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Survival and transport of Escherichia coli in the aquatic environment

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Survival and transport of *Escherichia coli* in the Aquatic Environment

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Abstract

In recent years, there has been increasing occurrence of environmentally associated illness caused by pathogenic organisms, such as *Escherichia coli* O157, introduced through faecal pollution. The presence of *E. coli* and other coliform bacteria in surface waters is an indication of possible contamination with faecal material, which may contain these pathogenic organisms. Understanding the behaviour of *E. coli* is key to provide accurate data for monitoring and regulatory purposes. This would allow improved assessment of health risk and, potentially, implementation of mitigation measures. This thesis investigates both survival and transport aspects of *E. coli* behaviour in the aquatic environment.

The transport of *E. coli* within the environment is very complex with many influential environmental factors. One aspect investigated was the native stream biofilm that proved to have little influence over the transport of *E. coli* during the short term. Over the long term, these native biofilms did accumulate *E. coli* as part of their community and thus provide in-stream stores that may be mobilised during high flow events.

Binding of *E. coli* to clays also proved to be an important in the transport of the organism within the aquatic environment as this processes reduces distance travelled and improves survival. Electrostatic forces seemed to have some influence on this process under controlled conditions but would be unlikely to have a large impact in the environment. The clays themselves may also play a small role with some providing a more hospitable surface to bind on than others.

The differences in binding behaviour and survival between *E. coli* found in manure and those found living in the stream environment suggest that the latter group have become

adapted to living under these hostile conditions. These naturalised *E. coli* potentially cause problems for monitoring and regulatory efforts as they are a constant source of *E. coli* within this environment and relatively little is known about potential pathogens in this group.

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Contents

Chapter 1. Introduction

Escherichia coli plays an important role in both the natural and human environment as it is a commensal organism for most mammalian guts, including humans, and thus widely distributed throughout most environments. Of particular importance is the role *E. coli* plays as an indicator for the presence of faeces and that some of these *E. coli* strains can cause disease.

The most well known of the pathogenic strains is *E. coli* O157, although other strains such as *E. coli* O104 have caused widely publicised outbreaks recently (DEFRA, 2008). Although there are relatively low numbers of cases every year (~1000, HPA, 2008) in comparison to other enteric pathogens (e.g. *Campylobacter* (~50,000 per year in the UK (HPA, 2008)) the severity of the disease prompts detailed investigation of these organisms.

E. coli is typically found within the guts of mammalian animals and thus the principal route of infection lies through contamination of food with the faeces of these animals, with the highest risk of this occurring during the butchering process. However, the increasing cases of *E. coli* O157 infection have proven to be acquired from contact with contaminated environmental waters (Howie et al., 2003) and many outbreaks have also been linked with environmental water (e.g. (Thurston-Enriquez et al., 2005; Hathaway and Hunt, 2011). Some of these cases also arise due to insufficient treatment of private water supplies exposed to contaminated run-off from the surrounding area.

The monitoring of surface waters has become standard to reduce the risk of outbreaks from these and other organisms (EA, 2002; SEPA, 2007). The standard practice is to monitor for faecal pollution through indicator organisms, of which *E. coli* is one. Some

pathogenic organisms can be difficult to detect against large numbers of background organisms and made harder due to the need to establish their presence in very low numbers, as some pathogens have very low infectious doses (Quilliam et al. 2011).

The survival and transport of *E. coli* in the natural environment has therefore become very important due to the resulting implications for human health. How long *E. coli* survives in the environment is the result of a wide range of interacting factors. However there is not one defining factor that determines survival. The principal factors include nutrient availability (Lim and Flint,1989) , the presence of metal ions (Kershaw et al., 2005; Borkow and Gabbay, 2005), oxygen availability (Avery et al., 2008), exposure to sunlight (Sinton et al., 2002), sediment binding (Davies et al., 1995) and the presence of competing organisms (Wang and Doyle, 1998). There is also variation in survival within the *E. coli* species itself (Watterworth et al., 2006) adding further complexity. All these factors vary when investigating different environments and thus it can be difficult to predict survival rates for one combination of factors using another.

The transport of *E. coli* in the environment primarily occurs through the water medium (Madsen and Alexander, 1982) and of particular interest is the interaction between *E. coli* and sediment particles, as bound *E. coli* tend to persist longer than those that are non-attached (Davies et al., 1995). The binding process is not fully understood with many influencing factors, as is the case with survival. Areas of investigation focus on the physio-chemical properties of both the bacterial cell and the sediment particle, in addition to the chemistry of the medium in which this interaction is taking place.

Understanding these aspects of *E. coli* ecology would allow much better prediction of water containing high levels of pathogens. This means that more effective measures can be taken to reduce the risk to human health.

Aims and Objectives

The aim of this thesis is to gain further understanding into the behaviour of *Escherichia coli* in the environment at both a generic and at a strain level. This study will investigate both survival and transport behaviours of several strains of *E. coli* and provide information that can be incorporated into further studies. These aims will be achieved through the following objectives:

- The study of the behaviour of *E. coli* within a stream environment and the influence that the native biofilm has on this behaviour
- The study of the influence of water chemistry variation on the surface characteristics of *E. coli* and its subsequent behaviour
- The study of the binding behaviour of several strains of *E. coli* to investigate the influence of strain type
- The study of clay types within the environment that may influence the survival of *E. coli*

Chapter 2. Literature Review

2.1.*E. coli* and illness

Escherichia coli forms part of the typical gut flora for humans and mammals and within this group are a number of *E. coli* serotypes (e.g. O157, O26, O103, O104 and O111) that can cause human disease (Mead and Griffin, 1998).

E. coli O157 is the serotype most frequently associated with human infections (Tarr et al., 2005), and therefore much of the research into the *E. coli* species has centred on this one serotype. This pathogen along with other members of the Enterohaemorrhagic *Escherichia coli* (EHEC) group produces one or both of two very similar Shiga-like toxins, termed shigatoxin 1 (Stx1) and shigatoxin 2 (Stx2). *E. coli* strains that produce these shiga-toxins are thus also described as Shigatoxigenic *E. coli* (STEC). Other virulence genes are encoded within the locus of enterocyte effacement (LEE Island) or on the EHEC virulence plasmid (Figure 1). Most human infections of *E. coli* O157 are self-limiting; however, infection may occasionally culminate in serious symptoms such as Haemorrhagic Colitis, Haemolytic Uremic Syndrome (HUS), kidney failure, and occasionally, death (Mead and Griffin, 1998).

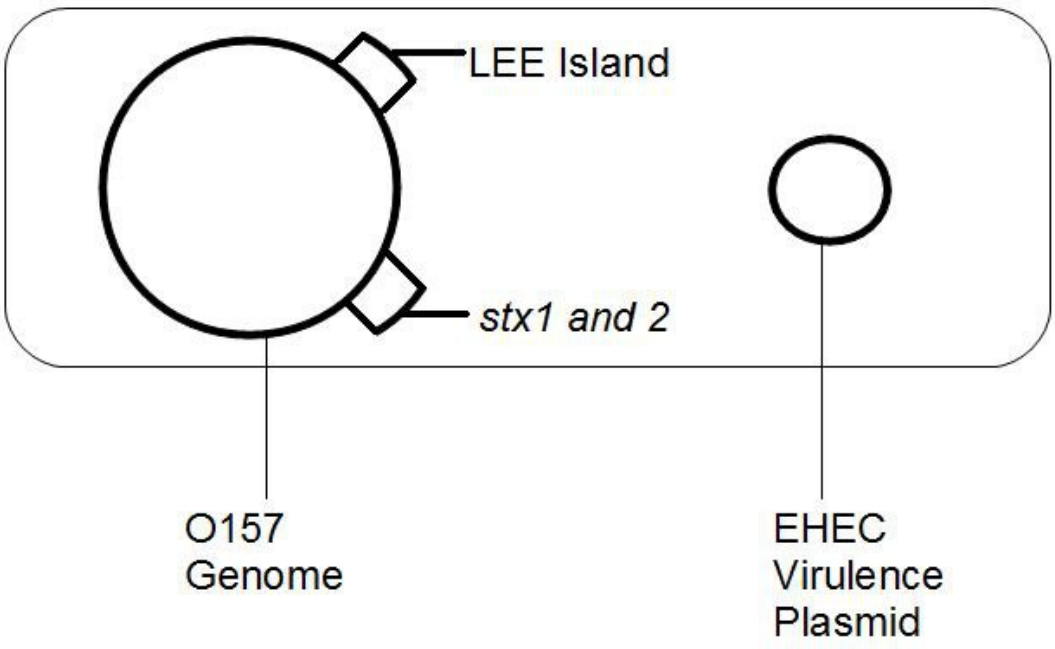


Figure 1.1: *E. coli* cell with key virulence genes highlighted.

E. coli O157 infections arise in the form of both outbreaks and sporadic cases. Depending on the source, outbreaks can affect thousands of people (Karmali, 2004). In the US alone, *E. coli* O157 is responsible for approximately 70,000 illnesses, 2,000 hospitalizations, and 60 deaths per year (Mead et al, 1999). In the UK, this figure is approximately 1000 cases per year. The rate per head of population for each country varies, with approximately 1.6 cases per 100,000 in the UK (DEFRA, 2008) but approximately 23 cases per 100,000 in the USA (Mead et al., 1999). While the number of *E. coli* O157 infections per year is relatively low in comparison to other enteropathogens such as *Campylobacter* (~50,000 per year in the UK (HPA, 2008)), the possible severity of the disease means that *E. coli* O157 is of high importance with regards to human health.

2.1.2 *E. coli* and *E. coli* O157 sources within the environment

The principal environmental reservoir for *E. coli* in general and *E. coli* O157 specifically is the gastrointestinal tract of cattle, with the bacterium being excreted in faeces (Yoon and Hovde, 2008). Carriage rates of *E. coli* O157 within cattle herds vary widely; from 0.2% to 48.8% of dairy cattle (Hussein and Sakuma, 2005) and 0.2% to 27.8% of beef cattle (Hussein and Bollinger, 2005). Carriage rates also appear to vary with season (Chapman et al., 1997) and diet (Caprioli et al., 2005). Other livestock, including sheep (Cornick and VuKhac, 2008) and pigs (Cornick and Helgerson, 2004) have also been shown to harbour the pathogenic form of the organism. As a result, outbreak sources are usually associated with meat or milk products or through cross-contamination of these with other foods (Rangel et al., 2005; Gyles, 2007). Outbreaks have also been linked to consumption of vegetable produce irrigated with contaminated water (Tyrrel and Quinton, 2003), highlighting the importance of environmental water in the *E. coli* O157 infection cycle.

Although ingestion of contaminated foodstuffs is the most frequent instigator of *E. coli* O157 infection, there are an increasing number of infections from environmental sources (Howie et al., 2003). The likelihood of human contact with *E. coli* O157 in the environment is augmented due to its ability to survive extended periods in faeces (Kudva et al., 1998; Williams et al., 2008), soil (Maule, 2000), on vegetation (Abdulraouf et al., 1993) and in water (e.g. Wang and Doyle, 1998; Avery et al., 2008). In the USA, environmental contact accounts for at least 9% of the total number of outbreaks and the majority of these are associated with recreational use of water (Rangel et al., 2005). Summarising the *E. coli* O157 outbreak sources for Scotland, Strachan et al. (2006) found that between 1994 and 2003 over half (54%) were environmentally associated. However

when examining the number of cases associated with each outbreak there were many more associated with food outbreaks (83% of the total number of cases) with only 16% of the total associated with the environment (Strachan et al., 2006).

Figure 2.2 illustrates sources and pathways through which *E. coli* O157 may pass through the agricultural environment and into watercourses. Rapid transfer of potentially infective *E. coli* O157 to watercourses may occur during periods of high rainfall due to overland flow and sub-surface carriage from soil or animal waste (Muirhead et al., 2004; Jamieson et al., 2005). In addition to causing human infections, it has been suggested that contamination of water sources may also be important in the cycle of re-infection of livestock (Jones, 1999; Avery et al., 2008). It is therefore essential to understand the ecology of the organism in the aquatic environment in order to understand and possibly interdict routes of infection.

Studies have tried to establish risk of *E. coli* O157 infection in the environment using dose-response models (e.g. Haas et al., 2000; Strachan et al., 2005: Vinten et al., 2009). However, the variation in risk factors between the various outbreaks investigated in these studies is quite large, making it difficult to apply to situations outside those of the study (Teunis et al., 2008).

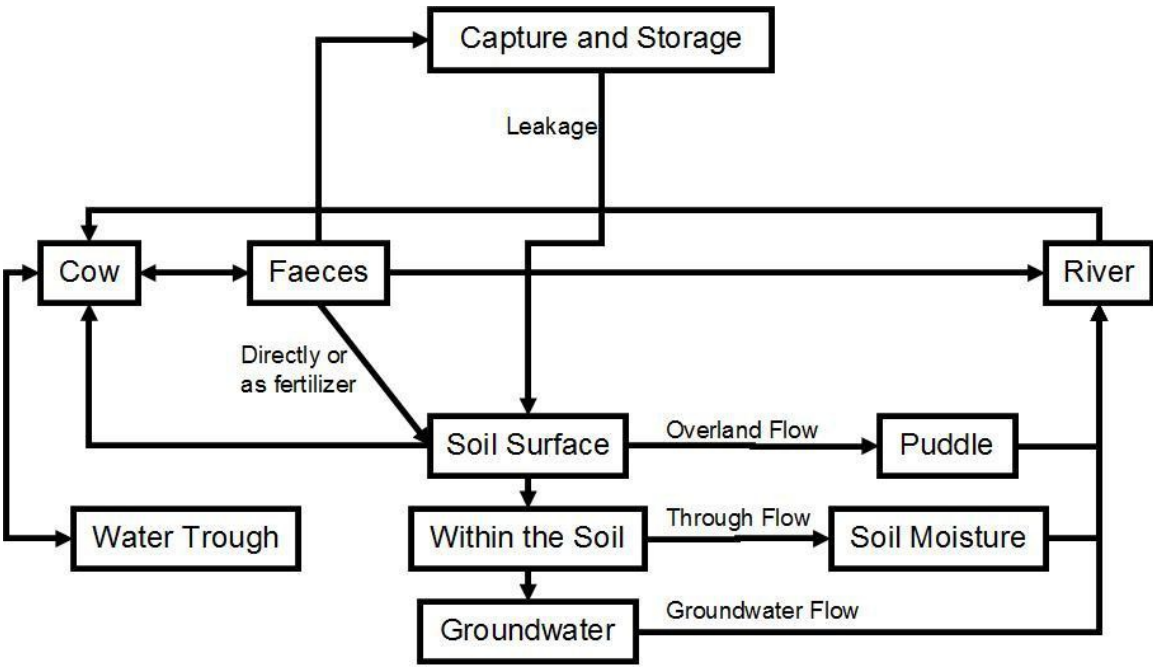


Figure 2.2: Simplified schematic of *E. coli* O157 transmission routes within the agricultural environment.

2.1.3 Environmentally associated *E. coli* O157 cases

The cycle through water sources may contribute to the persistence and the cycling of *E. coli* O157 in a geographical area and a clustering of infection cases. North-East Scotland is an area with seemingly high levels of infections and where recent outbreaks have been associated with drinking from contaminated private water supplies (HPS, 2008).

Indeed, a considerable number of human *E. coli* O157 infections have been linked to water sources (Table 2.1). These cases occur through direct contact with environmental waters contaminated with *E. coli* O157 (Thurston-Enriquez et al., 2005; Hathaway and Hunt, 2011) or by the use of contaminated drinking water from private water supplies that have undergone insufficient (or no) treatment (Rutter et al., 2000). As most countries

stipulate specific treatment and legislative controls for mains drinking water, it is only likely to be a source of *E. coli* O157 where the treatment process has failed. Although infrequent, outbreaks from contaminated mains water supplies are potentially very serious, affecting large numbers of people (e.g. Hrudey et al., 2002; Auld et al., 2002). Private water supplies, however, are frequently at risk of contamination by agricultural run-off containing animal faeces and this risk is increased during periods of heavy rain due to greater transport to wellheads and spring-fed sources. Further problems occur through poor regulation of private water supplies and are often exacerbated by the owners who may not appreciate the dangers posed by these supplies. As a result, private water supplies have been a proven source in many outbreaks worldwide (Table 1).

Table 2.1. Outbreaks of *E. coli* O157 infections related to environmental water**.**

Country	Source	Year	Cases	Reference
Scotland	Drinking Water	1991		Dev et al., 1991
USA	Drinking Water	1989	243	Swerdlow et al., 1992
Japan	Drinking Water	1990	174	Akashi et al., 1994
USA	Lake	1991	21	Keene et al., 1994
Netherlands	Lake	1993	4	Cransberg et al., 1996
USA	Lake	1994	12	Ackman et al., 1997
Canada	Drinking Water	1995	1	Jackson et al., 1998
USA	Lake	1995	8	Levy et al., 1998
USA	Lake	1995	6	Levy et al., 1998
USA	Lake	1995	2	Levy et al., 1998
USA	Lake	1996	6	Levy et al., 1998
USA	Drinking Water	1999	781	Rangel et al., 2005
Finland	Lake	1997	14	Paunio et al., 1999
USA	Lake	1997	8	Barwick et al., 2000
USA	Public Water Supply	1997	157	Barwick et al., 2000
USA	Lake	1998	5	Barwick et al., 2000
Scotland	Private Water Supply	1999	6	Licence et al., 2001
USA	Lake	1999	7	Feldman et al., 2002
USA	Lake	1999	5	Lee et al., 2002
USA	Ditch Water	1999	2	Lee et al., 2002
Canada	Public Water Supply	2000	2,300	Hrudey et al., 2003
Canada	Swimming Beach	2001	4	Bruneau et al., 2004
England	Beach	1999	14	Harrison and Kinra, 2004
Ireland	Private Water Supply	2005	18	Mannix et al., 2005
USA	Lake	2001	20	Dziuban et al., 2006
England	Stream on a beach	2004	7	Ihekweazu et al., 2006
Scotland	Private Water Supply	2008	15	HPS, 2008
USA	Lake	2005	4	Yoder et al., 2008
USA	Lake	2006	3	Yoder et al., 2008
USA	Lake	2006	3	Yoder et al., 2008

2.1.4 Monitoring of *E. coli* in the environment

To reduce the likelihood of outbreaks, water supplies and their sources need to be monitored for pathogens such as *E. coli* O157. This can be difficult as most pathogens are found in relatively low numbers in comparison to background bacteria and their signal can be swamped. However it is still essential to find them as *E. coli* O157 in particular has a very low infectious dose with ingestion of as few as 10 organisms thought to be sufficient to cause disease (Chart, 2000). With this in mind, much research has been done and is continuing on various methods for pathogen detection against high background numbers of non-pathogenic organisms (as reviewed in Quilliam et al., 2011).

Current standard practice relies on the detection of faecal events through monitoring of indicator organisms, such as generic *E. coli* (EA, 2002; SEPA, 2007), from this the likelihood of there being pathogens present can be estimated. For example, if there are high numbers of *E. coli* present then there has probably been a large faecal pollution event meaning that there is a high likelihood of pathogens being present. This is why it is imperative to not only understand the survival and transport characteristics of pathogenic *E. coli* but also generic *E. coli* that are used as their indicators.

2.2. Survival of *E. coli* in freshwater

Following excretion within faeces, the survival and transport of *E. coli* in the wider environment is dependent upon many factors. By evaluating the findings of laboratory and field-based studies on generic and pathogenic *E. coli* in a range of aquatic environments (Table 2.2), we can begin to elucidate the nature of the key drivers for survival in diffuse pollution-impacted freshwaters and where knowledge gaps exist. It is

apparent from such studies that persistence depends upon a range of interacting factors which fall into three main categories: chemical, physical and biological.

2.2.1 Chemical factors

Water chemistry can affect *E. coli* directly or indirectly by changing the degree of competition and predation from other microorganisms. As typical of many enteric bacteria, *E. coli* O157 is tolerant of acidic pH found in the mammalian stomach, normally around pH 2.5 (Leyer et al., 1995; Foster, 2004). UK rivers and lakes typically range from pH 4 to 8.5 (Environmental Change Network, 2009), resulting in very little impact on the *E. coli* numbers (Foppen and Schivjen 2006; Avery et al., 2008). Acidification events may occur in aquatic environments as a result of large precipitation events and increased snowmelt (Davies et al., 1992).

It is broadly accepted that *E. coli* persists rather than proliferates in environmental waters, with rapid die-off occurring over time (Table 2.2; Avery et al., 2008). However, there is substantial variation in published die-off rates (Table 2.2), which depend on the nature of the study or the matrix involved. There is some evidence that where conditions allow, *E. coli* can adapt to, and multiply in, environmental matrices (Brennan et al., 2010). For example, the growth of faecal indicator bacteria in microcosms generated from mixtures of urban runoff, treated wastewater and storm drains appeared to be at least partly controlled by dissolved organic carbon (DOC) and phosphate concentrations (Surbeck et al., 2010). Vital et al. (2008) showed that *E. coli* O157 is able to grow in conditions with low levels of available C (10-1000 μg L^{-1}) typical of natural freshwaters, indicating that it is able to efficiently capture and metabolise environmental sources of

carbon. However, the same study also found that *E. coli* O157 was unable to grow at the same rate as the indigenous microbial community, possibly due to the relatively larger *E. coli* cells requiring more carbon for assembly, suggesting that the pathogen would be out-competed.

Surface water nutrient concentrations can be expected to vary widely depending upon catchment and hydrological characteristics (e.g. soil type, land-use and management, diffuse and point-source pollution and rainfall (Ekholm et al, 2000; Helliwell et al., 2007), for example, nitrate concentrations between 1994 and 2007 in selected UK rivers ranged from 0.1-7.1 mg L^{-1} (Environmental Change Network, 2009).

Lim and Flint (1989) studied the effects of additions of carbon, nitrogen and phosphate sources, as well as synthetic sewage on survival of *E. coli* in filtered and unfiltered lake waters. They reported greater T_{90} values with increasing BOD from 0-50 mg L^{-1} (increasing synthetic sewage addition) in both filtered and unfiltered samples. The increase in viable counts was greater at 37 °C than 15 °C, and the addition of various C sources demonstrated that *E. coli* cells were quickly out-competed by the indigenous microbial community as they were limited by the range of carbon sources they could metabolise linking with the work mentioned earlier by Vital et al. (2008). Although the addition of P did not affect survival of *E. coli* in unfiltered lake water, the addition of amino acids or N in the form of $NH_{4\,(2)}\,SO_4$ did lead to increased survival times. Different environmental-dependent survival strategies of *E. coli* O157 have previously been demonstrated in freshwaters (Avery et al., 2008). The impact of nutrient addition may differ depending on whether water bodies are already nutrient impacted or not, and if

enough is added to exceed thresholds governing survival strategies or indigenous biota interactions.

Table 2.2. Studies investigating survival of generic and pathogenic *E. coli*.

