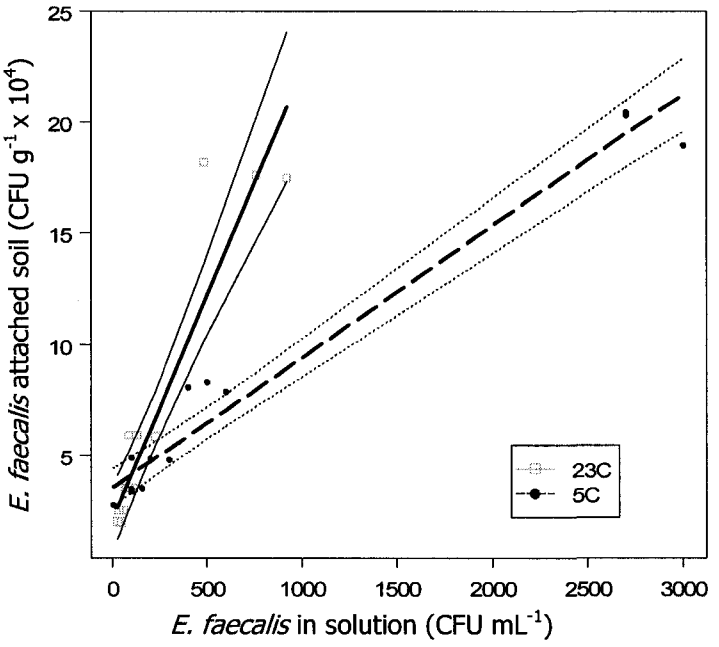

Figure 3.12: Adsorption regression of *E. faecalis* on the Rocky Mountain House Bt horizon at two temperatures. Lighter lines indicate 95% confidence intervals.

The significance of the temperature treatment as analyzed by an ANCOVA is displayed in Table 3.6. As with *E. coli*, the slopes or K_d of each equation are significantly different which confirms once more the significant effect of the temperature treatment.

Table 3.6: ANCOVA temperature treatment significance on the adsorption of *E. faecalis*

		Probability of different slopes		Probability of Parallel Slopes		[x]Probability of different intercepts	
[z]Soil	Horizon	F value	[y]Pr(>F)	F value	[y]Pr(>F)	F value	[y]Pr(>F)
Ellerslie	Ah	10.6	<0.001	34.0	<0.001	-	-
Ellerslie	Bt	62.2	<0.001	3.3	0.081	5.2	0.030
RMH	Ah	101.3	<0.001	16.2	<0.001	-	-
RMH	Ae	179.5	<0.001	0.8	0.376	33.3	<0.001
RMH	Bt	254.5	<0.001	67.2	<0.001	-	-

[z]Ellerslie= Black Chernozem; RMH= Rocky Mountain House, Gray Luvisol
[y]p-values <0.05 indicate a significant regression fit
[x]If probability of parallel slopes is significant, it is not necessary to continue to test the probability of different intercepts are the regression models have proven to be significantly different.

As a final step to confirming the treatment significance of both temperature and horizons, a MANOVA was constructed with the regression data. The results in Table 3.7 show that all treatment effects were significant.

Table 3.7: MANOVA results for treatment significance

Treatment	D.F.	[z]p-value
Soil	1	<0.001
Horizon	2	<0.001
Temperature	1	<0.001
Soil:Horizon	1	<0.001
Soil:Temperature	1	<0.001
Horizon:temperature	2	<0.001
Soil:Horizon:Temperature	1	<0.001
Residuals	140	

[z]p-value < 0.05 is significant.

3.4.4. Principal Components Analysis of All Soil Properties and Adsorption of Indicator Organisms

Eleven soil properties were chosen for their possible effect on the K_d values of the indicator organisms: percent clay, percent sand, organic matter percentage, estimated porosity (f), concentration of Hydrogen ions expressed as pH, electrical conductivity (dS/m), total cations (meq/100g), magnesium (meq/100g), calcium (mEq/100g), sodium (mEq/100g), and potassium (mEq/100g). These graphs are presented in Figure 3.5 for 23°C and Figure 3.6 for 5°C.

The PCA (Legendre and Legendre 1998) shown as Figure 3.13 represents the different tendencies of the data above along with the adsorption partitioning coefficients for 23°C. The main axis or data tendencies are along axis 1, representing 45.81% of the data while axis 2 represents 21.81% of the data. Both *E. faecalis* and *E. coli* exhibit similar behaviour trends with respect to each other and the other data and both tend more with the second axis than the first. The adsorption of these two organisms seems to be more correlated with the potassium, sodium and magnesium content, while only slightly inversely correlated with the organic carbon content, total exchangeable cations, calcium and electrical conductivity.

Figure 3.14 represents the same ordination biplot for 5°C. In this case, the main axis represents 46.97% of the data while axis 2 represents 20.86%. Once again, both organisms tend very closely together. The adsorption behavior at this temperature is inversely correlated to the cations magnesium, sodium and potassium. The clay content in the soil and porosity tend in an opposite direction to the organisms. The electrical conductivity, calcium content, concentration of hydrogen ions expressed as pH, and sand are not correlated at all with the organisms at 5°C.

An ordination biplot such as this is constructed to discover possible tendencies between the data. In both of the PCAs, the organisms tend in similar directions. The clay content and porosity were thought to be of major importance to adsorption, but they do not seem to play a major part in the tendencies of both indicators. Another soil component thought to be important is the organic carbon content. This particular soil property tended opposite of the organisms. More analysis on different soils is necessary to fully understand how the indicator organisms interact with soil properties.

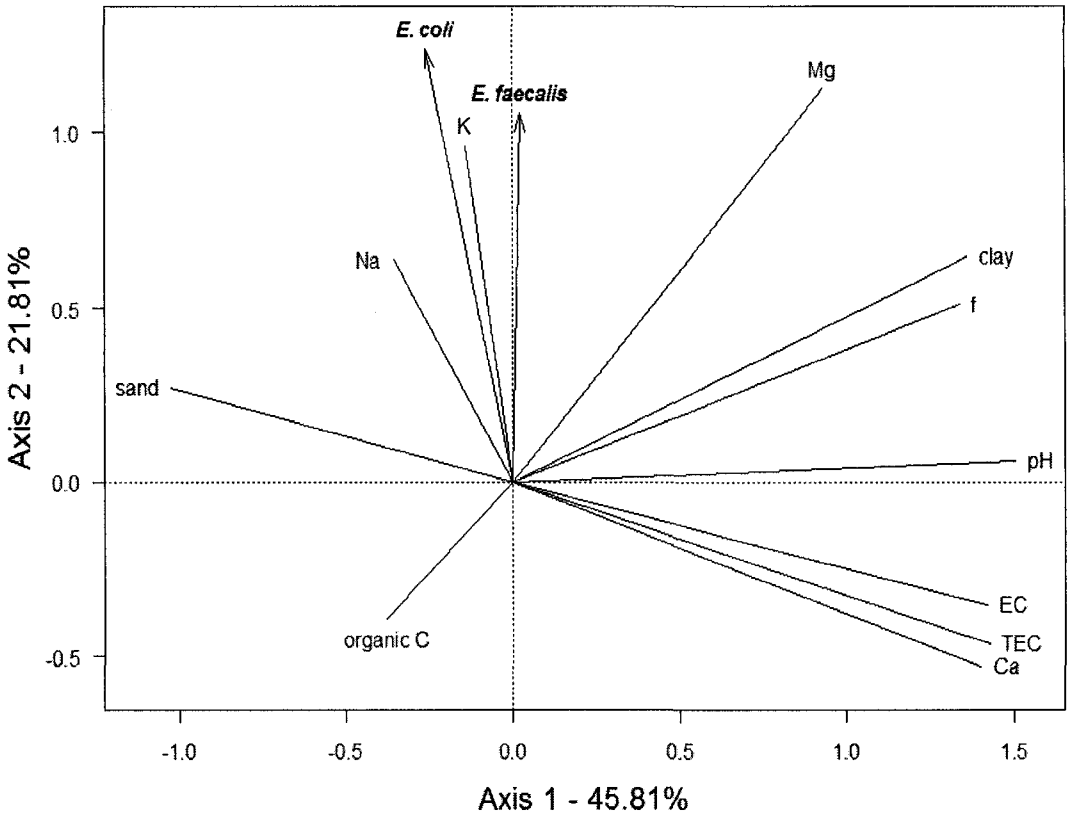

Figure 3.13: Principal component analysis (PCA) ordination biplot of experimental data determined at 23°C. Soil Components: electrical conductivity (EC), total exchangeable cations (TEC), magnesium (Mg), potassium (K), sodium (Na), calcium (Ca), hydrogen ion concentration expressed as pH, estimated porosity (f), percentage of organic carbon in soil (organic C), percentage of clay in soil (clay), percentage of sand in soil (sand). Bold arrows represent the indicator organism: *E. coli* adsorbed by the soil (*E. coli)*, *E. faecalis* adsorbed by the soil (*E. faecalis).*

