OCCURRENCE AND SURVIVAL OF ZOONOTIC BACTERIAL PATHOGENS AND INDICATOR ORGANISMS IN GROUNDWATER AND SANDY SOIL FOLLOWING FIELD APPLICATION OF HOG MANURE

by

Janice Rogasky

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

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ABSTRACT

With growing concerns regarding the human and animal safety of manure application to agricultural fields, it is imperative that work be done to address and investigate the microbiological safety of this practice. Several organisms of concern, such as *Salmonella*, *Escherichia coli* and *Yersinia* are capable of survival in manure, soil and water environments. The objectives of the research were to examine coliform and *Pseudomonas* levels in well water from the experimental site following the application of manure, to examine survivability of pathogenic organisms in the well water and to determine if *Salmonella* could travel through soil columns taken from the study field. Results obtained indicated that only 2.3% of 603 water samples over 20 months contained confirmed positive coliforms, with only 2 instances of fecal coliform detection (one positive sample in the unmanured section of the field). The presence of coliforms appeared to be unrelated to the application of manure during these experiments. However, many of the well water sampling times were >100 d after manure application. The survival of pathogenic organisms in well water at 5°C revealed that *Yersinia* persisted throughout the 91 d testing period in the highest numbers, while *Salmonella* survived the least well, between 21 to 56 d. *E. coli* was detectable until the end of 91 d, but in lower numbers than *Yersinia*. From the soil column perfusion experiments, *Salmonella* were detected in the effluent from 4/6 columns, however in low numbers (0.01-0.11% of the added inoculum). Recovery of *Salmonella* in soil from dissected columns revealed the highest concentrations of the organism in the top 20 cm of the column. From these results, it was determined that the sandy soil was capable of filtering

large quantities of *Salmonella* from inoculated water, which may aid in the prevention of contamination of groundwater following the application of manure to agricultural fields.

Chapter 1

INTRODUCTION

There is concern regarding the human and animal health effects of manure application on agricultural land. Agricultural waste application to agricultural soil can serve as a potential source of pathogenic bacterial contamination of surfacewater, groundwater and soil (Abu-Ashour et al, 1994; Bicudo and Goyal, 2003). It is therefore recommended that there is a holding period of a minimum of 30 d between manure application and land use, to reduce survival of zoonotic bacteria in manure-treated soil (Holley et al. 2006). Organisms of concern, such as *Salmonella*, are capable of extended survival at 4°C when present in manure slurries and have been recovered >300 d (Arrus et al. 2006).

Many pathogenic bacteria are also capable of survival at cool water temperatures similar to those of the aquifers present in Manitoba. *E. coli* has been reported to survive in water for 100 to >300 d at 5°C (Bogosian et al., 1996; Jones, 1999; Rattray et al, 1992; Olson, 2001). *Salmonella* has been reported to survive > 6 months in water at 5°C (Olson, 2001). *Yersinia* is highly resistant to environmental stress, such as low temperature, and has been recovered in water samples up to 64 weeks at 4°C (Karapinar and Gonul, 1991).

Pathogenic organisms such as *Salmonella* have also been recovered from liquid manure-treated soil samples, up to 20 cm deep, 54 d following manure application (Cote and Quessy, 2005). Nicolson et al. (2005) also recovered *Salmonella* in 15 cm deep soil

sample columns 32 d following manure application. Therefore the potential for pathogenic organisms to travel into shallow wells or groundwater is a significant concern, especially since they are capable of survival if they reach the groundwater.

The objectives of this research were three fold. The first objective was to assess the transport and survival of coliform bacteria and *Pseudomonas*, and their potential effects on water quality following the field application of manure. The second part of the research was to determine if *Salmonella*, *E. coli* and *Yersinia* isolated from hog manure could survive in well water at temperatures consistent with the Assinibione Delta aquifer that ran under the experimental site. The third objective was to determine if *Salmonella* could travel through drilled, perfused soil columns taken from the Green Farm experimental field.

Chapter 2

LITERATURE REVIEW

2.0 Introduction

Since the beginning of the twentieth century there have been environmental and public health problems observed associated with the spreading of liquid waste on land (Abu-Ashour et al., 1998). However, there are advantages to this process. Application of animal/human waste to agricultural soils can restrict movement of undesirable contaminants and provide opportunity for their degradation. Soil application may remove some of the pollutants from the applied sewage which might otherwise end up in water supplies and it can also increase crop yields by supplying essential nutrients and improving soil organic matter quality (Abu-Ashour et al., 1994; 1998). Disadvantages include compromising surface and groundwater quality through chemical or microbial contamination and by heavy metal accumulation, which could occur if the amounts applied are excessive or if application is timed incorrectly. There is the possibility of environmental problems occurring even when guidelines and application procedures are followed because of frequent or heavy rainfall following application (Abu-Ashour et al., 1994; 1998). Agricultural waste application to soil can be a source of pathogens for contamination of surfacewater, groundwater and soil (Abu-Ashour et al., 1994; Bicudo and Goyal, 2003). Contamination of water following manure application to fields is a serious issue but is largely avoidable. Bacterial pathogens and disease causing human viruses have been detected in groundwater following manure treatment of adjacent land.

The potential for water contamination is increased if soil structure allows microorganisms to migrate through the soil and is further increased if the microorganisms present have the capacity to survive for an extended period of time (Abu-Ashour et al., 1994).

2.1 Zoonotic pathogens of concern in hog manure

2.1.1 *Escherichia coli* O157:H7

Escherichia coli is the major facultatively anaerobic bacterium present in the human and animal gut and can be found at levels of 9 log colony forming units (cfu)/g (wet weight) of feces (Mawdsley et al., 1995). There are many biovars of this organism with a few being responsible for diarrhoeal outbreaks. Pathogenic *E. coli* can be found in water, soil and manure with < 1 % being potentially harmful (verocytotoxigenic, VTEC) strains, and with the most common of these being O157:H7. In healthy pigs the frequency of VTEC *E. coli* is between 0.4% and 7.5% and in pork products the frequency is about 1.5% (Olson, 2001). The danger to humans arises when *E. coli* contaminates food or water supplies. The symptoms vary in degree of severity and include bloody diarrhea, abdominal cramps and in the young and elderly it can lead to the hemolytic uremic syndrome (kidney failure due to destruction of red blood cells) which can lead to death, kidney failure, seizures, blindness or high blood pressure (Olson, 2001). Hog manure is not considered a consistent source of this organism since hogs rarely excrete verotoxin producing *E. coli* O157:H7 (Olson, 2001). Verotoxins produced by *E. coli* O157:H7 can cause hemorrhagic colitis (diarrhea that becomes profuse and bloody), hemolytic uremic

syndrome (bloody diarrhea that leads to renal failure) and thrombocytopenic purpura (similar to previous symptoms with central nervous system involvement) (Bicudo and Goyal, 2003; Pell, 1997).

Infection with *E. coli* in adults is usually an inconvenience, but the infection in the very old or very young or in immunocompromised individuals may become a life threatening challenge (Mawdsley et al., 1995). Outbreaks of illness in humans caused by *E. coli* O157:H7 have been attributed to consumption of ground beef, raw milk, water, unpasteurized apple cider or fruits and vegetables contaminated with the organism (Chapman et al., 1997; Lung et al., 2001). Recognition that *E. coli* O157:H7 was a foodborne pathogen occurred in 1982 and since then it has been implicated as a causative agent of serious human illness worldwide (Wang et al., 1996). For clinical symptoms to develop in humans an infectious dose of only 10 to 50 cells of *E. coli* O157:H7 is required (Jones, 1999).

It has been well established that the primary reserviour of *E. coli* O157:H7 is cattle, however, other farm animals such as sheep and goats have also been shown to be carriers of *E. coli* O157:H7 (Jones, 1999). *E.coli* O157 was isolated from 752 (15.7%) of 4800 cattle, 22 (2.2%) of 1000 sheep and from 4 (0.4%) of 1000 pigs (samples collected from the same abattoir), however it was not isolated from 1000 chickens (samples collected from same processing plant) (Chapman et al., 1997).

Even though many cases of *E. coli* O157:H7 food poisoning are related to contaminated meat and dairy products, there is also evidence that human infections have been the result of contamination through soil, fruit and vegetables, unpasteurized juices, fresh apple cider, and water has also been implicated (Jones, 1999; Yu and Bruno, 1996). *E. coli* O157:H7 illness outbreaks have also been linked to lettuce, radish sprouts and alfalfa sprouts (Beuchat, 1999). Other sources of infection include mayonnaise, delicatessen foods, lamb, venison, deer jerky, cured salami and by direct contact between contaminated animals and humans (Kudva et al., 1998).

Contamination by this organism can occur when cattle or other animals enter fields when improperly composted manure has been applied as a fertilizer (Beuchat, 1999). Runoff water from recently fertilized fields, the application of sewage sludge that has been improperly managed or the use of non-potable irrigation water on fields can also be potential contamination sources (Beuchat, 1999). Animal manure contains many viable bacteria, including pathogens such as *E. coli* O157:H7 and the number present is dependent upon animal diet, association with other animals that may carry the organism, age and physical condition of the animal and the season, with animal shedding of this organism being higher in the summer. Composting and storage of manure at >25°C will reduce the numbers of this organism in manure (Gagliardi and Karns, 2000; Guan and Holley, 2003).

It has been shown that in response to environmental stress, *E. coli* cells can lose their ability to grow on selective media but still remain viable (McKay, 1992). Since *E. coli*

has the ability to survive environmental stresses at low temperature, the organism can persist for extended periods in water, soil or manure. There is potential for its contamination of surface waters and even percolation through the soil with repeated manure applications (Olson, 2001).

2.1.2 *Salmonella*

Salmonella is a Gram negative rod-shaped lactose-negative bacterium which can reside asymptomatically in the intestines of mammals, birds and reptiles (Mawdsley et al., 1995; Olson, 2001). Disease (salmonellosis) caused by these organisms is characterized by septicaemia, acute or chronic enteritis. Other symptoms include nausea, vomiting, cramps, diarrhea and in some cases arthritis (Pell, 1997).

The feces of infected animals have the potential to contaminate feed, water, milk, fresh and processed meats or plant and animal products (Olson, 2001). Traditionally, *Salmonella* has been associated with foods of animal origin such as poultry, eggs, meat and dairy products (Beuchat, 1995; Guo et al., 2002). However, manure used for fertilization of crops consumed without cooking (fruits and vegetables) may be a source of human infection since it can often be contaminated with *Salmonella* (Olson, 2001). There have been many outbreaks of human gastroenteritis due to the consumption of contaminated fresh vegetables and fruits. Human cases of salmonellosis have been linked to the consumption of contaminated tomatoes, mustard cress, bean sprouts, cantelope and watermelon (Beuchat, 1995). *Salmonella* has been found in eggplant,

cauliflower, peppers, endive and lettuce (Beuchat, 1995). Fresh fruits and vegetables are a staple part of the diets of many people around the globe, whether grown themselves or purchased. Pathogens such as *Salmonella* can contaminate produce by their presence in irrigation waters or in the soil. Contamination can occur during plant growth, during harvesting, postharvest handling, processing or distribution (Beuchat, 1995). Water contaminated with runoff from manure-treated fields following heavy rains or directly by animal defecation may also be a source of human infection if it is consumed or used for washing of uncooked or unprocessed foods (Olson, 2001). Salmonellosis has also been associated with different types of seed sprouts as well as unpasteurized apple and orange juices (Guo et al., 2002).

2.1.3 *Yersinia*

Yersinia is an infrequent human pathogen (a Gram negative bacterium) which has the capability to grow at normal refrigeration temperatures (Chao et al., 1998; Karapinar and Gonul, 1991; Lee, 1997), even in nutritionally poor conditions (Karapinar and Gonul, 1991).

Y. enterocolitica has been recovered from the feces of pigs (Chao et al., 1988; Lee, 1977; Olson, 2001; Walker and Grimes, 1985), dogs (Chao et al., 1988; Walker and Grimes, 1985) and cattle (Olson, 2001). There have been several studies which implicate *Y. enterocolitica* contaminated food and water with human infections (Chao et al., 1988). Swine are the predominant natural reservoir for *Y. enterocolitica*, but the pathogen has

also been isolated from raw vegetables as well (Beuchat, 1995). A study examining 1420 healthy finishing pigs from Ontario, Quebec and Manitoba showed that 5.2% were positive for *Yersinia*, and 24.6% were carriers for one or both *Salmonella* or *Yersinia* (Letellier et al., 1999).

The symptoms of infection vary depending on the age of the patient. *Y. enterocolitica* causes fever, abdominal pains and diarrhea (Olson, 2001). In infants, fever and diarrhea are the primary symptoms, whereas children who are older exhibit mesenteric lymphadenitis and ileitis (Lee, 1977). Adults may have abdominal pains, acute enteritis arthritis and erythema nodosum. Septicemia can develop in the immunocompromised and the aged (Lee, 1977). Similar to *Salmonella*, *Yersinia* infections can be systemic following invasion of tissues other than the digestive tract, leading to formation of abcesses, other lesions and eye infections (Lee, 1977).

2.1.4 Occurrence of zoonotic bacterial pathogens in hog manure

The prevalence of *Salmonella* spp., *Escherichia coli* O157:H7 and *Yersinia enterocolitica* in pigs was reported to be 38%, 0.4% and 18%, respectively (Olson, 2001).

The association of human foodborne disease with hog products is a public health concern and can have significant negative economic impacts (Baloda et al., 2001). Bacterial transport in porous media like soils has been an area of great interest for community health, for its influence on soil and water pollution and in the biological control of plant

root diseases. The disposal of domestic and industrial wastes by application to the land has generated tremendous concern. Pathogenic bacteria and viruses present in the waste have the potential to contaminate the groundwater and numerous outbreaks of illness from this source have been reported (Tan et al., 1992). The application of manure to land is the most frequent disposal method used in Canada by dairy, swine and beef operations (Joy et al., 1998). The application of liquid sewage to land is increasingly practical because of concerns regarding surface water pollution and the realization that sewage can be a good source of nutrients for crops (Lance et al., 1982). However, waste from livestock, which can contain many pathogenic organisms including bacteria, viruses and protozoa, can be a significant source of environmental contamination following its application to land (Mawdsley et al., 1995). Even with guidelines which stipulate maximum application rates, there have been many cases of beach closures and contamination of rural wells where liquid manure application has been identified as the cause of contamination (Joy et al., 1998).

Animal waste is a valuable plant nutrient and due to the high cost associated with its transport, manure is generally applied near its origin (Lung et al., 2001). Raw fruits and vegetables contaminated with manure or fecal material during growth, harvest or processing have led to foodborne illness outbreaks caused by *Salmonella* and *E. coli* (Lung et al., 2001).

Manured fields have been shown to have higher bacterial numbers than non-manured fields. On treated fields the highest bacterial concentrations were present in surface

water, compared to tile drain water (Joy et al., 1998). A variety of conditions can influence the survival of pathogenic bacteria and these include: the source of manure, temperature, dry matter content, pH, bacterial numbers and type, aeration and the length of time which the manure or slurry is held before application (Himathongkham et al., 1999; Kudva et al., 1998; Pell, 1997). The survival of microorganisms in manure is dependent upon exposure to sunlight, drying, freezing and thawing cycles, high temperatures, high or low pH and oxygen (Pell, 1997). Because these factors interact to influence bacterial survival, it is often difficult to accurately predict the length of time pathogens will survive in manure (Joy et al., 1998).

Contamination of food and water by microorganisms from animal manure can occur as a result of non-point or point sources (Gagliardi and Karns, 2000). Non-point source contamination occurs during the free roaming of animals or by spreading manure on fields as fertilizer or when fields are used as a waste disposal site. Point source contamination develops at animal feedlot locations, sites of animal housing, or at manure storage facilities (ie. lagoons). A point source can become a non-point source of manure or pathogens through run-off or leaching which can spread to other fields or a water supply (Gagliardi and Karns, 2000). Even if pathogens are initially present in low numbers in water, they have the potential to multiply when environmental conditions are appropriate in the presence of adequate nutrients (Gagliardi and Karns, 2000). Several pathogenic bacteria were reported to decrease only slightly in number during the first 100 days exposure in groundwater (Gagliardi and Karns, 2000), including *E. coli*, which in autoclaved filtered river water showed no loss of viability after storage at 4°C to 25°C for

up to 260 d (Flint et al., 1987) as well as *S.* Typhimurium, *E. coli* and *P. aeruginosa* which survived up to 100 d in groundwater at 10°C (Filip et al., 1988).

Direct contamination of produce, water supplies, animals or humans also can result from improper manure handling (Guan and Holley, 2003). With regards to run-off and water supplies, a contaminated water supply has the capability to affect large numbers of people (Guan and Holley, 2003). The most important factor associated with spread of contaminated run-off from manure treated fields is the occurrence of a rainfall event shortly following the application of manure. This factor is more important than the manure application rate or the field condition before application in determining the potential for environmental contamination (Guan and Holley, 2003). Most of the manure-associated outbreaks of human illness are more frequently associated with bovine manure than with other types. This has led to increased study of pathogens in bovine manure compared to other types (Guan and Holley, 2003).

2.1.4.1 Manure as a vehicle for zoonotic pathogen distribution

It is a common and convenient practice in North America to apply manure in a liquid form to fields. There are few adverse environmental consequences with this process under the proper conditions (Joy et al., 1998). If weather conditions are adverse and excessively high manure rates are used, bacteria or other contaminants that may be present can contaminate ground and surface waters (Joy et al., 1998; Pell, 1997). There is evidence that many bacteria possess the capacity to contaminate surface water after land application through infiltration of the soil and movement to subsurface tile drains

and then flow to streams and lakes (Joy et al., 1998). From their experiments, Joy et al. (1998) used biotracers and found 100 cfu/100ml nalidixic acid resistant *E. coli* in tile drain water samples at several sites, with one sample containing 1000 cfu/100ml. They concluded that with the close location of drains to a ditch, it was possible that contamination of surface waters could occur if conditions were appropriate (such as rain 24 h following application). Within 24 h of application, the biotracer generally penetrated the soil column to 900 mm (Joy et al., 1998).

2.1.4.2 *Escherichia coli* survival in manure

Escherichia coli can be problematic in manure because the organism can cause serious human health problems and has the capacity to survive under adverse conditions (Pell, 1997). A potential risk from the disposal of animal fecal waste is the spread of enteric pathogens (Jiang et al., 2002).

In 1998, an Ontario farm well supplying water to the farmhouse became contaminated with surface water containing bovine manure. The well had a shallow design and subgrade location with a defective well head. About 63% of the cattle on the farm were positive for *E. coli* O157:H7. This situation also led to an outbreak of *Escherichia coli* O157:H7 diarrhoeal infections in humans (Jackson et al., 1998). Manure from cattle used to fertilize soil for raising potatoes has also caused human illness (Chapman et al., 1997).

It has been shown that *E. coli* O157:H7 present in bovine feces was able to survive for approximately 70 days with their ability to produce verotoxin intact when inoculated at 5 log cfu/g at 5°C (Beuchat, 1999; Kudva et al., 1998; Wang and Doyle, 1996). It has been shown that *E. coli* can survive for up to 11 weeks in cattle slurry (Mawdsley et al., 1995). The length of survival of *E. coli* in soil was lower than in cattle manure slurry, with survival in soil ranging from 7-8 d to several weeks (Mawdsley et al., 1995). When *E. coli* was present in cattle feces, the organism survived > 100 d at low temperatures (< 0°C, 5°C) but only about 10 d at 30°C (Olson, 2001).

2.1.4.3 *Salmonella* survival in manure

In cattle manure, *Salmonella* was found able to survive > 6 months, 12-28 weeks and 4 weeks at frozen, cold (5°C) and warm (30°C) temperatures, respectively (Olson, 2001). *Salmonella* at 22°C was able to survive for 13-75 d in cattle manure slurry but survived in cattle feces at 5°C for 12-28 weeks, but only for 4 weeks at 30°C. *Salmonella* has been shown to survive for 286 days at 22°C in cattle manure slurry, however, it is important to note that in many cases there were 90% reductions of *Salmonella* within a month (Pell, 1997).

2.1.4.4 *Yersinia* survival in manure

In cattle feces, *Yersinia* was found to survive for more than a year at temperatures below 0°C, between 30-100 d at 5°C and for 10-30 days at temperatures of 30°C (Olson, 2001).

Swine feces were collected and analyzed for *Y. enterocolitica* from three farms over two sampling periods. From the samples, 34 strains of *Y. enterocolitica* were isolated with all but one coming from swine feces. The latter was isolated from the runoff water in a stream located 100m away from the swine facility (Walker and Grimes, 1985). Through biochemical analysis, it was shown that the isolate from water was identical with a swine isolate, and it was concluded that swine feces could serve as a vehicle for the introduction of *Y. enterocolitica* into water (Walker and Grimes, 1985).

In 1984, heavy springtime rains may have contributed to animal fecal contamination of a well used for drinking water, where *Y. enterocolitica* was isolated from the well and from infected family members (Thompson and Gravel, 1986). This observation implies that *Yersinia* can be transported to water supplies from fecal material through runoff water or seepage into the groundwater (Chao et al., 1988).

2.2 Pathogen survival in soil

There are many factors that affect the survival of pathogens in the soil environment, including the strain of microorganism and its physical state. The physiological and chemical nature of the soil containing the organism, including: pH (a neutral pH favours bacterial survival, whereas acidic soil conditions increase bacterial lethality), soil porosity, organic matter content (an increase in organic matter allowed for increased survival), texture and particle size distribution (finer soils have increased water retention and allow increased bacterial survival), soil hydrophobicity, available carbon in

rhizospheres, clay content and bulk density, pore size distribution, soil elemental composition, temperature (increased survival is associated with lower temperatures, whereas higher temperatures - especially when combined with dry conditions, increase bacterial lethality). If freezing and thawing occur, there will be a decrease in bacterial viability as well. Soil moisture content (survival increases with increasing moisture), soil adsorption and filtration properties, as well as the availability of nutrients (bacterial survival decreased in lower nutrient environments due to the inability of enteric bacteria to lower their metabolic requirements when exposed to lower nutrient environments outside animals), and microbial interactions (Abu-Ashour et al., 1994; Benckiser and Simarmata, 1994; Crane and Moore, 1984; Guan and Holley, 2003; Jiang et al., 2002; Mawdsley et al., 1995; Moore et al., 1981; Muribu et al., 2000; Reddy et al., 1981; Terzieva and McFeters, 1991; Unc and Goss, 2003; Wang and Doyle, 1998). Atmospheric conditions such as: sunlight (shorter survival at surface), humidity, precipitation (increased survival in wet conditions), and temperature (lower temperatures favour increased survival) can all affect the survival of bacteria in soil (Abu-Ashour et al., 1994; Benckiser and Simarmata, 1994; Crane and Moore, 1984; Gagliardi and Karns, 2000; Jiang et al., 2002; Mawdsley et al., 1995; Moore et al., 1981; Muribu et al., 2000; Reddy et al., 1981; Terzieva and McFeters, 1991). Biological interations among organisms such as competition from the indigenous microflora, as well as production of antibiotics and toxic substances all hinder the survival of bacteria in soil (Abu-Ashour et al., 1994; Benckiser and Simarmata, 1994; Crane and Moore, 1984; Jiang et al., 2002; Muribu et al., 2000; Reddy et al., 1981; Terzieva and McFeters, 1991). The method used to add organisms to the soil, whether surface applied or injected, the nature of the

biosolid (manure slurry or solid manure from different animal species), the frequency and amount of manure applied, as well as the organism density in the added material, all contribute to the length of survival of bacterial pathogens in soil (Crane and Moore, 1984; Jiang et al., 2002; Jones, 1999; Reddy et al., 1981; Unc and Goss, 2003).

2.2.1 *Escherichia coli* survival in soil

It has been shown that *E. coli* O157 has the capability to remain viable in soil for 4 months or longer. The organism exhibits high resistance to environmental stresses, such as low temperatures of 4°C, and has even shown the capacity for growth at 6°C (Jones, 1999). Non-O157 strains of *E. coli* have been shown to survive for more than 60 d at 25°C and 100 d at 4°C (Jones, 1999). Since *E. coli* O157:H7 has a low infectious dose, even though the risk of contamination by this organism diminishes after the first rainfall event, as long as viable organisms remain in the soil there is some risk (Ogden et al., 2001). Chandler and Craven (1980) added *E. coli* and *Salmonella* to dry soil and showed that these organisms had the ability for regrowth when the soil was moistened after 14 d (Chandler and Craven, 1980). In other work, *E. coli* survived in soil for more than 300 d, 100 d and 2 d at frozen temperatures, 5°C and 30°C, respectively (Olson, 2001). Under dry weather conditions on well drained sandy soil, *E. coli* present on the soil or grass will decline in numbers due to desiccation and natural UV radiation exposure (Ogden et al., 2001).

In manure-amended autoclaved soil incubated at 5, 15 and 21°C, it was seen that *E. coli* O157:H7 cells were able to survive for up to 77, >226 and 231 d, respectively (Jiang et al., 2002). When experiments were conducted with non-autoclaved soil with added manure, *E. coli* cells were able to survive for 56, 152 and 193 d when incubated at 5, 15 and 21°C, respectively. The more rapid decrease in cell numbers in non-autoclaved soil was attributed to antagonistic interactions among the natural soil microorganisms (Jiang et al., 2002).

E. coli and *Salmonella* numbers were studied following the application of liquid hog manure on surface soil used for the growth of pickling cucumbers (Cote and Quessy, 2005). Upon analysis of the manure applied, *E. coli* was present in years 2 and 3 at 5.43 and 6.11 log cfu/g, respectively. *Salmonella* was detected in the third year application of liquid hog manure, at undisclosed numbers. The pathogens were not recovered from the cucumbers, but were detected in the soil up to 8 weeks from application in some samples. The numbers of *E. coli* recovered was between 1.78 and 2.5 log cfu/g at day 56. *Salmonella* was present in one sample at day 54 (Cote and Quessy, 2005).

2.2.2 *Salmonella* survival in soil

The survival of S*almonella* in soil that has been amended with animal waste can pose a potential risk for the transmission of infection by transfer of the pathogen back to animals which graze on treated soil (Baloda et al., 2001). Of 30 soil samples tested for the presence of *Salmonella,* by the analysis of the upper 10 cm layer of the soil surface, a

total of 26 were positive. Of 10 samples tested immediately after spreading of the contaminated slurry, 9 were positive at day 0 and 7, 5 and 5 were positive 2, 6 and 14 d respectively, after application of the contaminated slurry on soil (Baloda et al., 2001). All 10 of the treated soil samples tested on day 21 for *Salmonella* were negative. It was concluded that the recovery of viable *Salmonella* cells from soil under natural environmental conditions even after 14 d represents significant risk (Baloda et al., 2001).

At 5°C and 30°C in soil, survival of *Salmonella* was longer than *E. coli*. In frozen, cold (5°C) and warm (30°C) soil, *Salmonella* was able to survive longer than 12 weeks, 12-28 weeks and 4 weeks, respectively (Olson, 2001). In other work, *Salmonella* Typhimurium was found to survive in soils for at least 110 d (Mawdsley et al., 1995). It was also shown that *Salmonella* could survive in high numbers in moist soil for more than 45 d at 20°C, with numbers decreasing by 1.4 log cfu/g (from an initial population of 8.15 log cfu/g) (Guo et al., 2002).

In experiments conducted with differing soil moisture and temperatures, it was observed that *S.* Typhimurium died within 1 week in dry soil at 39°C. However, the survival at 5 and 22°C was similar (up to 63 d) and was generally longer than that at 39°C (Zibilske and Weaver, 1978).

The capacity of *Salmonella* to survive in soil can allow for its spread to grazing farm animals, birds, cats, dogs, rodents and even humans. Therefore, there must be effective waste management protocols devised which take into account the potential long term

survival of pathogens such as *Salmonella* in soil which has been treated with slurry (Baloda et al., 2001).

2.2.3 *Yersinia* survival in soil

Yersinia enterocolitica was able to survive in frozen soil or in soil at temperatures of 5°C for a year or longer (Olson, 2001). At soil temperatures of 30°C, the organism survived for approximately 10 d (Olson, 2001).

2.3 Bacterial movement in soil

Any soil characteristic or factor which favours bacterial survival has the potential to have an affect on the numbers of bacteria leached through the soil environment. These factors include pH, temperature, rainfall, amount of organic matter and the presence of other organisms in the soil (Abu-Ashour et al., 1994; Abu-Ashour and Lee, 2000; Bicudo and Goyal, 2003; Crane and Moore, 1984; Huysman and Verstraete, 1993; Kemp et al., 1992; Mawdsley et al., 1995; Reddy et al., 1981; Unc and Goss, 2003).

Physical soil characteristics also will have an affect on the numbers of organisms and their movement through the soil environment. The texture (sand, silt and clay) and structure of the soil (porosity) are also major factors that affect movement of organisms. Finer textured soils possess smaller pore sizes and therefore greater movement of bacteria is seen in coarse soils with large pore spaces (Abu-Ashour et al., 1994; Bicudo and Goyal, 2003; Crane and Moore, 1984; Gannon et al., 1991; Mawdsley et al., 1995).