Organism	Water source/info	Factor/Variable investigated	Measure of decline/survival	Reference
E. coli O157	Sterilized well water (4 Sources)	Survival in different waters	2 log reduction after 35 days in all waters	Geldreich et al., 1992
E. coli O157 #C4195 and #932 *E. coli* #R1	Portable groundwater source	5 °C 20 °C	3.5 log reduction after 70 days 5 log reduction after 35 days	Rice et al., 1992
Generic *E. coli*	Sterile seawater	Rate of die-off under light conditions Turbidity Sewage Mixing Temperature	Significant effect Significant effect Significant effect Non-significant effect	Alkan et al., 1995
E. coli K-12 (MC4100)	Filter sterile esturine water	Linear regression slopes Organic Matter (Presence / Absence) Salinity (Artificial Seawater / Physiological Water) Light (Presence / Absence)	OM+ OM- S - L - +0.050 -0.005 S + L - -0.006 -0.020 S - L + -0.060 -0.110 S + L + -0.120 -0.100	Troussellier et al., 1998
E coli O157	Filtered and autoclaved municipal water, in reservoir water, and in water from two recreational lakes	8 °C 25 °C	1-2 log reduction after 91 days >3 log reduction within 49-84 days	Wang and Doyle, 1998
E. coli O157:H7 (NCTC 12900)	Bottled natural drinking water	Survival in: Unsterilised mineral water Sterile mineral water Sterile distilled water	3 log reduction after 70 days 3.5 log reduction after 70 days 4.5 log reduction after 70 days	Kerr et al., 1999
E. coli O157	River water	Survival in river water	Detection limit reached 27 days	Maule, 2000
E. coli O157	Cattle drinking water (2 sources)	Temperature (5 and 15 °C) Water Source	First water source – no difference between temperatures Second water source – 5 °C reached detection limit after 8 days, 15 °C reached detection limit after 4 days	Rice and Johnson, 2000
E. coli O157 (Environmental)	Cattle water troughs (473)	Water characteristics that encourage survival	Presence/absence 6/473	LeJeune et al., 2001
E. coli O157 #3704 Tn5 *lux CDABE*	Well water from four different sites	Variation in several factors between sites + the presence of different organism	Number of *E. coli* O157 present reduced by copper, predation by protozoa and in competition with other micro-organisms	Artz and Killam, 2002

E. coli DH5α	Unsterilised and sterile groundwater	Influence of microflora	Unsterile 1 log reduction 2 days Sterile 1 log reduction 82 days	Banning et al., 2002
E. coli O157:H7 (NCTC 12900)	River water – with and without faeces Sterile distilled water (SDW)	Difference between temperature and water sources	River water w/o faeces - <15 °C – 7 log reduction in 12 days - 15 °C – 6 log reduction in 23 days River water w/ faeces - <15 °C – 7 log reduction in 18days SDW w/o faeces - <15 °C – 6 log reduction in 14 days - 15 °C – 4 log reduction in 31 days SDW w/ faeces - <15 °C – 7 log reduction in 29 days	McGee, 2002
Escherichia coli Famp (ATCC 700891)	Dechlorinated water	10 °C 22 °C 37 °C	1 log reduction in 7.7 days 1 log reduction in 5.7 days 1 log reduction in 3.0 days	Allwood et al., 2003
E. coli O157 #3704 Tn5 *lux CDABE* and *E. coli* O157 #3704	Sterile artificial groundwater	Difference between the strains at 15 °C	Both showed a 5-log reduction over 70 days	Ritchie et al., 2003
E. coli O157	Surface water from lakes and rivers	6 °C 24 °C	Detection limit reached 32-51 days Detection limit reached 21-32 days	Czajkowska et al., 2005
7 strains of *E. coli* O157	Untreated well water	10 °C 22 °C	2 strains – 1-2 log reduction after 56 days 2 strains – 4 log reduction after 56 days 3 strains – Detection limit reached <42 days 1 strain – 6-7 log reduction after 56 days 2 strains - Detection limit reached <56 days 4 strains – Detection limit reached <42 Days	Watterworth et al., 2006
E. coli O157 #3704	Non-sterile: Lake Faecally Contaminated Puddle River Drinking Trough	Variation in several factors between sites	2 log reduction after 12.9 days 2 log reduction 17.8 days 2 log reduction 6.0 days 2 log reduction 6.3 days	Avery et al., 2008
E. coli O157:H7	Pond and holding tank water	Difference between water sources	Pond – 3-4 log reduction within 12 days Holding tank – 3-4 log reduction within 6 days	Suhalim et al., 2008
6 clinically isolated ETEC strains	Sterile-filtered sea water and freshwater	Induction of VBNC state in water	2 log drop after 12 weeks	Lothigius et al., 2009

Metal ions are to be found naturally in surface waters or can be present as anthropogenic pollutants. Many of these metals are essential for bacteria function at trace levels as they have roles in the functioning of enzymes (e.g. copper) but become toxic at higher concentrations as they can damage cell structure and function (Kershaw et al., 2005; Borkow and Gabbay, 2005). There is a significant body of evidence reporting the genetic basis of mechanisms for metal ion resistance in environmental isolates of *E. coli* O157 and in many other *E. coli* strains (e.g. Cu^{2+} exporter *copA*; Rensing and Grass, 2003). However, the pattern of resistance genes can vary between isolates because they are usually encoded on highly mobile plasmids that are regularly gained and lost (Muhldorfer and Hacker, 1994). Despite the paucity of information on the importance of heavy metals on survival and metabolic activity of *E. coli* O157 in environmental waters, Avery et al. (2008) found that there were significant negative correlations between mean $\log_{10}$ CFU *E. coli* O157:H7 and $\log_{10}$ concentration of both Zn and Al. Furthermore, metal toxicity has been implicated in the die-off of *E. coli* O157:H7 in well water where persistence with high concentrations of Cu (up to 3.9 mg L^{-1}) and Zn (up to 0.1 mg L^{-1}) was less than that in well waters with low concentrations of Zn (<0.015 mg L^{-1}) and Cu (<0.01 mg L^{-1}) (Artz and Killham, 2002). Grey and Steck (2001) reported that toxic concentrations of copper (500 μM) led to die-off of *E. coli* but also induced a fraction of a population to enter the viable but nonculturable (VBNC) condition. As the concentration of metals is known to influence abundance and structure of freshwater microbial communities (Hemme et al., 2010), there may also be indirect effects upon *E. coli* survival.

Agrochemicals are in extensive use in many agricultural areas and include pesticides, antihelminthics, fertilisers and various antibiotics. These can enter the water environment through surface run-off from fields, impacting the intrinsic microbial community within the water (Kolpin et al., 2002). Ng et al. (2005) demonstrated that three *E. coli* serotypes (including O157) were able to grow in some reconstituted herbicide solutions even at full application strength, rather than at the low concentrations likely to be present in environmental waters suggesting that concentrations used on farms would have limited impact on *E. coli* survival. Veterinary drugs and their residues are often present in agricultural run-off from livestock areas, while waste water treatment plant and septic tank effluent contribute human antibiotics and their residues, which can lead to the build-up of resistant bacterial strains of *E. coli* (Reinthaler et al., 2003).

Oxygen concentration is thought to influence survival of *E. coli*. For example, Avery et al. (2008) reported greater recovery of the *E. coli* O157 serotype from waters with an oxygen saturation of between 98-100% compared with samples from the same sources that had between 2-5% oxygen saturation. This was not unexpected, as *E. coli* O157 is a facultative anaerobe and therefore can use oxygen as a terminal electron acceptor, increasing the efficiency of energy generation (Ingledew and Poole, 1984). However, in the natural environment, increased oxygen could favour protozoal predators and competitive microflora, leading to an increase in predation or die-off of *E. coli* O157. Nevertheless, Ravva et al. (2006) found that aeration had no significant effect on the survival of *E. coli* O157 in dairy wastewaters and hypothesised that in this environment other factors have a greater influence on the survival of *E. coli* O157. Increased oxygen availability in water has an additional effect on the inherent water chemistry, causing

changes in the availability of compounds and the concentrations at which are actually present at different depths of water (Labaugh, 1980); however this effect is likely to be overshadowed by other depth-dependant factors (e.g. UV exposure as discussed below).

2.2.2 Physical factors

Direct sunlight has been shown to have an antimicrobial effect on freshwater and one study found that when waters are exposed to sunlight, the die-off caused by the light overshadows any warming effect sunlight has on survival (Alkan et al., 1995). Penetration depth of UV radiation in natural waters can range from a few centimetres to several metres (Morris et al., 1995) and is inversely related to turbidity. Sinton et al. (2002) provide evidence that this has the greatest effect on faecal organisms present in water, including *E. coli*. The susceptibility of faecal organisms to UV radiation is due to the induction of repair mechanisms for UV damage taking longer in faecal organisms than in environmental organisms (Sinton et al., 2002). The antimicrobial effect is reduced when particulates in the water provide shade to the surrounding bacteria therefore shielding them from the UV light (Figure 2.3). The shielding properties of suspended particulates is greatly influenced by their size and, as expected, larger particles (mean size of 262 μm) have been shown to induce a greater shielding effect than smaller particles (mean size of 119 μm) for faecal coliforms in wastewater (Winward *et al*., 2008). The use of sunlight for microbial inactivation has been investigated as a method for cheap disinfection of water in areas where large scale water treatment and distribution is not currently practicable (Walker et al., 2004). However, its use is limited to waters with low numbers of particles to avoid the survival of organisms that are shaded.

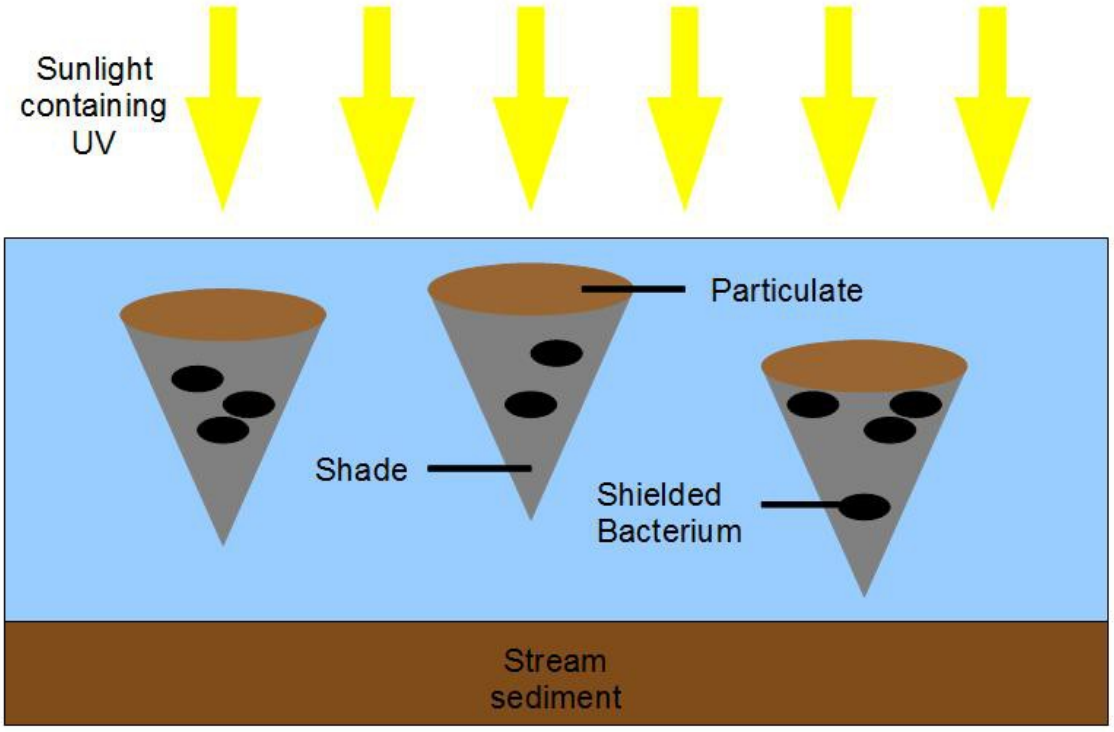


Figure 2.3: Shielding of bacteria by particulates in the water column.

In addition to the shielding effects, particulates such as sediment and colloids play a complex role in the survival of *E. coli* within the aquatic environment. The binding of *E. coli* cells to sediment and clay colloids is also a key factor in the transport of the organism (See Chapter 4).

The persistence of waterborne microorganisms can be affected by the presence of clay colloids, with studies showing better survival of pathogens when bound to sediment compared to free-floating in freshwater (e.g. Davies et al., 1995). When bound to sediment particles, *E. coli* are partially sheltered from any antimicrobial properties or processes that might occur in the water through physical shielding, in addition to any effects of the chemical properties of the colloid on the immediate environment and upon the attached bacterium. Sediment particle size plays a role in this with thermotolerant

coliforms being able to survive for longer in sediment with predominantly clay-sized particles compared with coarser sediments (Howell et al., 1996). However, LaLiberte and Grimes (1981) report that a lab culture of *E. coli* inoculated in lake sediments survived longer in sandy than silty sediment (the latter with a higher clay content), although interestingly, naturally present populations of *E. coli* monitored in the same experiment did not differ between sediment types. Similarly, longer survival times for *E. coli* have been reported in sediments containing at least 25% clay than in those with larger particles (Burton et al., 1987). It is not clear whether these effects are related to the presence of particles within the colloidal size fraction or directly to the clay content of sediments, which appear to have specific effects on some microorganisms, including pathogens.

Clay types have also been found to affect pathogen survival. The levels of Montmorillonite in soil has been associated with reduced occurrence of some human pathogens and greater survival of indigenous bacteria (Filip, 1973); whilst Bentonite clays have been shown to inhibit protozoal grazing of *Rhizobium* in liquid culture (Heijnen et al., 1991) and a particular Illite clay ($CsAgO_2$) antagonises *E. coli* through loss of outer membrane integrity by the action of Fe^{2+} ions (Williams et al., 2011).

Several studies have investigated the effect of temperature on survival (Table 2.2) as it is one of the most important factors in the persistence of *E. coli* in the environment. It appears that survival is greater in waters held at lower temperatures than those at higher temperatures (Wang and Doyle, 1998; Watterworth et al., 2006). This may be due to the viable but nonculturable (VBNC) state being induced much quicker at colder temperatures (e.g. 5 °C) for the temperature is further from the optimum temperature of this organism. In comparison, warmer temperatures (e.g. 20 °C) may be conducive to

survival in most environments so the protective state of the cell is not induced until much later. Antagonistic effects from other micro-organisms may be reduced at lower temperatures (e.g. protozoa have lower activity) and therefore *E. coli* may survive longer. The mean environmental surface water temperature is approximately 10 °C in the UK (Environmental Change Network, 2009) which facilitates persistence of *E. coli* O157 compared with areas where the water temperature is much higher (e.g. tropics).

2.2.3 Biological factors

Competition for nutrients to survive and replicate, and the degree of inter- and intra-species competition is a critical factor in the survival of water-borne pathogens. Although known for its resilience in water, Avery et al. (2008) and Wang and Doyle (1998) showed that survival of *E. coli* O157 was reduced when grown in the presence of the resident water micro-organism population in comparison to autoclaved (sterile) samples; indicating the influence of the resident microbial population. Foppen and Schivjen (2006) calculated that, on average, there is an increase in the die-off rate of approximately one log-unit due to the predation or antagonistic effects of indigenous communities on *E. coli* and thermotolerant coliforms.

Zooplankton (protozoa, rotifers, cladocerans and copepods) are known to be major regulators of bacterial population dynamics and community structure in freshwaters and can be expected to be a major determinant of pathogen survival (Pace et al., 1990; Berninger et al., 1991). This was highlighted by LeJeune et al. (2001) who found that there was an inverse relationship between counts of *E. coli* O157 and protozoa in water troughs, indicating a possible predator/prey relationship. Another study

providing further evidence found the rate of decrease in *E. coli* O157 numbers was much less in well waters from which the protozoa had been removed (Artz and Killham, 2002). However, intriguing evidence brought forward by Barker et al. (1999) shows, instead of the protozoal species utilising *E. coli* as a food source, some are only able to ingest, but not digest *E. coli*. Gourabathini et al. (2008) corroborated this for *E. coli* O157. The resulting relationship offers protection for the organism from environmental influences including potential bactericidal processes. The mechanisms of survival during internalisation are unknown, and this relationship may provide a route of transport and infection. Similarly, the adhesion of faecal coliforms to the external surface of zooplankton increases persistence in aquatic environments and has been reported to stimulate the VBNC state, which may form a vital part of the transmission pathway (Signoretto et al., 2004, 2005; Cellini et al., 2005).

Biofilm formation and quorum sensing are important microbial survival strategies in aquatic systems and are often produced where nutrients are deposited (Costerton et al., 1995). Typically this formation consists of five stages (Van Houdt and Michiels, 2005). The first stage is a reversible attachment between the bacterium and the solid surface when brought together through bacterial movement or through flow of the medium. The second stage of the process is the production of exopolysaccharide (EPS), an important bacterial surface determinant of attachment and fimbriae that anchor the bacterium irreversibly to the solid surface (Junkins and Doyle, 1992). During the third and fourth stages, the EPS matrix matures with the addition of macromolecules that also include proteins and DNA to its structure (Sutherland, 2001). The fourth stage is distinguished by the alteration of the biofilm to trap and funnel nutrients to those bacteria immobilized in

that matrix. The final stage is the steady release of bacteria from the fully mature biofilm, ready to repeat the process at another location.

E. coli O157 is known to produce biofilms on a range of solid surfaces (e.g. mineral, plastic, steel, wood, plant roots and leaves), which prevents it from being readily displaced by flowing water and facilitates a long-term reservoir in aquatic environments (Cooper et al., 2007).

The ability of *E. coli* O157 to produce biofilms, however, appears to be critically dependent on the presence of other bacteria (Bauman et al., 2009; Klayman et al., 2009), and it is likely that surface roughness and the age of biofilm are major determinants for resistance to predation (Korber et al., 1997). Biofilms also provide further protection from the surrounding environment, such as from antimicrobial compounds and UV exposure, (Ryu and Beuchat, 2005), while the significance of biofilm formation and *E. coli* O157 survival have been demonstrated in simple systems, its relative importance in natural environments is still unclear.

Bacteriophage are highly abundant in the freshwater environment, ranging from $10^4 – 10^8$ mL^{-1} (Bergh et al., 1989) and are in sufficient numbers to potentially influence the survival of *E. coli*. Phage exclusively infecting indigenous microflora would present a competitive advantage to *E. coli*, however, coliphage or less host-specific phage may have a detrimental effect. Looking specifically at *E. coli* O157 it has been shown that *E. coli* O157-infecting bacteriophage are naturally present in cattle and, therefore, are released concurrently with the pathogen (Niu et al., 2009). Generally, bacteriophage are remarkably stable in the environment and can be readily recovered from soil, sewage, water, farm effluents and faeces. The presence of host-specific phage may provide a

means for adaptation to environmental change, although while bacteriophage are capable of infecting *E. coli* O157, their capacity to infect cells present within a biofilm is greatly reduced (Sharma et al., 2005, 2009). There is also evidence to suggest that phage-resistant mutants of *E. coli* O157 could evolve and that they may facilitate the transfer of undesirable characteristics between strains (Greer, 2005). It is known, for example, that bacteriophage with double-stranded DNA and lambdoid morphology that are naturally present in aquatic ecosystems can carry *stx* genes 1 and 2 leading to the potential for inter-strain transfer (Herold et al. 2004). *Stx*-phages represent highly mobile genetic elements that play an important role in the expression of *stx* genes by horizontal transfer. However, Dumke et al. (2006) concluded that the low abundance of *stx*-carrying phage, the low metabolic activity of the host bacteria together with unsuitable conditions for gene transduction made this unlikely to be significant from a public health perspective.

It could be hypothesised that macroinvertebrates found in the water environment, such as amphibians, fish and insects, may provide an environment that is somewhat similar to that of the primary habitat of *E. coli* (i.e. the mammalian gut) therefore creating an in-stream store for *E. coli,* potentially including pathogenic strains. Furthermore, these animals may facilitate the spread of pathogens to the wider environment via the food chain. Indeed, Gray et al. (2007) provide some preliminary evidence that tadpoles of the species *Rana catesbeiana* (American Bullfrog) are able to act as a source for *E. coli* O157; indicating that *E. coli* O157 hosts are more varied and widespread than previously thought. Bivalves may also act as passive carriers of human pathogens (Canesi et al., 2001). Nevertheless, many screens of *E. coli* O157 in shellfish and crabs have been undertaken over the last decade and have revealed a very low abundance which is

probably of low risk to human health unless eaten raw (Guyon et al., 2000; Gourmelon et al., 2006). Although there have been few studies on wild fish, data from farmed fish have indicated the potential for GI tract, internal organs and fish skin (particularly the mucus covering) to support high population densities of *E. coli* O157 that can persist for weeks after inoculation (Suhalim et al., 2007, 2008). There are conflicting reports of fish muscle contamination, although current evidence suggests this could occur at sufficient levels to induce human infection if eaten raw (Fattal et al., 1992; Guzman et al., 2004). Fish have been implicated as long-distance vectors and a transient reservoir for *E. coli* O157 (Guzman et al., 2004); however further work is needed to verify this.

E. coli cells, specifically *E. coli* O157 cells, are known to readily bind to plant surfaces and can also become internalized in plant tissues (Mitra et al., 2009). While most experiments have focussed on crop plants, aquatic plants may also have the same potential to bind and harbour the pathogen (Mootian et al., 2009). Further, the presence of plants may indirectly increase persistence by promoting biofilm formation, reducing zooplankton and reducing UV exposure through shading (MacIntyre et al., 2006). However, well managed reedbed treatment systems have been shown to dramatically reduce, but not eliminate coliforms from wastewaters (Morgan et al., 2008) and filtration via plants forms part of some on-farm mitigation measures against diffuse pollution (Avery et al., 2009).

Relatively little is known about the physiological state of *E. coli* or *E. coli* O157 when introduced into the aquatic environment. The metabolic activity of bioluminescent *E. coli* O157 cells in aqueous environments is critically dependent upon water quality (Williams et al., 2007). In most fresh and marine waters, metabolic activity rapidly

declines after release from faeces, which may result from the lack of available carbon or cessation of host factors. Knowledge of the physiological state of *E. coli* is particularly important as inactive cells (i.e. stationary phase) possess greater resistance to environmental stresses such as acidity and anoxia thereby increasing the probability of survival rather than proliferation (Cheville et al., 1996; Saby et al., 1999). Experimental evidence shows that in the exponential phase, *E. coli* O157 appears to be much more vulnerable to biocides and environmental stress (Arnold and Kaspar, 1995); however, if the environment is sufficiently benign then being in this state facilitates quick resource exploitation and proliferation. Current evidence suggests that *E. coli* O157 enters a stationary phase in its ruminant host once it becomes detached from the intestinal margins and enters the lumen (Poulsen et al., 1995). Subsequently, the bacterium leaves its host in the stationary phase, increasing its chances of survival in the environment. There is also evidence that many *E. coli* present in cattle faeces are present in a VBNC state (Wu et al., 2009a). It is well established that *E. coli* may enter a VBNC state in response to a range of external stresses including: desiccation, inorganic nutrient and carbon starvation, suboptimal temperature, extreme pH, visible light irradiation and high salinity (Asakura et al., 2007; Muela et al., 2008). However, identifying cells that have entered a VBNC state is crucial for identifying pathways of infection, particularly as most routine surveillance techniques are based on the enumeration of culturable bacteria only.

E. coli O157 is traditionally thought to enter a VBNC state as a means of shutting down metabolic function in times of stress until conditions for growth are experienced. A counter argument is that in the case of serious damage by stress factors, cells will donate their nutrients to other members of the population to ensure species survival until better

environmental conditions prevail (Arana et al., 2007). The surviving culturable cells can "cheat at the expense of the altruism" displayed by the majority. The phenotypic change into a VNBC state involves alterations to membrane lipid composition and fluidity and a rearrangement of the outer membrane subproteome (Scherber et al., 2009). Changes in the outer membrane occurs in response to the detection of stress via the osmosensor gene *EnvZ* (a transmembrane histidine kinase protein which is capable of detecting changes in external solute concentration), possibly modulated by *MzrA*, and upregulation of some outer membrane proteins (e.g. *OmpW*; Darcan et al., 2009; Asakura et al., 2008). The porin protein encoded by *OmpW* is known to be upregulated by extremes of pH (Wu et al., 2009b). By analogy with other food-borne pathogens, *OmpW* may also be triggered by a range of other stresses (e.g. salt and oxidative stress; Gil et al., 2009; Wu et al., 2006). Although not shown in *E. coli*, there is evidence that *Pseudomonas* exhibits reduction in nutrient transport, respiration rates and macromolecular synthesis from similar organisms; and that VBNC cells can still actively divide in a VBNC state, albeit at a reduced rate (Peneau et al., 2007). Favourable growth conditions with a source of energy and an ideal stoichiometric ratio of carbon to inorganic elements appear to reverse the VBNC state, although this remains largely unproven (Arana et al., 2007) and may take days to occur (Scherber et al., 2009). Reversion to a culturable state probably involves a resuscitation-promoting or anti-dormancy factor which can cleave peptidoglycan, altering the mechanical properties of the cell wall to facilitate cell division or release lysis products that function as antidormancy signals. Whether VBNC cells are capable of causing infection is an area which is poorly understood as it is probably highly dependent on the reactivation time and external conditions and whether a secondary

vector is involved prior to human consumption. Research in to *Salmonella* has indicated that newly formed VBNC cells are either weakly able or unable to cause infection (Passerat et al., 2009). Further, recovery from a VBNC state may be strain-specific. The degree to which environmental changes are likely to impact on the proportion of potentially infective but undetected VBNC cells in a given population warrants further consideration.

