EVALUATION OF METHODOLOGY FOR DETECTION AND QUANTIFICATION OF COLIFORM BACTERIA AND THEIR SURVIVAL IN SOIL AND WATER

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Abstract

In Aberdeenshire, approximately 25,000 people rely on potable water from private water supplies. Many of these supplies are of questionable quality with regards to microbiological contamination, which is often due to a lack of maintenance and protection of these supplies. Agricultural practices such as the spreading of slurry and grazing animals in the proximity of supplies all increase the risk of contamination.

The presence of coliform bacteria and in particular *E. coli*, in private water supplies is an indication of the potential for the water supply to be contaminated with pathogenic microorganisms. With the increasing occurrence of severe illness from pathogens such as *E. coli* O157, which have been found in a number of private water supplies, there is an increasing concern with regards to groundwater quality.

This study was conducted initially to determine the magnitude of private water supply contamination in Aberdeenshire and to investigate any links between this contamination with seasonality and rainfall. To enable analysis of a large number of water samples to be carried out within an accepted timeframe, Colilert 18™ was validated for the detection and enumeration of coliform bacteria in potable water and was compared to the accepted standard method, membrane filtration. Colilert 18™ was further validated for use with soil samples to allow the study of factors affecting coliform survival in water and soil to be carried out. Coliform survival in

soil was found to be influenced particularly by temperature and soil texture. The transport of coliform bacteria in soil was investigated using repacked and undisturbed soil columns, with transport enhanced by rainfall. However even with heavy rainfall coliform bacteria can become attached to or trapped within the soil structure. It was evident from these studies that coliform bacteria and therefore pathogenic microorganisms have the potential to survive in the environment and to travel through the soil structure and enter groundwater.

Declaration

I, the undersigned, declare that the work presented in this thesis is my own, except where otherwise stated and with due reference made. This work has not been submitted in any form for any other higher degree or qualification.

Elaine Bannister

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Abbreviations

APHA	-	American Public Health Association
BSTC	-	British Soil Texture Classification
CFU	-	Colony forming unit
DEFRA	-	Department for Environment, Food and Rural Affairs
DH	-	Department of Health
DNA	-	Deoxyribonucleic acid
DoE	-	Department of Environment
DST	-	Defined substrate technology
DWI	-	Drinking Water Inspectorate
EEA	-	European Environment Agency
EHEC	-	Enterohaemorrhagic *Escherichia coli*
FC	-	Faecal coliform
HMSO	-	Her Majesty's Stationery Office
HUS	-	Haemolytic uraemic syndrome
ISO	-	International Organisation for Standardisation
LPW	-	Lactose peptone water
MF	-	Membrane filtration
MLSB	-	Membrane lauryl sulphate broth
MMM	-	Modified Miles Misra
MPN	-	Most probable number
MTFM	-	Multiple-tube fermentation method
MUG	-	4-methylumbeliferyl-β-D-glucuronide
NHMRC	-	National health and medical research council
OM	-	Organic matter
ONPG	-	ortho-nitrophenol-β-D-galactopyranoside
ORS	-	Old Red Sandstone
PHLS	-	Public Health Laboratory Service
pI	-	Isoelectic point
PWS	-	Private water supply
RNA	-	Ribonucleic acid
SCA	-	Standing Committee of Analysts
SSI	-	Scottish Statutory Instrument
TC	-	Total coliforms
UN	-	United Nations
UNICEF	-	United Nations Children's Fund (formerly United Nations International Children's Emergency Fund)
UV	-	Ultra violet
VTEC	-	Vero-cytotoxin producing *E. coli*
WHO	-	World Health Organisation

Chapter 1 Introduction

1.1 Global water distribution and water usage

Seventy one percent of the earth's surface is covered by water. The majority of this is saltwater (97.5%) which is found in the oceans and seas. The remaining 2.5% is freshwater yet two thirds of this is unavailable as it is trapped in the polar ice caps and glaciers. The majority (96%) of the available freshwater is found as groundwater (Shiklomanov and Rodda 2003). The rest is distributed between lakes, rivers, wetlands, soil and the atmosphere with a small quantity held by plants. Figure 1.1 shows a graphical representation of the percentage distribution of the world's water.

Globally the majority of freshwater is used for agricultural irrigation with 70% used for this purpose. Industrial use and energy production utilises a further 20% and only 10% is used for domestic purposes including potable water. Of the total global water usage, 20% is obtained from groundwater (UN 2009).

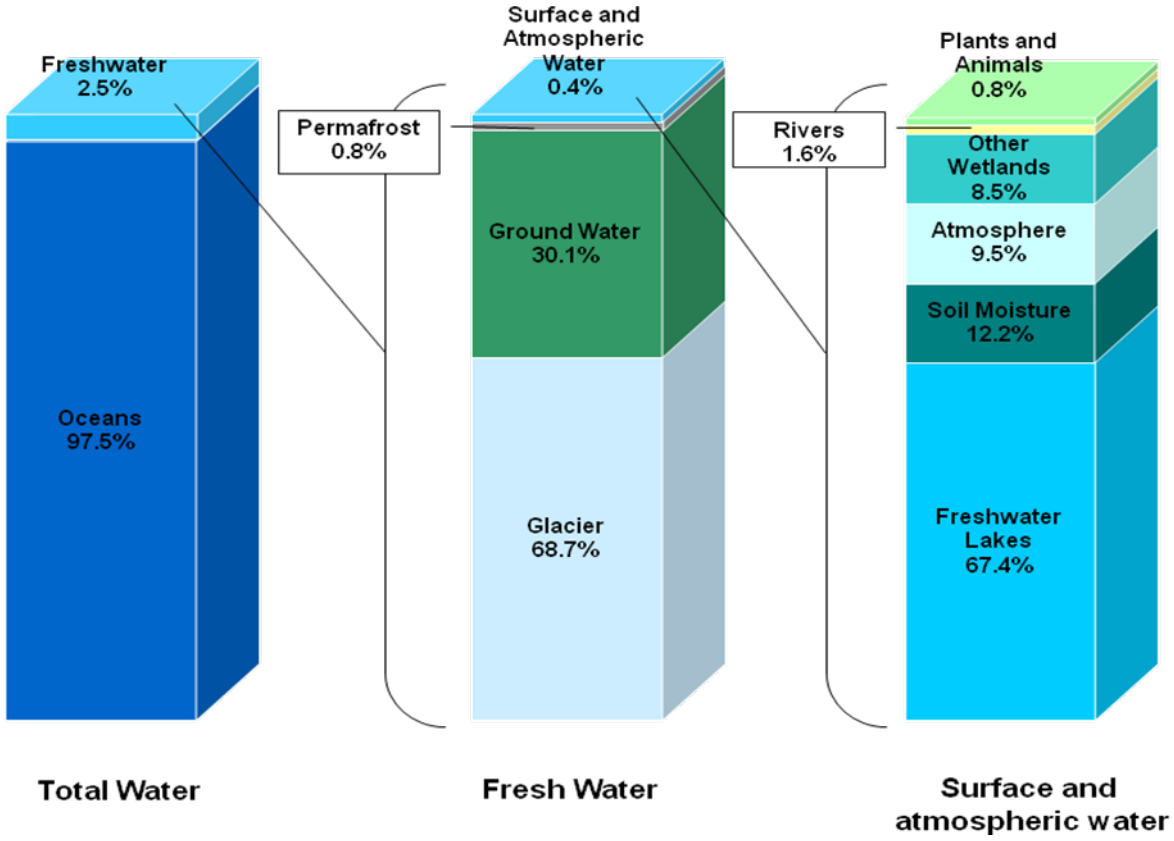


Figure 1.1 Global distribution of water (Data from Shiklomanov and Rodda 2003). Sources of drinking water: ( ) groundwater = 30.1 % of global freshwater (0.75% of global water); ( ) freshwater lakes = 0.27 % of global freshwater (6.74 x 10^{-3} % of global water); ( ) rivers = 0.0064% of global freshwater (1.60 x 10^{-4} % of global water)

Water is a basic necessity for life itself. In many countries worldwide, the availability of drinking water of acceptable quality is often taken for granted. However safe drinking water in many parts of the world is not something that is easily accessible or available. With the effects of climate change likely to increase drought in places already suffering from water shortage (UN 2009), the scarcity of fresh water is likely to be an increasing problem.

It was estimated in 2006 that 13% of the world's population, approximately 884 million people, did not have access to safe drinking water (WHO/UNICEF 2008). The world population is projected to increase between 70 to 80 million people per year to reach approximately 9 billion by 2050 (UN 2006; UN 2009). This in itself will mean an increased demand on the world's water resources for use in agriculture, energy production and for personal use.

1.2 Water and disease

The link between water and disease with regards to public health has been recognised since the 19th century. If the quality of drinking water is compromised it can lead to serious ill health in the general population. There are an estimated 3 million deaths per annum linked to water and disease. The majority of these are from diarrhoea which causes 1.8 million deaths, and malaria which accounts for 1.3 million deaths (WHO/UNICEF 2008). Most of those dying from water-related disease are small children struck by virulent but preventable diarrheal diseases (UN 2009; WHO 2000).

Water-related diseases are typically placed in four classes: waterborne, water-washed, water-based, and water-related disease. The first three are associated with lack of clean domestic water supplies while the latter class is often related to large scale water systems which create suitable breeding grounds for their hosts (Hassan, Scholes and Ash 2005).

Waterborne diseases are enteric diseases caused by the ingestion of pathogens which are present in water contaminated by human or animal faeces or urine. Waterborne diseases include typhoid, cholera, hepatitis, salmonellosis, leptospirosis, giardiasis, ameobiasis, roundworm, tapeworm and hookworm (Hassan, Scholes and Ash 2005).

Water-washed diseases are caused by poor personal hygiene and skin or eye contact with contaminated water and are usually due to scarcity of water. These include scabies, typhus, trachoma and flea, lice and tick-borne diseases (Hassan, Scholes and Ash 2005).

Water-based diseases are caused by parasitic worms that require an intermediate aquatic host for part of their life cycle. These diseases are usually passed to humans when they drink contaminated water or use it for washing. The most prevalent examples are schistosomiasis and drancunculiasis (Hassan, Scholes and Ash 2005).

Water-related diseases are caused by insect vectors, especially mosquitoes that breed in water. As well as malaria it includes two viral diseases, yellow fever and dengue (Hassan, Scholes and Ash 2005).

It has been assessed that by the year 2015 there will remain almost 1 billion people around the world who lack access to an “improved water supply” and more than 2.4 billion will still lack access to “improved sanitation” (WHO/ UNICEF 2008). Improved water supply refers to water sources which are

protected from contamination particularly by faecal matter. Improved sanitation ensures that human excreta are disposed of hygienically, thereby preventing human contact. This includes flush and pour/flush toilets that discharge into a piped sewer, septic tank or pit latrine and does not included waste which enters open drains or other water bodies. Access to safe water and acceptable sanitation as well as improving water resource management all play a huge part in improving human health and reducing deaths linked to water (WHO 2009).

1.3 Water related health issues in the UK

Waterborne disease is not just a problem seen in underdeveloped countries but has also been linked to disease outbreaks in developed countries. In the UK, there have been numerous outbreaks of illness related to potable water, from both public and private water supplies. Contamination of potable water supplies can lead to serious illness and disease and sometimes death.

During the early part of the 20th century many recorded cases of disease were caused by Salmonella typhi and Salmonella paratyphi, which are bacteria causing typhoid and paratyphoid fever respectively. These diseases were linked to the inadequate chlorination of water supplies. A reduction in the number of these infections was most likely linked to better hygiene and sanitation (Galbraith, Barrett and Stanwell-Smith 1987; Furtado et al. 1998). During the latter part of the 20th and into the 21st century the majority of waterborne diseases recorded in the UK have been linked to contamination of water

supplies by campylobacter, cryptosporidium, and more recently *Escherichia coli* (*E. coli*) O157 (Furtado et al. 1998).

Although less common in the UK it is possible that drinking water contamination can be chemical, for example nitrates, heavy metals and other toxins, and must also be considered (Galbraith, Barrett and Stanwell-Smith 1987; Pretty et al. 2000; Reimann and Banks 2004; Scottish Executive 2006; Hooda et al. 2000). Naturally occurring nitrate is released when bacteria in the soil break down organic matter. The normal concentration in areas without intense agriculture is between 0 and 10 mg/l (EEA 1999). This level is increased by the addition of fertilisers and manure. Any nitrate not utilised by plants is liable to be carried through soil by rainwater as nitrate has a high solubility, making it highly mobile given the right conditions. Nitrate (NO_3) should not exceed 50 mg/l in any drinking water sample as stated in the Private Water Supplies (Scotland) Regulations, 2006 and also stated in guidelines set by the World Health Organisation (WHO 2008).

Methaemoglobinaemia, which is a condition resulting in the blood being unable to carry oxygen to cells in the body, is the main health issue linked to nitrate. It can occur in adults and children as a result of extremely high nitrate intake however it is more likely to occur with bottle-fed infants and is known as blue-baby syndrome. A small percentage of ingested nitrate is converted to nitrite by the action of bacteria in the mouth, and also in the stomach. Available nitrite then binds with haemoglobin in the red blood cells to form methaemoglobin, which binds oxygen preventing oxygen transport, and thus causing cyanosis.

Bottle-fed infants are considered to be at greater risk because the intake of water in relation to body weight is high and, in infants, the development of repair enzymes is limited (Fewtrell 2004).

1.4 Pathogens and sources

There are many human pathogens that can be transmitted orally by drinking water, including *Salmonella spp*, *Shigella spp*, parasitic protozoa such as *Giardia lamblia*, and viruses. However *Campylobacte*r, *Cryptosporidium parvum* and pathogenic *E. coli,* in particular *E. coli* O157, are the pathogens of most concern in this present day.

1.4.1 *Campylobacter*

Campylobacter has been a recognised human enteric pathogen since the early 1970's. It is the most common reported bacterial cause of infectious intestinal disease in the UK with 49,880 cases recorded in England and Wales during 2008 (Health Protection Agency 2009) and 4878 cases recorded in Scotland over the same period (Health Protection Scotland 2009). *Campylobacter jejuni* and *Campylobacter coli* are the two species which account for the majority of infections.

Campylobacter is a Gram-negative motile bacterium which has the ability to enter a viable but non-cultivable state when subjected to adverse environmental conditions. Optimal growth occurs at temperatures between 30 and 45˚C. The main reservoir of *Campylobacter* is within the gastrointestinal tract of many wild and domestic animals, in particular cattle. Deposition of contaminated faecal material can enter the environment and lead to human exposure through contaminated soil or water (WHO 2008).

1.4.2 *Cryptosporidium*

Cryptosporidium spp. are protozoan parasites that cause an infection called cryptosporidiosis. In 2008, 4151 cases were recorded in England and Wales (Health Protection Agency 2009) and a further 613 cases in Scotland (Health Protection Scotland 2009). Oocysts are the infectious stage of the *cryptosporidium* spp. life cycle and are shed in the faeces of infected animals and humans and then ingested by a suitable host. The organism is extremely virulent and a low dose can result in infection. Oocysts are destroyed by temperature extremes (Dubey, Speer and Fayer 1990; Fayer and Nerad 1996), with their optimal survival occurring between 0 and 20^{o}C (Fayer, Trout and Jenkins 1998b). Oocysts can tolerate chlorination which can result in their persistence in treated water supplies (Fayer 1995).

Cryptosporidium spp. can be found in soil, food, water or surfaces that have been contaminated with infected human or animal faeces. Infection occurs

following consumption of contaminated water or food, or by swimming in contaminated water such as lakes or rivers. Transmission also occurs through direct animal-to-human or human-to-human contact (Medema, Bahar and Schets 1997; Pell 1997).

1.4.3 *E. coli* O157

There are many strains of the *E. coli* bacterium most of which are harmless organisms found in the intestinal tract of warm-blooded animals. However, serotypes belonging to the category referred to as enterohaemorrhagic *E. coli* (EHEC) or often referred to as verocytotoxin producing *E. coli* (VTEC) are recognised as causing the most severe illness. They are known to produce potent toxins and can cause a range of illnesses which may be severe and sometimes fatal, particularly in infants, young children and the elderly. The most important toxin-producing strain associated with human illness is known as *E. coli* O157:H7 (Meng et al. 2001).

E. coli O157 can cause a range of symptoms from mild diarrhoea to bloody diarrhoea (haemorrhagic colitis) with a small percentage (5 %) developing haemolytic uraemic syndrome (Parry and Palmer 2000). Haemolytic uraemic syndrome (HUS) is characterised by acute renal failure, haemolytic anaemia and thrombocytopaenia (Jones, Campbell and Kaspar 2002; Meng et al. 2001; Chalmers, Aird and Bolton 2000; Parry and Palmer 2000).

The infectious dose of *E. coli* O157 appears to be very low, probably between 10 and 100 organisms. Humans become infected through the consumption of contaminated foods or water although transmission can also occur following direct contact with animals, particularly cattle. The main reservoir for *E. coli* O157 is the intestine of healthy cattle but carcasses can become contaminated through contact with intestinal contents at slaughter. Unlike the majority of *E. coli* strains, *E. coli* O157 is not thermotolerant therefore does not confirm as a faecal coliform using standard methods for detection. It also lacks the ability to produce β-D-glucaronidase therefore cannot be detected using many of the newer rapid enzymatic detection methods (Meng et al. 2001).

The incidence of *E. coli* O157 infections is variable throughout the UK with the highest rate in Scotland, 243 cases being recorded in both 2006 and 2007 and 241 cases recorded in 2008 (Health Protection Scotland 2009). In England and Wales 1003 cases were recorded in 2006 (Health Protection Agency 2009).

1.5 Private water supplies in the United Kingdom

In the United Kingdom the majority of potable water is provided from public mains supply by the water utilities in England and Wales and government owned Scottish Water in Scotland. However, throughout the UK there are also approximately 140,000 private water supplies providing potable water to many rural homes and communities, with 38,000 of these located in Scotland (DWI 1993).

A private water supply is any water supply not provided by a water utility or government authority. Private water supplies can originate from a variety of sources. The water is often groundwater and is commonly abstracted from deep or shallow wells, boreholes or spring sources, but can also be surface water from a loch or lake, stream or river. Often these sources can be shallow and unprotected from contamination by livestock and other agricultural activities. The majority of private water supplies receive little or no treatment or maintenance (Reid et al. 2003).

In Scotland it is estimated that over 60,000 people are dependent upon private water supplies for their drinking water (Reid et al. 1999). Approximately 50% of these people are drawing water from supplies which are not monitored under any legislation. As the responsibility for the upkeep and maintenance of the supply and water testing lies with the home owner, many people are drinking water of unknown quality and therefore putting themselves at risk of illness due to water contamination.

1.6 Water quality and contamination issues

The presence of pathogenic microorganisms in drinking water supplies is usually due to faecal contamination either at the water source or during its distribution. Field application of manure or slurry, human sewage from septic tanks in the vicinity of the supply, grazing animals in the vicinity of the water

supply and contamination from birds and wildlife are all possible sources of faecal contamination (Davies and Mazumder 2003).

The management of animal waste is of major importance in the prevention of microbial water contamination. Traditionally farmyard manure and straw used for animal bedding was composted (Jones 1982). Composting is an aerobic process where temperatures can rise as high as 70˚C which eradicates most pathogens. Farmyard manure would typically be applied annually in the spring when new plant growth would make use of available nutrients. However due to intensive farming methods, both herd size and housed animal numbers have increased. This has changed the management of waste collection and storage, with waste generally collected as slurry containing only a minimum amount of solid bedding material. This slurry is stored in large tanks and rapidly becomes anaerobic, hence temperature does not rise and pathogens are not destroyed (Mawdsley et al. 1995). Slurry is then applied to land often by spraying. Nicholson, Webb and Moore (2002) found that pathogens including *E. coli* O157 and *Campylobacter* could not be detected in an unturned manure heap after 1 week while the same pathogens could still be detected in batched stored dairy slurry 3 months later. In contrast to this, Maule (1997) and Kudva, Blanch and Hovde (1998) found that *E. coli* O157 survived longer in cattle manure than in cattle slurry, survival in the slurry being less than 10 days. Following manure and slurry application to land, pathogen numbers will reduce due to exposure to UV light and the drying effects of weathering. However if these organic wastes are readily exposed to rainfall the potential for runoff is greatly increased (Crane et al. 1983).

The Control of Pollution (Silage, Slurry and Agricultural Fuel Oil) (Scotland) Regulations 2003 (SSI. 2003/531) regulates farms where slurry is produced. Slurry facilities should usually have a 6 month storage capacity. Guidelines offering best practice advice are given in the Code of Good Practice (Scottish Executive 2005, DEFRA 2009) on storage and application to land of manure and slurry. Application of solid manure or slurry should not be made to waterlogged, frozen or snow covered ground or where there is a likelihood of heavy rain in the 48 hours following application. Organic waste should not be applied within 10 m of surface waters and 50 m of springs, boreholes or wells. Application rates are specific to each site depending on loading and crop requirements. However the rate should never exceed either 50 m^3/ha for surface spreading although the normal rate is 25-30 m^3/ha (Scottish Executive 2005; DEFRA 2009).

Direct deposition of faecal material by farmed animals also poses a risk to the contamination of surface waters and private water supplies where there is no protection provided for the water supply. The Scottish Executive (2005) and DEFRA (2009) give guidelines on grazing animals in the vicinity of surface waters and wells, springs or boreholes.

Microorganisms inside faecal waste deposits have protection against the effects of weathering and predators. Faecal coliforms have been shown to survive intense sunlight and heat for at least one summer (Buckhouse and Gifford 1976). Direct deposition of faecal material by birds and wildlife is much more difficult to manage with regards to surface waters. However private water

supplies can be protected by ensuring they are encased in a secure unit to prevent access by small animals. Regular checks and maintenance is also of utmost importance.

Septic tank storage of human waste has the potential to contaminate water supplies. Recommendations for private water supplies state that they should be sited uphill of and at least 50 m away from potential sources of pollution (McGaw et al. 1998).

1.7 Survival of Bacteria

Bacteria that have the ability to survive for long periods of time in water and soil environments increase the potential for groundwater contamination which may in turn contaminate potable water supplies.

The introduction of microbes into water or soil often has a dramatic effect on their survival and activity. There is often a rapid decline in their numbers and a decrease in the average activity per cell of the surviving introduced microbes. This has been attributed to the scarcity of available nutrients and the hostility of the soil environment to incoming microbes including competition from indigenous microorganisms (Van Veen, Overbeek and Van Elsas 1997).

In general, the survival of microorganisms depends on a number of factors such as temperature, pH, soil moisture and texture, nutrient availability and the

presence of predators and competing microbial species. Temperature is thought to be one of the major factors influencing bacterial survival (Davenport, Sparrow and Gordon 1976; Gerba and Bitton 1984). Survival of microorganisms has been found to be prolonged at low temperatures. *E. coli* has been shown to survive longer at 4˚C than at 25˚C (Rattray et al. 1992; Bogusian et al. 1996; Cools et al. 2001). Increased exposure to UV light can also reduce bacterial survival (Crane et al. 1983).

Temperature can affect microbial activity by altering the moisture content of soils (Entry et al. 2000). Microorganisms require a certain amount of moisture to avoid desiccation. Bacterial survival has been shown to be greater in finer soils as water retention is increased (Abu-Ashour et al. 1994). However, an excessive increase in moisture content can lead to oxygen depletion as pore spaces become saturated. This has been shown to lead to a decrease in microbial numbers (Postma and van Veen 1990). In addition, microbial predators such as protozoa tend to be more active at higher soil moisture contents, perhaps because increased water tends to provide a mechanism for movement between pores, although protozoa may be excluded from some pores due to their size (Abu-Ashour et al. 1994). In clay-rich soils bacteria adsorb more readily to particles and gain increased protection from predation (Abu-Ashour et al. 1994; Stotzky 1989).

1.8 Transport of bacteria through soil

The soil serves as a natural filter by providing adsorption sites for the removal of bacteria and viruses. It can therefore reduce microbial transport to a certain extent but its retention capabilities are finite (Gerba and Bitton 1984).

There are many factors which are thought to have an effect on bacterial transport through soil. The soil itself is of utmost importance with factors such as soil texture, particle size distribution, organic matter content, pH, ionic strength of soil solution and bulk density, all shown to impact microbial transport (Bitton, Lahav and Henis 1974; Smith et al. 1985; Paterson et al. 1993). Soil is comprised of a combination of sand, silt, clay and organic matter and the combination of these helps to determine its adsorptive properties and physical structure. The organic matter and clay particles have a significant effect on bacterial movement due to microbial attraction to their negatively charged surfaces (Mawdsley et al. 1995). The highest organic matter content is found in the surface layer of soil which explains why the surface layer is very effective at removing bacteria by both filtration and adsorption mechanisms (Gerba, Wallis and Melnick 1975; Crane et al. 1983). Gerba, Wallis and Melnick (1975) found that 92% or greater of the bacteria studied were removed in the top 1 cm of soil.

One of the most important environmental factors with regards to bacterial movement in soil appears to be the water content of the soil, and water movement through the soil. In unsaturated soil, water movement is primarily vertical because of the force of gravity, however, water movement in the

saturated zone or groundwater is horizontal because of the differences in pressure or elevation. Madsen and Alexander (1982) found that there was almost no transport in soil microcosms when there was no water movement but when water was present vertical transport occurred. Increased movement of bacteria in saturated soils has been found in a number of studies (Wong and Griffin 1976; Worrall and Roughley 1991).

Many studies of bacterial movement show rapid movement and a high concentration of bacteria reaching receiving waters. This is thought to be due to preferential flow of microorganisms through macropores in the soil. These can be cracks, fractures, worm holes or channels formed by plant roots or animals. Preferential flow through macropores has been observed in both laboratory and field studies (Chandler, Farran and Craven 1981; Thomas and Phillips 1979). Smith et al. (1985) found greater retention of *E. coli* in disturbed soil compared to a corresponding intact soil. The disturbed soil had been mixed which removed most of the macropores. The role of macropores in bacterial transport was also indicated by van Elsas, Trevors and van Overbeek (1991) when it was shown that *Pseudomonas fluorescens* was transported to greater depths in undisturbed soils than in those which had been repacked.

1.9 Monitoring methods

The identification of pathogens in drinking water can be difficult due to their low numbers. Their detection may require the examination of large volumes of water

using time consuming methods. It is recognised that contamination of water may be intermittent and so may not be revealed by the examination of a single sample. It is therefore of greater value to examine a supply frequently by a simple test than occasionally by a more complicated test (Anon 1994).

When pathogens are present in water they are usually greatly outnumbered by the normal commensal bacteria of the human or animal intestine and therefore detection of an indicator organism is used to show the likelihood of pathogens being present in a water supply. An indicator organism should be present in the intestinal flora of healthy people, and in higher numbers than those of the pathogens they are intended to indicate. They should be unable to grow out with the intestine and must die off at a slightly slower rate than that of the pathogens. They should also be non-pathogenic and easy to isolate, identify and count (Oliveri 1982).

Coliform bacteria meet most of these criteria. Coliforms are recognized by their ability to ferment lactose at 37 °C and the presence of the enzyme β-galactosidase. Coliforms do not possess the enzyme cytochrome oxidase and therefore are oxidase negative (SCA 2002). Coliforms are members of the Enterobacteriaceae family. Genera which belong to this family are *Escherichia, Citrobacter, Enterobacter* and *Klebsiella*. Many coliforms are ubiquitous to soil but the coliform *E. coli* is known to be present in the gut of warm-blooded animals. Some species of *Escherichia*, C*itrobacter, Enterobacter* and *Klebsiella* are thermotolerant however some thermotolerant species may not be faecal in

origin unlike *E. coli* which is solely faecal and is regarded as the only true faecal coliform (Waite 1985).

The detection and enumeration of coliform bacteria is the standard microbiological analysis used in the UK to determine water quality, however there are a number of different methods used. The two most common methods are membrane filtration (MF) and the multiple tube fermentation method (MTFM). Both standard methods have their advantages and disadvantages. The membrane filtration is quicker and easier to perform than the multiple tube method, however high background bacterial numbers can interfere with the results as the growth of indicator organisms may be inhibited (Fricker, Illingworth and Fricker 1997). In a 100 ml sample of drinking water, coliforms must not be detected. The presence of one or more coliform bacteria indicates a fail for that water sample.

There have however been advances in more rapid detection techniques of the coliform organisms. Chromogenic and fluorogenic compounds have been developed following the understanding of the biochemical actions of the β-D-galactosidase and β-D-glucoronidase enzymes which are primary characteristics of coliforms and *E. coli* respectively. Media has been developed by several companies which contain a specific substrate for each of these enzymes. These include Colilert™ (IDEXX), Coliquick™ (Hach) and Colisure™ (Millipore). Colilert 18™ gives results in 18 to 22 hours. It utilises the most probable number (MPN) method of statistical analysis providing a counting range from one to 2419 colony forming units per 100 ml.

1.10 Statement of aims

The aim of this thesis is to evaluate methodology that can reliably detect and quantify coliform bacteria in potable water and soil and to use this method to study factors which influence the survival and transport of coliform bacteria in the environment. The extent and severity of drinking water contamination from private water supplies in the north east of Scotland will be determined along with potential factors increasing the likelihood of contamination. These aims will be achieved through the following objectives:

- Background research of private water supplies in Aberdeenshire, Scotland using data provided by Aberdeenshire council

- Study of a number of private water supplies in Central Aberdeenshire to investigate microbiological quality of potable water from supplies

- Evaluation of a rapid testing method (Colilert 18™) for the detection and enumeration of coliform bacteria in potable water and soil samples

- The application of this testing to potable water samples and soil samples to study factors affecting coliform survival

- The application of this testing to study factors affecting the transport of coliform bacteria in repacked and intact soil columns

Chapter 2 Evaluation of Colilert 18™ as a replacement method for detection of coliform bacteria in water compared to the standard method, membrane filtration

2.1 Introduction

The detection and enumeration of coliform bacteria particularly *E. coli* is well recognised as the standard microbiological analysis to indicate the presence of faecal contamination of drinking water. Coliform bacteria particularly *E. coli* are present in large numbers in the gut of humans and warm blooded animals. The presence of these 'indicator' bacteria in drinking water highlights the potential for waterborne pathogens to be present. Pathogens, if present, are usually found in low numbers and therefore are more difficult to detect whereas coliforms are relatively easy to test for. Not all coliform bacteria are of faecal origin, many are found naturally in the environment. However the presence of any coliform bacteria in potable water can be an indication of the failure of a treatment system or a poorly protected groundwater supply. Coliforms have similar survival patterns to many waterborne pathogens and are effective at indicating the potential presence of these pathogens (Geldreich 1978; Gleeson and Gray 1997; SCA 2002).

The two most commonly used tests for the detection and enumeration of coliforms in potable water are the membrane filtration method (MF) and the multiple-tube fermentation method (MTFM). In these methods, the ability of

coliform bacteria to ferment lactose at 37°C is used as a means of identifying them. Faecal coliforms including *E. coli* are further identified by their ability to ferment lactose at 44°C. These established methods initially give a presumptive count with further confirmatory tests required to eliminate any false positive results. However these tests are time consuming and labour intensive, with the final confirmed result taking up to 72 hours to achieve.

The historic definition of coliform bacteria based on their ability to ferment lactose (Anon 1994) was revised and now includes their enzymatic properties, coliform bacteria possessing the enzyme β-D-galactosidase and *E. coli* possessing β-D-glucoronidase. By understanding the actions of the enzymes, chromogenic and fluorogenic compounds have been developed which can result in colour changes and fluorescence respectively in specific growth media. This has seen advances in more rapid detection techniques of the coliform bacteria. Such media are now being used as an alternative method for the detection and enumeration of coliforms and *E. coli.*

One of these methods is Colilert 18™ which is also referred to as Defined Substrate Technology (DST). This method identifies bacteria through the effect of their constitutive enzymes on chromogenically labelled specific substrates. For β-D-galactosidase, the substrate galactopyranoside is present, conjugated with ortho-nitrophenol as a chromogenic indicator, in the form of the molecule ortho-nitrophenol-β-D-galactopyranoside (ONPG). When coliforms grow in Colilert 18™ the enzyme β-D-galactosidase allows them to metabolise the

galactopyranoside component of ONPG, thereby releasing free ortho-nitrophenol which changes the solution from colourless to bright yellow.