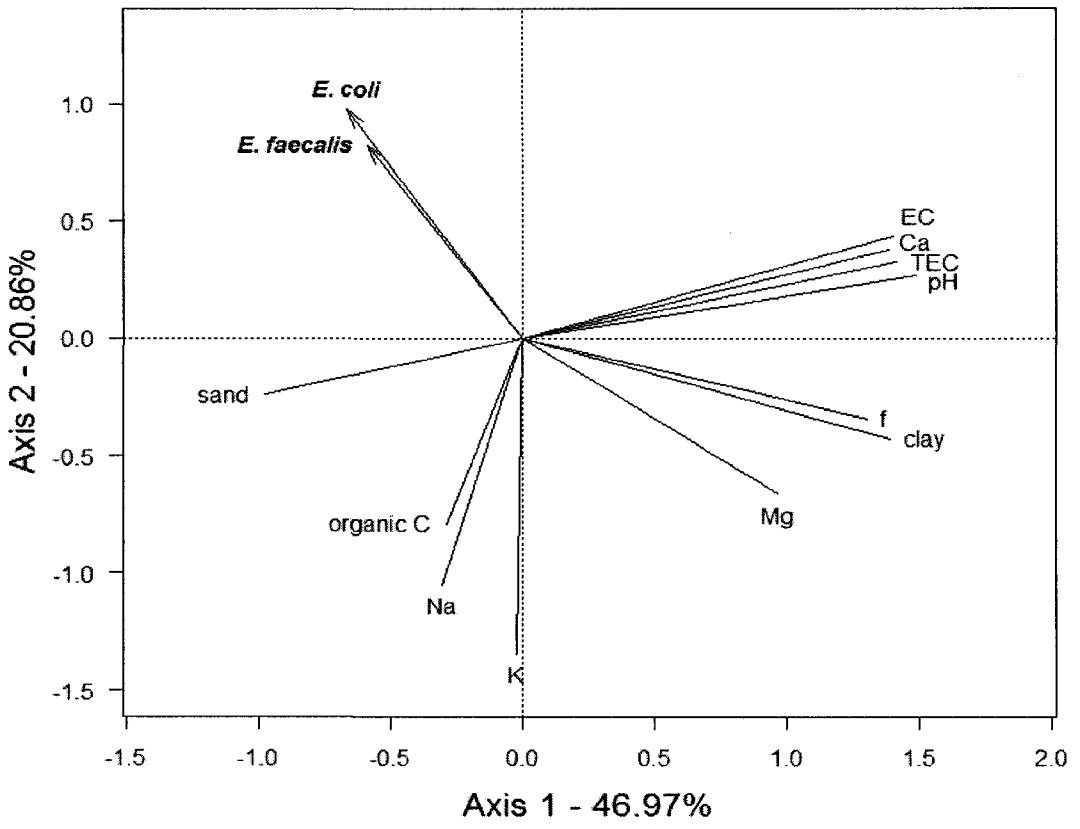

Figure 3.14: Principal component analysis (PCA) ordination biplot of experimental data determined at 5°C. Soil Components: electrical conductivity (EC), total exchangeable cations (TEC), magnesium (Mg), potassium (K), sodium (Na), calcium (Ca), hydrogen ion concentration expressed as pH, estimated porosity (f), percentage of organic carbon in soil (organic C), percentage of Clay in soil (clay), percentage of sand in soil (sand). Bold arrows represent the indicator organism: *E. coli* adsorbed by the soil (*E. coli)*, *E. faecalis* adsorbed by the soil (*E. faecalis).*

3.5 Discussion

The soil's capacity to retain inorganic and organic compounds, indigenous and introduced non-pathogenic and pathogenic organisms is directly related to its adsorption capacity and the partitioning coefficients of each material retained. This retention capacity is an essential part of its function as a reservoir or pollutant filter, especially for the treatment of effluent from onsite wastewater treatment systems (OWTS). This discussion is organized under two themes: the adsorption of indicator organisms in the major horizons of two soils at two temperatures, and the relationship between adsorption rates and OWTS.

3.5.1 Adsorption of Indicator Organisms in the Major Horizons of Two Soils at Two Temperatures

It was generally thought that the adsorption capacity of soil was over 1×10^9 CFU/gram (Britton and Marshall 1980). However, Huysman and Verstraete (1993) observed that even at equilibrium, the adsorption sites are not fully saturated with bacteria.

They also found that the higher adhesion could be due to higher clay content or a mixture between the latter plus greater cation exchange capacity, greater surface area or a higher Ca content in the clay loam soil they used compared to the sandy soil they used. In this experiment, clay content, cation exchange capacity, and calcium content was not found to have such a profound impact as temperature on the microbial adsorption. The PCA biplot did not show a strong correlation between higher K_d values of the indicator microorganisms and higher clay content.

As adsorption is an exothermic reaction, it tends to increase as temperature decreased for inorganic colloids. However, there is limited information on the effect of temperature on bacterial adsorption in soil (Jiang et al. 2007). In this study, the PCA showed that the K_d values for both organisms were highly correlated; however, their behavior at two temperatures 23°C and 5°C were quite different. In general, the K_d values for *E. coli* for the Ah and Bt horizons on the soil from Ellerslie were higher at 23°C than 5°C (Table 3.3). Similar trends were also observed for *E. coli* on the Ah horizon of the soil from Rocky Mountain House. However, an inverse trend was observed on the Ae and Bt horizons of this soil; the K_d values were higher at 5°C.

In contrast to *E. coli,* the K_d values for *E. faecalis* were higher on the Ah and Bt horizons of the soil at Ellerslie at 5°C and an inverse trend was observed on the thee horizons of the soil from Rocky Mountain House.

As the soil property effects could not completely explain these trends, there is a possibility of explaining them though properties of the indicator organisms. The key difference between both organisms their cell structures as *E. coli* is Gram negative while *E. faecalis* is Gram positive. The presence of teichoic acids and lipoteichoic acids in the surface of the peptidoglycan layer of Gram-positive bacteria is known to promote adherence. In addition to this, medical research has shown that *E. faecalis* have been found to have the capability to form surface pili to aid in the formation of biofilm. This, however, is unlikely in the soil environment as stressed organisms seldom produce external appendages.

In contrast, Gram-negative bacteria do not have teichoic acids, but instead, their outer membrane protects them from substances such as antibiotics and detergents. As they are protected, these organisms are less likely to adsorb in favourable environments. In the Bt horizon, however, the lower energy and lower nutrient availability in the environment could have promoted active adsorption as a means of protection, especially in Luvisolic soil (Bengtsson and Ekere 2001).

Overall, *E. faecalis* showed higher adsorption rates than *E. coli* in the Bt horizons.. Overall, this organism seemed to prefer a more sessile life in comparison to *E. coli* and adsorbed to soil more readily.

From this study, the adsorption of both indicator organisms occurred in the major horizons of two soils but their behaviour was markedly different. This reflects the reality of differences in the structure and function of individual organisms. This is also a reminder that developing an indicator for environmental monitoring is a very complex task which requires thorough test of the behaviour of each indicator species in a wide range of soils.

3.5.2 Experimental Adsorption Rates and OWTS

One of the main problems in regulating onsite wastewater treatment systems is the inability to distinguish which organisms are immobile and which have the potential to move through a soil profile. Earlier studies by Juma et al. (2007) have

found a significant number of organisms to a depth of 60 cm (Bt horizon) under a dispersal orifice, but they were unable to assess the hazard potential. By using K_d values found in this study, it may be possible to develop a preliminary assessment of threats with more accuracy.

In this experiment, the K_d values were examined from the perspective of a soil horizon instead of soil depth. This is because soil properties can vary dramatically depending on the horizon in which they are located. This is demonstrated by the significant difference in net die-off rates and adsorption rates of each horizon in a same soil.

Here is an idealized example of how K_d values can be used to predict sorption in a soil profile. Using the K_d values of *E. faecalis* in the Gray Luvisol at 5°C, it is possible to calculate the number of organisms which would be adsorbed to the soil and those which would travel with the soil solution after a one-time dose of 10,000 CFU/g (Figure 3.15). If this dose were applied to the Ah horizon with a K_d of 5.7, approximately 8,000 of the organisms are expected to be adsorbed while around 1750 of the organisms would remain in solution with the potential to be transported to the next horizon. The Ae horizon has a K_d of 15.6; therefore, of the from the estimated 1750 CFU in solution from the Ah horizon, about 1600 CFU would then be adsorbed to the Ae horizon while 110 CFU could pass to the Bt horizon with the soil solution. This horizon has the highest K_d value of the three of 59.1. Here, approximately 110 CFU of the organisms would be expected to adsorb while two could still be in solution.