Studies have shown that there is increased movement of bacteria in water-saturated soils. Percolating water, either through irrigation or rainfall, will affect the movement of organism through the soil matrix (Mawdsley et al., 1995; Unc and Goss, 2003). The intensity of rainfall affects the rate and extent of translocation of microorganisms, and thus the faster the water flows the greater is the movement of bacteria. Saturated water flow (water flow is through large pores and channels) overcomes the filtering effect of the soil and thus the risk of both chemical and biological pollution is increased (Mawdsley et al., 1995).

Much of the movement of contaminants through the soil occurs as particulate flow. This is because they are often inherently particulate (perhaps due to low solubility) or because they are adsorbed onto mobile particles already present in the soil (McGechan and Lewis, 2002). Smaller microorganisms (bacteria, viruses) are mainly transported via adsorption to mobile colloidal clay particles or soil organic matter (McGechan and Lewis, 2002).

The migration of microorganisms through the soil can pose a serious threat to both surface and groundwater quality and safety (Abu-Ashour et al., 1994). Their migration can occur over significant distances, particularly if preferential flow occurs (Abu-Ashour et al., 1994). Preferential flow occurs when there is rapid transport of water and solutes through soil channels (Bundt et al., 2001). This phenomenon can allow solutes to bypass a large part of the soil matrix and reduces the effectiveness of soil filtration which normally reduces bacterial mobility (Bundt et al., 2001).

Migration of microorganisms through soil is largely by preferential flow through macropores (Abu-Ashour et al., 1994, 1998; Unc and Goss, 2003). Macropores are formed in agricultural soils in a number of ways (McGechan and Lewis, 2002). They can be created by worm movement (Abu-Ashour et al., 1994, 1998; Mawdsley et al., 1995; McGechan and Lewis, 2002), from cracks or fractures in dry soil (Abu-Ashour et al., 1994, 1998; Mawdsley et al., 1995; McGechan and Lewis, 2002), or from channels left by decayed plant roots. The growth of plant roots in general, increases the translocation of bacteria through the soil and this may be increased even further by percolating water (Bundt et al., 2001; Kemp et al., 1992; Mawdsley et al., 1995), and animal activity in the soil (Abu-Ashour et al., 1998; Gannon et al., 1991) especially if there are spaces which can become water filled under wet soil conditions (McGechan and Lewis, 2002). The pathways of preferential flow are more susceptible to drying and wetting when compared to the soil matrix, and because of these changes, serve as a better source of nutrients compared to the soil matrix (Bundt et al., 2001; Gannon et al., 1991). Due to the size of bacterial cells, it is suggested that they are primarily transported through larger soil pores (Unc and Goss, 2003) however, gravity influences the direction of their travel.

The geometry of macropores in a field is dynamic and changes occur in response to wetting, drying, freezing and thawing events, or burrowing of animals (Gannon et al., 1991).

It was concluded following a study performed by Abu-Ashour et al. (1998), that if the amount and rate of rainfall was high enough following manure application, bacteria

present in the soil could reach the groundwater, leading to contamination, especially if there were macropores present in the soil. It was also shown that macropores in wet soil allowed up to 83% more movement of nalidixic acid resistant (NAR) *E. coli* through unsaturated soil columns than when compared to soil without macropores (Abu-Ashour et al., 1998). Preferential flow effects upon microbial mobility have been observed in both the laboratory and field settings (Abu-Ashour et al., 1994). Research has indicated that once bacterial cells enter the macropores they are relatively unrestricted during passage through the profile of the soil and their movement is maximized. In undisturbed soil cores there was increased transport observed as the water bypassed the filtering effects of the soil and flowed through macropores (Mawdsley et al., 1995).

Reductions in bacterial viability, competitive interactions within the soil, retention of organisms within applied manure or constricted macropore paths, and adhesion to soil surfaces result in reduced contaminant dispersion (Unc and Goss, 2003). The total soil porosity was found to be a poor indicator for estimating the potential for deep transport of bacteria because of size exclusion effects (Unc and Goss, 2003). When compared to the measured average pore water velocity, bacteria present moved up to 35 times faster than expected, with greater speeds observed in finer textured soils, even though the latter had a smaller total porosity. This indicates that a pore size exclusion effect occurred which resulted in the relatively large bacterial cells moving through the soil macropores at an accelerated speed (Unc and Goss, 2003). Macropore flow can be induced by a small amount of added liquid and can mediate transport of a highly concentrated suspension of bacteria regardless of soil moisture (Unc and Goss, 2003).

It was concluded that the application of manure to fields presents a potential risk for groundwater contamination by fecal bacteria especially if fields are located on shallow water tables and contain a high percentage of macropores (Unc and Goss, 2003).

The use of soil columns for bacterial transport studies is considered by many to be prone to generate misleading results (Gannon et al., 1991). Soil which has been mixed or sieved prior to use will show little structural resemblance to undisturbed soil from the field, and may show significantly less microbial movement than intact soil cores (Mawdsley et al., 1995).

In general, except for *E. coli* O157:H7 where there is little difference, the ability of pathogenic organisms to survive is better in water than in soil or manure. In all three environments the organisms survive longer at lower temperatures (Guan and Holley, 2003).

2.3.1. Laboratory studies of bacterial transport in soil

The presence of macropores limits the retention of bacteria, viruses and solutes in the soil profile and increases the possibility of groundwater contamination (Smith et al., 1985). It was observed that when channels and pores were removed from soils in a laboratory setting by mixing part or all of the soil in the column, they became more effective bacterial filters, regardless of the type of soil (Smith et al., 1985).

Bacterial pathogen movement through the soil profile is usually required before groundwater can be contaminated, however runoff also can contaminate groundwater directly, particularly where shallow soils are located above groundwater (McMurry et al., 1998). Therefore the depth of soil to the water table or bedrock is important in determining the potential for contamination (McMurry et al., 1998).

When transport studies were performed on disturbed cores (uniformly packed and thus destroying macropore presence) they retained a minimum of 93% of applied cells while intact cores (macropores present) retained 21 to 78% of bacteria (Smith et al., 1985). Up to 96% of *E. coli* that were added onto the surface of 280 mm deep columns were recovered in the effluent when the columns were intact (Smith et al., 1985). Experimental results from soil columns obtained by Smith et al. (1985), showed that with moderate to high rates of water addition, suspended bacteria travelled rapidly through the profiles of well-structured soils. It was concluded that this was a result of macropore transport (Smith et al., 1985).

There are two aspects to water movement through the soil. One of these is represented by flow through the soil matrix, where movement of *E. coli* is delayed and is preceeded by Cl^- ions, when the latter are added as a marker. The second is flow through macropores where Cl^- ions would move slower or at the same rate as *E. coli* and because each travels in water with little interaction with the soil (McMurry et al., 1998; Smith et al., 1985). To explain the latter, when Br^- or Cl^- were used as tracers in experiments conducted by Harvey and George (1989), it was found that the peak breakthrough of

DNA-specific fluorochrome labelled bacteria was in advance of the ion tracers used. The faster transport of the bacteria compared to the tracer may have been due to their larger size since bromide (chloride) would be subjected to the effects of particle surface roughness to an increased degree than the stained bacteria. It was concluded that even though the Br^- and Cl^- tracers were easy to detect and gave well defined peaks, they were not the most useful tracers since their behaviour differed from the bacteria (Harvey and George, 1989). There appears to be enhanced bacterial movement in soil columns with a high degree of macropores where the majority of water flow is through preferred flow pathways (Harvey, 1989).

The distribution of water flowing through the soil matrix or macropores is variable and depends upon the soil structure and the rate of addition of the water (Smith et al., 1985). Therefore it was concluded that the degree of macropore flow not only influences the rate of movement of water and Cl^- but also determines the efficiency or extent of *E. coli* transport (Smith et al., 1985). It was also shown by Smith et al. (1985) that the more macropores there were present in the soil, the greater was the movement of *E. coli*.

Since microorganisms have been found to travel in groundwater faster than that of chemical tracers under the same flow conditions, it is possible that there are other factors that influence the movement of organisms in water in addition to those which are responsible for the transport of chemical tracers (Abu-Ashour et al., 1994; McMurry et al., 1998; Smith et al., 1985). There are several transport processes that may assist

microbial movement through the soil such as dispersion, filtration, adsorption/desorption, sedimentation, as well as growth, death and chemotaxis (Abu-Ashour et al., 1994).

Experiments conducted using fine and coarse sand repacked in columns showed that the recovery of bacteria during elution with water was lowest for the fine sand and highest for the coarse sand (56 and 84%, respectively) from a starting inoculum of 8 log cells/ml. It was concluded that the retardation of bacterial movement relative to Cl^- in the column experiments was caused by adsorption of bacteria onto solid particles (coarse sand has a smaller surface area for bacterial adsorption than fine sand) (Tan et al., 1992).

Larger diameter (>12 cm) soil columns (50 cm in length) can allow for the study of bacterial movement to the deeper layers of the soil and model the distances found in the natural environment to the groundwater (van Elsas et al., 1991). Laboratory transport experiments generate relevant results when tests are conducted under conditions similar to those found in the agricultural setting. Unfortunately, due to the diversity found in nature, it is difficult to accurately predict all of the possible results of bacterial movement in the field (van Elsas et al., 1991).

2.4 Pathogen survival in water

An important source of enteropathogenic organisms in water is believed to be fecal contamination from animals (Terzieva and McFeters, 1991). Pathogens can be introduced in various ways to groundwater and often septic tanks, landfills and crop

irrigation with sewage effluent are sources responsible (Snowdon and Cliver, 1989). Regardless of the time or rate of application the greatest bacterial concentrations in surface waters has been observed during spring runoff (Joy et al., 1998). Much of the contamination of groundwater was found to be associated with shallow wells (<31 m) since these are more susceptible to movement of water through the soil (Olson, 2001).

In untreated surface waters there are factors which may cause bacterial stress such as ultraviolet radiation, metals and nutrient restriction. Should stress cause bacterial injury, bacterial populations may be underestimated, even though injury can be reversible under the proper circumstances (Terzieva and McFeters, 1991). The detection of pathogens in the environment is often difficult since bacteria can undergo physiological changes when exposed to hostile conditions in order to survive (Wang and Doyle, 1998). There are a number of factors which influence the survival of microorganisms in groundwater which include temperature, pH value, chemical and mineralogical properties of the water (Filip et al., 1988).

It has been shown that there are other factors which affect bacterial survival in water such as protozoa, antibiosis, organic matter, algal toxins, dissolved nutrients, heavy metals, and the physiochemical characteristics of the water (Burton et al., 1987). Much of the variation in results from studies on bacterial survival in water may be due to different strain responses to stress, use of different methodologies for bacterial recovery and incomplete understanding of the environmental conditions during conduct of the work (Burton et al., 1987).

2.4.1 Survival of *Escherichia coli* in water

In agricultural settings, *E. coli* O157 can contaminate surface and groundwaters via surface runoff, lateral and vertical flow from and through contaminated soil (Jones, 1999). It has been found that protozoan predation and/or competition for nutrients affects the survival of *Escherichia coli* and *Campylobacter jejuni* in untreated and filtered lake water (Kohonen and Martikainen, 1991). It was found that *E. coli* survived better at 4°C than 25°C over a 260 d period (Flint, 1987; Korhonen and Martikainen, 1991). Flint (1987) also showed that the competition for nutrients had a greater negative effect on the survival of *E. coli* than did protozoan predation (Korhonen and Martikainen, 1991). There have been instances of *E. coli* O157 infections worldwide from groundwater supplies contaminated by animal feces (Jones, 1999). Reports have shown that non-O157 *E. coli* strains can survive from 1 to 7 d in non-sterile natural river water and that the greatest survival was at 4°C (Bogosian et al., 1996; Jones, 1999). There also have been observations of *E. coli* O157 survival in river water for up to 90 d, where the major factor affecting survival was temperature (greater survival was seen at 8°C than 25°C) (Jones, 1999). When survival was compared in water from different sources at 8, 15 or 25°C, the survival of *E. coli* O157:H7 populations were greatest at 8°C regardless of the water source. *E. coli* was resilient in water and numbers decreased by 1 to 2 log at 8°C after 91 d (Wang and Doyle, 1998). There was also a period of time in these tests where *E. coli* could not be detected by plate counts; however, it was detected by overnight TSB enrichment (37°C) during a 4 to 5 week period. This occurred in at least one water

sample stored 21 d at both 15°C and 25°C (Wang and Doyle, 1998). These authors found that sunlight and organic matter affected *E. coli* survival in water.

In water that was frozen or kept at 5°C, *E. coli* was able to survive for > 300 d but at warmer temperatures (30°C) it survived for 84 d (Olson, 2001).

The retention of virulence by pathogenic *E. coli* in drinking water supplies indicates that they are hardy organisms and results from a variety of studies showed that even in water only a low number of organisms are required to cause human infection (Yu and Bruno, 1996).

When comparing the survival of *E. coli* in filtered and untreated lake water, it was found that *E. coli* survived better in the filtered water. These results suggested that predation and/or competition for nutrients does affect survival of *E. coli* in aquatic environments (Korhonen and Martikainen, 1991).

E. coli O157:H7 populations were shown to decrease by 3 to 3.5 log in municipal well water at 5°C over a 45 to 70 d period, whereas at 20°C, a 5 log reduction was observed after 35 d (Rice et al., 1992). Die off was also faster at 20°C than 5°C when *E. coli* was monitored in a potable groundwater source, however, there was no significant reduction at either temperature until after 7 d (Rice et al., 1992). Studies have shown non-O157 *E. coli* survival at 25°C was > 60 d, and the organism survived 100 d at 4°C (Bogosian et al., 1996; Jones, 1999; Rattray et al., 1992).

The risk of water pollution with *E. coli* is greatest after application of animal waste slurry (cattle), where the first increments of drainflow carry the greatest concentration of the bacteria (Ogden et al., 2001). It was concluded that it is unlikely that there would be significant losses of organisms to drains if precipitation was minimal after the application of slurry to well-drained sandy soils (Ogden et al., 2001).

A stream in Ontario, which flows into Lake Huron, was found to have significant manure contamination in winter and spring facilitating the survival of *E. coli* and other organisms because of the presence of carbon, nitrogen and phosphorous (Palmateer et al., 1989).

In inoculated artificial sea water, two of three strains of *E. coli* were recovered by direct plating for 31 d, while a third strain was recovered by plating until day 65 during storage at 5°C (Rigsbee et al., 1997). In filter sterilized river water the three strains decreased gradually at 5°C with the most dramatic decreases occurring within the initial two week period. All three stains were recovered by plating up to day 20 (Rigsbee et al., 1997).

The influence of temperature, sedimentation, UV light or a combination of these effects was thought to be responsible for the disappearance of *E. coli* from marine environments (Flint, 1987). However, the beneficial effects of reduced UV light intensity in turbid estuarine conditions was thought to have been reversed by increased predation or adverse high temperature in estuaries (Flint, 1987; Lim and Flint, 1989). In freshwater the major conditions responsible for the disappearance of *E. coli* are not fully understood. In autoclaved, filtered river water, *E. coli* was able to survive for up to 260 d. The survival

of *E. coli* was dependent upon temperature with survival being reduced in order of 4°C> 15°C > 25°C > 37°C. However, it was concluded that competition with the natural microbial flora of the water was the primary cause of the decrease in the inoculated population. Autoclaving water samples removed viral predators (bacteriophage) and bacterial competitors, giving the longest survival regardless of temperature (Flint et al., 1987). In a laboratory setting the temperature and nutrient availability played primary roles in survival (Flint, 1987). In the absence of competitors (autoclaved river water) *E. coli* was able to survive < 260 d 25°C. There was no decline in numbers under these conditions and no apparent injury (Flint, 1987; Lim and Flint, 1989).

In work conducted by Bogosian et al (1996) using non-sterile river water, *E. coli* levels dropped to < 10 cfu/ml within 10 d when an initial inoculum of > 10^7 cfu/ml was used and held at 4, 20 or 37°C. In sterile water, added *E. coli* survived for 50 d at 20 and 37°C without a significant reduction. In sterile soil at 4, 20 and 37°C there was no decline in numbers observed after 100 d (Bogosian et al., 1996). The *E. coli* numbers also remained the same over 50 d in sterile artificial seawater at 4°C or sterile river water at 4 or 20°C. From these studies it was suggested that *E. coli* present in the inoculum added to non-sterile soil or river water were unable to compete with and were perhaps being consumed by the predatory organisms in the samples (Bogosian et al., 1996).

Karapinar and Gonul (1991), found that after inoculation and 13 weeks of incubation in sterile spring water at 4°C, no *E. coli* was detected. When cultures of *E. coli* and *Yersinia* were incubated together under the same conditions, the viability of *E. coli* was futher

reduced. After 7 weeks, at an inoculum of 9.1×10^2 cells/ml water, no *E. coli* were detectable. At the higher inoculum of 9.0×10^4 cells/ml water, *E. coli* were not detectable after 13 weeks by direct plate counts (Karapinar and Gonul, 1991).

It has been speculated that loss of *E. coli* viability is directly related to temperature, however, interference by indigenous microorganisms or the low sensitivity of the method used for recovery may have contributed to this conclusion (Rhodes and Kator, 1988). Centrifugation, washing, holding at cool temperatures and other harsh manipulations may stress organisms before exposure to the test environment and the use of selective media can underestimate the population that does survive (Rhodes and Kator, 1988).

2.4.2 Survival of *Salmonella* in water

Salmonella Enteritidis numbers were shown to decrease from 5 log cfu/ml to < 1 cfu/ml in culturable counts when *Salmonella* Enteritidis was incubated in filtered autoclaved river water for 48 h (McKay, 1992; Roszak et al. 1984).

In water, *Salmonella* was shown to be able to survive > 6 months regardless of whether frozen or held at 5°C or 30°C. Holding at lower temperatures allowed for prolonged survival in water, as the cold water can act to sustain viability (Olson, 2001). One ml of four strains of *Salmonella* at approximately 9 log cfu/ml were inoculated individually into 9 ml of twice filtered river water, as well as untreated river water or autoclaved-filtered river water and held at room temperature. In the autoclaved water treatment there was a minor decrease in numbers during the first week. Three of four strains used survived at 5

log cfu/ml after 45 d. However, in the other treatments (untreated and filtered) there was a decrease of 3 log cfu/ml after 45 d. Protozoan predators did not seem to influence the results since survival was better in untreated river water compared to filtered water (Santo Domingo et al., 2000). At temperatures of <10°C inoculated *Salmonella* spp. exhibited less die-off and stress in water when compared to *E. coli* (Rhodes and Kator, 1988). *Salmonella* spp. were shown to be more persistent than *E. coli* in filtered water at < 10°C (Rhodes and Kator, 1988). A series of unpublished studies showed a rapid decline in the survival of several enteric bacteria including *Salmonella* and *E. coli* in river water within 72 h (Santo Domingo et al., 2000). In addition, Rhodes and Kator (1988) showed that *E. coli* and *Salmonella* spp. were capable of survival and multiplication in the estuarine environment, with population decreases attributed to either the low water temperatures or predation by the natural microflora at warmer temperatures (Rhodes and Kator, 1988). Enteric bacteria in estuarine environments are affected by an interaction of physical, chemical and biological factors and processes, such as low nutrients, predation and competition (Rhodes and Kator, 1988). *E. coli* and *S.* Newport survival was greater than that shown by *P. aeruginosa* and *K. pneumoniae* in freshwater sediments containing a minimum of 25% clay, when studied at 16°C to 21°C (Burton et al., 1987). In water there was a faster reduction in bacterial viability which was attributed to an inability to compete with the natural microflora for low levels of nutrients (Burton et al., 1987).

Protozoan predation and the competition for nutrients are important factors which affect the survival of bacteria in water (Korhonen and Martikainen, 1991). It was found by Rhodes and Kator (1988), that the survival of *E. coli* and *Salmonella* spp. decreased with

an increasing density of microflagellates (Korhonen and Martikainen, 1991; Rhodes and Kator, 1988).

2.4.3 Survival of *Yersinia* in water

Yersinia is highly resistant to some extreme environmental conditions and survived for a year or longer in frozen water or at temperatures of 5°C (Olson, 2001). At a water temperature of 30°C, *Yersinia* survived for approximately 10 d (Olson, 2001). *Yersinia* is capable of limited reproduction and survival in distilled water since it is adapted to a temperate aquatic environment and is psychrotrophic (Terzieva and McFeters, 1991). *Yersinia enterocolitica* showed the greatest survival among all pathogens examined in water at 4-8°C (Guan and Holley, 2003). The endurance of *Y. enterocolitica* in river water is primarily controlled by predators and toxin producers, in contrast with the beneficial effects of low temperature on survival (Chao et al., 1988).

In natural river water, the rapid disappearance of *Yersinia* was believed due to the presence of eukaryotic predators (Chao et al., 1988). Since groundwater possesses a low microbial population, *Yersinia* has the capability for greater survival (Chao et al., 1988). In sterile spring water held at 4°C, *Yersinia enterocolitica* increased 2 or 3 logs during the initial 3 weeks, and numbers were highest at 7 weeks of storage. After 64 weeks, viable cells were still detectable at the initial inoculum level (Karapinar and Gonul, 1991). In a previous study it was reported that *Yersinia enterocolitica* was able to survive in sterile distilled water at 4°C for over 18 months (Highsmith et al. 1977; Karapinar and Gonul,

1991). In untreated lake water the survival of *Yersinia enterocolitica* biovar 0:3, was dramatically reduced when other microorganisms were present at 4°C (Lund, 1996). When inoculated at 6 log cfu/ml, at 4°C there was a 3 log, or 99.9%, reduction in *Yersinia enterocolitica* within 17-18 d, while at 10°C, only 14-15 d were required to achieve the same reduction (Lund, 1996). *Y. enterocolitica* survived better than *E. coli* at low temperatures (Lund, 1996). In autoclaved water the period required for a 4 log inactivation of *Y. enterocolitica* was about five times longer than was seen in the natural lake water regardless of whether 4 or 10°C was used (Lund, 1996). In autoclaved water at 4°C, 99.9% of *E. coli* were inactivated within 55 d, but 95 d were required to achieve the same reduction of *Y. enterocolitica*. At 10°C, 99.9% of *E. coli* were inactivated within 42 d. These results suggest that naturally present water organisms may cause significant reductions in numbers of fecal pathogens in water (Lund, 1996). In survival studies with *E. coli* and *Yersinia* at 6°C and 16°C where membrane diffusion chambers were immersed in stream waters, it was found that there was little change in bacterial numbers within the first 4 d, followed by a decline in colony forming units (Terzieva and McFeters, 1991). Greater persistence of both bacteria was seen at 6°C than at 16°C. The reduced temperature resulted in an extended survival of two of the three *Yersinia* strains and all three *E. coli* strains tested after 14 d (Terzieva and McFeters, 1991). Temperature was clearly a major factor influencing persistence of these bacteria in aquatic conditions. Even though the test organisms became injured and appeared to die, a significant portion persisted in a culturable state for up to 14 d (Terzieva and McFeters, 1991). The survival of bacteria at 10°C in groundwater showed that test bacteria could survive for up to 100 d (Filip et al., 1988). Inoculated *Y. enterocolitica and P. aeruginosa* survived < 50 d with a

reduction of < 1 log, whereas *E. coli* and *Salmonella* Typhimurium showed reductions of up to 4 log cfu/ml (Filip et al., 1988). Even after 100 d the four organisms were still detectable (Filip et al., 1988). In cold water (4 to 8°C) *Y. enterocolitica* had the greatest survival among all pathogens studied (Guan and Holley, 2003) with detection of *Yersinia* up to 64 weeks in sterile spring water held at 4°C (Karapinar and Gonul, 1991).

2.4.4 Survival of *Pseudomonas* in water

Pseudomonas spp. are common in soil and groundwater (Jay, 2000; Leclerc and Moreau, 2002; Teixeira et al., 2001) due to their ability and adaptability to survive and grow in a wide range of conditions. They are capable of growth on various organic substrates, they do not require any specific vitamins or amino acids and can utilize a number of carbon sources (Lecler and Moreau, 2002). Pseudomonads are also able to survive in water environments with low concentrations of available nutrients (Stetzenbach et al., 1986). *Pseudomonas* is an opportunistic pathogen and can cause infections through contact or inhalation. They are widely distributed in nature and can colonize water environments (Hernandez et al, 1997).

Unfortunately, there is little information regarding *Pseudomonas* levels in groundwater. However, Filip et al. (1988), showed that *Pseudomonas* was capable of survival at 10°C for 100 d using groundwater, with less than a one log reduction in numbers up to 50 d.

2.4.5 Use of coliphage in water as an indicator of fecal pollution

E. coli present in the intestines and feces of all warm blooded animals is specifically infected by viruses known as coliphages (Ronner and Cliver, 1990). These phage, because of their close relationship with *E. coli* can serve as indicators of possible water contamination by fecal material containing pathogenic bacteria or other viruses of public health significance (Snowdon and Cliver, 1989; Stetler, 1984).

The importance of coliphages in the indication of fecal contamination was first established by Guelin, who during his early work established a correlation between coliform and coliphage levels in fresh and marine waters (Armon and Kott, 1996; Wentsel et al., 1982). A linear relationship exists between coliphages detected using the host *E. coli* C and fecal or total coliforms present in samples (Wentsel et al., 1982). Coliphage levels can be equal to or greater than the number of coliforms or enteric bacteria present in contaminated waters (Grawbow and Coubrough, 1986; Wentsel et al., 1982).

Coliphages are not pathogenic to man, are specifically found in the gastrointestinal tract and are easily detected, which also allows them to be used as suitable indicators for the presence of human enteric viruses. Because of their small round form, they move with water in the same way as enteroviruses and they are likely to be present in the same if not greater number as when human enteric viruses are present. Coliphages can persist and

survive in contaminated waters as well as their host bacteria and human viruses (Jay, 2000; Snowdon and Cliver, 1989).

Contamination of drinking water sources by enteric viruses is a major public health concern (Schwab et al., 1996). Consumption of contaminated groundwater is responsible for half of the outbreaks of disease with waterborne causes (Snowdon and Cliver, 1989; Yates et al., 1985). Of additional concern is the possibility for survival and accumulation of pathogens in soil and their movement to the groundwater (Moore et al., 1981). Several factors can affect the survival and migration of viruses in soil once they have been introduced. These factors include: pH, temperature/climate, rainfall, soil characteristics, chloride, total organic carbon, water hardness, turbidity, presence of other aerobic organisms, chemical and microbiological content of groundwater, sewage pollution, moisture holding capacity and the type of soil, ultra-violet radiation at the soil surface and viral characteristics (Hurst et al., 1988; Moore et al., 1981; Snowdon and Cliver, 1989; Yates et al., 1985). Viruses have the capacity to migrate great distances (Yates et al., 1985). However, coliphages are less likely to be found in groundwater due to the lack of nutrients for hosts, dilution of bacterial hosts and cool temperature (Snowdon and Cliver, 1989).

Numerous studies have shown that indicator bacteria are inadequate for the prediction of the presence of enteric viruses in water (Schwab et al., 1996). An indicator is a tracer or an organism that possesses behavioural characteristics similar to the pathogen and possesses the ability to withstand environmental stresses equal or better than the target

bacteria (Armon and Kott, 1996). There is concern that when coliphages are used as indicators of fecal pollution, the host strains used may not detect the array of viable phages present (Jay, 2000).

From 147 samples of drinking water tested for total coliforms, fecal coliforms and coliphage, 78 were positive for coliphage and negative for total and fecal coliforms; 65 were negative for total and fecal coliforms and coliphage; and 4 were positive for both total coliforms and coliphage. It was concluded from this study conducted in Egypt that coliform-free potable water may not be necessarily free of pathogens or coliphages (El-Abagy et al., 1988). In other work coliphage-free water was found to be a reliable indication of acceptable virological and hygienic quality (Grabow and Coubrough, 1986).

In 1987, a study performed by Sim and Dutka (1987), challenged the view that coliform-free drinking water is pathogen safe water. In their experiments, they tested water samples for total coliforms, fecal coliforms and coliphage concentrations. Of the 30 potable water samples tested, there were two samples which were negative for coliphage after 24 h. There were no samples that were negative for coliphage and positive for total or fecal coliforms. There was however, nine samples found to be positive for coliphage and negative for total and fecal coliforms. The conclusion according to their study was that coliform-free water is not necessarily pathogen-free potable water, however, the authors recommend that coliphage testing be including as part of any potable water testing regime.