For β-D-glucuronidase, the substrate glucuronide is present in Colilert 18™ conjugated with methyl umbeliferone as a fluorogenic indicator, in the form of the molecule 4-methylumbeliferyl-β-D-glucuronide (MUG). When *E. coli* grows in Colilert 18™ the enzyme β-D-glucuronidase allows metabolism of the glucuronide component of MUG, thereby releasing methyl umbelifererone, which can be seen as a bright blue fluorescence under UV light at 365 nm. Organisms that do not possess the target enzymes cannot utilise the specific substrate used in DST media therefore are unable to grow and cause interference (Sartory and Watkins 1999). Colilert has a special formulation which suppresses non- coliforms which do have these enzymes, minimizing false positives and false negatives (IDEXX 2007).

An alternative method for the microbiological assessment of private water supplies in Aberdeenshire was required for this project. The water to be analysed was non-chlorinated groundwater collected from private water supplies. The technique to be used had to make the workload more manageable. In particular, preparation and analysis time had to be reduced. The replacement technique had to be shown to be at least as reliable as the standard techniques. The cost also had to be comparable with standard methods. It is recognised that water contamination can be of a sporadic nature and that a single water sample may not give an accurate representation of the quality of a body of water. Faster analysis of samples would increase the

potential for a greater number of samples to be tested and so gaining a clearer picture of the microbiology of the water.

Validation of Colilert 18™ for the microbiological analysis of water in this project required the use of Colilert 18™ in parallel with an existing method for the examination of drinking water, which in this case was the membrane filtration method, with further confirmation using a modified Miles Misra (MMM) method. Methods were compared using several Gram negative bacterial isolates which have a widespread occurrence in the environment, namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Aeromonas hydrophila*.

E. coli and *E. aerogenes* are both coliform bacteria, but can be differentiated between due to *E. coli* being a faecal coliform. *P. aeruginosa* and *A. hydrophila,* although not coliform bacteria, are opportunistic potentially pathogenic bacteria which can occur in drinking water.

2.2 Material and methods

2.2.1 Preparation of bacterial cultures

Stock cultures were prepared from Cultiloops supplied by Oxoid (Basingstoke, UK). The organisms used were *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27583), *Enterobacter aerogenes* (ATCC 13048) and *Aeromonas hydrophila* (ATCC 7966).

Stock cultures were prepared under aseptic conditions by streaking the Cultiloop onto nutrient agar (Oxoid, Basingstoke, UK)) plates and placing them in an incubator at 37±0.5°C for approximately 24 hours. This allowed for visible growth of the bacteria.

2.2.2 Preparation and quantification of bacterial suspensions of stock cultures for serial dilution using turbidity measurements

Sterile ¼ strength Ringers solution (Oxoid, Basingstoke, UK) (10 ml) was pipetted into a sterile universal container. A turbid suspension of each of the cultures was prepared by removing bacterial culture from the nutrient agar plates and mixing it with the ¼ strength Ringers solution.

A turbidity meter was used to standardise bacterial suspensions. An aliquot (3 ml) of the 100% turbid suspension was removed and mixed with 10 ml ¼ strength Ringers solution in a turbidity tube. More Ringers solution was added if required to produce a stock suspension of 60 % transmittance measured on the turbidity meter. Further dilutions were prepared from this stock suspension. Serial dilutions were prepared by removing 1 ml of the stock suspension and placing it in a sterile universal container, containing 9 ml of sterile ¼ strength Ringers solution. The bottle was inverted to ensure mixing of the organism. This process was continued, each time obtaining a tenfold dilution. The stock suspension was referred to as ‘0’ dilution, each successive dilution being labelled accordingly (1, 2, 3 etc).

Working suspensions were prepared by removing 1 ml from the serial dilutions, and adding each to a separate 1 litre of ¼ strength Ringers solution. This resulted in a 100 ml of sample with an approximate bacterial count for *E. coli* and *E. aerogenes* which would lie within the range of detection of Colilert 18™ i.e. between 0 and 2419 cfu. Each study required the preparation of fresh serial dilutions however it was important to note that using the turbidity meter could result in some variation in bacterial counts between different batches of serial dilutions.

2.2.3 Preparation of glycerol stocks of bacteria for long term storage

Glycerol (0.15 ml; 100%) was added to a 2 ml screw-cap vial. This was sterilised by autoclaving at 121°C for 20 minutes. The glycerol was allowed to cool prior to adding 0.85 ml of a logarithmic-phase bacterial culture to the vial of pre-sterilised glycerol. The vial was vortexed vigorously to ensure even mixing of the bacterial culture and the glycerol. This was then frozen and stored in a minus 70°C freezer. The glycerol stocks were defrosted prior to use. Repeated thawing and re-freezing of glycerol stocks was avoided to prevent a reduction in the viability of the bacteria.

2.2.4 Modified miles misra (MMM) for bacterial quantification

Serial dilutions of each of the bacterial cultures were used to obtain an actual number of bacteria present in each dilution series. An aliquot (20 µl) from each one litre dilution was pipetted onto a separate, recorded position on the surface of a pre-dried (37°C) agar plate. This was repeated nine times for each dilution. The drops were allowed to dry. The plates were then incubated at 37°C for 24 hours until visible colonies developed in the small circular areas corresponding to the drops. If a sufficient number of dilutions had been prepared, a drop from one of the dilutions gave rise to a countable number of discrete colonies. Colonies were counted, and the mean and standard deviations calculated to obtain a direct bacterial count in colony forming units (cfu) per 1 ml. Spots containing between 20 – 40 colonies were used to calculate the colony count accurately (Singleton and Sainsbury 1996; Miles and Misra 1938). The Miles Misra technique has been described by Baker and Breach (1980) as “probably the most accurate of the viable count techniques”.

2.2.5 Membrane filtration method for bacterial quantification

Using sterile membrane filtration apparatus, a measured volume of water for analysis was filtered through a sterile cellulose nitrate membrane of pore size 0.45 µm. The membrane was then transferred aseptically, onto a pad in a 55 mm Petri dish which had been soaked in membrane Lauryl Sulphate Broth (MLSB) (MERCK pharmaceuticals, West Drayton, UK). This is a selective

media containing lactose and a pH indicator that allowed for the detection of acid production from the fermentation of lactose. The Petri dish containing the membrane and pad was then placed in a 30°C incubator for 4 hours then transferred to a 37°C incubator for 14 hours. This procedure was duplicated; however the second sample was transferred to a 44°C incubator after the initial 4 hour incubation at 30°C.

After incubation it is assumed that the organisms under investigation will be retained on or near the surface of the membrane and will form colonies of characteristic morphology and colour. The other organisms will either be inhibited or can be distinguished by their appearance. When coliform colonies grow on this medium they produce yellow colonies. Pink colonies were not counted as they are classified as non-lactose fermentors and by definition are not coliforms, although they are noted as they may interfere with coliform growth. It is assumed that a single bacterium will generate a colony. The yellow colonies are counted as colony forming units (cfu) to give a numerical value for membrane filtration (Anon 1994).

Following incubation any yellow colonies can only be presumed to be coliforms (at 37°C) or faecal coliforms (at 44°C) and are termed presumptive colonies. Further tests to confirm the presumptive bacteria as coliforms are required.

2.2.6 Tests used to provide confirmation of coliform bacteria

A minimum of 10 yellow colonies from each membrane were selected randomly for confirmation (or all colonies if less than ten on the membrane). The following tests were carried out for each individual colony used for confirmation:

2.2.6.1 Lactose peptone water

Capped test tubes containing 5 ml of Lactose peptone water (LPW) were prepared and sterilised in advance. For each colony two LPW test tubes were required. A single yellow colony from the membrane was used to inoculate two tubes of LPW. This was also used to inoculate tryptone water, a nutrient agar plate and a McConkey agar plate.

One LPW test tube was placed in an incubator at 37°C for 24 hours. A colour change from yellow to pink/red was observed if coliforms were present due to acid production from the fermentation of lactose. One LPW was placed in an incubator at 44°C for 24 hours. Again a colour change due to the fermentation of lactose was observed from yellow to pink/red if *E. coli* was present.

2.2.6.2 Tryptone water

Tryptone Water was prepared and 5 ml placed into capped test tubes and sterilised. The Tryptone water was inoculated with a single colony following

inoculation of the LPW. The test tube was placed in an incubator at 44°C for 24 hours. Following incubation, the test tubes were placed in a fume cupboard, and examined for indole production from tryptophan by adding two drops of Kovac's reagent (Fisher Scientific, Loughborough, UK). A positive reaction will produce a red ring at the meniscus of the liquid. This confirms the colony as *E. coli* as opposed to other coliform bacteria.

2.2.6.3 Oxidase test

A nutrient agar plate was prepared and streaked with the same colony as was used to inoculate the LPW and the Tryptone water. The plate was then incubated at 37˚C for 24 hours after which time growth should be visible. An oxidase stick (Oxoid, Basingstoke, UK) was placed on a single colony on the nutrient agar. If the bacterial colony was oxidase positive the oxidase stick changed to purple. There should be no colour change reaction for coliforms as they are oxidase negative.

2.2.6.4 Examination of pure culture

McConkey agar plates were prepared and streaked with the same colony as was used to inoculate the LPW, Tryptone water and nutrient agar plate. The plate was then incubated at 37˚C for 24 hours to allow for visible growth. McConkey agar allows the growth of Gram-negative bacteria and inhibits most

Gram-positive bacteria. Gram-negative bacteria that can ferment lactose grow as red/pink colonies due to utilisation of the available lactose in the agar. Non-lactose fermenting bacteria will form white/colourless colonies by utilising peptone. For each of the colonies plated onto McConkey agar, the appearance of the colonies was noted to determine whether the sample was a pure strain of lactose fermenting, Gram-negative bacteria.

2.2.6.5 Confirmation of coliform bacteria

A number of tests were carried out to confirm that a subcultured colony was a coliform. Table 2.1 summarises these tests and the interpretation of results. To confirm as coliform bacteria each of the tests must meet all the criteria.

Table 2.1 Summary of tests used to confirm colonies as coliform bacteria

Test	Confirmation of coliform	Confirmation of faecal coliform
Lactose peptone water	Acid produced at 37˚C	Acid produced at 44˚C
Tryptone water	No indole production from tryptophan	Indole production from tryptophan
Oxidase test	Oxidase negative	Oxidase negative
Growth in McConkey agar	Gram negative bacteria produce red / pink colonies	Gram negative bacteria produce red / pink colonies

The number of colonies in a sample was then calculated using the following equation (SCA 2002):

$$\text{Confirmed coliform count (cfu / 100 ml)} = \frac{\text{No. of confirmed colonies}}{\text{No. of presumptive colonies set up for confirmation}} \times \text{Total no. of presumptive colonies}$$

2.2.7 Protocol for use of Colilert 18™

Colilert 18™ (IDEXX, Buckinghamshire, UK) was used as instructed by the manufacturer and as outlined here. A water sample (100 ml) was poured into a sterile Colilert 18™ bottle. The Colilert 18™ reagent was then added to the sample (Figure 2.1) and shaken vigorously to aid the reagent to dissolve.

Figure 2.1 Addition of Colilert 18™ reagent to 100 ml sample of water in a sterile Colilert 18™ bottle (Reproduced with permission from IDEXX)

Once the reagent had completely dissolved, the sample was poured into the Quanti-tray™. This was then placed into the rubber tray carrier, wells facing down and sealed using the Quanti-tray™ heat sealer unit. The Quanti-tray™ was then placed into an incubator, wells facing down, and incubated at 37 ±

0.5°C for 18 hours. Control blanks containing only ¼ strength Ringers solution for each analysis were prepared and incubated. After 18 hours the tray was removed from the incubator and examined visually for any yellow wells (Figure 2.2) which indicate the presence of coliform bacteria.

The number of wells were counted and referred to the Most Probable Number (MPN) table (IDEXX), to determine the coliform count per sample. If wells were very pale or no colour was present in any of the wells, the trays were placed back into the incubator and examined again after a further 4 hours.

Figure 2.2: Quanti-tray™ containing water and dissolved reagent. Yellow wells indicate presence of coliform bacteria (Reproduced with permission from IDEXX)

To determine the number of *E. coli* present, the tray was examined under ultra-violet light (365 nm) using a UV viewing cabinet containing a 6-Watt fluorescent UV lamp (IDEXX). Wells that fluoresced indicated the presence of *E. coli*. Using the same MPN tables the number of *E. coli* per sample was determined. The MPN per 100 ml was calculated taking into account any dilutions made.

A Quanti-tray Colilert 18™ comparator (IDEXX) was supplied with the Colilert 18™ media. If any wells were particularly pale from a sample after a maximum of 22 hours incubation, they would be compared with the comparator wells. If the sample wells were more yellow than the comparator they were regarded as a positive result, however if they were less yellow than the comparator wells they were ignored. The comparator tray was kept in the dark in a refrigerator at 4-8°C for a maximum of 9 months according to the manufacturers use by date.

2.2.8 Preparation of antibiotic resistant strains of *E. coli and E. aerogenes*

An antibiotic resistant strain of *E. coli* and *E. aerogenes* was selected to enable differentiation from the original strain and ultimately to allow them to be used as tracer organisms within water and soil samples containing natural organisms. They each had to have resistance to two antibiotics and this resistance had to differ for each bacterium so as to be able to distinguish between them. It has been estimated that within bacterial populations one cell in every 10^8 cells is spontaneously resistant to any one antibiotic, whereas one cell in 10^{12} cells may be resistant to two combined antibiotics (Linton 1983). It was also essential that there was minimal or no natural resistance to these antibiotics by bacteria present in the soil.

E. coli and *E. aerogenes* were cultured in nutrient broth to produce a concentrated broth culture of each bacterial strain. *E. coli* was prepared with

resistance to streptomycin and rifampicin. *E. aerogenes* was prepared with resistance to chloramphenicol and erythromycin.

Prior to adding the antibiotics to the nutrient agar, the antibiotics were sterilised in the following ways:

- streptomycin was prepared with water and filter sterilised
- rifampicin was prepared in methanol
- erythromycin and chloramphenicol were prepared in ethanol

Petri dishes were prepared containing various concentrations of the antibiotic using gradient plates. For *E. coli*, nutrient agar solutions were prepared which contained streptomycin concentrations ranging from 50 µg/ml to 500 µg/ml. For *E. aerogenes*, nutrient agar solutions were prepared which contained chloramphenicol concentrations ranging from 20 µg/ml to 200 µg/ml. These were poured into Petri dishes to make a thin layer. The Petri dishes were then tilted by slightly raising up one end. The agar was allowed to set before pouring in nutrient agar to make up to the normal volume of agar. The plate was placed level and the agar left to set. The antibiotic diffuses through the agar allowing a gradient to be set up i.e. a higher concentration of antibiotic at one end decreasing to a lower concentration.

Using a sterile, bent glass rod, 0.1 ml aliquots of concentrated bacterial broth culture were spread onto the agar surface containing antibiotic. The plates were inverted and incubated at 37°C for 24 hours.

Plates were observed under good lighting conditions. Those Petri dishes giving confluent lawns of growth represented concentrations of antibiotic that were below the minimum inhibitory concentration for that particular strain. A clear background represented complete inhibition of the parent strain. Plates that gave distinct colonies arising from a clear background were used to further purify the bacteria as a potential antibiotic resistant strain (Hagedorn 1994).

Resistant colonies were removed using a sterile loop and streaked onto another antibiotic-supplemented plate of the same medium. In this case *E. c*oli was streaked onto nutrient agar plates containing 400 µg/ml of streptomycin and *E. aerogenes* was streaked onto nutrient agar plates containing 100 µg/ml chloramphenicol. This simple purification step was sufficient for obtaining a pure culture of the antibiotic-resistant strain.

This process was repeated but with nutrient agar supplemented with two antibiotics. For *E. coli*, nutrient agar was prepared which contained a streptomycin concentration of 400 µg/ml and rifampicin concentrations ranging from 40 µg/ml to 200 µg/ml. For *E. aerogenes*, nutrient agar was prepared which contained a chloramphenicol concentration of 100 µg/ml and erythromycin concentrations ranging from 50 µg/ml to 400 µg/ml. These were poured into Petri dishes to make a thin layer. The Petri dishes were then tilted by slightly raising up one end. The agar was allowed to set before pouring in nutrient agar to make up to the normal volume of agar. The plate was placed level and the agar left to set.

Aliquots (0.1 ml) of concentrated bacterial broth cultures containing bacteria already resistant to the initial antibiotics (streptomycin resistant *E. coli* and chloramphenicol resistant *E. aerogenes*) were spread onto the agar surface containing antibiotics. The plates were inverted and incubated at 37°C for 24 hours. Plates that gave distinct colonies arising from a clear background were used to further purify the bacteria as a potential antibiotic resistant strain.

Resistant colonies were removed using a sterile loop and streaked onto another antibiotic-supplemented plate of the same medium. The final concentrations of antibiotic used to prepare antibiotic resistant *E. coli* were 400 µg/ml streptomycin and 80 µg/ml rifampicin. The final concentrations of antibiotic used to prepare antibiotic resistant *E. aerogenes* were 100 µg/ml chloramphenicol and 200 µg/ml erythromycin.

2.2.9 Isolation and characterisation of an antibiotic resistant strain of *E. coli* and *E. aerogenes*

Confirmation tests were carried out to determine whether selected antibiotic resistant coliform bacteria behaved in the same way as non-antibiotic resistant coliform bacteria, i.e. original culture. The tests were carried out as described previously using the tests for the confirmation of coliform bacteria (method 2.2.6). Ten colonies were confirmed from the original culture and twenty from the antibiotic resistant colonies. Additionally the growth on both the nutrient agar

and McConkey agar plates with and without antibiotics were examined to compare growth rate and visual similarity.

The Colilert 18™ method was carried out on the same colonies to ensure they could be detected by this technique. A colony was added to 100 ml of sterile ¼ strength Ringers solution and analysed as described in method 2.2.7.

2.3 Material and methods: Evaluation of Colilert 18™ for the detection of coliform bacteria

To evaluate the detection and quantification of coliforms using Colilert 18™ four bacterial strains (*E. coli*, *E. aerogenes*, *P. aeruginosa*, *A. hydrophila*) were quantified using Colilert 18™, membrane filtration method and by the drop plate method, using a modified Miles Misra (MMM).

2.3.1 Detection and quantification of single bacterial cultures

Serial dilutions of *E. coli*, *E. aerogenes*, *P. aeruginosa* and *A. hydrophila* were prepared using the methods described in sections 2.2.1 and 2.2.2. Dilutions tested for *E. coli* and *E. aerogenes* ranged from 3 to 8 dilutions. *P. aeruginosa* and *A. hydrophila* dilutions tested ranged from 3 to 5. From each dilution, 1 ml was removed and added to a separate sterile 1 litre of ¼ strength Ringers

solution. These one litre bacterial suspensions were then tested using MMM, MF and Colilert 18™.

MMM was carried out as described in 2.2.4. The average value for the nine replicates of MMM was calculated and given as cfu per 20 µl. For each of the bacteria, the MMM result was extrapolated to determine the expected number of bacteria in 100 ml of the same solution.

The bacterial suspensions were analysed using MF (2.2.5 and 2.2.6) and Colilert (2.2.7). For both methods, two, 100 ml of sample from each bacterial suspension was used. Each method was used to detect the total coliform and *E. coli* count per 100 ml of sample.

2.3.2 Detection and quantification of mixed bacterial cultures

To determine if mixed bacterial populations affected the detection of coliform bacteria the four selected bacterial isolates were tested in a series of experiments as shown in Table 2.2.

Fresh dilutions of *E. coli*, *E. aerogenes*, *P. aeruginosa* and *A. hydrophila* were prepared using the method described in section 2.2.2. Serial dilutions tested ranged from 4 to 7. From each dilution, 1 ml was removed and added to a separate sterile 1 litre of ¼ strength Ringers solution. These bacterial suspensions were then analysed using MMM as described in 2.2.4. The

average value for the nine replicates of MMM was calculated and extrapolated to determine the expected number of bacteria in 100 ml of the same solution.

To quantify bacterial numbers in mixed suspensions, sterile 1 litre solutions of ¼ strength Ringers were prepared for each experiment in Table 2.2. *E. coli* and *E. aerogenes* from dilution 7 (1 ml) and *P. aerogenes* and *A. hydrophila* from dilution 4 (1 ml) were added to the solution when required. Low dilutions (7) were used to determine the ability of each method to accurately detect low colony counts of each bacteria whereas high dilutions (4) were used to determine if excessive concentrations of bacteria caused interference with the detection methods.

Table 2.2: Combinations of bacteria and dilution factor used for analysis of bacterial solutions by modified Miles Misra, membrane filtration and Colilert 18™

Bacterium	**Experiment and dilution factor**										
	EC EA	**EC PA**	**EC AH**	**EA PA**	**EA AH**	**PA AH**	**EC EA PA**	**EC EA AH**	**EC PA AH**	**EA PA AH**	**EC EA PA AH**
***E. coli* (EC)**	7	7	7				7	7	7		7
***E. aerogenes* (EA)**	7			7	7		7	7		7	7
***P. aeruginosa* (PA)**		4		4		4	4		4	4	4
***A. hydrophila* (AH)**			4		4	4		4	4	4	4

For analysis using MF (method 2.2.5 and 2.2.6) and Colilert 18™ (method 2.2.7) two, 100 ml of sample from each bacterial suspension was used to determine the total coliform and *E. coli* count per 100 ml of sample. The averages of these values were used for comparison.

2.3.3 Comparison of membrane filtration and Colilert 18™ for analysis of coliform bacteria in groundwater

Thirty seven samples were collected from 14 private water supplies in Aberdeenshire where the domestic water supply was from a groundwater source. These supplies are briefly described in Table 2.3 along with the code allocated to it. Twenty two samples were taken from kitchen cold water taps, 15 of which were fed directly from the well or spring source with no additional storage prior to use, and 7 which were fed from a storage tank or external reservoir. Two samples were taken from a storage tank supplying the kitchen cold water tap and a further 13 samples were taken directly from the well or spring supplying potable water.

Tap samples were collected in a sterile 500 ml glass bottle. The tap was sterilised with hypochlorite solution prior to running the water for one minute prior to collecting the sample.

Well samples were collected in a sterile 500 ml glass bottle. The bottle was weighted by fastening a heavy chain around it, prior to lowering into the well.

Once the bottle was filled with water it was removed and sealed. The storage tank samples were collected in the same way as the well samples.

Table 2.3 Details of private water supplies used for potable water sample collection

Code	Property Location	Supply Type	Details
A	Tarves	Well	Direct supply to kitchen tap
B	Ardallie	Borehole	Borehole supplies storage tank which feeds kitchen tap
C	Arnage	Well	Direct supply to kitchen tap
D	Craigdam	Well	Direct supply to kitchen tap
E	Methlick	Spring	External reservoir which supplies kitchen tap
F	Kemnay	Well	External reservoir which supplies kitchen tap
G	Kemnay	Well	Direct supply to kitchen tap
H	Kemnay	Well	Direct supply to kitchen tap
I	Thainstone	Well	Direct supply to kitchen tap
J	Kintore	Well	Direct supply to kitchen tap
K	Dyce	Borehole	Direct supply to kitchen tap
L	Udny	Well	Direct supply to kitchen tap
M	Kinellar	Well	Direct supply to kitchen tap
N	Kinellar	Well	Direct supply to kitchen tap

All samples were placed in a cool box and taken back to the laboratory for analysis. The analysis was carried out within 4 hours of sample collection. Samples were analysed by both membrane filtration (method 2.2.5 and 2.2.6) and Colilert 18™ (method 2.2.7). Analysis was carried out using a 10 and 100 ml volume of each sample, making the 10 ml sample up to 100 ml with ¼ strength Ringers solution.

2.4 Results and Discussion

2.4.1 Detection and quantification of single bacterial cultures

All bacteria were grown and could be quantified using the modified Miles Misra. Results are shown in Table 2.4. This would be expected as this is a non-specific method for bacterial quantification. In contrast, the selective methods, membrane filtration and Colilert 18™ only detected and quantified *E. coli* and *E. aerogenes* which are specific methods for coliform detection.

Both methods successfully distinguished between *E. coli* and *E. aerogenes,* with the Colilert 18™ observation under UV light being significantly more rapid compared to the need for confirmatory tests when membrane filtration is used. Both methods gave similar detection limits down to the 8^{th} dilution.

Table 2.4 Detection and quantification of single bacterial cultures using modified Miles Misra, membrane filtration and Colilert 18™

Bacterium	Dilution	Modified Miles Misra		Membrane Filtration		Colilert 18™	
				TC[2]	FC[3]	TC[2]	*E. coli*
		cfu / 20µl[1]	cfu/ 100 ml	(cfu / 100 ml)		(MPN[4]/ 100 ml)	
E. coli	3	73	365000	C[5]	C[5]	>2419[6]	>2419[6]
E. coli	4	8	40000	C[5]	C[5]	>2419[6]	>2419[6]
E. coli	5	0.89	4450	C[5]	C[5]	>2419[6]	>2419[6]
E. coli	6	0.11	550	>200, >200	>200, >200	488, 461	488, 461
E. coli	7	0	**55**[7]	25, 28	25, 28	46.5, 44.3	46.5, 44.3
E. coli	8	0	**5**[7]	1, 0	1, 0	4.1, 4.0	4.1, 4.0
E. aerogenes	3	59	295000	C[5]	0	>2419[6]	0
E. aerogenes	4	6.11	30550	C[5]	0	>2419[6]	0
E. aerogenes	5	0.67	3350	C[5]	0	>2419[6]	0
E. aerogenes	6	0	**335**[7]	150, 161	0	272.3, 307.6	0
E. aerogenes	7	0	**33**[7]	19, 22	0	28.2, 32.4	0
E. aerogenes	8	0	**3**[7]	1, 0	0	2.0, 1.0	0
P. aeruginosa	3	48	240000	0	0	0	0
P. aeruginosa	4	6	30000	0	0	0	0
P. aeruginosa	5	0.44	2200	0	0	0	0
A. hydrophila	3	45	225000	0	0	0	0
A. hydrophila	4	8	43500	0	0	0	0
A. hydrophila	5	0.78	3900	0	0	0	0

[1] mean of 9 samples [2] TC (total coliforms) [3] FC (faecal coliforms)

[4] MPN (most probable number) [5] C (confluent – unable to read due to excessive growth)

[6] > 2419 (exceeds the readable MPN count) [7] approximate extrapolated values

MF limits of detection (cfu / 100 ml): upper = 200, lower = 0

Colilert 18™limits of detection (cfu / 100 ml): upper = 2419, lower = 0

The linearity of all three methods was assessed using a linear regression plot for *E. coli* (figure 2.3) and *E. aerogenes* (figure 2.4). Quantification of both organisms gave R^2 values of between 0.98 and 1.0 indicating a good correlation across the dilution range.

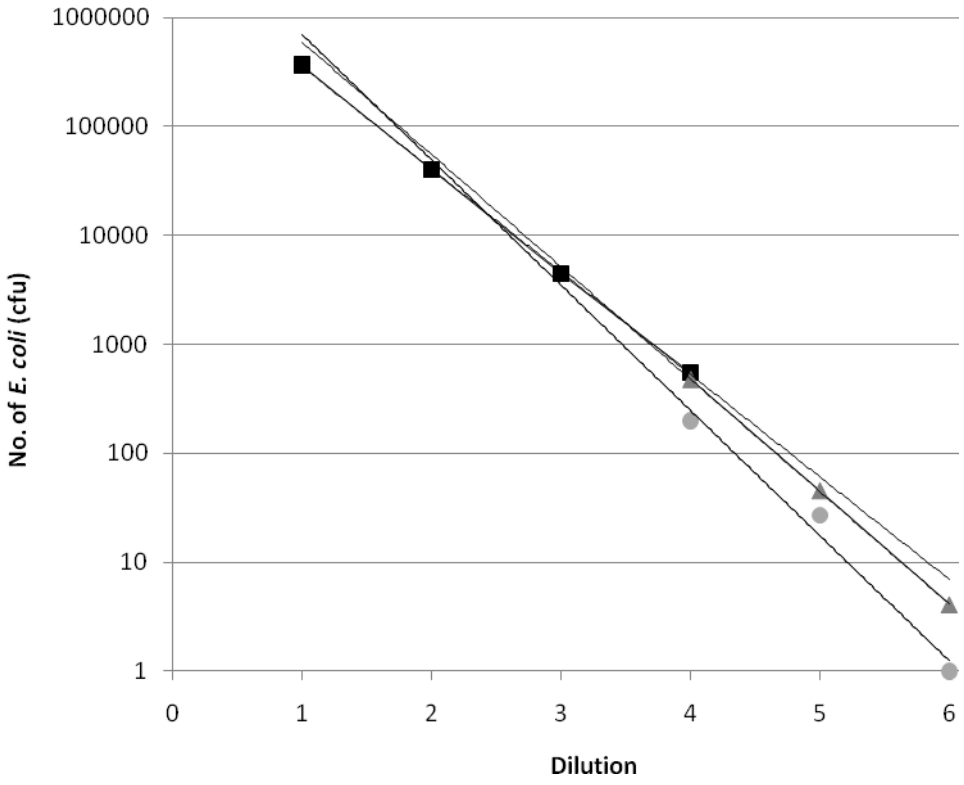


Figure 2.3: Linear regression of results for the detection of *E. coli* by modified Miles Misra (MM), membrane filtration (MF) and Colilert 18™ methods

(■ MM (R^2 = 0.9998); ● MF (R^2 = 0.9805);

▲ Colilert 18™ (R^2 = 1))

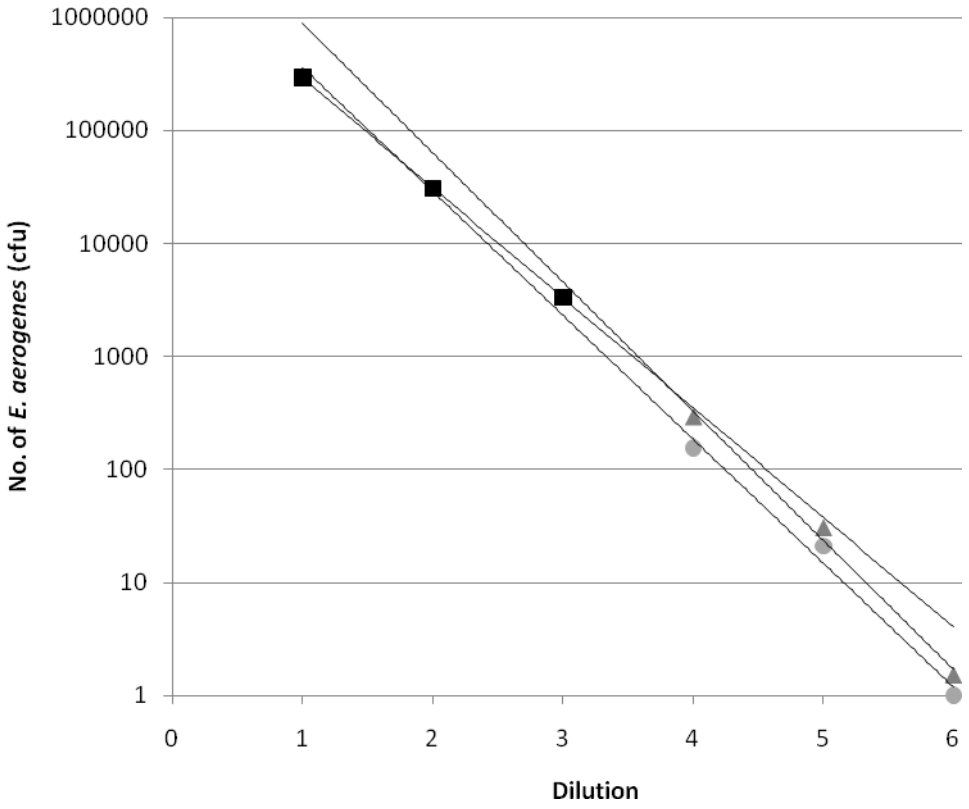


Figure 2.4: Linear regression of results for the detection of *E. aerogenes* using modified Miles Misra (MM), membrane filtration (MF) and Colilert 18™ methods

(■ MM (R^2 = 0.9999); ● MF (R^2 = 0.9861);

▲ Colilert 18™ (R^2=9933))

In this study Colilert 18™ detected a greater number of bacteria than membrane filtration. The values obtained can be compared to the quantification of bacteria by modified Miles Misra. For *E. coli*, the predicted numbers for the 7th dilution would be around 55 cfu/100 ml which compares favourably with the *E. coli* counts using Colilert 18™ (46 and 44 MPN/100 ml) than by comparison with detection by membrane filtration which was approximately fifty percent less (25 and 28 cfu/100 ml). The trend is repeated for *E. aerogenes* with a predicted

number for the 7th dilution of approximately 33 cfu; Colilert 18™ detecting 28 and 32 MPN/100 ml and membrane filtration lower at 19 and 22 cfu /100ml. These findings cause some concern as the membrane filtration method is one of the standard methods used to monitor water for faecal contamination however the results here suggest it may be underestimating their occurrence.