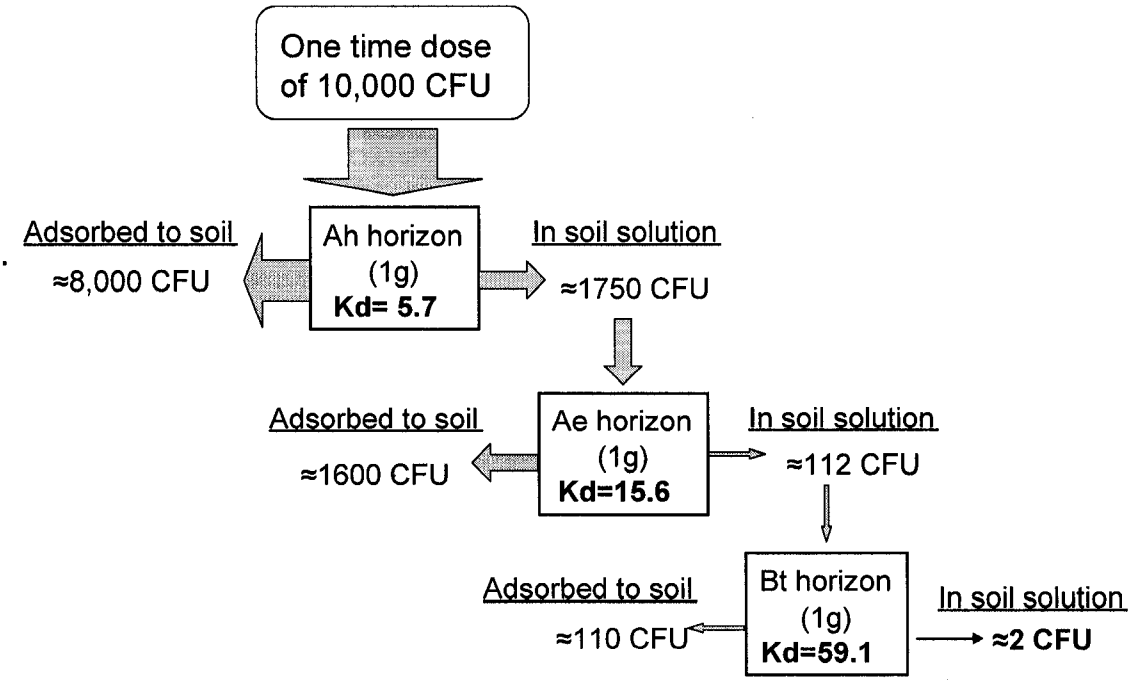

Figure 3.15: Theoretical representation of the number of organisms available for transport after being adsorbed by three Luvisolic horizons, Ah, Ae, and Bt, successively. This diagram represents a one-time dose and does not take into account factors such as water flow, temperature variance, depth of the horizon, or bulk density.

Though this is an idealized situation and does not take into account water flow, temperature variance, depth of horizons or bulk density, it does illustrate that as effluent passes though the various horizons of a soil profile, fewer and fewer organisms are expected in suspension.

Unlike wastewater regulations in urban water treatment facilities, there is no regulation of the number of introduced microorganisms which can be found in a soil. If the adsorption rates can be compiled for other soil types and for other microorganisms, the regulation of the number of organisms that can be allowed in a soil at a given moment would be much easier to obtain. Finding organisms does not necessarily mean there is a contamination threat; instead, finding a concentration of pathogenic organisms in the soil solution of a certain soil horizon would be cause for

concern because these can be transferred out of the soil (Figure 3.15). However, in order for this to occur, correlations must first be made between this clean soil laboratory experiment and the soil found under functioning OWTS. The next chapter ventures into this territory by investigating the adsorption rate of the indicator organisms in contaminated soil from under failed OWTS.

3.6 Conclusions

In order to understand the adsorptive capability of the major horizons of two clean soils, the K_d, soil water partitioning coefficients of two indicator organisms was calculated. Variances in temperature significantly affect the adsorption of these organisms in different manners. In general, the Bt horizon adsorbed the most organisms. *E. faecalis* was adsorbed at much higher rates than *E. coli*; however, since both organisms were affected by treatment effects in a different way, it is recommended that both organisms should be analyzed when testing soil from OWTS.

3.4 References

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Chapter 4

Adsorption of Indicator Organisms in Soil Samples Obtained from a Failed Septic Field, a Failed Mound and a Failed At-grade Dispersal System

4.1 Introduction

The onsite wastewater treatment systems (OWTS) are soil-based systems that are affected by a complex variety of factors because they are used year round and receive multiple doses of wastewater daily. Temperature, water cycles, and soil type are some of the many factors that influence soil adsorption efficiency in dispersal fields.

One of the best ways to analyze the adsorption rates of the indicator organisms in the field is to take soil samples from systems that are in operation. The adsorption of the indicator microorganisms has been studied in laboratory conditions on clean soil samples (Chapter 3). The samples taken from dispersal fields would differ from the clean soil samples because this soil has been receiving effluent continuously. Effluent contains not only the pathogens but also anions and cations which can compete for adsorption sites and reduce the adsorption efficiency of the soil. On the other hand, the continual passage of microorganisms that adhere to the soil surfaces combined with the higher nutrient content from the passing effluent may cause the formation of a biofilms which adsorb passing organisms. The sum of these effects add to the already complex mechanisms which operate in situ to treat the effluent.

In order to get soil samples from dispersal fields that have most likely been contaminated, soil samples from three failed systems were chosen from Leduc County: a failed septic field, a failed mound, and a failed at-grade system. Failed systems were chosen for two reasons: to test the levels of indicator organisms that may be present, and to assess the adsorption of newly added indicator organisms to these soils.

4.2 Three Examples of OWTS

The easiest system to sample was a septic tank and field. This system provides the least amount of treatment to the effluent prior to dispersing it onto the field. Though the dispersal pipes lie on top of a gravel bed, this system relies on the soil to complete the effluent treatment. The dispersal pipes are buried within the ground to a depth of at least 1 metre (Figure 4.1).

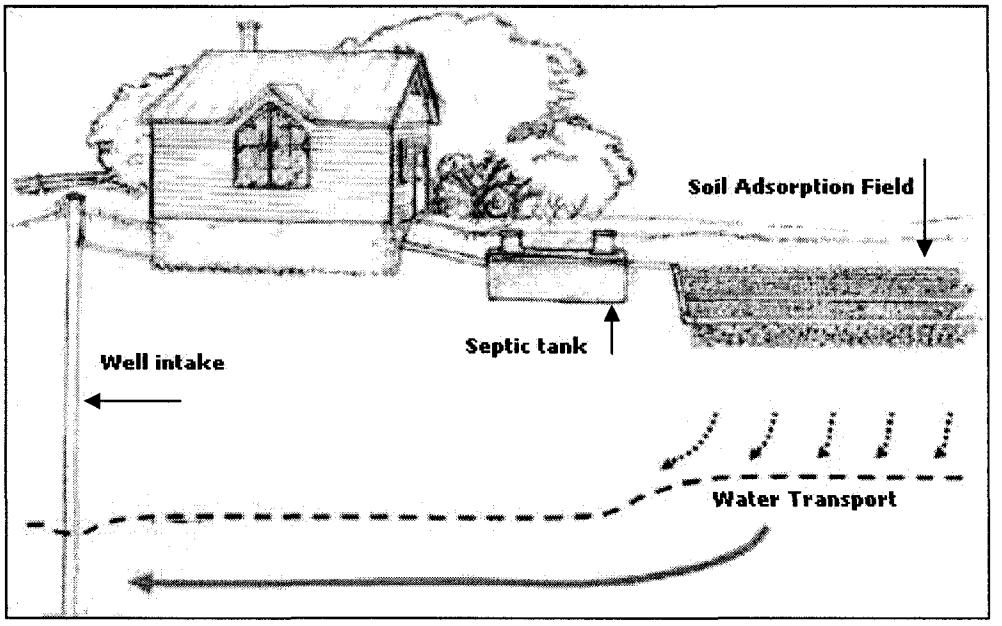

Figure 4.1: Diagram of a traditional septic tank with dispersal field. (adapted from the Canada Mortgage and Housing Corporation website.)

One of the more advanced systems is a mound. It is usually placed in areas where there is a restrictive soil layer close to the surface or the property does not have enough room for a traditional field. This type of system is raised above a natural soil surface (Figure 4.2). A network of pipes is placed on top of a gravel or sand bed though which effluent passes. The effluent usually passes though this system to be fully treated and then enters the soil for final polishing of the wastewater.

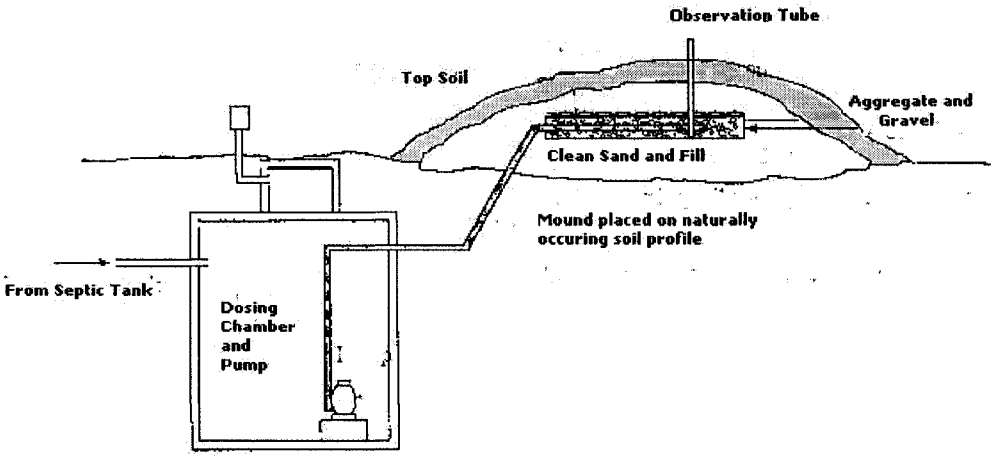

Figure 4.2: Diagram of a mound onsite treatment system. (Adapted from the University of Nebraska-Lincon extension website.)

One of the most advanced OWTS is the at-grade system. This system is usually placed in areas with a limited lot size or restrictive layers. In order to reduce the number of pathogens, the effluent is treated with an advanced treatment plant before being dispersed onto the surface of a soil. The dispersal pipes are protected by plastic PVC chambers and a layer of mulch to prevent the dispersal field from freezing in winter.