The main advantage in using coliphage indicators over traditional microbiological and virological testing is that it is economical (compared to enteric bacterial detection methods), simple to perform and rapid (resulting phages produce plaques within 4-6 h under optimal conditions) (El-Abagy et al., 1988; Grabow and Coubrough, 1986; Leclerc et al., 2000; Mariam and Cliver, 2000; Sim and Dutka, 1987; Stetler, 1984) and the sensitivity of the test can be increased easily (Sim and Dutka, 1987). Bacteriophage can be easily used and detected in water samples (Sim and Dutka, 1987; Snowdon and Cliver, 1989). The test is readily applicable to the majority of water types and samples can be tested for false positives easily (Stetler, 1984). However, the sensitivity of these tests needs to be improved beyond current levels (one coliphage/100 ml) so that even smaller numbers of coliphages can be detected (Wentsel et al., 1982). Due to the number of different methods available for coliphage enumeration, there is the possibility of contradictory results being generated. This can result in confusion and underestimation of the overall test value due to the differences in sensitivity, selectivity and reproducibility (Grabow and Coubrough, 1986).

Since there is usually a low level of enteric viruses in environmental waters, virus concentration steps are needed and methods sometimes pose an analytical problem (Schwab et al., 1996). The concentration of large sample volumes has been shown to decrease coliphage recovery (Stetler, 1984). Beef extract was found to be a poor coliphage eluant at 1%, however, a better coliphage eluant has been shown to be 4% beef extract in 0.5 NaCl (Stetler, 1984).

Standard Methods for the Examination of Water and Wastewater (1989) specifies *E. coli* C for use as the host for coliphage detection and enumeration since it is sensitive to a broad range of coliphages in sewage and also lacks the restriction and modification systems (Ijzerman et al., 1994). This method also uses 2, 3, 5-triphenyltetrazolium chloride (TTC), to identify non-infected *E. coli* cells (which reduce TTC and colour the colony a pale pink) while infected cells generate plaques which remain colourless (Ijzerman et al., 1994). Phage diffusion in the soft agar (0.7% w/v) overlay yields further cell lysis, forming plaques that appear as "holes" which are counted as plaque forming units (Armon and Kott, 1996). There are several limitations of this method. There is sometimes a lack of significant colour contrast, sometimes there is difficulty in detection of low numbers of *E. coli* and there is also the possibility that clear plaque-like areas may form for reasons other than phage infection, which can generate false positive results (Ijzerman et al., 1994). Another reason for the slow adoption of coliphages for use as indicators of fecal contamination and the presence of enteric viruses is that the phage may lack specificity for the target bacterial host in environmental samples (Leclerc et al., 2000).

The ability to predict enteric virus presence in groundwater is limited by low rate of occurrence of all types of bacteriophages. Comparisons are difficult to make in the literature because standardized detection methods are infrequently used (Leclerc et al., 2000).

Chapter 3

MATERIALS AND METHODS

3.0 Experimental Field Site Treatments and Sampling

The experimental field site used for this study was approximately 32.8 ha of almost flat land that overlies part of the shallow sandy Assinibione Delta Aquifer near Carberry, MB. and was termed the Green Farm Site. There was uniform brown sand with scattered silt to silty clay lenses (1 cm to 0.5 m thick), with local thin stringers of coal, in the first 6 m of soil. From 6 to 10.5 m soil depth there was fine to medium coarse gray sand. The total expected sand thickness was 15 m. Soil geology was provided by Manitoba Conservation (Winnipeg, MB.). The field was divided into four north / south sections, each measuring approximately 100.6 m wide by 804.7 m long. The two westerly fields (designated as field 1 and field 2) had an annual crop rotation (winter wheat, oats or canola), and the two easterly fields (designated as field 3 and field 4) had a forage crop (alfalfa) planted. The application of liquid hog manure, from an above ground storage reservoir supplied from a grower-finisher barn located near the fields, was rotated to some extent among the fields with one annual and one forage field being fertilized at a time, and the other two fields being fertilized at the next seasonal application. In the third year of the study all four fields were fertilized at the same time.

Availability of equipment and labour, as well as weather influenced application of manure to fields. Manure application dates were October 30, 1999 (fields 2 and 3) at an application rate of ~56170 L/ha (76 kg N/ha); June 3, 2000 (fields 1 and 4) at an application rate of ~41566 L/ha (112 kg N/ha); May 3, 2001 (all 4 fields) at an application rate of ~28085 (4260), ~48306 (70), ~88749 (127) and ~88749 (127) L/ha (kg N/ha) in fields 1 through 4, respectively; October 22, 2001 (fields 2 and 3) at application rates of ~65157 L/ha (111 kg N/ha) and ~61787 L/ha (105 kg N/ha), respectively, and April 24, 2002 (only field 4) with an application rate of ~69089 L/ha (118 kg N/ha). Manure application rate determinations and nitrogen analyses were provided by Manitoba Conservation (Winnipeg, Manitoba).

3.1 Water quality monitoring

3.1.1 Groundwater sampling sites

The groundwater monitoring system consisted of sampling wells at 67 sites on the fields (Table 3.1). There were a total of 26 piezometer nests (a piezometer is a non-pumping well, generally of small diameter, that is used to measure the elevation of the water table). There were six in each of the four fields and two additional nests at the south eastern edge, an area of lower elevation, to determine background water levels and quality. Each field was subdivided into three subsections for management reasons and piezometers were located within each subsection. The wells were professionally installed using hollow stem auger drilling techniques. In each of the nests there was a well drilled

to the level of the water table (termed a shallow depth well) and a piezometer was located 2-3 m below the water table (termed an intermediate depth well). At every second nest and at the background nests there was an additional piezometer 5-6 m below the water table (termed a deep depth well). Each of the individual wells had a manually activated Waterra pump (Mississauga, ON) for sampling. Each of fields 2, 3, and 4 contained 15 wells. Field 1 had 16 wells and the background field had 6 wells, giving a total of 67 sampling wells. The water from the wells was sampled 9 times over a 20 month period.

Table 3.1: Tabulated description of relative location of wells drilled at the Green Farm experimental area

North

Background section One (BGD-1)[1] W962 (D)[2] / W963 (I)[3] / W964 (S)[4]		Background section Two (BGD-2) W965 (D) / W966 (I) / W967 (S)		
Field One (F1)	Field Two (F2)	Field Three (F3)	Field Four (F4)	
W916 (S)				
W915 (I)	W931 (S)	W946 (S)	W961 (I)	
W914 (S)	W930 (I)	W945 (I)	W960 (S)	Subsection
W913 (I)	W929 (S)	W944 (S)	W959 (D)	Three (S3)[5]
W912 (D)	W928 (I)	W943 (I)	W958 (I)	
W911 (S)	W927 (D)	W942 (D)	W957 (S)	
W910 (I)	W926 (S)	W941 (S)	W956 (I)	
W909 (I)	W925 (I)	W940 (I)	W955 (S)	Subsection
W908 (S)	W924 (S)	W939 (S)	W954 (D)	Two (S2)
W907 (I)	W923 (I)	W938 (I)	W953 (I)	
W906 (D)	W922 (D)	W937 (D)	W952 (S)	
W905 (S)	W921 (S)	W936 (S)	W951 (I)	
W904 (I)	W920 (I)	W935 (I)	W950 (S)	Subsection
W903 (S)	W919 (S)	W934 (S)	W949 (D)	One (S1)
W902 (I)	W918 (I)	W933 (I)	W948 (I)	
W901 (D)	W917 (D)	W932 (D)	W947 (S)	

South

[1] Background sections (BGD-1 and BGD-2) each contained 3 wells (W962 to W967)

[2] D = deep well (5-6 m below water table)

[3] I = intermediate well (2-3 m below water table)

[4] S = shallow well (drilled to water table)

[5] Each subsection represented one third of each field and contained 5 wells

3.1.2 Water sampling procedure

Before water sampling, each well was purged of three well volumes of water. The tip of the Waterra pump hose was wiped with a sterile cloth saturated with a 70% aqueous isopropanol solution before samples were taken. Samples of 100 to 125 ml of well water were taken in sterile bottles from each well at each sample time. The samples were placed in a cooler with ice packs and stored at 4-5°C until analysis. Coliforms were analyzed within 24-72 h after sampling while *Pseudomonas aeruginosa* were analyzed within 5-7 d.

Water sampling dates (including days since last manure treatment) were: October 13, 2000 (fields 1 and 4: 132 d and fields 2 and 3: 349 d); March 14 (fields 1 and 4: 284 d and fields 2 and 3: 349 d); April 30 (fields 1 and 4: 331 d and fields 2 and 3: 548d); May 24 (all four fields: 21 d); July 13 (all four fields: 71 d); September 7 (all four fields: 127 d), in 2001; March 15 (fields 1 and 4: 186 d and fields 2 and 3: 14 d) and May 9 (field 1: 371 d, fields 2 and 3: 199 d, field 4: 15 d) in 2002. Coliform analysis was performed on all samples, whereas *Pseudomonas* was analyzed in water samples from March 14, July 13, September 7, and November 5, 2001 as well as on March 15 and May 9, 2002.

3.1.3 Coliform analysis of water samples

The protocol used was the standard total coliform multiple tube most probable number (MPN) fermentation technique from Standard Methods for the Examination of

Water and Wastewater (Standard Methods, 1989). In brief, for the presumptive phase, double strength lauryl sulfate tryptose broth (LST, BBL, Becton Dickinson, Cockeysville, MD) was used. From each sample five 10 ml portions were inoculated into 5 separate tubes of double strength LST containing inverted Durham fermentation vials. Inoculated tubes were incubated at 35°C for 24-48 h, with the presence of any amount of gas production regarded as a positive result.

For confirmation of coliforms, 0.1 ml from the positive LST tubes was transferred into brilliant green lactose bile broth (BGLB, BBL) tubes with inverted fermentation vials. Tubes were incubated at 35°C for 24-48 h, where gas production represented a positive result.

Fecal coliform determination was carried out by transferring 0.1 ml from the positive LST tubes into EC broth (EC, BBL) tubes containing inverted fermentation vials. Tubes were incubated at 44.5°C for 24-48 h, and gas production indicated a positive result. Results were then evaluated using an MPN table (Standard Methods, 1989) to determine the sample MPN index/100 ml of sample. Positive cultures were streaked onto Eosin Methylene Blue agar for confirmation (EMB, Difco Becton Dickinson, Sparks, MD) and incubated at 35°C for 24-48 h.

3.1.4 *Pseudomonas aeruginosa* analysis of water samples

The procedure followed was a multiple tube MPN technique for *Pseudomonas aeruginosa* (Standard Methods, 1989). From each water sample five 10 ml portions were inoculated into 5 separate tubes containing 10 ml double strength Asparagine broth (Standard Methods, 1989). Tubes were incubated at 35-37°C for 24-48 h. Positive results were determined under long wave ultraviolet light (Spectro F15T8-BLB 22 watt bulb, Lamp model 1801-3, L&L Manufacturer, Spectro Electric Ind., Scarborough, ON.), where the production of a greenish fluorescent pigment indicated a positive result.

A confirmed test was performed on presumptive positive tubes by spread plating 0.1 ml onto Acetamide agar plates (Standard Methods, 1989). A positive result occurred with the development of a purple colour on the plates within 24-36 h of incubation at 35-37°C. Results were evaluated using an MPN table (Standard Methods, 1989) to determine the MPN of *P. aeruginosa*/100 ml of water sample.

3.1.5 Coliphage analysis of well water

Analysis for coliphage was performed on well water collected at the field site on May 9, 2002 from the following wells: 901, 903, 905, 929, 931, 934, 936, 957, 960 and 964. Samples were collected using 1000 ppm chlorine-sterilized 4 litre plastic jugs and stored at 4-5°C for 5 days until analysis. Each sample was also analyzed for coliforms as described previously.

From each container 100 ml well water was divided in half, placed in sterile containers and was frozen at -18°C until analysis. The remaining sample was concentrated by aluminum hydroxide adsorption/precipitation as described in Standard Methods (1989) and analyzed for coliphage presence. The concentrated samples were tested using *E. coli* C (ATCC 13706) and *E. coli* C3000 (ATCC 15597) as the host cultures. *E. coli* C was obtained from the Dept. Microbiology (Univ. Manitoba) and *E. coli* C3000 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Concentrated samples of well water were analyzed undiluted and enumerated on host-inoculated modified tryptic soy agar (Standard Methods, 1989). The plates were incubated at 35°C for 4 to 6 h and examined for coliphages according to Standard Methods (1989). The method for coliphage recovery was evaluated by inoculating well water samples with 10^5 or 10^6 plaque forming units (pfu) of MS2 bacteriophage (ATCC 15597-B1) which is specific for *E. coli* C3000. Samples were concentrated as previously described.

3.2 Pathogen presence in manure

3.2.1 Analysis of manure for *Salmonella*

The liquid hog manure analyzed was aseptically sampled from the manure applicator tank reservoir on May 3, 2001. Eight samples were taken and stored for 48 h at 4-5°C until analysis.

The procedure followed was after Hu and Gibbs (1995), where 60 g of sample was diluted tenfold with buffered peptone water (Difco, Becton Dickinson, Sparks, MD) and incubated overnight at 37°C. One ml of this pre-enrichment culture was then transferred to Rappaport-Vassiliadis R10 broth (Difco) and incubated at 43°C for 48 h. The sample was then sub-cultured on Xylose Lysine Desoxycholate (XLD) agar (BBL) and incubated for 24 h at 35°C. Colonies were subsequentially transferred to MacConkey agar without salt (Difco) and incubated at 35°C for 24 h. Colonies were then inoculated onto slants of Triple Sugar Iron agar (TSI, Difco) and Lysine Iron agar (LIA, BBL). Both sets of slants were incubated at 35°C for 24 h. Growth from tubes which both showed positive results on TSI and LIA (TSI-alkaline (red) slant and acid (yellow) butt, with or without H_2S (blackening) production; LIA-alkaline (purple) reaction with or without H_2S blackening reaction), was transferred to Trypticase Soy Agar (TSA, BBL) plates and incubated 48 h at 35°C. Single colonies were then tested for agglutination with *Salmonella* O poly A antiserum (Bacto, Difco, Detroit, MI). Cultures which were positive on both TSI or LIA and agglutinated with poly A *Salmonella* antiserum were characterized biochemically with API 20E strips (bioMerieux, Hazelwood, MO) after further growth on TSA.

3.2.2 Analysis of manure for *Yersinia*

The liquid hog manure analyzed was aseptically sampled from the manure applicator tank reservoir on May 3, 2001. Eight samples were taken and stored for 48h at 4-5°C until analysis.

The procedure used was from the Bacteriological Analytical Manual (BAM, 1998). Ten g of manure was added to 90 ml of peptone sorbitol bile broth (BAM, 1998), and incubated at 10°C for 10 d. Following incubation, 0.1 ml was inoculated into 1 ml of 0.5% KOH in 0.5% saline and mixed for 10 s. Following this, the solutions were streaked onto MacConkey agar (Difco, Becton Dickinson, Sparks, MD) and Cefsulodin Irgasan Novobiocin (CIN) agar, which is also known as *Yersinia* Selective agar base (Difco), containing *Yersinia* antimicrobic supplement CN (Difco). An additional 0.1 ml of enrichment culture was inoculated into 1ml of 0.5% saline, mixed for 10 s and streaked again on MacConkey and CIN agars. All plates were incubated at 30°C for 24 h. After the plates were examined for growth, a single colony was inoculated on a Lysine Arginine Iron agar slant (LAIA, BAM, 1998), a Christensen's Urea agar plate (BAM, 1998) and a bile esculin agar plate (Difco, Becton Dickinson, Sparks, MD) and incubated at 22°C for 48 h. Positive cultures (isolates which gave an alkaline slant and acid butt, no gas or H_2S in LAIA, which were also urease positive and negative for esculin hydrolysis

(no blackening of agar) were grown on TSA (BBL) and then further chacterized on API 20E strips (bioMerieux).

3.2.3 Analysis of manure for *Escherichia coli*

The liquid hog manure analyzed was aseptically sampled from the manure applicator tank reservoir on May 3, 2001. Eight samples were taken and were stored for 48h at 4-5°C until analysis.

A 50 g sample was placed in 450 ml buffered peptone water (Difco). After mixing one ml was then inoculated into 3 separate LST (BBL) tubes with inverted Durham fermentation vials and incubated at 35°C for 24 h. Positive tubes (showing any gas production) were streaked onto *E. coli* Rainbow agar (Biolog, Hayward, CA) and at the same time tenfold serial dilutions of samples in 0.1% peptone were inoculated onto Petrifilm *E. coli*/coliform count plates (3M Microbiology, St. Paul, MN). Both were incubated at 35°C for 24-48 h. As well, 0.1 ml from LST tubes was inoculted into EC broth (BBL) and kept at 44°C for 24-48 h. After examination, serial dilutions in 0.1% peptone were performed from the gas positive EC broth tubes. These were also inoculated onto Petrifilm *E. coli*/coliform count plates. From the inoculated Petrifilm plates which originated from the LST tubes, colonies were streaked onto Eosin Methylene Blue agar (EMB, Difco, Becton Dickinson, Sparks, MD) and incubated at 35°C for 24-48 h. Afterwards the cultures were inoculated into TSB (BBL) and

incubated at 35°C for 24 h. Cultures were then subjected to indole, methyl red, Voges-Proskauer and citrate (IMViC) testing as outlined in the BAM manual (BAM, 1998).

3.3 Analysis of field soil samples for potential pathogens

Analysis of soil for *Salmonella*, *Yersinia* and *E. coli* was carried out as described for the manure analysis. The exception was that 10 ml (50 units) of alpha amylase (Sigma-Aldrich Co., St. Louis, MO) enzyme was added to 10 g of soil with incubation at 35-37°C for 18 h (Brisou, 1995). Then buffered peptone water (Difco) or peptone sorbitol bile broth (BAM, 1998) was added to create a 1 in 10 dilution of samples. Other steps followed are as previously outlined.

The first set of soil samples was collected November 17, 2000 by Maple Leaf Drilling (Carberry, MB). The samples were stored at 4-5°C until analysis. The sample designation was GGB 1 through 10. The location of the samples (along with GPS location) were as follows: GGB 1 (0454303/5534565) is approximately west of well 904, GGB 2 (0454310/5534072) is approximately west of well 912, GGB 3 (0454348/5534968) is west of well 912, GGB 4 (0454351/5534564) is just west of well 904, GGB 5 (0454445/5534562) is west of well 931, GGB 6 (0454452/5534962) is west of well 921 (Fig. 3.1). The remaining four samples were collected in the farm field immediately south from the Green Farm study site near the Green Farm hog barn and had

the following GPS locations: GGB 7 (0454424/5533708), GGB 8 (0454437/5533704), GGB 9 (0454478/5533702) and GGB 10 (0454517/5533708).

The second set of soil samples were collected on May 21, 2001 by Maple Leaf Drilling (Carberry, MB). The samples were stored at 5°C until analysis. Sample designation consisted of each of the fields 1-4 (F1, F2, F3 and F4) in combination with each subsection 1-3 (S1, S2 and S3) being sampled. A subsection consisted of one third of a field and included 5 wells (with the exception of field 1, where subsection 3 contained 6 wells). The two background sections (BGD-1 and BGD-2), each containing three wells, were also sampled.

Soil cores (8 cm diameter), to a 1 m depth were collected from the site on Oct. 10, 2002 by Maple Leaf Drilling (Carberry, MB) from the experimental field. These soil columns were transferred to 9 cm diameter polycarbonate tubes (sleeves), which were previously sterilized with 1000 ppm chlorine and capped. The columns were kept at 4-5°C until analysis. The outside plastic sleeve of each soil column was sterilized with 70 % ethanol prior to opening by cutting with a dremmel saw lengthwise down opposite sides of the column. Once the sleeve of the column was cut open, the top 5 cm of soil was removed from columns taken from the 0-60 cm depth and placed inside a sterile stomacher bag (Fisher Scientific Ltd., Nepean, ON.). In addition, a mixture of 50 g of each of the top (0-5 cm), middle (28-32 cm) and bottom (56-60 cm) sections of each of

the columns were placed in a sterile stomacher bag (Fisher Scientific Ltd., Nepean, ON), using a spoon sterilized with flamed 95 % ethanol. The bags of soil were stored at 4-5°C until analysis.

The first set of soil core samples was analyzed for *Yersinia*, *Salmonella* and *E. coli*. These sections were designated GGB 1 through 10 with the number of samples for each depth as follows. These soil samples were sectioned into 3 depths: top 5 cm (cores of GGB 1 through 10), 5-90 cm (cores GGB 1 through 10) and 90-180 cm (cores GGB 1 through 6). Cores GGB 7-10 were not long enough to allow sampling at the 90-180 cm depth.

The second set of soil core samples were taken from the experimental site, and were from all the four fields (with 3 subsections identified within each field designated S1, S2 and S3). The background soil areas were also analyzed and samples were designated BGD-1 and BGD-2. These core samples were also divided into the top 5 cm, 0-60 cm, 60-120 cm, 120-180 cm and 180-240 cm. Samples were not taken in some cases at all depths. For the 120-180 cm depth and 180-240 cm there was not a complete range of samples because the drilling company had difficulty obtaining intact cores from greater depths.

3.4 Survival of zoonotic pathogens in water

3.4.1 Standardization of bacterial cultures

One culture of *Salmonella*, 2 cultures of *E. coli* and 2 cultures of *Yersinia* (*fredereksenii* and *intermedia*) each isolated and purified from hog manure before application to the fields, as well as one culture of *E. coli* from the soil at the field site, were used in this study. Cultures were inoculated from TSA (BBL) plates into 5ml Tryptic Soy Broth (TSB, BBL) and incubated at 35-37°C for 4 h (*Salmonella* and *E. coli*) or 8 h (*Yersinia*). One ml of each culture was transferred into a fresh flask containing 200 ml of TSB and reincubated for 4 h (*Salmonella* and *E. coli*) or 8 h (*Yersinia*). Cultures were then placed into sterile 250 ml bottles and centrifuged at 3000 x g for 10 min at 22°C. The supernatants were discarded and the pellets were resuspended in 100 ml sterile distilled water. Cultures were centrifuged again at the same speed and the supernatant discarded. Cell pellets were resuspended again in 50 ml sterile distilled water and were adjusted to an optical density (OD) of 0.6-0.8 at 600 nm with a spectrophotometer (Ultrospec 2000, Pharmacia Biotech Baie d'Urfe, QC) using sterile distilled water as a blank. To confirm bacterial numbers present at specific OD, cultures were serially diluted in peptone water, spread-plated on TSA (BBL), incubated at 35°C for 48 h (25°C for *Yersinia*) and counted. These cultures were used in the next section to study their survival in water.

3.4.2 Pathogen survival in sterile and non-sterile water

Cultures (1 ml) of the three organisms with an OD_{600} of 0.65-0.7 (approximately 6-7 log cfu/ml) were separately placed in duplicate sterile flasks containing either 200 ml untreated non-sterilized well water (obtained from the Green Farm Site) or 200 ml autoclaved well water (obtained from the Green Farm Site). This represented the high inoculum study. For the low inoculum study, OD_{600} adjusted cultures were diluted with 0.1% peptone to 3 log cfu/ml before inoculation into similar flasks of water. Inoculated samples were held at 5°C and regularly sampled as noted below for up to 91 d.

At sampling, one ml of water was taken, serially diluted in 0.1% peptone and 0.1 ml was spread plated on TSA (BBL) and a selective agar as described below, in duplicate. For *Yersinia*, *Yersinia* selective agar base (Difco, Becton Dickinson, Sparks, MD) was used with the *Yersinia* antimicrobic supplement CN (Difco). For *Salmonella*, the selective agar was Brilliant Green agar (BGA, Difco). For *Escherichia coli*, Eosin Methylene Blue agar (EMB, Difco) was used as the selective medium. Plates were incubated at 35-37°C for *Salmonella* and *E. coli*, and at 25°C for *Yersinia*. Colonies on plates were counted after 24-48 h of incubation.

Sampling of the high inoculum *Salmonella* treatment was done for 63 d at regular 3 or 4 d intervals. For the high inoculum *Yersinia* tests, sampling was done at 3 or 4 d intervals for 31 d and at weekly intervals thereafter until day 91. Sampling for *E. coli* at

the high inoculum level was done at 3 or 4 d intervals for the first week and at weekly intervals thereafter until day 91.

For the low inoculum treatments with all organisms, samples were taken weekly for three weeks. For *Yersinia*, sampling continued weekly for 7 weeks. Since initial numbers of *Yersinia* were low, plating of samples for this organism up to 21 d was duplicated and the volume used was increased (0.1 ml/plate *Yersinia* selective agar x 5 plates) to increase recovery sensitivity.

3.5 *Salmonella* survival in inoculated soil

Salmonella Typhimurium 02-8421 (a human isolate obtained from R. Ahmed, Health Canada, Winnipeg, MB.), resistant to ampicillin and tetracycline, was used in these tests. Its preparation and optical density adjustment were performed as mentioned previously. The culture was adjusted to an OD_{600} of 0.720 to yield about 7 log cfu/ml. Both dry (moisture unadjusted) and moisture saturated soil treatments were used. Soil was saturated with water by slowly adding sterile water to one kg soil in a Buchner funnel. Soil was saturated when water dripped from the funnel after mixing the soil. Soil was aseptically weighed and 250 g of each sample in duplicate was placed in sterile stomacher bags (Fisher Scientific Ltd., Nepean, ON). One ml of the *Salmonella* culture was added to the high moisture soil and mixed. *Salmonella* culture addition to the dry soil was made slowly in 0.1 ml increments followed by mixing until 1.0 ml was reached.

Soil used was from a soil profile from the Green Farm Site and consisted of a mixture of the top, middle and bottom portions of the soil column. The sample moisture content was 11.6% for the unsaturated soil and 30.99% for the saturated soil sample. Inoculated soil stored in sterile stomacher bags at 22°C was sampled up to 12 d.

Numbers of *Salmonella* were determined on MacConkey agar (Difco) supplemented with 100 ppm each of tetracycline (Sigma-Aldrich Co., St. Louis, MO) and ampicillin (anhydrous) (Sigma-Aldrich Co.). At sampling a tenfold dilution was prepared by placing 25 g soil in 225 ml of buffered peptone water (Difco). Further ten fold dilutions were prepared with 0.1% peptone and 0.1 ml of the samples were spread-plated in duplicate on the antibiotic-supplemented plates. Incubation was for 48 h at 35-37°C.

Samples were taken and analyzed on days 0, 1, 2, 4, 6, 8, 10 and 12. When bags of soil were not being sampled they were stored loosely sealed with a twist tie at 22°C.

3.6 *Salmonella* transport through soil columns

Soil cores measuring about 8 cm diameter x 1 m were taken from the experimental site near Carberry using a commercial mobile well drill (Maple Leaf Drilling, Carberry, MB). One m cores were removed from the upper 2 m of the soil profile from fields 3 and 4 planted with alfalfa. Intact cores were transferred to sterile polycarbonate tubes of similar internal diameter, sealed and brought to the laboratory

where they were stored at 4°C before use. For the experiments where the movement of *Salmonella* through the columns was to be studied, a single core in a plastic tube (a column) was removed from storage and a rubber stopper, the same diameter as the column, was used to plug the bottom of the column. Each stopper contained a small hole which allowed glass tubing to be inserted through it to allow passage of water. The glass tubing was secured with water resistant caulking to prevent leakage. From the bottom of the soil column a small amount of some soil was removed to ensure the stopper fit tightly without any space remaining. The stopper was secured with several layers of duct tape. The column was then placed upright and clamped to a stand.

Tygon tubing with two different diameters was used to pass water through the column using a Technicon II auto analyzer proportioning pump (Pulse Instrumentation, Saskatoon, Sask.). Tubing with a flow rate of 0.32 ml/min, (Pulse Instrumentation) was used to carry tap water from a glass reservoir to the top of the column where it was allowed to drip on the centre of the soil column. Tubing with a flow rate of 0.42 ml/min. (Pulse Instrumentation) was used to drain the column at the bottom and collect the water sample in a sterile flask for microbial analysis.

3.6.1 Measurement of *Salmonella* movement through the soil columns

Before *Salmonella* was added to the column, the pump was run for approximately 4 h, prior to addition of inoculum a sample of 100 ml of water was collected to verify

Salmonella absence from the soil. After the *Salmonella* 02-8421 inoculum was standardized at OD_{600} to contain 10^6 CFU/ml in 0.85% saline, 10 ml was applied using a sterile syringe to the centre of the top of the column. A 250 ml, 500 ml or 1 L sterile collection flask was placed at the bottom of the column and a sterile foam stopper held the column exit tubing in place during sample collection. There were 8 perfusion tests preformed. Six columns were studied with the addition of *Salmonella* and 2 columns were controls run with saline. There were no columns which were run twice. The experiments were conducted with the columns at 25°C during testing. Samples not analyzed for *Salmonella* immediately were stored at 4°C and were plated within 12 h of collection.

Sample collection intervals were 3-4 h prior to the addition of *Salmonella*, 0-10 h after addition, every 2-4 h until 28 h and approximately every 8 h thereafter until completion of the run at 48-62.5 h. Pooled sample volumes in the exit flask ranged from 60 to about 250 ml.