Other recent studies comparing Colilert 18™ with membrane filtration have observed similar results (Bonadonna, Cataldo and Semproni 2007; Hörman and Hänninen 2006; Pitkänen et al. 2007). Bonadonna, Cataldo and Semproni (2007) compared Colilert 18™ with the membrane filtration reference method ISO 9308-1. It was found that Colilert 18™ detected higher *E. coli* counts but was in fact more sensitive than the reference method. The study also called into question the use of indole production as a reliable method for *E. coli* confirmation. Hörman and Hänninen (2006) suggest that higher numbers of *E. coli* are detected with Colilert 18™ due to its ability to recover injured and stressed coliforms.

Two bacteria were used to assess the potential for false positives, namely *P. aeruginosa* and *A. hydrophila*. Both of these were easily quantified by the modified Miles Misra but neither gave any response by membrane filtration or Colilert 18™ confirming the suitability of these methods for water quality analysis. This is particularly useful in providing assurance that Colilert 18™ is an appropriate simple and rapid replacement for the more laborious membrane filtration, a conclusion also reached by Bonadonna, Cataldo and Semproni (2007), Hörman and Hänninen (2006) and Buckalew et al. (2006).

2.4.2 Detection and quantification of mixed bacterial cultures

Each of the bacteria at the various dilution factors were analysed by modified Miles Misra. The values calculated from each dilution are shown in Table 2.5. These values were then used to make a comparison with the actual number of bacteria detected by each method for the mixed bacterial samples.

Table 2.5 Quantification of bacterial samples using modified Miles Misra

Organism	cfu / 100 ml			
	Dilution 4	**Dilution 5**	**Dilution 6**	**Dilution 7**
E. coli	105000	9450	1100	95 – 110*
P. aeruginosa	225000	25000	2200	220 – 250*
A. hydrophila	140000	13350	1650	134 – 165*
E. aerogenes	90000	15000	1500*	90 – 150*

*estimated values

Following this quantification, the bacterial samples were combined then analysed by MF and Colilert 18™. Figure 2.5 shows the comparison between MF and Colilert 18™, using the modified Miles Misra results as the reference method.

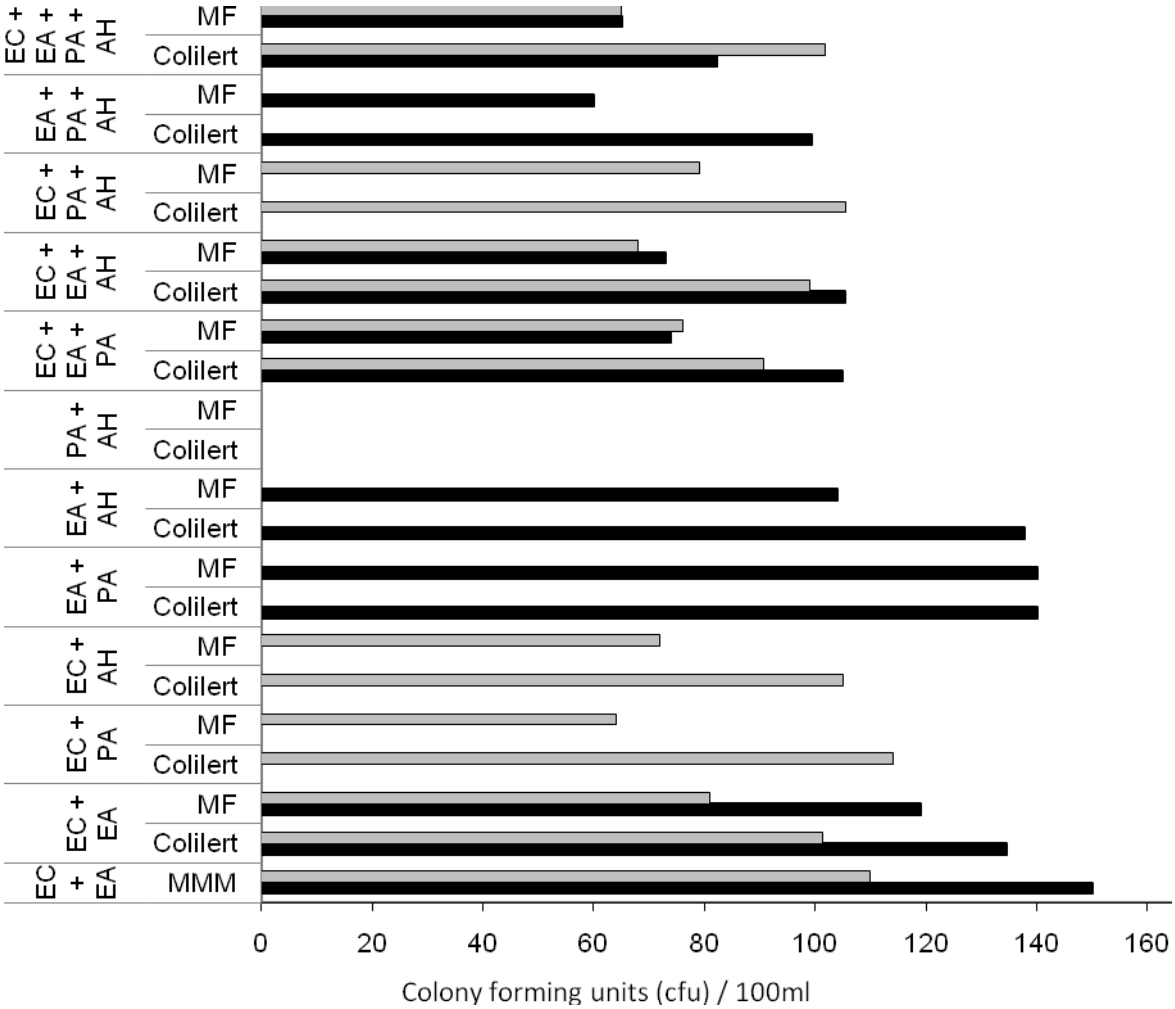


Figure 2.5: Comparison of membrane filtration and Colilert 18™ for the detection and quantification of *E. coli* (EC ■), *E. aerogenes* (EA ■), *P. aeruginosa* (PA), and *A. hydrophila* (AH), in spiked water samples. A modified Miles Misra is used for reference. (EC and EA – dilution 7, PA and AH – dilution 4)

Note: *P. aeruginosa* and *A. hydrophila* were enumerated using MMM and were found to contain approximately 225,000 and 140,000 cfu / 100 ml respectively at dilution 4, however were not detected using MF or Colilert 18™.

Membrane filtration and Colilert 18™ both detected *E. coli* and *E. aerogenes* in each sample in which they were present. Neither method detected *P. aeruginosa* or *A. hydrophila* in any of the samples even though there were

large numbers present. This was also shown in 2.4.1 where large numbers of single culture samples were examined. These findings contradict Cowburn et al. (1994) and Landre, Gavriel and Lamb (1998) who found that false positive results can occur attributed to non-coliform organisms when present at high cell densities. Landre, Gavriel and Lamb (1998) found that *A. hydrophila* gave false positive results with Colilert when cell counts were greater than 1×10^5 cfu ml^{-1} and fresh regent used although when aged reagent was used it was found that cell counts as low as 1×10^1 cfu ml^{-1} gave false positive results. However the method used for detection of ortho-nitrophenol production was to measure absorbance at 420 nm. This causes some confusion as ortho-nitrophenol production using Colilert as stated in the study by Landre, Gavriel and Lamb (1998) is a “ready detectable yellow” which can be seen visually. This raises some question as to what was detected in the study.

Colilert 18™ consistently detected higher numbers of coliform bacteria, both *E. aerogenes* and *E. coli*, than membrane filtration. The values obtained for Colilert 18™ are closer to those of the reference values found with modified Miles Misra, as also observed with the single cultures. As mentioned previously this may be due to the greater ability of Colilert 18™ to recover injured or stressed coliforms in comparison to MF. It has been noted that it is possible to detect enzyme activity from coliform bacteria even when the bacteria are no longer culturable (Davies, Apte and Peterson 1995).

The membrane filtration method requires the colonies to be easily counted using the naked eye. While Colilert 18™ contains media which limits the growth

of all bacteria except coliforms and *E. coli*, MF media allows the growth of non-coliform bacteria. This increases overcrowding on the membrane which may hinder the growth of coliforms and is likely to lead to an underestimation of numbers of the targeted bacteria. It is recommended that approximately 100 colonies should be the maximum grown on a single membrane to prevent a decrease in the accuracy of colony counts (SCA 2002).

The presence of *P. aeruginosa* and *A. hydrophila*, although not shown to cause false positive counts of bacteria, do appear to influence the bacterial counts of *E. coli* and *E. aerogenes* detected in samples. An increase in bacteria particularly non-coliform bacteria is likely to increase competition for nutrients within samples.

2.4.3 Comparison of membrane filtration and Colilert 18™ for analysis of coliform bacteria in groundwater

Water samples were taken from 14 sites (A – N). Each of the water samples were tested using the membrane filtration and Colilert 18™ methods to detect coliforms and *E. coli*. A 10 ml and a 100 ml sample of each were analysed. The results for this study are shown in Table 2.6. The results for Colilert 18™ have been rounded up to the nearest whole number.

Table 2.6 Comparison of membrane filtration and Colilert 18™ for the analysis of groundwater

		Membrane Filtration				Colilert 18™			
		Original Sample Volume (ml)							
Site	Sample	10	100	10	100	10	100	10	100
		Total Coliforms (cfu / 100 ml)		*E. coli (cfu / 100 ml)*		*Total Coliforms (cfu / 100 ml)*		*E. coli (cfu / 100 ml)*	
A	T1	90	22	50	7	100	34	31	14
	T2	50	32	30	40	63	94	20	34
	T3	0	4	0	0	121	80.9	0	0
	T4	70	22	40	8	1585	>2420	20	12
	T5	20	44	0	2	86	51	0	2
	W1	260	C	300	75	7270	>2420	2460	326
	W2	660	C	800	C	4106	>2420	2014	1987
B	T1	0	0	0	0	0	0	0	0
	T2	10	1	0	0	0	3	0	0
	T3	0	0	0	0	0	0	0	0
	T4	0	0	0	0	0	0	0	0
	T5	0	22	0	0	0	3	0	0
	S1	0	0	0	0	0	1	0	0
	S2	0	0	0	0	0	0	0	0
C	T1	10	0	0	0	0	1	0	0
	W1	30	5	0	0	20	35	0	0
D	T1	110	18	0	0	10	91	0	0
	W1	30	30	200	5	594	462	72	29
E	T1	30	7	0	0	20	11	0	0
	SP1	0	0	0	0	0	0	0	0

Continued

Table 2.6 Comparison of membrane filtration and Colilert 18 for the analysis of groundwater

		Membrane Filtration				Colilert 18™			
		Original Sample Volume (ml)							
Site	Sample	10	100	10	100	10	100	10	100
		Total Coliforms (cfu / 100 ml)		*E. coli (cfu / 100 ml)*		*Total Coliforms (cfu / 100 ml)*		*E. coli (cfu / 100 ml)*	
F	T1	70	30	0	0	31	25	0	0
	W1	80	44	0	0	52	62	0	0
G	T1	10	11	20	4	20	11	10	5
	W1	150	50	80	74	464	687	31	14
H	T1	600	C	0	0	9804	>2420	0	0
	W1	900	C	0	0	2613	>2420	0	0
I	T1	10	2	0	0	10	10	0	0
	W1	90	28	0	2	296	173	0	1
J	T1	50	40	100	83	2359	1987	135	82
	W1	600	C	300	100	2595	>2420	160	96
K	T1	20	5	0	0	0	1	0	0
L	T1	40	20	0	0	52	30	0	0
	W1	460	C	0	0	1019	525	0	0
M	T1	0	0	0	0	0	0	0	0
	W1	0	2	0	0	0	5	0	0
N	T1	40	20	0	3	63	17	0	5
	W1	150	C	20	32	594	326	31	23

T = kitchen tap; W = well; S = storage tank; SP = spring; C = confluent sample

There are discrepancies observed between the 10 and 100 ml samples for both systems. One would expect these to be around the same order of magnitude

difference however they are often not. This may be due to a problem of sample homogeneity however Gale, Pitchers and Gray (2002) stated that 100 ml ‘spot’ samples reliably indicate the quantity of coliform bacteria in a water supply. It is likely that in the 100 ml sample there will be a greater number of heterotrophic bacteria than compared with the 10ml sample which has been made up to 100 ml with sterilised ¼ strength Ringers solution. The presence of non-coliform bacteria in the groundwater samples is likely to influence growth and survival of the coliform bacteria as there will be increased competition for nutrients and potentially predation by the natural heterotrophic bacteria or protozoa present in the groundwater.

The results show a large amount of variability between sites and more interestingly, between well and tap samples from the same site. Of the 37 samples taken only 6 had no coliforms or *E. coli* present when analysed by both methods at the two concentrations. Approximately 35% of the total samples contain *E. coli*. However, only 19% of the tap water samples contain E. *coli*. It is possible that the well had recently been contaminated and that due to the lag time this contamination had yet to reach the kitchen tap. However this seems unlikely as the majority of contaminated water samples had higher bacterial counts from the source compared to the tap and it is unlikely they had all been recently contaminated. Natural settling of sediments and bacteria in the well supply may account for the bacterial reduction. However, injury or death of the coliform bacteria due to predation or environmental stresses could also provide some explanation for the differences observed between the supply and the tap.

The variability between MF and Colilert 18™ for analysis of coliform bacteria and *E. coli* present in groundwater may be explained by the different detection capabilities of each method. MF relies on the ability of coliforms to produce acid from lactose and faecal coliforms to produce indole from tryptophan. However Colilert 18 ™ detects coliform bacteria and *E. coli* by their possession of the enzymes β-D-galactosidase and β-D-glucoronidase, respectively. Enzyme based methods have allowed the detection and quantification of a much broader range of environmental coliforms in comparison to the more historic standard methods (NHMRC 2003).

To further compare the two methods, the samples (10 and 100 ml) were grouped into the quantity of coliforms detected in 100 ml of samples. Figure 2.6 shows a comparison between the two methods and their ability to detect coliforms and *E. coli* in non-chlorinated groundwater samples.

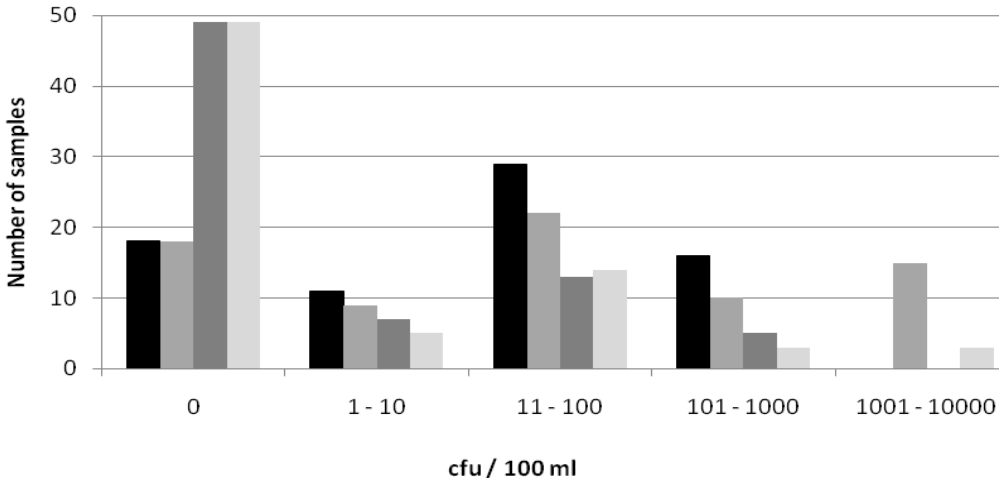


Figure 2.6 Comparison of membrane filtration and Colilert 18™ for the detection of coliforms and *E. coli* in groundwater

MF (TC); Colilert 18™ (TC);
MF (FC); Colilert 18™ (E. coli)

Colilert 18™ and MF are comparable at low quantities of bacteria but MF underestimates coliform numbers at high densities due to the limitations of growth on the membrane. Due to the design of the Colilert 18™ method, it has the ability to quantify large numbers of coliforms with a high degree of accuracy while continuing to successfully detect small numbers of coliform bacteria. This is supported by studies carried out by Buckalew et al. (2006), Katamay (1990), Edberg, Allen and Smith (1991) and Berger (1991).

A number of studies have found Colilert 18™ to be more accurate and sensitive than the ISO 9308-1 standard membrane filtration method for coliform detection maintaining that the ISO reference procedure fails to detect a significant proportion of coliforms and *E. coli* in drinking water (Bonadonna, Cataldo and Semproni (2007), Niemela, Lee and Fricker (2003), Eckner (1998), Fricker, Illingworth and Fricker (1997), Cowburn et al. (1994)). The ability of Colilert 18™ to assist the recovery of injured and stressed coliforms and *E. coli* in the samples is likely to be responsible for this (Hörman and Hänninen 2006).

Although in many of the groundwater samples, *E. coli* numbers are small, it does cause concern that *E. coli* is detected in drinking water at all. However, the aim of this study was to compare the ability of MF and Colilert 18™ to detect coliforms when using real samples. The presence of coliforms and *E. coli* in private water supplies will be investigated in Chapter 3, so will not be discussed in depth here. However, the fact that both methods have the ability to detect a single bacterium in a sample is probably the most important fact regarding the

monitoring of water quality and the reliability of both the MF and Colilert 18™ methods.

2.4.4 Isolation and characterisation of antibiotic resistant strains of *E. coli* and *E. aerogenes*

Antibiotic resistant strains of *E. coli* (resistant to Streptomycin 400 µg per ml / Rifampicin 80 µg per ml of nutrient agar) and *E. aerogenes* (resistant to Chloramphenicol 100 µg per ml / Erythromycin 200 µg per ml of nutrient agar) were compared to the original strains of *E. coli* and *E. aerogenes* (2.2.1) to determine if their behaviour was the same. The comparisons made of *E. aerogenes* and *E. coli* bacteria to their antibiotic resistant equivalents are given in Tables 2.7 and 2.8 respectively.

At these concentrations there was noted to be no growth of the non coliform bacteria used in the trial, therefore these concentrations were selective to the individual bacteria. There was also found to be no growth after inoculating it with a fresh soil sample solution prepared in ¼ strength Ringers solution. Further studies of the survival and transport of *E. coli* and *E. aerogenes* in soil and water was planned. It was concluded that antibiotic resistant strains could be easily detected in soil samples and be distinguished from any background counts of bacteria.

Table 2.7 Comparison of behaviour of *E. aerogenes* and antibiotic resistant *E. aerogenes*

	E. aerogenes	**Antibiotic resistant *E. aerogenes***
LPW 37°C	10 confirmed	10 confirmed
LPW 44°C	0 confirmed	0 confirmed
Tryptone	10 negative for *E. coli*	10 negative for *E. coli*
NA oxidase	20 negative	20 negative
McConkey	Large red colonies	small pink colonies
NA growth rate	good growth overnight	growth overnight
Visual appearance	creamy coloured round colonies	creamy coloured small round colonies
Colilert 18™	10 confirmed as coliforms but not as *E. coli*	10 confirmed as coliforms but not as *E. coli*

Table 2.8 Comparison of behaviour of *E. coli* and antibiotic resistant *E. coli*

	E. coli	**Antibiotic resistant *E. coli***
LPW 37°C	10 confirmed	10 confirmed
LPW 44°C	10 confirmed	10 confirmed
Tryptone	20 confirmed	20 confirmed
NA oxidase	20 negative	20 negative
McConkey	Large red colonies	small red colonies
NA growth rate	good growth overnight	growth overnight
Visual appearance	creamy coloured round colonies	creamy coloured round colonies
Colilert 18™	20 confirmed as coliforms and *E. coli*	20 confirmed as coliforms and *E. coli*

However it was found during experiments to determine survival of these bacteria that they behaved quite differently from the original isolates. They were much more resistant to low temperatures and grew exceptionally well even though the colonies were much smaller. Growth of the antibiotic resistant bacteria was often increased and the survival time was extended. It was decided to discontinue use of the antibiotic strains as their behaviour would be difficult to relate back to real samples and events.

2.5 Conclusion

Colilert 18™ proved to be a suitable method for water testing. It has the ability to detect coliforms and *E. coli* when bacterial counts are very low and can also quantify large numbers of coliforms and *E. coli* when present in a sample. In this study there was no evidence to suggest that Colilert 18™ detects false positives due to interference from *A. hydrophila* or *P. aeruginosa*.

The utilisation of Colilert 18™ for enumeration of coliform bacteria is further supported by the approval and inclusion of Colilert 18™ into the United Kingdom reference methods in The Microbiology of Drinking Water (SCA 2002). In the United States of America Colilert 18™ was given approval and included in the Standard methods for examination of water and wastewater (APHA, AWWA and AEF 2005). Colilert 18™ has also been introduced as a reference method in other countries including Germany, Italy and Denmark.

Colilert 18™ requires less time for preparation and analysis than the membrane filtration method with the advantage of faster results. Overall, the use of Colilert 18™ appears to be a good alternative to the membrane filtration method when used to quantify coliform bacteria in groundwater.

Chapter 3 Investigation of drinking water quality from private water supplies in Aberdeenshire, Scotland

3.1 Introduction

In the United Kingdom the majority of potable water is provided from a public mains supply, by the water utilities in England and Wales and Scottish Water in Scotland. However, throughout the UK there are also approximately 140,000 private water supplies providing potable water to many rural homes and communities, with 38,000 of these located in Scotland (DWI 1993). A private water supply is any water supply not provided by a water utility or Scottish Water.

Private water supplies can originate from a variety of sources. The water is often groundwater and is commonly abstracted from deep or shallow wells, boreholes or spring sources, but can also be from a loch or lake, stream or river.

In Scotland private water supplies are principally governed by the Private Water Supplies (Scotland) Regulations 2006 which came into force in July 2006. This transposes the European Drinking Water Directive (98/83/EC) which sets out specific quality standards which apply to all public and private water supplies intended for drinking, cooking, food preparation and other domestic purposes.

The 2006 Regulations place responsibilities on Local Authorities to regulate and enforce Private water supplies. Local authorities are required to maintain a register of every private water supply to premises in its area and to categorise each private water supply as either Type A or Type B, and to complete risk assessments of each supply.

Type A private water supply

This refers to a private water supply for human consumption purposes which meets one or more of these criteria:

- on average, provides 10 or more cubic metres of water per day
- serves 50 or more persons
- is supplied or used as part of a commercial or public activity. (Commercial or public activity includes food producers, hotels, holiday let accommodation, bed and breakfast establishments, village halls etc.)

Type A supplies are further classified by the maximum average daily volume of water provided during any period of maximum supply for human consumption purposes. This is shown in Table 3.1 along with the sampling frequency for coliform bacteria and *E. coli* which is dependent on the level of supply.

Table 3.1 Classification and sampling regime of Type A private water supplies (from the Private Water Supplies (Scotland) Regulations 2006)

Category of Type A supplies	Maximum average daily volume of water supplied for human consumption purposes in m^3/day	Sampling frequency for coliforms and *E. coli* (per annum)
Level 1	≤ 100	1
Level 2	> 100 – ≤ 1,000	4
Level 3	> 1,000	X[1]

[1]The sampling frequency (X) shall be determined as X = 4 + (3 for each 1,000 m^3/d and part thereof of the total volume).

Type B private water supply

Type B private water supplies are domestic supplies providing less than 10 m^3 of water per day, or supply less than 50 people. Risk assessments are not required to be carried out however Local Authorities have discretionary powers to complete a risk assessment and to sample and monitor water quality on these supplies if concerns are raised. They must also provide owners / users with advice and assistance if required. The water supplies are still subject to nationally set quality parameters.

Prior to June 2006 private water supplies in Scotland were regulated under the Private Water Supplies (Scotland) Regulations 1992. Private water supplies were grouped into two categories. Category 1 supplies were purely used for domestic purposes. Category 2 supplies provided water to be used in commercial food production or to premises with changing populations for

example, hotels or camp sites. Each of these categories was further sub divided into classes depending on the number of people or the volume of water supplied as shown in Table 3.2.

Table 3.2 Classification of private water supplies (from the Private Water Supplies (Scotland) Regulations 1992)

Category/Class	Number of Persons Supplied	Consumption (m^3d^{-1})	Sampling Frequency for coliforms (per annum)
One/A	> 5000	> 1000	24
One/B	501 –5000	101 – 1000	12
One/C	101 – 500	21 – 100	2
One/D	25 – 100	5 – 20	1
One/E	<25	<5	0.2[1]
One/F	1 dwelling	n/a	n/a
Two/1	n/a	>1000	24
Two/2	n/a	101 – 1000	12
Two/3	n/a	21 – 100	6
Two/4	n/a	2 – 20	2
Two/5	n/a	<2	1

[1] From 1992 – January 1995 samples were required to be taken annually, from January 1995 the sampling period changed to once every five years (i.e. 0.2 samples per annum)
n/a – not applicable

As is presently the case, the local authorities had a duty to monitor and regulate private water supplies under the Water (Scotland) Act 1980.They were also responsible for classifying supplies relevant to their use and the number of people served by each supply.

Under the new legislation, in most cases less water samples will be analysed for the presence of coliform bacteria. However, Type A supplies will now be better regulated in that risk assessments of these supplies together with a

minimum of annual sampling will be carried out. Although Type B supplies are still regulated under provisions of the Water (Scotland) Act 1980 (as amended), a high proportion of the rural population was and still is consuming water of unknown quality.

Private water supplies within the UK have been linked to a number of outbreaks of infection, primarily with *Campylobacter* being the causative organism but there have also been cases linked to *Cryptosporidium*, *Giardia* and *E. coli* (Furtado et al. 1998; Galbraith, Barrett and Stanwell-Smith 1987). Between 1937 and 1986, Galbraith, Barrett and Stanwell-Smith (1987) detailed 13 outbreaks of illness related to private water supplies which resulted in over 1904 individual cases. Over half of these cases were between 1977 and 1986. Shepherd and Wyn-Jones (1997) identified 31.7% of waterborne disease outbreaks to have an association with private water supplies with a further 37.2% of disease outbreaks being suspected to have an association with private water supplies.

Agricultural practices such as storage of manure, spraying of slurry and livestock in the area surrounding a water supply have the potential to contaminate water supplies. Septic tanks in the vicinity of supplies are also a possible source of contamination (Goss, Barry and Rudolph 1998; Rudolph, Barry and Goss 1998). There are also factors which may have an impact on the level of contamination of a water supply such as temperature and rainfall (Howard et al. 2003; Petrie et al. 1994; Rodgers et al. 2003) and the type of water supply, its depth and the level of repair and maintenance it receives.

The incidence and severity of contamination of private water supplies in Aberdeenshire was investigated and causes of contamination were considered for individual sites. This study was undertaken prior to the updated regulation for private water supplies so supplies were classified according to The Private Water Supplies (Scotland) Regulations 1992.

3.2 Material and methods

3.2.1 Aberdeenshire council water quality data from private water supplies in Central Division, Aberdeenshire

Of the Scottish local authorities, Aberdeenshire has the highest percent of population relying on private water supplies for their drinking water. This accounts for around 11,000 properties and over 25, 000 people (Reid et al. 2003; Reid et al. 1999).

Aberdeenshire Council is made up of three distinct divisions which are referred to as North, Central and South Divisions. Prior to 1996 these areas were referred to as the District Councils of Banff and Buchan, Gordon, and Kincardine and Deeside respectively. Aberdeenshire Council has a duty under the Water (Scotland) Act 1980 to check the quality of private water supplies in their area. They are also responsible for classifying supplies relevant to its use and the number of people served by each supply. Samples were taken as part of routine monitoring with regards to the regulations for the type of supply.

Some samples would have been requested from homeowners if for example there was concern over the water quality due to illness or often for selling homes. However there was no statutory requirement on the local authority to monitor water quality from category 1F supplies.

The primary focus for this study was the data for the Central division of Aberdeenshire council. It contained the most complete record of supplies and properties in comparison to the other divisions. Central division contains over half of the approximate 11,000 properties relying on private water supplies in Aberdeenshire (Table 3.3). Records held by Aberdeenshire Council, Central division, of monitoring and water quality data were attained. Data were available from 1992 to 1998. All data were entered into a Microsoft Access database and collated to determine the extent of the sampling and analysis carried out.

Table 3.3 Details of private water supplies in Central Division, Aberdeenshire

	Properties	Supplies
Category one		
Class A	0	0
Class B	0	0
Class C	87	1
Class D	278	28
Class E	1280	447
Class F	3793	3793
Category two		
Class 1	0	0
Class 2	31	1
Class 3	127	8
Class 4	183	30
Class 5	41	21

The data were sorted and multiple entries of the same property were removed by only taking the first entry on the register within the statutory sampling period. Multiple entries can occur when a sample has failed and a repeat sample taken to monitor the effectiveness of remedial action. Although no testing of Category one F supplies were required by statute, multiple entries of data for these supplies were treated using the same approach as Category one E supplies.

Microbiological analyses of drinking water prior to 1994 were obtained in accordance with the methods described in DoE/DH/PHLS (Anon 1983). Subsequent samples (i.e. post 1994) were obtained in agreement with the methods described by the Report on Public Health and Medical Subjects No 71 (Anon 1994).

3.2.2 Random data set of Category 1F private water supplies in Central Division, Aberdeenshire

In Central Aberdeenshire 300 Category 1F supplies were randomly selected from the Central Division water quality database to participate in a study on microbiological quality of the water supply. Homeowners were given information on the study and asked if they would participate. This resulted in 82 supplies taking part in the monitoring programme. The study also included a questionnaire regarding the private water supply such as type and depth of supply, water storage facilities, water treatment and the surrounding land use. An example of this questionnaire is provided in Appendix 1.

The monitoring programme consisted of 3 samples from each supply, taken over the period of one year. As well as gaining more information on water quality, this sampling programme hoped to reflect changes in quality due to seasonal variation and rainfall. Following investigation of these supplies it was noted that thirty of them provided water for more than one property, therefore were not category 1F supplies. However, they were still included in the survey. Due to a change of circumstance for a number of residents, such as commencing employment or moving home, access could not be gained to a number of properties resulting in only 64 of the 82 supplies being sampled on all three occasions. Microbiological analyses of water taken from the random data sets were carried out using membrane filtration as described by the Report on Public Health and Medical Subjects No 71 (Anon 1994).

3.2.3 Short term intensive sampling of nine 1F private water supplies

Of the 82 supplies monitored in the Random Data Set, 9 category 1F supplies were selected to undergo further monitoring. The supplies were chosen due to their high incidence of microbiological contamination. They were also logistically suitable, had easy access to both the water supply and the kitchen tap, and the homeowner was willing to continue with the study. The supplies were studied over a 6 week period and were sampled on a minimum of 3 occasions. At this time samples were taken from both the water supply and the kitchen tap.

Coliform counts for the short term intensive study were carried out using the Colilert 18™ method as described in section 2.2.7. This method was selected following the trial which indicated its suitability as an alternative to the more time-consuming membrane filtration.

3.2.4 Longitudinal study of two private water supplies

Over a two year period from January 1998 to November 1999, two supplies in rural Aberdeenshire were monitored regularly to determine microbiological water quality changes with an aim to determine rainfall and seasonal impacts and the effect surrounding land use has on their water quality. The supplies were both Category 1F, one of the supplies being a shallow well, the other a deep borehole.

Initially the water samples were analysed using membrane filtration in accordance with the Report on Public Health and Medical Subjects No 71 (Anon 1994). Microbiological analysis of water samples from August 1999 were carried out using Colilert 18™.

3.3 Results and discussion

3.3.1 Aberdeenshire council water quality data from private water supplies in Central Division, Aberdeenshire

Central Division of Aberdeenshire council has the legal responsibility to monitor over 4000 private water supplies which provide water to over 5800 homes. Over the 7 year sampling period central division were legally bound to collect and analyse almost 3000 samples. It is recognised that there is a huge demand on central division of Aberdeenshire council to monitor this number of supplies, many of which are in remote areas. Central division's remit would also include follow up samples for supplies that have failed and requests for sampling of Category 1F supplies by homeowners. Table 3.4 summarises the sampling of private water supplies between 1992 and 1998 in Central division of Aberdeenshire.