2.2.4 *E. coli* strain differences

The limitation with most survival studies is that they tend to focus on only one strain of interest. While it is still possible to draw conclusions from these studies and apply to the *E. coli* spp. as a whole, it is important to recognise that *E. coli* strains can be geneotypically very different and thus asimilar in phenotype; indeed one study found only 40% of the proteins were found in all three of the *E. coli* strains examined (Welch et al., 2002). For example, Watterworth et al. (2006) found evidence that different strains of *E. coli* O157 varied in their ability to compete with background organisms despite showing equal ability to survive when in isolation; and Cook et al. (2011) found wide variation in binding efficiency of different strains (Section 2.4). Environmental isolates of STEC can show substantial variation in chromosomal, phage- and plasmid-encoded virulence factors. Many of these virulence factors are encoded on potentially mobile elements and, therefore, diversity in virulence to both humans and animal hosts is expected. This is exemplified by a study of 73 STEC isolates by Slanec et al. (2009), who found that 71% contained *stx*2, 9% harbored *stx*1, 57% contained *iha* (adherence-associated protein), 40% contained *ehxA* (EHEC hemolysin), 28% contained *espP* (serine

protease), 25% contained *subAB* (subtilase cytotoxin) and only 5% possessed *eae* (intimin). While *E. coli* O157 is typically characterised by the possession of its *stx* genes and those of the type III secretion system including genes encoding for intimin (*eae*), the intimin receptor (*tir*), and other secreted proteins (*esp*), most clinical isolates also contain an F-like 92-kb plasmid, pO157. Putative virulence factors encoded by pO157 include enterohemolysin (*ehxA*), the general secretory pathway (*etpC* to *etpO*), and attaching and effacing gene-positive conserved fragments (*ecf*). Experiments using *E. coli* O157 strains in which pO157 was either present or absent have shown that the pO157 deficient strain survived better during passage through the bovine gastrointestinal tract but did not colonize the bovine rectoanal junction mucosa as well as that when pO157 was functional (Lim et al., 2007).

2.3. Transport of *E. coli* in freshwater

Bacterial transport in soil relies almost exclusively on water providing the matrix (Madsen and Alexander, 1982), with very little movement achieved by the bacteria alone. Within surface waters, approximately 40% of *E. coli* and faecal coliforms are reported to be associated with particles, although this can increase considerably during storm events as run-off enters surface waters (Characklis et al., 2005; Krometis et al. 2007; Jamieson et al, 2005a).

The factors that control the binding process are not fully understood. However, research in some areas has been able to pick out factors worth further investigation. Hermansson (1999) has shown that increasing the ionic strength tends to promote adsorption between particulates and bacteria, as demonstrated by Jiang et al. (2007). Cao

et al. (2011) investigated the adsorption exopolysaccharide (EPS), and biofilm formation (Danese et al., 2000), to Montmorillonite, Kaolinite and Goethite. They observed a promotive effect of sodium ions on EPS binding and attributed this to two possible factors; i) the tendency of cations to suppress the repulsive electrostatic charges of clay particles, leading to increased contact between EPS and mineral surfaces, or ii) the formation of cation bridges between functional groups of the EPS molecules and the negatively charged sites of clays. However, this final process only occurs in the presence of divalent ions.

Characteristics of the bacterium, the particulate material and the environment influence attachment and detachment processes. Bacteria-associated factors include intrinsic physico-chemical characteristics arising from the cell wall constituents, in particular extracellular lipopolysaccharides and the presence or absence of surface appendages which contribute to surface charge, hydrophobicity and topography (Gilbert et al, 1991). Foppen and Schivjen (2006) provide a comprehensive review of generic *E. coli* surface characteristics important for interactions with aquifer substrates. Like most bacteria, *E. coli* has an overall negative surface charge (Foppen and Schijven, 2006). Surface characteristics, for example the exposed portions of the flagellar antigen, can vary significantly with *E. coli* strain (Bilge et al., 1996; Pachepsky et al, 2008; Foppen, 2010) and may be implicated in attachment processes.

Zeta potentials (charge measured at the Stern Layer (Figure 2.4.)) have also been shown to differ significantly among *E. coli* serotypes and even within serotypes (e.g. *E. coli* O157) (Tufenkji and Castro, 2009; Foppen et al., 2010). However, in contrast it has also been suggested that there is little difference in the bulk functional group chemistry of

bacteria (Jiang et al., 2004). *E. coli* tends to be hydrophilic and for hydrophilic cells, zeta potential is thought to be important in determining adhesion to surfaces (Van Loosdrecht et al., 1987, Gilbert et al., 1991), although studies are contradictory (Tufenkji and Castro, 2009; Foppen et al., 2010). Surface characteristics of *E. coli* cells may also be affected by biological aspects such as the metabolic state of the organism. For example, both hydrophobicity and zeta potential have been shown to be related to the growth rate or phase (Allison et al., 1990; Smets et al., 1999). A comparison of seventeen *E. coli* strains, all isolated from livestock or water sources, showed a wide range of attachment efficiencies (0.039-0.44) when binding to quartz sand, with the most efficient having the highest number of genes associated with adhesion, toxin production, iron acquisition or capsular synthesis.

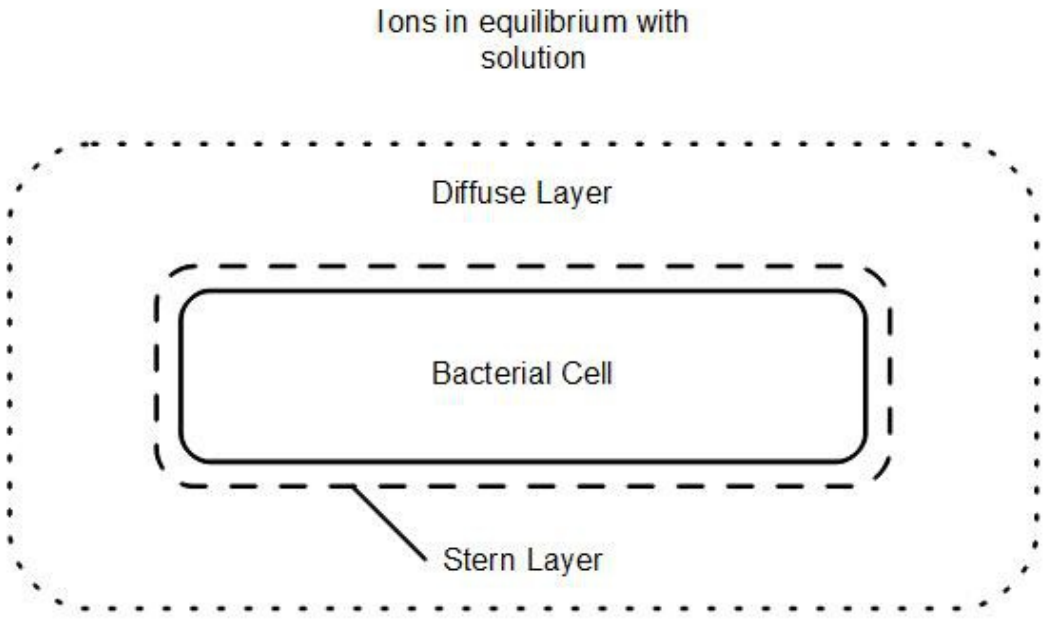


Figure 2.4: Different layers of ions surrounding a bacterial cell. The Stern Layer is where the permanently attached ions border the more fluid, diffuse layer.

Characteristics of the particulate material are also important in the attachment process and as for bacterial cells, physico-chemical properties of particle surfaces influence attachment, as discussed in detail elsewhere (Mills and Powelson, 1996; Foppen et al., 2006). Mineral composition, organic content and particle size (Pachepsky et al., 2008) affect the propensity of bacterial cells to adhere or release. Indeed, Scholl and Harvey (1992) studied the interactions between bacterial transport and sediment surfaces within a sandy aquifer and indicated that for relatively hydrophilic microorganisms, it was the mineral surface charge that controlled the initial adhesion of bacteria. Mineralogy often differs between size fractions, the smaller particles of the clay fraction providing a larger and more reactive surface area for adsorption. Cation exchange capacity also tends to increase with decreasing particle size and most colloids are coated in other reactive groups such as metals, metal oxides and hydroxides and organic material. This surface conditioning can alter the surface characteristics (Mills and Powelson, 1996) and influence interaction with cells.

The above cell and particle characteristics involved in attachment processes are further mediated by the nature of their environment. For example, electrolyte conditions (concentration, specific cations and valency of cations present) and pH directly influence the zeta potential of both cells and particles and increase collision frequency in saturated porous media (e.g. van Loosdrecht et al., 1987; Zita and Hermansson, 1994; Foppen et al., 2006). In general, increasing electrolyte concentrations lead to increased flocculation, although this depends on a number of other factors and the literature is contradictory for bacteria-colloid binding (Yee, 2000). In addition, the presence of phosphate in the

external aquatic environment had been shown to hinder the ability of *E. coli* O157 to bind to surfaces and potentially reverse previous binding events (Wang et al., 2011).

Changes in the external environment, such as concentrations of nutrients and organic material are likely to trigger changes in bacterial gene expression leading to associated differences in cell surface structures (Foppen et al., 2010). Colloidal particles are also likely to interact with organic matter from soils and from the stream environment. Depending on the type and structure of the organic matter, this can lead to stabilisation of colloids or can predispose them to sedimentation (Wilkinson et al., 1997) and can decrease bacterial attachment to soil or sediment particles (Guber et al., 2007). In addition to this, simple changes in the ratio of cells to particulates are likely to influence aggregation. Shear forces generated during high flow conditions lead to increased mixing and collision frequency, but also could potentially increase shearing of existing cell-particle interactions and biofilms. Turbulence during peak flows is also likely to increase oxygenation and generate bubbles, which can themselves differentially influence detachment rates depending on bacterial shape and strain (Gómez-Suárez et al., 2001). Environmental controls on cell-particle binding have been reviewed (e.g. Filip, 1973) but are not well understood for aquatic freshwater systems.

E. coli predominantly attaches to small particles (<2 μm), increasing the ease by which they are transported and dispersed in the environment (Muirhead et al., 2006; Goldscheider et al., 2010). Bacterial binding to surfaces, including sediment particles, can be reversible or more permanent (Van Houdt and Michiels, 2005) and it is not clear whether bacteria remain attached to the same particulates upon entering water bodies, or whether some are released and reattach to other particles, or remain free. However, it is

evident that pathogens and indicator organisms entering aquatic systems are more frequently associated with finer sediments and particles (Wilkinson et al., 1995; Borst and Selvakumar, 2003) than the water column itself (Crabill et al., 1999; Davies and Bavor, 2000). This association with particles is important for transport processes: cells attached to larger particles fall to the stream bed, whilst unattached cells, or those attached to small buoyant particles, are likely to be transported further, e.g. to shellfish harvesting waters (Jamieson et al., 2005a). Several authors have specifically demonstrated the presence of an in-stream or lake bottom store of faecally-derived bacteria (Jamieson et al., 2005b, Czajkowska et al., 2004, Muirhead et al., 2004; Droppo et al., 2009). Subsequent sediment re-entrainment during storm events or recreational water use disturbance can, therefore, lead to the deterioration in microbiological water quality (Crabill et al., 1999).

2.4. Conclusions

Understanding the ecology of *E. coli* in the water environment is essential as it forms a key proxy for the presence of pathogens in the environment. Sediment, biofilm and indigenous biota are potential and little-investigated stores and vectors for both faecal indicators and pathogenic organisms, including *E. coli* O157.

Although there have been studies investigating factors that control the survival of *E. coli* in water environments (e.g. Wang and Doyle, 1998; Williams et al., 2007; Avery et al., 2008), none of these have been able to identify the key factor that governs survival. From this it seems that there is no clear single factor that governs the survival of *E. coli* in the aquatic environment. Rather, the balance between survival and die-off seems to be

driven by the interaction of multiple stressors. Literature evidence suggests that the key chemical factors include pH, nutrient availability and the concentrations of metal ions in the environment. However, these are strongly linked to biological factors affecting both pathogens of faecal origin and indigenous microbial communities and thus affecting the competitive balance between the two. Important physical effects include temperature, the presence of sediment stores and the availability of oxygen. Interactions with indigenous microbiota, particularly protozoa have been highlighted in the literature as important biological factors.

It is clear that transport relies hugely on the interaction between *E. coli* and sediment. This interaction is very complex with the variation in the bacterial cell properties and sediment properties making massive differences to the outcome.

Unless all of these factors are understood and factored into current conceptual models (Brookes et al., 2004), a complete transport model cannot be created. This is required to understand which factors generate the variability in a quantitative microbial risk assessment to establish the risk to human health based on various environmental variables. Furthermore, if there are environmentally adapted *E. coli* in the aquatic environment, their use as indicators of faecal pollution can be questioned.

Chapter 3. Influence of native biofilm on the behaviour of *Escherichia coli* within an aquatic system

3.1. Introduction

Understanding the behaviour of *E. coli* in the aquatic environment is important as it is an important faecal indicator organism (EA, 2002). Furthermore, some *E. coli* strains entering surface waters are pathogenic (Mead and Griffin, 1998), therefore it becomes critical to comprehend their fate.

Principally, *E. coli* enters the environment through faeces of animals and birds and humans, particularly in livestock farming areas where the high density of cattle causes correspondingly high levels of manure to be deposited on the soil surface. This is easily transferred to watercourses by rainfall washing the manure from the soil surface and into streams and rivers (Muirhead et al., 2004; Jamieson et al., 2005). This process is a particular problem during storm events were high levels of rainfall cause the surrounding areas to be scoured of manure with it ending up in these surface waters and resuspension of stream sediment causing a spike in bacterial numbers that gets transferred downstream. *E. coli* has been shown in many studies to be able to survive for long periods of time within the aquatic environment (e.g. Wang and Doyle, 1998; Avery et al., 2008) and thus has the potential to be transported a substantial distance downstream if it remains suspended within the water column. However, there are many competing factors that control transport and most are not fully understood.

This study investigates the natural biofilm that forms in the stream environment. Biofilms form from one organism species or a collection of different species collaborating to create a community on a surface in their environment. Being part of this community improves

the survival chances of the participating organisms, protecting them from antimicrobial compounds and UV exposure (Ryu and Beuchat, 2005) and predation (Korber et al., 1997). Pathogenic *E. coli* have been found in these natural biofilms but the ability of *E. coli* to produce biofilms, however, appears to be critically dependent on the presence of other bacteria (Bauman et al., 2009; Klayman et al., 2009).

Currently the interactions between the biofilm on the stream surfaces and the planktonic *E. coli* in the water column above are not fully characterised. This is important for there is a need to know more about the balance between *E. coli* becoming trapped in stream biofilms leading to die-off verses the protection they receive once contained within the biofilm leading to a stream pool that can later be resuspended. Also, the issue of *E. coli* becoming stable in the environment has implications for its usefulness as an indicator (Brennan et al., 2010). The aim of this study was to investigate whether natural stream biofilm influences attachment of *E.coli* or coliforms to sandy sediment, thus potentially affecting survival and downstream transmission.

3.2. Methods

3.2.1 Experimental Site

Stream channels were constructed at the James Hutton Institute Research Station at Glensaugh Farm on the eastern edge of the Grampians, Scotland, UK (NGR NO 671783). The surrounding area is well characterised with ongoing environmental monitoring. The channels were constructed alongside the Slack Burn, a stream that runs through farmland, with grazing for cattle and sheep on both banks. There is an average annual rainfall of approximately 1200mm.

3.2.2 Channel Construction and Biofilm growth

Channels were constructed with rectangular plastic ducting, made watertight with waterproof sealant. Each channel was 10 m long with baffles set at either end and two within the channel to maintain water depth along the channel (See Chapter 8.2). In total there were 12 channels, these were fed from four header tanks, which in turn were gravity fed from Slack Burn, passing through one of two settling tanks. After exiting the channels, the water was returned to the stream *via* filtration through grass. The flow rate for the channels was approximately 1.2 L per minute, however, there was some variation due to the use of a gravity only system. Two runs of the experiment were conducted during June and July 2010.

Sand was added to six of the channels, determined through random selection) four weeks before the start of the experiment to allow natural biofilm to grow during that time (Table 3.1). Ceramic tiles were placed in the top sections of each channel for later sampling of the biofilm. Two of these channels were used for preliminary experiments testing the methodology. Three days before the start of the experiment the remaining channels were scoured of any biofilm growth and sand was added to allow washing off of any fine sand. Throughout the growth period, water flow in the channels was monitored and adjusted as necessary to maintain even flow across them all as far as practically possible.

TABLE 3.1: Channels

Channel Number	Run 1	Run 2
1	Biofilm	Not Used
2	No Biofilm	Not Used
3	Test Channel	Not Used
4	Biofilm	Biofilm
5	No Biofilm	No Biofilm
6	Biofilm	Biofilm
7	No Biofilm	No Biofilm
8	No Biofilm	No Biofilm
9	Test Channel	Test Channel
10	No Biofilm	No Biofilm
11	Biofilm	Biofilm
12	Biofilm	Biofilm

3.2.3 Slurry Addition

Slurry was added to the channels to simulate faecal run-off during a storm event and to give detectable numbers of *E. coli* and coliforms in sediment. This was made by collecting fresh manure, sieving through a 2 mm sieve and adding streamwater. In total, 80 L was made at a rough concentration of 1 kg (wet weight) per 10 litres.

For the first run, Slurry was added to all ten experimental channels consecutively by a simple method of buckets with holes in the bottom. Preliminary tests gave an approximate initial flow rate of 4 L per hour. These sat at the top end of the channels and were topped-up when they reached half empty. For the second run, a peristaltic pump was used to added the slurry to one channel at a time to provided a more even flow rate.

3.2.4 Sampling Procedures

3.2.4.1 Water

From the start of slurry addition, water samples were taken from the channel effluent in vessels previously washed out with channel effluent every 15 minutes. The gaps between the sample times increased to every 30 minutes after 45 minutes. Samples were taken for coliform and *E. coli* enumeration using Colilert mixing bottles, diluting as needed, using sterile de-ionised water and for water chemistry in clean plastic bottles.

3.2.4.2 Sediment

Sediment samples were taken from the sand surface using 5 mL scoops and placed into sterile containers. Care was taken to ensure that sampling did not occur at previously disturbed sites. Samples for *E. coli* and coliform analysis were stored at cool temperatures in a cooler box and analysed within an hour. Water chemistry samples were also stored at 5 °C at the end of each day. These samples were analysed within a week being taken.

3.2.4.3 Biofilm

Biofilm samples were obtained through scraping any biofilm off that had accumulated on the ceramic tiles into sample bottles using sterile de-ionised water to wash any excess of the tiles.

3.2.5 *E. coli* and Coliform enumeration

E. coli and coliforms were enumerated using Colilert 18™. This method identifies bacteria through the effect of their constitutive enzymes on chromogenically labelled substrates and is therefore referred to as a Defined Substrate Technology. Colilert specifically tests for coliforms and *E. coli* through the effect of two enzymes, coliforms possessing β-D-galactosidase and *E. coli* also possessing β-D-glucoronidase, have on

substrates within the test medium. The presence of coliforms causes the test medium to turn yellow from colourless. These are then viewed under UV to look for fluorescence that indicates the presence of *E. coli.* The actual number of coliforms and *E. coli* present in a sample was calculated using Most Probable Number based on the number of wells in each Colilert Tray that showed the presence of the organism of interest.

For the sediment samples, 15 mL sterile de-ionised water was added to each sample, then were subjected to 20 seconds vortexing to knock off recently bound bacteria from the sediment. The sample was then added into the Colilert mixing bottle with the remainder of the required volume made up with sterile de-ionised water.

3.2.6 Water Characteristics

Water samples were analysed for nitrogen (ammonia, nitrate and organic), phosphorus (phosphate and organic), dissolved organic carbon (DOC), pH and conductivity. All analysis was conducted to ISO 9001 standard.

3.2.7 Bromide Tracer

Prior to the first run, a bromide tracer was used to investigate the retention time of the channels.

3.2.8 Microcosm Experiment

A microcosm experiment was undertaken to establish if there were any observable effects of a biofilm on the binding of *E. coli.* An overnight culture of cells of *E. coli* O157:H7 #3704 and an environmental strain of *E. coli* were grown in LB broth (100 rpm). These

were washed twice @ 5000 x gravity in filter sterilised (2 μm Whatman) river water from Slack Burn (Glensaugh) giving two separate cell inocula.

Sand with biofilm was collected from channels after three weeks of biofilm growth. Fresh sand was placed in one of the stream channels, allowed to equilibrate for four hours to remove fines and then samples were also taken from this channel to provide experimental substrate. For each microcosm five g wet-weight sediment was weighed into sterile plastic universal bottles, half using the biofilm coated sediment, half using the uncoated sediment and 15 mL sterile river water was added to each microcosm. 500 μL cell inoculum of either the *E. coli* O157 or the environmental isolate was added to microcosms. This gave six different groups (See Table 3.1) within which there were three replicates.

All microcosms were placed on their side, stabilised in a rack and shaken at 125 rpm on an orbital shaker for 45 minutes at 10 °C. Microcosms were left to stand for 40 minutes to allow the majority of large particulates to settle. 100 μL was sampled from the top of each microcosm for serial dilution and plating. Sediment free controls (three for each strain) were also incorporated and shaken as per treatment.

All samples were plated on ES Chromocult Agar and incubated at 37 °C for 18 hrs. Samples containing *E. coli* O157 were also plated on Sorbitol Maconkey Agar and incubated at 37 °C for 18 hrs.

3.2.9 Statistical analyses

All data were checked for meeting the assumptions of t-test and ANOVA. Normality of the data was tested using Shappiro-Wilk and all the data came from a normal distribution.

For all the experiments, to determine any difference between the channels with and without biofilm coverage, the mean numbers of *E. coli* for the two groups were compared using independent t-test (SPSS 14 (PC/Windows). Further analysis into the variation within the groups was conducted using one-way ANOVA (SPSS 14 (PC/Windows).