To approximate the movement of *Salmonella* through the column, 10 ml 0.85% NaCl without *Salmonella* was added to the top of a new column prior to its elution with water. Conductivity readings of the column eluant were taken using a conductivity meter (YSI Model 32 Conductance Meter, Yellow Springs Instrument Co. Inc., Yellow Springs, OH.) and the volume of sample collected at each interval was measured. The volume at

which elution of NaCl occurred (as measured by an increase in conductivity) was taken as the point where *Salmonella* should exit the column. For *Salmonella* analysis, 0.1 ml of column eluant was spread-plated in duplicate on MacConkey agar supplemented with 100 ppm of each tetracycline and ampicillin as previously described. Colonies were enumerated after incubation at 35-37°C for 48 h.

When colonies were recovered on agar plates from the column eluant, plates were sealed and stored at 4°C until further biochemical analysis could be performed. Biochemical tests were performed on colonies transferred and grown on TSA. Catalase, oxidase, Gram stain reaction and microscopic morphology were determined for typical suspect colonies, as well as reactions of the isolates on TSI and LIA agar slants to verify that organisms recovered were *Salmonella*.

3.6.2 Examination of column soil for residual *Salmonella*

The soil from *Salmonella* inoculated columns was removed from each of the four columns within 24 h of completion of the *Salmonella* transport experiment. The outer surface of the columns was sterilized with 70% (v/v) ethanol and were cut into 10 cm sections using an alcohol-sterilized hacksaw. Each soil section was aseptically removed from the column and placed into sterile stomacher bags. The bags of soil were sealed and stored at 4°C until analyzed for *Salmonella*. Tenfold dilutions were made by placing 10 g of soil into 90 ml of buffered peptone water. Further serial dilutions of samples were

made by transferring 1 ml to 9 ml 0.1% peptone. Samples were spread-plated on MacConkey agar containing 100 ppm each of tetracycline and ampicillin. Colonies were enumerated after incubation at 35-37°C for 48 h.

Chapter 4

RESULTS

4.0 Water analysis quality and monitoring

4.0.1 Presumptive, confirmed and fecal coliforms in water samples

The first manure application to fields 2 and 3 occurred on October 30, 1999, whereas fields 1 and 4 received manure on June 3, 2000. Upon the first water sampling on October 13, 2000 fields 2 and 3 showed a combined total of 3 wells with presumptive positive coliforms but no confirmed coliforms were present. The interval between the last previous manure application and water sampling was 349 d. Fields 1 and 4 contained a total of 12 wells which were presumptive positive for coliforms at 132 d from the last manure application (Table 4.1). For confirmed coliforms, among these samples, one well in each of field 1 and field 4 showed a positive result at 2 MPN/100ml (Table 4.2). Field 4 also showed a positive result for fecal coliforms with an MPN/100ml of 2 (Table 4.3).

The water sampling performed on March 14, 2001, occurred 284 d after the last manure application to fields 1 and 4 and 501 d following manure application to fields 2 and 3. Tests showed presumptive positive coliform results in 1 or 2 wells in each of fields 1 to 4 regardless of the number of days since the last manure application. However fields 1 and 4 had average MPN/100ml values of 10.8, which were higher than the 5.8 MPN/100ml values found in fields 2 and 3 (Table 4.1). The higher MPN values of presumptive

coliforms seemed unrelated to manure application. For confirmed coliforms, field 3 showed 2 positive wells, and each of the other 3 fields (1, 2 and 4) had 1 well positive for confirmed coliforms. These were highest in field 1 at 8.0 MPN/100ml (Table 4.2). None of the wells in any field were positive for fecal coliforms (Table 4.3).

The April 30, 2001 water sampling occurred 331 d after the last manure application to fields 1 and 4, and 548 d after the last manure application to fields 2 and 3. Field 4 had 5 wells which were presumptive positive for coliforms, with an average MPN of 4/100ml. Each of the other 3 fields had a single well positive for presumptive coliforms (Table 4.1). However, field 3 had the highest number present in a single well at 13 MPN/100ml. Fields 1 and 2 had no wells positive for confirmed or fecal coliforms (Tables 4.2, 4.3). Fields 3 and 4 each had one and three wells, respectively, which were positive for confirmed coliforms but not for fecal coliforms (Tables 4.2, 4.3).

The May 24, 2001 sampling occurred 21 d after the last manure application to all four fields. Field 1 contained wells which were negative for presumptive coliforms, whereas fields 2, 3 and 4 had wells which were presumptive positive (Table 4.1). Wells in fields 1 and 3 were negative for confirmed and fecal coliforms, whereas fields 2 and 4 each had one well which was positive for confirmed coliforms at 2 MPN/100ml (Table 4.2), but both were negative for fecal coliforms (Table 4.3).

Seventy-one days after the last previous manure application, water sampling was performed on July 13, 2001. For presumptive positive coliforms, wells in field 1 were

negative, but field 2 had 1 well, and fields 3 and 4 had 2 wells which were positive (Table 4.1). Each of fields 2, 3 and 4 had one well which was positive for confirmed coliforms (Table 4.2). Fecal coliforms were absent from all wells tested at this sampling time (Table 4.3).

At the September 7, 2001 sampling, there was only one field (field 4) that had positive results for presumptive and confirmed coliforms (Tables 4.1, 4.2). In this field there were three wells with both presumptive and confirmed positive coliforms at a level of 2.7 MPN/100ml. This and other fields last received manure 127 d prior to this date. There were no positive results for fecal coliforms (Table 4.3).

From the November 5, 2001 water sample analysis, Fields 1 and 4, which received manure treatment 186 d previously, showed presumptive positive coliforms in a total of 7 wells, with field 1 only having one well positive. Fields 2 and 3 last received manure 14 d prior to sampling and had 6 and 7 presumptive positive wells, respectively (Table 4.1). However upon performing the confirmational testing, field 2 and field 4 each showed a single well with a confirmed coliform result at a level of 2 MPN/100ml (Table 4.2). There were no positive results for fecal coliforms (Table 4.3).

The March 15, 2002 water sampling yielded no positive results for presumptive, confirmed or fecal coliforms, regardless of the last manure application date (144 or 316 d previously) or well depths (Tables 4.1, 4.2, 4.3).

On May 9, 2002 fields 1 and 2 showed no presumptive or confirmed coliforms even though the two fields differed in their last manure application dates (Tables 4.1, 4.2). Field 3 showed 2 presumptive positive wells and was manured 199 d prior to sampling, and Field 4 showed one well presumptive positive and it was manured 15 d prior to the sampling date. There was also a presumptive positive result from one of the background wells (Table 4.3). However, the only confirmed coliform was found in a background well and the sample was also positive for fecal coliforms (Tables 4.2, 4.3).

Over the nine sampling dates from 603 possible positive results (67 wells multiplied by the 9 nine sampling dates), there were only 69 water samples presumptive positive for coliforms. Of the total of 67 wells, there were 37 which were positive for presumptive coliforms at least once over the nine sampling dates. Therefore there were 30 wells which never contained presumptive positive coliforms. These wells were: 902, 903, 904, 905, 906, 908, 909, 910, 912, 914, 915 (field 1); 920, 921, 925, 927, 928, 930, 931 (field 2); 933, 937, 942, 943, 944, 946 (field 3); 949, 959 (field 4); and 962, 963, 964 and 966 (background wells).

For confirmed coliforms, there were 14/67 wells which showed positive results over the nine sampling dates (again, 603 possible positive results). There were 11 shallow wells which were confirmed positive for coliforms (911, 916, 919, 926, 929, 941, 950, 952, 955, 960 and 967) and two intermediate depth wells (953 and 961) which were also positive for these organisms. Well 932 (field 3) was the only deep well which was positive for confirmed coliforms.

Table 4.1: Presumptive coliforms in groundwater from the Green Farm site over 20 months

Sample Date	Field Number	Crop Type	Number of Wells per Field	Number of Positive Wells	Average MPN / 100ml	Well(s) Depth	Interval (d) since Last Manure Treatment
October 13, 2000	Field 1	Winter Wheat	16	5	7	S, I, D	132
	Field 2	Winter Wheat	15	1	4	Shallow	349
	Field 3	Alfalfa	15	2	12.5	I and D	349
	Field 4	Alfalfa	15	7	10.4	S and I	132
	Background	Alfalfa	6	1	4	Deep	-
March 14, 2001	Field 1	Oats	16	1	13	Shallow	284
	Field 2	Oats	15	1	4	Shallow	501
	Field 3	Alfalfa	15	2	7.5	S and D	501
	Field 4	Alfalfa	15	2	8.5	S and I	284
	Background	Alfalfa	6	0	-	-	-
April 30, 2001	Field 1	Oats	16	1	2	Shallow	331
	Field 2	Oats	15	1	2	Shallow	548
	Field 3	Alfalfa	15	1	13	Deep	548
	Field 4	Alfalfa	15	5	4	S and I	331
	Background	Alfalfa	6	0	-	-	-
May 24, 2001	Field 1	Oats	16	0	-	-	21
	Field 2	Oats	15	2	2	Shallow	21
	Field 3	Alfalfa	15	3	2.7	S and D	21
	Field 4	Alfalfa	15	2	2	Shallow	21
	Background	Alfalfa	6	0	-	-	-
July 13, 2001	Field 1	Oats	16	0	-	-	71
	Field 2	Oats	15	1	4	Shallow	71
	Field 3	Alfalfa	15	2	2	Shallow	71
	Field 4	Alfalfa	15	2	3	Shallow	71
	Background	Alfalfa	6	0	-	-	-
September 7, 2001	Field 1	Oats	16	0	-	-	127
	Field 2	Oats	15	0	-	-	127
	Field 3	Alfalfa	15	0	-	-	127
	Field 4	Alfalfa	15	3	2.7	S and I	127
	Background	Alfalfa	6	0	-	-	-

Table 4.1 (cont'd): Presumptive coliforms in groundwater from the Green Farm site over 20 months

Sample Date	Field Number	Crop Type	Number of Wells per Field	Number of Positive Wells	Average MPN / 100ml	Well(s) Depth	Interval (d) since Last Manure Treatment
November 5, 2001	Field 1	Oats	16	1	2	Intermediate	186
	Field 2	Oats	15	6	4.5	S, I, D	14
	Field 3	Alfalfa	15	7	2.6	S, I, D	14
	Field 4	Alfalfa	15	6	3.3	S, I, D	186
	Background	Alfalfa	6	0	-	-	-
March 15, 2002	Field 1	Canola	16	0	-	-	316
	Field 2	Canola	15	0	-	-	144
	Field 3	Alfalfa	15	0	-	-	144
	Field 4	Alfalfa	15	0	-	-	316
	Background	Alfalfa	6	0	-	-	-
May 9, 2002	Field 1	Canola	16	0	-	-	371
	Field 2	Canola	15	0	-	-	199
	Field 3	Alfalfa	15	2	2	S and I	199
	Field 4	Alfalfa	15	1	2	Shallow	15
	Background	Alfalfa	6	1	2	Shallow	-

Shallow (S) < 4.5 m b/g, Intermediate (I) 4.5 - 8.0 m b/g, Deep (D) >8.0 m b/g

"-" denotes not applicable test due to zero positive wells to be tested for an MPN value

Table 4.2: Confirmed coliforms in groundwater from the Green Farm site over 20 months

Sample Date	Field Number	Crop Type	Number of Wells per Field	Number of Positive Wells	Average MPN / 100ml	Well(s) Depth	Interval (d) since Last Manure Treatment
October 13, 2000	Field 1	Winter Wheat	16	1	2	Shallow	132
	Field 2	Winter Wheat	15	0	0	-	349
	Field 3	Alfalfa	15	0	0	-	349
	Field 4	Alfalfa	15	1	2	Shallow	132
	Background	Alfalfa	6	0	0	-	-
March 14, 2001	Field 1	Oats	16	1	8	Shallow	284
	Field 2	Oats	15	1	4	Shallow	501
	Field 3	Alfalfa	15	2	7.5	Deep, Shallow	501
	Field 4	Alfalfa	15	1	4	Shallow	284
	Background	Alfalfa	6	0	0	-	-
April 30, 2001	Field 1	Oats	16	0	0	-	331
	Field 2	Oats	15	0	0	-	548
	Field 3	Alfalfa	15	1	4	Deep	548
	Field 4	Alfalfa	15	3	2	Shallow	331
	Background	Alfalfa	6	0	0	-	-
May 24, 2001	Field 1	Oats	16	0	0	-	21
	Field 2	Oats	15	1	2	Shallow	21
	Field 3	Alfalfa	15	0	0	-	21
	Field 4	Alfalfa	15	1	2	Shallow	21
	Background	Alfalfa	6	0	0	-	-
July 13, 2001	Field 1	Oats	16	0	0	-	71
	Field 2	Oats	15	1	4	Shallow	71
	Field 3	Alfalfa	15	1	2	Shallow	71
	Field 4	Alfalfa	15	1	2	Shallow	71
	Background	Alfalfa	6	0	0	-	-
September 7, 2001	Field 1	Oats	16	0	0	-	127
	Field 2	Oats	15	0	0	-	127
	Field 3	Alfalfa	15	0	0	-	127
	Field 4	Alfalfa	15	3	2.7	Shallow, Intermediate	127
	Background	Alfalfa	6	0	0	-	-

Table 4.2 (cont'd): Confirmed coliforms in groundwater from the Green Farm site over 20 months

Sample Date	Field Number	Crop Type	Number of Wells per Field	Number of Positive Wells	Average MPN / 100ml	Well(s) Depth	Interval (d) since Last Manure Treatment
November 5, 2001	Field 1	Oats	16	0	0	-	186
	Field 2	Oats	15	1	2	Shallow	14
	Field 3	Alfalfa	15	0	0	-	14
	Field 4	Alfalfa	15	1	2	Intermediate	186
	Background	Alfalfa	6	0	0	-	-
March 15, 2002	Field 1	Canola	16	0	0	-	316
	Field 2	Canola	15	0	0	-	144
	Field 3	Alfalfa	15	0	0	-	144
	Field 4	Alfalfa	15	0	0	-	316
	Background	Alfalfa	6	0	0	-	-
May 9, 2002	Field 1	Canola	16	0	0	-	371
	Field 2	Canola	15	0	0	-	199
	Field 3	Alfalfa	15	0	0	-	199
	Field 4	Alfalfa	15	0	0	-	15
	Background	Alfalfa	6	1	2	Shallow	-

Shallow (S) < 4.5 m b/g, Intermediate (I) 4.5 - 8.0 m b/g, Deep (D) > 8.0 m b/g

Table 4.3: Fecal coliforms in groundwater from the Green Farm site over 20 months

Sample Date	Field Number	Crop Type	Number of Wells per Field	Number of Positive Wells	Average MPN / 100ml	Well(s) Depth	Interval (d) since Last Manure Treatment
October 13, 2000	Field 1	Winter Wheat	16	0	-	-	132
	Field 2	Winter Wheat	15	0	-	-	349
	Field 3	Alfalfa	15	0	-	-	349
	Field 4	Alfalfa	15	1	2	Shallow	132
	Background	Alfalfa	6	0	-	-	-
March 14, 2001	Field 1	Oats	16	0	-	-	284
	Field 2	Oats	15	0	-	-	501
	Field 3	Alfalfa	15	0	-	-	501
	Field 4	Alfalfa	15	0	-	-	284
	Background	Alfalfa	6	0	-	-	-
April 30, 2001	Field 1	Oats	16	0	-	-	331
	Field 2	Oats	15	0	-	-	548
	Field 3	Alfalfa	15	0	-	-	548
	Field 4	Alfalfa	15	0	-	-	331
	Background	Alfalfa	6	0	-	-	-
May 24, 2001	Field 1	Oats	16	0	-	-	21
	Field 2	Oats	15	0	-	-	21
	Field 3	Alfalfa	15	0	-	-	21
	Field 4	Alfalfa	15	0	-	-	21
	Background	Alfalfa	6	0	-	-	-
July 13, 2001	Field 1	Oats	16	0	-	-	71
	Field 2	Oats	15	0	-	-	71
	Field 3	Alfalfa	15	0	-	-	71
	Field 4	Alfalfa	15	0	-	-	71
	Background	Alfalfa	6	0	-	-	-
September 7, 2001	Field 1	Oats	16	0	-	-	127
	Field 2	Oats	15	0	-	-	127
	Field 3	Alfalfa	15	0	-	-	127
	Field 4	Alfalfa	15	0	-	-	127
	Background	Alfalfa	6	0	-	-	-

Table 4.3 (cont'd): Fecal coliforms in groundwater from the Green Farm site over 20 months

Sample Date	Field Number	Crop Type	Number of Wells per Field	Number of Positive Wells	Average MPN / 100ml	Well(s) Depth	Interval (d) since Last Manure Treatment
November 5, 2001	Field 1	Oats	16	0	-	-	186
	Field 2	Oats	15	0	-	-	14
	Field 3	Alfalfa	15	0	-	-	14
	Field 4	Alfalfa	15	0	-	-	186
	Background	Alfalfa	6	0	-	-	-
March 15, 2002	Field 1	Canola	16	0	-	-	316
	Field 2	Canola	15	0	-	-	144
	Field 3	Alfalfa	15	0	-	-	144
	Field 4	Alfalfa	15	0	-	-	316
	Background	Alfalfa	6	0	-	-	-
May 9, 2002	Field 1	Canola	16	0	-	-	371
	Field 2	Canola	15	0	-	-	199
	Field 3	Alfalfa	15	0	-	-	199
	Field 4	Alfalfa	15	0	-	-	15
	Background	Alfalfa	6	1	2	Shallow	-

Shallow (S) < 4.5 m b/g, Intermediate (I) 4.5 - 8.0 m b/g, Deep (D) > 8.0 m b/g

For fecal coliforms, only two wells showed a positive result from all 67 wells over the nine sampling dates. These wells were 960 in field 4 on October 13, 2000 (almost a year after the last manure application) and 967 in the background field on May 9, 2002 (field 4, which is adjacent, and had been manured 15 d previously). Both of these wells were classified in the shallow depth range.

4.0.2 Presumptive and confirmed *Pseudomonas aeruginosa* in water samples

Following the analysis of the water samples from March 14, 2001 for *Pseudomonas* it was found that every well was presumptive positive (Table 4.4). However after performing confirmational testing, the numbers of positive wells decreased (Table 4.5). The background wells had the highest MPN/100 ml value at 21.0 (Table 4.5). Fields 1 and 4, which were manured 284 d prior to sampling, showed 10 confirmed positive wells each. Fields 2 and 3, manured 501 d previously, together had 12 wells showing positive results (Table 4.5).

From the July 13, 2001 samples (last manured 71 d prior to sampling), the number of presumptive positive wells were 54/67 (Table 4.4). Once samples were confirmed, field 2 had the highest number of positive wells at 7, but field 3 showed the highest MPN/100ml value at 4.7. The background well MPN/100ml was greater than the field 1 value and these were 4.0 and 3.6, respectively (Table 4.5).

After 127 d following the last manure application to all fields, the water samples from September 7, 2001 were analyzed. Fields 1 and 3 each showed 10 wells which were presumptive positive (Table 4.4). Upon confirming for *P. aeruginosa* presence, field 1 had only one well positive, whereas field 3 showed the most wells positive at 6. However, the average MPN/100ml was 2.0 and was the same for fields 1, 4 and background wells (Table 4.5).

The November 5, 2001 water sampling showed fields 2 and 3 with a lower number of presumptive positive wells (9 and 6, respectively), than fields 1 and 4 (14 and 12, respectively) (Table 4.4). Fields 1 and 4 were manured 14 d prior to sampling, whereas fields 2 and 3 were last manured 186 d prior to sampling. Again upon confirmation, it was seen that the less recently manured fields (2 and 3) had a slightly lower number of positive wells (3 and 1, respectively). However, the average MPN/100ml for both pairs of fields was similar (3.9 and 4.0 for fields 1 and 4, and fields 2 and 3, respectively) (Table 4.5).

For the March 15, 2002 samples, fields 2 and 3 showed a total of 22 presumptive positive *P. aeruginosa* wells after their last manure treatment 144 d prior to sampling. Fields 1 and 4 (treated with manure 316 d earlier) showed 19 presumptive positive wells (Table 4.4). Field 2 had the lowest MPN/100ml confirmed value at 2.0 and only had a single well positive, which was the same as the background wells (Table 4.5). Field 3 had the highest number of positive wells at 5, whereas field 1 had the highest average MPN at 7.7/100ml for its 3 wells that were confirmed positive for *P. aeruginosa* (Table 4.5)

Field 1 and 4 from the May 9, 2002 samples showed the same number of wells presumptive positive at 11. However, the last manure application to these fields was 371 d and 15 d prior to sampling for field 1 and 4, respectively. Fields 2 and 3 showed the same number of wells presumptive positive at 12 each (199 d following manure application) (Table 4.4). Following the confirmation testing, field 1 had no positives, whereas field 4 had 4 confirmed positive wells, but also had the lowest average MPN/100ml (other than the background wells) at 2.5 MPN/100ml (Table 4.5).

Each of the 67 wells was presumptive positive for *P. aeruginosa* on at least one of the 6 sampling dates. There were 55/67 wells where confirmed positive *P. aeruginosa* were found over the 6 sampling dates. There were 24 shallow wells, 22 intermediate depth wells and 9 deep wells which were confirmed positive for this organism. The 12 wells which were not positive for confirmed *P. aeruginosa* on any sample date were: 901, 903, 908, 909, 912 (field 1), 922 (field 2), 932, 933, 937, 940, 945 (field 3), and well 956 (field 4). The wells in the background field (962-967) were all positive at least once for confirmed *P. aeruginosa*.

4.0.3 Coliphage recovery

No coliphage were found in the concentrated water samples from the shallow wells. Each concentrated well water sample was plated in quadruplicate using two different *E. coli* hosts (*E. coli* C and *E. coli* C3000) for the two different sets of four plates. All 9 concentrated shallow well water samples from all four fields (wells 901, 903, 905, 929,

Table 4.4: Presumptive *Pseudomonas* occurrence in groundwater from the Green Farm site over 20 months

Sample Date	Field Number	Crop Type	Number of Wells per Field	Number of Positive Wells	Average MPN / 100ml	Well(s) Depth	Interval (d) since Last Manure Treatment
March 14, 2001	Field 1	Oats	16	16	20.1	S, I, D	284
	Field 2	Oats	15	15	20.9	S, I, D	501
	Field 3	Alfalfa	15	15	21.6	S, I, D	501
	Field 4	Alfalfa	15	15	23.0	S, I, D	284
	Background	Alfalfa	6	6	23.0	S, I, D	-
July 13, 2001	Field 1	Oats	16	14	4.6	S, I, D	71
	Field 2	Oats	15	13	3.9	S, I, D	71
	Field 3	Alfalfa	15	10	4.0	S, I, D	71
	Field 4	Alfalfa	15	13	5.2	S, I, D	71
	Background	Alfalfa	6	4	3.5	S, I, D	-
September 7, 2001	Field 1	Oats	16	10	5.3	S, I, D	127
	Field 2	Oats	15	6	3.0	S, I, D	127
	Field 3	Alfalfa	15	10	2.4	S, I, D	127
	Field 4	Alfalfa	15	4	2.5	S, I, D	127
	Background	Alfalfa	6	4	2.0	S and I	-
November 5, 2001	Field 1	Oats	16	14	2.7	S, I, D	186
	Field 2	Oats	15	9	4.4	S, I, D	14
	Field 3	Alfalfa	15	6	3.3	S, I, D	14
	Field 4	Alfalfa	15	12	4.6	S, I, D	186
	Background	Alfalfa	6	3	2.8	S and D	-
March 15, 2002	Field 1	Canola	16	9	4.6	S, I, D	316
	Field 2	Canola	15	10	2.6	S, I, D	144
	Field 3	Alfalfa	15	12	3.0	S, I, D	144
	Field 4	Alfalfa	15	10	3.0	S, I, D	316
	Background	Alfalfa	6	4	3.0	S, I, D	-
May 9, 2002	Field 1	Canola	16	11	2.6	S, I, D	371
	Field 2	Canola	15	12	2.5	S, I, D	199
	Field 3	Alfalfa	15	12	3.5	S, I, D	199
	Field 4	Alfalfa	15	11	2.7	S, I, D	15
	Background	Alfalfa	6	3	3.3	S and D	-

Shallow (S) < 4.5 m below/ground, Intermediate (I) 4.5 - 8.0 m b/g, Deep (D) > 8.0 m b/g

Table 4.5: Confirmed *Pseudomonas* occurrence in groundwater from the Green Farm site over 20 months

Sample Date	Field Number	Crop Type	Number of Wells per Field	Number of Positive Wells	Average MPN / 100ml	Well(s) Depth	Interval (d) since Last Manure Treatment
March 14, 2001	Field 1	Oats	16	10	13.9	S, I, D	284
	Field 2	Oats	15	7	19.4	S, I, D	501
	Field 3	Alfalfa	15	5	12.6	S and D	501
	Field 4	Alfalfa	15	10	20.0	S, I, D	284
	Background	Alfalfa	6	5	21.0	S, I, D	-
July 13, 2001	Field 1	Oats	16	5	3.6	S and I	71
	Field 2	Oats	15	7	4.1	S and I	71
	Field 3	Alfalfa	15	3	4.7	S and I	71
	Field 4	Alfalfa	15	4	4.5	S and I	71
	Background	Alfalfa	6	1	4.0	Deep	-
September 7, 2001	Field 1	Oats	16	1	2.0	Intermediate	127
	Field 2	Oats	15	5	3.2	S and I	127
	Field 3	Alfalfa	15	6	2.3	S and I	127
	Field 4	Alfalfa	15	2	2.0	S and I	127
	Background	Alfalfa	6	2	2.0	S and I	-
November 5, 2001	Field 1	Oats	16	2	2.0	S and I	186
	Field 2	Oats	15	3	4.0	S and I	14
	Field 3	Alfalfa	15	1	4.0	Shallow	14
	Field 4	Alfalfa	15	4	5.8	S and I	188
	Background	Alfalfa	6	2	2.0	Shallow	-
March 15, 2002	Field 1	Canola	16	3	7.7	S and I	316
	Field 2	Canola	15	1	2.0	Shallow	144
	Field 3	Alfalfa	15	5	2.4	S and I	144
	Field 4	Alfalfa	15	4	4.0	S and I	316
	Background	Alfalfa	6	1	2.0	Intermediate	-
May 9, 2002	Field 1	Canola	16	0	-	-	371
	Field 2	Canola	15	6	2.7	S and I	199
	Field 3	Alfalfa	15	5	4.0	S and I	199
	Field 4	Alfalfa	15	4	2.5	S and I	15
	Background	Alfalfa	6	1	2.0	Shallow	-

Shallow (S) < 4.5 m b/g, Intermediate (I) 4.5 - 8.0 m b/g, Deep (D) > 8.0 m b/g

931, 934, 936, 957, and 960) were plated undiluted. Field 1 was manured 371 d, fields 2 and 3 was manured 199 d and field 4 was manured 15 d prior to the water sampling. There was no plaque formation on any of the four plates of each sample plated with each *E. coli* host. When MS2 bacteriophage was used in water and plated on its specific *E. coli* host (C3000) in control tests, there was some clearing or plaque formation. This confirmed that the protocol used was capable of detecting coliphage if they had been present and that the assay did work. The well water used from the experimental site did not contain detectable coliphage. All of the samples tested negative for confirmed and fecal coliforms using the MPN fermentation technique.

4.1 Analysis of Manure for Potential Pathogens

4.1.1 *Salmonella* identification

Of 8 manure samples taken from the tanker before field application of the manure, one sample was positive for *Salmonella* and was identified according to API 20E as *Salmonella* spp. subgroup 3 (bioMerieux, Hazelwood, MO).

4.1.2 *Yersinia* identification

Eight manure samples taken May 3, 2001 from the manure spreader tank reservoir just prior to field application were analyzed for *Yersinia* species. Six of the 8 samples were positive for the presence of *Yersinia*. Identity confirmation and serotyping of *Yerinia*

spp. were conducted by the Enteric and Environmental Microbiology and Special Procedures Laboratories Branch in Etobicoke ON. Seven isolates were confirmed to be *Yersinia* , with 5 cultures identified as *Y. frederiksenii*, one as *Y. intermedia* and one confirmed as non-pathogenic *Y. enterocolitica* biotype 1A (with untypable serotype).

4.1.3 *Escherichia coli* identification

The same manure samples were also analyzed for *E. coli* and all 8 samples were positive. The average level of *E. coli* when plated on Petrifilm from LST broth was 8.2 log cfu/ml, and coliforms were present at 7.3 log cfu/ml. When the EC broth tubes were plated on Petrifilm the average result for all 8 samples was 7.6 log cfu/ml. When the 8 samples were diluted and plated on Rainbow agar for *E. coli* O157:H7 no black colonies were present and thus this organism was absent from the sampled manure.

4.2 Analysis of soil for potential pathogens

4.2.1 Soil sample set 1

The first set of soil core samples taken November 17, 2000 were analyzed for *Yersinia, Salmonella*, coliforms and *E. coli*. These soil cores were sectioned into 3 depths: top 5 cm, 0-90 cm and 90-180 cm. The most recent manure addition prior to the soil sampling occurred October 30, 1999 (fields 2 and 3) and June 3, 2000 (fields 1 and 4).