Table 3.4 Sampling compliance and supply fails of private water supplies in Central division, Aberdeenshire

Category / Class	Expected number of samples	Actual number of samples	Compliance (%)	Supplies never sampled (%)	Total coliform fails (%)	Faecal coliform fails (%)
One A	n/a	-	-	-	-	-
One B	n/a	-	-	-	-	-
One C	14	8	57	0	12.5	12.5
One D	196	57	29	14	37	28
One E	1788	360	20	35	42	31
One F	-	476	n/a	n/a	37	26
Two 1	n/a	-	-	-	-	-
Two 2	84	20	24	0	25	25
Two 3	336	94	28	25	36	27
Two 4	420	116	28	20	33	22
Two 5	147	13	9	76	46	31

Of the 1144 samples taken from kitchen taps, total coliforms were found in 38%, and faecal coliforms found in 27% of samples. Overall there was only a 22% compliance of required sampling and analysis of private water supplies in central division, with compliance being as low as 9% for the category Two, class 5 supplies. This is of particular concern as it had the highest failure rate for total coliforms, and one of the highest for faecal coliforms within the supply groups. It is also interesting to note that 34.3 % of supplies covered by the legislation have never been sampled during the 7 years of the study, again with the majority of these being in Category Two, class 5. It is possible then, that the number of supplies failing due to the presence of coliform bacteria including *E. coli* could in fact be much higher than indicated.

Samples taken from kitchen taps for microbiological analysis were recorded by the month each sample was taken (Figure 3.1). Samples failing on the presence of total coliform and faecal coliform bacteria were shown as a percent of the overall samples taken.

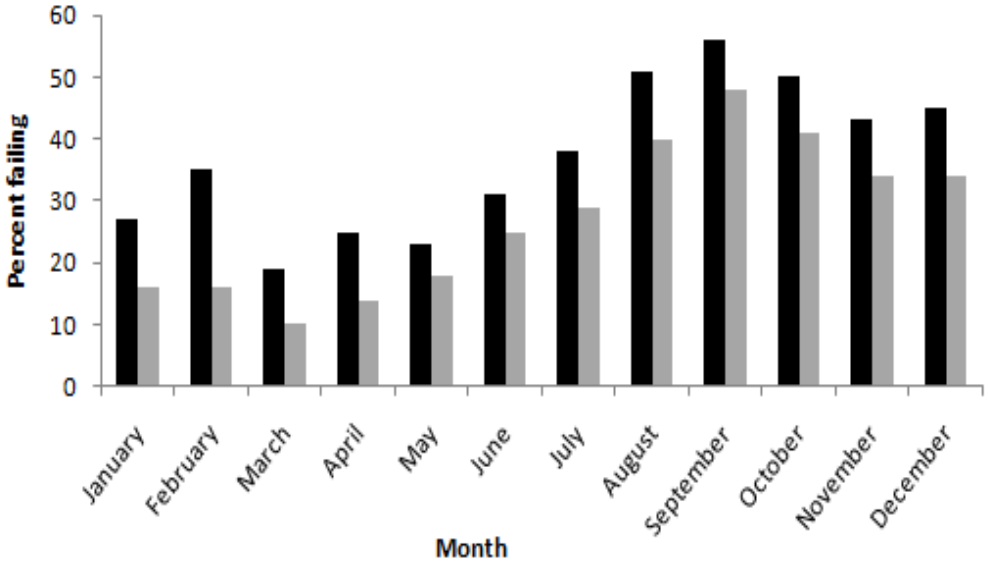


Figure 3.1 Seasonal trend for samples from Aberdeenshire Central Division collected from 1992 to 1998 and grouped according to the month of sampling; TC fails (■) FC fails (■)

Water quality was most likely to fail the statutory limits for coliforms, both total and faecal, during late summer and autumn, with the highest percentage of fails being found in September. Findings are similar to those documented by Rutter et al. (2000) who compiled statutory water test results from nine Public Health Laboratories in England. Although Rutter et al. (2000) found the highest percentage of fails for *E. coli* to be during November, there was also a small increase in February along with lowest number of fails being recorded during March and April. Perhaps the slight increase in fails in February for central Aberdeenshire and in the study by Rutter et al. (2000) can be explained by an increase in surface runoff as snow melts.

3.3.2 Random data set of Category 1F private water supplies in Central Division, Aberdeenshire

Microbiological analysis was carried out on 220 samples of which 71% tested positive for total coliforms and 29% contained faecal coliforms. In 88% of the cases a supply failed on at least one occasion for total coliforms and 52% of supplies failed at least once for faecal coliforms. Only 11% of supplies that had analysis carried out on three occasions had no coliforms present. The microbiological analysis was grouped into months to determine seasonal trends (Figure 3.2).

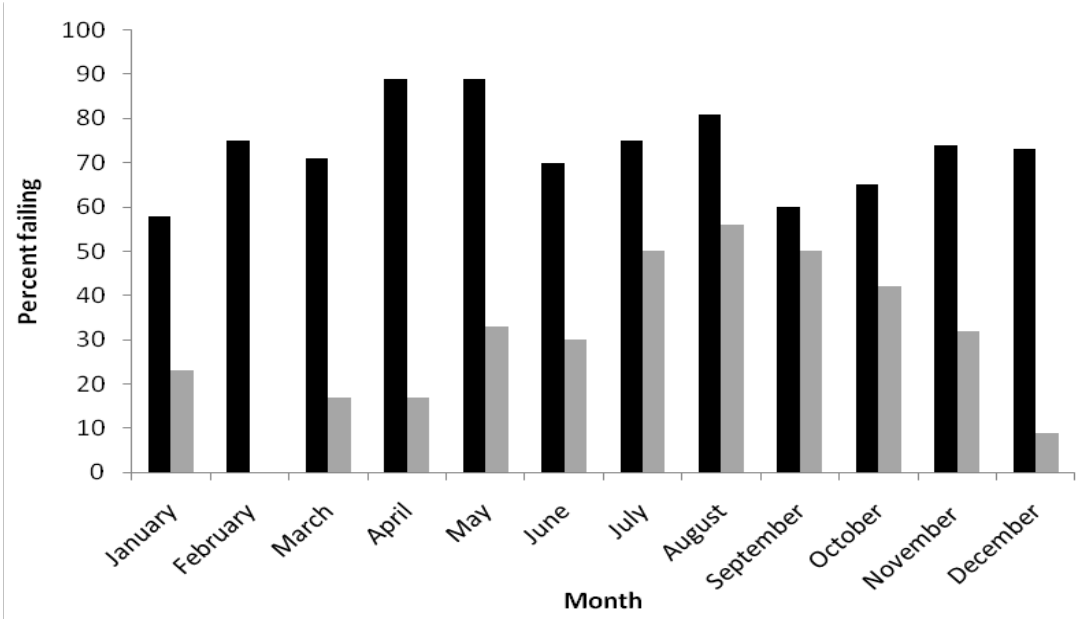


Figure 3.2 Random samples from Central Division, Aberdeenshire, showing fails per month as a percentage of the total samples taken, TC fails (■), FC fails (■)

Unlike the Aberdeenshire council seasonal trend, the majority of FC fails for the random samples are during the summer months and into early autumn decreasing during the winter months and with no faecal coliforms detected during February. The results found were in agreement with Conboy and Goss (1999), who carried out sampling in Ontario, Canada. Although sampling was only carried out in spring and late summer they also found that faecal coliform results were higher during late summer.

The types of supplies were investigated regarding the number of samples failing and the presence of faecal coliforms indicating faecal contamination (Table 3.5).

Table 3.5 Association of supply type with samples failing on the presence of coliforms

	Spring	Well	Borehole
Number of Supplies	41	39	2
Total number of samples taken	110	104	6
Samples with total coliforms (%)	72	74	17
Samples with faecal coliforms (%)	34	27	0

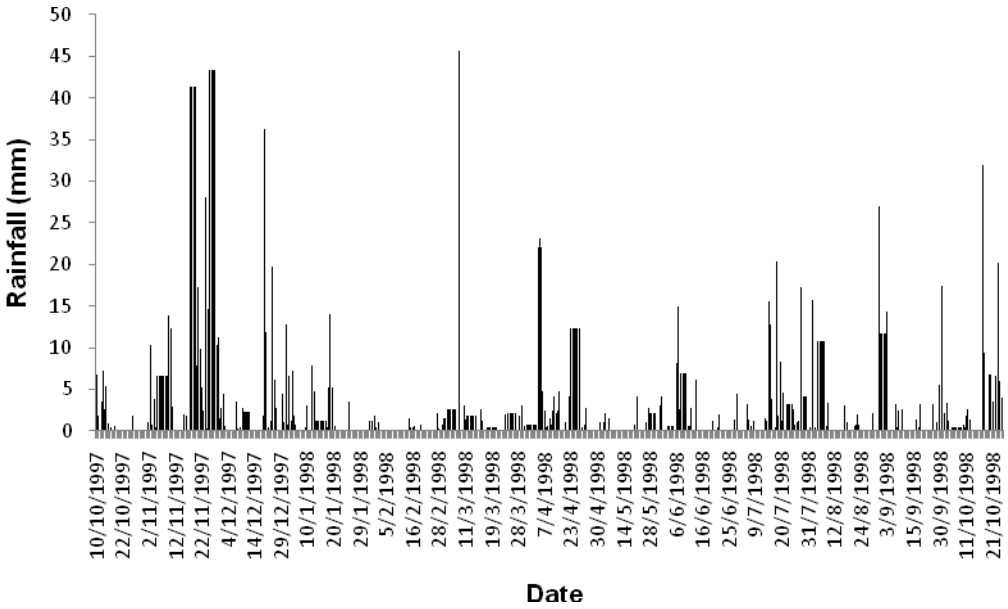
This study may not give a true representation of quality of borehole supplies due to the small number of samples, however it does show a good comparison of the spring and well supplies with springs more likely to have faecal contamination. This is consistent with a study by Rutter et al. (2000) who found that boreholes were the least likely to be contaminated, with springs then wells being the most susceptible to contamination. Fewtrell, Kay and Godfree (1998) carried out a similar study of 91 private supplies and also found that boreholes

were the least likely to be contaminated, with 64% of spring supplies contaminated and 100% of well supplies. It should be noted however that only three well supplies were examined compared to 28 springs and 56 boreholes.

There are a number of studies which investigate the impact rainfall has on the contamination of private water supplies. Petrie et al. (1994) showed that there was a rapid increase in the contamination of spring supplies following rainfall with the lag time being between 1 to 3 days, the effect of rainfall diminishing with time. During dry spells there was no faecal contamination. Howard et al. (2003) also showed a strong correlation of spring water contamination by coliform bacteria (TC and FC) to rainfall in Kampala, Uganda.

In this present study of 82 private water supplies in central Aberdeenshire, coliform counts in water samples were compared to rainfall events from October 1997 to October 1998 (Figure 3.3).

Rainfall was recorded on more than 60% of the 385 days over which the study was carried out. From figure 3.3 (b) it can be seen that there is an increase in the total coliforms and more likely to be faecal coliforms detected following a period of high rainfall. It is likely that due to the frequency of rain in Aberdeenshire there is often contamination of private water supplies which gives low levels of coliforms in many samples.



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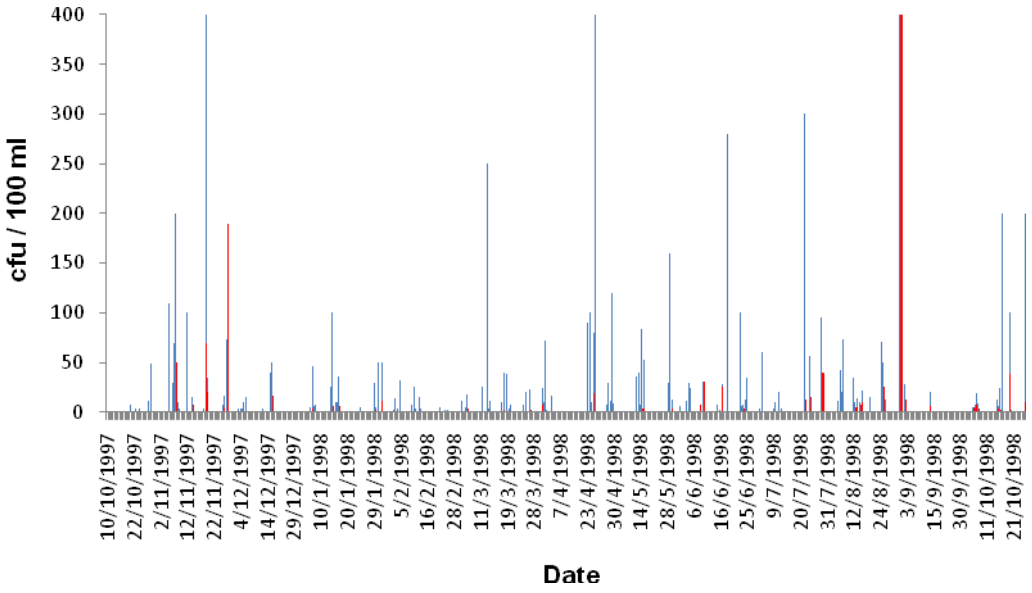


(b)

Figure 3.3 Association of rainfall (a), with the presence of coliform bacteria (b), in samples from 82 private water supplies in central Aberdeenshire (Rainfall (–), TC (–), FC (–))

One of the most severe drinking water contamination events occurred in Walkerton, Ontario, Canada, where 2300 people were affected with

gastroenteritis, resulting in 27 people developing haemolytic uraemic syndrome (HUS), a potentially fatal kidney ailment, and the death of 7 people. *E. coli* O157 and *Campylobacter jejuni* were held responsible. The subsequent inquiry highlighted that the most likely contamination was from manure entering a shallow well following a period of heavy spring rainfall. Heavy rainfall or heavy-snow melt was also directly implicated in other waterborne infection outbreaks in Canada and the USA (Hrudey et al. 2003).

The properties from which samples were taken were also investigated with reference to the storage of water prior to reaching the kitchen tap. Storage type, if any, can vary and is generally either an external reservoir for the water or a header tank within the property. Properties without storage have either a pumping system for the water on demand or are gravity fed directly from the water source. In this study results for storage revealed a similar proportion of contamination for all properties suggesting that storage has little influence on the degree of contamination. However although samples were examined quantitatively there was no comparison made of the level of contamination found at the water source and the kitchen tap.

3.3.3 Short term intensive sampling of nine 1F private water supplies

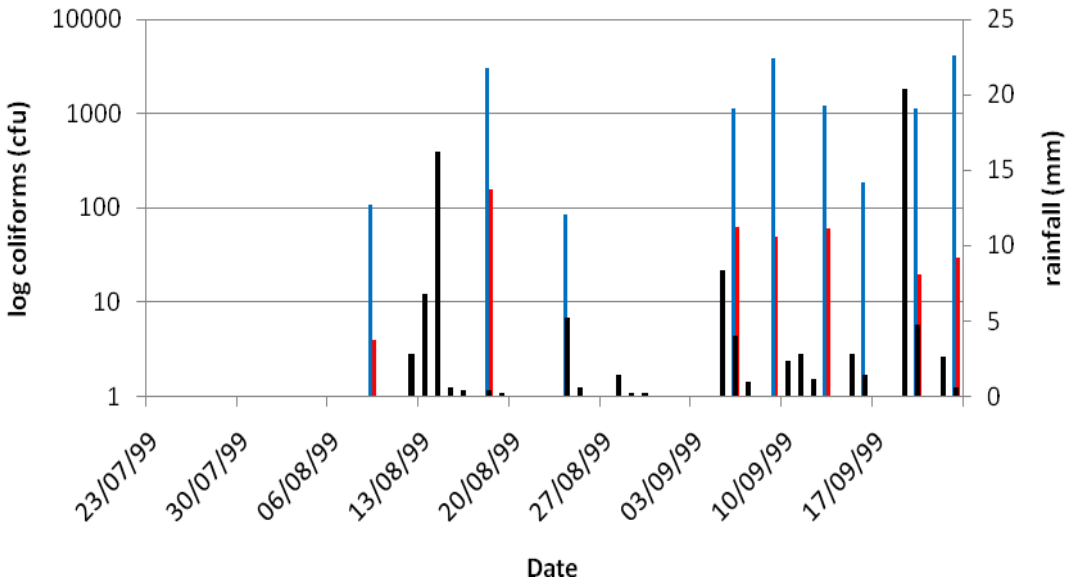
Samples were taken from the well and from the kitchen tap of 9 Category 1F supplies (Table 3.6). Of the well samples 97% contained coliform bacteria (TC) while 65% contained faecal coliforms (FC). The kitchen tap samples were lower

with 82% containing TC and 38% containing FC. It must be recognised that the supplies taking part in the short term intensive study were chosen due to the likelihood that they would fail therefore the failure rates for coliform bacteria although high are not unexpected. What is more surprising is the actual number of coliform bacteria including faecal coliforms that were present in samples.

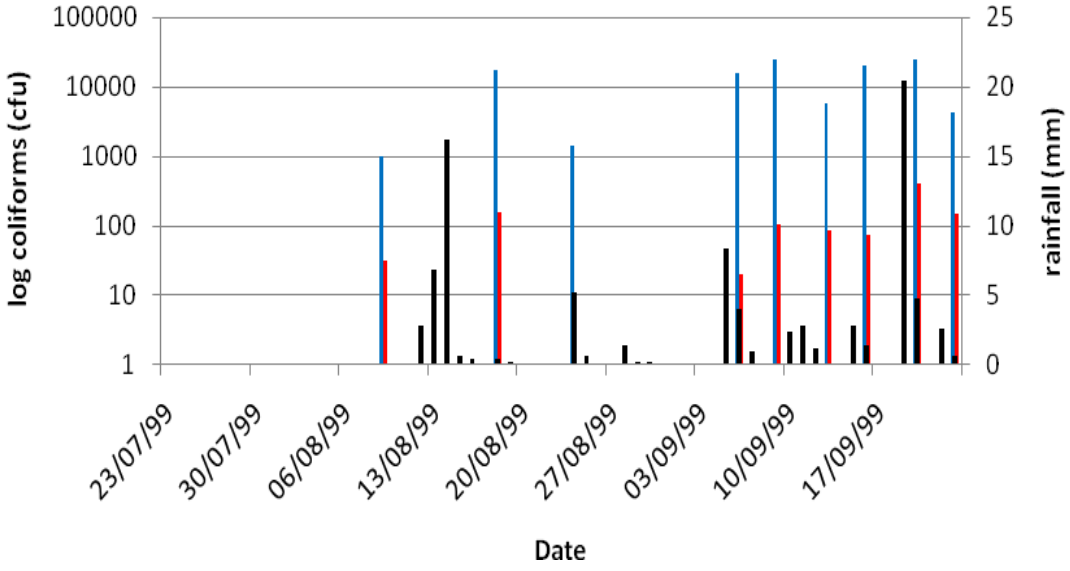
Table 3.6 Summary of samples taken from 1F supplies investigating well and kitchen tap water samples

	Kitchen samples			Well samples		
Private Water Supply	No of samples	TC fail	FC fail	No of samples	TC fail	FC fail
1	3	1	0	3	3	2
2	4	4	2	4	3	2
3	4	4	2	4	4	3
4	4	3	0	4	4	1
5	4	4	4	4	4	4
6	4	2	0	4	4	2
7	4	4	1	4	4	2
8	3	2	0	3	3	2
9	4	4	4	4	4	4

The well and kitchen tap samples were compared and the association with rainfall was investigated. It was found that in many cases there is a tenfold decrease in coliform counts at the kitchen tap compared to the well as indicated in Figure 3.4.



(a)



(b)

Figure 3.4 **Association of rainfall with coliform bacteria detected in kitchen tap samples (a) and well samples (b) taken from nine private water supplies in central Aberdeenshire. Rainfall for one week prior to sampling is shown.**

(■) TC, (■) FC, (▬) rainfall

There will be some lag time before the sample from the well reaches the kitchen tap. This is especially true for supplies that have an external storage tank and/ or a header tank. There are a number of studies that indicate the reduction of coliform bacteria following storage. Pope et al. (2003) found that a reduction in *E. coli* was more likely as storage time increased. Temperature was also seen to influence *E. coli* counts with temperatures above 10˚C having a greater impact in reduction than temperatures below 10˚C.

The primary concern for these 9 supplies is the source of the contamination and how it enters the private water supply. Many of the wells are contaminated even when there is little or no rainfall. To try to explain the frequency and extent of contamination of the private water supplies, the appearance and the individual features of each supply as recorded in Table 3.7 was examined.

Table 3.7 Well data of private water supplies

Supply	Type	Date built	Type of construction	Height of lip (m)	Depth of supply (m)	Depth of water (m)	External reservoir	Header tank	Land use in immediate vicinity	Source Fenced	Filter	Distance to streams in vicinity (m)
1	Well	1968	concrete rings	0.44	1.87	1.00	N	Y	woodland / bog	N	N	5
2	Spring	1970's	concrete rings	0.18	1.68	0.80	Y	Y	pasture land	N	N	2
3	Well	1975	concrete rings	0.14	3.74	1.22	N	Y	pasture land (cows)	Y	Y mesh	30
4	Well	1978	concrete rings	0.30	3.60	1.40	N	Y	crops / pasture land	N	Y gauze	n/a
5	Well	Not known	stone (glazed clay)	0.12	0.95	0.82	N	Y	moor / heath / woodland	N	Y mesh	1
6	Well	1970's	stone (granite)	0.09	7.15	2.55	N	Y	woodland / grass	N	N	n/a
7	Well	1970's	concrete rings	0.62	5.33	2.55	N	N (gravity fed)	crops	Y	N	n/a
8	Well	1970's	stone	0.47	5.50	2.2	N	Y	garden / woodland	N	Y mesh	30
9	Well	1970's	concrete rings	0.31	2.77	1.85	N	Y	pasture land	N	Y mesh	n/a (flooding in immediate vicinity

PWS 1

This well is 40 years old, receives no maintenance and has no filter in place

The well is shallow at 1.87 m deep, with water of 1 m depth. There is a reasonable depth of lip to the well, probably preventing surface water flow from directly entering the well. It is not fenced off or stock proof and although not surrounded by grazing animals, wildlife is likely to be in the vicinity of the well. The low levels of contamination may be due to preferential flow of surface water, entering the well through cracks.

Figure 3.5 Private water supply 1

PWS 2

This spring supply is very shallow at 1.68 m, with a river nearby at a distance of 2 m. In general the spring has low or no levels of pollution except following a heavy rainfall. It is interesting to note that on one occasion the tap sample is more

contaminated than the spring. It is possible that the external reservoir is more prone to contamination which is then taken to the tap supply without ever entering the spring. (No photo available of this supply)

PWS 3

This well consistently had high levels of contamination and often with faecal coliforms present. With reference to figure 3.6, the well is on the right covered with grass and moss. The chamber on the left is the pump. The well although of a reasonable depth at 3.74 m, has a very small lip. Cattle frequently graze in this field so it is likely the faecal material is washed into the well between the top of the lip and the lid.

Figure 3.6 Private water supply 3

PWS 4

This supply had low levels of coliform bacteria present but only one episode of low levels of faecal coliforms which occurred after heavy rainfall. The source is relatively deep at 3.6 m with the lip of the well slightly above ground preventing surface water flow directly into well

Figure 3.7 Private water supply 4

PWS 5

High levels of faecal contamination were found in this well and at the kitchen tap. It is extremely shallow at only 0.95 m deep, with water filling approximately 0.8 m of this. There is a stream at 1m distance from the well. It is most likely this well is severely contaminated by surface water and stream water. A mesh filter is in place

but it is likely that cleaning and maintenance of the filter is not carried out. (No photo available of this supply).

PWS 6

There were no faecal coliforms detected in any tap samples from this supply however faecal coliforms were detected in two of the well samples, both following heavy rainfall. For a well supply the source is very deep (7.15 m), with water only present to a depth of 4.6 m.

Figure 3.8 Private water supply 6

Although the source is found within the garden area of the house, and not surrounded by grazing animals, there is still potential for contamination of the

supply. Contamination following heavy rainfall is possible as the lip of the well only sits at 0.09 m above the ground.

PWS 7

This well supply had high coliform numbers at the source and at the kitchen tap but faecal contamination only following heavy rainfall. The kitchen tap is gravity fed from the source having no header tank and no filter present.

Figure 3.9 Private water supply 7

Although the source has a fence surrounding it, it offers very little protection from wildlife. Therefore the potential for faecal contamination to enter the water supply from faecal deposits on the soil or near the well is possible.

PWS 8

The wooden lid on the top of this water source is in need of repair and offers virtually no protection to the water supply. Although there is some protection from rainfall entering the well due to the peaked roof, there is no protection from small animals and birds.

Figure 3.10 Private water supply 8

Faecal contamination was only detected at the well. This supply does have a mesh filter in place which may be the reason that no faecal coliforms were found in the kitchen tap samples.

PWS 9

This supply was always heavily contaminated with coliform bacteria including faecal coliforms. The depth to water is less than 1 metre, although the depth of the well is 2.77 m. There was evidence of flooding at the source site and the lid is of a split design which makes the well more prone to contamination. The site is used for grazing animals so faecal deposits are likely. This supply has a mesh filter in situ which appears to offer no protection from bacterial contamination.

Figure 3.11 Private water supply 9

Evidence suggests that contamination of many of these private water supplies is due to lack of protection from grazing animals and wildlife, and poorly designed and maintained wells. The supplies are all around forty years old with a number of them likely to be receiving contaminated surface water especially following periods

of heavy rainfall. A number of the supplies have a physical filter in place however it is clear to see that unless regular maintenance of the filter is carried out, the filter offers very little protection to the potable water supply from the kitchen tap. The lack of maintenance and protection found with these supplies is not unusual. A study carried out in upstate New York by Schwartz et al. (1998) observed similar findings, with poor maintenance and contamination of the water supply being associated with a lack of education with regards to water quality.

3.3.4 Longitudinal study of two private water supplies

Over an approximate two year period, two private water supplies were investigated with regards to the presence of coliform bacteria and faecal coliforms detected in the kitchen tap sample (potable water). Laterally the study examined the presence of coliforms at the source.

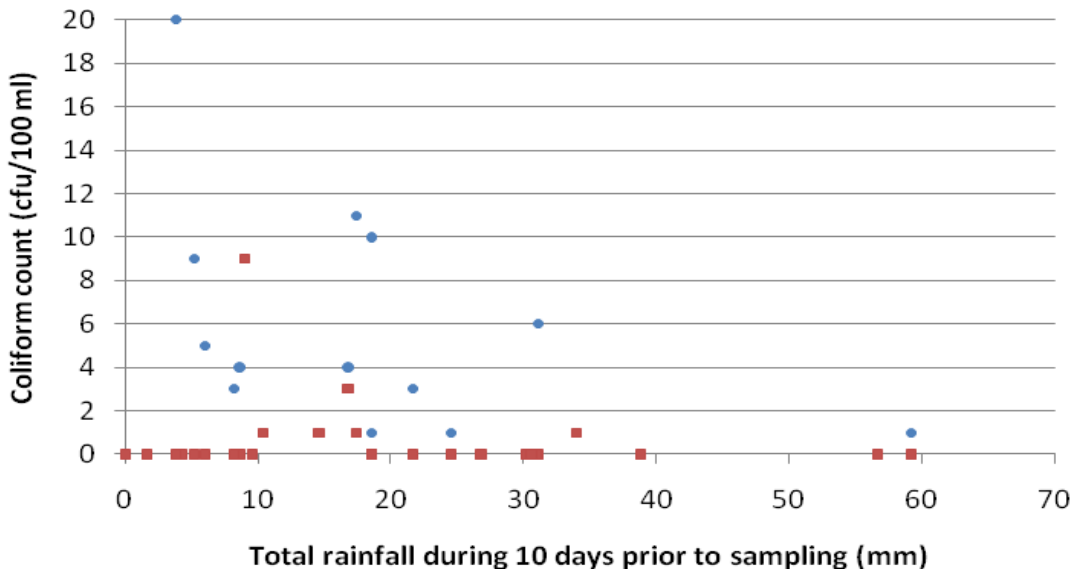
3.3.4.1 Longitudinal study of a borehole private water supply

The first supply is a borehole, approximately 33 m deep, which supplies water direct to the kitchen tap; there is also a storage tank in the attic which supplies the bathroom taps. The source is surrounded by grassland and sheep are evident near

the source and at times on the source lid itself. This water supply does not have any form of treatment or filter nor does it receive regular maintenance. Sampling commenced in June 1998 and finished in November 1999.

Figure 3.12 Borehole supply used for longitudinal study

The borehole supply was sampled at the kitchen tap on 32 occasions. Twenty four of these samples were positive for coliforms while 11 were positive for faecal coliforms. October had the highest percentage fails for faecal coliforms. Results were plotted against total rainfall for the 5 days prior to sampling to examine any links between fails and rainfall. These results are shown in Figure 3.13.



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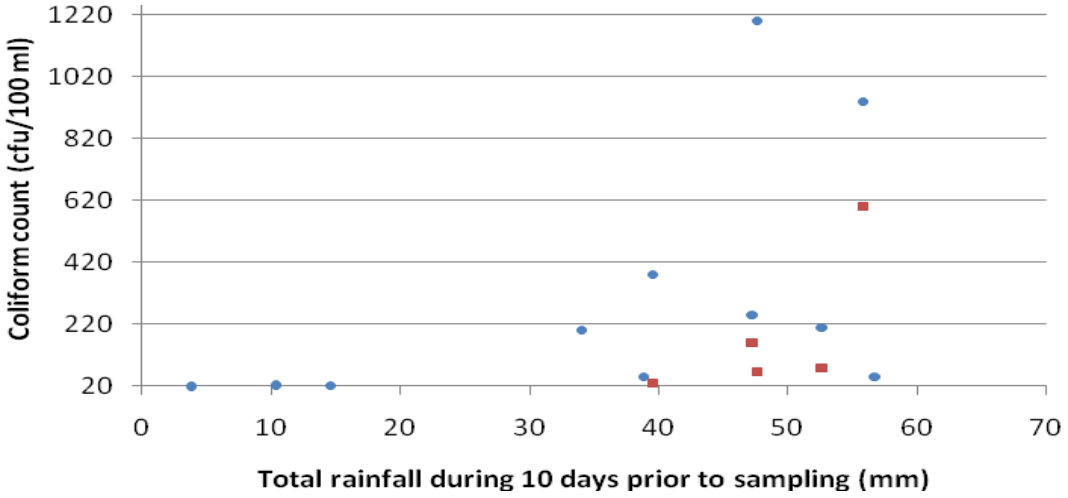


(b)

Figure 3.13 Borehole supply: the relationship between rainfall over ten days prior to sampling and number of coliform bacteria

(a) Less than 20 cfu/100 ml (b) Greater than 20 cfu/100 ml

TC (●), FC (■)

The presence of high levels of coliform bacteria and in particular *E. coli*, in the tap samples indicate that contamination may be directly linked to the level of rainfall over a 10 day period prior to sampling. High coliform counts (> 20 cfu/100 ml) were only found when rainfall over the 10 day period exceeded 38 mm. When rainfall was less than this faecal coliforms were often not detected or were present at very low levels. However two samples taken following rainfall exceeding 55 mm during the preceding 10 day period had no faecal coliforms present. One of these samples was taken in March, the other in July. There is no available information detailing the land use surrounding the borehole during these times. It is possible that there were no animals grazing in the vicinity or weather conditions may have been detrimental to coliform survival.