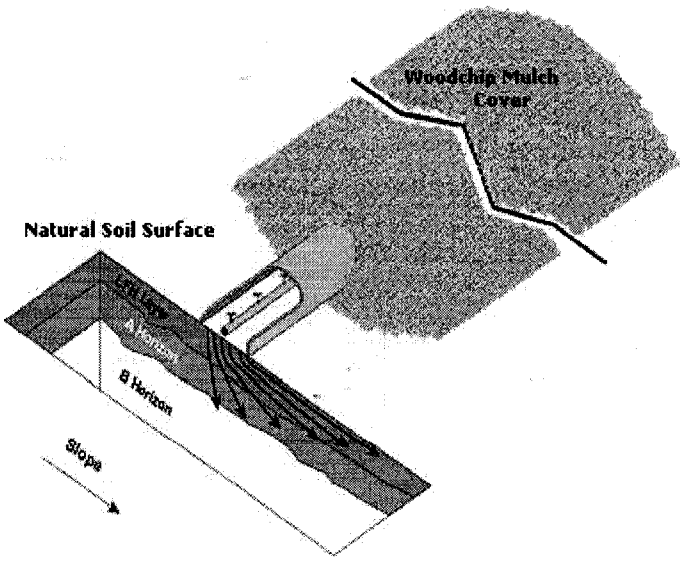

Figure 4.3: Diagram of an at-grade OWTS. (Adapted from Durnie 2002)

4.2 Objectives

The objective of this study was to quantify the presence of indicator organisms in soil samples obtained from a failed septic field, a failed mound, and a failed at-grade, and to assess the adsorption of newly added indicator organisms to these soil samples.

4.3 Materials and Methods

4.3.1 Site Description and Soils

The soils used in these experiments were obtained from three different sites located in the Leduc County, Alberta. In order to protect the privacy of the homeowners, the exact address is not given but instead a brief description of the area is provided.

The failed septic field had been operational for over 24 years, but had recently stopped adsorbing wastewater from a septic tank equipped with an aeration device. The owner of the property has since installed a trench field on a separate area of the property. The area itself is of hummocky terrain, however, for the establishment of the original septic field, the soil was removed, a PVC wastewater dispersal system was installed and the soil was replaced.

The samples taken from this area were obtained with a soil sampler to a depth of 90 cm. The soil texture from 10 to 30 cm horizon was a loam. The texture of the 60-90 cm horizon was a clay loam.

The failed mound is located around the area of Brenda Vista Estates where a number of properties are being developed. Due to insufficient space on the property, a septic mound was chosen as a final disposition point. It had only been operational for a few weeks when the homeowner noticed that the mound was leaking from the sides at the mound-soil interface. Samples of the top soil were taken adjacent to these breakout areas. The soil textural class was a loam.

The failed at-grade dispersal system was placed on top of undisturbed soil. Therefore, this was the only site where the soil was not disturbed. Instead of locating the dispersal field in a levelled portion of the property, the initial portion of the lateral was placed in an upslope position. The effluent was dispersed through 0.25 in. (60 mm) orifices which were drilled at a spacing of 1 ft (30 cm) in the lateral. This design did not permit the effluent to be evenly distributed and caused it to drain back and pond at a lower slope position of the lateral. The fault was noticed right after the

installation and continued for two months. The surface ponding indicated high levels of wastewater contamination. The texture of the Ae horizon was a loam and Bt horizon was a clay.

4.2.2 Collection and Microbial Characterization of Soil Samples

All tools and corers were surface sterilized and then used to take soil samples under aseptic conditions. These soil samples were then placed in sterile bags and transported in a cooler. Soil samples were stored in the refrigerator at 5°C before being analyzed for initial counts and adsorption rates of the indicator organisms.

Initial concentration of the indicator microorganisms were analyzed using five-tube, six-dilution Most Probable Number analysis. *E. coli* were determined using LTB broth and confirmed by EC broth for fecal coliforms. Positive tubes on EC were confirmed by BGB and the GAD procedure to ensure accurate numbers. *E. faecalis* was determined using Azide Dextrose broth, and brain-heart infusion broth with 6.5% NaCl (incubated at 35 ± 0.5°C for 48 hours). A detailed description of methods is presented in Chapter 3.3.

4.2.3 Adsorption Experiment

The adsorption experiment was conducted according to the method described in Chapter 3. Due to the smaller soil sample size, this experiment was only carried out at room temperature, 23°C.

The indicator organisms *E. coli* and *E. faecalis* were incubated overnight in the commercially available media Tryptic Soy Broth (TSB) and Brain-Heart Infusion Broth (BHIB), respectively. These cells were then suspended, washed twice in phosphate buffered solution (PBS), pH 6.8, and resuspended together in the same suspension to a density of approximately 10^4 to 10^5 cells per ml of each, calculated by using direct count with a Petroff-Hausser chamber. These counts were verified by the using the Most Probable Number method (MPN).

Five mL of a combined suspension of *E. coli* and *E. faecalis* were added to the 15 mL centrifuge tubes and shaken with a vortex to ensure the contact of the suspension with all the soil particles. This mixture was left standing for one minute and then shaken gently at 180 rpm for 5 minutes.

Separation of the soil particles was achieved using slow differential centrifugation, 48 x g for 3 minutes. The centrifugal speed was determined using $g=n^2\Phi/1800$ where n^2 is the bowl speed (RPM) and Φ is the bowl maximum inner diameter (m).

4.2.4 Microbiological Analysis

Due to the background microorganism counts and soil variability, the MPN method was once again used for determining bacterial concentrations in the supernatant.

After centrifugation was completed, 1 ml of the supernatant was diluted in 10 ml of phosphate buffered solution. Depending on the initial bacterial counts, various dilutions of three tubes were then inoculated and incubated.

E. coli numbers were determined using Laryl Tryptose broth (LTB) with inverted Durham tubes and incubated for 48 ± 3 hours at 35 ± 0.5°C. Positive tubes would show both growth and presence of gas. Verification of these microorganisms was completed using the GAD method.

E. faecalis numbers were determined using Azide dextrose broth. The same dilutions used with LTB were used with this broth and incubated at 35 ± 0.5°C for 48 ± 3 hours. Portions of growth from the positive tubes were verified by streaking on EI agar plates then incubating for 24 ± 2 hours at 35 ±0.5°C. Brownish-black colonies with brown halos indicated the presence of fecal streptococci.

All results were tabulated and analyzed according to the APHA (APHA 2005) method based on most probable number of microorganisms in 1mL of effluent.

4.2.5 Statistical Analysis

As in Chapter 3, the water partitioning coefficient of the adsorption process was determined by a linear isotherm: (Travis and Etnier, 1991;Huysman and Verstraete 1993; Ling et al 2002; Pachepsky et al. 2006):

$$S = K_d C \qquad \text{[Equation 4.1]}$$

where S is the concentration of bacteria adhering to soil (CFU/g soil), C in the concentration in the suspension (CFU/mL), and K_d is the partitioning coefficient. This equation assumes instantaneous equilibrium. Therefore, the K_d was found by calculating the slope of the regression line when the colony forming units of each microorganism in suspension (x-axis) versus the concentration in soil (y-axis) were plotted. The statistical analysis of these curves was done using the R statistical language (R Development Core Team, 2008). After this analysis, all data were tested to confirm a normal distribution using the Shapiro-Wilks test. If this test was negative, a permutation test of 999 permutations was used to calculate the correct probability value and regression coefficients.

The data collected were used to construct a principal component analysis ordination biplot such as the biplots constructed for Chapter 3. It was expected that the increased variance of the soil property data could give a better explanation of the behaviour of these indicator microorganisms in these soils.

4.3 Results

The highest number of *E. coli*, 5.4 x 10^6 MPN/ml, and *E. faecalis*, 4.4 x 10^2 MPN/ml, were measured in the soil sample taken from the Ae horizon of the failed at-grade site (Table 4.1). The concentration of the indicator organisms in soil samples taken from the foot of the mound were below detection limits. This indicated that the mound was performing properly but the there were problems at the mound/soil interface which caused the breakout of the effluent. In the failed septic field the surface soil horizon has low numbers of *E. coli* but in other soil samples, the indicator organism numbers were below detection limits. The soil pH in all cases ranged from neutral tending to alkaline. The EC ranged from 0.18 to 0.79 (dS/m); therefore, these soil samples were not saline.

Table 4.1: Initial concentrations of *E. coli* and *E. faecalis*, Ph, and EC of soil samples from contaminated failed OWTS.

Soil	Horizon	pH	EC (dS/m)	[z]MPN *E. coli*/g	[z]MPN *E. faecalis*/g
Failed At-grade	Ae	7.31	0.61	5.4×10^6	440
Failed At-grade	Bt	7.17	0.18	<3	<3
Failed Mound	Top	7.52	0.80	<3	<3
Failed Septic Field	10-30 cm	8.40	0.62	78	<3
Failed Septic Field	60-90 cm	8.36	0.45	<3	<3

[z]MPN = most probable number.

The overall organic matter content of the surface soil samples ranged from 1.31% to 5.08% while those in the sub-surface ranged from 0.53% to 1.01%. The values for these soil samples are lower than those found from Ellerslie and Rocky Mountain House.

Table 4.2: Texture and soil organic carbon content of soil samples from contaminated, failed OWTS.