TABLE 3.2: Experimental Groups for Microcosm Experiment

Organism	Sediment Present (Y/N)	Biofilm Present (Y/N)
E. coli O157	N	N
E. coli O157	Y	N
E. coli O157	Y	Y
Environmental Isolate*	N	N
Environmental Isolate*	Y	N
Environmental Isolate*	Y	Y

*of *E. coli*

3.3. Results

3.3.1 Coliform and *E. coli* numbers recovered from Channel effluent (First Run)

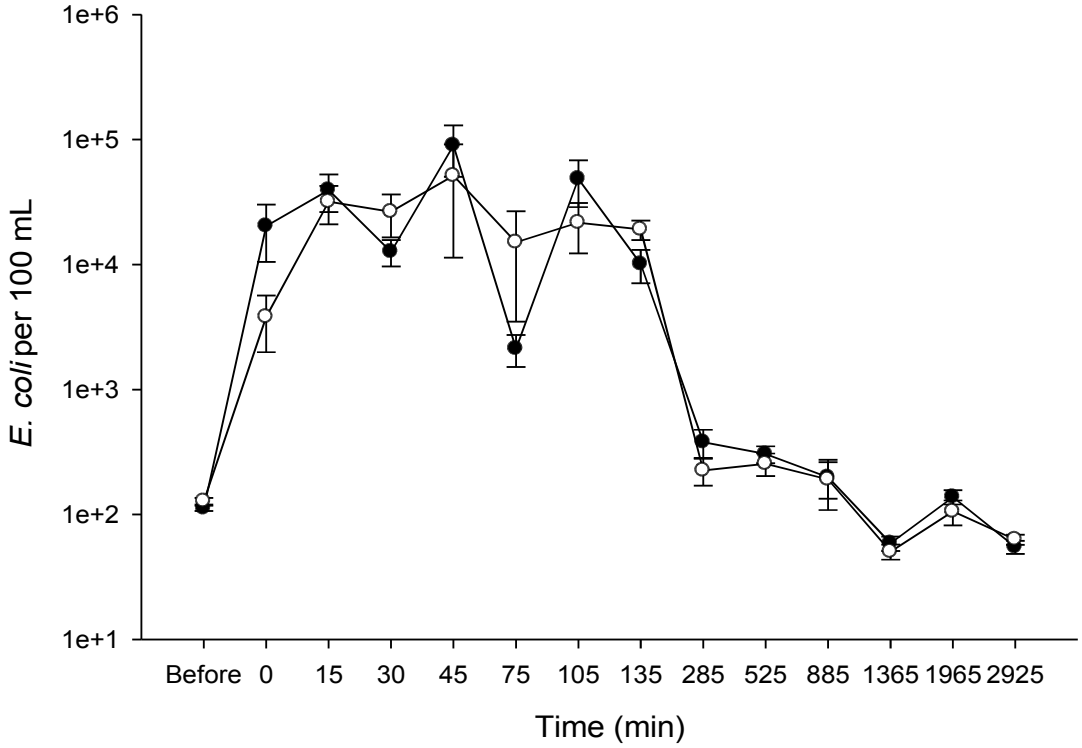


Figure 3.1. Run 1. The number of free E. coli detected exiting the channels per 100 mL (●) Channels with biofilm and (○) channels without biofilm. Data points represent treatment means for each timepoint (n=5) ± SE. Slurry addition started at time 0 and ended at time 135.

E. coli numbers before addition were approximately 100 CFU per 100 mL for both biofilm and non-biofilm channels (Figure 3.1). The source stream water also had this level of *E. coli* contamination indicating that the syphoning system did not unduly influence bacterial load.

After addition started, *E. coli* numbers spiked immediately up to ~2.0 x 10^4 CFU ± 9.81 × 10^3 per 100 mL for the biofilm channels and ~4 x 10^3 ± 1.83 × 10^3per 100 mL for the non-biofilm channels. However, much variation was observed within the two groups resulting in no statistical significance (p=>0.05) possibly due to small sample size. By 15 min after addition started, the two groups had both reached levels of 3 x 10^4 -4 x 10^4 ± 1 × 10^4 per 100 mL and remained at this level until addition had ceased. The large error values seen may be attributable to the variation in application speed when using the bucket method (i.e. the rate of addition dropped as the level of the slurry within the bucket decreased) .

Within 3.5 hours of addition ceasing, the *E. coli* numbers had returned to background levels for both groups with no statistical difference between them. Subsequent monitoring showed that there were no short-term differences in *E. coli* numbers in channel effluent between the biofilm coated and clean channels.

Total coliform numbers mirrored that of *E. coli* rising immediately from a background level of ~9 x 10^2 ± 5.0 × 10^1 per 100 mL to a peak of over 3 x 10^4 ± 1.52 × 10^4 per 100 mL during application (Figure 3.2). However, the proportion of *E. coli* within the total coliform counts increased to almost one from approximately 0.25 indicating that the majority of coliforms added within the slurry were *E. coli*.

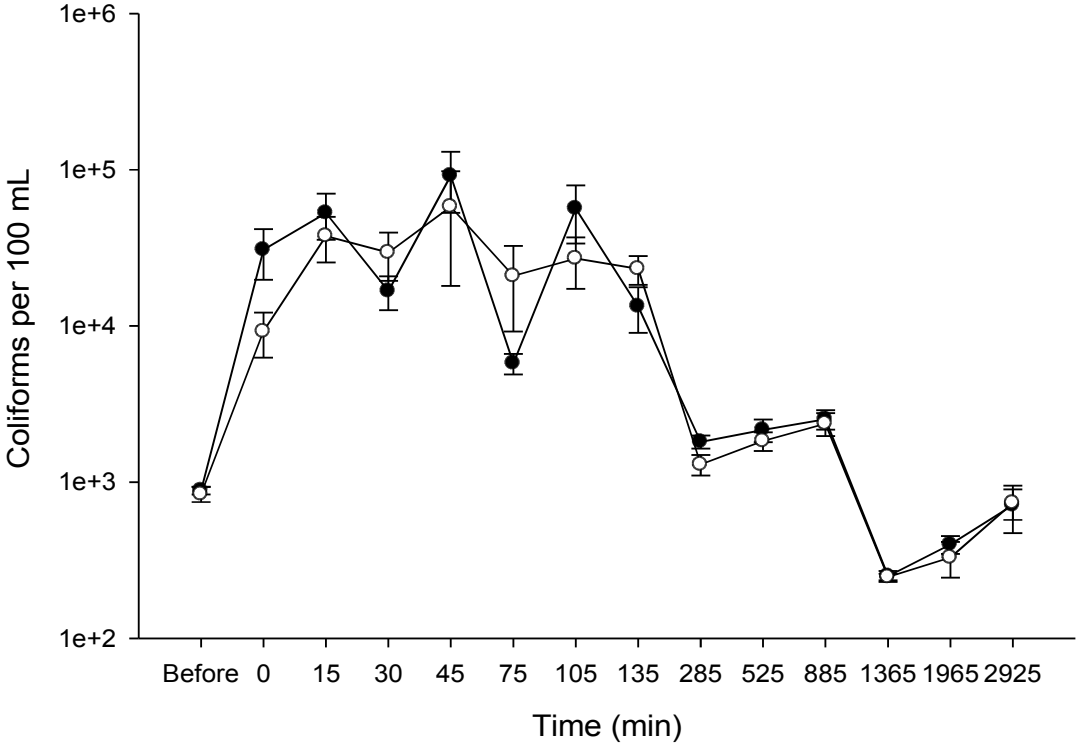


Figure 3.2. Run 1. The number of free coliforms detected exiting the channels per 100 mL (●) Channels with biofilm and (○) channels without biofilm. Data points represent treatment means for each timepoint (n=5) ± SE. Slurry addition started at time 0 and ended at time 135.

3.3.2 Coliform and *E. coli* numbers recovered from Channel effluent (Second Run)

The second run of the experiment using a peristaltic pump to add the manure slurry showed the same pattern as the first run. There was a large increase in both *E. coli* and coliform numbers a soon as the addition began and returned to background (~2 x 10^1 per 100 mL for *E. coli* and ~7 x 10^2 per 100 mL for Total Coliforms) within 2.5 hours (Figures 3.3 & 3.4).

The variation between the channels within the same group (i.e. Biofilm coated or not) was much less than during the first application. However, there was no statistically significant difference between the two groups.

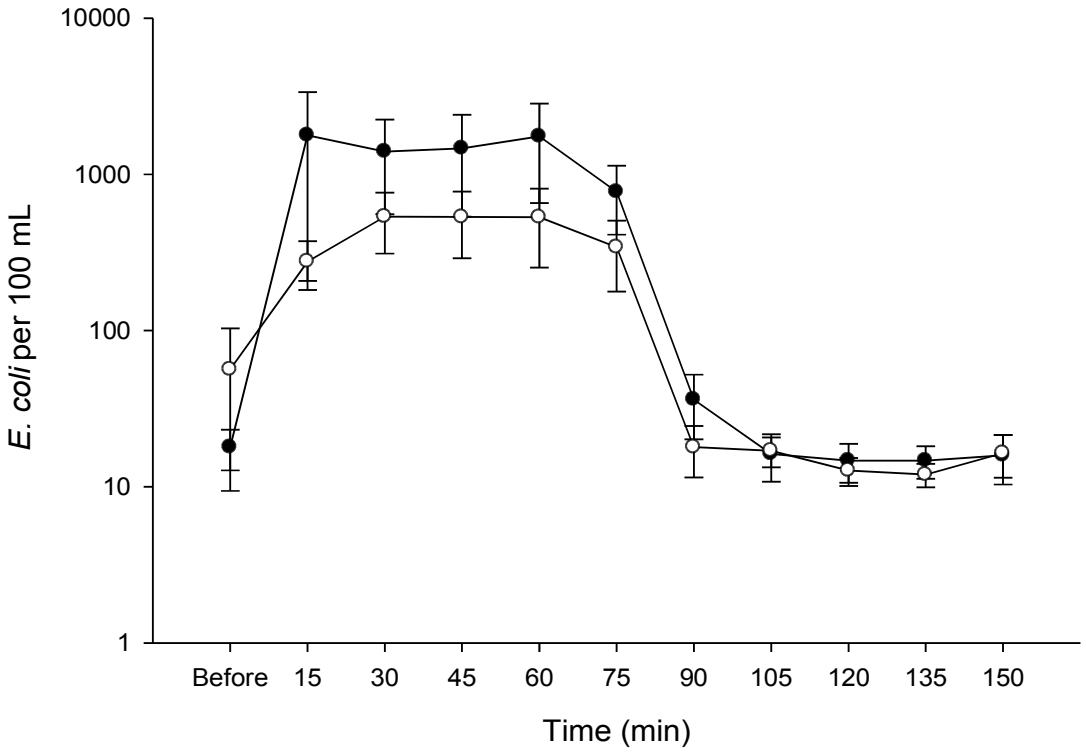
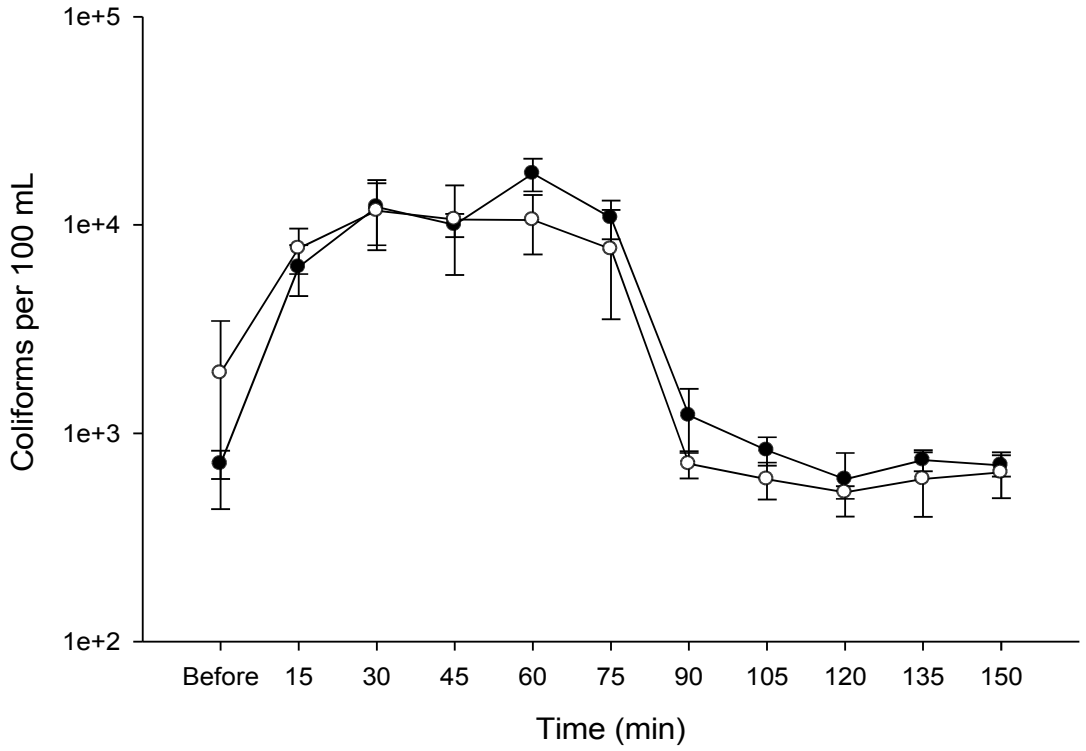


Figure 3.3. Run 2. The number of free *E. coli* detected exiting the channels per 100 mL (●) Channels with biofilm and (○) channels without biofilm. Data points represent treatment means for each timepoint (n=5) ± SE. Slurry addition started at time 0 and ended at time 60.

Figure 3.4. Run 2. The number of free coliforms detected exiting the channels per 100 mL (●) Channels with biofilm and (○) channels without biofilm. Data points represent



treatment means for each timepoint (n=5) ± SE. Slurry addition started at time 0 and ended at time 60.

3.3.3 Coliform and *E. coli* numbers recovered from sediment (First Run)

Before addition of manure, the concentrations of *E. coli* and coliforms were significantly lower in the sediment from biofilm free channels that had only been exposed to the water for a few days when compared to the biofilm coated channel the had been exposed for several weeks (Figures 3.5 and 3.6). The non-biofilm channels had ~1 ± 0.4 *E. coli* per g sediment and ~ 30 ± 7 coliforms per g sediment, whereas the biofilm coated sediment had

~15 ± 5 *E. coli* per g and over 2 x 10^2 ± 40 coliforms per g. This demonstrates that over time, faecal bacteria were accumulated from the stream water.

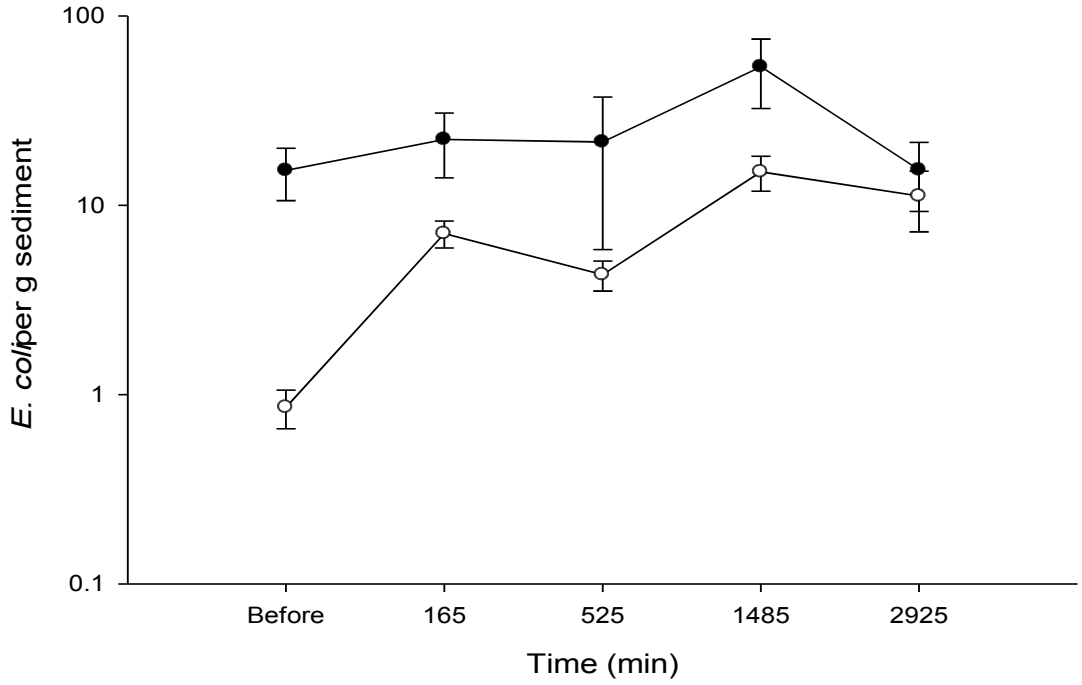


Figure 3.5. The number of *E. coli* detected per g sediment (dry weight) (●) Channels containing biofilm and (○) uncoated channels. Data points represent treatment means for each timepoint (n=5) ± SE. Slurry addition started at time 0 and ended at time 135.

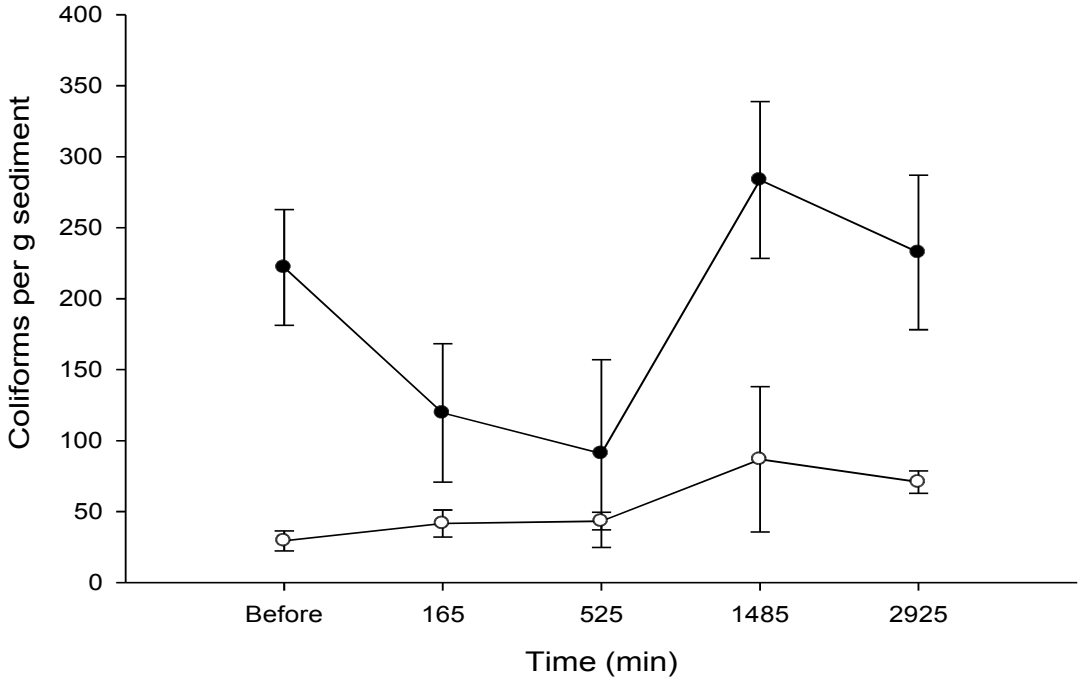


Figure 3.6. The number of Coliforms detected per g sediment (dry weight) (●) Channels containing biofilm and (○) uncoated channels. Data points represent treatment means for each timepoint (n=5) ± SE. Slurry addition started at time 0 and ended at time 135.

Sediment samples were taken immediately after slurry addition had ceased and showed an increase in *E. coli* and coliform numbers for both groups. The number of *E. coli* detected in the sediment of the biofilm coated channels increased by approximately eight per g of sediment up to ~22 per g sediment. The uncoated channels only showed an initial increase of 6 up to ~7 *E. coli* per g of sediment. Numbers of *E. coli* continued to increase in the uncoated channels to a point where there was no significant difference between the two groups after two days.

The coliform numbers proved to be more variable with a decrease in coliform numbers observed in the biofilm coated channels from over 200 coliforms per g sediment to below 120 per g sediment between the start value and the first sampling taken after slurry addition. The coliform numbers then climbed back to over 200 and remained above this value. The uncoated channels showed a small increase of coliform numbers during the slurry application, starting from ~30 per g sediment and reaching ~ 40 per g sediment. Coliform numbers then doubled over the next day but remained significantly lower than the coliform levels in the biofilm coated sediment channels two days later.

Taken in conjunction with the fact that *E. coli* levels for the uncoated channels do reach the levels seen in the biofilm coated channels it suggests that *E. coli* bind more to sediment than the coliform group as a whole.

3.3.4 Coliform and *E. coli* numbers recovered from sediment (Second Run)

As for the first run, both *E. coli* and coliform numbers were measured before and after addition, however, during the second run there was not any further monitoring after this point. The initial sampling before slurry addition again showed significantly lower numbers of both *E. coli* and coliforms in the uncoated channels for the same reasons as in Run 1 (Figures 3.7 and 3.8).

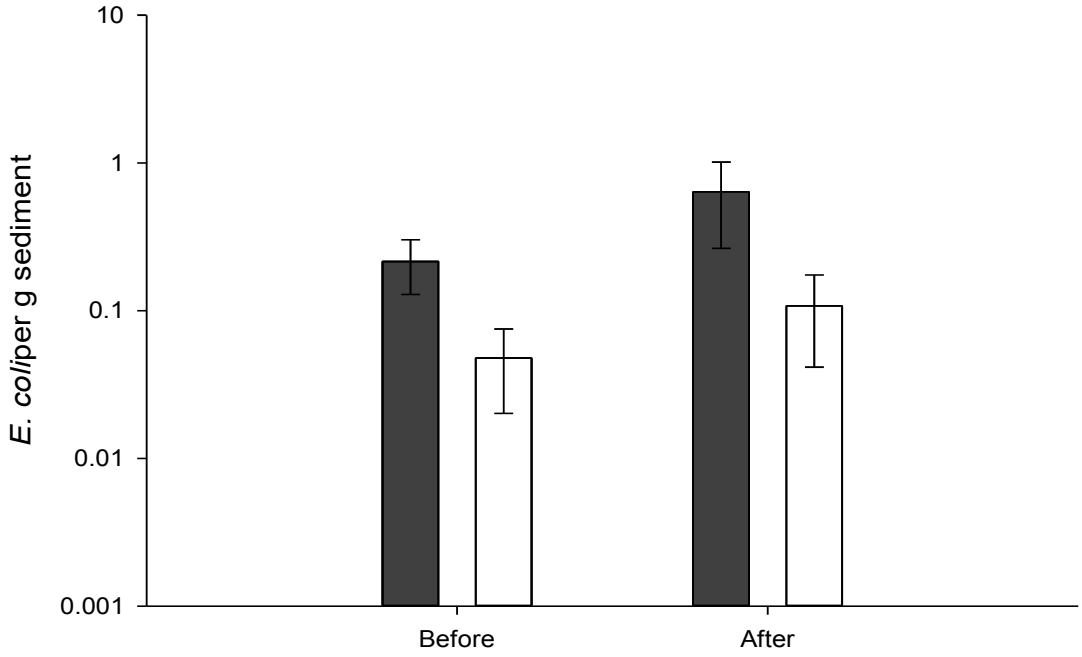


Figure 3.7. The number of *E. coli* detected per g sediment (Dry weight)before and after slurry addition. (■) Channels containing biofilm and (□) uncoated channels. Data points represent treatment means for each timepoint (n=4) ± SE.

Analysis of data showed that there were no significant differences in numbers before and after addition for either *E. coli* (p=0.734) or coliforms (p=0.445) and there were no significant differences between the biofilm coated and the uncoated sediment using this approach (p=0.510).