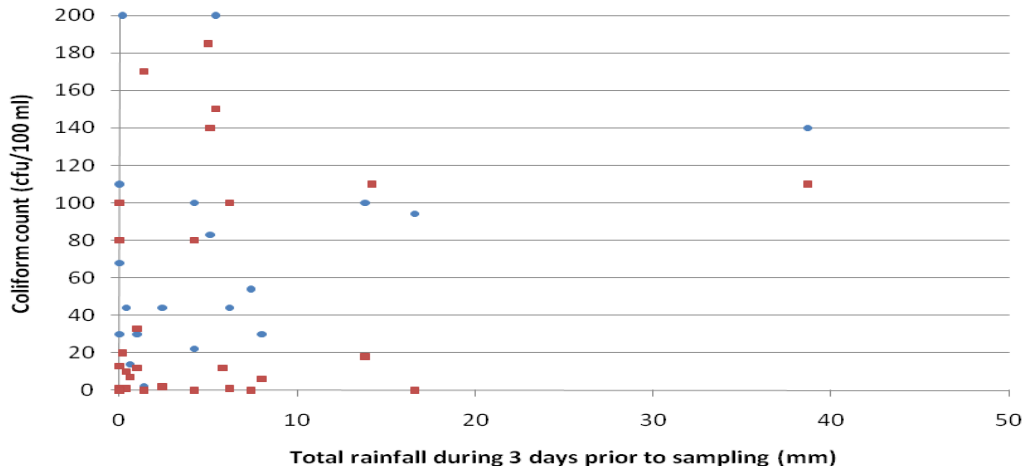
There is always the possibility that the groundwater abstracted from the borehole is contaminated at the source. Further investigation would be required to determine where the source of the groundwater originates and if there is potential for contamination. However Glanville, Baker and Newman (1997) believe that contamination of deep wells by coliform bacteria is likely to be caused by faulty casings allowing preferential flow of water to reach the supply without undergoing any natural filtration by soil. It is possible that this would also be the case for this borehole and indeed any borehole supply.

3.3.4.2 Longitudinal study of a well private water supply

The second supply is a well similar to many of those in the short intensive study. It is 3.76 m deep with over 2.6 m depth of water. It is made of concrete rings and has a 0.12 m lip. The source is fenced and stock proof however the ground surrounding the well is soft and a stream is evident at approximately 3 m distance. There is woodland surrounding the well. After 10 m there is pastureland and crops. The well supplies a header tank prior to reaching the kitchen tap. The water from this supply does not receive any form of treatment, it does not have a filter and it does not receive regular maintenance. Sampling commenced in June 1997 and finished in September 1999.

Figure 3.14 Well supply used for longitudinal study

The well supply was sampled at the kitchen tap on 38 occasions. All of the samples were positive for coliforms while 32 were positive for faecal coliforms. Results were plotted against total rainfall for the 3 days prior to sampling to examine any links between fails and rainfall (Figure 3.15).



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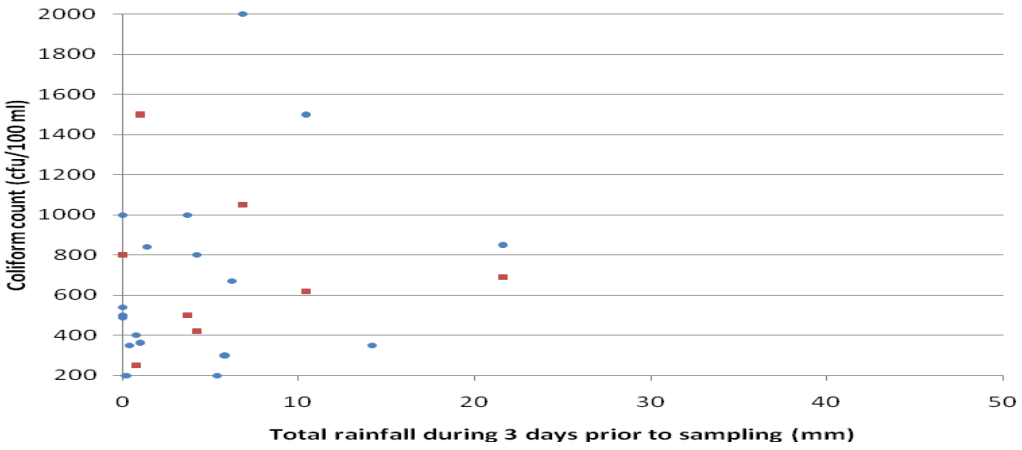

(b)

Figure 3.15 Well supply: the relationship between rainfall over three days prior to sampling and number of coliform bacteria (a) 0 – 200 cfu/100 ml (b) Greater than 200 cfu/100 ml
TC (●), FC (■)

Coliform bacteria were always detected in the well supply and faecal coliforms were frequently detected. The number of coliform bacteria detected was often exceptionally high. There appears to be some correlation to rainfall during the 3 days prior to sampling although even when there has been no rainfall for 3 days, coliform numbers can still be high. On a number of occasions coliforms were detected at counts exceeding 200 cfu per 100 ml and on one occasion coliform and *E. coli* counts were detected as high as 1000 and 800 cfu per 100 ml of sample respectively. There is potential for contamination from the nearby stream which seems likely taking into consideration the softness of the ground surrounding the well. This may help to account for the persistent contamination and the high counts of faecal coliforms in the water samples.

The results obtained from sampling of the well and borehole supplies showed very different levels of contamination at each supply. Faecal coliform contamination from the borehole was generally detected at levels far below 200 cfu, however the well supply was regularly found to have faecal coliform counts in excess of 200 cfu The sampling data obtained is supported by the findings of Goss, Barry and Rudolph (1998) who showed that deeper wells are less susceptible to contamination and have lower levels of contamination than shallow wells.

3.4 Conclusion

The evidence obtained to date confirms that a high proportion of private water supplies do not meet statutory requirements for water quality. It appears that smaller supplies, often those not closely regulated under legislation are most likely to have questionable water quality.

There are a number of guidance papers giving advice on how to best protect a water supply from contamination however it is ultimately the responsibility of the homeowner or tenant to maintain the water supply. The importance of protecting private water supplies from contamination by surface water runoff is indicated. The water supply should be constructed with non-porous material and the lip must be situated well above ground level. The lid should be watertight and have a good seal. It is very important to protect the water supply from livestock and wildlife. It would appear that further promotion of good water supply management is required to ensure appropriate water quality is obtained and maintained (DWI 1993).

There are of course ways to treat water which is contaminated with microorganisms, such as UV treatment, chlorination and boiling of water, but primarily a properly maintained and protected water supply has the potential to provide good quality potable drinking water.

The Private Water Supplies (Scotland) Regulations 2006 should increase the monitoring and protection offered to many water supplies, particularly Type A, therefore safeguarding the health of many people who rely on these water supplies for potable water. The requirement that local authorities carry out risk assessments and water quality analysis on Type A private water supplies in line with legislation will give a better understanding of each supply and give an indication of treatment and maintenance required to reduce and remove contamination.

Local authorities have a duty to provide advice and assistance to homeowners with a Type B supply to enable them to undertake a risk assessment of the potential threat to human health arising from their water supply. The local authority also has the right to carry out their own risk assessment if there are concerns with regards to the water quality from the supply. However as there are probably over 4000 Type B supplies in Aberdeenshire, the likelihood of the relevant local authorities carrying out risk assessments on many of these properties is questionable. The responsibility to maintain private water supplies ultimately lies with the homeowner however it would seem that in many cases better education is required before many of these homeowners come to understand the potential risks involved with an inadequately protected and poorly maintained private water supply.

Chapter 4 Survival of coliform bacteria in water and soil

4.1 Introduction

The microbiological quality of groundwater is affected by the presence of pathogenic bacteria, protozoa and viruses. Coliform bacteria particularly faecal coliforms, give an indication of the potential for pathogens to be present. In the preceding chapter it was shown that coliform bacteria are often present in water from private water supplies. Many of these coliform bacteria are ubiquitous to soil and the environment however *E. coli* is directly linked to faecal contamination as it lives in the intestines of warm blooded animals. *E. coli* is deposited on land through direct deposition of faecal deposits by grazing or wild animals, or spread on land in the form of slurry or manure. Faecal bacteria and pathogens then either stay within the soil matrix or have the potential to find their way into water sources. In both cases there are implications for human health if drinking water supplies become contaminated or food is grown on contaminated land.

There have been a number of studies which demonstrate the survival of coliforms, particularly *E. coli* in soil and groundwater. Ogden et al. (2002) found that *E. coli* O157 survived in soil for approximately 105 days during a time of heavy rainfall. Avery, Moore and Hutchison (2004) found that the *E. coli* from livestock faeces

could survive on grassland for more than 5 months while Gerba, Wallis and Melnick (1975) showed that in laboratory studies *E. coli* survived up to 4.5 months in groundwater maintained in darkness.

The survival of coliform bacteria in soil is influenced by a number of factors. These include the nature and application method of faecal waste, the nature of the soil, climatic effects and the nature and type of introduced microorganisms.

The survival of faecal coliforms differs between direct faecal deposits from grazing animals and wildlife to faecal material which has undergone some form of storage or treatment prior to deposition on the land. This can be in the form of compost heaps which can reach temperatures in excess of 55°C, or slurry storage tanks which are anaerobic and remain at air temperature. Composting of faecal material dramatically reduces numbers of faecal bacteria prior to spreading on the land whereas deposition in the form of slurry does not. Nicholson, Groves and Chambers (2005) studied the survival of *E. coli* O157 in dairy slurry tanks, where temperatures were generally less than 20°C and in a solid manure compost heap where temperature reached 60°C. It was found that *E. coli* O157 survived for approximately 1 week in the compost but survived for up to 90 days in the slurry.

UV light is well documented as detrimental to bacterial survival (Garvey et al. 1998; Sinton et al. 2002) but more protection of bacteria from UV light is afforded by solid

faecal deposits than by spraying of slurry. Conversely there is more likelihood of faecal bacteria entering a water supply if they are already in liquid form. The frequency of application of faecal waste and the organism density in the waste material also affects the survival of faecal bacteria (Crane et al. 1983; Gerba and Bitton 1984).

Bacteria contained within deposited animal wastes are usually retained in the upper layers of soil. The nature of the soil impacts the survival of bacteria. Of particular importance are texture, particle size distribution, moisture-holding capacity, cation-exchange capacity, pH and organic matter content (Gerba and Bitton 1984). Finer soils especially clay minerals and humic substances increase water retention by the soil which increases survival time of coliform bacteria (Gerba and Bitton 1984).

There are a number of climatic effects which influence bacterial survival in soil. Temperature, rainfall and UV light are of primary importance (Crane et al. 1983). Survival is prolonged at low temperatures, below 4°C they have been shown to survive for months or even years. At higher temperatures inactivation or die off is fairly rapid (Gerba and Bitton 1984). Rainfall mobilizes previously retained bacteria and greatly promotes their transport to groundwater. Several studies have shown that the greatest degree of well contamination occurs after periods of heavy

rainfall. Higher moisture content in soil gives a greater survival time for bacteria (Crane et al. 1983).

Microbial resistance to environmental factors varies between different species as well as different strains of bacteria. Many microorganisms cannot survive in the presence of antibiotics and toxic substances (Crane et al. 1983; Gerba and Bitton 1984). It has been shown that there is increased survival time in sterile soil due to the lack of antagonism from soil microflora. Soil moisture, temperature, pH and the availability of organic matter can also indirectly influence the survival of enteric bacteria by regulating the growth of antagonistic organisms (van Veen, van Overbeek and van Elsas 1997; Crane et al. 1983).

Within aquatic environments, enteric bacteria are often associated with sediments. Extended survival patterns have been noted for bacteria that have attached to sediment particles and settled to the bottom of streams and lakes. This is primarily attributed to the availability of soluble organics and nutrients and increased protection from predator protozoa within the sediments (Jamieson et al. 2004; Burton, Gunnison and Lanza 1987).

The more that is understood with regards to the survival of coliform bacteria in water and soil, the more we will recognise why coliform bacteria are more likely to be found in certain private water supplies and not others. Bacterial numbers in

water supplies do not remain constant, numbers increase because of further input into the water supply, or potentially the bacteria multiply, or numbers decrease due to death and predation. This study aims to determine the factors which influence the survival and growth of the coliform bacteria *E. coli* and *E. aerogenes,* in water and soil, with particular reference to the north-east of Scotland.

4.2 Material and methods

4.2.1 Bacterial cultures and serial dilutions

Bacterial cultures of *E. coli* and *E. aerogenes* were prepared as described in section 2.2.1. A turbid solution of each of the cultures was prepared by removing bacterial culture from the nutrient agar plates and mixing it with 10 ml of ¼ strength Ringers solution in a sterile universal container. Serial dilutions of the two bacteria were prepared using the method described in 2.2.2.

4.2.2 Collection and preparation of soil samples

A number of different soils were used to study the survival of coliform bacteria.

Soils A, B, C and D had been collected in association with other studies and had been stored for a number of years. All soils had previously had stones removed, and were sieved and dried. Soil E was a fresh sample collected for this study. Soils were sealed and labelled in soil sample bags.

4.2.2.1 Soil A

A freely draining Dystric cambisol with sandy loam texture belonging to the Countesswells Association and Series. This soil was collected as part of a field experiment started in 1965 and was located at Craigiebuckler, Aberdeen.

Cambisols are mineral soils which are only moderately developed due to their age. They are not confined to any particular region. Generally cambisols make good agricultural land and are intensively used. The term ‘dystric’ refers to the base saturation of the soil. When used with cambisol it indicates that it has, in at least some part between 20 and 100 cm from the soil surface, a base saturation (in 1 *M* NH_4OAc at pH 7) of less than 50 percent (ISRIC 2010).

4.2.2.2 Soils B and C

A freely draining Dystric cambisol with sandy loam texture belonging to the Countesswells Association and Series (Glentworth and Muir 1963). Soil was collected as part of a field experiment at Craibstone, Aberdeen. Soil pH had been manipulated in plots since 1950 through either the periodic addition of

$Al_2(SO_4)_3$ to lower the pH or through the periodic addition of $CaCO_3$ to increase the pH.

4.2.2.3 Soil D

A Dystric cambisol with sandy clay loam texture belonging to The Stonehaven Association and Series. The parent material is referred to as Old Red Sandstone (ORS). Samples were taken from a field south of Stonehaven (National Grid Ref. 793762).

4.2.2.4 Soil E

A Dystric cambisol with sandy loam texture belonging to the Countesswells Association and Series. This soil was located at Craigiebuckler, Aberdeen in 2004. A field sample was collected as part of this study to determine the effect of organic content on the survival of coliform bacteria (section 4.3.9) and further field samples were collected for a study of bacterial transport within undisturbed soil cores. Table 4.1 provides a summary of each soil.

The field sample (Soil E) was collected using a corer of diameter 6 cm and length 40 cm which was cleaned with ethanol and allowed to air dry preceding each core. The field had a grass covering of approximately 25 cm length which was trimmed to approximately two centimetres prior to taking the core sample. The sample was separated into two layers, the top layer of depth 0 to 10 cm, and the bottom layer of

depth 10 to 30 cm. Stones were removed and the soil mixed well. The soil pH was determined along with moisture content. Each layer was placed in a plastic sample bag, sealed, labelled and placed in a cool box until ready for analysis.

Table 4.1 Description and analysis of soil samples

Soil	Description and texture	Particle size (%)			pH	%C	%N	Organic matter (%)
		0.02 – 2 µm	2 – 60 µm	60 – 2000 µm				
A	Sandy loam topsoil	**3.49**	**70.32**	**26.19**	5.38	5.04	0.31	Not measured
B	Sandy loam topsoil with low pH	Not measured			4.50	5.1	0.33	Not measured
C	Sandy loam topsoil with high pH	Not measured			7.00	5.1	0.33	Not measured
D	Sandy clay loam topsoil	**24.6**[1]	**74.4**[1]		5.51	5.10	0.33	Not measured
E	Sandy loam 0 – 10 cm depth	**3.45**	**71.55**	**25.01**	4.66	5.67	0.42	8.47
	Sandy loam 10 – 30 cm depth	**3.61**	**70.56**	**25.83**	4.69	3.37	0.24	6.98
Sand	Fine grained, washed	Not measured			6.10	Not measured		

[1] Values taken from Glentworth and Muir 1964.

4.2.3 Measurement of soil moisture content

A clean evaporation dish was weighed. Soil sample (10 g) was weighed into dish and placed in an oven overnight at 105°C. The dish with dried soil was weighed and the dried soil weight calculated. The moisture content was calculated as a percentage of the soil weight using the following equation:

$$\text{Soil moisture content (\%)} = \frac{\text{weight of wet soil - weight of dried soil}}{\text{weight of wet soil}} \times 100$$

4.2.4 Measurement of soil pH

Soil pH was determined by placing 10 g of air dried soil in a pH cup and adding 20 ml of 0.01 M $CaCl_2$ to the soil. The sample was stirred and left for 30 minutes following which the sample was stirred again and the pH read using a pH meter.

4.2.5 Determination of organic matter content

The moisture content of a 10g sample of soil was determined (4.2.3). The soil sample was then transferred to a cold muffle furnace which was ignited and the temperature set at 400˚C. The sample remained in the muffle furnace overnight then was transferred to a desiccator prior to being reweighed. Any organic matter

was removed due to the high temperature. The percentage of organic matter content of the oven dried soil could then be calculated using the following equation:

$$\text{Organic matter content of oven dried soil (\%)} = \frac{\text{oven dry soil weight} - \text{ignited soil weight}}{\text{oven dry soil weight}} \times 100$$

4.2.6 Particle Size Analysis

Laser diffraction was used to determine the particle size distribution of soil samples within the range 0.02 to 2000 µm. Air dried soil was sieved to remove particles greater than 0.2 mm. A 5 g sample of sieved soil was then dispersed prior to analysis by laser diffraction. The dispersal procedure depended upon the samples being analysed. It can involve the addition of a dispersant agent, physical disaggregation, ultrasonication or a combination of these. Once dispersed the samples were introduced into the sample dispersion tank which pumps the liquid suspension of soil sample through the optical cell. A laser was then passed through this cell and was diffracted by the suspended particles. A series of detectors register the degree of diffraction. The angle of diffraction is directly proportional to the angle of incidence encountered by the laser therefore the

smaller the particle the higher the angle of incidence and the greater the diffraction. A mathematical model determined the required particle size distribution to account for the observed diffraction of the laser.

4.2.7 Determination of total N and C in soil sample using an elemental analyser

The soil sample for analysis was ground as fine as possible to ensure complete uniformity, using a ball mill. The sample was dried overnight at approximately 50°C in an oven. Once dried the sample was stored in a desiccator until ready for weighing, 15 mg of sample weighed out for analysis. The sample was analysed by the Thermo Finnigan Elemental Analyser (FlashEA 1112 Series).

With this method a sample contained in a tin capsule was dropped into a combustion reactor maintained at 900°C. The container melts and the tin promotes a ‘flash reaction’ in a helium atmosphere temporarily enriched with pure oxygen. This momentarily raises the temperature in the reactor to ~2000°C combusting the sample. The combustion products are carried by a constant flow of helium through an oxidation catalyst, copper oxide and platinised Alumina. CO_2, N_2, NO_x and H_2O then flow into a reduction reactor containing copper wires held at 680°C, where excess oxygen is removed and any nitrogen oxides are converted into nitrogen

gas. Water is then absorbed by magnesium perchlorate. A chromatographic column held at 40°C then separates the CO_2 and N_2 into defined peaks, and the relative amounts determined using a thermal conductivity detector. The detection limit of this method for a 15 mg sample ranges from a minimum of 0.03% N and 0.02% C, to a theoretical maximum of 100% dry weight for N or C.

4.3 Material and methods: Survival of *E. aerogenes* and *E. coli* in water and soil

4.3.1 Evaluation of Colilert 18™ for the detection and quantification of *E. aerogenes* and *E. coli* in soil

Colilert 18™ was evaluated to determine its suitability for the detection and quantification of coliform bacteria in soil. The ability of Colilert 18™ to detect coliform bacteria at high and low concentrations was investigated.

Bacterial cultures of *E. coli* and *E. aerogenes* were prepared as described in section 2.2.1. Serial dilutions of the two bacteria were prepared using the method described in 2.2.2

Soil A (as described in 4.2.2) was prepared and 2 g quantities placed in sterilised test tubes with lids. Sterile ¼ strength Ringer's solution (2 ml) was added to each sample. The samples were left for 1 hour to allow for wetting of soil. As a comparison, test tubes were prepared without soil, having only 2 ml of sterile ¼ strength Ringer's solution in each. To each test tube 1 ml of each of the chosen serial dilution of both *E. aerogenes* and *E. coli* were added. The samples were vortexed for 1 minute before a selected volume was removed for analysis by Colilert 18™ using the method described in section 2.2.7. A number of different sample dilutions and volumes for analysis were tested to determine the suitability of Colilert 18™ for use with soil samples. These are shown below.

(i) Serial dilution 5 was used for both *E. coli* and *E. aerogenes*. Five samples were prepared in ¼ strength Ringers solution and 14 samples prepared as soil suspensions. For Colilert 18™ analysis, 0.5 ml was taken from each sample and made up to 100 ml with ¼ strength Ringers solution.

(ii) Serial dilution 6 was used for both *E. coli* and *E. aerogenes*. Five samples were prepared in ¼ strength Ringers solution and 16 samples prepared as soil suspensions. For Colilert 18™ analysis, 0.1 and 1 ml was taken from each sample and made up to 100 ml with ¼ strength Ringers solution.

4.3.2 Effect of temperature on the survival of coliform bacteria in water

To investigate the effect of temperature on the survival of the selected bacteria (*E. coli* and *E. aerogenes*), aqueous suspensions of each organism were prepared, and maintained at a range of temperatures and sampled over time.

Nine, 1 litre solutions of sterile ¼ strength Ringers solution were prepared. One ml of serial dilution 5 of *E. coli* and *E. aerogenes* (4.2.1) was added to each 1 litre solution of ¼ strength Ringers solution. Each 1 litre solution was inverted a number of times to ensure thorough mixing of the contents. To determine the initial total coliform and *E. coli* counts, 10 ml was sampled from each 1 litre solution and made up to 100 ml with ¼ strength Ringers solution for analysis by Colilert 18™ as described in section 2.2.7. When the bacterial count for samples at 37˚C was expected to be above the maximum count detected by the Colilert 18™ method, 1 ml samples were taken for analysis. The bacterial count per 100 ml of sample was then calculated accordingly.

Three of the bacterial solutions were placed in each of the pre-set incubators at 37°C, 10°C and 4°C. All of the samples were kept in the dark. The bacterial solutions were resampled at 6, 24 and 48 hrs, then at 7, 14, 21 and 28 days using Colilert 18™ to determine the total number of coliforms, and the number of *E. coli*

present in each solution. The mean of the three samples at each temperature was calculated.

4.3.3 Effect of pH on the survival of coliform bacteria in water

The effect of pH on the survival of selected bacteria (*E. coli* and *E. aerogenes*) was investigated. Aqueous suspensions of each organism were prepared and maintained at a range of pH and sampled over time.

Seven, 1 litre solutions of sterile ¼ strength Ringers solution were prepared. The pH of all the solutions was measured. The pH of three solutions was lowered using hydrochloric acid to pH's ranging from 4.09 to 4.56. The pH of three solutions was increased using sodium hydroxide to pH's ranging from 9.07 to 9.39.

One ml of each of the serial dilution 5 of *E. coli* and *E. aerogenes* (4.2.1) was added to each 1 litre solution of ¼ strength Ringers solution. Each 1 litre solution was inverted a number of times to ensure thorough mixing of the contents. To determine the initial total coliform and *E. coli* counts, 10 ml was sampled from each 1 litre solution and made up to 100 ml with ¼ strength Ringers solution for analysis by Colilert 18™ as described in section 2.2.7.

All the 1 litre samples were placed in a dark temperature controlled room at 10°C. The solutions were resampled at 6, 24 and 48 hrs, then at 7 and 14 days using Colilert 18™ to determine the total number of coliform bacteria and *E. coli* present in each sample.

4.3.4 Effect of temperature on the survival of coliform bacteria in soil

To investigate the effect of temperature on the survival of *E. coli* and *E. aerogenes* in soil, aqueous suspensions of the selected coliform bacteria were prepared then added to moist soil. The soil solutions were maintained at a range of temperatures and sampled over time.

Bacterial cultures of *E. coli* and *E. aerogenes* were prepared as described in section 2.2.1, and serial dilutions of the two bacteria were prepared using the method described in 2.2.2

Soil A (2 g) was placed in each of 21 sterile test tubes. Sterile ¼ strength Ringer's solution (2 ml) was added to each soil sample. The samples were left for 1 hour to allow for wetting of soil. After 1 hour, 1 ml of each of *E. aerogenes* and *E. coli* (both serial dilution 5) was added to each test tube. The samples were vortexed for 1 minute to ensure even mixing. An initial sample of 0.1 ml from each test tube was

taken immediately after vortexing, and made up to 100 ml with ¼ strength Ringers solution for analysis by Colilert 18™ as described in section 2.2.7.

Three of each soil solutions were placed in incubators at 20°C, 10°C and 4°C and 14 were placed in the freezer at - 20°C. All of the samples were kept in the dark. These solutions were sampled at 24, 48 and 72 hrs then at 7, 14 and 21 days. Prior to sampling all solutions were vortexed for 1 minute then 0.1 ml was removed and sampled immediately. For the frozen samples at - 20°C, 2 samples were removed for each analysis. Once the sample had defrosted it was vortexed and analysed immediately and the remaining sample discarded. The results for all analyses were then calculated as cfu / 100 ml of suspension to make them consistent and comparable.

4.3.5 Effect of freeze / thaw conditions on the survival of coliform bacteria in soil

To determine the effect of freeze / thaw conditions on the survival of coliform bacteria in soil, aqueous suspensions of *E. aerogenes* and *E. coli* were prepared and added to moist soil and the samples subjected to repeated freezing and thawing and sampled over time.

Bacterial cultures of *E. coli* and *E. aerogenes* were prepared as described in section 2.2.1, and serial dilutions of the two bacteria were prepared using the method described in 2.2.2

Three 2 g samples of soil A were weighed out and placed in sterile test tubes. Sterile ¼ strength Ringer's solution (2 ml) was added to each sample. The samples were left for 1 hour to allow for wetting of soil. After 1 hour, 1 ml of each of *E. aerogenes* and *E. coli* (both serial dilution 5) was added to each test tube. The samples were vortexed for 1 minute to ensure even mixing. An initial sample of 0.1 ml was taken from each test tube immediately after vortexing, and made up to 100 ml with ¼ strength Ringers solution for analysis by Colilert 18™ as described in section 2.2.7.

Samples were placed in a –20°C freezer. After 24 hours samples were removed and left to thaw. Samples were then vortexed and resampled immediately. The samples were returned to the freezer and this procedure repeated at 48 and 72 hrs and again after 7 days.

4.3.6 Effect of pH on survival of coliform bacteria in soil

The effect of pH on the survival of coliform bacteria in soil was investigated.

Aqueous suspensions of *E. coli* and *E. aerogenes* were prepared and added to moist soil at a range of pH and sampled over time.

Three soil samples of differing pH were obtained as described in 4.2.2. Soils B and C had pH's of 4.50 and 7.00 respectively. Soil A (pH 5.38) was used as a comparison. Three samples of each soil were weighed out (2g) and placed in sterile test tubes. Sterile ¼ strength Ringer's solution (2 ml) was added to each sample. The samples were left for 1 hour to allow for wetting of soil. One ml of each of *E. aerogenes* and *E. coli* (both serial dilution 5) was added to each test tube. The samples were vortexed to ensure even mixing. A 0.1 ml sample was taken immediately from each test tube and made up to 100 ml with ¼ strength Ringers solution for analysis by Colilert 18™ as described in section 2.2.7.

All samples were placed in the dark at 10°C. Each soil solution was resampled at 24, 48 and 72 hrs and then at 7 days. Prior to sampling all solutions were vortexed for 1 minute prior to removing 0.1 ml for analysis. The results for all analyses were then calculated as cfu / 100 ml of suspension to make them consistent and comparable.

4.3.7 Effect of colloids on the survival of coliform bacteria in soil

To determine the role colloids play on the survival of *E. coli* and *E. aerogenes* in soil, aqueous suspensions of each organism were prepared and added to moist soil. Bacterial cultures of *E. coli* and *E. aerogenes* were prepared as described in section 2.2.1, and serial dilutions of the two bacteria were prepared using the method described in 2.2.2

Soil A was weighed out and 2 g placed in each of three test tubes. This was repeated 3 times. Sterile ¼ strength Ringers solution (2 ml) was added to each sample. These samples were left for 1 hour to allow for wetting of soil. After 1 hour, 1 ml of each of *E. aerogenes* and *E. coli* (both serial dilution 5) was added to each test tube. The samples were vortexed for 1 minute to ensure even mixing. An initial sample of 0.1 ml was taken from each test tube immediately after vortexing, and made up to 100 ml with ¼ strength Ringers solution for analysis by Colilert 18™ as described in section 2.2.7.

Samples were placed in the dark at 10°C. After 24 hours samples were resampled. The soil solution had settled leaving clear liquid at the top of the sample. A 0.1 ml sample of this liquid was removed for analysis, prior to vortexing the sample. After vortexing another 0.1 ml of solution was removed immediately and analysed. This

procedure was repeated at 24, 48 and 72 hours and at 7 days. The results for all analyses were calculated as cfu / 100 ml of suspension.

4.3.8 Effect of soil texture on the survival of coliform bacteria in soil

To determine the effect of soil texture on the survival of *E. coli* and *E. aerogenes* in soil, aqueous suspensions of each organism were prepared and added to clay soil and sand combinations. Bacterial cultures of *E. coli* and *E. aerogenes* were prepared as described in section 2.2.1, and serial dilutions of the two bacteria were prepared using the method described in 2.2.2.

Five combinations of soil D and sand were prepared to provide a variety of soil textures (Table 4.2). Three 2 g samples of each soil/sand combination were weighed out and placed in sterile test tubes.

Table 4.2 Soil and sand combinations used to determine effect of soil texture on survival of bacteria

Sample	% Soil D (by weight)	% Sand (by weight)
1	100	0
2	75	25
3	50	50
4	25	75
5	0	100

Sterile ¼ strength Ringer's solution (2 ml) was added to each sample. The samples were left for 1 hour to allow for wetting of soil. After 1 hour, 1 ml of each of *E. aerogenes* and *E. coli* (both serial dilution 5) was added to each test tube. The samples were vortexed for 1 minute to ensure even mixing. An initial sample of 0.1 ml was taken from each test tube immediately after vortexing, and made up to 100 ml with ¼ strength Ringers solution for analysis by Colilert 18™ as described in section 2.2.7.

Samples were placed in the dark at 10°C and resampled at 24, 48 and 72 hours and at 7 days. Samples were vortexed for 1 minute immediately prior to sampling. The results for all analyses were calculated per 100 ml of solution.

4.3.9 Effect of organic matter on the survival of coliform bacteria in soil

As depth of soil increases within the soil profile, the proportion of organic matter decreases. To determine the role organic matter has on the survival of coliform bacteria in soil, aqueous suspensions of *E. coli* and *E. aerogenes* were prepared as described in section 2.2.1 and added to moist soil. Serial dilutions of the two bacteria were prepared using the method described in 2.2.2.

A 30 cm soil core (soil E) was separated into two layers; top layer (0 – 10 cm; high organic) and bottom layer (10 – 30 cm; lower organic). Stones were removed and the soil mixed well. Three 2g samples of each were prepared and placed in sterile test tubes. Sterile ¼ strength Ringers solution (2 ml) was added to each test tube and left for 1 hour to allow for wetting of soil. After 1 hour, 1 ml of each of *E. aerogenes* and *E. coli* (both serial dilution 5) was added to each test tube. The samples were vortexed for 1 minute to ensure even mixing. An initial sample of 0.1 ml was taken immediately after vortexing, from each test tube and made up to 100 ml with ¼ strength Ringers solution for analysis by Colilert 18™ as described in section 2.2.7.

Samples were placed in the dark at 10°C and were resampled at 24, 48 and 72 hours and at 7 and 21 days. All samples were vortexed immediately prior to sampling.

4.4 Results and discussion

4.4.1 Evaluation of Colilert 18™ for the detection and quantification of *E. aerogenes* and *E. coli* in soil

Colilert 18™ was evaluated for use in detection of coliform bacteria in soil. Colilert 18™ was previously evaluated and found suitable for the detection of coliform bacteria in ¼ strength Ringers solution and groundwater samples (Chapter 2). Colilert 18™ has also been approved for use in the analysis of sewage sludge (SCA 2003) which although different to soil has similar issues when detecting microorganisms.

In this study, samples were taken from ¼ strength Ringers solution and from the soil suspension both of which had been inoculated with serial dilution 5 of freshly prepared bacterial culture of *E. aerogenes* and *E. coli*. From each sample 0.5 ml was removed for analysis. This study was repeated using serial dilution 6 of bacterial culture, and sampling 1 ml and 0.1 ml from both the soil suspension and from the ¼ strength Ringers solution. In all cases the number of colony forming units per 100 ml of sample was calculated. Results were compared following data analysis which is shown in Tables 4.3 and 4.4.