Location	Horizon	Sand	Silt	Clay	Organic C	Soil Texture
		Percent (%)				
Failed At-grade	Ae	41.8	35.4	22.8	1.54	Loam
Failed At-grade	Bt	26.4	22.8	50.8	0.53	Clay
Failed Mound	Top Soil	44.3	41.5	14.2	5.08	Loam
Failed Septic Field	10-30 cm	36.6	41.5	21.9	1.31	Loam
Failed Septic Field	60-90 cm	34.1	27.1	38.8	1.01	Clay loam

Exchangeable and total cations of soil samples from contaminated OWTS showed that sodium ions were 8.0 meq/100g in the 10 cm to 30 cm of the failed septic field and 8.98 meq/100 g for the top soil of the failed mound. These values were almost two orders of magnitude higher than the highest value from the

previous adsorption experiment (0.089 meq/100 g from Ah soil horizon sample from Ellerslie).

Table 4.3: Exchangeable and total cations of soil samples from contaminated failed OWTS.

Location	Horizon	Na	K	Mg	Ca	Total cations
		meq/ 100g soil				
Failed At-grade	Ah	0.12	0.96	2.97	12.04	15.77
Failed At-grade	Bt	0.36	0.83	2.26	20.38	23.83
Failed Mound	Top Soil	8.98	0.39	1.80	24.41	35.56
Failed Septic Field	10-30 cm	8.00	0.17	2.36	13.00	23.53
Failed Septic Field	60-90 cm	0.12	0.96	2.97	12.04	15.77

Tables 4.4 and 4.5 show the results from the adsorption experiment for *E. coli* and *E. faecalis* respectively. The adsorption regressions for *E. coli,* and four of the regressions for *E. faecalis* did not consist of normally distributed data and therefore a data permutation was employed to correct this problem. All of the adsorption regressions are significant with a p-value less than 0.05.

The highest K_d value for *E. coli,* 64.0, was measured in the Bt horizon from the failed at-grade. The lowest K_d values was measured on both horizons of the septic field (K_d = 2.9 for the 10 cm to 30 cm depth sample; K_d = 1.6 on the 45 cm to 60 cm depth sample)

Table 4.4: Adsorption partitioning coefficients (K_d) for *E. coli* on soil samples from OWTS

Soil Sample	Horizon	[z]SW p-value	Intercept	Slope ([y]K_d)	[x]R^2	Permuted [w]p-value
Failed At grade	Ae	0.017	5353000	39.1	0.92	0.006
Failed At grade	Bt	0.002	-18049	64.0	0.90	0.005
Failed Mound	Top	<0.001	31959	7.3	0.87	0.004
Failed Septic Field	10-30 cm	<0.001	27157	2.9	0.93	0.005
Failed Septic Field	45-60 cm	0.001	113900	1.6	0.68	0.006

[z]SW= Shapiro-Wilks test of normality; data considered not normal if p-value<0.05
[y]K_d=water partition coefficient
[x]R^2 is the coefficient of determination
[w]p-values <0.05 indicate a significant regression fit

The K_d values for *E. faecalis* was generally higher than that for *E. coli.* The highest K_d, 633.1 was observed for the failed mound soil sample. This K_d value was higher than those obtained from soil samples from Ellerslie, Rocky Mountain House, and the soil samples from OWTS. As with *E. coli,* the lowest adsorption rate is from both horizons of the failed septic field (K_d = 3.7 for the 10 cm to 30cm depth; K_d = 7.8 for the 45 to 60 cm depth).

Table 4.5: Adsorption partitioning coefficients (K_d) for *E. faecalis* on soil samples from OWTS

Horizon	Horizon	[z]SW p-value	Intercept	Slope ([y]K_d)	[x]R^2	[w]p-value	Permuted [w]p-value
Failed At grade	Ae	<0.001	443252	17.2	0.75		0.005
Failed At grade	Bt	<0.001	-406	123.7	0.90		0.008
Failed Mound	Top	<0.001	5442	633.1	0.85		0.003
Failed Septic Field	10-30 cm	0.001	604800	3.7	0.70		0.003
Failed Septic Field	45-60 cm	0.16	828700	7.8	0.63	0.002	

[z]SW= Shapiro-Wilks test of normality; data considered not normal if p-value<0.05
[y]K_d=water partition coefficient
[x]R^2 is the coefficient of determination
[w]p-values <0.05 indicate a significant regression fit

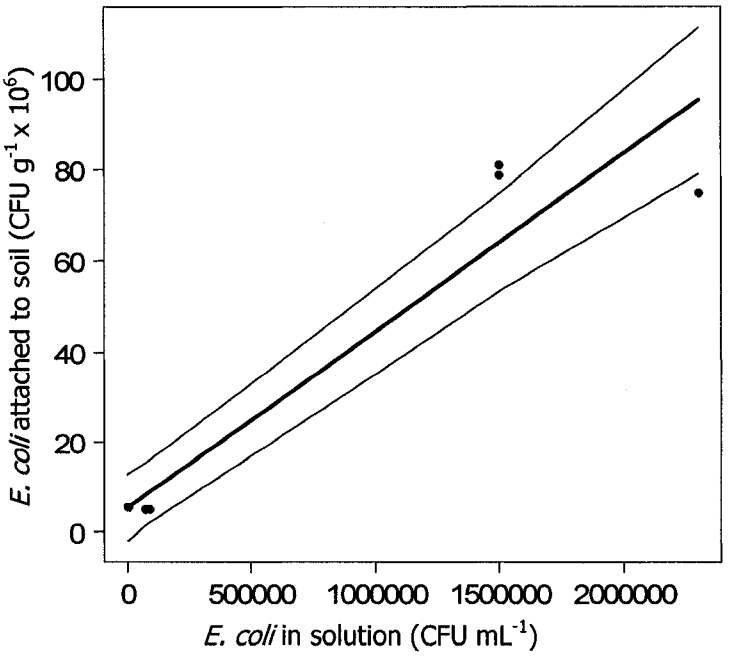

Figure 4.4: Adsorption isotherm for *E. coli* on soil samples from the Ae horizon of the failed at-grade. Lighter lines indicate the 95% confidence intervals.

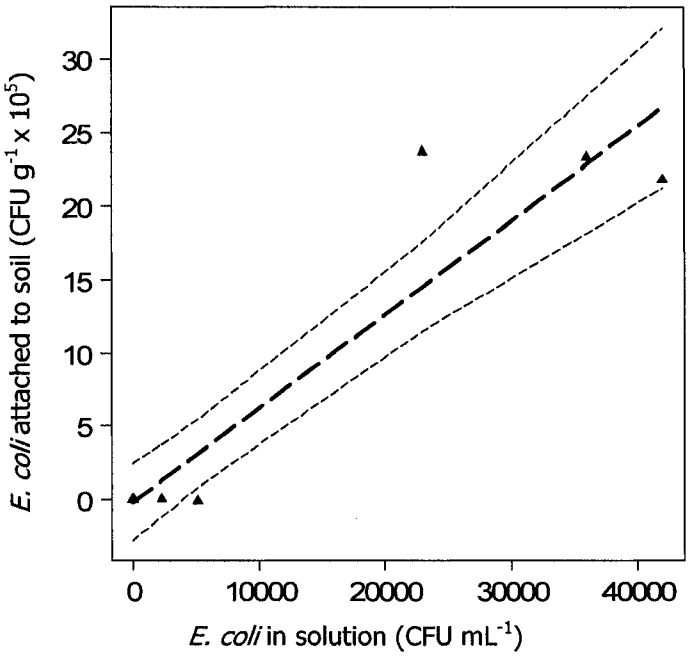

Figure 4.5: Adsorption isotherm for *E. coli* on soil samples from the Bt horizon of the failed at-grade. Lighter lines indicate the 95% confidence intervals.

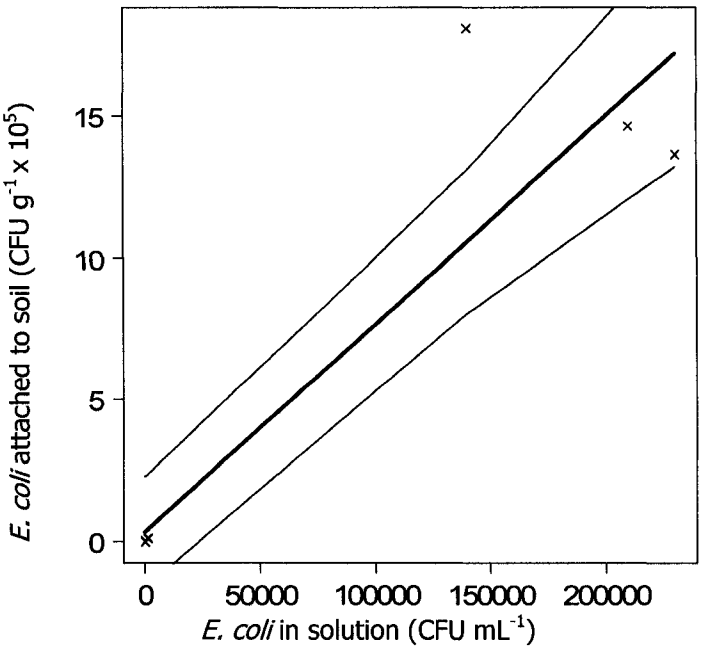

Figure 4.6: Adsorption isotherm for *E. coli* on soil samples from the top soil of the failed mound. Lighter lines indicate the 95% confidence intervals.