Table 4.6: Microbiological analysis of set 1 soil core samples (0-5cm depth) for coliforms, *E. coli*, *Salmonella* and *Yersinia.* [a]

Soil sample	coliform	*E. coli*	*Salmonella*	*Yersinia*
GGB-1	+	-	-	-
GGB-2	+	-	-	-
GGB-3	+	-	-	-
GGB-4	+	-	-	-
GGB-5	+	$1.1x10^9$	-	-
GGB-6	+	-	-	-
GGB-7	+	$5.0x10^8$	-	-
GGB-8	+	$1.6x10^9$	-	-
GGB-9	+	$1.0x10^9$	-	-
GGB-10	+	$1.0x10^9$	-	-

[a)] Soil samples were taken at the Green Farm site near Carberry, MB. Values reported indicate colony forming units per gram soil sample

Table 4.7: Microbiological analysis of set 1 soil core samples (5-90cm depth) for coliforms, *E. coli*, *Salmonella* and *Yersinia*.[a]

Soil sample	coliform	*E. coli*	*Salmonella*	*Yersinia*
GGB-1	+	-	-	-
GGB-2	+	-	-	-
GGB-3	+	-	-	-
GGB-4	+	-	-	-
GGB-5	+	-	-	-
GGB-6	+	-	-	-
GGB-7	+	-	+[b]	-
GGB-8	+	-	-	-
GGB-9	+	-	+[b]	-
GGB-10	+	-	-	-

[a)] Soil samples were taken at the Green Farm site near Carberry, MB. Values reported indicate colony forming units per gram soil sample

[b)] The organisms were confirmed as H_2S positive *E. coli*

Table 4.8: Microbiological analysis of set 1 soil core samples (90-180cm depth) for coliforms, *E. coli*, *Salmonella* and *Yersinia*. [a]

Soil sample	coliform	*E. coli*	*Salmonella*	*Yersinia*
GGB-1	-	-	-	-
GGB-2	-	-	-	-
GGB-3	-	-	-	-
GGB-4	-	-	-	-
GGB-5	-	-	-	-
GGB-6	-	-	-	-

[a)] Soil samples were taken at the Green Farm site near Carberry, MB.

In the top 5 cm, all samples, GGB-1 through GGB-10 were positive for coliforms. The level of *E. coli* present in the top 5 cm samples varied substantially. Samples GGB 1, 2, 3, 4 and 6 contained no *E. coli*. Sample GGB-7 contained 8.7 log cfu/g, samples GGB 5, 9 and 10 contained 9.0 log cfu/g while sample GGB-8 contained 9.2 log cfu/g *E. coli* (Table 4.6).

At the 0-90 cm depth, all samples (GGB-1 through GGB-10) were positive for coliforms but were negative when tested for *E. coli* and *Yersinia* (Table 4.7). Two samples tested positive for *Salmonella* (GGB-7 and GGB-9) however, when the cultures were sent for confirmation to the National Laboratory for Enteric Pathogens (Winnipeg, MB), they were found to be strains of *E. coli* (H_2S positive).

At the 90-180 cm depth, samples designated GGB-1 through GGB-6 and were all negative for coliforms, *E. coli*, *Salmonella* and *Yersinia* (Table 4.8).

4.2.2 Soil sample set 2

The second set of soil cores samples analyzed were taken from the Green Farm on May 24, 2001. The Green Farm was divided into 4 fields (F1, F2, F3 and F4), with three subsections identified within each field (S1, S2 and S3). There were two background sections sampled (BGD-1 and BGD-2). The core samples were divided into the top 5 cm, 0-60 cm, 60-120 cm, 120-180 cm and 180-240 cm and analyzed separately. All four fields had manure applied on May 3, 2001.

All the samples, regardless of depth or location at the farm tested negative for *Salmonella* and *Yersinia*, but were positive for presumptive coliforms.

Table 4.9: Microbiological analysis of set 2 soil samples at the 0-5 cm depth from the Green Farm

Sample	Coliforms (LST)	Fecal Coliforms (EC Broth)	*E. coli* (cfu/g) Petrifilm	*Salmonella*	*Yersinia*
F1S1	+	-	-	-	-
F1S2	+	+	-	-	-
F1S3	+	-	-	-	-
F2S1	+	-	-	-	-
F2S2	+	+	$4.0x10^6$	-	-
F2S3	+	-	-	-	-
F3S1	+	+	$1.2x10^8$	-	-
F3S2	+	+	$1.6x10^8$	-	-
F3S3	+	+	$6.4x10^8$	-	-
F4S1	+	+	$5.0x10^7$	-	-
F4S2	+	-	-	-	-
F4S3	+	+	-	-	-
BDG-1	+	-	-	-	-
BGD-2	+	-	-	-	-

F= field; S= site location within field
BGD= background location (untreated with manure)

Table 4.10: Microbiological analysis of set 2 soil samples at the 5-60 cm depth from the Green Farm

Sample	Coliforms (LST)	Fecal Coliforms (EC Broth)	*E. coli* (cfu/g) Petrifilm	*Salmonella*	*Yersinia*
F1S1	+	+	$1.0x10^9$	-	-
F1S2	+	+	$1.2x10^9$	-	-
F1S3	+	-	-	-	-
F2S1	+	+	-	-	-
F2S2	+	+	$8.5x10^8$	-	-
F2S3	+	+	-	-	-
F3S1	+	+	$4.5x10^9$	-	-
F3S2	+	+	$3.4x10^9$	-	-
F3S3	+	+	$5.0x10^9$	-	-
F4S1	+	+	-	-	-
F4S2	+	+	$8.0x10^9$	-	-
F4S3	+	-	-	-	-
BDG-1	+	-	-	-	-
BGD-2	+	-	-	-	-

F= field; S= site location within field
BGD= background location (untreated with manure)

Table 4.11: Microbiological analysis of set 2 soil samples at the 60-120 cm depth from the Green Farm

Sample	Coliforms (LST)	Fecal Coliforms (EC Broth)	*E. coli* (cfu/g) Petrifilm	*Salmonella*	*Yersinia*
F1S1	+	-	-	-	-
F1S2	+	+	$1.0x10^9$	-	-
F1S3	+	-	-	-	-
F2S1	+	-	-	-	-
F2S2	+	+	$2.2x10^9$	-	-
F2S3	+	-	-	-	-
F3S1	+	+	$5.0x10^8$	-	-
F3S2	+	+	$4.2x10^9$	-	-
F3S3	+	+	$2.1x10^9$	-	-
F4S1	+	+	$3.4x10^9$	-	-
F4S2	+	+	$2.4x10^9$	-	-
F4S3	+	-	-	-	-
BDG-1	+	-	-	-	-
BGD-2	+	-	-	-	-

F= field; S= site location within field
BGD= background location (untreated with manure)

Table 4.12: Microbiological analysis of set 2 soil samples at the 120-180 cm depth from the Green Farm

Sample	Coliforms (LST)	Fecal Coliforms (EC Broth)	*E. coli* (cfu/g) Petrifilm	*Salmonella*	*Yersinia*
F1S2	+	-	-	-	-
F1S3	+	-	-	-	-
F2S1	+	+	$1.3x10^9$	-	-
F2S2	+	+	$9.5x10^8$	-	-
F2S3	+	-	-	-	-
F3S1	+	+	$8.3x10^8$	-	-
F3S2	+	-	-	-	-
F3S3	+	+	$1.6x10^9$	-	-
F4S3	+	-	-	-	-
BDG-1	+	-	-	-	-
BGD-2	+	-	-	-	-

F= field; S= site location within field
BGD= background location (untreated with manure)

Table 4.13: Microbiological analysis of set 2 soil samples at the 180-240 cm depth from the Green Farm

Sample	Coliforms (LST)	Fecal Coliforms (EC Broth)	*E. coli* (cfu/g) Petrifilm	*Salmonella*	*Yersinia*
F2S3	+	-	-	-	-
F4S3	+	-	-	-	-

F= field; S= site location within field
BGD= background location (untreated with manure)

Soil samples from the 0-5 cm depth contained confirmed fecal coliforms and the numbers of *E. coli* on Petrifilm found (Table 4.9) were as follows: F2S2 6.6 log cfu/g, F3S1 8.1 log cfu/g, F3S2 8.2 log cfu/g, F3S3 8.8 log cfu/g and F4S1 had 7.7 log cfu/g. Five samples in three fields (2, 3 and 4) contained fecal coliforms at levels between 6.6 and 8.8 log cfu/g. Seven of 12 samples from all four fields contained between 8.9 and 9.9 log cfu fecal coliforms/g soil fecal coliforms in the 5-60 cm depth (Table 4.9). Soil from F1S1 at the 5-60 cm depth contained *E. coli* 0157:H7, since colonies on Chrome and Rainbow agars were typical of this organism.

As sampling progressed deeper through the soil columns (Tables 4.10, 4.11, 4.12) beyond 120 cm fewer samples were found positive for fecal coliforms and *E. coli*, and when positive for the latter organism, numbers were slightly lower. An exception was sample F2S1 which was positive for *E. coli* at 120-180 cm (Table 4.12) but negative for this organism at depths more shallow in the soil column (Tables 4.9, 4.10, 4.11). Soil samples taken at the 180-240 cm depth were negative for fecal coliforms and *E. coli* (Table 4.13).

4.3 Well water survival studies (high inoculum)

4.3.1 *Salmonella* survival

Immediately following inoculation of sterilized water samples, 6.5 log cfu/ml were recovered when plated on TSA and 6.3 log cfu/ml were found when plated on BGA after 48 h at 35-37°C. After 49 d storage at 5°C, viable numbers had decreased to 5.0 and 1.0

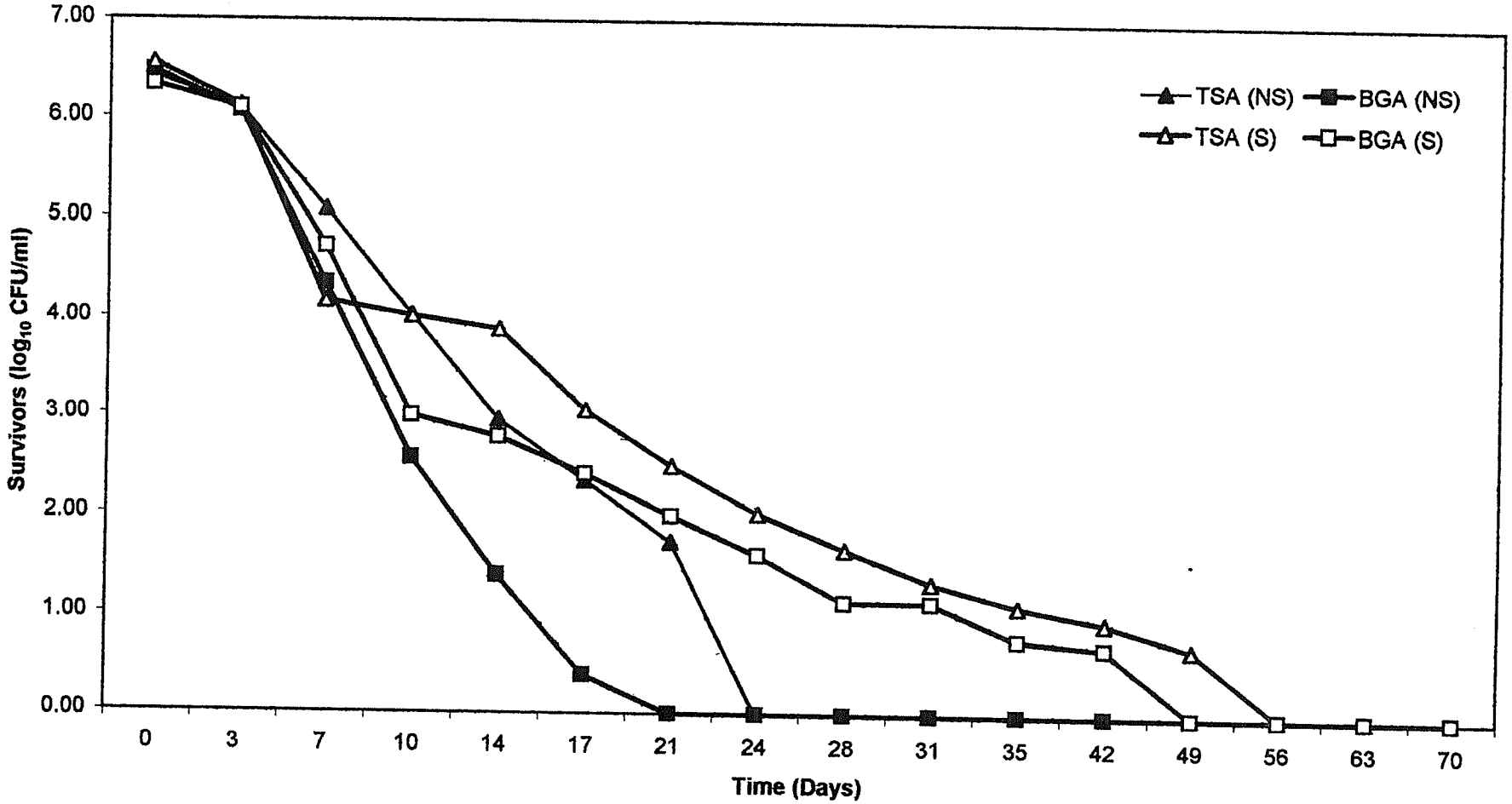

Figure 4.1: *Salmonella* survival in sterile (S) and non-sterile (NS) well water at 5°C plated on TSA and BGA

cfu/ml when plated on the two agars, respectively. When water was sampled at 56 and 70 d and plated on TSA and BGA agars, neither agar contained viable colonies, even when enriched in TSB for 3 h prior to plating on the agars (Fig. 4.1). For the native or non-sterile water experiments, following *Salmonella* inoculation the initial numbers were 6.4 log cfu/ml when plated on TSA and 6.5 log cfu/ml when plated on BGA. After 21 d storage at 5°C there were 55 cfu/ml on TSA and 2.5 cfu/ml on BGA. Neither of the non-sterile water samples contained viable *Salmonella* at 28 d of storage, even after enrichment (Fig. 4.1).

Thus, *Salmonella* numbers decreased more rapidly in non-sterile inoculated water than in sterile samples. In untreated well water viable *Salmonella* disappeared 25 d earlier than in inoculated sterile water stored at 5°C.

4.3.2 *Escherichia coli* survival

In the first replication of the *E. coli* experiment, in initially sterile water, using a manure isolate (manure 1), initial levels of *E. coli* were 6.4 log cfu/ml when plated on TSA and 6.5 log cfu/ml when plated on EMB agar. After 77 d the plate counts were 1.0 cfu/ml when plated on TSA and after 70 d the numbers on EMB were 2.0 cfu/ml (Fig. 4.2). When native well water was inoculated, the initial plate counts were 6.5 log cfu/ml when plated on both TSA and EMB agars. At the completion of the experiment on day 91, the viable cells numbered 25 cfu/ml and 1.0 cfu/ml, respectively, on TSA and EMB agars (Fig. 4.2).

A second replication was performed with another *E. coli* isolate from manure (manure 2). For the sterile water samples, the initial numbers were 6.2 log cfu/ml and 6.1 log cfu/ml, when recovered on TSA and EMB agars, respectively. On day 63, the final recovery on TSA was 2.0 cfu/ml. On EMB at day 56 numbers recovered were also 2.0 cfu/ml (Fig. 4.3). For the non-sterile water, the initial plate counts were 6.2 log cfu/ml and 6.1 log cfu/ml for samples plated on TSA and EMB agars, respectively. *E. coli* remained viable in these water samples at least until day 91. On TSA the final plate count was 43 cfu/ml while on EMB the final plate count was 2.0 cfu/ml (Fig. 4.3).

The third replication of the *E. coli* water survival experiment was performed with an isolate recovered from the soil section F2S2 (0-2). For the sterile water samples, the initial plate count was 6.3 log cfu/ml for both TSA and EMB agars. The final recoverable plate counts on these samples on days 49 and 42 were 1.0 cfu/ml on TSA and EMB agars, respectively. In the non-sterile water, the initial plate counts were 6.2 log cfu/ml for both TSA and EMB. *E. coli* in these non-sterile water samples were recoverable on both agars until the experiment was ended on day 91. The final TSA count was 1.6 cfu/ml and the final EMB count was 1.0 cfu/ml (Fig. 4.4).

Overall, all three *E. coli* strains showed greater persistence in the non-sterile water samples, with organisms in three replications surviving until day 91. When sterile water samples were plated on EMB, recovery of organisms from stored samples was for a shorter period than when plated on TSA. This may have been be due to the selective nature of the agar making it harder for the organism to grow over time. Differences in

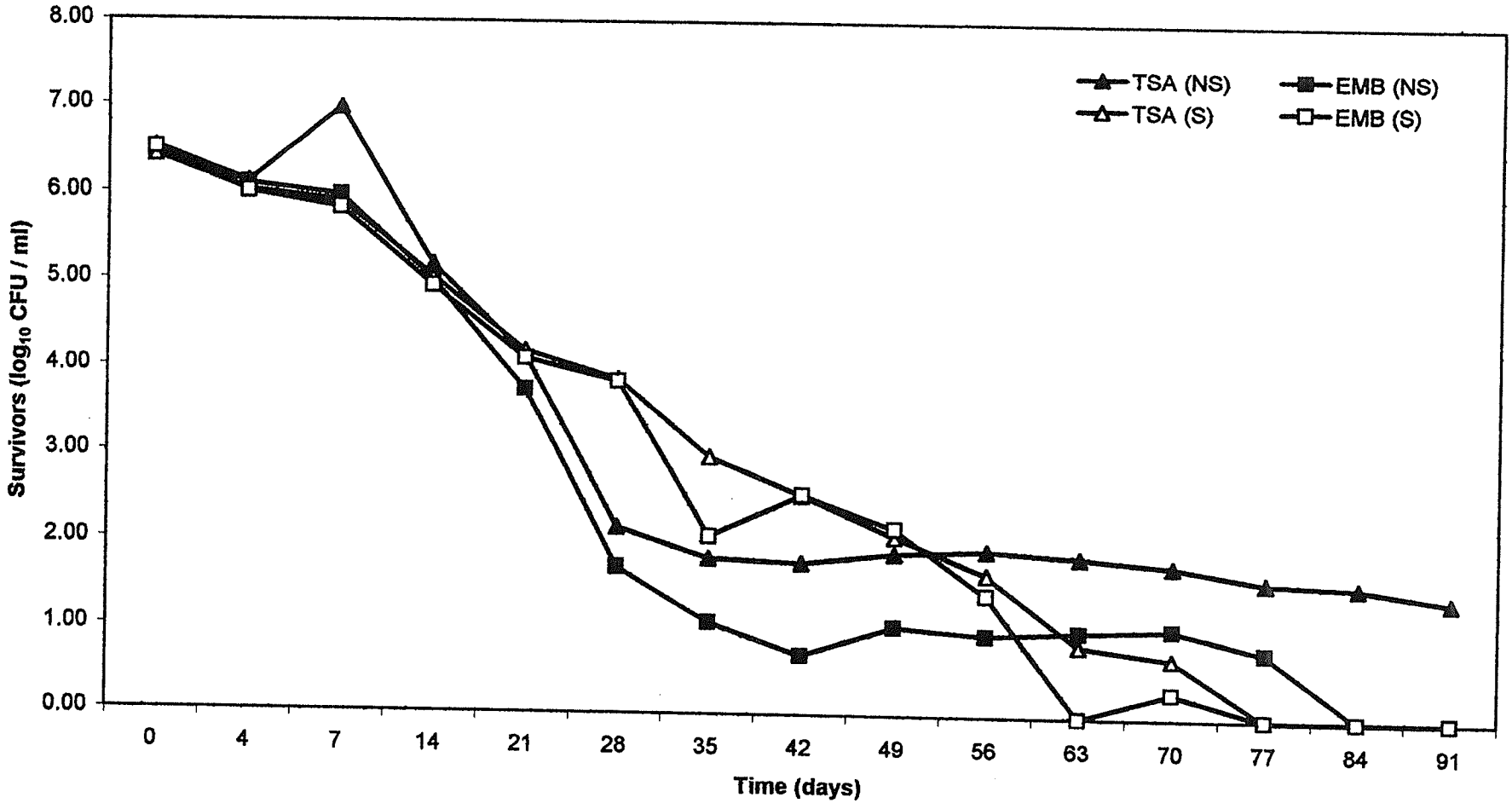

Figure 4.2: *E. coli* (manure isolate 1) survival in sterile (S) and non-sterile (NS) well water at 5°C plated on TSA and EMB

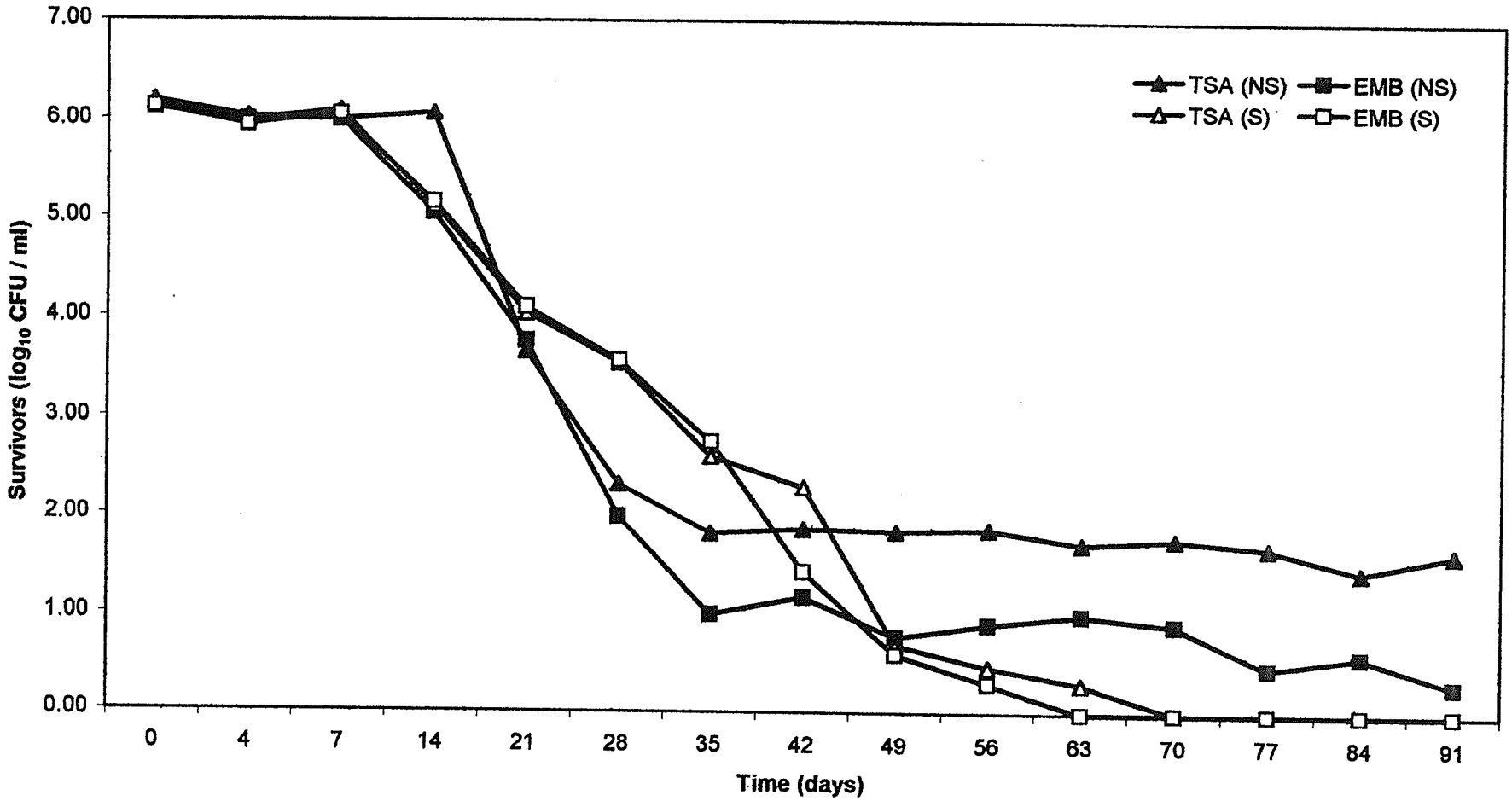

Figure 4.3: *E. coli* (manure isolate 2) survival in sterile (S) and non-sterile (NS) well water at 5°C plated on TSA and EMB

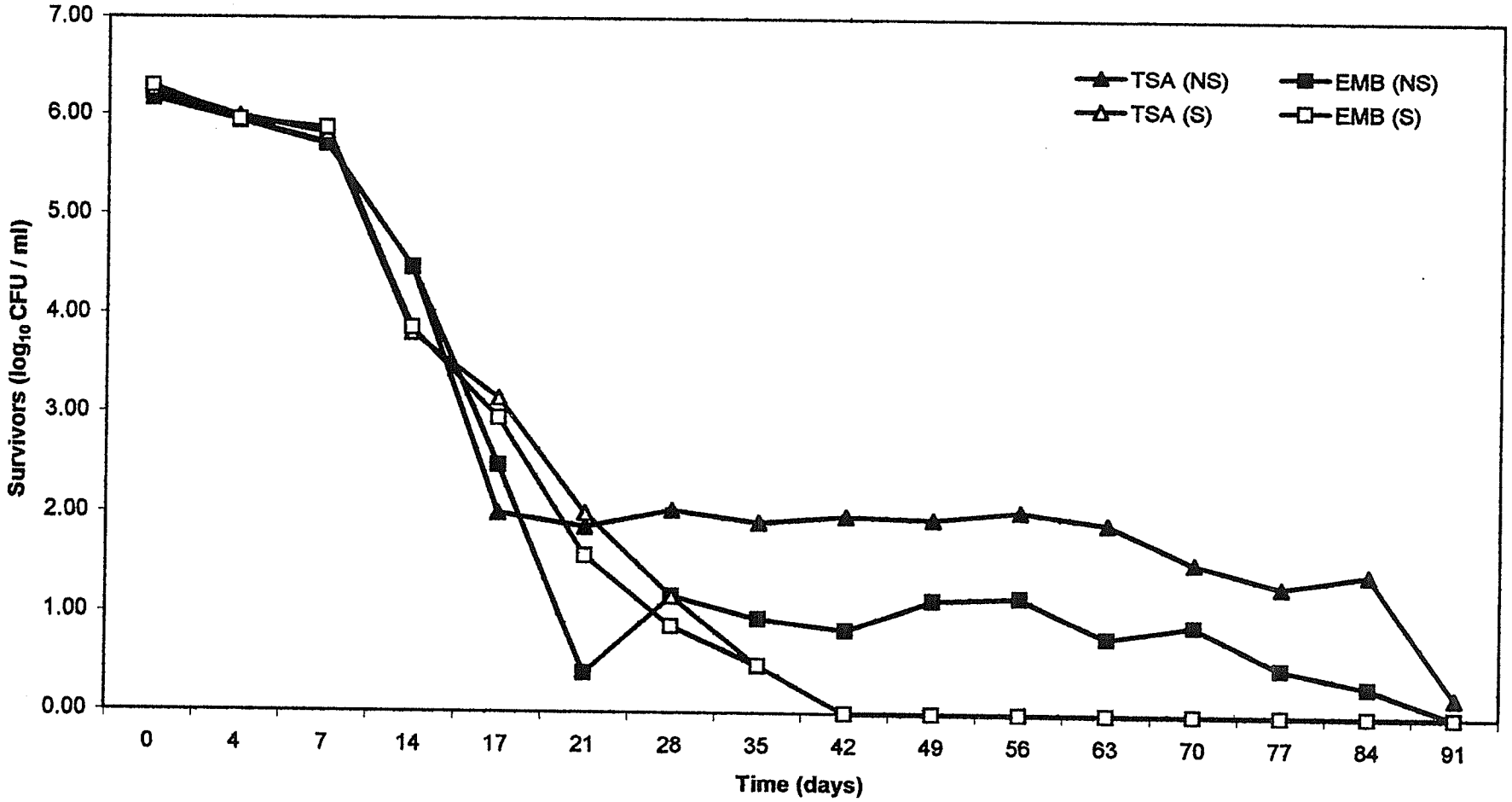

Figure 4.4: *E. coli* (soil isolate) survival in sterile (S) and non-sterile (NS) well water at 5°C plated on TSA and EMB

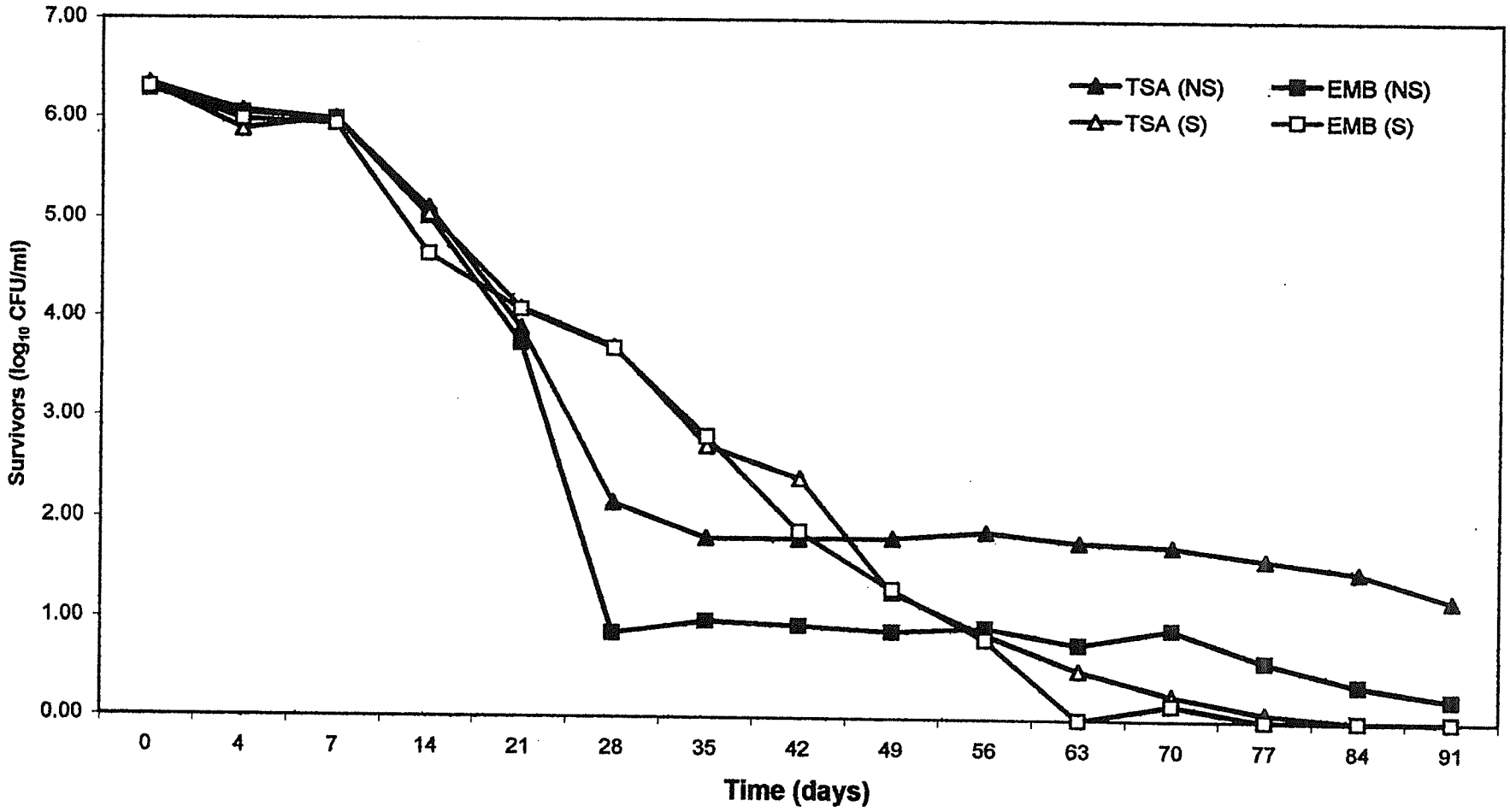

Figure 4.5: *E. coli* survival (mean of 2 manure isolates, Figs. 4.2 and 4.3) at 5°C when recovered on TSA and EMB

the survival of the manure isolates in the two types of water are shown in Fig. 4.5 as a mean of the results presented in Figs. 4.1, 4.2, 4.3. Bacterial numbers recovered from sterile water on TSA were significantly higher ($p \leq 0.05$) than from non-sterile water from day 29 to 45, but thereafter to day 91 the reverse was true. With bacteria recovered on EMB, numbers from sterile water were significantly higher ($p \leq 0.05$) than from non-sterile water at day 28 to 49 and again, the reverse was true from day 63 to 91 (Fig. 4.5).