Table 4.3 Descriptive statistics following analysis of ¼ strength Ringers solution for coliform bacteria using Colilert 18™ (cfu / 100 ml)

	1/4 strength Ringers solution					
	SD 5 (0.5 ml)		SD 6 (0.1 ml)		SD 6 (1 ml)	
	TC	EC	TC	EC	TC	EC
No. of samples	5	5	5	5	5	5
Mean	121312	95068	14880	3240	10382	2008
Standard Deviation	6063	8172	3222	885	1095	568
Range	1480	2024	9000	2100	2900	1350
Minimum	11496	8320	10900	2000	8820	1080
Maximum	12976	10344	19900	4100	11720	2430
Confidence Level (95.0%)	7528	10147	4001	1099	1360	706

(TC = total coliforms; EC = *E. coli*)

Table 4.4 Descriptive statistics following analysis of soil suspension for coliform bacteria using Colilert 18™ (cfu / 100 ml)

	Soil solution					
	SD 5 (0.5 ml)		SD 6 (0.1 ml)		SD 6 (1 ml)	
	TC	EC	TC	EC	TC	EC
No. of samples	14	14	16	16	16	16
Mean	111821	89064	10344	2113	9216	1803
Standard Deviation	16845	12724	3487	1676	2397	504
Range	5014	4054	13400	5200	8190	1820
Minimum	8720	6896	6300	0	6310	1100
Maximum	13734	10950	19700	5200	14500	2920
Confidence Level (95.0%)	9726	7347	1858	893	1277	268

(TC = total coliforms; EC = *E. coli*)

A lower number of coliforms and *E. coli* were consistently recorded from soil samples compared to ¼ strength Ringers solution. This ranged from 6% less with *E. coli* from serial dilution 5, 0.5 ml sample to 35% less with serial dilution 6, 0.1 ml sample. However when the analysis was carried out there was no allowance made for the different suspensions. The water suspension had a total of 4 ml of liquid while the soil suspension had 4 ml liquid and 2g of soil, therefore the bacteria was distributed within a greater volume in the soil suspension. Removing the same quantity of suspension from each sample will give rise to some degree of error when determining the bacterial count.

There is a much greater variation in the range of results from the soil suspension than the ¼ strength Ringers suspension. The variation of results would normally reduce as the sample numbers increase. However in this case the sample variation may be due to a lack of homogeneity within the soil samples compared to the ¼ strength Ringers solutions. Vortexing of the soil samples prior to analysis should increase homogeneity however as soon as the vortexing ceases there will be some settling of the sample.

There were a number of difficulties when using Colilert 18™ to detect coliform bacteria within soil suspensions. Soil quantities must be low enough so as not to produce a high discolouration in the sample for analysis as this may make reading

of the Colilert 18™ difficult. However when too little soil was used when setting up the experiments, the soil - bacteria interaction was not investigated.

It was decided to continue using Colilert 18™ to detect coliform bacteria within soil solutions. However it was decided to use serial dilution 5 and take 0.1ml of sample for analysis. This would give a higher number of organisms inoculated into the sample but a reduction in the amount of soil suspension removed for analysis therefore preventing a discoloration in the sample.

4.4.2 Effect of temperature on the survival of coliform bacteria in water

It was found that the survival of *E. coli* and *E. aerogenes* in water was influenced by temperature. Figure 4.1 shows the survival behaviour of *E. coli* and *E. aerogenes* at varying temperatures over a four week period.

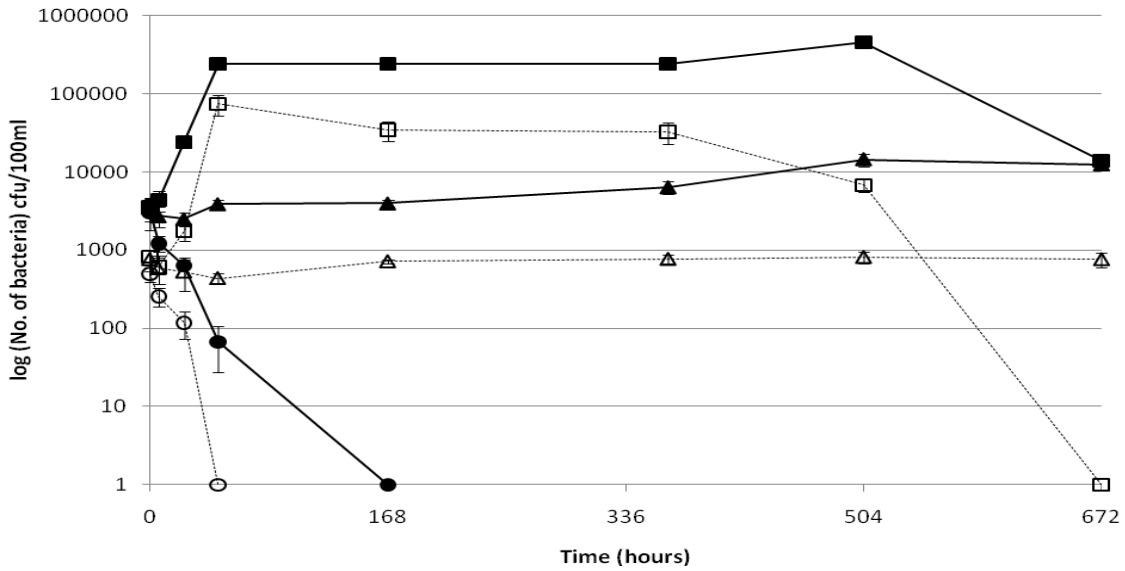

Figure 4.1: Survival of coliform bacteria at varying temperatures in water over a 28 day period. Error bars equivalent to one standard deviation, (n = 3), (TC at 37°C (■), TC at 10°C (▲), TC at 4°C (●), *E. coli* at 37°C (□), *E. coli* at 10°C (△), *E. coli* at 4°C(○)

In general, the number of coliform bacteria initially decreases over a six hour period following addition of *E. coli* and *E. aerogenes* to the ¼ strength Ringers solution and incubation at chosen temperatures. However in the case where the sample is incubated at 37°C there is a rapid increase over the first six hours in both *E. coli* and *E. aerogenes*. This is perhaps not surprising since they are enteric bacteria and as such naturally survive and grow at this temperature. It was however observed that at 37°C *E. coli* survived for a shorter period of time than

those at 10°C. At 28 days and a temperature of 37°C there are no surviving *E. coli* and numbers of *E. aerogenes* are decreasing.

Survival was lowest at 4°C, *E. aerogenes* surviving from 2 to 7 days however *E. coli* survived for less than 48 hours. The optimum temperature for survival was noted at 10°C from the 3 temperatures studied with coliforms continuing to survive and maintain their numbers to at least 28 days. McFeters and Stuart (1972) studied the effect of temperature in natural waters and found that *E. coli* survival was inversely proportional to temperature changes between 5 and 15°C. At 5°C a 50% reduction in *E. coli* counts was found after 4.5 days, however at 15°C this took less than 1.5 days. Pope et al. (2003) studied the impact of storage over 48 hours on natural waters. It was found that reduction of *E. coli* in samples was greater at temperatures of 20°C and 35°C than at 4°C and 10°C.

Medema, Bahar and Schets (1997) found that the die off rate of *E. coli* was greater at 5°C than at 15°C in autoclaved river water. At 15°C *E. coli* numbers increased over a two week period then remained constant to at least 77 days. The presence of indigenous microorganisms in the natural water may be the reason for the discrepancy between the two studies. At higher temperatures the quantity of indigenous bacteria are likely to increase thereby having a negative impact on the survival of coliform bacteria. Whereas in sterile water there will be no competition or predation by intrinsic bacteria.

The minimum temperature for growth of *E. coli* was indicated as 7.5 to 7.8°C by Shaw, Marr and Ingraham (1971). This substantiates results found in this study and supports the short survival time and absence of growth recorded at 4°C for *E. coli* and *E. aerogenes.*

The fact that coliform bacteria can survive and possibly grow at temperatures of 10°C indicates that they also have the ability to survive in groundwater as the typical groundwater temperatures in the UK are 10°C with very little variation throughout the year. Although it is recognised that coliform bacteria have a finite lifespan in water, their ability to survive for even short periods of time could impact on human health due to contamination of water supplies.

4.4.3 Effect of pH on the survival of coliform bacteria in water

The effect of pH on coliform survival in water was investigated using two extremes of pH at 10°C. The mean of the samples taken at low pH (pH 4.09 – 4.56) and at high pH (pH 9.07 – 9.39) were plotted against time. Figure 4.2 shows the survival of coliform bacteria and *E. coli* in water at these pH values over a 14 day period.

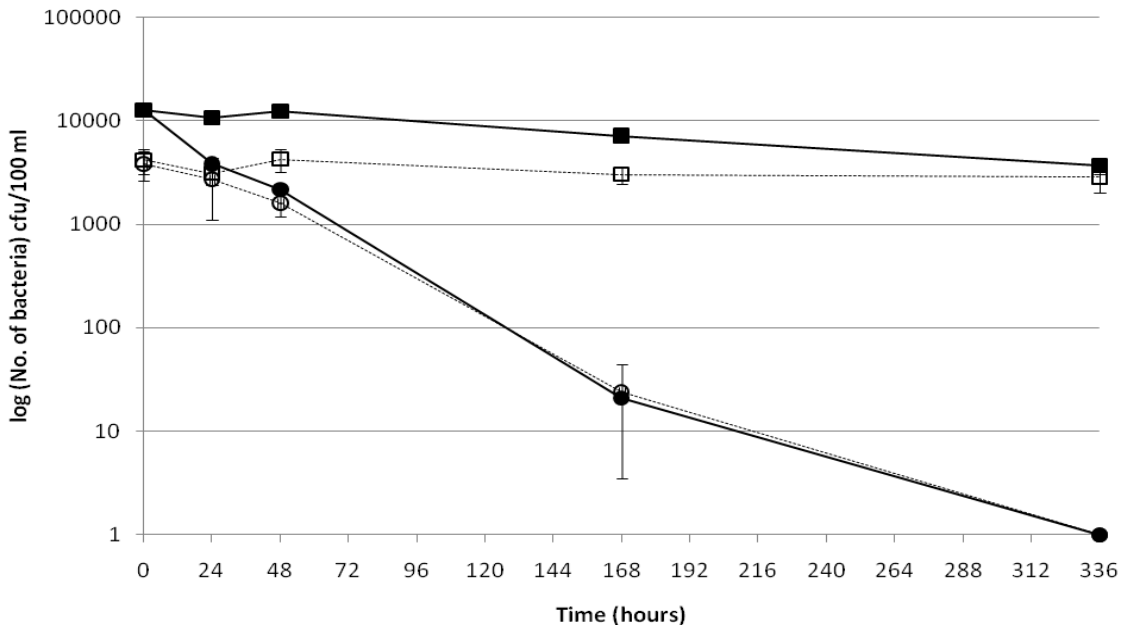

Figure 4.2: Comparison of the survival of coliform bacteria in water at high pH (9.07 – 9.39) and low pH (4.09 – 4.56) over a 14 day period. Error bars equivalent to one standard deviation (n = 3)

TC at high pH (●), TC at low pH (■),

***E. coli* at high pH (○), *E. coli* at low pH (□)**

In comparison to the temperature study on coliform survival where the ¼ strength Ringers solution had a pH of 7.58, it is clear to see that a high pH has a detrimental effect on the survival of both *E. aerogenes* and *E. coli* in water. Both bacterial counts remain relatively stable at low pH over the 14 day period. McFeters and Stuart (1972) found that *E. coli* survival in natural waters at 10°C was most favourable at pH between 5.5 and 7.5 and numbers reduce dramatically at pH higher and lower than this. Sjogren and Gibson (1981) found that *E. coli* survival

increased in natural waters at pH 5.5 compared to pH 7.5. It was suggested that this was linked to increased mobilisation of energy resources by enzyme activity, specifically ribonuclease at the lower pH. The study also notes that pH 5 is the optimal pH for adenosine triphosphate synthesis by the proton motive force therefore increasing the potential for survival.

4.4.4 Effect of temperature on survival of coliform bacteria in soil

The survival of coliform bacteria in soil was studied at four temperatures over a 28 day period. In water samples at 4°C, coliform bacteria were no longer present after 7 days. However in soil samples at the temperatures studied, coliform bacteria including *E. coli* were still present at day 28 (Figure 4.3).

The soil had previously been sampled using Colilert 18™ and was found to have no background level of Coliform bacteria, however it was important to note however that unlike the water samples used previously, the soil was not sterile.

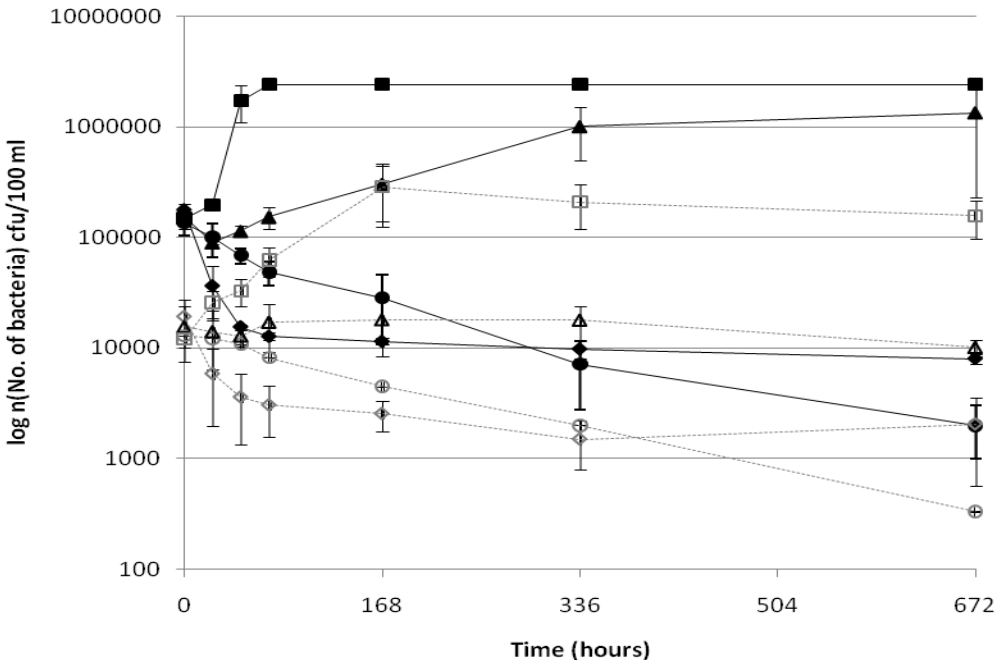

Figure 4.3 Survival of coliform bacteria in soil at varying temperatures recorded over a 28 day period. Error bars equivalent to one standard deviation (n = 3)

(TC: 20°C (■), TC: 10°C (▲), TC: 4°C (●), TC: -20°C (◆)

***E. coli* : 20°C (□), *E. coli* : 10°C (△), *E. coli* : 4°C (○),**

***E. coli* : -20°C (◇)**

At all temperatures studied, the coliform bacterium in soil survives longer than in water at the same temperatures. After 28 days at all 4 temperatures both *E. coli* and *E. aerogenes* were still present in samples. This suggests a protective effect afforded by the soil. Vargas and Hattori (1986) have shown that bacteria are better protected from protozoan grazing, when they were positioned within soil

micropores. Bacteria distributed within a water column are not protected in the same way as bacteria retained within soil pores which may indicate why these bacteria in water survived for a shorter period of time. Bacteria at - 20°C survive better than those at 4°C, possibly because they are likely to be in a suspended state.

Temperature influences the moisture content of the soil; as temperature increases soil moisture is likely to decrease. The moisture content of soil is widely recognised as being influential in bacterial survival, consequently if soil moisture content is low bacterial survival tends to be reduced (Entry et al. 2000). This is supported by Garcia-Orenes et al. (2007) who found that coliforms survived in moist soil at temperatures exceeding 20°C for 80 to 100 days; however the length of survival decreased to less than 45 days when the soil was not irrigated. Cools et al. (2001) noted that *E. coli* survived longer at 5°C than at 25°C.

In summary, temperature and moisture content of the soil play an important role in the growth and survival of coliform bacteria in soil. These factors also influence the presence of intrinsic soil bacteria which will in turn impact on coliform survival.

4.4.5 Effect of freeze / thaw conditions on the survival of coliform bacteria in soil

Coliform bacteria including E. coli in soil was studied in section 4.4.4 to determine the effect of temperature on their survival. This study showed that coliform bacteria were found to still be present and viable at 28 days when subjected to a constant - 20°C. The aim of this study was to determine the impact of repeated freezing and thawing on the survival of *E. coli* and *E. aerogenes* compared to soil maintained at a constant - 20°C. It can be seen in Figure 4.4 that survival time is reduced quite dramatically by repeated freezing and thawing, particularly in the case of *E. coli*.

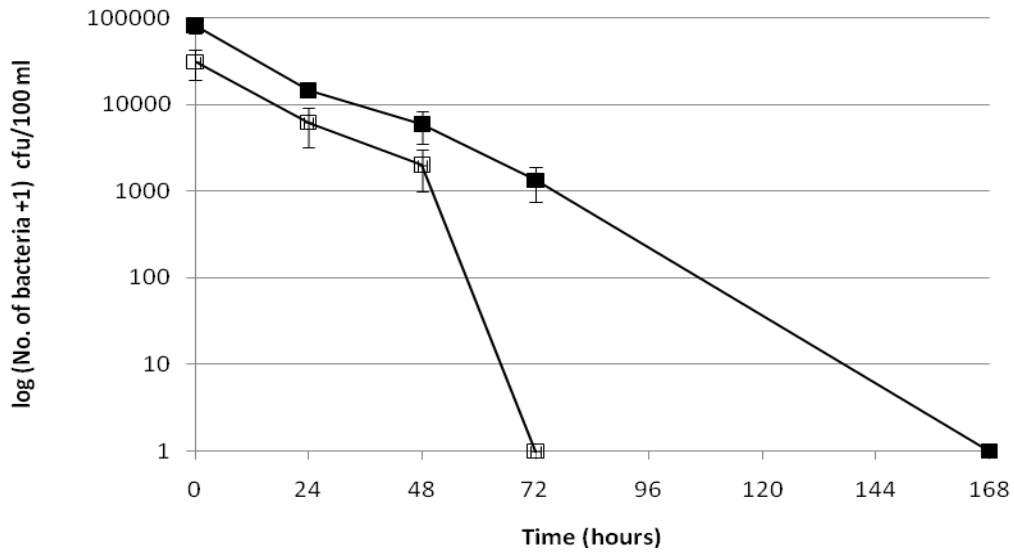

Figure 4.4 Effect of repeated freeze / thaw conditions on the survival of coliform bacteria in soil. Error bars equivalent to one standard deviation (n = 3), TC (■), *E. coli* (□)

The effects of freezing are well documented with regards to the impact on microbial survival. A number of factors are influential to the degree of damage suffered by bacteria in soil when subjected to freezing conditions. These include the rate and temperature of freezing, the length of frozen storage, thaw conditions, moisture content of the soil and soil composition.

In the case of slow freezing of aqueous suspension, solute concentration increases in the unfrozen section which causes diffusion of water from bacterial cells. The concentration of cellular liquids changes the pH and ionic strength within the cell which causes inactivation of enzymes, denatures proteins and impedes the function of DNA, RNA and cellular organelles. As water freezes externally, ice crystals are produced which cause mechanical injury to the cell membrane.

When freezing rates are increased the survival of microorganisms increases. This is due to a reduction of the osmotic effects. However if freezing rates are too high, crystal formation occurs intracellularly as well as extracellularly, causing greater injury to cells resulting in a decrease in survival rate. Gram negative bacteria particularly mesophiles, appear to be more susceptible to cold shock than gram positive bacteria.

Bacterial cells which survive freezing are subjected to further osmotic effects during thawing. Many injured cells die gradually during frozen storage however

some may have reversible injuries which cells are able to repair if nutrients, energy sources, and specific ions are available and metabolism can commence.

This study found that repeated freeze / thaw episodes were much more destructive than a single freezing event over a long period of time. This is supported by Kibbey, Hagedorn and McCoy (1978) who studied the effect of freezing on *Streptococcus faecalis* and found that repeated freeze-thaw episodes caused greater cellular damage than one extended period of freezing. In the UK, soil can be subjected to repeated freezing and thawing during the winter months. This would appear to be advantageous to the reduction and removal of enteric bacteria from the environment and therefore reducing the likelihood of contamination of groundwater and private water supplies.

4.4.6 Effect of pH on survival of coliform bacteria in soil

As with the study on coliform survival in water at varying pH, soil pH also affects coliform survival. This effect however is much less dramatic than in the water samples. Figure 4.5 shows the comparison between soils of different pH values.

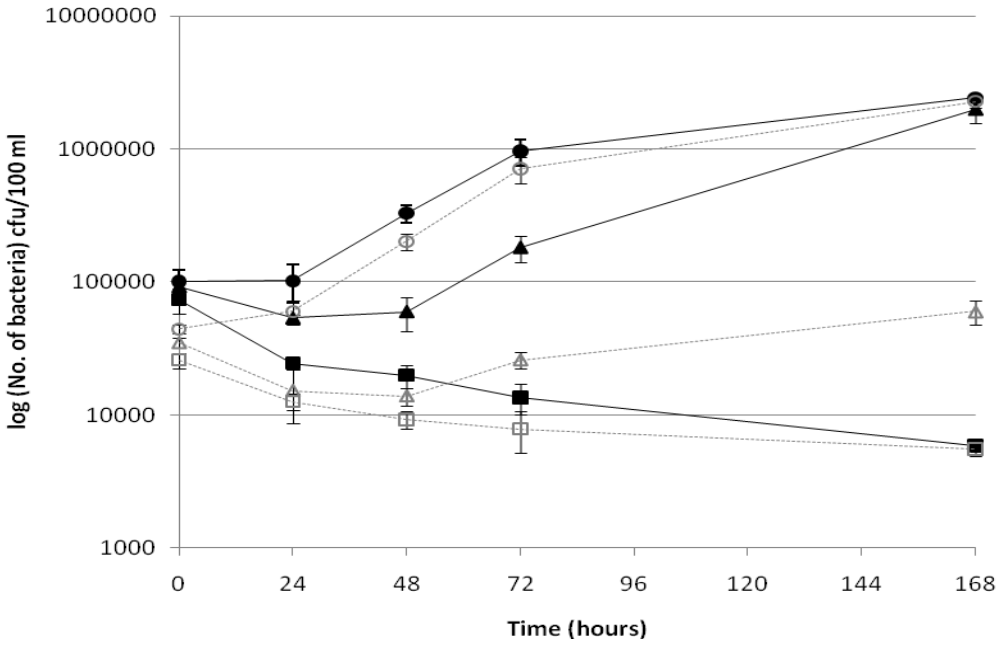

Figure 4.5 Effect of soil pH on survival of coliform bacteria in soil

Error bars equivalent to one standard deviation, (n = 3)

(TC pH 7.00 (●), TC pH 4.50 (■), TC at pH 5.38 (▲),

***E. coli* pH7.00 (○), *E. coli* pH 4.50 (□), *E. coli* pH 5.38 (△)**

Coliform bacteria in soil at pH 4.50 appear to decrease slightly faster than coliforms in water at pH 4.09 – 4.56 as shown in section 4.4.3, while bacteria in soil of pH 7.00 are increasing in numbers. This is similar to a study by Estrada et al. (2004) who found that soil with pH less than 6 or greater than 8 was likely to have an adverse effect on most bacteria, in contrast a positive effect on growth and survival of enteric bacteria was found in neutral soils. Gerba, Wallis and Melnick (1975) also noted shorter survival in acidic soils of pH 3 to 5.

This is unexpected as bacteria in soil are normally expected to have greater protection from adverse conditions due to the presence of pore spaces within the soil, whereas bacteria in water do not have this protection.

It is possible that this difference in the survival of coliforms in low pH soil compared to water of a similar pH is due to the presence of clay minerals in the soil. It is known that at low pH values (< pH 5), clay minerals may dissolve and release high levels of silica (and alumina) to the water which in high concentrations can be detrimental to bacterial survival.

4.4.7 Effect of colloids on the survival of coliform bacteria in soil

The aim of this study was to determine whether *E. coli* and *E. aerogenes* were more likely to be found suspended within the water layer or retained within the settled soil particles. The samples removed from the settled soil sample were removed from the liquid column above the settled soil. The other samples were removed following vortexing of the samples to ensure thorough mixing.

It can be seen from Figure 4.6 that more bacteria are detected following mixing of the soil solution than from the water column above the settled suspension. This indicates that the bacteria are retained within the settled soil with possible

attachment to colloidal material. It can also be noted that coliforms found within the settled soil have increased growth and survival in comparison to coliforms found within the water suspension.

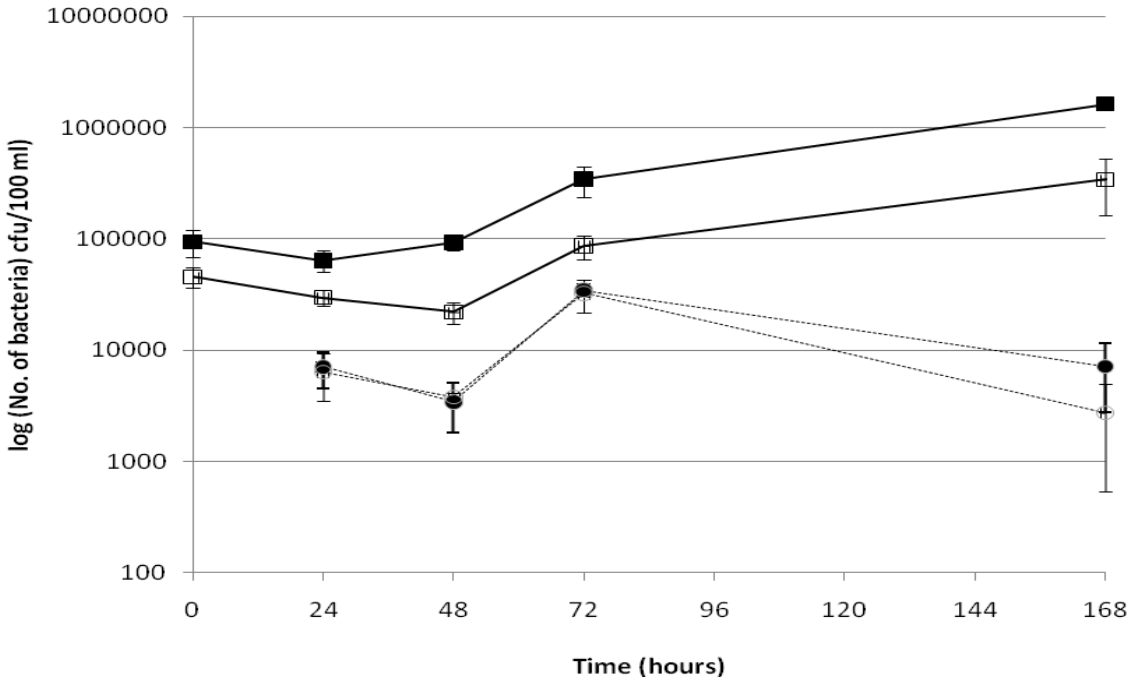

Figure 4.6 Effect of colloids on the survival of coliform bacteria in soil
Error bars equivalent to one standard deviation, (n = 3)
TC soil A (vortexed) (■), TC settled soil A (●),
***E. coli* soil A (vortexed) (□), E. coli settled soil A (○)**

A number of studies have shown increased numbers and greater survival of coliform bacteria in sediments than in the overlying water column and have linked this with bacterial association with the sediments (Gerba and McLeod 1976; Karim

et al. 2004; An, Kampbell and Breidenbach 2002; Burton, Gunnison and Lanza 1987). This effect was found to be particularly increased with clay sediments (Burton, Gunnison and Lanza 1987). Craig, Fallowfield and Cromar (2004) studied bacterial survival and found that at all temperatures survival was greater in sediment than water. It was also recognised that higher quantities of silt, clay and organic carbon improved bacterial survival. This agrees with Karim et al. (2004) who inferred that increased faecal coliform survival in artificial wetlands may be due to the increased organic matter content of sediments.

4.4.8 Effect of soil texture on the survival of coliform bacteria in soil

Soil texture has been documented as having an effect on the survival of bacteria in soil. The effect of increasing sand content with decreasing clay content on the survival of coliform bacteria was studied and results shown in Figure4.7.

It is generally thought that fine textured soils such as clays are more favourable to coliform survival than coarse textured sandy soils due to the presence of small pore spaces where bacteria can be protected from predation and adverse soil conditions. Under the same conditions clay soils would have greater moisture content than sand which is also conducive to coliform survival.

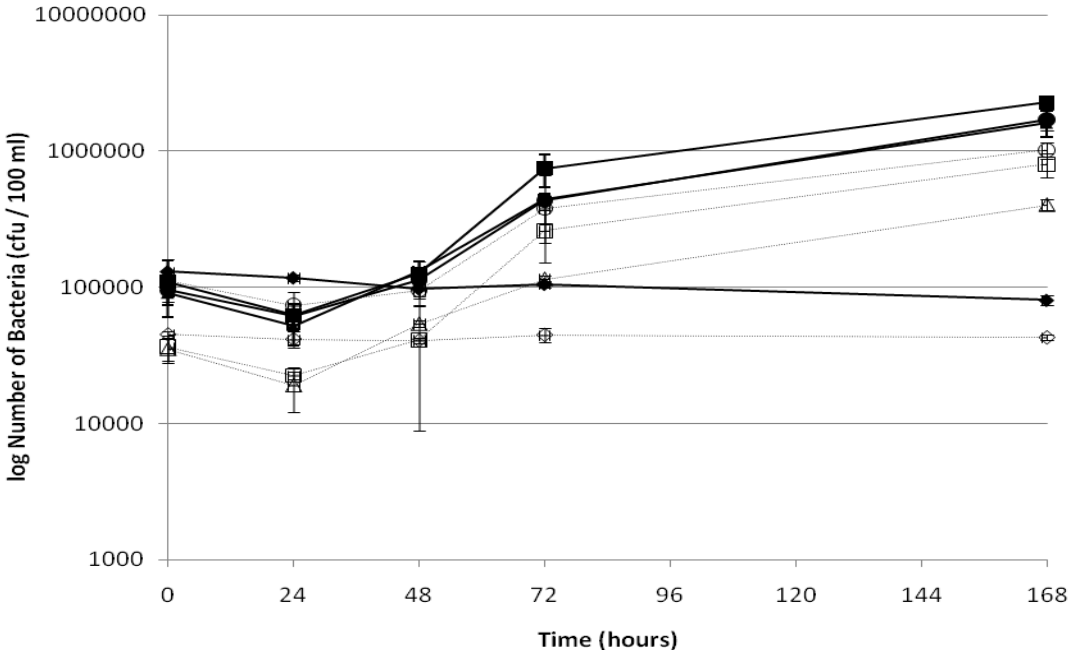

Figure 4.7 Effect of soil texture on the survival of bacteria in soil

Error bars equivalent to one standard deviation, (n = 3)

100 % soil: TC (●), *E. coli* (○),

66 % soil / 33 % sand: TC (▲), *E. coli* (△),

33 % soil / 66 % sand: TC (■), *E. coli* (□)

100 % sand: TC (◆), *E. coli* (◇)

The findings of this study do indicate that the presence of clay particles enhances survival and growth of coliforms, whereas in the presence of only sand, the numbers of coliform bacteria start to decline. Burton, Gunnison and Lanza (1987) found greater survival in sediments with higher clay content as did Fenlon et al. (2000) who studied the survival of *E. coli* O157 in various soils and concluded that

E. coli O157 could survive considerably longer in clay and loam soils compared to sandy soils. However, Cools et al. (2001), studied *E. coli* survival in soils of different texture (sandy soil, loamy soil, loamy sand) and found the sandy soil to be the best for *E. coli* survival. The water holding capacity of the 3 soils studied was similar however the organic matter content was much higher in the sandy soil. Organic matter is known to be important for water retention, the formation and stabilisation of aggregates and the formation of microhabitats which could explain the increased survival in the sandy soil.