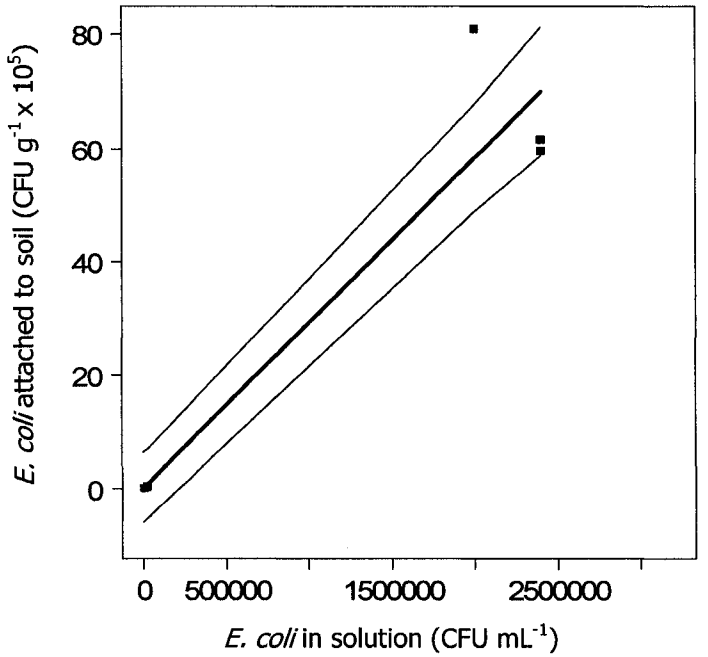

Figure 4.7: Adsorption isotherm for *E. coli* on soil samples from the failed septic field, 10-30 cm. Lighter lines indicate the 95% confidence intervals.

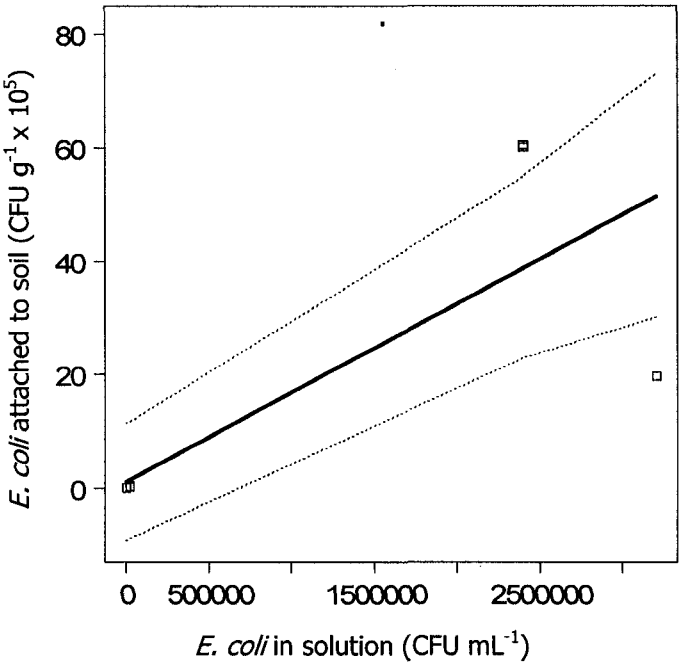

Figure 4.8: Adsorption isotherm for *E. coli* on soil samples from the failed septic field 60-90 cm. Lighter lines indicate the 95% confidence intervals.

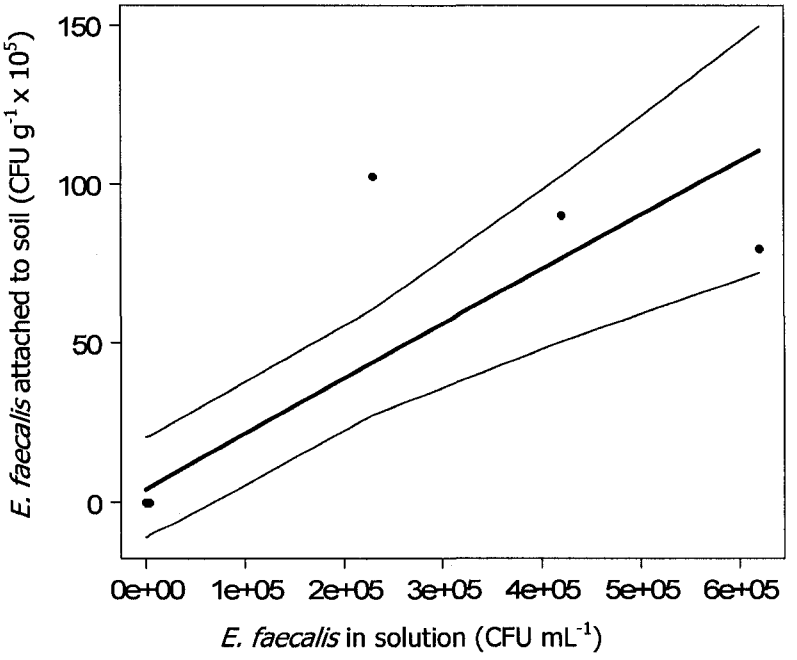

Figure 4.9: Adsorption isotherm for *E. faecalis* from the Ae horizon of the failed at-grade site. Lighter lines indicate the 95% confidence intervals.

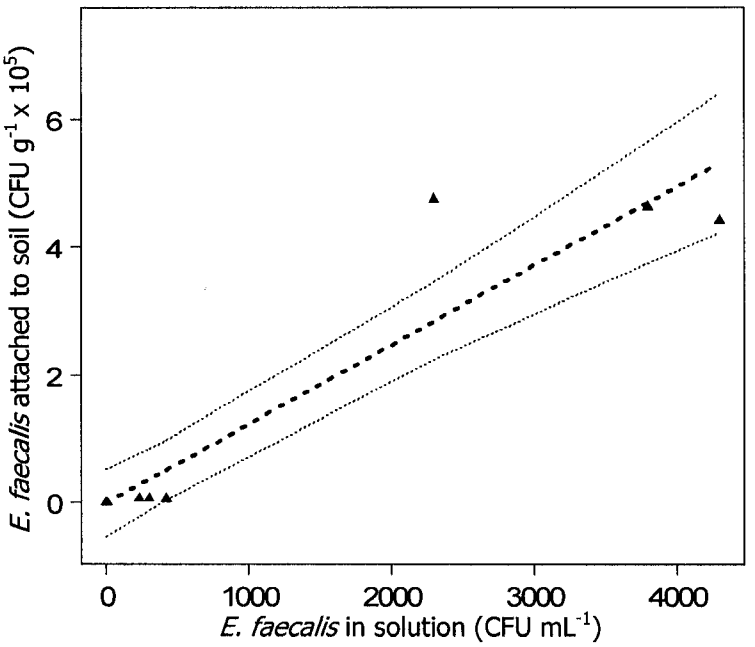

Figure 4.10: Adsorption isotherm for *E. faecalis* from the Bt horizon of the failed at-grade site. Lighter lines indicate the 95% confidence intervals.

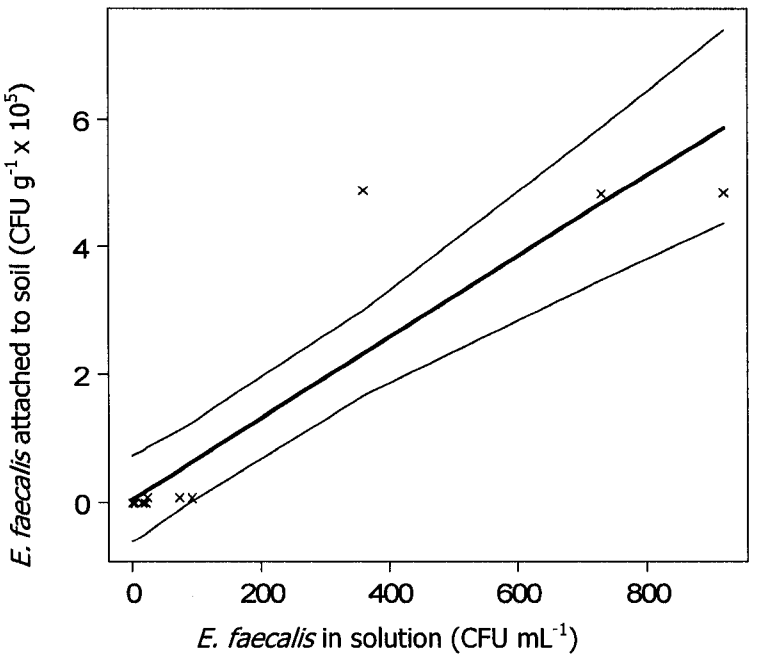

Figure 4.11: Adsorption isotherm for *E. faecalis* on soil samples from the top soil of the failed mound. Lighter lines indicate the 95% confidence intervals.