4.3.3 *Yersinia intermedia* survival

Following inoculation of sterile water the initial numbers of *Yersinia intermedia* were 6.4 log cfu/ml when plated on both TSA and YSA. After 91 days the final numbers were 5.4 log cfu/ml and 4.3 log cfu/ml when plated on TSA and YSA agars, respectively. For the non-sterile water studies, the initial numbers were 6.4 log cfu/ml on both TSA and YSA. Completion of the experiment was on day 91 where the final plate counts were 5.0 log cfu/ml and 5.6 log cfu/ml on TSA and YSA, respectively. It was observed that *Yersinia* was able to survive in all four samples (two sterile and two non-sterile samples) of water beyond day 91 (Fig. 4.6).

4.3.4 *Yersinia frederiksenii* survival

For *Yersinia frederiksenii*, the initial numbers in sterile water were 6.2 log cfu/ml and 5.5 log cfu/ml on TSA and YSA, respectively. After 91 days the final plate counts were 6.4 cfu/ml and 4.6 cfu/ml on TSA and YSA, respectively. In the native well water

experiments, the initial numbers on TSA and YSA were 6.1 log cfu/ml and 5.1 log cfu/ml, respectively. After 91 days, when the experiment was terminated, the final numbers were 4.8 log cfu/ml and 4.1 log cfu/ml, when plated on TSA and YSA, respectively (Fig. 4.7).

Overall, *Yersinia* was able to survive storage in sterile or non-sterile water in greater numbers than *Salmonella* or *E. coli*. This may have been due in part to the psychrotrophic nature of *Yersinia,* since samples of water were stored at 5°C.

Yersinia were recovered from the two test media (TSA and YSA) and from both sterile and non-sterile water until the termination of experiments on day 91 and were probably viable for much longer. Both *Yersinia* strains (Fig. 4.6 and 4.7) lost viability during the first three weeks but then grew back to initial numbers by day 70. *E. coli* were recovered at least until day 91 from the non-sterile samples with shorter survival in sterile water. In contrast, *Salmonella* was able to survive longer in the sterile water samples, but only until day 49-52.

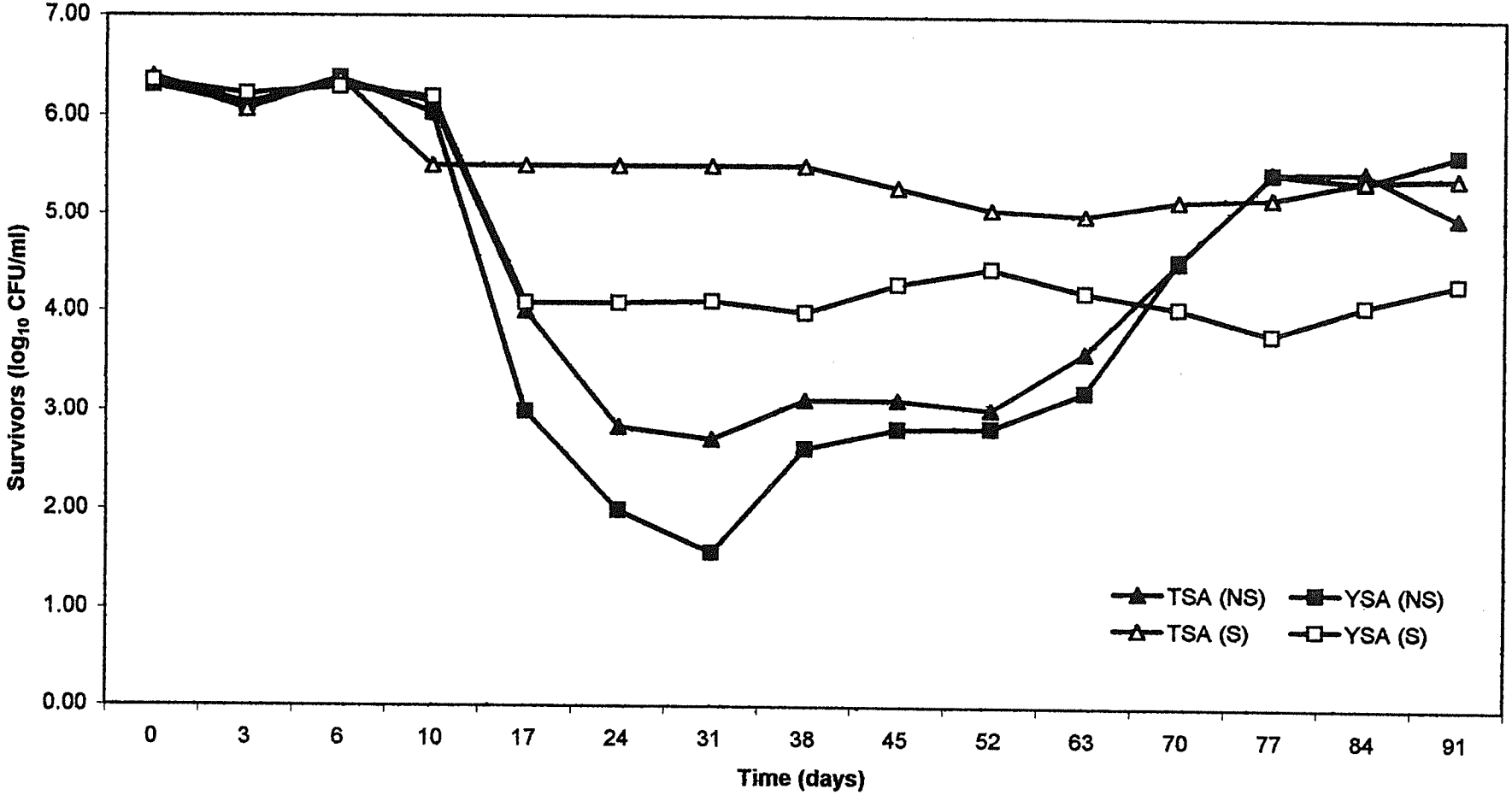

Figure 4.6: *Yersinia intermedia* survival in sterile (S) and non-sterile (NS) well water at 5°C plated on TSA and YSA

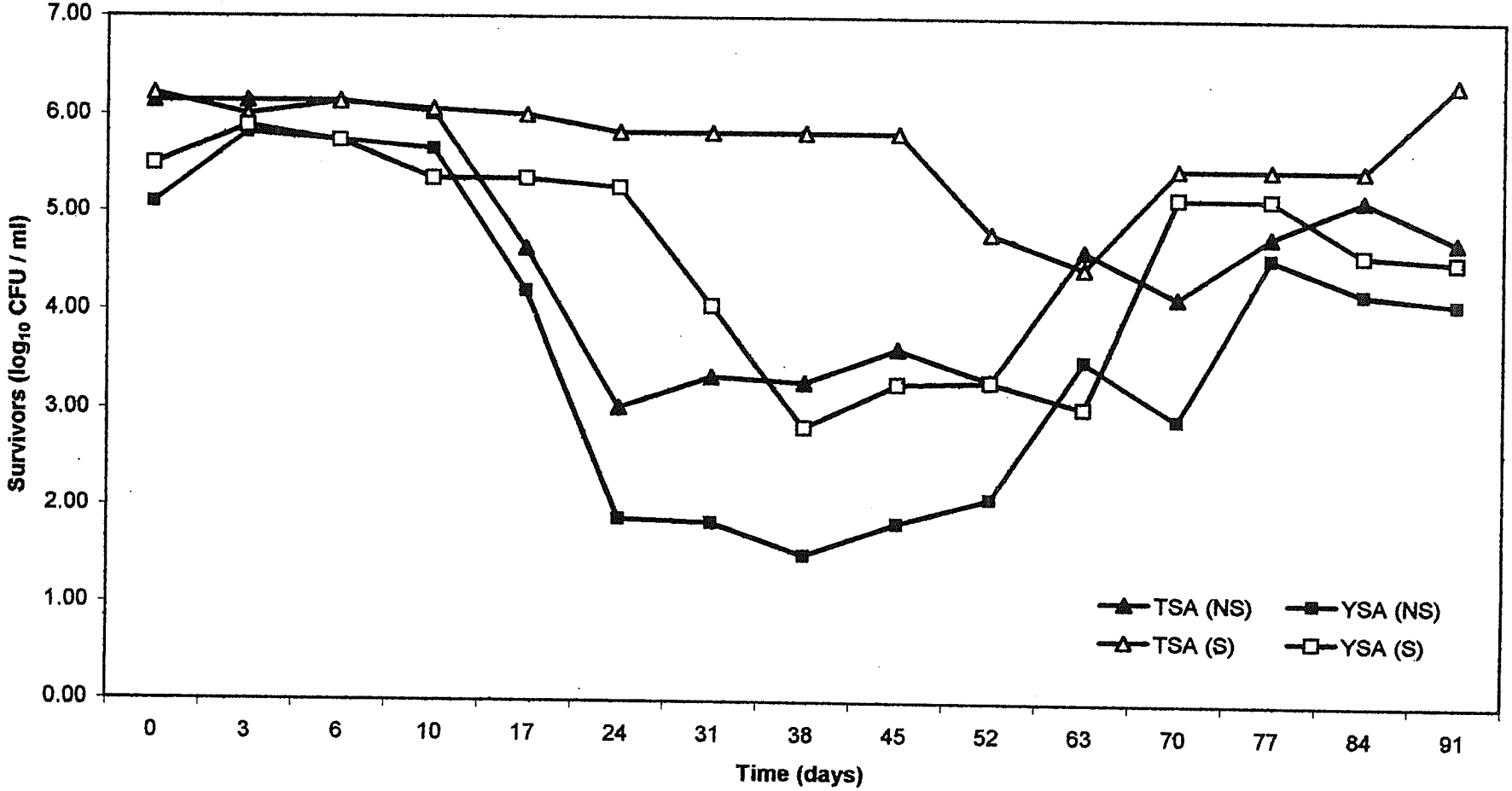

Figure 4.7: *Yersinia frederiksenii* survival in sterile (S) and non-sterile (NS) well water at 5°C plated on TSA and YSA

4.4 Survival of *Salmonella*, *E. coli* and *Yersinia* spp. in well water at low initial inoculation

The water experiments were repeated with a low inoculum level in unautoclaved well water. The initial *Salmonella* level was 4.2 log cfu/ml, and after 21 d it reached 44 cfu/ml when plated on TSA agar. This represented a reduction of > 3 log cfu/ml (Fig. 4.8).

The initial number of *E. coli* on TSA in this test was 4.1 log cfu/ml. On day 21, the final plate count was 2.4 log cfu/ml. This represented a reduction of 2 log cfu/ml (Fig. 4.9).

The *Yersinia intermedia* initial plate count on TSA was 4.2 log cfu/ml and after 49 d, numbers reached 5.3 log cfu/ml. This represented an increase in viable bacteria of 1.3 log cfu/ml (Fig. 4.10).

The *Yersinia intermedia* experiment was repeated using a selective agar (YSA) for plating. Initially the number of *Yersinia* present was 2.9 log cfu/ml, and by 21 d, numbers reached 3.3 log cfu/ml. This represented an increase of 0.4 log cfu/ml (Fig. 4.11).

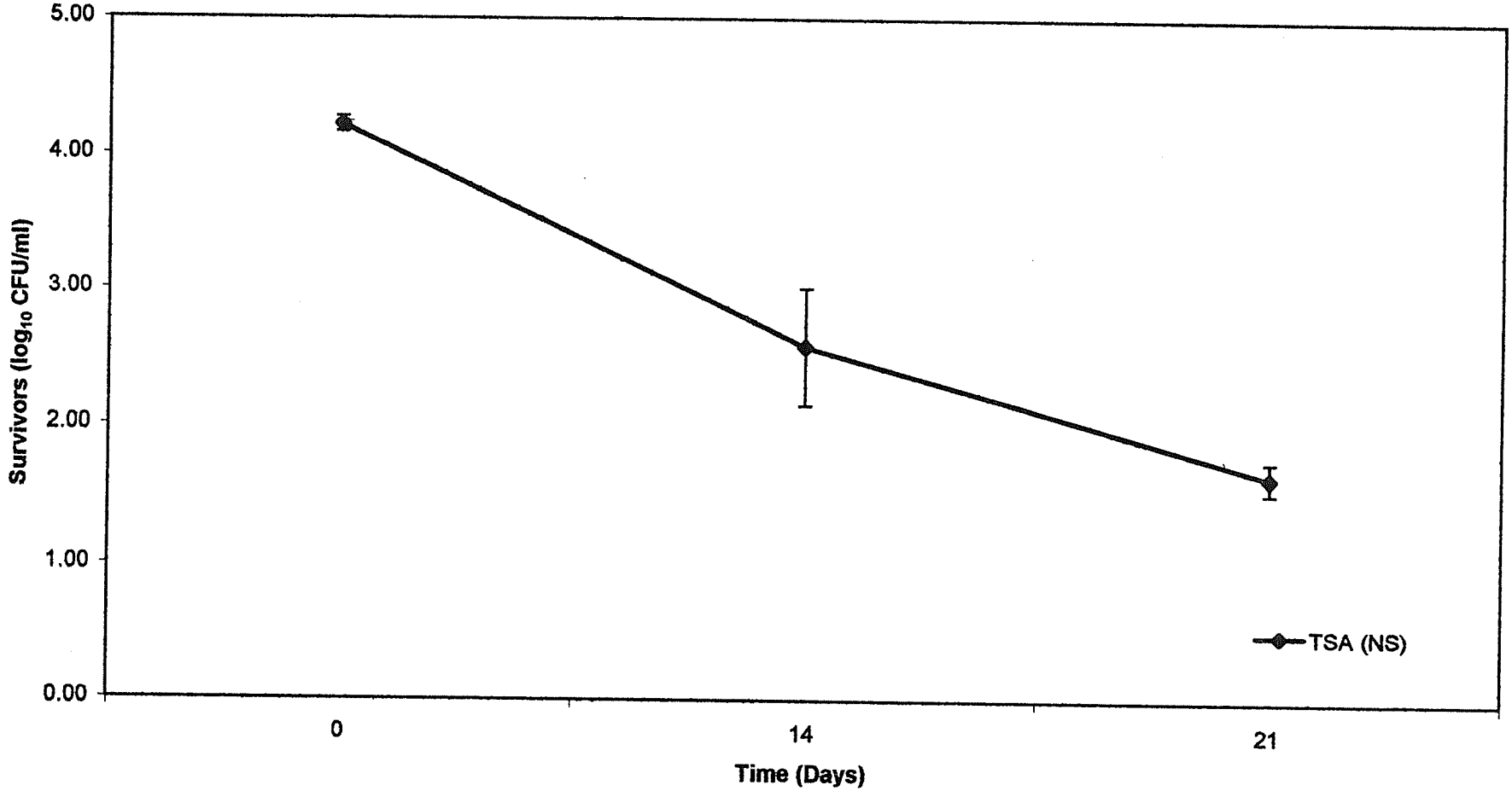

Figure 4.8: *Salmonella* survival in non-sterile well water (low inoculum) at 5°C when plated on TSA

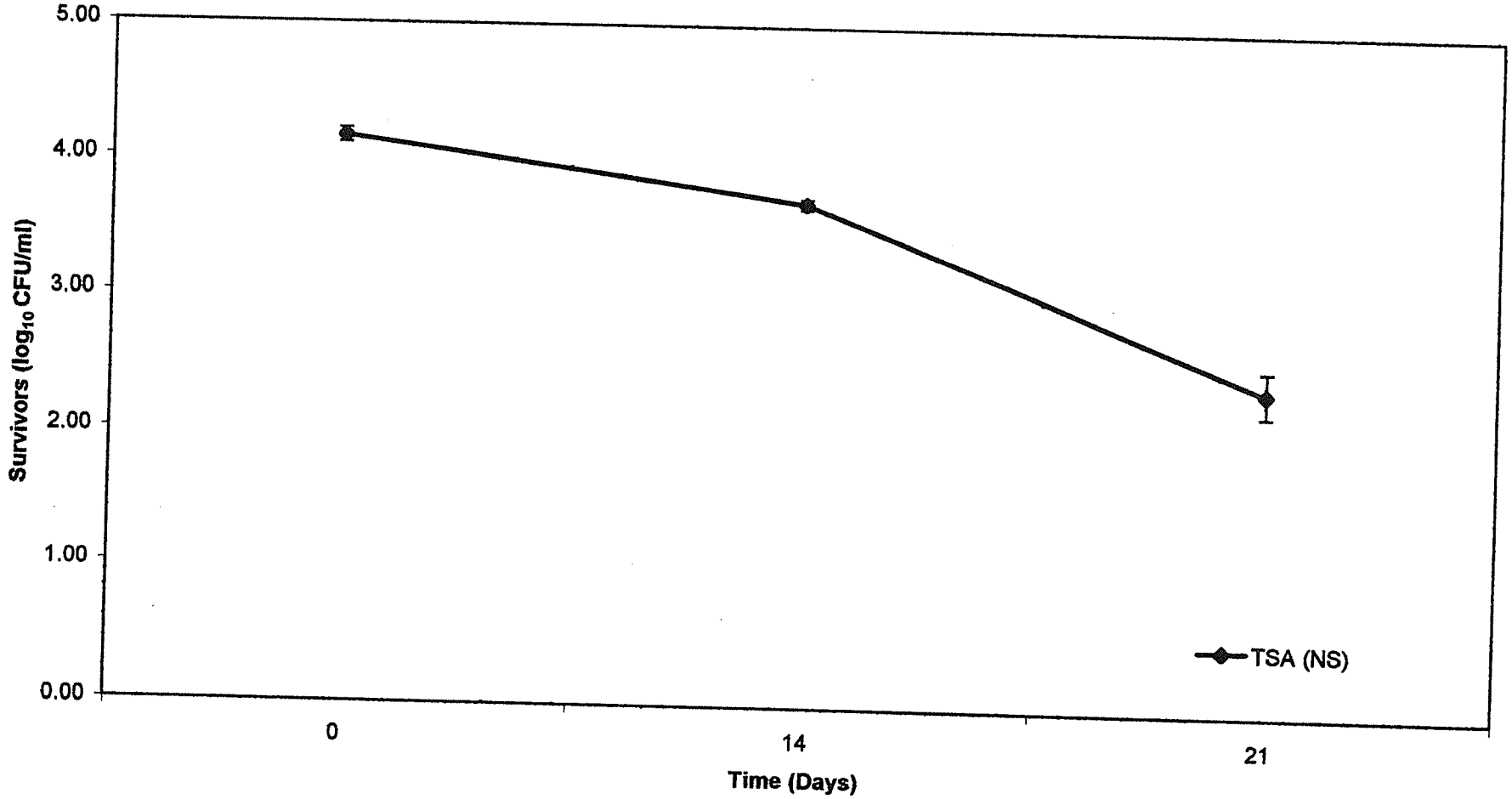

Figure 4.9: *Escherichia coli* survival in non-sterile well water (low inoculum) at 5°C when plated on TSA

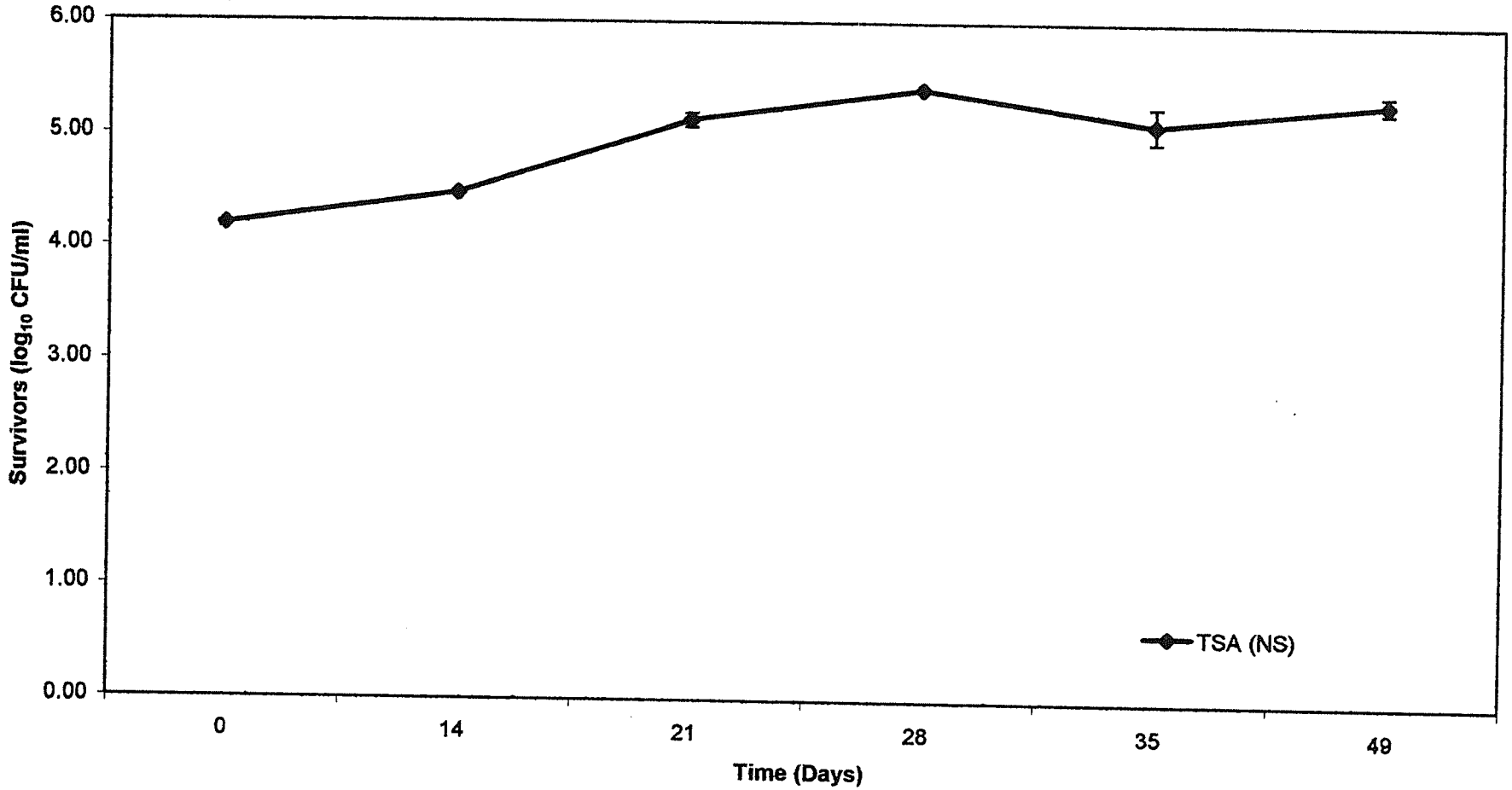

Figure 4.10 : *Yersinia* survival in non-sterile well water (low inoculum) at 5°C when plated on TSA

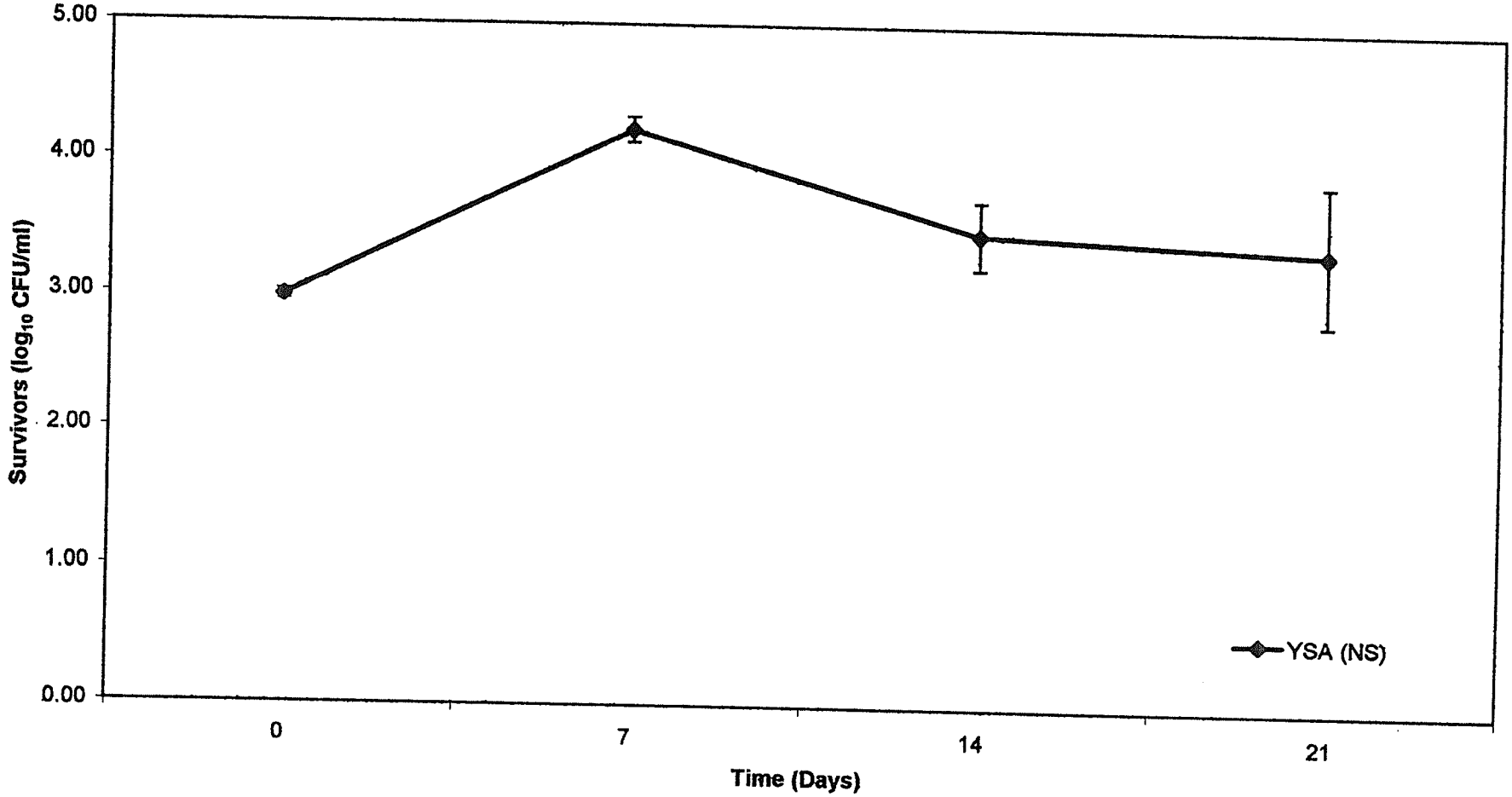

Figure 4.11: *Yersinia intermedia* survival in non-sterile well water (low inoculum) at 5°C when plated on selective agar (YSA)

Overall, *Yersinia* had the greatest survival in the untreated well water at 5°C over the experimental period. However, at the low inoculum level, all three organisms (*E. coli*, *Salmonella* and *Yersinia*) were able to survive for at least 21 d.