The importance of soil moisture was indicated in a study by García-Orenes et al. (2007). All irrigated soils were found to have a greater coliform survival than all soil types without irrigation. Of the irrigated soils, the soil with the highest clay content had the highest coliform survival

4.4.9 Effect of organic matter on the survival of coliform bacteria in soil

In this investigation two soil depths were studied to determine the effect of organic matter on the survival of *E. coli* and *E. aerogenes*. Soil E was collected as described in section 4.2.2.4. Soil description and soil analysis is shown in Table 4.1. The top 10 cm of the soil profile has an organic matter content of 8.47%, while the soil from a depth of 10 – 30 cm has 6.98% organic matter. Comparison of the

two soil layers which have different quantities of organic matter is shown in Figure 4.8.

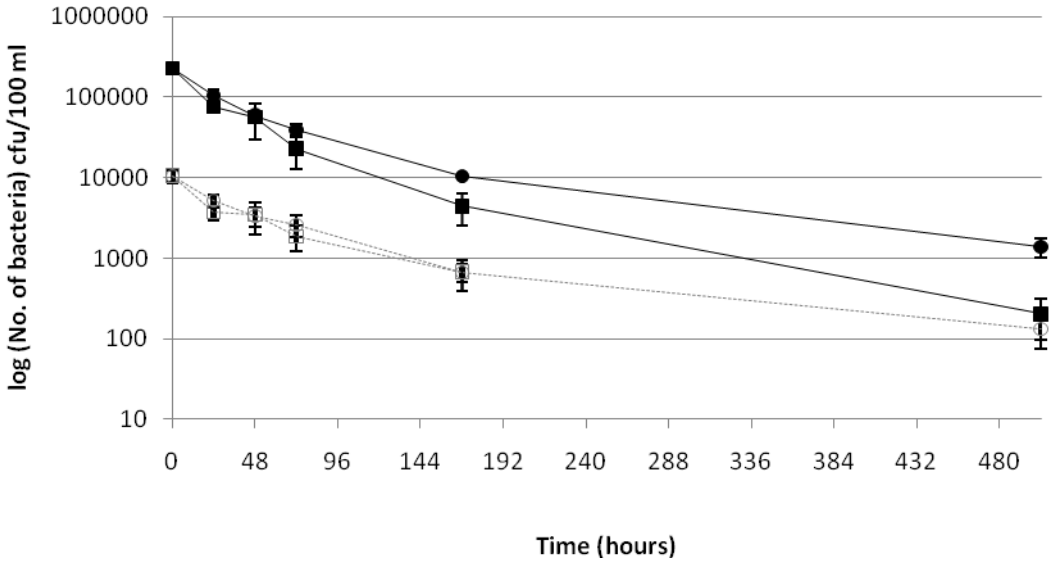

Figure 4.8: Comparison of the survival of coliform bacteria in soil taken from different depths with different organic matter contents
Error bars equivalent to one standard deviation (n = 3)
Soil from 0 – 10 cm depth: TC (●), E. coli (○),
Soil from 10 – 30 cm depth: TC (■), *E. coli* (□)

Total coliforms have a slightly increased survival time with soil from the top 10 cm of the soil profile when compared to soil from a depth of 10 to 30 cm. However in this study the difference in survival for *E. coli* in the two soils is insignificant.

A number of studies have shown that increased organic matter can increase coliform survival in soil and possibly also allow regrowth of bacteria (Dazzo, Smith and Hubbel 1973; Tate 1978). However Temple, Camper and McFeters (1980) also recognised that the extended survival and growth in organic soils may be due not only to the presence of organics but to the high moisture holding capacity of these soils.

The soil used in this study was fresh with no coliform bacteria detected prior to the study. It is interesting to compare the behaviour of the coliform bacteria in this study to the previous studies carried out at the same temperature. In the other studies there is generally growth of coliform bacteria however in this study there is a fairly rapid decrease in coliforms numbers. An explanation for this decrease can probably be explained by the presence of indigenous soil microorganisms which will reduce coliform numbers either through predation or competition. Although the soil used in the other studies was unsterile, due to long term storage, the presence of indigenous soil microorganisms would have been very low or absent.

4.5 Conclusions

The primary aim of this study was to recognise factors affecting the survival of coliform bacteria in water and soil. In laboratory based studies using sterile water

and aged soil it was very easy to recognise distinct factors which have an effect on coliform survival. However this proved to be more challenging when using a fresh soil sample.

There are many factors which have an impact upon the survival of coliform bacteria in both water and soil. In this study the effect of temperature and pH were investigated with regards to coliform survival in the water environment. A summary of these findings are shown in Table 4.5.

Table 4.5 Summary of effect of environmental conditions on growth and survival of *E. aerogenes* and *E. coli* in water over a specified period of time

Environmental Conditions		***E. aerogenes***	***E. coli***
Temperature (28 days)	37˚C	⬇	⬇
	10˚C	no observed effect	no observed effect
	4˚C	⬇	⬇
pH (14 days)	4.09 – 4.56	no observed effect	no observed effect
	9.07 – 9.39	⬇	⬇

Factors influencing coliform survival within the soil environment are summarised in Table 4.6.

Table 4.6 Summary of effect of environmental conditions on growth and survival of *E. aerogenes* and *E. coli* in soil over a specified period of time

Environmental Conditions		*E. aerogenes*	*E. coli*
Temperature (28 days)	20˚C	⬆	⬆
	10˚C	⬆	⬆
	4˚C	⬇	⬇
	0˚C	⬇	⬇
Repeated freeze/thaw (7 days)		⬇	⬇
pH (7 days)	7.00	⬆	⬆
	5.38	⬆	No observed effect
	4.50	⬇	⬇
Colloids (7 days)	Presence	⬆	⬆
	Absence	⬇	⬇
Clay content (7 days)	High	⬆	⬆
	Low	No observed effect	No observed effect
Organic matter[1] (21 days)	presence	⬇	⬇
	absence	⬇	⬇
Soil	aged	⬆	⬆
	fresh	⬇	⬇

[1] Fresh soil used

It is important to take into account the fact that the majority of these investigations were carried out using sterile water and aged soil which is likely to have a much lower indigenous soil bacterial community, if any, than a fresh sample. The presence and effect of natural soil bacteria was evident in the study using fresh soil samples where the survival of coliform bacteria was reduced significantly. The extent of this observation was surprising as increased levels of organic matter have been shown to enhance coliform survival. However the response is attributed to the presence of indigenous soil bacteria.

The importance of recognising differing effects found in laboratory based experiments compared to those found in the natural soil and water environments need to be understood. This study looked at individual factors affecting longevity of coliform bacteria whilst in the natural environment many factors can and do interact to influence their survival. One of the factors having the greatest influence on coliform survival was soil texture, with the presence of clay particles promoting not only survival but growth. Within the UK, groundwater is generally maintained at 10°C, which permits the survival of coliform bacteria. However within the natural soil environment in the UK, coliform bacteria have the potential to not only survive but to increase in numbers. The main limiting factor recognised during this study is the competing effects shown by presence of indigenous bacteria.

Chapter 5 Movement of bacteria through soil

5.1 Introduction

Movement of bacteria through soil increases the potential for water supplies to become contaminated which may then pose a risk to human health. The study and subsequent understanding of bacterial transport through soil is therefore of great importance.

Within the soil profile the surface soil and the vadose zone (area lying between the ground surface and the saturated zone) are usually unsaturated whereas the groundwater zone is usually saturated. Underlying this there will be an impermeable layer or bedrock. The soil and vadose zones offer protection for groundwater by shielding it from contamination by microorganisms or chemicals. The distance between the surface soil and the groundwater is often significant for determining the likelihood of groundwater contamination; the greater the distance the more opportunity for natural filtration through the soil to occur. However variations in each site such as soil type and treatment must also be taken into account as they may affect the potential filtering effects of the soil (Conboy and Goss 2000).

Soil is classified according to the size of its individual particles. This classification is made on the relative amounts of each of the sand, silt, and clay particles within a soil. With reference to the British Soil Texture Classification system (BSTC) sand is larger in diameter than silt and clay particles and its particles are visible to the naked eye. The diameter of particles ranges from very fine (0.06 mm) to very coarse (2 mm) in diameter. Sandy soils tend to be limited in nutrients because nutrients leach out from the large pore spaces between sand grains. Silt contains silicate minerals like sand but the diameter of silt particles are smaller (0.06 – 0.002 mm). Pore spaces between silt particles are smaller than sand therefore silt has the ability to hold water between particles and can retain nutrients for plant use. Clay contains silicates, mica, quartz, carbonate and metal oxides. Clay particles are 0.002mm in diameter or smaller so the pore spaces between clay particles are very small. Thus water and air movement through clay particles is significantly decreased. When clay becomes wet it swells, cohesion occurs and it feels sticky. As wet clay dries, it shrinks and cracks. Clay also becomes dense, hard and brittle making it difficult for plant roots to grow through (Ashman and Puri 2002).

The key processes involved in physical transport of microorganisms through soil have been summarised by Gerba, Yates and Yates (1991) as advection, dispersion, adsorption and filtration. The extent each of these processes has on the transport of microorganisms often depends on factors such as soil type, soil

porosity, the microorganism in question and weather conditions.

Advection is the primary process by which bacteria are transported through the soil profile. In unsaturated permeable soil, water movement is primarily vertical because of the force of gravity. Particles are carried in the soil solution at a rate equal to the average velocity of the water flow. The flow rate and degree of saturation of the soil can play a significant role in determining transport potential. In general, higher water content and greater flow velocities results in increased transport (Wong and Griffin 1976; Worrall and Roughley 1991). Irrigation and rainfall have been shown to increase movement through soil. Trevors et al. (1990) showed that bacterial movement in soil columns was negligible when there was an absence of downward water flow. However, following percolation bacteria could be detected throughout the columns and in leachate.

Bacteria can also be transported in overland flow which can occur if the intensity of rainfall is exceptionally high and no further soil saturation is possible. Overland flow can transport bacteria significant distances often with their ultimate destination being surface waters. Surface or subsurface runoff is likely to occur if the soil is saturated or is heavy clay. In fine textured clay soils, macropores such as shrinkage cracks, fissures, root holes and earthworm channels encourage the occurrence of preferential flow. These flow pathways can cause rapid movement of solutes through soil. Water flow occurring through large pores and channels results

in bypassing of the natural filtering effect of the soil (McCoy and Hagedorn 1979). Unc and Goss (2003) found that macropores associated with clay soils was one of the most important factors for increased flow and therefore the transport of bacteria through soil. Smith et al. (1985) compared intact and corresponding disturbed soil. It was found that at least 93% of the inoculated *E. coli* cells were retained in disturbed soil. In contrast only 21 to 73% were retained in intact cores. Similarly van Elsas, Trevors and Overbeek (1991) showed greater transport in undisturbed soils than in repacked soils probably due to macropores in the undisturbed soil.

Dispersion is the spreading of the organisms as they pass through the sub-surface medium. This is a function of the variation in actual compared with average, pore water velocity and the effect of Brownian movement. As matrix potential falls, water will drain from pores and water content together with pore size will determine the ability of microorganisms to move through soil either by active movement or Brownian motion (Wong and Griffin 1976; Worrall and Roughley 1991).

Soil porosity depends on the ratio of the volume of void spaces to the total soil volume. Generally the porosity of clay soils which have small particle size is larger than that of sandy soils which have large particle size but because of the arrangement of particles, not all of the void space is available for water flow (Dighton et al. 1997). There is greater potential for microbial movement in coarse than fine soil because of the larger pore spaces (Bitton, Lahav and Henis 1974).

Microbes naturally adhere to surfaces such as soil particles. This is referred to as adsorption. It is thought that the majority of enteric bacteria in soil and aquatic systems are associated with sediments and that these associations influence their survival and transport. There are factors that control microbial adsorption to and detachment from solid surfaces. Microbial adsorption is the influence of cell surface properties however there is wide variation in these properties among genera and even species. The key cell surface factors influencing adsorption are charge and hydrophobicity. There are two types of bacterial adsorption, these being weak adsorption which is due to van der waals forces exceeding repulsive forces, and strong adsorption due to cellular appendages such as fimbriae and pili, or extracellular polymers excreted from the cell (Palmateer et al. 1993; Marshall 1980; Marshall 1986).

Different soil types have varying adsorptive properties associated with their colloidal matter. Therefore soil type is a major factor influencing microbial transport in soils (Bitton, Lahav and Henis 1974; Smith et al. 1985, Paterson et al. 1993). The large surface area per given volume of clays make them ideal adsorption sites for bacteria in soils. Thus adsorption plays a more important role in the removal of microorganisms in soils that contain clays. Ling et al. (2002) found in adsorption trials that four times more *E. coli* was adsorbed in clay loam than silt loam. However, bacteria sorbed to soil can become resuspended and travel significant distances under saturated conditions (Rahe et al. 1978).

Filtration limits the movement of bacteria through soil by removing organisms from the pore water by size exclusion. Filtration becomes an important mechanism when the limiting dimension of the microbe is greater than 5% of the mean diameter of the soil particles. This is particularly relevant in a soil containing a significant portion of silt or clay particles where filtration will be a major mechanism of bacterial cell removal. When suspended particles, including bacteria, accumulate on the soil surface, these particles become the filter as water passes through the soil. Such a filter is capable of removing even finer particles by bridging or sedimentation before they reach and clog the original soil surface (Gerba and Bitton 1984). Sedimentation is frequently ignored when discussing microbial transport through the subsurface as advective processes are generally thought to overwhelm any significant effect of sedimentation. However sedimentation may occur when the density of the microbe is greater than that of the liquid medium and flow velocities are low (Gerba and Bitton 1984).

Other factors that may be influential in the movement of microorganisms within soil are the ionic strength of the soil solution, pH of the soil solution, the physiological state and activity of the microorganisms and the presence of macro organisms (Gerba and Bitton 1984).

Soil solution ionic strength influences bacterial transport. Transport is promoted when ionic strength decreases and when flow rate increases. Rainfall events can

influence the ionic strength in a porous medium as it increases the water flow rate. The added water will also generally lower the ionic strength of the soil solution. With particular relevance to clay soils, in the presence of high concentrations of monovalent cations such as Na^{+}, clays tend to be dispersed. Dispersed clays create puddled soils, which are sticky when wet and hard when dry. As a dispersed soil dries, compaction may occur, which reduces pore spaces, inhibiting soil aeration and reducing the capacity for water flow. This adversely affects the transport potential of microbes. The reverse occurs in the presence of divalent cations such as Ca^{2+} and Mg^{2+}, with smaller radii of hydration, which leads to flocculated soils, which have increased pore space and thus favour transport (Maier, Pepper and Gerba 2009).

The pH of the matrix solution within a porous medium does not seem to have a large effect on bacterial transport. Bacteria have very chemically diverse surfaces, and thus a change in pH would not be expected to alter the net surface charge to any great extent. The isoelectric point (pI) is the pH at which the molecule has a net charge of zero. For bacteria, the isoelectric point (pI) usually ranges from 2.5 to 3.5, so the majority of cells are negatively charged at neutral pH. At pH values more acidic than the isoelectric point, a microbe becomes positively charged. This will reduce its transport potential because of increased sorption. This will not happen often with bacterial cells in environmental matrices, given their low pI values (Davet 2004).

The physiological state and activity levels of bacteria may affect their survival which will subsequently affect their potential transport in the soil. The size and shape of bacteria play a role in bacterial removal by filtration. Bacteria may also have a variety of appendages such as flagella, pili or fimbriae. Pili and fimbriae are involved in attachment whereas flagella are concerned with bacterial motility. However the influence of bacterial motility on overall transport is generally minimal because extensive continuous water films would be needed to support microbial movement. Although continuous films exist, they are present only in soils with high soil moisture contents. In flowing systems, the primary mechanism of transport is advection. Advective transport will be many orders of magnitude greater than transport due to motility (Maier, Pepper and Gerba 2009).

There is also evidence that bacteria can travel through soil due to earthworm activity (Opperman, McBain and Wood 1987). Joergensen et al. (1998) showed that faecal coliforms were transported by earthworms to depths of 40 to 80 cm. Rusek (1985) found that earthworms could reach depths of approximately two metres. Thorpe et al. (1996) also showed that movement of bacteria in unsaturated soil is aided by earthworms.

The focus of this research was to determine the effect of soil texture and rainfall on the transport of bacteria through soil. This was initially carried out using repacked

soil columns of varying soil textures. This study was then extended to investigate intact soil cores and the effect of rainfall.

5.2 Materials and methods

5.2.1 Bacterial cultures and serial dilutions

Bacterial cultures of *E. coli* and *E. aerogenes* were prepared as described in section 2.2.1. A turbid solution of each of the cultures and subsequent serial dilutions were prepared as required using the method described in section 2.2.2.

5.2.2 Measurement of *E. coli* and *E. aerogenes* within bacterial suspensions using Colilert 18™

The coliform count in the bacterial stock suspension added to soil columns was analysed using Colilert 18™ to give an approximate number of coliform bacteria applied to the soil columns. A sample of 0.1 ml was removed from bacterial suspensions of dilution 5 and 6. This sample was made up to 100 ml using ¼ strength Ringers solution and analysed using the method described in 2.2.7.

5.2.3 Measurement of *E. coli* and *E. aerogenes* within soil samples using Colilert 18™

Soil (10 g) was weighed out from each section and 20 ml of sterile ¼ strength Ringers solution added. Each sample was shaken for 20 minutes. Soil solution (1 ml) was removed and made up to 100 ml with ¼ strength Ringers solution and analysed using Colilert 18™as described in Section 2.2.7. For each layer of soil, samples were taken in triplicate for analysis. The colony forming units (cfu) per section was calculated thus allowing an approximate cfu per column to be determined.

5.2.4 Collection and preparation of soil for repacked columns

Soil used for the preparation of repacked soil columns had been collected previously in association with other studies and had been stored for a number of years. This was referred to as Soil A as described in Section 4.2.2.

The repacked columns were prepared using plastic pipe of 4 cm diameter which was cut into 12 lengths each measuring 40 cm long. One end was sealed with mesh (pore size 500 µm) and 20 g sand added to the bottom of each column to aid drainage and prevent loss of soil particles.

Columns were filled with a combination of soil A and dried washed sand. The 4 variations of soil and sand mixtures are detailed in Table 5.1. Soil and sand mixtures were prepared by weighing out the required quantity of each into plastic bags, sealing and mixing thoroughly. The mixture was spooned into the column to ensure even packing and mixing. Each column was tapped 4 times to encourage some degree of settling. Three replicate columns of each combination were prepared.

Table 5.1 Soil and sand content of repacked soil columns

	Weight of Soil (g)	Weight of Sand (g)
Column 1	381	0
Column 2	254	127
Column 3	127	254
Column 4	0	381

The columns were saturated with water by placing them inside 2 litre measuring cylinders. Deionised water was added to the cylinder being careful not to wet the top layer of the soil column. Water was continually added over time as the soil soaked up the liquid until it was observed that the top of the soil was completely saturated with water. The columns were left for a further 30 minutes to ensure saturation. The soil columns were then removed and the bottom of the columns

sealed prior to reweighing the column. The volume of water taken up by the soil column was measured and recorded.

The columns were attached to a clamp stand and the water allowed to drain from the columns. When no further draining was observed, the ends of the columns were sealed with plastic film, covered with aluminium foil and stored in the cold room until required.

5.2.5 Collection of undisturbed soil cores

Soil cores were removed from grass covered land at the Macaulay Institute, Craigiebuckler, Aberdeen. The land had not received any treatment for a number of years and was not used for grazing animals. This soil in the cores is the same as that described as Soil E in Section 4.2.2.

The soil cores were taken using rigid, cylindrical PVC pipes which were 40 cm long with an internal diameter of 15 cm. The leading edge of the pipe was tapered to allow it to enter the soil easily. Using mallets, each core was gently driven into the ground to a depth of 32 cm, ensuring that the cores entered the soil vertically. The

area surrounding each core was dug away to enable the core to be removed with minimal disturbance. The grass covering on each core was trimmed to a height of 2.5 cm. From the bottom of the core, 1.5 cm depth of soil was removed and replaced with sand. The base of each core was supported by nylon mesh (500 µm pore size) and fitted to a funnel. The soil cores were sealed with foil and kept in the cold room for 2 weeks prior to use.

5.2.6 Destruction of soil columns for soil analysis

Prior to using the undisturbed soil columns, one column was destroyed to obtain soil for analysis and to determine the background concentration of coliform bacteria. For this purpose a composite sample from the entire column was used. Similarly the repacked and the undisturbed soil columns were destroyed following completion of the studies to determine the presence and quantity of coliform bacteria within the column layers.

Each column to be destroyed was cut open lengthways using an electric rotary saw (Figure 5.1). The soil layers were separated into sections (100 and 66% soil mixtures separated into 4 sections and the 33% and 0% soil mixtures separated into 3 sections). The undisturbed soil cores were separated into four layers, each approximately 8 cm depth. Each section was placed in individual plastic bags and

mixed thoroughly. Soil was removed as required for soil analysis. Measurement of soil moisture was determined using the method described in section 4.2.3.

Figure 5.1 Destruction of undisturbed soil cores

5.2.7 Investigation of dye transport through repacked soil columns

The study of dye transport through soil columns was carried out to obtain information on the potential flow rate and movement of coliform bacteria through the columns. Three repacked soil columns of each soil / sand mixture were prepared as described in section 5.2.4.

The columns were saturated then attached to clamp stands and drained until they were at field capacity. (Field capacity is the amount of soil moisture retained in the soil after excess water has drained away for example following heavy rainfall). Filter paper was placed on each column on top of the soil. Blue food dye (1.5 ml) was added directly onto each filter paper and the start time noted. Water was applied to the surface of the soil columns (surface area = 12.56 cm^2) at a rate of 3.4 ml per minute via a pump and allowed to drip approximately 2 – 3 cm above the filter paper (Figure 5.2).

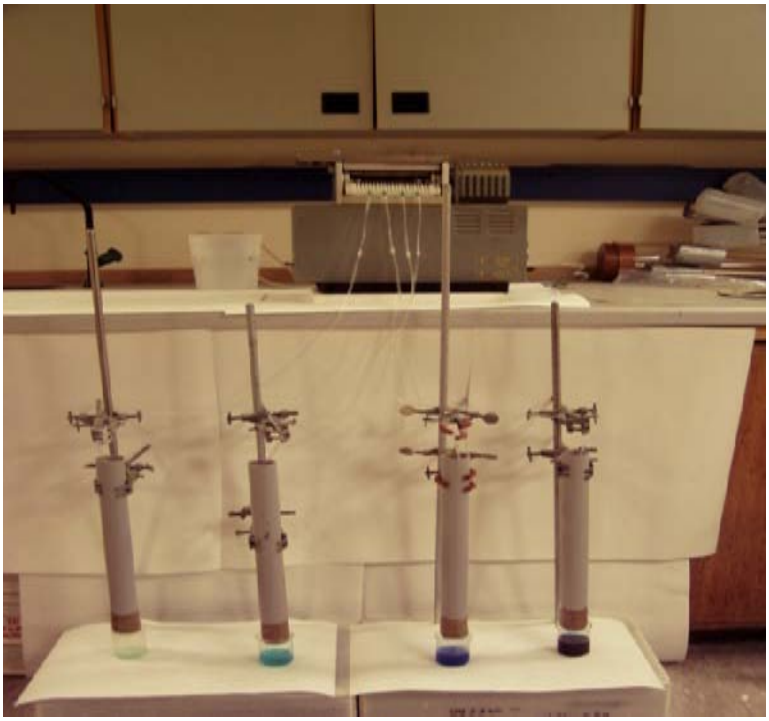

Figure 5.2 Soil columns for dye transport study

The rate of simulated rainfall onto the soil columns was calculated using the following equation:

$$\text{Rate of simulated rainfall (mm / hr)} = \frac{\text{Flow rate of water (ml / min)}}{\text{surface area of soil (cm}^2\text{)}} \times 60$$

The rate of simulated rainfall was calculated as 16.2 mm / hr. The drainage water was observed for signs of the dye. It was noted when the drainage water started to change colour, the degree of colour, and when it was observed that all dye colour had visibly disappeared.

5.2.8 The effect of soil texture on transport of *E. coli* and *E. aerogenes* through repacked soil columns

Repacked soil columns of each soil / sand mixture were prepared in triplicate. Each column was saturated, noting the volume of water required in each case. The columns were attached to clamp stands and drained until they were field moist. All cores needed to be at field capacity prior to the start of the experiment to enable them to be compared. Each column was attached to a clean funnel and a clean collection vessel placed underneath. One ml of serial dilution 5 of both *E. coli* and *E. aerogenes* was pipetted directly onto the soil surface. Filter paper of 4 cm

diameter was placed on top of each column directly onto the soil mixture. A peristaltic pump was set up to feed water continuously to each column at a flow rate of 3.4 ml per minute giving a flow rate of 16.2 mm/hr as in 5.2.7.

The water was dripped onto the filter paper, which allowed for its uniform dispersal over the soil mixture. Drainage water was collected at pre determined intervals. For the 100% soil columns and the 67% soil columns, samples were taken every 15 minutes initially. The 33% and 0% soil columns had samples taken every 10 minutes. As time progressed the time between sample collections was increased. At each designated collection time a clean (although not sterile) container was placed under the funnel to collect 10 ml of solution. This was then analysed for coliform bacteria using Colilert 18™ as described in method 2.2.7. The number of coliforms in each sample volume was determined. The drainage water from each column was measured to ensure the flows through each column were the same.

On completion of the study, the soil column was destroyed as described in section 5.2.6. From each soil layer 10 g was removed and mixed with 20 ml of ¼ strength Ringers solution. One ml of sample was removed and analysed using Colilert 18™ as described in section 5.2.6. The approximate number of coliforms within each soil column could then be calculated.

5.2.9 Investigation of transport of *E. coli* and *E. aerogenes* through undisturbed soil cores

Undisturbed soil cores each with a funnel attached to the bottom were placed into a wooden frame to support the cores and to keep them vertical (Figure 5.3). Using a template to ensure even distribution, 1 ml of serial dilution 4 of both *E. coli* and *E. aerogenes* was pipetted onto the grass as near to the soil surface as possible. Filter paper of diameter 15 cm was placed on top of the grass.

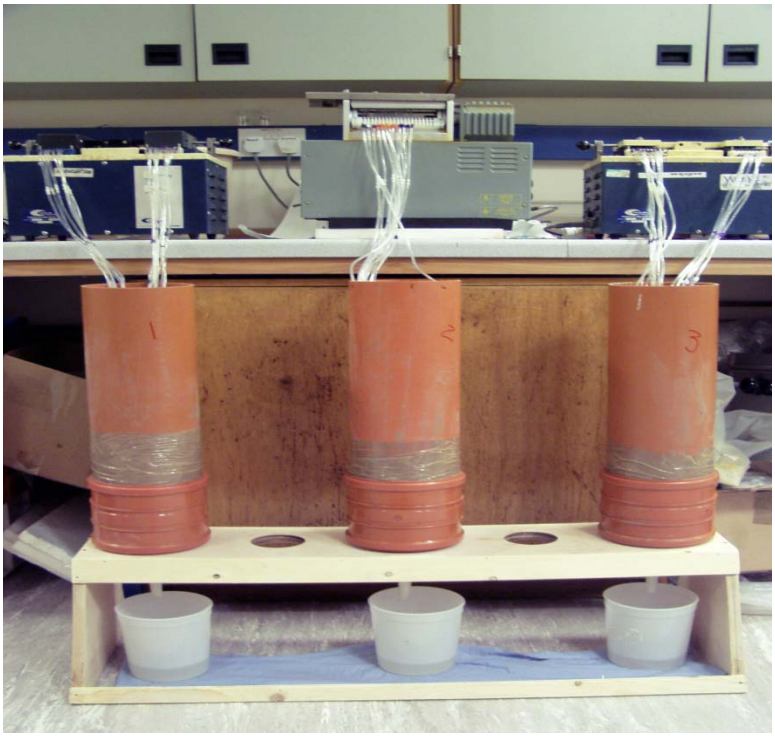

Figure 5.3 Study of coliform transport through undisturbed soil cores

A peristaltic pump was set at a rate comparable to that used for the repacked soil columns to give a simulated rainfall rate of 16.2 mm / hr. In this case the surface area of the column was 176.63 cm^2 which is 14 times greater than the repacked soil columns. Therefore the flow rate was set at 47.6 ml per minute to each soil column. The pump fed four tubes to each soil column at a height of 5 cm above the grass allowing water to be dripped onto the filter paper and allowing for its uniform dispersal over the soil column. Drainage water was collected at pre-determined intervals, initially this was every 15 minutes. At each designated collection time a clean collection container was placed under the funnel to collect 10 ml of solution which was analysed for coliform bacteria using Colilert 18™ as described in method 2.2.7. The drainage water from each column was measured to ensure an equivalent flow travelled through each column.

On completion of the study, the soil column was destroyed as described in section 5.2.6. From each soil layer 10 g was removed and mixed with 20 ml of ¼ strength Ringers solution. Analysis of 1 ml of sample was carried out as described in section 5.2.3 using Colilert 18™. The approximate number of coliforms retained within each soil column could then be calculated.

5.3 Results and discussion

5.3.1 Concentration of *E. coli* and *E. aerogenes* in bacterial suspensions used in the study of soil columns

The quantity of coliform bacteria in serial dilutions 5 and 6 of the bacterial suspensions used in the study of repacked soil columns and undisturbed soil cores was determined. These results are shown in Tables 5.2 and 5.3. Colilert 18™ was used for analysis prior to adding a known quantity of the bacterial suspensions to soil columns.

Table 5.2 Quantification of coliform bacteria in serial dilutions 5 and 6 used for the study of repacked soil columns.

Organism	Dilution 6 (cfu / 0.1 ml)	Dilution 5 (cfu / 0.1 ml)	Dilution 5[1] (cfu / 1 ml)
E. coli	106.0	913.9	9139
E. aerogenes	364.9	>2419.2	36490

[1]Estimated values

Table 5.3 Quantification of coliform bacteria in serial dilutions 4, 5 and 6 used for the study of undisturbed soil cores

Organism	Dilution 6 (cfu/ 0.1 ml)	Dilution 5 (cfu/0.1 ml)	Dilution 5[1] (cfu/1 ml)	Dilution 4[1] (cfu/1 ml)
E. coli	136.7	1299.65	12996.5	129965
E. aerogenes	271.7	>2419.2	27170.0	271700

[1]Estimated values

5.3.2 Background concentration of coliform bacteria in soil columns

The background level of coliform bacteria in soil A, used for the repacked columns, and in the undisturbed soil column was determined. Three soil samples were taken from each. Results are shown in Table 5.4.

Table 5.4 Background concentrations of coliform bacteria in soil

Column Type	Sample	Total coliforms in soil column (cfu)	*E. coli* in soil column (cfu)
Undisturbed	1	324800	0
	2	873600	0
	3	708960	0
Repacked (Soil A)	1	0	0
	2	0	0
	3	0	0

Each sample taken from the undisturbed soil core contained coliform bacteria. Coliform bacteria are indigenous to the soil environment therefore it was expected that they would be present. No coliforms were detected in Soil A probably due to the length of time it had been stored. There was no *E. coli* detected in any sample.

5.3.3 Dye transport through repacked soil columns

The time taken for dye to travel through repacked soil columns of varying soil and sand quantities was investigated. Observations made are shown in Table 5.5. The information gained from the study was used to plan an investigation into coliform bacteria transport in soil columns.

Colour was first noted in all 4 columns between 20 and 32 minutes following commencement of water flow. In most of the columns the initial colour was yellow / green then turning to a bright blue colour. However in the sand columns the blue colour was constant throughout. It must be noted that observing for colour change is very subjective. Dye may have been travelling out of the column before and after these times particularly in columns containing soil, but in such small quantities so as not to be seen by the naked eye.

The pump flow rate was equivalent to 16 mm/hr. Storm flow rate is normally 8 – 10 mm / hr, therefore this rate is 1.5 – 2 times greater than storm flow. To be able to compare transport through each column, the time taken for the dye to pass through 1 cm of soil / sand mixture at a water flow rate of 3.5 ml per minute was calculated. It was shown that the dye passed through sand almost 4 times faster than through the soil used in this experiment.

Table 5.5 Observations on dye transport through repacked soil columns

Soil : sand ratio	Depth of soil (cm)	Time for dye to be visible (min)	Time for dye to travel through column (min)	Average time for dye to travel one cm (min)
1:0	37.5	32	422	11.25
2:1	32.5	31	297	9.14
1:2	27.5	27	211	7.67
0:1	22.5	20	64	2.84

Although the same weight of soil was used in each column it is clear to see the importance of particle size when investigating transport through the columns. The same weight of soil compared to sand gives almost double the volume in the column. The dye moves through the sand column very quickly, almost in a

constant plug, however as the soil volume increases the dye time in the column is extended.