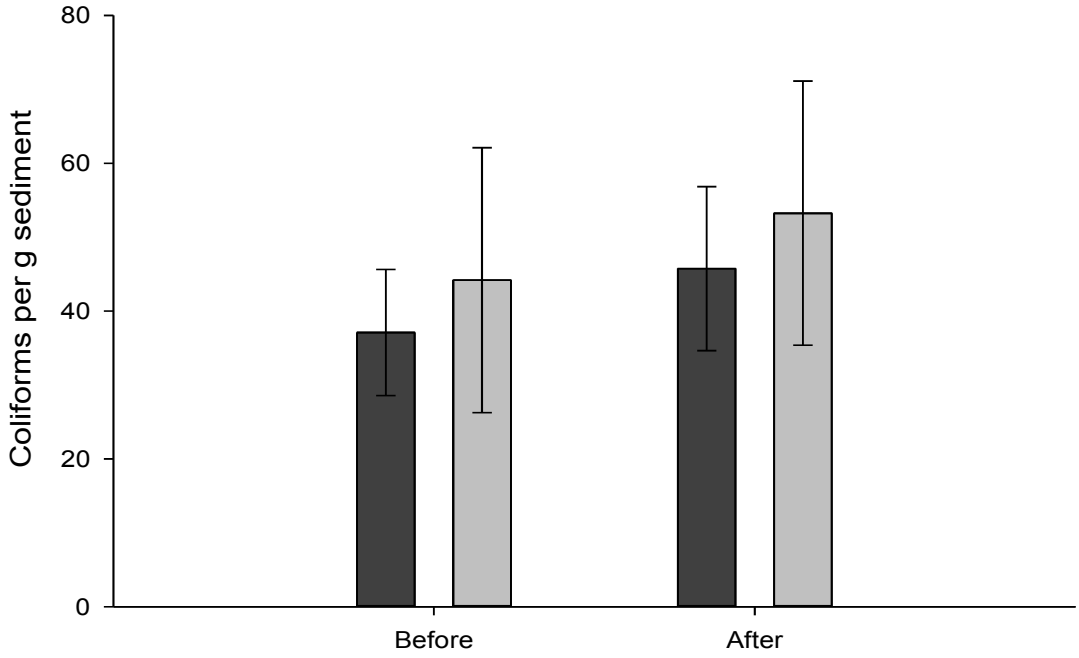


Figure 3.8. The number of Coliforms detected per g sediment (dry weight) before and after slurry addition. (■) Channels containing biofilm and (□) uncoated channels. Data points represent treatment means for each timepoint (n=4) ± SE.

3.3.5 Microcosm Experiment

For the environmental isolate of *E. coli,* there was a significant difference between the group with neither sediment nor biofilm and the other two groups (p=<0.05) (Figure 3.9). There was no significant difference between the group with the sediment and the group with both sediment and biofilm (p=0.650), indicating that for this organism it is purely the presence of the sediment that influences the number of organisms in the water column above it.

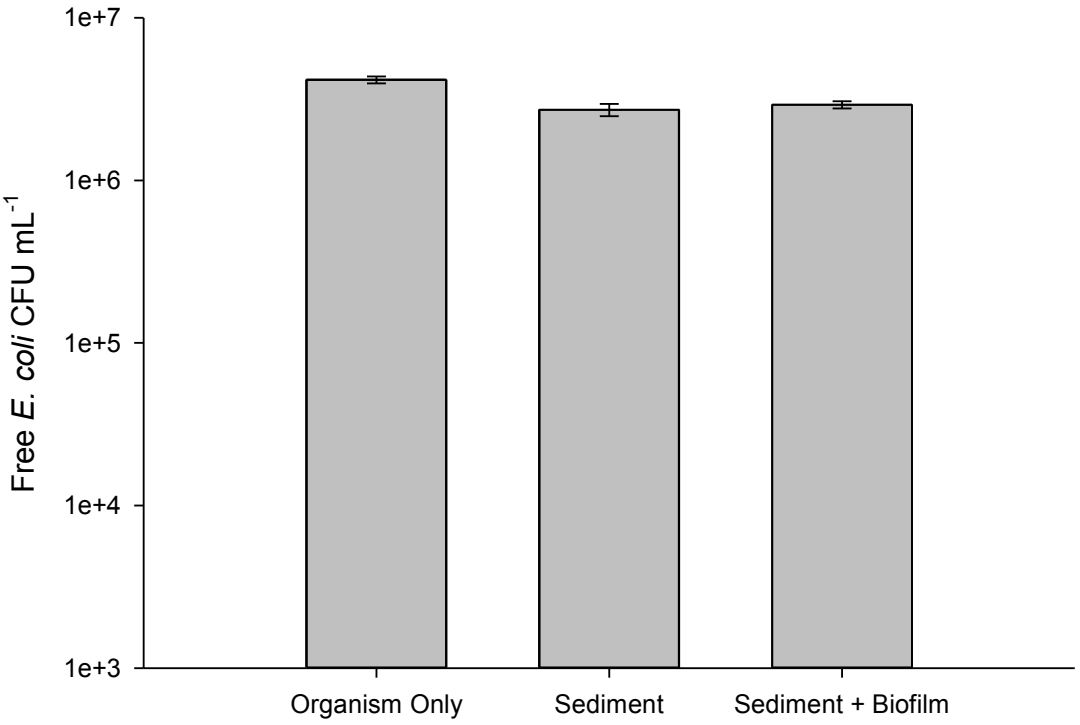


Figure 3.9. The number of free Environmental Isolate E. coli detected after shaking for 45 minutes at 10 °C; 100 rev min^{-1}. Data points represent treatment means for each timepoint (n=4) ± SE. Plated on ES Chromocult Agar.

E. coli O157 produced unusual results; instead of the expected drop in numbers as seen with the environmental isolate of *E. coli*, the numbers of *E. coli* O157 actually increased (Figure 3.10). No *E. coli* were introduced to the microcosms through the sediment or the biofilm suggesting that something is happening to alter the metabolic state of the organisms so that they would not be able to grow when plated out. The most likely explanation is that there is an unknown factor within the streamwater used in the experiment that compromises the *E. coli* O157 cells or even kills them. The presence of

sediment and the biofilm can protect the *E. coli* O157, thus boosting numbers. In the other microcosms, both the sediment and the biofilm provide sufficiently benign conditions so that the influence on *E. coli* O157 is less pronounced. However, there is a significant difference between these two groups, suggesting the described effect could be occurring here as well. What negates this idea is that it does not seem to affect the environmental isolate also used in this experiment that has been shown in previous experiments to be less hardy than *E. coli* O157. One further possible explanation is that the low nutrient condition of the organism-only microcosms that only contain streamwater cause *E. coli* O157 to begin to enter a viable but non-culturable (VBNC) state and thus fewer numbers are seen when cultured. However, previous research on the VBNC state of *E. coli* O157 suggests that it can take many days for the organism to transition to this state and the length of this experiment is simply not long enough.

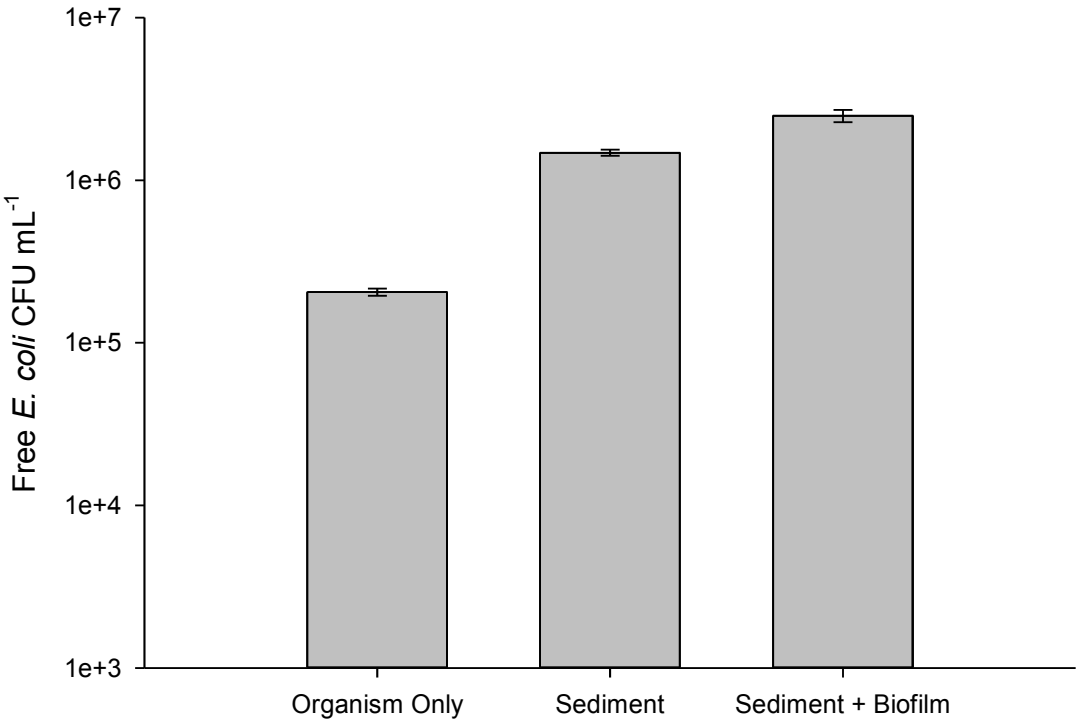


Figure 3.10. The number of free *E. coli* O157 detected after shaking for 45 minutes at 10 °C; 100 rev min^{-1}. Data points represent treatment means for each timepoint (n=4) ± SE. Plated on ES Chromocult Agar.

3.4 Discussion

Biofilms are known to play an important role in bacterial survival for within them bacteria can resist predation (Korber et al., 1997) and are protected from the surrounding environment, such as antimicrobial compounds and UV exposure (Ryu and Beuchat, 2005). Biofilms also resist displacement by flowing water and facilitate a long-term reservoir in aquatic environments (Cooper et al., 2007). Natural biofilms have been found to carry pathogenic *E. coli* O157 expressing virulence factors associated with human disease (Cooper et al., 2007) along with other pathogens (Balzer et al., 2010), raising the importance of understanding these communities further.

Competition for nutrients to survive and replicate, and the degree of inter- and intra-species competition is a critical factor in the survival of any water-borne pathogen and their indicators. Avery et al. (2008) and Wang and Doyle (1998) demonstrated that survival of *E. coli* O157 was reduced when grown in the presence of the resident water micro-organism population in comparison to autoclaved (sterile) samples; indicating the influence of the resident microbial population. On average, there is a die-off rate of approximately 1 log-unit due to the predation or antagonistic effects of indigenous communities on *E. coli* (Foppen and Schivjen, 2006). Further studies by LeJeune et al. (2001) and Artz and Killham, (2002) confirm the importance the indigenous population play in removing *E. coli* from the environment. Therefore it becomes important to understand how *E. coli* survive and form stores within this hostile environment.

There have been several studies investigating the location of these faecal indicator stores within the environment. For example, Hirotani and Yoshino (2010) identified the natural

biofilm as a store for *E. coli*. In addition, there was evidence in their study that it was possible for the *E. coli* within the biofilm to reproduce, potentially creating a constant supply into the surface water. Further evidence also suggested that the attachment of planktonic *E. coli* to biofilm was considered negligible in the river environment. Several authors have specifically demonstrated the presence of an in-stream or lake bottom store of faecally-derived bacteria (Jamieson et al., 2005b, Czajkowska et al., 2004, Muirhead et al., 2004; Droppo et al., 2009; Balzer et al., 2010) and that subsequent sediment re-entrainment during storm events or recreational water use disturbance can, therefore, lead to the deterioration in microbiological water quality (Crabill et al., 1999).

The stream channels used in these experiments provided the opportunity to investigate the effects of biofilms on the *E. coli* and coliform levels in surface waters within an environmental setting. Overall, the presence of the biofilm does not seem to have any short-term effect on the numbers of *E. coli* or coliforms during a faecal pollution event. However, over the long-term *E. coli* and coliforms seem to be part of the natural biofilm and therefore act as a store within the surface water system. This effect can be seen in the high levels of both *E. coli* and coliforms in the channels that have encouraged biofilm growth over the channels that have only been exposed to the natural environment for a few days (e.g. Figures 3.5 and 3.6). In addition, there seems to be a background level at which the population density equilibrates within the biofilm, for despite adding many times than average for the stream neither *E. coli* nor coliform numbers significantly increase. As a consequence it is unlikely to see any further accumulation of bacteria in this case. However, exposure to low levels of *E. coli* over a period of time, as seen in the experimental preparation, may allow a gradual accumulation in comparison to a short

exposure to high numbers. There is also the possibility that the stream flow was too quick to allow *E. coli* to settle within the short distance of the channels, only collecting in areas where the water is still enough to allow settling to occur. In fact, a study by Fries et al. (2006) suggested that only 38% of the entrained *E. coli* would be able to settle out of the water column.

The only significant increase is seen in the uncoated channels where the addition of manure slurry containing high levels of *E. coli* causes a 10-fold increase in *E. coli* numbers associated with the sediment (Figure 3.5). In contrast, coliform levels do not significantly change, highlighting that almost all of the coliforms within the cattle slurry are of the *E. coli* species. This is corroborated by the water samples that had a ratio of *E. coli*:coliforms approaching 1.

The microcosm experiment provided a more controlled environment in which to study the effects of the biofilm and also provide an insight into how the phenotypic variation between different *E. coli* strains influences the reactions within this environment. In this controlled environment, we again saw that uncoated sediment encourages the binding of *E. coli* from the water column for the environmental isolate *E. coli* examined corresponding with previous research indicating the importance of *E. coli* – sediment binding.

The behaviour seen in the microcosm experiment and the high variability seen between the stream channels highlights the problems investigating bacterial binding for there are many complex factors that can influence this process.

Investigating this process without a faecal pollution event it seems clear that an established biofilm would have no effect on bacterial contamination in the water column

above. Based on the microcosm experiment, it seems that early stage biofilms and recently exposed sediment would provide areas where it is possible for *E. coli* and other organisms to attach.

This adds further complication to trying to predict the final destination of *E. coli* and other organisms once released into surface waters. Other factors that could potentially influence this process, range from the flow rate to water chemistry to bacterial surface characteristics without one single controlling factor. For example, the presence of strongly charged ions in the surrounding matrix had some influence over the binding process (Hermansson, 1999) but in this system the charged ions would be in too low concentrations to have an impact. Bacterial products such as exopolysaccharide (EPS) also play a role in adsorption and biofilm formation (Cao et al., 2011) and so does clay type (Danese et al., 2000). However, further work in needed to known how *E. coli* integrates into the established biofilm.

The findings of this study have relevance beyond the natural environment to man-made environments such as water distribution systems (Pachepsky et al., 2012) and food manufacture (Ryu and Beuchat, 2005) where biofilms are found along with *E. coli.* Particularly, it highlights the need to remove these biofilms containing these organisms for they do not provide a positive role in health protection and potentially harbour organisms that cause serious illness.

To summarise, this study has provided further evidence to support the view that the ability of biofilms to reduced bacterial load in the water column above is negligible over short periods of time. However, over a long period of time biofilms do provide stores of faecal indicators and their associated pathogens and through either resuspension or

reproduction can release these important organisms into the water column potentially impacting public health.

Further research needs to investigate the specific *E. coli* strains associated with the biofilm in order to identify the source, either from faecal pollution events or, perhaps more importantly, if the *E. coli* within these biofilms are sourced from this habitat rather than the mammalian gut.

Chapter 4. Investigation of how electrolyte type and concentration regulate the binding of an environmental *Escherichia coli* isolate onto kaolinite and montmorillonite clays

4.1. Introduction

Environmental dissemination of zoonotic pathogens through the rural environment and transport to water courses is typically facilitated through hydrological events, primarily arising from agricultural run-off (*via* overland flow or percolation into ground water) and through storm overflows from wastewater treatment plants. Large numbers of faecal organisms can be transported from the surrounding catchment into watercourses (Thurston-Enriquez et al., 2005; Hathaway and Hunt, 2011), ultimately contaminating downstream bathing and recreational waters and presenting a potential risk of infection.

In order to reduce risk, faecal pollution is monitored through testing waters for indicator organisms, principally *E. coli*, therefore warnings can be given during high risk periods. Thus, it is important to understand the reaction of these organisms in the environment in addition to pathogenic organisms.

Cells are frequently associated with organic material at source (faeces) and can become attached to soil particles as they are washed across soil surfaces (Tyrrel and Quinton, 2003) or to sediment particles following entry to the stream environment. Interactions with soil particles have been well studied (Muirhead et al., 2006; Oliver et al., 2007), however bacteria-particle interactions are also important within aquatic systems (Muirhead et al., 2004; Jamieson et al., 2005: Wu et al., 2009). Binding to

sediment particles can provide physical shielding against environmental stressors such as damaging ultraviolet light and protozoal predation (Langenheder and Jurgens, 2001; Winward et al., 2008). These protective effects are seen through increased survival of *E. coli* within stream bed sediments in comparison to free cells in the water column (Davies et al., 1995). Natural (environmental) waters typically contain colloidal clay particles which can interact with bacterial cell surfaces. Studies have shown that the outer cell walls of bacteria from river sediment are often encrusted with fine-grained (<1 μm) particles of clays (Konhauser et al., 2009); eliciting profound impacts on their mobility, respiration, predation, infection by bacteriophages and transfer of genetic material (e.g. Roper and Marshall, 1979; Singleton, 1983; Jiang et al., 2007).

Bacterial attachment to particles is influenced by both physical and chemical characteristics of the sediment, the bacterial cell surface and the surrounding water. Sediment particle size is particularly important, as smaller particles have a greater surface area-to-volume ratio, providing greater scope for binding. It is broadly accepted that *E. coli* cells are predominately associated with small particles of soil or sediment; for example, Muirhead et al. (2006) and Goldscheider et al. (2010) reported that most *E. coli* cells in stream water, and indeed other bacteria present in storm water, were associated with colloidal particles <2 μm. Clay colloids are the most influential inorganic particles present in stream bed sediment and therefore understanding the fundamental mechanisms of attachment of indicator microorganisms to these particles is essential to determine their influence on transport, survival and stream storage.

Attachment of *E. coli* to surfaces is understood to occur through several stages (Van Houdt and Michiels, 2005), beginning with reversible attachment between the

bacterium and the surface. Exopolysaccharide (EPS) and fimbriae are then produced, anchoring the bacterium irreversibly (Junkins and Doyle, 1992). In order for this initial attachment to take place, the bacterial cell needs to come close enough in order to overcome the electrostatic repulsion due to both sediment (Zhuang and Yu, 2002) and bacteria (Soni et al., 2008) generally having a net negative surface charge. The charge of the sediment is concentrated in the smaller particles (<2 μm) (Mattson, 1931) therefore they are of key importance when considering the role of electrostatic charge.

Storm events can resuspend particles from the stream bed, releasing pathogens and faecal indicator organisms associated with these sediment stores (Muirhead et al., 2004; Jamieson et al., 2005; Wu et al., 2009). Stream sediments can harbour up to 10^8 CFU of *E. coli* m^{-2} of streambed area and if resuspended during storm events, the concentration of *E. coli* in the water column has been shown to increase by two orders of magnitude (Muirhead et al., 2004). Rainfall events can also alter stream water chemistry through the influx of surface run-off. For example, Caissie et al. (1996) noted the progressive dilution of electrolytes in stream water, reporting a negative correlation between stream discharge and electrolyte concentration. Some chemical characteristics (e.g. concentrations of Na^+, K^+ and Ca^{2+} ions) also follow this trend while others (e.g. conductivity, Dissolved Organic Carbon and Cl^-) correlate positively with stream discharge (Caissie et al., 1996). These relationships are influenced by geologic structure and land-use within the catchment, with chemical input through human activity of particular importance (Siwek et al., 2011).

Discharge-associated changes in water chemistry have the potential to alter adsorption-desorption behaviour of any pathogenic or indicator bacteria with colloidal

material within the stream water. For example, lower electrolyte concentrations may reduce the likelihood of binding, as it influences the surface charge of cells and particles and affects collision frequency (van Loosdrecht et al., 1987), although this depends on a number of other factors and the literature is contradictory for bacteria-colloid binding (Yee, 2000). This binding influences transportability of pathogens as larger aggregates are likely to be transported shorter distances as they settle more rapidly, whereas free bacteria can potentially travel further. The influence of electrolytes may become exaggerated where salinity is greater, such as tidal or estuarine environments where agricultural run-off interfaces with bathing waters.

The low infectious dose of many zoonotic pathogens (Chart, 2000) and their relative scarcity in environmental samples leads to a reliance on the more abundant faecal indicator organisms to provide information on pathways for entry of pathogens (and other faecal pollutants) into watercourses and their subsequent transport. Standard practise is to monitor *E. coli* and faecal coliforms (EA, 2002; SEPA, 2007) within the water column, however, a more fundamental understanding of the impacts of changing aquatic conditions during rainfall events and impacts on pathogen or indicator binding and transport will strengthen targeted monitoring and risk assessment approaches.

In this study, laboratory model system was used to investigate the effect of electrolyte type and concentration on the binding of an environmental strain of *E. coli*. It was hypothesized that divalent cations would induce greater biding of *E. coli* to colloids and that this would be enhanced at higher electrolyte concentrations.

4.2. Materials and Methods

4.2.1 Microcosm preparation

Electrolyte solutions (Sodium chloride (NaCl), Potassium chloride (KCl) and Calcium chloride ($CaCl_2$) were prepared at concentrations of 0.1, 1, 3, 10 and 100 mM plus 0.01, 0.03 and 0.3 mM for Calcium chloride (due to the lower flocculation point for this electrolyte; Goldberg and Glaubig, 1987; Garcia-Garcia et al., 2007) in Milli Q water and autoclaved at 121 °C for 15 minutes. Forty-five mL of each electrolyte solution was added to triplicate sterile microcosms (250 mL polypropylene screw cap bottles). Montmorillonite SWy-1 and Kaolinite KGa-1b (van Olphen and Fripiat, 1979) clay solutions were prepared by sonicating 0.125 g of clay in 250 mL Milli Q water for five min at 15 Hz using a 24 mm diameter low intensity solid horn probe (600 W Ultrasonic Processor, Sonics and Materials Inc., CT, USA). Five mL of clay solution was added to each microcosm giving a final concentration 0.05 g L^{-1} clay representative of that seen in freshwaters under storm flow conditions. Controls containing no clay (i.e. electrolyte only) were also prepared in triplicate with the clay solution being replaced with an equivalent volume of Milli Q water. Particle size analysis of stock clay solutions was also performed (Mastersizer, Malvern, Worcs, UK).

4.2.2 Preparation and quantification of bacterial inoculum

An environmental isolate of non-pathogenic *E. coli* (Avery et al., 2009) was shaken (100 rev min^{-1}; 37 °C) in Luria-Bertani (LB) Miller broth (Difco) for 18 h. One mL of culture was washed three times in sterile electrolyte solution (5,000 × *g* for 2 min) and resuspended in 1 mL of sterile 0.1 mM electrolyte solution (NaCl, KCl or $CaCl_2$

according to experimental treatment). The final inoculum was prepared by performing a further ten-fold dilution of the washed cell suspension, again using the appropriate electrolyte at 0.1 mM concentration. The concentration of *E. coli* cells was confirmed by performing serial dilutions in maximum recovery diluent (MRD) and subsequent duplicate plate counts using the Miles Misra technique on ES Chromocult agar (Merck, Hoddesden, UK) (incubated at 37 °C for 18 hours) and by absorbance at 600 nm (Eppendorf Biophotometer, Eppendorf, Cambridge, UK) and comparison with previously generated growth curves.

Each microcosm was inoculated with 50 μL of *E. coli* inoculum to a final concentration of 10^6 CFU mL^{-1}. The microcosms were then placed randomly into a temperature-controlled chamber at 10 °C and shaken for 45 minutes at 100 rev min^{-1}.

4.2.3 Quantification of free and total *E. coli*

A centrifugation approach, developed through preliminary work, was used to distinguish between particle-bound and free *E. coli* cells (Avery et al., 2009). Each microcosm was gently inverted to mix the contents and 8 mL of the *E. coli*-clay solution was carefully removed using cut-off pipette tips (to minimise shear force). These aliquots were centrifuged in 14 mL sterile centrifuge tubes at 500 × *g* for two minutes (Beckman Coulter Allegra X-22R centrifuge with C1015 rotor) and the top 1 mL was carefully removed avoiding any mixing. Using calculations based on Stokes' Law (Equation 1) applied with the values shown below, alongside experimental trials, this procedure removed clay particles greater than 1 μm diameter while still retaining free cells within the top 1 mL of liquid. The number of *E. coli* in this fraction was determined by serial

dilutions and plating on ES Chromocult agar as described previously (Section 2.2). This represented the "free" non-bound cells.