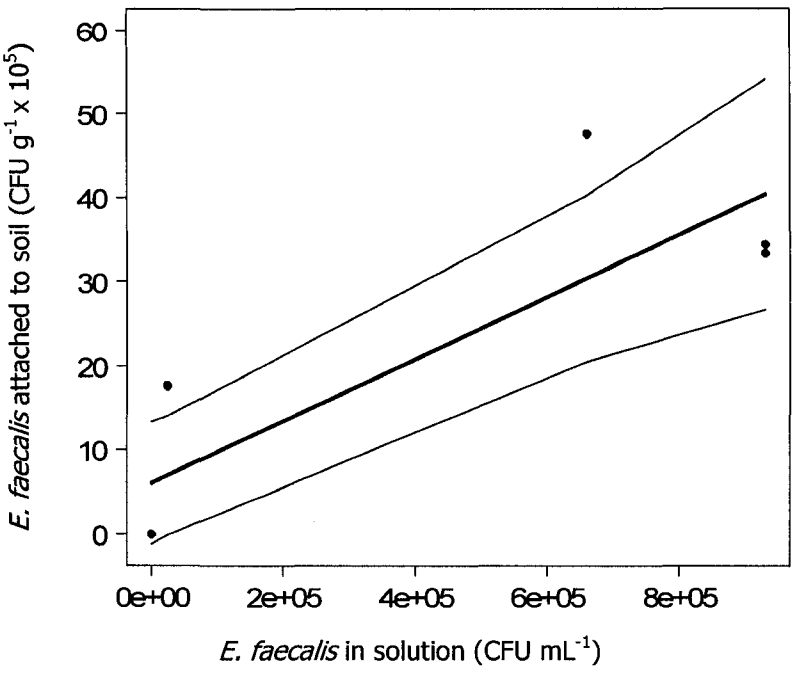

Figure 4.12: Adsorption isotherm for *E. faecalis* on soil samples from the failed septic field, 10-30 cm. Lighter lines indicate the 95% confidence intervals.

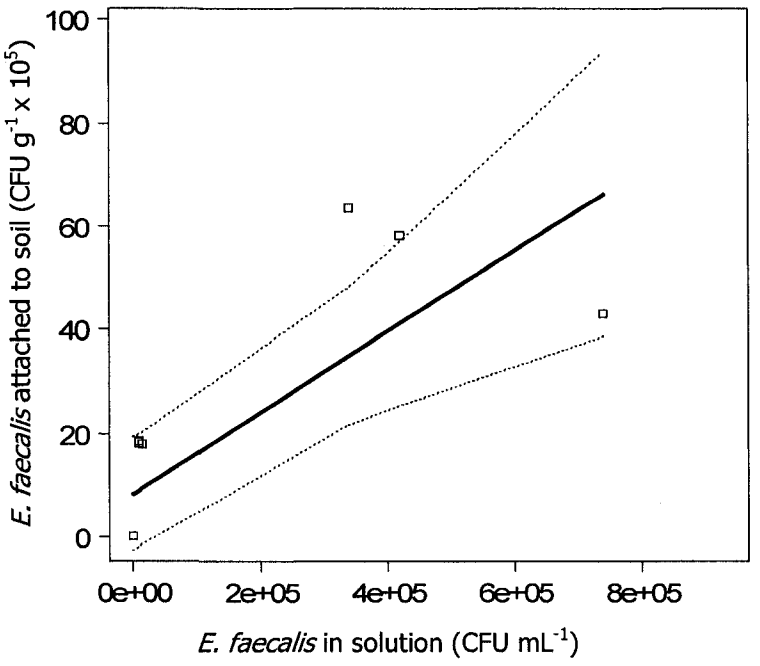

Figure 4.13: Adsorption isotherm for *E. faecalis* on soil samples from the failed septic field, 60-90 cm.

The biplot ordination of the PCA (Figure 4.13) shows the major tendencies of the soil properties and the adsorption of each indicator organism. The first axis explains 50.88% of the variance of the data while the second axis explains 23.54%.

As was the case in Chapter 3, both indicator organisms act in a similar manner and are better explained by the second axis. The most correlated data are the concentration of hydrogen ions (pH) and the concentration of magnesium in soil (Mg). The total exchangeable cations (TEC) and soil calcium content (Ca) are inversely correlated to the adsorption of the indicator organisms. Correlations are considered negligible between adsorption of the organisms and the other variables considered in this study.

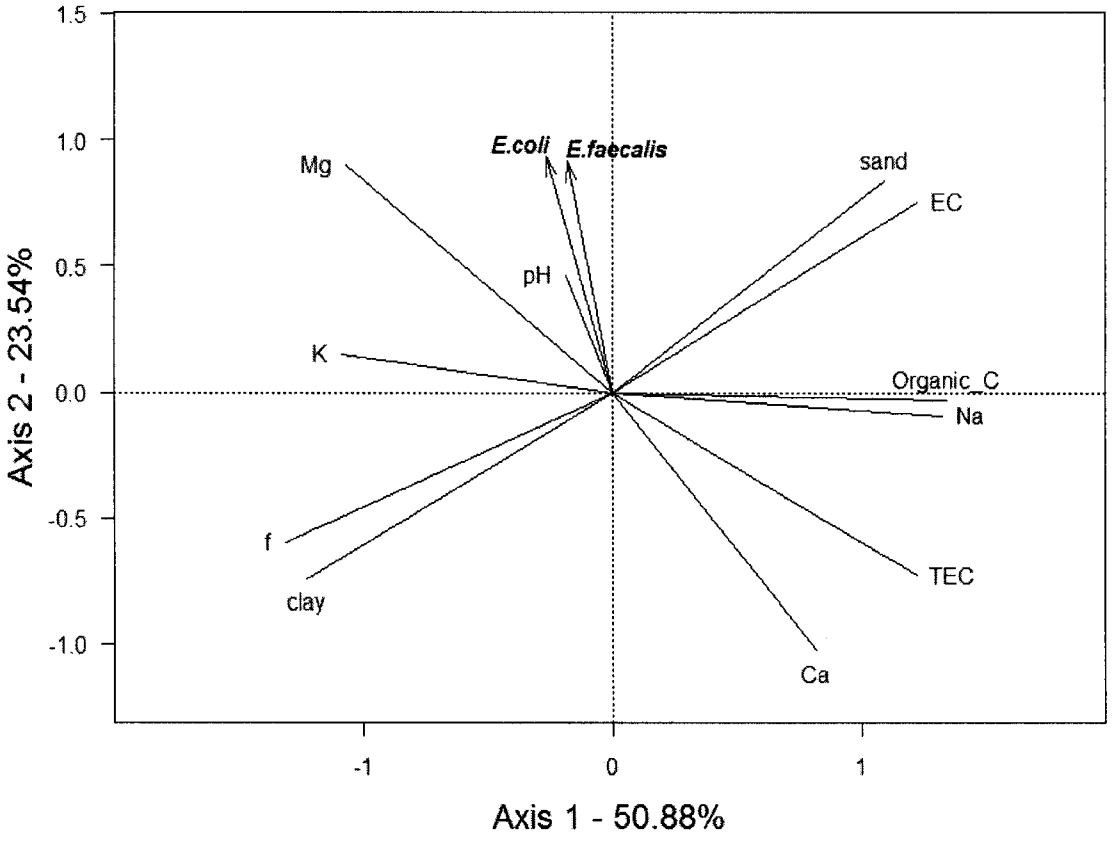

Figure 4.14: Principal component analysis (PCA) ordination biplot of experimental data from the field. Soil Components: electrical conductivity (EC), total exchangeable cations (TEC), magnesium (Mg), potassium (K), sodium (Na), calcium (Ca), concentration of hydrogen ions expressed as pH, estimated porosity (f), total organic carbon, percentage of clay in soil (Clay), percentage of sand in soil (Sand). Bold arrows represent the indicator organism: $CFUg^{-1}$ of *E. coli* adsorbed by the soil samples, and $CFUg^{-1}$ *E. faecalis* adsorbed by the soil samples.

4.4 Discussion

This application aspect of the study will be discussed under two themes: the adsorption potential of two indicator organisms and the adsorption partitioning coefficients in soil sampled from failed OWTS.

4.4.1 Adsorption Potential of Indicator Organisms in Contaminated Soils

In this study, soil samples were taken from three different failed OWTS. This is only a limited dataset; however, it can be used to make an initial assessment of the challenges that have to be overcome when conducting this type of study.

The first problem is identification of sites that have failed to meet the guidelines of the Safety Codes Committee. For this study, the Safety Codes Officers in Leduc County provided tremendous support with in identifying sites and obtaining permission from homeowners to sample their systems.

The second challenge was to search sites with similar history. This proved to be very difficult for which only three sites were sampled. Therefore, the observations and experimental results need to be interpreted with great caution.

One of the most interesting measurements was presence of *E. coli* and *E. faecalis* in the Ae horizons of the failed at-grade system. This horizon had been saturated with effluent when it drained back from a faulty lateral. The soil was sampled after the system had been moved to an alternative location. The magnitude of *E. coli* was 5.4 $x10^6$ CFU/gram of soil and 440 CFU/gram of soil of *E. faecalis.* These data show that the indicator organisms chosen for the clean soil were present in the contaminated soil. The relative magnitudes also show the differential behaviour of the indicator organisms.

E. coli was also present in the 10 to 30 cm depth of the failed septic system at a concentration of 78 CFU/ gram, but the number of *E. faecalis* was below the detection limit. There was a detectable number of *E. coli* in the abandoned, failed septic field, which indicates that this field did receive wastewater.

The surface soil at the mound/soil interface did not have any detectable numbers of indicator organisms. The mound itself was considered a failed system because treated effluent was leaking at the soil mound interface. Since this system

was still in use at the time of sampling, the mound itself was not sampled as this could ruin the integrity of the mound.