4.5 *Salmonella* survival in inoculated soil

Salmonella Typhimurium 02-8421 was inoculated into water-saturated soil (moisture content of 31.0 % w/w) and also into dry (unamended) soil with a moisture content of 11.2 % w/w. Soil was obtained from a soil column taken from the experimental site. The soil and *Salmonella* mixture was kept at 22°C. Following inoculation, *Salmonella* remained viable and was recovered throughout the 12 d sampling period, but at decreased numbers (Fig. 4.12). The saturated (wet) soil allowed for slightly better survival of the organism compared to the dry soil. The initial plate count of the dry soil on MacConkey agar containing tetracycline and ampicillin, was 5.9 log cfu/g. After 12 d there were 3.9 log cfu/g present. The initial numbers present in the saturated wet soil were 6.1 log cfu/g (6.0 log cfu/g on a dry weight basis). After 12 d storage at 22°C final numbers reached 4.2 log cfu/g (4.1 log cfu/g on a dry weight basis). In both cases the organism survived throughout the 12 d storage period.

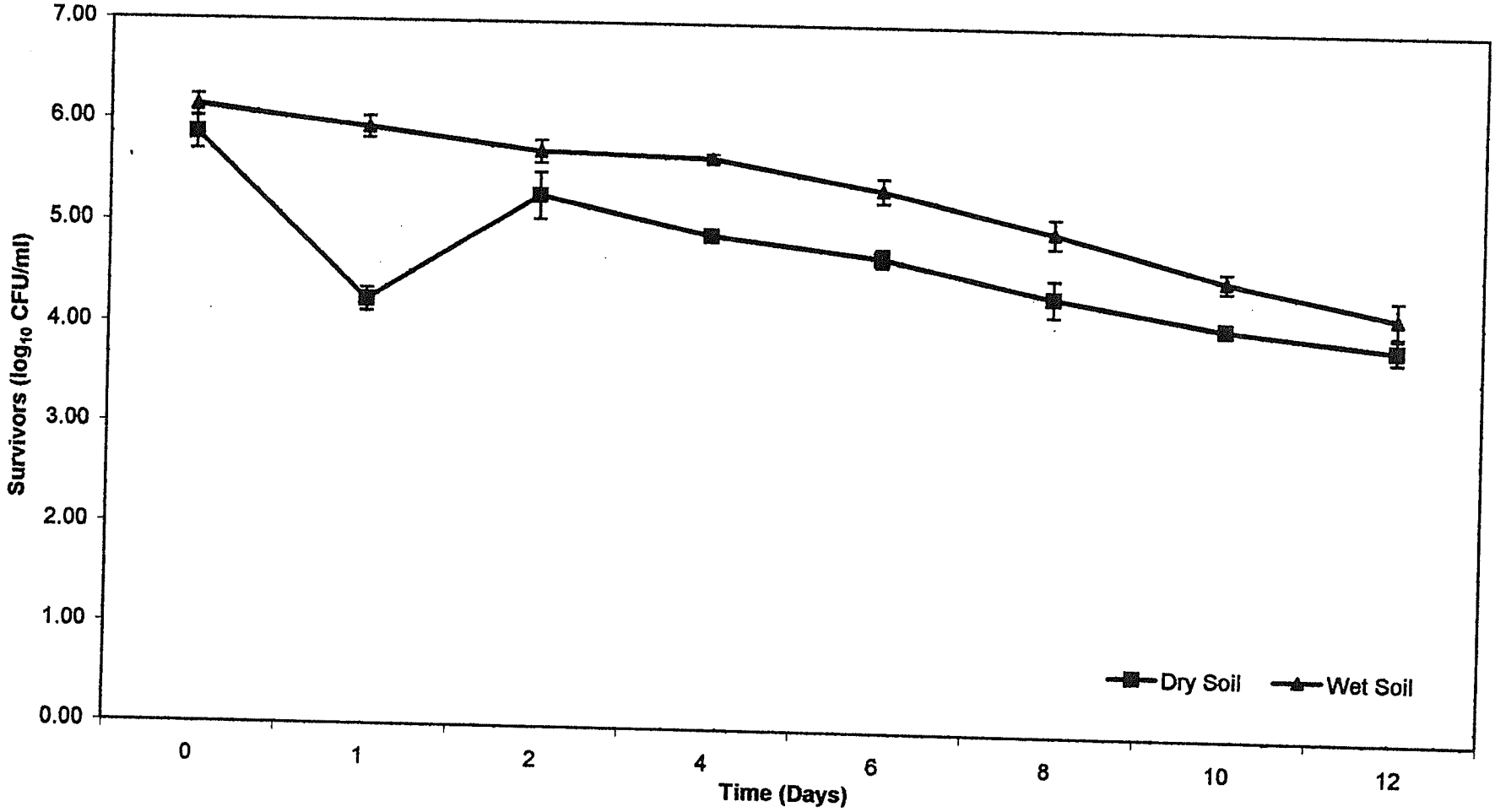

Figure 4.12: *Salmonella* survival in wet and dry soil at 22°C

4.6 *Salmonella* transport through soil columns

The first column tested was inoculated with 10 ml of 0.85% NaCl containing approximately 8.0 log cfu/ml of *Salmonella* Typhimurium 02-8421. Unfortunately, no *Salmonella* were recovered from the column effluent when plated on MacConkey agar supplemented with 100 ppm each of ampicillin and tetracycline. The peak conductivity reading occurred 14-16 h after the inoculum was added (Table 4.14).

Salmonella were recovered from the effluent taken from a second column during a repeated experiment. Numbers recovered represented only 0.11% of the total inoculum added (9.0 log cfu/10 ml). The highest recovery of organisms occurred within the first 10 h, and this represented 65.6 % of recovered *Salmonella*. Thus *Salmonella* moved through the column faster than the added NaCl (Table 4.15).

During a third trail (Table 4.16) a control column containing only added saline was run. No *Salmonella* were recovered from the column effluents.

During the fourth trial of the experiment (Table 4.17), *Salmonella* were recovered from the column effluent. The peak conductivity reading was between 16-20 h of the 62.5 h column run. About <0.01 % of *Salmonella* added were recovered from the initial inoculum of 9.0 log cfu/10 ml. Within the first 10 h of adding the inoculum, 47.3% of the recovered organisms were found.

During a fifth replication (Table 4.18), <0.01% of the organisms added were recovered from the column effluent. The greatest percentage (49.2 %) was found within the effluent collected within the first 10 h of the start of the test. Again, *Salmonella* traversed the column faster than the NaCl.

During a sixth replication (Table 4.19), a control test was done with no *Salmonella* addition. No *Salmonella* were recovered from the column effluent over a period of 59 h. The peak conductivity reading was found in the effluent discharged from the column between 12-20 h.

During a seventh replication (Table 4.20), following inoculation, no *Salmonella* were recovered from the column effluent samples. The conductivity peak occurred at 20-28 h.

During an eighth replication of the experiment (Table 4.21) 0.02% of the total inoculum of 9.0 log cfu/10 ml was recovered in the column effluent. The greatest percentage of recovered organisms was found between 8-10 h and represented 30.69 % of the organisms recovered. The peak conductivity reading occurred at 10-20 h after addition of the initial inoculum.

Colonies recovered from MacConkey agar containing ampicillin and tetracycline were subjected to biochemical testing, including oxidase, catalase and Gram stain testing, as well as morphological determination by phase contrast microscopy. All colonies examined were Gram negative, oxidase negative and catalase positive. Cells were rod

shaped. Isolated colonies were transferred to TSI and LIA agars. Polyvalent O antiserum agglutination testing was also carried out. All isolates gave results typical of *Salmonella.*

Control columns were also run (Tables 4.16, 4.19) to ensure that no background organisms were being detected. There were no additional organisms recovered from the control column runs where 0.85 % saline was added and passed through the column without added *Salmonella*. Thus the plating medium used was able to prevent interference from naturally present organisms eluted from the soil columns.

4.7 Analysis of soil from the *Salmonella*-inoculated columns

After the columns were run, soils from 4 of the columns (Tables 4.17, 4.18, 4.20, 4.21) were separately divided into sections 10 cm in length and analyzed for *Salmonella*.

The first set of soil sections (Table 4.17) analyzed yielded a total of 6.9 log cfu *Salmonella*. The majority (96.1%) of the *Salmonella* recovered were found in the top 20 cm of the soil column. When this number was added to the number recovered from the column effluent, a total of 6.9 log cfu *Salmonella* were accounted for. This represented an overall recovery of 0.78% of the added organisms (Fig. 4.13).

Table 4.14: *Salmonella* 02-8421 movement through a soil core perfused with 0.85% saline at 22°C (Experiment #1)

Sample Time (h)	Collected Volume (ml)	Cumulative Volume (ml)	Conductivity	Plate Count (per 1.0 ml)	Total Plate Count	% of Total Recovered
4 hours prior	78	-	0.319	0	0	0
0 to 10	210	-	0.243	0	0	0
10 to 12	44	254	0.429	0	0	0
12 to 14	40	294	0.794	0	0	0
14 to 16	41	335	0.9	0	0	0
16 to 18	44	379	0.843	0	0	0
18 to 20	44	423	0.621	0	0	0
20 to 22	42	465	0.548	0	0	0
22 to 24	44	509	0.547	0	0	0
24 to 28	84	593	0.476	0	0	0
28 to 36	164	757	0.394	0	0	0
36 to 48	242	999	0.349	0	0	0
48 to 54	117	1116	0.297	0	0	0
Totals	1194 ml				0	

Column experiment performed from March 14 - 17, 2003

Table 4.15: *Salmonella* movement through a soil core perfused with 0.85% saline at 22°C (Experiment #2)

Sample Time (h)	Collected Volume (ml)	Cumulative Volume (ml)	Conductivity	Plate Count (per 1.0 ml)	Total Plate Count	% of Total Recovered
4 h before inoculation	83	-	0.299	0	0	0.00
0 to 10	212	-	0.309	>3000	>636000	65.56
10 to 12	42	254	0.508	>3000	>126000	1.30
12 to 14	43	297	0.807	1930	82990	8.55
14 to 16	45	342	0.916	720	32400	3.34
16 to 18	43	385	0.818	405	17415	1.80
18 to 20	44	429	0.712	290	12760	1.32
20 to 22	44	473	0.646	255	11220	1.16
22 to 24	43	516	0.543	130	5590	0.58
24 to 28	85	601	0.445	125	10625	1.10
28 to 36	174	775	0.366	80	13920	1.43
36 to 40	86	861	0.298	105	9030	0.93
40 to 48	174	1035	0.29	70	12180	1.26
Totals	1118 ml				284330	

Column experiment performed from March 28 - 30, 2003

Table 4.16: Saline movement through a soil core perfused with 0.85% saline at 22°C (Experiment #3)

Sample Time (h)	Collected Volume (ml)	Cumulative Volume (ml)	Conductivity	Plate Count (per 1.0 ml)	Total Plate Count	% of Total Recovered
3.5 h before saline added	73	-	0.236	0	0	0
0 to 10	214	-	0.225	0	0	0
10 to 12	43	257	0.339	0	0	0
12 to 16	84	341	0.959	0	0	0
16 to 20	84	425	0.582	0	0	0
20 to 24	86	511	0.398	0	0	0
24 to 28	84	595	0.294	0	0	0
28 to 36	148	743	0.246	0	0	0
36 to 40	84	827	0.196	0	0	0
40 to 48	170	997	0.196	0	0	0
48 to 62.5	310	1307	0.234	0	0	0
Totals	1380 ml				0	

Control Column was run from April 4 - 7, 2003

Table 4.17: *Salmonella* movement through a soil core perfused with 0.85% saline at 22°C (Experiment #4)

Sample Time (h)	Collected Volume (ml)	Cumulative Volume (ml)	Conductivity	Plate Count (per 1.0 ml)	Total Plate Count	% of Total Recovered
3.5 h before inoculation	72	-	0.263	0	0	0
0 to 10	210	210	0.259	45	9450	47.30
10 to 12	42	252	0.297	175	7350	36.79
12 to 16	84	336	0.361	15	1260	6.31
16 to 20	82	418	0.828	0	0	0
20 to 24	83	501	0.696	0	0	0
24 to 28	83	584	0.447	0	0	0
28 to 36	146	730	0.327	0	0	0
36 to 40	82	812	0.246	5	410	2.05
40 to 48	166	978	0.228	0	0	0
48 to 62.5	302	1280	0.26	5	1510	7.56
Totals	1352 ml				19980	

Column #1 was run from April 4 - 7, 2003

Table 4.18: *Salmonella* movement through a soil core perfused with 0.85% saline at 22°C (Experiment #5)

Sample Time (h)	Collected Volume (ml)	Cumulative Volume (ml)	Conductivity	Plate Count (per 1.0 ml)	Total Plate Count	% of Total Recovered
3.5 h before inoculation	73	-	0.245	0	0	0
0 to 10	214	214	0.229	35	7490	49.23
10 to 12	43	257	0.264	160	6880	45.22
12 to 16	85	342	0.285	5	425	2.79
16 to 20	84	426	0.866	5	420	2.76
20 to 24	84	510	0.693	0	0	0
24 to 28	85	595	0.369	0	0	0
28 to 36	148	743	0.281	0	0	0
36 to 40	83	826	0.217	0	0	0
40 to 48	168	994	0.204	0	0	0
48 to 62.5	310	1304	0.228	0	0	0
Totals	1377 ml				15215	

Column #2 was run from April 4 - 7, 2003

Table 4.19: Saline movement through a soil core perfused with 0.85% saline at 22°C (Experiment #6)

Sample Time (h)	Collected Volume (ml)	Cumulative Volume (ml)	Conductivity	Plate Count (per 1.0 ml)	Total Plate Count	% of Total Recovered
2.5 h before saline addition	51	-	0.408	0	0	0
0 to 2	32	-	0.407	0	0	0
2 to 4	49	81	0.399	0	0	0
4 to 6	44	125	0.392	0	0	0
6 to 8	41	166	0.324	0	0	0
8 to 10	44	210	0.347	0	0	0
10 to 12	37	247	0.415	0	0	0
12 to 20	168	415	0.775	0	0	0
20 to 28	167	582	0.53	0	0	0
28 to 36	167	749	0.303	0	0	0
36 to 44	166	915	0.298	0	0	0
44 to 52	168	1083	0.371	0	0	0
52 to 59	146	1229	0.352	0	0	0
Totals	1280 ml				0	

Control Column run from May 3 - 5, 2003

Table 4.20: *Salmonella* movement through a soil core perfused with 0.85% saline at 22°C (Experiment #7)

Sample Time (h)	Collected Volume (ml)	Cumulative Volume (ml)	Conductivity	Plate Count (per 1.0 ml)	Total Plate Count	% of Total Recovered
2.5 h before inoculation	49	-	0.35	0	0	0
0 to 2	39	-	0.332	0	0	0
2 to 4	43	82	0.326	0	0	0
4 to 6	44	126	0.315	0	0	0
6 to 8	42	168	0.267	0	0	0
8 to 10	42	210	0.27	0	0	0
10 to 12	43	253	0.238	0	0	0
12 to 20	173	426	0.485	0	0	0
20 to 28	174	600	0.752	0	0	0
28 to 36	176	776	0.274	0	0	0
36 to 44	172	948	0.239	0	0	0
44 to 52	176	1124	0.3	0	0	0
52 to 59	152	1276	0.283	0	0	0
Totals	1325 ml				0	

Column # 1 run from May 3 - 5, 2003

Table 4.21: *Salmonella* movement through a soil core perfused with 0.85% saline at 22°C (Experiment #8)

Sample Time (h)	Collected Volume (ml)	Cumulative Volume (ml)	Conductivity	Plate Count (per 1.0 ml)	Total Plate Count	% of Total Recovered
2.5 h before inoculation	53	-	0.403	0	0	0
0 to 2	39	-	0.375	0	0	0
2 to 4	43	82	0.364	0	0	0
4 to 6	44	126	0.35	385	16940	9.27
6 to 8	42	168	0.303	1255	52710	28.83
8 to 10	43	211	0.414	1305	56115	30.69
10 to 12	43	254	0.633	290	12470	6.82
12 to 20	172	426	0.633	60	10320	5.65
20 to 28	172	598	0.538	40	6880	3.76
28 to 36	172	770	0.271	45	7740	4.23
36 to 44	172	942	0.259	25	4300	2.35
44 to 52	172	1114	0.304	45	7740	4.23
52 to 59	152	1266	0.295	5	7600	4.16
Totals	1319 ml				182815	

Column # 2 run from May 3 - 5, 2003

From the second set of column soil sections (Table 4.18) a total of 6.7 log cfu *Salmonella* were recovered. When this was combined with the number recovered from the water samples eluted from the column it gave a total of 6.7 log organisms. Therefore 0.57% of the added *Salmonella* were recovered from this column, with 96.2% of the organisms recovered within the first 20 cm depth of soil analyzed (Fig. 4.13).

During analysis of the third set of soil sections (Table 4.20), where there had been no *Salmonella* recovered from the column effluent, there were 7.2 log cfu *Salmonella* in the soil. The majority of the organisms (99.4%) recovered were in the top 20 cm depth of the column (Fig. 4.13).

The final soil column sections analyzed were from the eighth replication of the experiment (Table 4.21). The total number of *Salmonella* from the column soil was 6.8 log cfu. When this number was added to the number recovered from the column effluent, a total of 6.8 log cfu *Salmonella* were accounted for, which represented 0.77% of the organisms added to this column. The majority of the organisms recovered from the soil sections (87.1%) were in the top 20 cm of the column (Fig. 4.13).

Colonies were subjected to oxidase, catalase, Gram staining and microscopic morphology testing. All colonies were Gram negative, oxidase negative and catalase positive. Colonies were also streaked onto TSI and LIA agars and following incubation were tested for agglutination with poly O *Salmonella* antiserum. All isolates gave reactions typical of *Salmonella*.

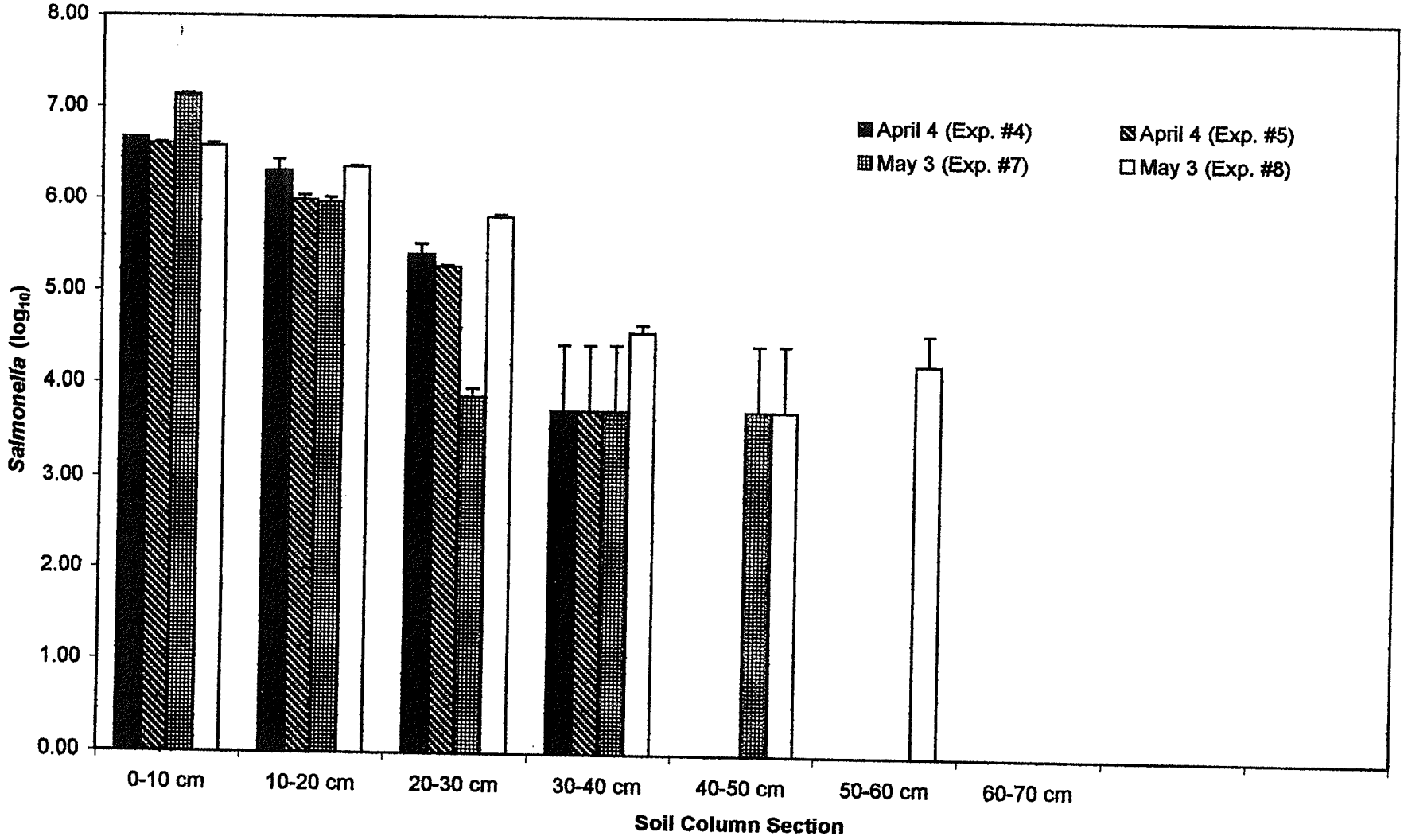

Figure 4.13: Distribution of *Salmonella* through perfused soil in 4 separate inoculated columns at various depths following plating on BGA

Chapter 5

DISCUSSION

5.0 Indicator organisms in wells at the Green Farm

5.0.1 Coliform occurrence

Only 2.3 % of 603 water samples from wells at the Green Farm over 20 months contained confirmed positive coliforms. The shortest interval between manure application and water sampling of all four fields was 21 d, and at this sampling time it was found that two wells contained 2 MPN/100ml confirmed coliforms each. With fields 2 and 3 following an interval of 14 d from manure application to sampling, one positive well was found in field 2, but there were none in field 3. In other water samples taken from fields 1 and 4 at the same time (which had been manured 186 d previously) there was one well positive in each field. Thus, there was no apparent effect of manure addition upon the frequency with which wells were positive for confirmed coliforms. There were more confirmed coliform samples from the shallow wells, which is reasonable given the proximity of the water to probable sources of contamination. Since on one occasion a well in the untreated background field contained a confirmed coliform, it is probable that there were sources of confirmed coliforms other than the manure applied to the treated fields (wild or uncontrolled domestic animals), which may also be true for the soil samples as well. When field cropping practices and occurrence of well contamination were examined, 5.5% of water samples were found positive for confirmed

coliforms from wells in fields planted with alfalfa, and 2.7, 3.2 and 0% of well water samples from fields containing annual crops (oats, winter wheat and canola, respectively) were positive for these organisms. It is possible that bacterial movement through the undisturbed soil under the alfalfa crop was greater than through soil under the annual crops which was periodically tilled at planting (Mawdsley et al., 1995; McGechan and Lewis, 2002). However, since differences in coliform recovery were small it is unlikely that the type of crop present on the field affected the bacterial status of well water.

In water samples, there were only two instances where fecal coliforms were detected. One positive fecal coliform result was in a shallow well in field 4, sampled 132 d following manure application and the other positive result for fecal coliforms was in the untreated background field in 2002 of the study.

Although the presence of coliforms and fecal coliforms in well water seemed unrelated to manure application at the Green Farm site it is important to note that intervals between manure application and water sampling were mostly >100 d after manure application. This interval may have provided opportunity for bacterial contamination from manure application to disperse, die off in the water or be adsorbed to soil particles and thus not be recovered by water analysis. Unfortunately there is little information in the literature on the levels of coliforms and fecal coliforms present in similar environments for comparison. It is important to note that the Canadian guideline for drinking water is 0/100ml for both *E. coli* and for total coliforms (Health Canada, 2002).

5.0.2 *Pseudomonas aeruginosa* occurrence

There were only 12 wells that were not positive for confirmed *P. aeruginosa* over the 6 sampling dates. The remaining 55 wells (including the background wells) were all positive on at least one occasion. Of confirmed *P. aeruginosa* in samples, 29.4% were found in fields 3 and 4, which were planted with alfalfa, and *P. aeruginosa* were identified in 32.3 and 16.1% of samples from fields containing oats and canola, respectively. The water samples from background wells were positive for confirmed *P. aeruginosa* in 33.3% of samples. *P. aeruginosa* recovery appeared unrelated to manure application to the soil. While most wells including those in the background (untreated) plots were periodically positive, there was one well in each of fields 2 and 4 (922 (D) and 958 (I), respectively) which were consistently negative for *P. aeruginosa*. There were 5 wells in each of fields 1 (901 (D), 903 (S), 908 (S), 909 (I), and 912 (D)) and 3 (932 (D), 933 (I), 937 (D), 940 (I), and 945 (I)) which were consistently negative for *P. aeruginosa*.

Difference in levels of *Pseudomonas* between the background and manure treated field wells were not evident. Even when manure was applied 21 d prior to sampling, there was minimal difference between the numbers present in the fields and background sections. There were several occasions where the number of *Pseudomonas* cells in the background wells remained constant at 2.0 MPN/100ml. When manure was applied 14 d (Nov. 2001) or 15 d (May 2002) prior to testing, we observed that those fields appeared to have higher numbers when compared to the background untreated samples. This leads to the conclusion that the application of manure to fields can cause an increase in the number of *Pseudomonas* cells present. The organism is very versatile and has been shown to

colonize groundwater systems (Leclerc and Moreau, 2002) and has been shown to survive >100 d in groundwater at 10°C (Filip et al., 1988). In the latter work, organisms isolated from two wells (151 and 182 meters in depth) pumping groundwater had 1 and 3 cfu of *Pseudomonas*/ml present, respectively. However, these represented only 0.4 and 1.2 % of the total bacteria present in each of the wells. *Pseudomonas* is capable of growth in water with low available nutrient concentrations (Stetzenbach et al., 1986). Again, it is unfortunate that there is little information reported on the levels of *Pseudomonas* found in the agricultural environment. These organisms can cause illness, especially in immunocompromised individuals (Teixeira et al., 2001).

5.0.3 Coliphage occurrence

No coliphage was detected in the water samples analyzed. This observation is consistent with the absence of confirmed and fecal coliforms in the well water analyzed by the MPN fermentation method. The absence of coliphage from the shallow wells tested is also not surprising in view of the low frequency of coliform and fecal coliform recoveries. Coliphage are considered to be a reliable indicator of water quality and groundwater contamination (Snowdon and Cliver, 1989; El-Abagy et al., 1988). Even though the method used (Standard Methods, 1989) has some drawbacks, such as a lack of colour definition on agar plates and is somewhat limited in its detection of low numbers (Ijzerman et al., 1994), this method is still considered the best technique available (El-Abagy et al., 1988). It has numerous advantages such as being simple to perform, is

inexpensive and provides results in a timely manner (El-Abagy et al., 1988; Sim and Dutka, 1987).