Equation 1

$$v_s = \frac{2}{9}\frac{(\rho_p - \rho_f)}{\mu} g\, R^2$$

Where v_s = settling velocity (cm s^{-1})

g = acceleration of gravity (cm s^{-2}) = 4.9×10^5

d = particle diameter (cm) = 1.0×10^{-4}

ρ_p = density of particle (g cm^{-3}) = 2.65

ρ_m = density of medium (g cm^{-3}) = 1.0

and μ = viscosity of medium (g cm s^{-1}) = 0.0091

Thus giving a settling distance of 5.9 cm for clay particles of 1 micron diameter at 500 × *g* for 120 seconds at 25 ºC.

4.2.4 Zeta potential and pH

The zeta potential of the clays was measured (Malvern Zetasizer nano ZS; Malvern Instruments, UK) under the different electrolyte conditions in microcosms treated exactly as above, without the addition of bacterial cells. Measurements were performed at 15 °C using a refractive index of 1.5 within the general purpose methodology and applying the Smoluchowski equation (Eisenberg et al. 1979). Where conductivity was over 10 mS cm^{-1}, monomodal analysis was applied. The Malvern Zeta Potential transfer standard was run at the start and end of analyses for quality control

(Malvern Instruments, UK). The pH of each solution was also recorded (Meterlab Ion 450 analyser, Radiometer Analytical, Lyon, France).

4.2.5 Microscopic analysis

Samples from each treatment were analysed microscopically by removing an aliquot from the top 1 mL of liquid after separation. This was placed into a well slide and examined under ×400 magnification in bright field mode and qualitatively assessed for aggregation of clay particles, bacteria and bacteria-clay interactions.

4.2.6 Flow cytometry

Flow cytometry was applied to a subset of samples (Kaolinite in a range of NaCl concentrations) in a miniaturised version of the microcosm experiment (Chapter 4.2.1). performed using a Biosciences LSRII flow cytometer (BD Biosciences, San Jose, USA) configured with a 488 nm blue argon-ion laser for the detection of forward scatter (FSC), side scatter (SSC) and Syto-9 in the green channel. The stained cell (*E. coli* alone) population was identified and gated and 10,000 events overall were recorded. Changes in the particle size (forward scatter) and complexity (side scatter) were noted through visualisation of the stained cell population and also by assigning arbitrary quadrants to the graphical data to allow clear differentiation between treatment effects.

4.2.7 Humic acids

The influence of humic substances on *E. coli*-clay binding was investigated using a similar method to that described for the investigation of electrolyte effects. Bacterial

inoculum was prepared as described in Section 2.2. Microcosms were prepared through the addition of 45 mL stream water to triplicate sterile microcosms (250 mL polypropylene screw cap bottles). Montmorillonite SWy-1 and Kaolinite KGa-1b (van Olphen and Fripiat, 1979) clay solutions were prepared by adding 0.125 g of clay in 250 mL sterile distilled water. The humic acid-clay complex was created as described in Ghosh et al. (2009); Humic acid (HA) was dissolved in a minimum volume of 0.5 mol L^{-1} NaOH to prepare a 5 g L^{-1} solution. 200 mg L^{-1} of sodium azide (NaN_3) was used as a background solution to avoid any microbial degradation and the pH was adjusted to 5 with 0.1 M HCl and 0.1 M NaOH. Mineral to HA ratio was maintained at 3:1 (w/w) to obtain mineral-humic complexes. HA solution was added to the minerals and allowed to shake for 24 h. Then the mixture was centrifuged at 7600 × g for 15 min. The precipitates were washed with deionized water several times to remove any unbound HA fraction followed by freeze drying Five mL of clay and 5 mL of organic acid-clay solution were added to separate microcosms giving a final concentration 0.05 g L^{-1} clay representative of that seen in freshwaters under storm flow conditions. Final quantification of free *E. coli* was performed as described previously in Chapter 4.2.3.

4.2.8 Statistical analyses

All plate count data were normalised to the same starting cell density and checked for meeting the assumptions of ANOVA. Normality of the data was tested using Shappiro-Wilk and all the data came from a normal distribution. To determine any impacts of clays on viability of *E. coli* cells during the experiment, the number of free *E. coli* from microcosms containing clay were compared to the control microcosms without

clay using REML variance component analysis (GenStat Release 11.1 (PC/Windows); VSN International Ltd., 2008). Zeta potential and pH interdependencies and relationship with free *E. coli* cells were analysed using a simple linear regression in the same software package.

A general analysis of variance was performed on normalised plate counts with clay (Montmorillonite or Kaolinite), Electrolyte (NaCl, KCl, $CaCl_2$) and electrolyte concentration (0-100 mM) as factors. Linear regression was performed in the same statistical package to test for any relationship between zeta potential and the number of free cells.

4.3. Results

4.3.1 Clay and electrolyte effects on *E. coli* attachment

Particle size distribution of the clay stock solutions in aqueous solution (Fig. 4.1) demonstrated the propensity for Montmorillonite to disperse more readily than Kaolinite following the same preparation and sonication. Analysis of the *E. coli* populations in clay-free controls showed no significant differences to clay treatment microcosms, demonstrating no difficulties with recovery of viable free cells within the 45 minute duration of the experiment (data not shown).

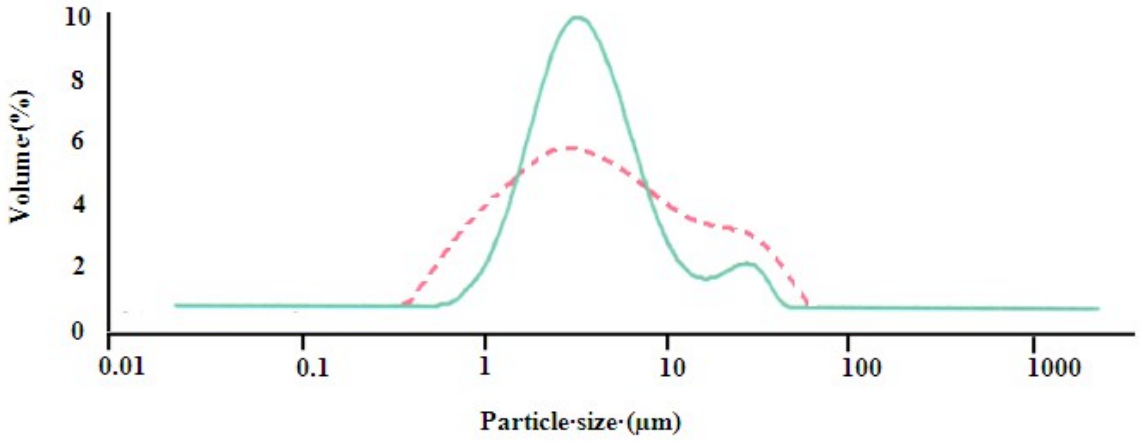


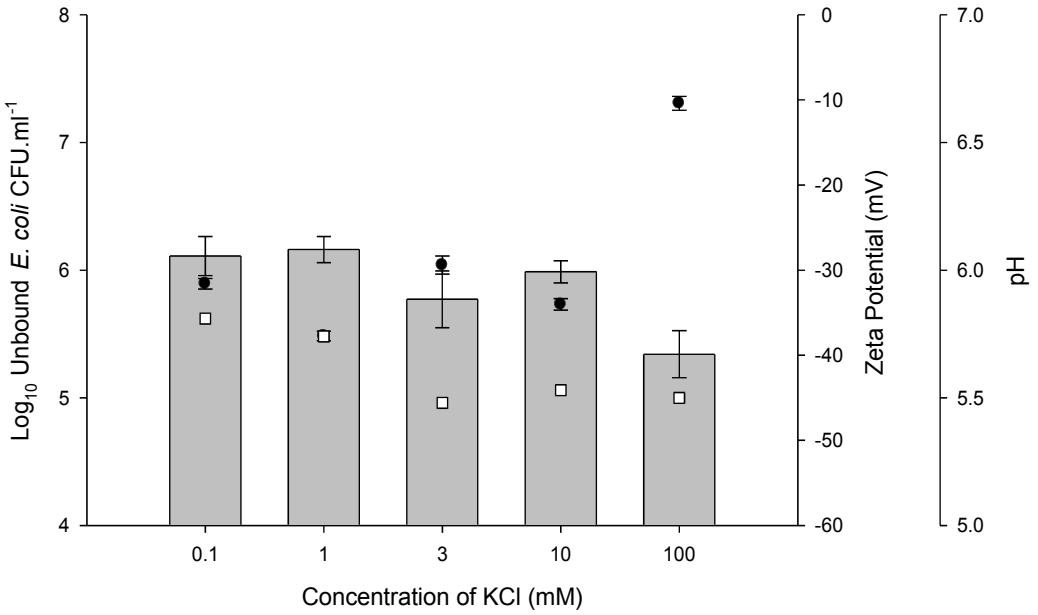
Figure 4.1. Volume-weighted particle-size distribution of Montmorillonite (solid line) and Kaolinite (dashed line) stock solutions in de-ionised water.

There was a significant interactive effect of clay type, electrolyte type and electrolyte concentration on the number of free *E. coli* recovered after the batch adsorption assay ($P<0.001$). Broadly, there was little difference in the number of free *E. coli* recovered between Kaolinite and Montmorillonite treatments with KCl and $CaCl_2$ electrolyte solutions (Fig. 4.2 a, b, d and e). However, the number of cells remaining unattached was significantly greater in Montmorillonite than Kaolinite treatments for NaCl (Montmorillonite mean $2.8 \times 10^6 \pm 2.7 \times 10^4$ CFU mL^{-1}; Kaolinite mean $9.7 \times 10^5 \pm 2.2 \times 10^4$ CFU mL^{-1}; Fig. 4.2c and f). Free cells in the Montmorillonite-NaCl treatment were also overall significantly greater than any of the other electrolyte-clay treatment combinations (means ranging from 7.8×10^5 CFU mL^{-1} to 1.1×10^6 CFU mL^{-1}). Although significant, this difference is of a relatively small magnitude of less than half a log unit (Fig. 4.2). Likewise, differences attributable to electrolyte concentrations were also small

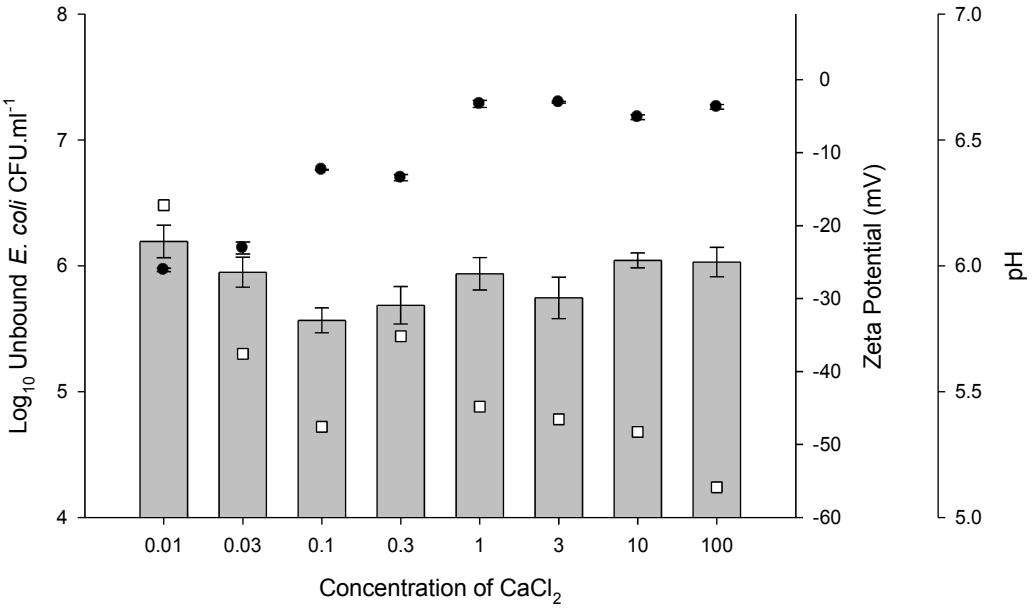
in magnitude and were most notable in the Kaolinite-$CaCl_2$ treatment, where the free cells recovered decreased between 0.01 mM to 0.1 mM (means $1.7 \times 10^6 \pm 5.1 \times 10^5$ CFU mL^{-1} and $3.9 \times 10^5 \pm 8.8 \times 10^4$ CFU mL^{-1} respectively). There were no further significant differences with increasing $CaCl_2$ concentration (Fig. 4.2 a and b), although the trend was towards a recovery in the number of free cells and this pattern was reflected in the NaCl-Kaolinite treatment.

Figure 4.2. The number of free *E. coli* detected after shaking for 45 minutes at 10 °C; 100 rev min^{-1} with 0.05 g L^{-1}; a) Kaolinite in KCl; b) Kaolinite in $CaCl_2$; c) Kaolinite in NaCl; d) Montmorillonite in KCl; e) Montmorillonite in $CaCl_2$ and f) Montmorillonite in NaCl solutions. (Grey bars) *E. coli* number ± SEM (n = 3), (●) Zeta potential ± SEM (n = 3) and (□) pH.

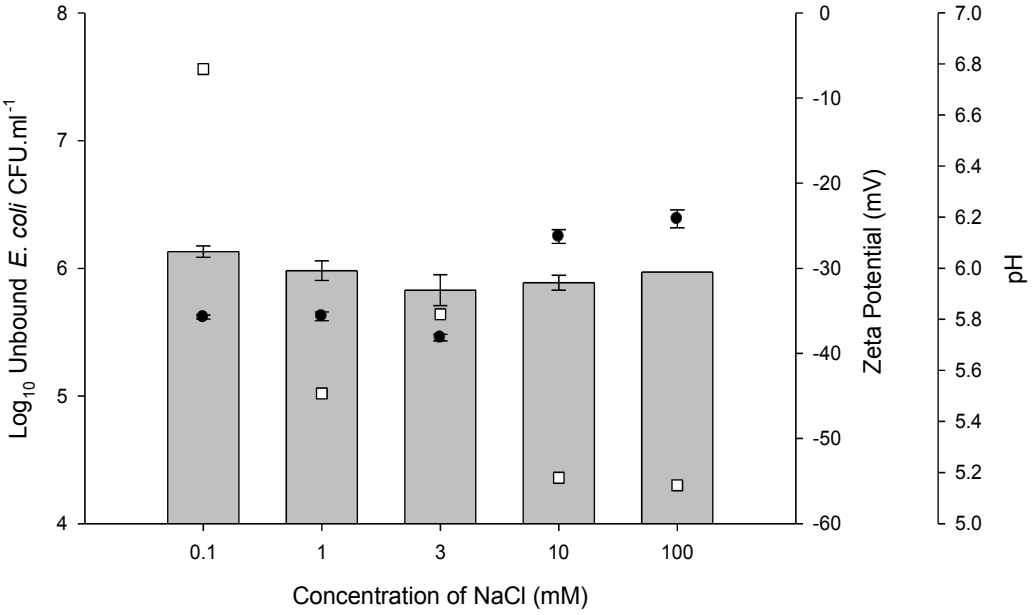
a



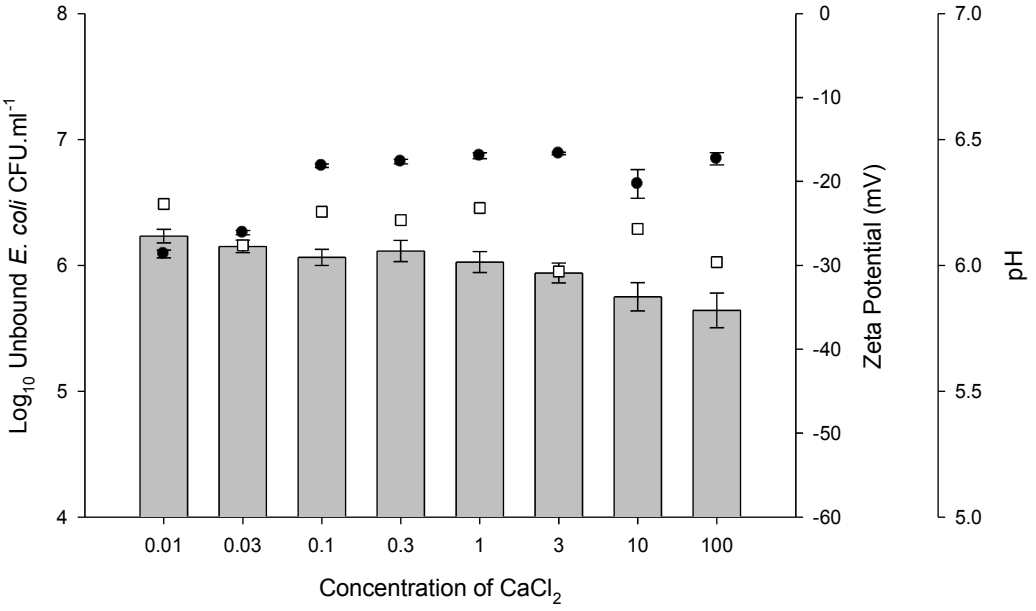
b.


Log10 Unbound E. coli CFU.ml-1
Zeta Potential (mV)
pH
8
7
6
5
4
0
-10
-20
-30
-40
-50
-60
7.0
6.5
6.0
5.5
5.0
0.01
0.03
0.1
0.3
1
3
10
100
Concentration of CaCl2

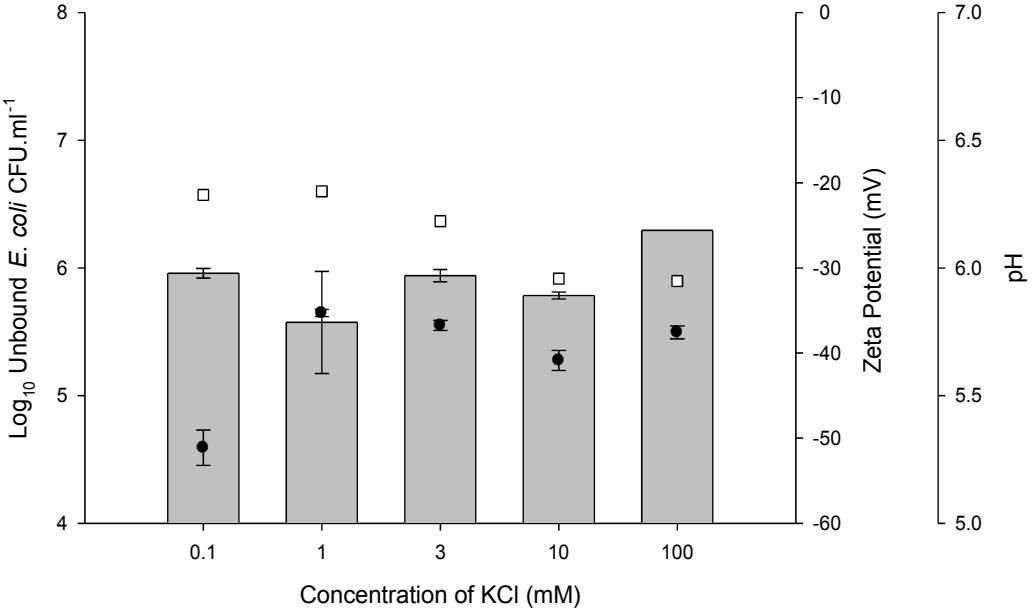
c


Log10 Unbound E. coli CFU.ml-1
Zeta Potential (mV)
pH
8
7
6
5
4
0
-10
-20
-30
-40
-50
-60
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
0.1
1
3
10
100
Concentration of NaCl (mM)

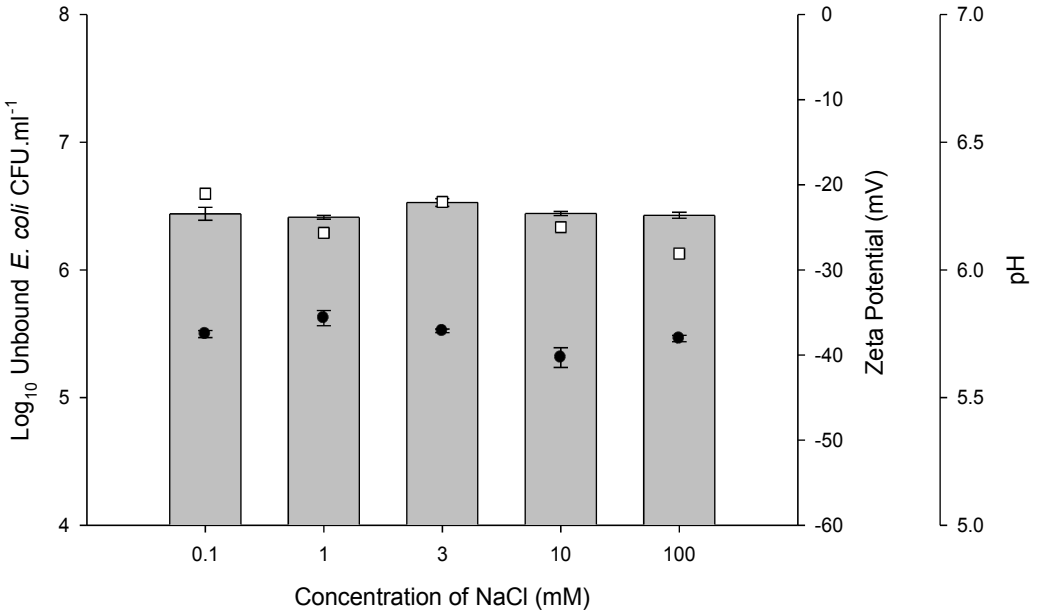
d


Log10 Unbound E. coli CFU.ml-1
Zeta Potential (mV)
pH
8
7
6
5
4
0
-10
-20
-30
-40
-50
-60
7.0
6.5
6.0
5.5
5.0
0.01
0.03
0.1
0.3
1
3
10
100
Concentration of CaCl2

e


Log10 Unbound E. coli CFU.ml-1
Zeta Potential (mV)
pH
8
7
6
5
4
0
-10
-20
-30
-40
-50
-60
7.0
6.5
6.0
5.5
5.0
0.1
1
3
10
100
Concentration of KCl (mM)

f



4.3.2 Zeta potential and pH

While the pH was relatively stable across treatments (range 5.1-6.7), it displayed an inverse relationship with zeta potential ($P_{regression}$ = 0.001; $P_{pH\ term}$ = 0.001; R^2 = 0.24), which became less negative with decreasing pH; however, pH only explained 20% of the variation in zeta potential. Significant interactions between clay type, electrolyte type and concentration influenced the zeta potential of clay solutions (P<0.001). Overall, zeta potential was more negative for Montmorillonite suspensions than for Kaolinite (means -34.3 mV and -27.7 mV, respectively) and broadly increased (became less negative) with increasing electrolyte concentration (Fig. 2). $CaCl_2$ facilitated a more rapid change with zeta potential becoming less negative at lower concentrations than the other electrolytes (e.g. zeta potential at 0.1 mM, Montmorillonite-$CaCl_2$ –18.1 mV, Montmorillonite-NaCl –

37.5 mV) and overall, zeta potentials were notably lower in $CaCl_2$ electrolyte solutions than in either KCl or NaCl (means -15.7, -38.4 and -38.8 mV, respectively).

Linear regression highlighted a significant ($P<0.001$) negative correlation between zeta potential and free *E. coli* numbers across all treatments. A positive correlation between pH and free *E. coli* cells was also evident ($p<0.001$), the effect being more apparent in Kaolinite suspensions than Montmorillonite. Zeta potential and pH accounted individually for 12.4 and 15.9% respectively of the variation in free *E. coli* (constant and parameter estimates significant to $P<0.05$).

4.3.3 Microscopic analysis and flow cytometry

Microscopic analysis suggests that there were no incidences of clay-clay binding occurring in the Montmorillonite suspensions (Table 1). The only incidence of clay-clay binding in the Kaolinite suspensions was observed at the highest concentration of $CaCl_2$, and therefore experimental results were generally not compromised by this effect.