Overall, the data from this study indicate that *E. faecalis* and *E. coli* can both be used to measure faecal contamination of soil from OWTS.

4.4.2 Adsorption Partitioning Coefficients of Indicator Organisms in Contaminated Soil Samples

Soil samples taken from under the soil profile surrounding the distribution laterals provided insights into the adsorption process that takes place on soil that has been exposed to effluent on a long-term basis under field conditions.

The K_d values of soil samples from both horizons of the failed at-grade system and from the failed mound are comparable to the values calculated on clean soil samples. However, the K_d values of both organisms on the older, failed septic field soil samples were very low. As with the clean samples, higher K_d values occurred in the lower depths.

The K_d values on the soil samples from OWTS varied depending on where the soil was collected. The K_d value was of *E. faecalis* on the top soil sample from the mound of 633.10; However, the K_d value for *E. coli* on this soil was very low, 7.34. The initial concentration of *E. coli and E. faecalis* did not seem to adversely affect the K_d values on the failed at-grade soil samples.

The older septic field had K_d values of only 2.9 and 3.69 for the 10 to 30 cm depth and 1.58 and 7.82 in the 40 to 60 cm depth for *E. coli* and *E. faecalis*, respectively. Since this OWTS had been in operation for the longest period of time, and it was not taking up wastewater at the time of sampling, there may be other reasons for limited adsorption. At present, we do not have sufficient data to explain why this dispersal field failed.

In this initial model of contaminated soils, soil properties did not influence organism behaviour as expected. The correlation between clay content, porosity and organic carbon and adsorption was negligible at best. Properties that did correlate to variance of adsorption are the concentration of hydrogen ions (pH) and the concentration of magnesium in the soil. The total exchangeable cation and calcium concentration were inversely correlated. In this case, the variance in pH was

correlated with the adsorption of the indicator organisms. It is, however, a point to consider further in subsequent investigations since the pH difference between samples is only of one order of log.

4.5 Conclusions

The overall outcome of this experiment is an indication that the adsorption capacity in newer systems is comparable to that of clean soil. However, there is an indication that this adsorption efficiency may change over time. It is unclear whether this due to the adsorption competition by previously existing organisms or constituents from the effluent of onsite wastewater treatment systems, or the altered soil characteristics, or that the soil had simply reached its carrying capacity. In order to verify or dismiss these assumptions, further investigation of previously contaminated soil must be realized.

4.5 References

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Chapter 5

Conclusions

5.1 Major Experimental Findings

Onsite wastewater treatment systems used to treat wastewater from rural homes can be a source of fecal coliform contamination. In order to better understand how the microorganisms interact with different soil types and their horizons, two experiments were set up: a net die-off experiment where the net-die off rates of two indicator organisms was measured after being introduced into the Ah and Bt horizons of an Eluviated Black Chernozem and into the Ah, Ae, and Bt horizons of a Dark Gray Luvisol; and an adsorption experiment where the two K_d values of the same two indicator organisms were measured in the same soils as net die-off. Another adsorption experiment was conducted using the same two organisms and contaminated soil collected from under failed systems. From these experiments, we have concluded that two of the major factors influencing the net die-off and K_d values were temperature and soil type.

5.1.1 Temperature

Temperature is a major factor to consider when contemplating the design of OWTS in Canada. The average soil temperatures decrease from the south to the north. Therefore, one of the objectives of these experiments was to determine the effect of lower soil temperature on indicator organism behaviour.

The net die-off of both organisms was significantly different at different temperatures. Theory holds that the survival time of the organisms will increase as the temperature decreases. This held true for only half the soil horizons of the experiment. The half-life of the *E. faecalis* population ranged from 5.0 to 11.9 days at 23°C, 12.8 to 56 days at 5°C, and 20.6 to 45.1 days at -18°C in both soils. There was an increase in half-life as temperature decreased in all samples but those from the Ae and Bt horizons from Rocky Mountain House, which had longer half-lives at 5°C. The half life of the *E. coli* population ranged from 5.8 to 13.3 days in at 23°C, 4.9 to 16.3 days at 5°C, and 9.8 to 32.8 at -18°C. A similar increase in half life as temperature decreased was observed in the Gray Luvisol, but the soil horizons from Ellerslie showed a shorter half life at 5°C.

In general, the K_d values for *E. coli* for the Ah and Bt horizons on the soil from Ellerslie were higher at 23°C than 5°C. Similar trends were also observed for *E. coli* on the Ah horizon of the soil from Rocky Mountain House. However, an inverse trend was observed on the Ae and Bt horizons of this soil; the K_d values were higher at 5°C. On the other hand, the K_d values of *E. faecalis* were higher on the Ah and Bt horizons of the soil at Ellereslie at 5°C, and an inverse trend was observed on the three horizons of the soil from Rocky Mountain House.

From this study, the adsorption of both indicator organisms occurred in the major horizons of two soils but their behaviour was markedly different. This reflects the reality of differences in the structure and function of individual organisms. This is also a reminder that developing an indicator for environmental monitoring is a very complex task which requires thorough test of the behaviour of each indicator species in a wide range of soils.

5.1.2 Soil Type

Of the clean soils used in Chapter 2 and 3, soil type and its horizons significantly affected both the adsorption and net die-off rates as these were different in each soil type. One of the most evident differences between horizons was their ability to support regrowth of the indicator organisms when the soil had been previously sterilized. The Gray Luvisol from Rocky Mountain House proved less favourable than the Chernozemic soil, especially for *E. coli*. The growth rates of *E. faecalis* in the Gray Luvisol were particularly low. This was most likely caused by the low concentration of available nutrients available in this soil. In contrast, the nutrient and carbon rich Black Chernozem was more prone to regrowth of the indicator organisms in the absence of competition and predation from other soil microorganisms.

A correlation between soil properties and K_d values was found using a principal component analysis. This biplot shows that the adsorption of both organisms is correlated roughly the same way with all the studied soil properties. The soil clay content was not correlated to the organism adsorption. The organic carbon content and sodium concentration were inversely correlated in the clean soil samples. The soil pH was correlated to the K_d values only in soil samples from OWTS. Overall,

there were no clear trends between the different soil properties and an increase or decrease in adsorption of both indicator organisms.

5.1.3 Indicator Organisms

The indicator organisms, *E. coli* and *E. faecalis* are commonly used as indictors of pathogen pollution in wastewater and in recreational water. Though these organisms behaved quite differently in the different soil samples and with temperature with respect to their net die-off and adsorption partition coefficients, the PCA demonstrated that their variance was equally proportional throughout the adsorption experiments. In general, *E. coli* had a much higher die-off and regrowth rate than *E. faecalis.* Adsorption of *E. faecalis* in clean soil was higher than the adsorption of *E. coli.*

Although *E. faecalis* could be seen as the more conservative indicator, surviving longer and adsorbing more to soil, the rapid growth rate of *E. coli* suggest that it is preferable to use these indicators in combination when analyzing fecal pollution in soil.

5.2 Data Contribution to OWTS Design and Regulation Problems

The final intention of these data was to contribute to the development of better regulation for the soil-based component of OWTS. The first step in reaching this goal was to better understand the survival and die-off of indicator organisms in soil. Adsorption was better understood by obtaining the K_d water partitioning coefficients of the indicator organisms in soil.

As we are interested in the number of organisms moving though the soil as a contamination potential, it is possible to calculate this number by looking at the number of organisms introduced minus the number of organisms that have been adsorbed or have died off. This efficiency rate will change as the soil properties change with horizon. As shown in Figure 3.15, we can expect that as the effluent passes though the various horizons of the soil system, there will be fewer pathogens traveling though. However, how the adsorption and die-off rates change over time remains to be seen.

The low K_d values in the older, failed septic field was evidence that continuous use of the soil system may cause a decrease in K_d values over time. The exact cause of the lower K_d values is unclear, but it could be caused by competition from other organisms or chemicals added to the soil from the OWTS. Further investigation of these rates in other contaminated soils is necessary to verify this information and create new K_d values from which adsorption can be inferred.

The influence of temperature on the treatment efficiency of OWTS is complex because of the variable and low soil temperatures found in the soil surrounding these systems in Alberta. Although freezing temperatures can be expected in control soils, the warmth of the effluent entering the soil can overcome this freezing process. As a result, the average soil temperature in these systems is much higher than the surrounding soils. As such, the information from the net die-off and adsorption experiments in clean soil at 5°C are the most interesting to expand and investigate in onsite conditions.

5.2.1 Soil Quality Analysis

There is a need to be able to regulate the number of organisms entering the soil system so as to avoid transportation of pathogens. In water, these regulations are made by measuring the concentrations of indicator organisms such as *E. coli* and *E. faecalis.* This type of regulation cannot be made for the soil under onsite systems as there is a lack of knowledge of how these organisms react in soil. We have seen that both organisms react differently in different soil types and at different temperatures. Therefore, quality analysis would best be made by analyzing the soil adsorption capacity as well as the concentration of both organisms. Further studies could be made with other organisms or groups of organisms to validate their use as indicators, as well as further studies of adsorption in other soils.

Another way of approaching the problem of quality analysis of soil from under onsite systems is to look at the population dynamics of predator organisms. The exponential growth of indicator organisms in a sterilized, unhealthy soil is proof of the importance of these a healthy soil ecosystem under these systems. Further studies could be made to classify and quantify these organisms as their health could indicate whether the soil system is capable of promoting die-off of pathogens from OWTS.