5.1 Pathogen analysis of soil samples

Results from microbial analysis of the hog manure applied to the test fields revealed the presence of *Salmonella*, several *Yersinia* spp. and *E. coli*. However, *E. coli* O157:H7 was absent from the sampled manure on this one occasion (May 3, 2001). It is probable these organisms (*Salmonella*, *Yersinia* and *E. coli*) were present at other times manure from this barn was spread on the test fields. Upon analyzing the effect of manure management strategies on pathogen detection, Hutchison et al. (2005) found that the age of the oldest manure in the storage area was not useful as an indicator of pathogens, since many farms are continually adding to their storage. It is important to reduce pathogen numbers applied to fields and therefore the storage of manure (slurry, solid or compost) prior to application is important (Guan and Holley, 2003). Manure application can lead to extended viability of pathogens due to greater nutrient availability and a 30 d day wait between manure application and use (planting) of the land is recommended (Holley et al., 2006). There has been much recent research in the area of manure handling and on factors affecting bacterial pathogen survival prior to the application of manure to fields. It has been shown by Arrus et al. (2006) that *Salmonella* is capable of survival at 4°C for >300 d in hog manure slurry, and therefore could contaminate fields after winter storage during springtime application. It is also important to consider using composting and drying methods, since these can decrease the number of pathogens (Kudva et al., 1998).

Aeration of manure can shorten pathogen survival. *E. coli* survival in aerated ovine and bovine manure was 4 months and 47 d, respectively, compared to more than one year when the manure was non-aerated (Kudva et al., 1998). Opportunity for contamination of produce with zoonotic pathogens is increased following use of untreated manure as a fertilizer. The potential hazard to human health could be significant (Hutchison et al., 2005).

5.1.1 Soil sample set 1

In the first set of soil samples taken from the Green Farm, there were no *Yersinia* or *Salmonella* present regardless of the depth from which the sample was taken. *E. coli* was recovered in 5 of 10 samples from the 0-5 cm depth. However, at the 5-90 cm depth there was no *E. coli* recovered from any of the samples. Therefore, these pathogens may have been injured through environmental exposure or unfavourable conditions were present for the movement of bacteria through the soil (for example reduced precipitation). Soil set 1 samples were taken 6 months to almost a year after manure addition. Thus, it was not surprising that *Salmonella* and *Yersinia* were not recovered from the soil. Isolation of *E. coli* from samples suggests a source of contamination (wild animals) other than the previously applied manure.

5.1.2 Soil sample set 2

There were no *Yersinia* or *Salmonella* recovered from any of the samples at any of the depths analyzed. *E. coli* was present in some of the soil samples. Some samples had *E. coli* present throughout consecutive depths (F2S2, F3S1, F3S3) up to 180cm. Other samples showed *E. coli* irregularly at only certain depths up to 180 cm. Since the second set of soil samples was taken from test fields within 21 d of manure application, the absence of *Salmonella* and *Yersinia* from samples suggests the organisms rapidly died following the application of manure. Fecal coliforms were present in the 0-60 cm samples of soil from all four fields and numbers were < 9 log cfu/g. These probably originated from the hog manure. These organisms did not penetrate >180 cm. The presence of *E. coli* O157:H7 in one soil sample was of interest since it occurs only rarely in hog manure and could have come from an undomesticated animal, such as deer.

It is unlikely that *E. coli* from manure was transferred to the aquifer water since they did not penetrate >180 cm in the soil. The same is probably true for *Salmonella* and *Yersinia*, but we do not have equivocal proof to support this statement since all soil samples were negative for these organisms.

5.2 Survival of pathogens inoculated in well water

Of the three pathogens studied for their survival in well water, *Yersinia* persisted in the highest numbers throughout the 91 d of the test. *E. coli* was also detectable in samples

until the experiment was terminated, but at lower numbers than *Yersinia*. *Salmonella* survived least well, with viable cells recovered from samples stored from 21 to 56 d. Initially, *Yersinia intermedia* and *Yersinia frederiksenii* declined over a period of 21 d but then recovered and their numbers increased during the remainder of the tests to reach initial numbers. Karapinar and Gonul (1991), found that *Y. enterocolitica* survived up to 64 weeks when inoculated into sterile spring water at 4°C, with an increase in cell count observed after 7 weeks. *Yersinia* has been shown to have extended survival at low temperatures in water (Terzieva and McFeters, 1991; Filip et al., 1988). *Yersinia* is capable of growth during storage at 5°C since it is psychrotrophic (Terzieva and McFeters, 1991) and could be problematic if the organism reached the groundwater.

E. coli survived significantly better in the non-sterile than in sterile well water and this was observed whether the isolate used was from soil or manure. When *E. coli* recovery was monitored by plating on EMB agar, numbers of viable cells were lower and the organism could be detected up to 63 d. Better recovery on TSA indicated that during storage in water at 5°C, *E. coli* cells were injured. The significantly ($p \leq 0.05$) greater survival of bacteria (on both TSA and EMB) in non-sterile water from 28-91 d storage at 5°C may reflect lower rates of predation by protozoans, their death and consequent increase in available nutrients, supporting longer *E. coli* survival in the non-sterile water. *E. coli* has been found to survive for 110 d at 10°C in groundwater (Filip et al., 1988). In contrast with the results presented above, Wang and Doyle (1988), found that *E. coli* O157:H7 survived 91 d at 8°C, with better survival in pure (filtered and autoclaved)

water, compared to lake or reservoir water, which can contain higher microbial counts and bacterial inhibitors.

Salmonella survived better in sterile autoclaved than non-sterile well water and use of TSA as a plating medium improved *Salmonella* recovery by 1.5 log at 56 d. *Salmonella* survived only 24 d in non-sterile well water with the selective medium BGA giving lower recovery, indicating a segment of the population was injured. In other work at 10°C, *S.* Typhimurium was found to survive for a period of 100 d in groundwater (Filip et al., 1988). Santo Domingo et al. (2000) found better survival of *Salmonella* in untreated water (non-filtered, non- autoclaved) compared to filtered river water, which led them to conclude that predators did not play an important role in *Salmonella* recovery. While results from the present study with *Salmonella* are in agreement with the previous work, it is unlikely that *E. coli* was affected by protozoan predation in unsterile water samples.

When pathogens were inoculated in non-sterile well water at 3 log cfu/ml, stored at 5°C, and plated on TSA, it was found that *E. coli* and *Salmonella* survived ≤ 21 d. *Yersinia* numbers increased by 1 log cfu during storage at 5°C up to 49 d (when plated on TSA) and by 0.5 log up to 21 d (end of test) when plated on YSA.

It is highly unlikely based on experiments in sterile and non-sterile water that *Salmonella* would survive 120 d in these waters. *Yersinia* species tested could persist longer at 5°C if present in the aquifer.

5.3 *Salmonella* transport through soil columns

The Cl^- tracer probably moved through columns faster than did *Salmonella*. Harvey and George (1989) found similar results with *E. coli*.

From the six soil columns inoculated with *Salmonella* in 0.85% saline, *Salmonella* was recovered in the column effluent in 4 instances. No *Salmonella* was recovered in the effluent collected from the other two columns. From two of the 4 positive columns <0.01% of the total inoculum was recovered, with the greatest number of organisms recovered in the first 10 h (which represented 47.3 and 49.2% of the total viable *Salmonella* found). From a third column 0.02% of *Salmonella* added were recovered within 8-10 h and this represented 30.69% of the remaining viable organisms. The greatest recovery of *Salmonella* was seen in the fourth column within 10 h where 0.11% of the added inoculum (which represented 65.6% of the viable organisms remaining) were recovered.

Four *Salmonella* positive columns were separately divided into 10 cm soil lengths and analyzed for *Salmonella* distribution within the column. The majority of *Salmonella* recovered (ranging from 87.1 to 99.4%) were found in the top 20 cm of the soil columns. This, when combined with the number of *Salmonella* recovered in the column effluent represented only 0.57-0.78% of the organisms added to the columns.

The recovery of viable *Salmonella* from total column effluents ranged from 0.01 to 0.11 % of the inoculum added. Times at which peak number of organisms were found ranged from 8-10 h following addition of the inoculum to the columns. Extremely low recoveries of viable organisms from columns was unexpected. The variation in peak elution times of *Salmonella* was not large in the four tests where *Salmonella* was recovered. In the other 4 positive columns total recovery of added *Salmonella* from both eluant and soil was less than 2%. Thus the organism used (a clinical isolate with ampicillin and tetracycline resistance, recovered on antibiotic containing media) seemed susceptible to soil/moisture exposure at 25°C. In spite of the observation that *Salmonella* were able to penetrate the 54-70 cm length of perfusion columns, results also indicated that the soil in columns was an effective bacterial filter (even though columns were from field 4, underlying a > 3 year old alfalfa crop) since during two soil perfusion tests all inoculated *Salmonella* were retained in the soil columns.

Cote and Quessy (2005) analyzed 20 cm depth soil samples for *Salmonella* and *E. coli* after the application of liquid hog manure. When the manure was analyzed over the 3 years of application, *E. coli* was recovered in the second and third years, while *Salmonella* was discovered in the third year of the experiment. In the sandy loam which was analyzed, *Salmonella* was present at 14 d (3/4 samples) and at 27 d (2/4 samples). *Salmonella* was also detected in some of the loamy sand sample replicates at 14, 27, 40 and 54 d. Therefore, there is the potential of crop contamination from the soil particles. Cote and Quessy (2005) recommend a 100 d timeframe between manure application and harvest. In the present work the majority of *Salmonella* were recovered in the top 20 cm

of the dissected soil columns, regardless of *Salmonella* presence or absence in the eluant. We also may have seen more consistent recovery of the organism if shorter columns had been used for our experiments. It was concluded by van Elsas et al. (1991), that as the column length increased, the numbers of organisms in the effluent decreased. *Salmonella* was found in 15 cm soil cores up to 32 d after the application of inoculated farmyard pig manure (Nicolson et al., 2005).

There could have been some improvement in *Salmonella* survival if the organism had been mixed with manure before addition to the column, since this would have provided additional protection from environmental stress. When intact 15-16 cm soil columns were used by Gagliardi and Karns (2000) for the recovery of applied *E. coli*, there was an increase of 0.53-1.32 log cfu in the total number of organisms recovered when manure was used compared to non-manured columns. It was felt that this difference was due to nutrient addition from the manure. *Salmonella* was shown to survive up to 120 d when inoculated into dairy cattle manure slurry and spread onto soil (Hutchison et al., 2004) and for up to 231 d when applied to soil with poultry manure compost (Islam et al. 2004). The present experiment was performed without manure addition because the organism was previously found to be able to survive for 12 d when added to soil.

Based on the results obtained it appears that the soil profiles from the Green Farm were capable of filtering large quantities of *Salmonella* from contaminated water.

Chapter 6

CONCLUSIONS

Evidence obtained from this study indicated that hog manure application to sandy soil did not result in the penetration of fecal coliforms to a depth greater than 180 cm through the soil profile. Growth of annual or perennial plants on test fields did not affect the extent of water or soil contamination by organisms from applied manure. Water samples from wells in the test fields were not contaminated by zoonotic pathogens found in the applied manure. Although repeated analysis were conducted over a period of 20 months, in 10/13 sample series collected after manure had been applied, it was > 4 months after manure application before samples were obtained for study. In 3 sample sets manure application was within 3 weeks of manure application.

In vitro laboratory work showed that *Salmonella, Yersinia* and *E. coli* were able to survive in water from the test site for up to 3 weeks, and *Yersinia* were able to grow. If organisms from manure were able to reach the groundwater underlying the test field, there would be significant potential for transport in the Assiniboine aquifer which passed under this field.

Results from microbiological testing showed that background wells located in field areas not treated with manure were contaminated with fecal coliforms periodically, and suggested that a source unrelated to manure application.

From soil percolation experiments it appeared that one meter of sandy soil from the test field was capable of removing or inactivating 99.9% of a challenge dose of *Salmonella* added in a saline solution. This ability was significant and may serve to prevent contamination of groundwater after application of hog manure of uncertain microbiological quality.

Chapter 7

RECOMMENDATIONS FOR FUTURE STUDIES

- Development of an effective monitoring method based on a single indicator organism for the determination of recent contamination of soil and water by manure would be useful. A standardized sampling protocol and reporting system, readily available for use by researchers, regulatory agencies, and industry would also be valuable.

- Research to determine the effect of the initial levels of pathogens applied to fields upon the period pathogens remain viable in the environment would help better characterize minimum intervals necessary between manure treatment and planting to prevent crop contamination. Further studies with regards to the cumulative effect of repeated manure application to agricultural fields upon the pathogen status of treated soil would be useful.

- Further study and implementation of better manure management strategies (e.g. limited co-mingling of manure) including monitoring of pathogens, so as to determine the influence of individual practices upon the survival and persistence of zoonotic pathogens is desired.

- Further study of manure storage practices in the field (such as alternating aeration/anaerobic treatments) upon pathogen survival and manure nutrient composition is needed.

- Establishment of safe intervals between manure application and harvesting of treated produce based on Manitoba data would be valuable.

- Further study of the survival of human pathogens in hog manure and their survival in soil and water environments is needed, especially for those organisms which have extended survival at cool groundwater temperatures such as *Yersinia*.

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Appendix 1: Presumptive coliform occurrence in groundwater over 20 months (Values Reported as MPN/100 ml)

		Sample Date									
Well Number	Depth	Oct, 00	Mar, 01	April, 01	May, 01	July, 01	Sept, 01	Nov, 01	Mar, 02	May, 02	Field #
901	Deep	4	<2	<2	<2	<2	<2	<2	<2	<2	1
902	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
903	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
904	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
905	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
906	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
907	Intermediate	2	<2	<2	<2	<2	<2	<2	<2	<2	1
908	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
909	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
910	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
911	Shallow	23	13	2	<2	<2	<2	<2	<2	<2	1
912	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
913	Intermediate	4	<2	<2	<2	<2	<2	2	<2	<2	1
914	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
915	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
916	Shallow	2	<2	<2	<2	<2	<2	<2	<2	<2	1
917	Deep	<2	<2	<2	<2	<2	<2	4	<2	<2	2
918	Intermediate	<2	<2	<2	<2	<2	<2	2	<2	<2	2
919	Shallow	<2	<2	<2	<2	<2	<2	2	<2	<2	2
920	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
921	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
922	Deep	<2	<2	<2	<2	<2	<2	4	<2	<2	2
923	Intermediate	<2	<2	<2	<2	<2	<2	13	<2	<2	2
924	Shallow	<2	<2	<2	<2	<2	<2	2	<2	<2	2
925	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
926	Shallow	4	4	2	2	4	<2	<2	<2	<2	2
927	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
928	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
929	Shallow	<2	<2	<2	2	<2	<2	<2	<2	<2	2
930	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
931	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2

Appendix 1 (cont'd): Presumptive coliform occurrence in groundwater over 20 months (Values Reported as MPN/100 ml)

932	Deep	23	13	13	2	<2	<2	2	<2	<2	3
933	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
934	Shallow	<2	<2	<2	4	2	<2	2	<2	2	3
935	Intermediate	<2	<2	<2	<2	<2	<2	2	<2	<2	3
936	Shallow	<2	<2	<2	<2	<2	<2	4	<2	<2	3
937	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
938	Intermediate	<2	<2	<2	<2	<2	<2	4	<2	<2	3
939	Shallow	<2	<2	<2	<2	<2	<2	2	<2	<2	3
940	Intermediate	2	<2	<2	<2	<2	<2	2	<2	<2	3
941	Shallow	<2	2	<2	2	2	<2	<2	<2	<2	3
942	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
943	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
944	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
945	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	2	3
946	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
947	Shallow	13	<2	2	<2	<2	<2	<2	<2	2	4
948	Intermediate	2	<2	<2	<2	<2	<2	<2	<2	<2	4
949	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
950	Shallow	2	<2	4	2	2	2	<2	<2	<2	4
951	Intermediate	<2	<2	<2	<2	<2	<2	4	<2	<2	4
952	Shallow	8	4	<2	<2	4	4	2	<2	<2	4
953	Intermediate	23	13	4	<2	<2	2	<2	<2	<2	4
954	Deep	<2	<2	<2	<2	<2	<2	4	<2	<2	4
955	Shallow	<2	<2	2	2	<2	<2	<2	<2	<2	4
956	Intermediate	<2	<2	<2	<2	<2	<2	4	<2	<2	4
957	Shallow	23	<2	<2	<2	<2	<2	<2	<2	<2	4
958	Intermediate	<2	<2	<2	<2	<2	<2	4	<2	<2	4
959	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
960	Shallow	2	<2	8	<2	<2	<2	<2	<2	<2	4
961	Intermediate	<2	<2	<2	<2	<2	<2	2	<2	<2	4
962	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
963	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
964	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
965	Deep	4	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
966	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
967	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	2	Bkgd

Appendix 2: Confirmed coliform occurrence in groundwater over 20 months (Values Reported as MPN/100 ml)

		Sample Date									
Well Number	Depth	Oct, 00	Mar, 01	April, 01	May, 01	July, 01	Sept, 01	Nov, 01	Mar, 02	May, 02	Field #
901	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
902	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
903	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
904	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
905	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
906	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
907	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
908	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
909	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
910	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
911	Shallow	<2	8	<2	<2	<2	<2	<2	<2	<2	1
912	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
913	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
914	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
915	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
916	Shallow	2	<2	<2	<2	<2	<2	<2	<2	<2	1
917	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
918	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
919	Shallow	<2	<2	<2	<2	<2	<2	2	<2	<2	2
920	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
921	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
922	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
923	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
924	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
925	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
926	Shallow	<2	4	<2	<2	4	<2	<2	<2	<2	2
927	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
928	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
929	Shallow	<2	<2	<2	2	<2	<2	<2	<2	<2	2
930	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
931	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2

Appendix 2 (cont'd): Confirmed coliform occurrence in groundwater over 20 months (Values Reported as MPN/100 ml)

932	Deep	<2	13	4	<2	<2	<2	<2	<2	<2	3
933	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
934	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
935	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
936	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
937	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
938	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
939	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
940	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
941	Shallow	<2	2	<2	<2	2	<2	<2	<2	<2	3
942	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
943	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
944	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
945	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
946	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
947	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
948	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
949	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
950	Shallow	<2	<2	2	2	<2	2	<2	<2	<2	4
951	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
952	Shallow	<2	4	<2	<2	2	4	<2	<2	<2	4
953	Intermediate	<2	<2	<2	<2	<2	2	<2	<2	<2	4
954	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
955	Shallow	<2	<2	2	<2	<2	<2	<2	<2	<2	4
956	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
957	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
958	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
959	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
960	Shallow	2	<2	2	<2	<2	<2	<2	<2	<2	4
961	Intermediate	<2	<2	<2	<2	<2	<2	2	<2	<2	4
962	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
963	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
964	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
965	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
966	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
967	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	2	Bkgd

Appendix 3: Fecal coliform occurrence in groundwater over 20 months (Values Reported as MPN/100 ml)

		Sample Date									
Well Number	Depth	Oct, 00	Mar, 01	April, 01	May, 01	July, 01	Sept, 01	Nov, 01	Mar, 02	May, 02	Field #
901	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
902	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
903	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
904	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
905	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
906	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
907	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
908	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
909	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
910	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
911	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
912	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
913	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
914	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
915	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
916	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	1
917	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
918	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
919	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
920	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
921	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
922	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
923	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
924	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
925	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
926	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
927	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
928	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
929	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
930	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	2
931	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	2

Appendix 3 (cont'd): Fecal coliform occurrence in groundwater over 20 months (Values Reported as MPN/100 ml)

932	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
933	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
934	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
935	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
936	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
937	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
938	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
939	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
940	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
941	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
942	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
943	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
944	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
945	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
946	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	3
947	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
948	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
949	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
950	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
951	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
952	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
953	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
954	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
955	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
956	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
957	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
958	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
959	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
960	Shallow	2	<2	<2	<2	<2	<2	<2	<2	<2	4
961	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	4
962	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
963	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
964	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
965	Deep	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
966	Intermediate	<2	<2	<2	<2	<2	<2	<2	<2	<2	Bkgd
967	Shallow	<2	<2	<2	<2	<2	<2	<2	<2	2	Bkgd

Appendix 4: Presumptive *Pseudomonas* occurrence in groundwater over 20 months (Values Reported as MPN/100ml)

		Sample Date						
Well	Depth	Mar, 01	July, 01	Sept, 01	Nov, 01	Mar, 02	May, 02	Field #
901	Deep	23	4	2	2	2	<2	1
902	Intermediate	13	4	4	2	2	2	1
903	Shallow	23	2	23	<2	4	4	1
904	Intermediate	23	13	4	2	<2	2	1
905	Shallow	23	2	2	2	13	2	1
906	Deep	8	2	8	4	<2	2	1
907	Intermediate	23	4	<2	2	<2	2	1
908	Shallow	23	2	4	4	2	<2	1
909	Intermediate	23	8	<2	<2	<2	<2	1
910	Intermediate	23	2	2	2	8	2	1
911	Shallow	2	8	<2	2	4	2	1
912	Deep	23	4	<2	2	2	4	1
913	Intermediate	23	2	<2	4	<2	<2	1
914	Shallow	23	<2	<2	4	<2	<2	1
915	Intermediate	23	<2	2	2	<2	4	1
916	Shallow	23	8	2	4	4	2	1
917	Deep	23	8	2	2	2	2	2
918	Intermediate	2	2	2	<2	2	2	2
919	Shallow	23	2	<2	<2	2	<2	2
920	Intermediate	23	4	<2	<2	4	4	2
921	Shallow	23	<2	<2	<2	<2	2	2
922	Deep	23	4	<2	<2	<2	2	2
923	Intermediate	13	2	<2	4	<2	<2	2
924	Shallow	23	13	<2	<2	<2	4	2
925	Intermediate	23	<2	2	2	4	<2	2
926	Shallow	23	4	2	8	2	4	2
927	Deep	23	2	<2	8	4	2	2
928	Intermediate	23	2	8	4	2	2	2
929	Shallow	23	2	<2	4	2	2	2
930	Intermediate	23	4	2	4	2	2	2
931	Shallow	23	2	<2	4	<2	2	2
932	Deep	23	<2	<2	4	2	4	3
933	Intermediate	2	2	2	4	4	<2	3
934	Shallow	23	<2	2	<2	<2	4	3
935	Intermediate	23	8	2	2	2	2	3
936	Shallow	23	4	<2	<2	<2	<2	3
937	Deep	23	<2	2	<2	4	<2	3
938	Intermediate	23	2	4	2	2	2	3
939	Shallow	23	4	2	<2	2	4	3
940	Intermediate	23	2	2	<2	8	4	3
941	Shallow	23	4	2	<2	2	4	3
942	Deep	23	8	<2	<2	<2	4	3
943	Intermediate	23	<2	<2	<2	2	8	3
944	Shallow	23	<2	2	4	2	2	3
945	Intermediate	23	4	4	<2	2	2	3
946	Shallow	23	2	<2	4	4	2	3

Appendix 4 (cont'd): Presumptive *Pseudomonas* occurrence over 20 months
(Values Reported as MPN/100ml)

Well	Depth	Sample Date Mar, 01	July, 01	Sept, 01	Nov, 01	Mar, 02	May, 02	Field #
948	Intermediate	23	8	<2	<2	8	<2	4
949	Deep	23	2	<2	8	<2	2	4
950	Shallow	23	13	2	4	2	4	4
951	Intermediate	23	2	<2	2	2	2	4
952	Shallow	23	<2	<2	2	<2	<2	4
953	Intermediate	23	4	<2	4	4	2	4
954	Deep	23	8	4	2	2	2	4
955	Shallow	23	4	<2	4	<2	<2	4
956	Intermediate	23	2	<2	<2	4	<2	4
957	Shallow	23	2	<2	4	<2	2	4
958	Intermediate	23	2	2	13	2	2	4
959	Deep	23	<2	<2	4	2	2	4
960	Shallow	23	8	2	<2	2	2	4
961	Intermediate	23	8	<2	4	<2	2	4
962	Deep	23	4	<2	4	2	4	Bkgd
963	Intermediate	23	<2	2	<2	<2	<2	Bkgd
964	Shallow	23	4	2	2	<2	2	Bkgd
965	Deep	23	2	<2	<2	4	4	Bkgd
966	Intermediate	23	4	2	<2	2	<2	Bkgd
967	Shallow	23	<2	2	2	4	<2	Bkgd

Appendix 5: Confirmed *Pseudomonas* occurrence over 20 months
(Values Reported as MPN/100ml)

		Sample Date						
Well	Depth	Mar, 01	July, 01	Sept, 01	Nov, 01	Mar, 02	May, 02	Field #
901	Deep	<2	<2	<2	<2	<2	<2	1
902	Intermediate	4	4	<2	<2	2	<2	1
903	Shallow	<2	<2	<2	<2	<2	<2	1
904	Intermediate	23	<2	<2	<2	<2	<2	1
905	Shallow	8	2	<2	<2	13	<2	1
906	Deep	8	<2	<2	<2	<2	<2	1
907	Intermediate	2	<2	<2	<2	<2	<2	1
908	Shallow	<2	<2	<2	<2	<2	<2	1
909	Intermediate	<2	<2	<2	<2	<2	<2	1
910	Intermediate	2	2	2	2	8	<2	1
911	Shallow	<2	8	<2	2	<2	<2	1
912	Deep	<2	<2	<2	<2	<2	<2	1
913	Intermediate	23	2	<2	<2	<2	<2	1
914	Shallow	23	<2	<2	<2	<2	<2	1
915	Intermediate	23	<2	<2	<2	<2	<2	1
916	Shallow	23	<2	<2	<2	<2	<2	1
917	Deep	23	<2	<2	<2	<2	<2	2
918	Intermediate	<2	<2	2	<2	<2	2	2
919	Shallow	23	2	<2	<2	2	<2	2
920	Intermediate	<2	4	<2	<2	<2	<2	2
921	Shallow	23	<2	<2	<2	<2	2	2
922	Deep	<2	<2	<2	<2	<2	<2	2
923	Intermediate	13	2	<2	4	<2	<2	2
924	Shallow	<2	13	<2	<2	<2	4	2
925	Intermediate	<2	<2	2	<2	<2	<2	2
926	Shallow	<2	4	2	<2	<2	4	2
927	Deep	23	<2	<2	<2	<2	<2	2
928	Intermediate	8	2	8	<2	<2	<2	2
929	Shallow	<2	<2	<2	<2	<2	2	2
930	Intermediate	23	<2	2	4	<2	2	2
931	Shallow	<2	2	<2	4	<2	<2	2
932	Deep	<2	<2	<2	<2	<2	<2	3
933	Intermediate	<2	<2	<2	<2	<2	<2	3
934	Shallow	<2	<2	2	<2	<2	4	3
935	Intermediate	<2	8	2	<2	<2	<2	3
936	Shallow	2	<2	<2	<2	<2	<2	3
937	Deep	<2	<2	<2	<2	<2	<2	3
938	Intermediate	<2	<2	4	<2	2	2	3
939	Shallow	23	4	2	<2	2	<2	3
940	Intermediate	<2	<2	<2	<2	<2	<2	3
941	Shallow	23	<2	2	<2	2	4	3
942	Deep	13	<2	<2	<2	<2	<2	3
943	Intermediate	<2	<2	<2	<2	<2	8	3
944	Shallow	<2	<2	2	<2	2	2	3
945	Intermediate	<2	<2	<2	<2	<2	<2	3
946	Shallow	2	2	<2	4	4	<2	3

Appendix 5 (cont'd): Confirmed *Pseudomonas* occurrence over 20 months
(Values Reported as MPN/100ml)

		Sample Date						
Well	Depth	Mar, 01	July, 01	Sept, 01	Nov, 01	Mar, 02	May, 02	Field #
947	Shallow	23	4	<2	<2	2	<2	4
948	Intermediate	8	8	<2	<2	8	<2	4
949	Deep	8	<2	<2	<2	<2	<2	4
950	Shallow	23	<2	2	<2	<2	4	4
951	Intermediate	23	<2	<2	<2	<2	<2	4
952	Shallow	23	<2	<2	2	<2	<2	4
953	Intermediate	<2	<2	<2	4	4	2	4
954	Deep	23	<2	<2	<2	<2	<2	4
955	Shallow	<2	4	<2	<2	<2	<2	4
956	Intermediate	<2	<2	<2	<2	<2	<2	4
957	Shallow	23	<2	<2	4	<2	<2	4
958	Intermediate	<2	2	2	13	<2	<2	4
959	Deep	23	<2	<2	<2	<2	<2	4
960	Shallow	<2	<2	<2	<2	2	2	4
961	Intermediate	23	<2	<2	<2	<2	2	4
962	Deep	23	4	<2	<2	<2	<2	Bkgd
963	Intermediate	13	<2	2	<2	<2	<2	Bkgd
964	Shallow	23	<2	2	2	<2	2	Bkgd
965	Deep	23	<2	<2	<2	<2	<2	Bkgd
966	Intermediate	<2	<2	<2	<2	2	<2	Bkgd
967	Shallow	23	<2	<2	2	<2	<2	Bkgd