TABLE 4.1: Microscopic observations of clay-bacteria interactions within the "free/total" fraction (at ×400 magnification).

Electrolyte (Concentration)		Montmorillonite	Kaolinite
NaCl	0.1 mM	Some bound *E. coli* to the clay but no clay-clay binding	Some bound *E. coli* to the clay but no clay-clay binding
	100 mM	Some bound *E. coli* to the clay but no clay-clay binding	Some bound *E. coli* to the clay but no clay-clay binding
KCl	0.1 mM	Some bound *E. coli* to the clay but no clay-clay binding	Some bound *E. coli* to the clay but no clay-clay binding
	100 mM	Some bound *E. coli* to the clay but no clay-clay binding	Some bound *E. coli* to the clay but no clay-clay binding
$CaCl_2$	0.1 mM	Some bound *E. coli* to the clay but no clay-clay binding	More bound *E. coli* to clay but no clay-clay binding
	100 mM	More bound *E. coli* to clay but no clay-clay binding	More bound *E. coli* to clay, many clay particles have bound together

Figure 4.3 illustrates the difference in the fluorescent population of *E. coli* cells stained with Syto 9 nucleic acid stain before and after addition of 0.05 g L^{-1} Kaolinite solution in 0.1 mM NaCl. With *E. coli* alone, the stained cell populations fell mainly within quadrant three of the arbitrarily applied quadrants (Fig. 4.3 a). This quadrant represents lower forward scatter and lower side scatter, which reflect lower particle size and low complexity. When Kaolinite was added (Fig. 4.3 b), the stained population extended into quadrant two (Q2), reflecting the larger particle size and complexity of aggregated material. Kaolinite-only controls (not shown) confirmed that the clay did not take up a significant amount of stain or exhibit significant autofluorescence, therefore any particles recorded within this fluorescent population must contain cellular material. *E. coli*-only

controls remained primarily within quadrant three therefore any cell-cell aggregation had little effect on the output data.

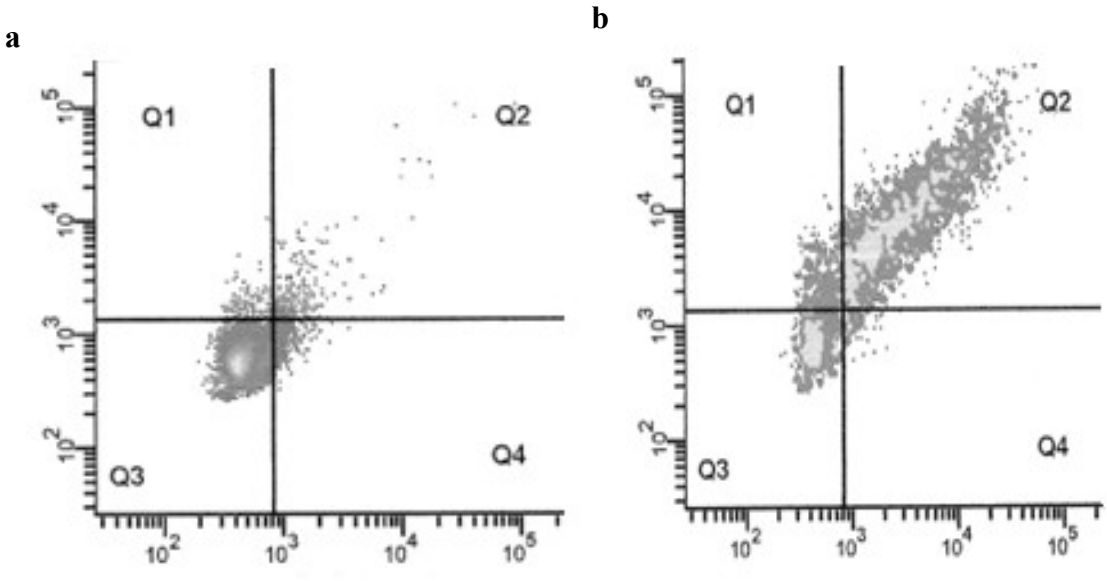


Figure 4.3. Forward Scatter (particle size) vs. Side Scatter (particle complexity) plots for Flow Cytometric analysis of Syto-7 stained *E. coli* a) alone and b) mixed with Kaolinite in 1 mM NaCl solution.

4.3.4 Humic acid effect

Figure 4.4 demonstrates the clear effect humic acid bound to the clays had in reducing the binding of *E. coli*. The presence of the bound humic acid resulted in an increased number of *E. coli* remaining in solution in comparison to the microcosms where the clays were clean. This was true of both types of clay tested (Montmorillonite and Kaolinite) indicating that the humic acid causes a direct effect rather than due to any clay-acid interaction ($p=<0.01$).

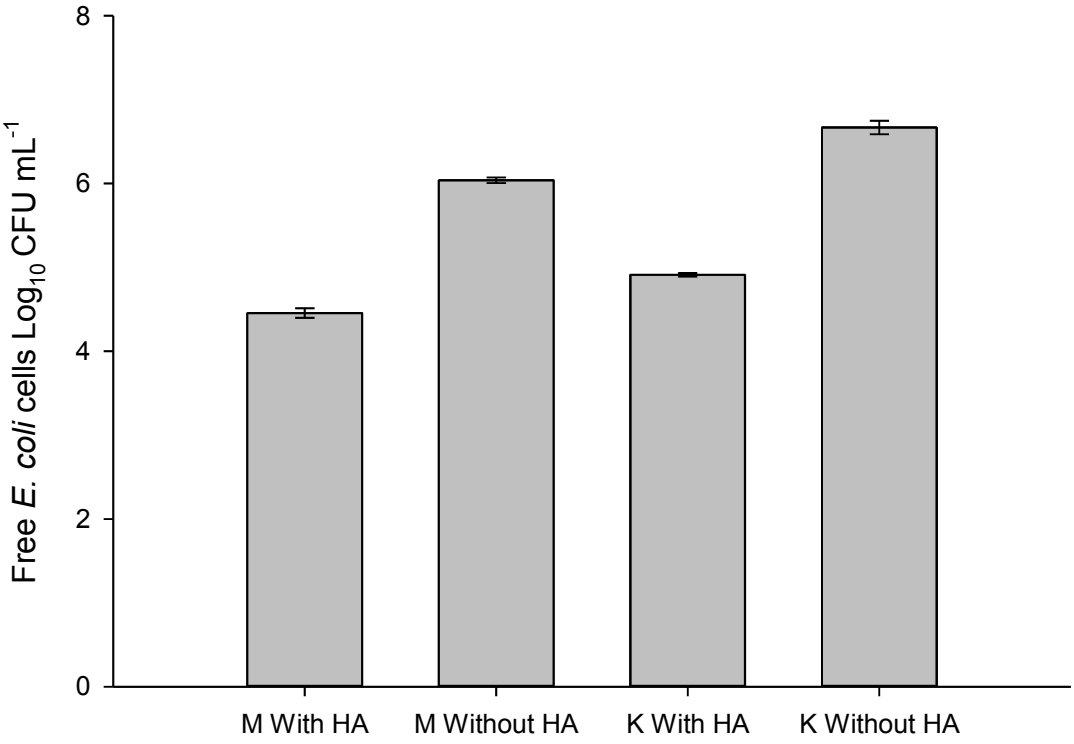


Figure 4.4. The number of free *E. coli* detected after shaking for 45 minutes at 10 °C; 100 rev min^{-1} with 0.05 g L^{-1} Montmorillonite (M) or Kaolinite (K) that are clean or have been coated with Humic acid (HA) ± SEM (n = 3).

4.4 Discussion

Overall, adsorption of *E. coli* cells was greatest within the Kaolinite treatments, primarily in the presence of NaCl electrolytes. Flow cytometric assessment confirmed that upon mixing of *E. coli* cells and clay colloid material (Kaolinite), clay-cell interactions occurred leading to larger, more complex particles. Similar findings of greater attachment to Kaolinite than Montmorillonite were reported by Rong et al. (2008) in a study of the adhesion of *Pseudomonas putida* onto the two clay types. These authors demonstrated that the enthalpy of adsorption onto Kaolinite was more favourable than that for adsorption onto Montmorillonite and concluded that non-electrostatic forces were the dominating factor in determining *P. putida* adsorption to clay minerals. Jiang et al. (2007) also found that *P. putida* adsorbed preferentially onto Kaolinite over Montmorillonite (and to Goethite in preference to both). In this current study, zeta potential was less negative for Kaolinite than Montmorillonite, which corresponds with increased likelihood of attachment to the bacterial cell surface which was negatively charged under the conditions in this experiment. Flow cytometric analysis served as a control to confirm that upon mixing of clay colloid material (Kaolinite) and *E. coli* cells, interactions occurred leading to the generation of larger more complex particles, indicative of aggregation. Because only the stained population was selected by flow cytometer gating, the movement of stained particles into quadrant two represented Kaolinite-cell aggregations leading to visualisation of fluorescently stained particles with increased granularity and size.

Montmorillonite SWy-1 and Kaolinite KGa-1b (van Olphen and Fripiat, 1979) were selected as model colloids for laboratory experiments to represent a range of surface

chemical characteristics of secondary clays. Kaolinite is a 1:1 phyllosilicate clay i.e. the aluminium layers alternate with silicate layers, whereas Montmorillonite is a 2:1 clay with each aluminium layer sandwiched between two silicate layers (van Olphen, 1977). Functionally, Montmorillonite has a higher cation exchange capacity (CEC = 66-123 cmol 100 g^{-1}) compared with Kaolinite (3-3.7 cmol 100 g^{-1}; Borden and Giese, 2001). Montmorillonite disperses more easily with more particles in the small particle size range and consequently provides greater surface area for binding (e.g. Battacharyya and Gupta, 2006) in contrast to Kaolinite which remains more aggregated under the same conditions. Hence differences in the aggregative properties were anticipated. Although the greater CEC and specific surface area of Montmorillonite may be assumed to lead to greater adsorption of bacteria, it is possible that the pH-dependent non-permanent charge associated with edge-faces of Kaolinite, rather than the permanent structural charge, influenced binding. Alongside the notably lower CEC and specific surface area, Kaolinite has approximately 50 times fewer structural charge sites, although over 20 % of its surface area is made up of edge rather than face sites, in contrast to <1 for Montmorillonite (Tombacz and Szekeres, 2006).

The influence of electrolyte concentration on the number of free cells remaining free in solution was also small in magnitude and most notable in the Kaolinite-$CaCl_2$ treatment. Increasing the ionic strength within the range of 0-100 mM tended to promote adsorption between particulates and bacteria (Hermansson, 1999; Jiang et al., 2007). Cao et al. (2011) investigated the adsorption of exopolysaccharide (EPS), an important bacterial surface determinant of attachment and biofilm formation (Danese et al., 2000), to Montmorillonite, Kaolinite and Goethite. They observed a promotive effect of sodium

ions on EPS binding and attributed this to two possible factors; i) the tendency of cations to suppress the repulsive electrostatic charges of clay particles, leading to increased contact between EPS and mineral surfaces, or ii) the formation of cation bridges between functional groups of the EPS molecules and the negatively charged sites of clays.

Within the range of concentrations typically encountered in freshwaters, electrolyte concentration changes are unlikely to play a major role in influencing cell-clay colloid interactions (Caissie et al., 1996). Effects of electrolyte concentration tended to become more evident at the higher molarities (10-100 mM) beyond those typical of freshwaters. For clays, colloidal destabilization increases as the ionic strength (salt content) and the charge of the cations increase. This occurs naturally when clays are transported from fresh waters into estuaries (Edzwald and O'Melia, 1975). Indeed, clay mineral distribution in transition to estuarine waters has been shown to reflect clay colloid stability, flocculation rates and associated deposition (Edzwal and O'Melia, 1975). Brackish or estuarine waters may be sufficiently saline to affect aggregative characteristics of cells and colloidal particles. For example, sea water is substantially above the range of molarities considered in this study at approximately 470 mM for Na^+ and around 10 mM for K^+ and Ca^{2+} (Karleskin et al., 2006). Aggregation and subsequent sedimentation and persistence of colloid-bacterial aggregations around coastal bathing waters has the potential to pose a risk of ingestion and infection, particularly as it is in the "swash" zone, where waves break onto the beach and sedimented material is readily resuspended and human contact can be expected (Verhougstraete, 2009). However, Singleton (1983) suggested that the concentration of sodium chloride in estuarine and

marine waters has minimal impact on binding of clay colloids to bacterial surface and the small magnitude of changes evident in this study tend to corroborate this assertion.

Scholl and Harvey (1992) studied the interactions between bacterial transport and sediment surfaces within a sandy aquifer and indicated that for relatively hydrophilic microorganisms, it was the mineral surface charge that controlled the initial adhesion of bacteria. It has been suggested that *E. coli* is hydrophilic rather than hydrophobic and that zeta potential is important in determining adhesion to surfaces (Van Loosedrecht et al., 1987; Gilbert et al., 1991). In a colloidal clay suspension, zeta potential is the difference in potential between the layer of ions attached to the surface of the dispersed clay and the dispersion medium. As the zeta potential approaches zero, and the electric double layer around particles is compressed by increasing electrolyte content, interaction between bacteria and mineral colloidal particles is more likely to lead to aggregation (Jiang et al., 2007). Thus, a high negative zeta potential may be expected to indicate electrostatic repulsion. In zeta potential measurements for each clay in the salt solutions at which the bacterial sorption assays were performed in this current study, Montmorillonite generally had a more negative zeta potential than Kaolinite and exhibited lower bacterial adhesion. At a given pH, increasing ionic strength can provide more ions to shield and neutralise cell surface charge, facilitating adsorption of negatively charged clay particles, but as charges on clays are also neutralised, attachment may decrease. This effect was evident in Kaolinite-$CaCl_2$ treatment and may be attributable to the lower CEC of this clay, neutralisation occurring more rapidly, particularly with a divalent cation.

Zeta potentials did not clearly explain bacterial adsorption, although there was evidence that they play a role in the process. As suggested by Rong et al. (2008), non-electrostatic

forces may be more important than electrostatic forces in bacterial adhesion to clay minerals. Adsorption has also been shown to be a function of bacterial surface characteristics. The bacterial zeta potential measured in the experiment increased gradually from -24 mV at 0 mM NaCl to -4 mV at 100 mM NaCl (data not shown). Weerkamp et al. (1988) noted that microorganisms with low zeta potentials had the greatest propensity to attach in higher numbers. They also suggested that an increased (more negative) substratum zeta potential may be responsible for the decrease in bacterial attachment. Binding of bacteria to attachment sites on the clays can also modify the surface and adsorption characteristics (Rong et al., 2008). It is possible that this, along with other factors, played a role in generating the differing adsorption patterns observed.

Despite displaying a weak positive correlation with free *E. coli* cells, pH was relatively stable across the Montmorillonite treatments. The greater proportion of pH dependent charge on Kaolinite compared to Montmorillonite leads to greater pH sensitivity of the aggregation of Kaolinite (Tombacz and Szekeres, 2006) which may have accounted for the slight effect of pH in the Kaolinite treatments.

Humic acid forms the largest part of dissolved organic matter in freshwater supplies (Zumstein and Baffle, 1989) and therefore it is likely that humic acid is to be bound to available surfaces, therefore its influence on *E. coli*-clay binding is very important. In this case the bound humic acid provides improved attachment for the planktonic *E. coli* possibly through similar mechanisms as discussed for the tested electrolytes, however further research is needed to test out the subtleties.

Differences in the composition and valency of electrolytes entering fresh (and saline) waters could be more important than concentration per se, as in this study, KCl and $CaCl_2$

led to a greater magnitude of differences in adsorption of *E. coli* cells onto Montmorillonite particles than NaCl. Divalent cations tend to promote suppression of the electrical double layer formation at lower concentrations than monovalent cations (van Olphen, 1977), thus the effect of $CaCl_2$ is not surprising and corroborates the findings of Jiang et al. (2007), who demonstrated increased binding in Mg^{2+} electrolyte solutions compared with NaCl. It is unclear why KCl should significantly increase the binding potential over NaCl, aside from possessing greater chemical reactivity.

Electrolyte type/valency had less impact on Kaolinite than Montmorillonite treatments. This may be due to the lower CEC of Kaolinite which would render the magnitude of change with electrolyte type less than where many more exchange sites are available. With higher valency or more reactive cations, there is potential for a change of greater magnitude with Montmorillonite as more ions can exchange and neutralise clay surface charges rendering attachment to negatively charged bacterial surfaces more likely. However, this effect was not explained by the zeta potentials, indicating that non-electrostatic mechanisms are important in clay-electrolyte interactions.

In environmental waters, the main short-term effect of storms is a dilution of the pre-storm stream concentration of major cations and geologically controlled anions (Na^+, Mg^{2+}, K^+ Ca^{2+}) generally correlate negatively with discharge (Giusti and Neal, 1993; Caissie et al., 1996). For example, Maruaoka and Hirata (2003) reported that concentrations of each element (including major cations and anions) of stormwater chemistry decreased during rainfall events in a forested basin catchment until peak flow, due to a dilution effect. They observed that subsequent increases during the recession period either returned to base flow levels (Na^+) or exceeded them (K^+, Ca^{2+}, Mg^{2+}). It can

take days for dilution of parameters to return to normal (Caissie et al., 1996), and high flows also facilitate the majority of faecally derived microbial pollutants to enter watercourses and promote resuspension of sediment particles. Furthermore, maximum adsorption of bacterial cells onto Montmorillonite and Kaolinite can occur at 15 ºC and considerable adsorption still occurs at 5 °C (Jiang et al., 2007) – both of which are consistent with typical river temperatures during the course of a year. Therefore, interactions between indicator organisms, colloidal particles and the chemical environment are key to understanding transport processes.

Cell-colloid interactions can influence the die-off and transport terms of faecal indicator transport models through interference with physiological processes and buoyancy. Clays also adsorb toxins which may enhance their antimicrobial activity. In some cases, they have also been shown to be protective to bacterial cells.

This study provided a simple model system to study *E. coli*-clay interactions. The 50 mg L^{-1} final clay concentration was in line with naturally occurring concentrations in aquatic systems (e.g. Kirk and Gilbert, 1990), and electrolyte concentrations spanned a range consistent with freshwaters and salinities which may be encountered across the mixing gradient towards estuarine environments (e.g. Giusti and Neal, 1993). The findings are also relevant to a range of other important clay-colloid applications, such as the removal of bacteria from wastewaters (Herrera et al., 2000). However, a model system cannot be directly extrapolated to the environment as, while permitting observation of some parameters, it deliberately does not reflect the complexity of environmental matrices. The principal limitations include the fact that environmental colloids are subject to surface modification and colloidal particles are likely to be coated with biofilm and organic

materials to varying degrees (Wu et al., 2011). Future studies will address the impact of organic materials and environmental modifications to clay colloid surfaces.

This is the first study to address *E. coli*-clay colloid interactions from a freshwater transport perspective. It provides important fundamental knowledge about the mechanisms involved and provides some indication of the likely importance of particular factors in a freshwater environment. Our findings are in accordance with other studies which suggest that electrostatic forces play a role but are not the most important factor governing interactions between colloidal clays and bacterial cells. Consequently, electrolyte concentrations are unlikely to play a major role in regulating *E. coli*-colloid attachment in surface waters. Increasing divalent cation concentrations during post-storm recession may exert a small influence on binding; however, the magnitude of such an effect is likely to be insignificant in terms of faecal indicator transport models and is likely to be mediated by environmental modifications to colloid surfaces.

Chapter 5. Influence of clay type on survival of *Escherichia coli* in freshwater

5.1. Introduction

Effective monitoring of faecal contamination of surface waters is key to protecting public health as faecal pollution introduces pathogens into the aquatic environment, contaminating drinking water sources and bathing areas. *Escherichia coli* and coliforms are used as indicators of faecal pollution as they are present in much higher numbers than pathogens and thus much easier to enumerate (Quilliam et al., 2011).

Although the majority of *E. coli* strains are harmless, some (notably the O157 strain) have been associated with outbreaks of human infections that occasionally lead to serious complications (Mead and Griffin, 1998). The organism is a typical part of the gut flora of mammals and is introduced into the environment through their faeces. Studies have shown that pathogenic *E. coli* can survive for relatively long periods in faeces (Williams et al., 2008) and with this longevity *E. coli* can be transferred through the environment by water flow (Thurston-Enriquez et al., 2005; Hathaway and Hunt, 2011). The speed of bacterial transfer is highly dependent on water flow with particularly high flows occurring during storm events, leading to large numbers of *E. coli* and other faecal organisms contaminating surface waters from the surrounding land (Jamieson et al., 2005: Wu et al., 2009).

Recently, focus has shifted to investigate in-stream stores of *E. coli* as sources of faecal organisms, for it has been shown that stream sediments harbour higher numbers of *E. coli* (Muirhead et al., 2004). Sediment resuspension during high flow events contributes to high *E. coli* numbers seen during storms (Muirhead et al., 2004 and Cho et

al., 2010). This has important implications for the monitoring of *E. coli* for it is very difficult to establish the source of the *E. coli* once suspended within the water column and there is limited knowledge on the survival of pathogens in sediment in comparison to their indicators.

The association between *E. coli* and sediment is very beneficial to the *E. coli* as studies show better survival of bacteria when bound to sediment in comparison to non-attached in freshwater (e.g. Davies et al., 1995). Through physical shielding, the sediment particle partially protects the bound *E. coli* from various antimicrobial stressors in water, e.g. protozoan predation (Jamieson et al., 2005), in addition to shading *E. coli* cells from damaging UV radiation (Sinton et al. 2002). Sediment particle size plays a role in this, with thermotolerant coliforms being able to survive for longer in sediment with predominantly clay-sized particles compared with coarser sediments (Howell et al., 1996). However, LaLiberte and Grimes (1981) report that a laboratory culture of *E. coli* inoculated into lake sediments survived better in sandy than silty sediment (the latter with a higher clay content), although interestingly, naturally present populations of *E. coli* monitored in the same experiment did not differ between sediment types. Similarly, longer survival times for *E. coli* have been reported in sediments containing at least 25% clay than in those with larger particles (Burton et al., 1987). It is not clear whether these effects are related to the presence of particles within the colloidal size fraction or directly to the clay content of sediments, which appear to have specific effects on some microorganisms, including pathogens.

Clay types have also been found to affect pathogen survival . The levels of montmorillonite in soil has been associated with reduced occurrence of some human

pathogens and greater survival of indigenous bacteria (Filip, 1973); whilst bentonite clays have been shown to inhibit protozoal grazing of *Rhizobium* in liquid culture (Heijnen et al., 1991), and illite clay antagonises *E. coli* through loss of outer membrane integrity by the action of Fe^{2+} ions (Williams et al., 2011). However, very little research has investigated the specific effect the clays have on *E. coli* survival within the aquatic environment.

Variation between *E. coli* strains can also lead to differences in survival as strains can be genotypically very different and thus dissimilar in phenotype; indeed one study found only 40% of the proteins were common between three strains (Welch et al., 2002). Further, different strains of *E. coli* O157 have also been reported to vary in their ability to resist predation by protozoa (Watterworth et al., 2006). In very few studies have the relative survival of different strains of *E. coli* in sediment been compared directly to establish the effect they have on *E. coli* numbers when sediment is resuspended.

The aims of this study were to establish the relative survival times of three *E. coli* strains and to determine the effect clay type has on survival time.

5.2. Methods and Materials

5.2.1 Microcosm preparation

5.2.1.1 Stream water preparation

Stream water was collected from the Afon Goch that runs past Bangor University's Henfaes Research Centre in North Wales, UK. This water was subsequently filtered through a sterile 0.45 μm filter in order to remove any microorganisms without

damaging the nutritional constituents. Water chemistry was analysed by MacaulayAnalytical (UKAS accredited).