The aim of this experiment was to get some guidance on expected flow rate through the soil columns. This can then be used in the preparation of the study using bacteria to give an expectation of their transport and movement through the column whilst taking into consideration some of the factors involved in their transport.

5.3.4 Effect of soil texture on movement of *E. coli* and *E. aerogenes* through repacked soil columns

The bulk density, porosity and volume of water required to saturate each column is given in Table 5.6, as well as the moisture contents of the repacked soil columns which were determined following completion of the transport study.

Table 5.6 Soil properties of repacked soil columns

Column Type	Bulk density (g/cm^3)	Porosity (%)	Volume of water to saturate (ml)	Soil sample depth (cm)	Moisture content[1] (%)
100% soil	0.85	69	208	0 – 19	32
				19 – 36	35
66% soil 33% sand	0.98	64	157	0 – 16	25
				16 – 32	29
33% soil 66% sand	1.15	57	130	0 – 9	14
				9 – 18	21
				18 – 27	24
100% sand	1.41	48	90	0 – 7.5	10
				7.5 – 15	13
				15 – 22.5	15

[1] on completion of experiment

The movement of coliform bacteria *E. coli* and *E. aerogenes* through repacked soil columns of varying soil and sand quantities was investigated. The number of coliform bacteria including *E. coli* transported through the four column types are shown in Figure 5.4 (a, b, c and d).

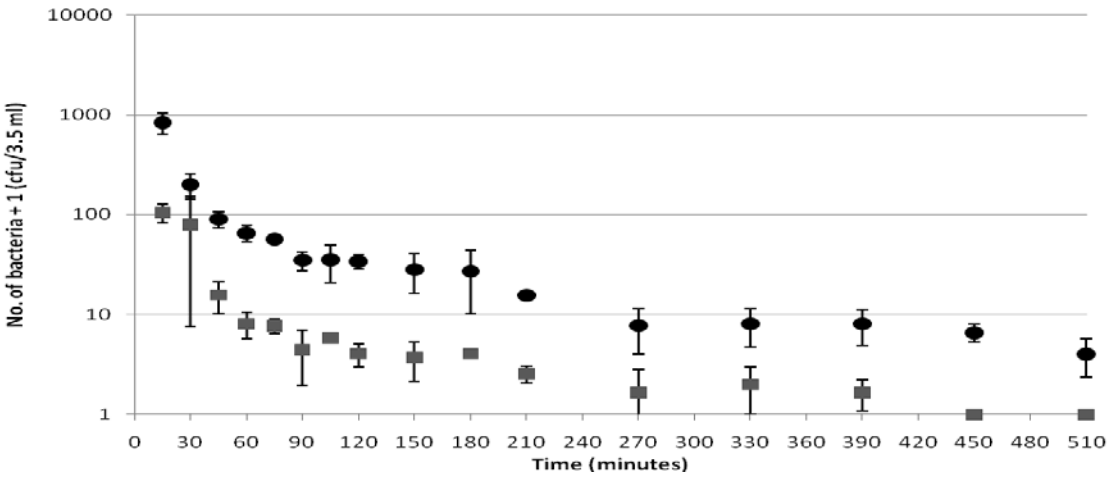

Figure 5.4(a) ***E. aerogenes*** **and** ***E. coli*** **eluted from repacked soil columns containing 100% soil.**
Error bars equivalent to one standard deviation, n = 3
Total coliforms (●) *E. coli* (■)

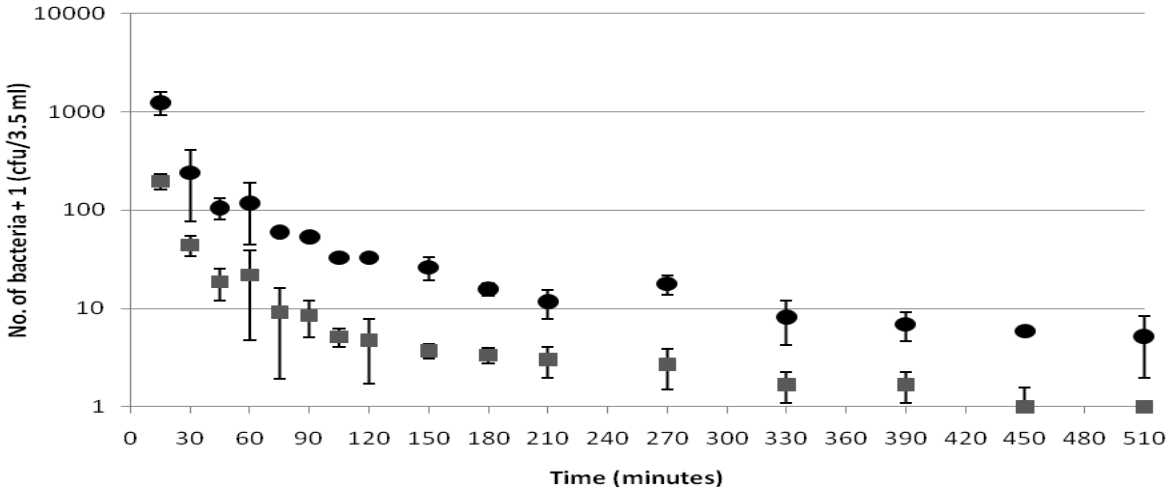

Figure 5.5(b) ***E. aerogenes*** **and** ***E. coli*** **eluted from repacked soil columns containing 66% soil and 33% sand.**
Error bars equivalent to one standard deviation, n = 3
Total coliforms (●) *E. coli* (■)

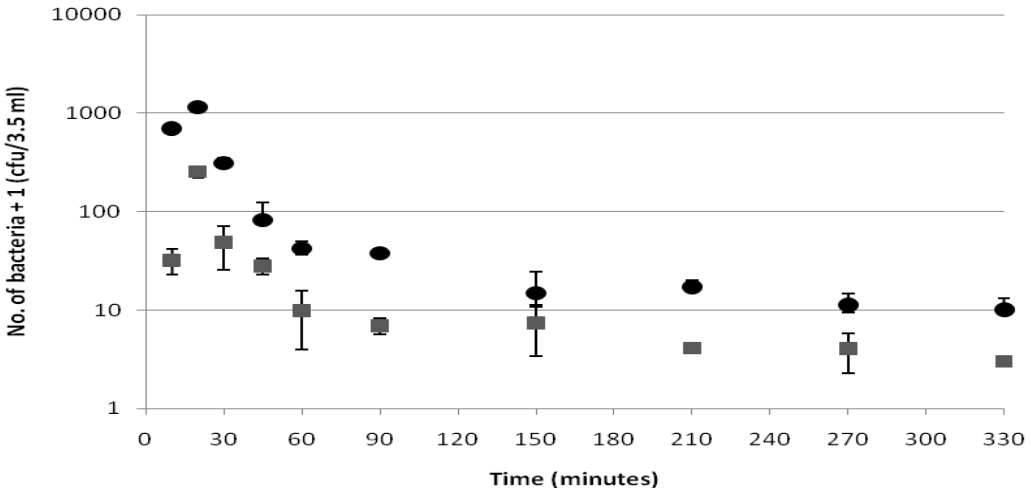

Figure 5.4(c) ***E. aerogenes*** **and** ***E. coli*** **eluted from repacked soil columns containing 33% soil and 66% sand.**
Error bars equivalent to one standard deviation, n = 3
Total coliforms (●) *E. coli* (■)

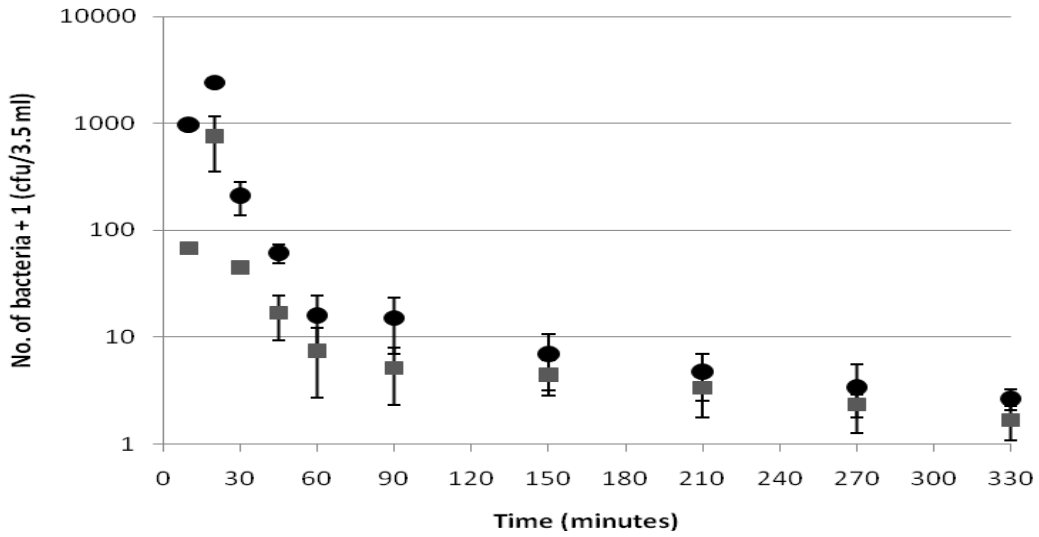

Figure 5.4(d) ***E. aerogenes*** **and** ***E. coli*** **eluted from repacked soil columns containing 100% sand. Error bars equivalent to one standard deviation, n = 3**
Total coliforms (●) *E. coli* (■)

The highest bacterial counts in drainage water were initially detected from the 100% sand column and counts remained high for the first 30 minutes before rapidly decreasing. Similarly with the columns containing soil, initial bacterial counts were high, noticeably reducing after 45 minutes. There was however faster drainage recorded with the sand column, with increasing soil in columns causing the water to travel through the column at a slower rate initially. The column with 100% soil had slightly lower bacterial counts in the initial drainage water than the other columns containing soil.

An estimate of the number of bacteria transported through the soil column during the study period could be calculated based on samples taken at intervals ranging from 10 minutes to hourly. The colony count per 3.5 ml could be extrapolated from the spot samples. Colony counts out with the sample times were estimated by taking the average value from the preceding spot sample and the following spot sample to give a cfu/3.5 ml for every minute of the study. These estimated values are shown in Table 5.7.

The number of bacteria remaining in the soil column on completion of the study could be estimated from soil samples taken following the destruction of the soil columns. These estimated values are also shown in Table 5.7.

Table 5.7 Extrapolated values of coliform bacteria transported through and remaining within the repacked soil columns

	Drainage water		Soil column		Total	
% soil	TC (cfu)	*E. coli* (cfu)	TC (cfu)	*E. coli* (cfu)	TC (cfu)	*E. coli* (cfu)
100	12868	1916	14850	610	27718	2526
66	16374	2682	10760	300	27134	2982
33	17951	3451	6680	100	24631	3551
0	24319	6797	2260	0	26579	6797

These extrapolated values allow the four column types to be compared. It is apparent that the greater the soil content the more likely the coliform bacteria are to remain within the soil column. It was also recognised during the soil investigation that the majority of coliform bacteria retained in the soil were found within the top layer of the soil column. In the case of the 100% and 66% soil columns the majority of the retained coliform bacteria were found in the top 25% (10 cm) of the soil. This accounted for 84 – 92% of the retained coliforms. In the 33% and 0% soil columns the majority of retained coliform bacteria were found in the top third of the soil. This accounted for 95% of the retained bacteria in these soil columns. A study by Gerba, Wallis and Melnick (1975) found that greater than 90% of bacteria applied to soil remain within the first centimetre. Even with the fast flow rate used in this study, the majority of bacteria retained within the soil were found in the upper part of the column which seems to suggest that they are either trapped or attached to particles within the soil. A greater number of coliforms are retained within the 100 % soil column than the 100 % sand column. The porosity of the soil is greater than

that of sand but generally the pore sizes within the soil are smaller so more likely to trap colloids and particulate matter.

The greatest moisture content within the repacked columns was seen in the lower layers of the column. The presence of organic matter increases the moisture holding capacity of soil however in this case the organic matter content of Soil A, although not determined was likely to be negligible due to the extended storage time of the soil. As the sand content increases within the repacked columns, the moisture content decreases due to good drainage and poor binding properties of sand.

The recovery of total coliforms is similar overall between the four column types. However there is a much greater recovery of *E. coli* from the drainage water from the sand columns than those containing soil. The presence of *E. coli* in the effluent decreases as the soil content increases. On destruction of the columns higher *E. coli* counts were detected within columns containing greater soil content, counts decreased as sand content increased. However counts detected within the columns were much lower than expected, with no *E. coli* detected in the sand column on destruction. This may be explained by the fast elution of coliforms from the sand column. McCaulou, Bales and McCarthy et al. (1994) found that retention of bacteria in sand columns is significantly increased at low water contents and that

it takes approximately 1 hour for bacteria (gram negative rods) to attach within the soil.

Although the highest *E. coli* numbers were found within the 100% soil columns, these were also the columns which showed the largest reduction overall of *E. coli*. It is possible that while this column allowed more attachment and retention of *E. coli* due to the nature of the soil, it also allowed increased moisture to be retained. Time spent in the cold room with the soil moist, could have encouraged the growth of microorganisms in the soil which have been detrimental to *E. coli* survival.

5.3.5 Evaluation of transport of *E. coli* and *E. aerogenes* through undisturbed soil cores

The movement of the coliform bacteria, *E. coli* and *E. aerogenes*, through undisturbed soil cores was investigated. The number of total coliforms and *E. coli* transported through three replicate soil cores following the addition of water to simulate heavy rainfall is shown in Figure 5.5.

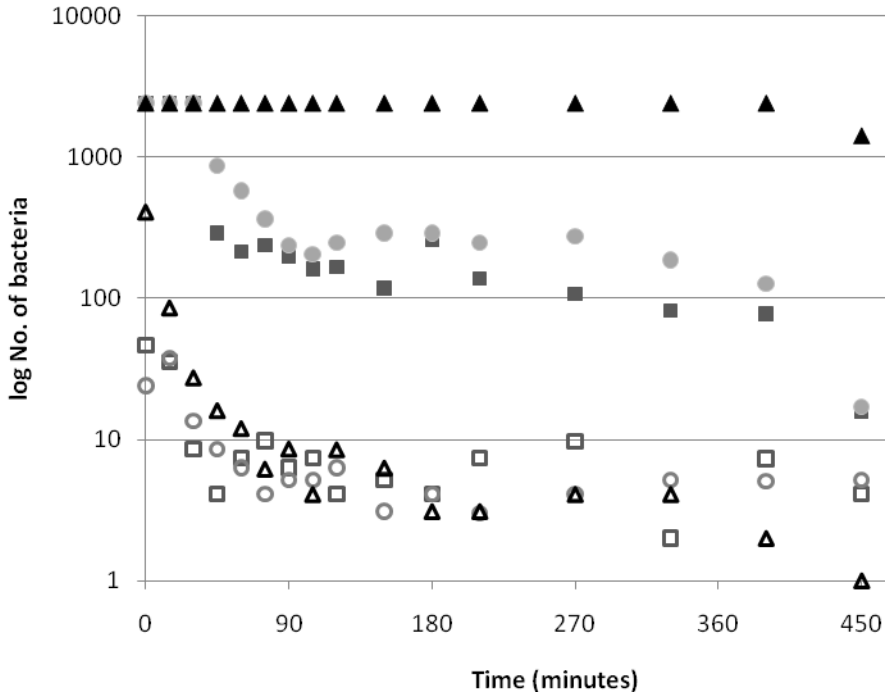

Figure 5.5 ***E. aerogenes*** **and** ***E. coli*** **eluted from undisturbed soil cores following simulated rainfall (Soil core 1: TC (■) *E. coli* (□), Soil core 2: TC (●) *E. coli* (○), Soil core 3: TC (▲) *E. coli* (△))**

Soil core 3 has a much greater concentration of coliform bacteria in the drainage water than the other two soil cores. Initially the *E. coli* counts within soil 3 are elevated in comparison to the other columns however they decrease to similar numbers after 15 minutes. It is possible that there is a pocket of faecal contamination within or on the surface of this core containing *E. coli* and other coliform bacteria.

An estimate of the number of bacteria transported through the soil column during the study period could be calculated based on samples taken at intervals ranging from 15 minutes to hourly. The colony count per 47.8 ml could be extrapolated from the spot samples. Colony counts between samples were estimated by taking the average value from the preceding spot sample and the following spot sample to give a cfu/47.8 ml for every minute of the study. These estimated values are shown in Table 5.8.

The number of bacteria remaining in the soil column on completion of the study could be estimated from soil samples taken following the destruction of the soil columns. These estimated values are also shown in Table 5.8.

Table 5.8 Extrapolated values of coliform bacteria transported through and remaining within the undisturbed soil cores

	Drainage water		Soil column		Total	Total
Column	**TC (cfu)**	**E. coli (cfu)**	**TC (cfu)**	**E. coli (cfu)**	**TC (cfu)**	**E. coli (cfu)**
1	164435	41815	208968	26378	373403	68193
2	220079	35737	237398	20809	457477	56546
3	1262959	78311	3215555	17585	4478514	95896

The quantity of coliform bacteria detected in the drainage water is an approximation as are the bacterial counts revealed within the soil columns. However this estimate does allow the three columns to be compared.

With reference to the number of coliform bacteria found in the soil columns, soil core 3 has a tenfold increase compared to the other cores. This validates the suggestion that there has been previous faecal contamination on or within this core, although there was no visible sign of contamination present. The source could have been a domestic dog or cat although wildlife such as deer and rabbits are also seen in this area.

The mean moisture content (n=3), within the upper layer of the undisturbed column was 33 % which was considerably higher than the lower layer which was found to be 23 %. The variation in moisture content can be explained by the higher organic matter content in the upper 8 cm of the undisturbed soil core which would allow for more water to be retained by the soil. As with the repacked soil columns the greatest concentration of coliform bacteria remaining in the soil were found in the top 25% (8 cm) of the soil core. The rate of water addition to replicate rainfall was greater than storm flow in the UK. It is likely to cause a much greater flushing effect than that likely in the natural environment. However it is interesting to find that even at this increased rate many bacteria remain trapped or adsorbed within the soil. This may be due to dissolved cations in the water which are known to promote adsorption of bacteria to solid surfaces.

In the two columns showing no evidence of background *E. coli* contamination, there were no coliform bacteria detected in the lower section (20 – 40 cm depth) of the destroyed soil column. The repacked soil columns in the previous study had coliform bacteria throughout the column although often in low quantities. This contradicts findings of van Elsas, Trevors and van Overbeek (1991) who found that introduced bacteria were transported to lower soil layers to a significantly higher degree in undisturbed soil cores than in repacked cores, with macropores in the undisturbed cores given as the most likely reason for this behaviour. Pang et al. (2003) used repacked columns to study bacterial removal in soil and concluded that the removal of bacteria in the study was primarily by filtration (87-88%) and die off (12 -13%).

There is also evidence to suggest that the initial rainfall has the most impact on bacterial transport as the quantity of coliform bacteria in the effluent reduces over time. This is in agreement with Trevors et al. (1990) and Stoddard, Coyne and Grove (1998) who found that even with recurring rainfall events, the initial event is the one that causes most displacement and movement through the soil column. It is thought that the majority of the introduced cells have adhered to soil particles or entered relatively protected soil pores following the first percolation.

There are a number of other factors which have been shown to influence the movement of coliform bacteria and other microorganisms within soil but which this study has not discussed. In soils without percolating water, Thorpe et al. (1996) found that earthworm activity could produce large pores in soil which assisted the downward movement of bacteria through the soil. The presence of plant roots in the absence of groundwater flow aided the movement of introduced *Pseudomonas fluorescens* (Trevors et al. 1990). It must also be recognised that agricultural activities such as ploughing can alter the soil structure and remove macropores, which in turn reduces the effects of preferential flow (Dighton et al. 1997).

5.4 Conclusions

In the studies of coliform transport through repacked soil columns and undisturbed soil cores there is clear evidence that water flow is of great important to the movement of bacteria from the soil surface throughout the soil column. In this study the application of water to the soil columns was rapid, however coliform bacteria still have the ability to remain within the soil matrix. It can then be assumed that if the flow of rainfall is less but still present, there is a greater potential for bacteria to become attached or trapped within the soil structure. The study using the four repacked columns with varying quantities of sand and soil

indicates that soil texture is influential in the retention of the bacteria. In columns where there is only sand the coliform bacteria are flushed through the column rapidly whereas in the column containing only soil this effect is greatly reduced with more coliforms being retained within the soil column. This highlights the importance of soil composition in the transport of microbes into groundwater supplies with areas where soils have high sand content being of greatest risk of groundwater contamination from surface applied faecal matter.

There is a need for careful consideration of results from laboratory based studies using repacked soil columns. It is evident that results can be very different from that occurring in the natural environment. Results from the repacked columns studied here only take into account the physical qualities of the soil and not the influence of intrinsic soil microorganisms due to the age of the soil. However, the study using undisturbed soil cores showed a major reduction of coliform bacteria most likely due to the presence of indigenous microorganisms. When bacteria are added to soil, the protozoan population will normally increase and the number of bacteria will reduce. This may be due to competition for nutrients or due to protozoa grazing on bacteria.

Chapter 6 Conclusions

6.1 Introduction

It is recognised that water is a precious resource which is essential to life itself. The entire human population has a responsibility to protect it. However human interaction and control of water resources in many cases has been detrimental to water availability and quality. Good quality freshwater resources are being diminished due to over abstraction of groundwater. Disease outbreaks linked to water supplies are well documented and outbreaks of potentially life threatening illness are increasingly common. A number of these outbreaks have been linked to the presence of pathogenic bacteria in private water supplies.

The aim of this research as stated in the objectives in section 1.9 was to investigate factors which influence the survival and transport of coliform bacteria in potable water and soil and to relate this to the microbiological quality of private water supplies in Aberdeenshire, Scotland. The study relied on methodology which could be repeated frequently, so it was essential that the method was quick, easy to carry out and would reliably detect and quantify coliform bacteria in potable water. The rapid testing method, Colilert 18™ was investigated and therefore had to be evaluated and comparison made to membrane filtration which is one of the recognised standard methods used today.

6.2 Key findings of study

The main findings of this thesis can be divided into sections which address the aims outlined in section 1.9.

6.2.1 Evaluation of Colilert 18™ for the detection of coliform bacteria in potable water and soil samples.

Colilert 18™ was successfully applied to this study. It was proven to be a suitable method for water testing and gave comparable results to the membrane filtration method. Colilert 18™ had the ability to detect coliforms and *E. coli* when bacterial counts were as low as 1 cfu and could also quantify large numbers of coliforms and *E. coli* to a greater degree of accuracy than membrane filtration. It has been suggested in a number of studies that Colilert 18™ gives false positives, however in this study with *A. hydrophila* and *P. aeruginosa* present, no false positives were detected. Colilert 18™ required less time for preparation and analysis than the membrane filtration method with the advantage of faster results. With Colilert 18™ results were available after 18 hours whereas membrane filtration can take over 42 hours to confirm the presence of *E. coli*. Overall, the use of Colilert 18™ was a good alternative to the membrane filtration method when used to quantify coliform bacteria in groundwater.

When using Colilert 18™ to detect coliform bacteria in soil, there was one significant issue. When too much soil was present in the soil solution taken for analysis there was difficulty reading Colilert 18™ due to samples being highly coloured. As Colilert 18™ gave positive confirmation of coliform bacteria by transformation of solution from clear to yellow, the soil quantity had to be low enough not to cause discolouration of the solution making accurate sample reading impossible. However enough soil must be used for analysis to ensure that the soil-bacterial interaction is investigated. Overall Colilert 18™ was found to be effective at detecting coliform bacteria within soil solutions.

6.2.2 Investigation of microbiological quality of private water supplies in Aberdeenshire, Scotland.

The historical evidence obtained from Aberdeenshire council confirmed that a high proportion of private water supplies did not meet statutory requirements for microbiological water quality. Often those supplies not regulated under legislation were most likely to have coliform bacteria present. Further research on a number of private water supplies in Central Aberdeenshire supported this data. The study indicated that over 50% of the 82 supplies studied, had *E. coli* present on at least one of three sampling events over a 1 year period.

It was concluded that contamination of private water supplies is primarily influenced by rainfall, temperature and land use. There was often an increase in both total and faecal coliforms detected in private water supplies following a period of heavy rainfall. Water sources surrounded by grazing animals were also likely to have an increased risk of contamination.

Ultimately, the contamination of private water supplies is linked to the type and depth of the supply, poor maintenance and supplies which are inadequately constructed and sealed. Boreholes are the least likely to be contaminated, with spring sources being the most susceptible to contamination. Protection of water supplies from contamination by surface water runoff is essential as is protecting the water supply from livestock and wildlife.

6.2.3 The analysis of water and soil samples using Colilert 18™ to study factors affecting coliform survival

Survival and movement of pathogenic bacteria is necessary for contamination of water supplies to occur. This study principally looked at individual factors affecting survival in both the water and soil environments.

Temperature was found to impact coliform growth and survival with the ideal temperature for survival found during this study to be 10°C. This corresponds with typical groundwater temperatures throughout the year in the UK. Little or no growth occurred at 4°C with eventual death of the bacteria over a number of days, however, while no growth occurred at freezing temperatures, coliform bacteria still remained viable for longer than coliforms at 4°C. However repeated freezing and thawing is known to damage the cell structure resulting in death. Coliform bacteria in soil survived as well as, and in many cases better than in water at the same temperatures.

Moisture content of the soil is often related to temperature. As temperature increases moisture content is likely to decrease which often has a detrimental effect on bacterial survival. The presence of organic matter in soil may increase coliform survival although this has also been linked to the increased water holding capacity of organic matter. Survival of coliform bacteria was reduced in intact soil columns which can be explained by the presence of indigenous microorganisms, either through predation or competition.

Other factors having a major influence on coliform survival were soil texture and the presence of colloids. Survival was enhanced as the clay particle content of soil increased and a greater percentage of coliform bacteria were detected attached to

colloid material than within the water column indicating that survival is likely to be greater within sediments.

It is however important to recognise that in the natural environment factors influencing coliform survival cannot be considered individually, as many factors interact to influence survival. This was recognised when comparing results from repacked soil columns and intact soil cores. It is also important to note that many of the factors important to coliform survival also play an intrinsic part in the transport of the bacteria.

6.2.4 The study of soil texture and rainfall and their influence on coliform transport in repacked and intact soil columns

Soil texture is influential in the retention and movement of coliforms with retention of coliform bacteria increasing as sand content decreases. In this study water flow was an important factor which increased bacterial transport through the soil columns. Furthermore, following deposition of bacteria on the soil surface, the initial rainfall event caused the greatest displacement of bacteria through the column. Preferential flow was also likely to play a significant role in bacterial transport but this study did not allow this to be observed individually.

Water quality of private water supplies is influenced by seasonality and rainfall. These influence temperature and soil moisture content which are essential for bacterial survival. Without survival there is no concern with regards to bacterial transport. With reference to private water supplies in Aberdeenshire, there was some discrepancy between seasonal effects observed for the Central Division database and the study of the 82 1F and 1E supplies. The study of the 82 supplies found that faecal coliforms increased during summer and early autumn while they decreased during the winter months with none detected in February. This disagrees with the Central Division database which showed that faecal coliforms were detected in February, and that faecal coliforms although detected during the summer months, continued to increase in autumn and remained high during the early winter months, finally decreasing in January. These discrepancies are almost certainly due to the variation in climatic effects found in Scotland. Links to seasonality although present are more closely connected to temperature and rainfall which is why differences will be found year to year. Contamination of potable water is more likely to occur following rainfall.

6.3 Future Work

- The development of rapid methods for water analysis which have the ability to detect pathogenic bacteria including *E. coli* O157.

- Continued development to decrease analysis time of current rapid methods for detection of coliform bacteria.

- The study of viral contamination of potable water and its impact on human health.

- Increase public awareness of potable water quality related to private water supplies, endeavouring to improve maintenance and treatment of supplies.

- Further studies of private water supplies with in situ monitoring following rainfall and detailed accounts of surrounding soil and land use.

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APPENDIX 1 SURVEY OF PRIVATE WATER SUPPLIES

WATER SOURCE DETAILS

Name of house-holder:

.....

Address:

.....

.....

Tel no: ..... Grid Ref:

.....

Date of visit: .....

Photograph reference number: .....

1. Water source – General

Six figure grid reference for water source: .....

Type of water source: Well ☐ Borehole ☐ Spring ☐
River / stream ☐ Unknown ☐

Does this water source supply: External reservoir / tank: Yes ☐ No ☐

If the water supply does have an external tank, comment on its construction (concrete, steel etc.) and its condition (cracks, leaks, uncovered hatches etc.)

:.....

.....

.....

How is the water supplied to the house?

Pump in well / borehole ☐ Pump remote ☐ Gravity ☐

Other (give details) ☐ Unknown ☐

Type of pipe work connecting the source to the house:

Lead ☐ Alkathene ☐ Other (specify) ☐ Unknown ☐

Is there a header tank in the house supplying the kitchen cold water tap?

Yes ☐ No ☐

Does the system supply animal troughs? Yes ☐ No ☐

Does the system prevent back siphoning from animal troughs?

Yes ☐ No ☐ Unknown ☐

Description of location:

Land use / land cover in immediate area (tick all land types that are appropriate):

Land use	Immediate vicinity	10 m radius	100 m radius
Crops / vegetables			
Pasture land			
Moor / Heath			
Woodland			
Set-aside			
Other (give details)			

Source protection:

Is the source area fenced off? Yes ☐ No ☐

Is the source area stock proof? Yes ☐ No ☐

Distance of fence from source (shortest) .....

Does the water system have:

UV filter .....

physical filter (specify) .....

chlorination facility .....

other .....

Give details of maintenance regime:

.....

.....

...

Location of waste water systems:

Does the house have a septic tank? Yes ☐ No ☐

Location of septic tank, grid reference:

.....

Location of mains sewage pipes:

.....

Additional sources of contamination in the vicinity of the supply (tick where appropriate and give details of distance from source):

- Agricultural steadings: .....
- Silage clamps: .....
- Manure storage: .....
- Slurry tank: .....
- Slurry lagoon, lined or unlined: .....
- Sheep dips: .....
- Fuel tanks: .....
- Streams / rivers / ditches: .....
- Sewage discharge into influent rivers: .....
- Subterranean field drains: .....
- Mining activity: .....
- Land fill / coup: .....
- Highway drainage: .....
- Evidence of flooding (standing water, sedges etc.): ....
- Is water used for irrigation: .....
- Others (give details): .....

Has sewage sludge been applied to the land in the vicinity of the source?

Yes (give details): ☐

No ☐ Unknown ☐

Has abattoir waste been applied to the land in the vicinity of the source?

Yes (give details): ☐

No ☐ Unknown ☐

2. Well Supply – Details

Date of construction: .....

Depth to water surface: .....

Depth of well: .....

Height of lip: .....

Internal diameter of well: .....

Well construction: Concrete rings ☐ Stone ☐ Other (give details) ☐

Is the well lined? Yes (give details) ☐ No ☐

Nature of well cover (comment on condition)

.....

.....

..

Is the well cover padlocked? Yes ☐ No ☐

Does the well have an apron? Yes ☐ No ☐

Maintenance regime:

Is the well cleaned? Yes ☐ No ☐

Is the pipework / tanks cleaned? Yes ☐ No ☐

How is the system cleaned?

How often is the system cleaned?

3. Borehole Supply – Details

Date of construction: .....

Depth of borehole: .....

Diameter of borehole: .....

Description of head works: .....

.....

Maintenance regime:

Is the borehole cleaned? Yes ☐ No ☐

Is the pipework / tanks cleaned? Yes ☐ No ☐

How is the system cleaned?

How often is the system cleaned?

4. Spring Supply – Details

Is the spring source protected? Yes ☐ No ☐

Maintenance regime:

Is the spring intake cleaned? Yes ☐ No ☐

Is the pipework / tanks cleaned? Yes ☐ No ☐

How is the system cleaned?

How often is the system cleaned?

5. Stream / River Supply – Details

Location of abstraction point? Bank ☐ River bed ☐ Other (specify) ☐

Maintenance regime:

Is the river collection point cleaned? Yes ☐ No ☐

Is the pipework / tanks cleaned? Yes ☐ No ☐

How is the system cleaned?

How often is the system cleaned?

6. Sketch Map of Area