5.2.1.2 Clay stock preparation

Montmorillonite SWy-1 and Kaolinite KGa-1b (van Olphen and Fripiat, 1979) and Illite clay stocks were prepared by mixing clay in 250 ml Milli Q water then sonicating for 5 min at 15 Hz using a 24 mm diameter low intensity solid horn probe (600 W Ultrasonic Processor, Sonics and Materials Inc., CT, USA). The fraction that remained suspended within the water was used as the stock. This gave an approximate concentation of 4 g L^{-1} for both Montmorillonite and Illite clays and 0.5 g L^{-1} for Kaolinite.

5.2.1.3 Microcosms

Microcosms were prepared by adding 5 ml clay stocks to 45 ml sterile streamwater in 250 ml sterile bottles. The final concentrations for Montmorillonite and Illite were approximately 400 mg L^{-1} and 50 mg L^{-1} for Kaolinite. In addition, microcosms containing streamwater only were also prepared. Each set of conditions was replicated in triplicate.

5.2.2 Preparation and quantification of bacterial inoculum

Three different strains of *E. coli* were used in this study: *E. coli* #25922 as a laboratory strain, an environmental isolate of non-pathogenic *E. coli* (Avery et al., 2009) and *E. coli* O157 #3704 (non-toxigenic). Each strain was incoculated into Luria–Bertani (LB) Miller broth (Difco) and shaken (100 rev min^{-1}; 37 °C) for 18 h.

One ml of culture was washed three times in filtered stream water (5,000 × *g* for 2 minutes) and resuspended in 1 ml filtered stream water to give an approximate concentration of 10^9 ml^{-1}. The final concentration was determined by performing serial

dilutions of the washed cell suspension and subsequent duplicate plate counts using Miles Misra technique on Sorbitol-Maconkey Agar (Sigma) (incubated at 37 °C for 18 h).

Each microcosm was inoculated with 50 µL of *E. coli* inoculum to a final concentration was approximately 10^6 CFU ml^{-1}. The microcosms were then placed randomly into a temperature controlled chamber at 10 °C and shaken at 100 rev. min^{-1}.

Starting concentrations of the two of the *E. coli* strains were the same, with the laboratory strain, 25922, slightly higher (O157 and environmental isolate, $1.2 \times 10^7 \pm 1.6 \times 10^4$ CFU mL^{-1} and 25922, $1.8 \times 10^7 \pm 2.0 \times 10^4$ CFU mL^{-1}).

5.2.3 Quantification of *E. coli*

Samples were removed for analysis 3, 6, 24, 48, 96 hours and 1, 2 and 4 weeks post-inoculation. Each sample was subjected to a 10-fold dilution series, if nesscessary, plated out on Sorbitol-Macconkey Agar and incubated for 18 h at 37 °C (as for inoculum enumeration). After incubation, the number of colonies at each concentration was recorded.

5.2.4 Statistical analyses

Analysis was undertaken using individual one-way analysis of variance (ANOVA) in SPSS (v. 16) as data met all assumptions. Normality of the data was tested using Shappiro-Wilk and all the data came from a normal distribution. To determine survival characteristics of the *E. coli* strains, numbers were analysed within each separate clay and water groups. Analysis across the clay and water group for each *E. coli* strain allows comparison of the effects of the clays on survival.

5.3. Results

5.3.1 Water characteristics

TABLE 5.1: Water chemistry of Afon Goch streamwater.

	Concentration (mg L^{-1})
Al	0.003
Ca	19.450
Cu	0.003
Fe	0.000
K	2.220
Mg	3.910
Na	16.800
P	0.018
Pb	0.000
Si	0.242
Zn	0.001

Analysis of the water used in the microcosms showed that the variables tested fell within typical environmental conditions as discussed in Chapter 2.2.1 and thus can be used to simulate a typical stream water.

5.3.2 Clay Influence

There was a significant effect of the clays on the survival of the three *E. coli* strains in comparison to the water only baseline. Simply looking at how many of the three strains survived the four weeks of the study it is quite clear that the Illite clay hindered survival, with none of the strains surviving (Figure 5.1), while Montmorillonite enhanced it, with two of the three surviving (Figure 5.2). Kaolinite (Figure 5.3) seems to give the

same result as the water only baseline (Figure 5.4), with one strain surviving both. However, this could be due to the concentration of the clay being too low to have any noticeable effect.

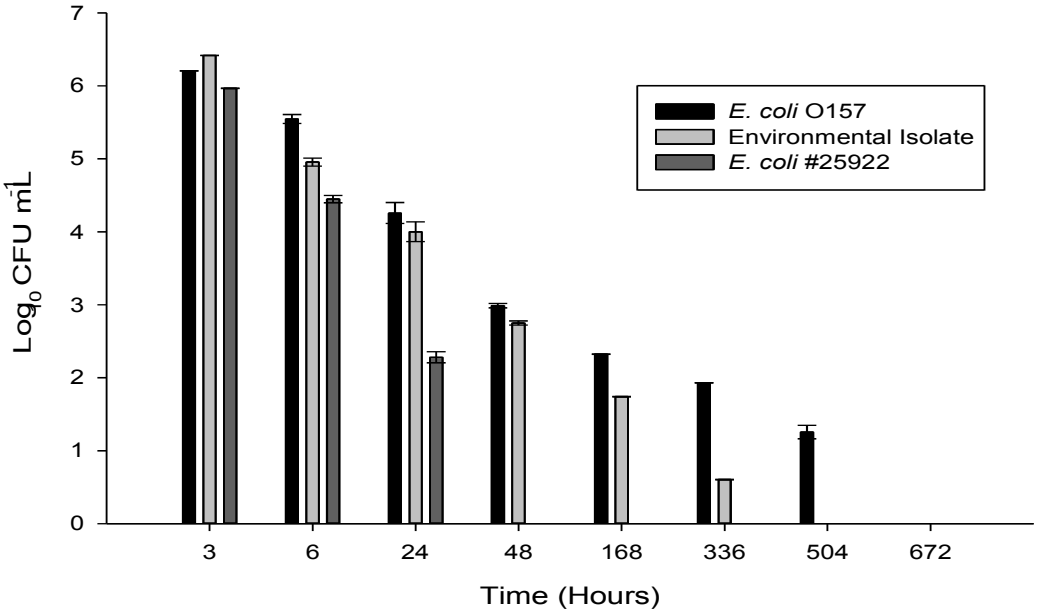


Figure 5.1. Number of *E. coli* left in microcosm containing 400 mg L^{-1} Illite. ±SEM (n=3)

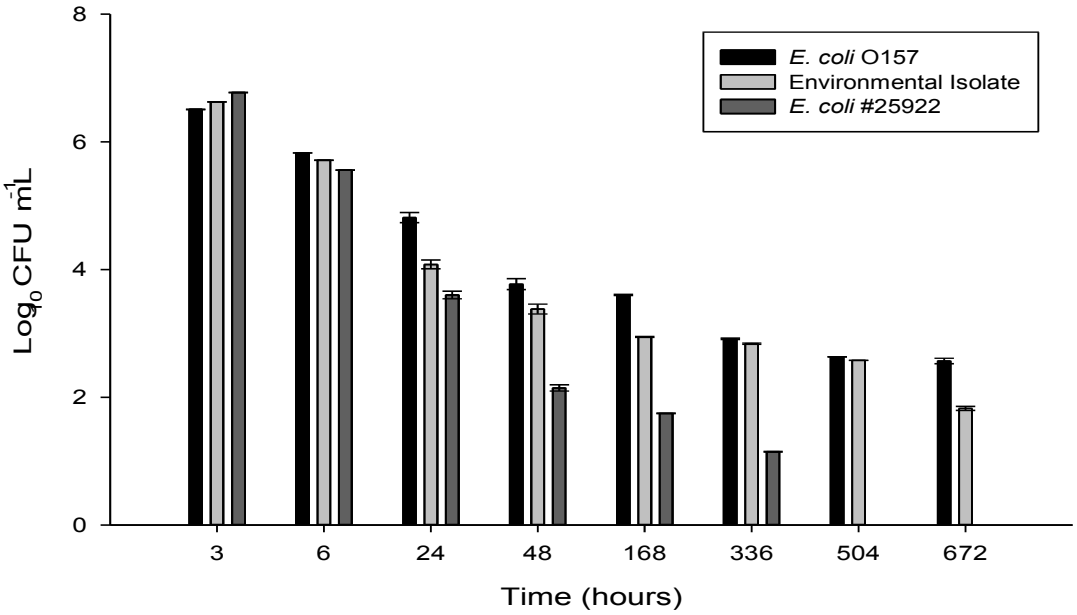


Figure 5.2. Number of *E. coli* left in microcosm containing 400 mg L^{-1} Montmorillonite over time. ±SEM (n=3)

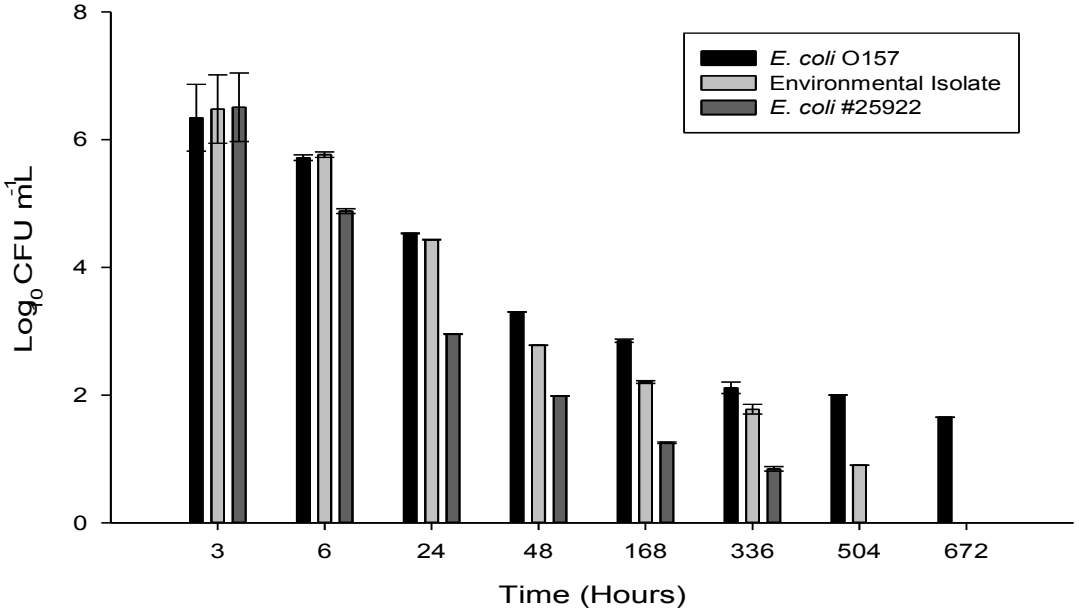


Figure 5.3. Number of *E. coli* left in microcosm containing 400 mg L^{-1} Kaolinite over time. ±SEM (n=3)

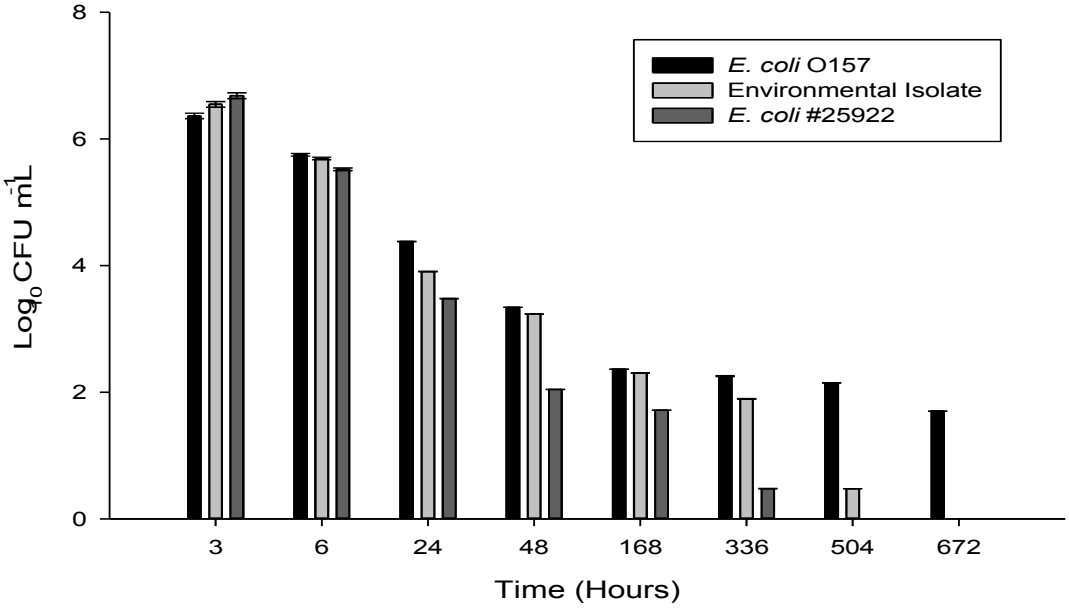


Figure 5.4. Number of *E. coli* left in microcosm without any clay over time. ±SEM (n=3)

Statistical analysis with an ANOVA of these data showed that both Montmorillonite and Illite were significantly different to the water only baseline from 24 hours post-inoculation with Montmorillonite improving survival ($p=<0.05$) and Illite hindering it ($p=<0.05$). Kaolinite did not prove to have significantly different results to that of the water baseline ($p=0.05$).

5.3.3 Strain Influence

The influence of the strain type was very significant ($p=<0.01$) with large differences between the strains for all environmental conditions tested. *E. coli* O157 proved to be the most resilient of the three strains, surviving at detectable levels for at least four weeks in Montmorillonite, Kaolinite and the Water only baseline (Figure 5.5). When exposed to the antimicrobial clay Illite, *E. coli* O157 was detectable for much

longer than either of the other two strains, it managed to survive for three weeks in comparison to two weeks for the environmental isolate (Figure 5.6) and just 24 hours for the laboratory strain #25922 (Figure 5.7).

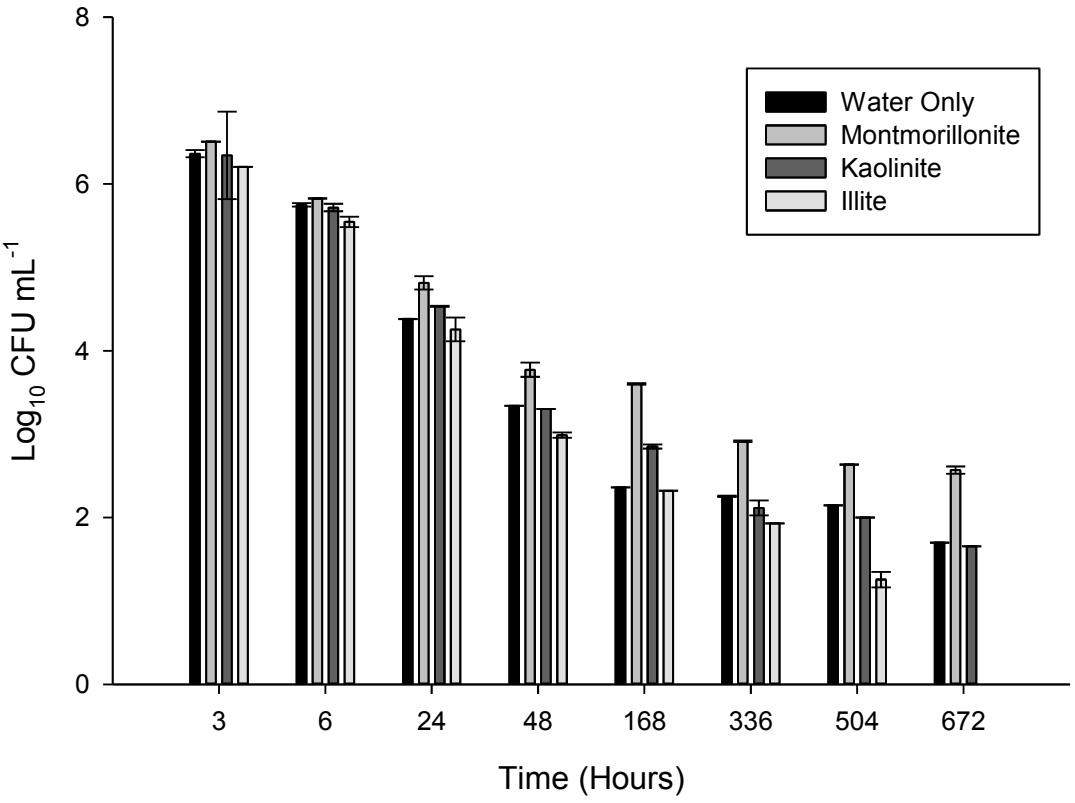


Figure 5.5. Number of *E. coli* O157 left in microcosm with each clay over time. ±SEM (n=3)

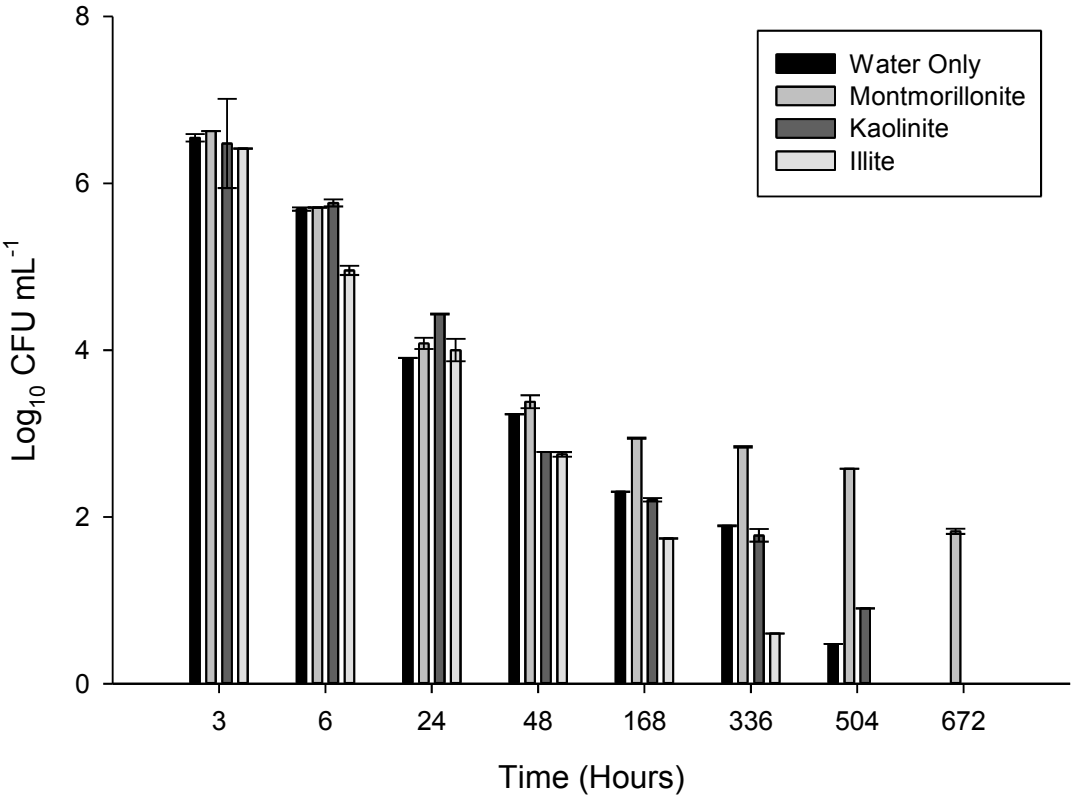


Figure 5.6. Number of the environmental isolate of *E. coli* left in microcosm with each clay over time. ±SEM (n=3)

The environmental isolate survived for three weeks when exposed to both the kaolinite clay and water only baseline and for at least 4 weeks when exposed to Montmorillonite (Figure 5.6). The laboratory strain, #25922, survived for a much shorter time, with no culturable cells detected at three weeks for any of the remaining tests (Figure 5.7).

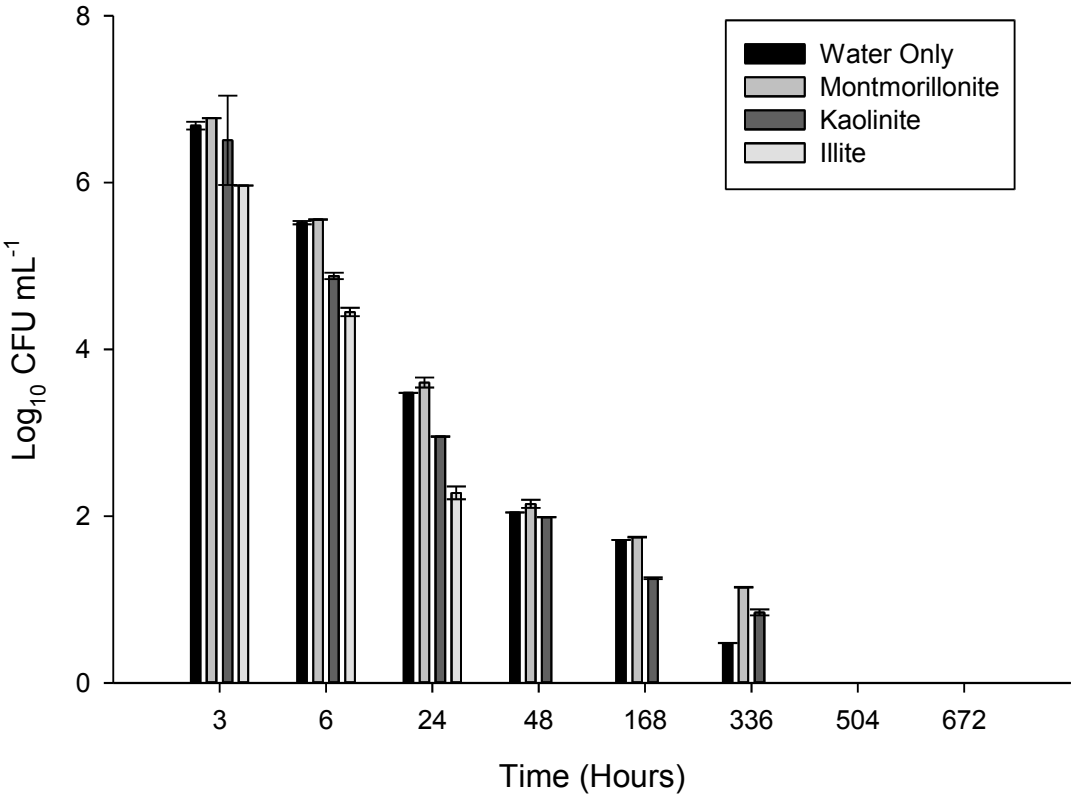


Figure 5.7. Number of the laboratory strain of *E. coli*, #25922, left in microcosm with each clay over time. ±SEM (n=3)

When looking at the individual sample points, for all conditions there was no significant difference between the strains after three hours. At six hours, strain #25922 had significantly less *E. coli* cells detected than the other two strains for all of the conditions. The environmental strain remained similar to the *E. coli* O157 up until two weeks post-inoculation when they started to diverge with fewer environmental isolate *E. coli* surviving. This was the case for all of the conditions with the one exception of the Montmorillonite clay where the two remained at similar numbers for at least four weeks when the experiment ended.

5.4 Discussion

With recent focus investigating the role sediments play in *E. coli* ecology, this study adds further weight to the importance of the sediment components on survival of *E. coli* once associated with the sediments.

Much research has focussed on survival of *E. coli* in water (e.g. Avery et al., 2008; Wang and Doyle, 1998) by trying to pick out the controlling factors. However, the survival proved to be very complicated with no one prominent factor having overall control. This study uses the survival in water as a baseline to investigate the influence clays have on survival. However, the influence of clays within the water column has not been investigated to any great depth.

Previous studies have shown that Illite clay possesses antimicrobial properties against several species of bacteria (Haydel et al., 2008) when applied in its dry form. When the three strains of *E. coli* were exposed to this clay suspended within the water column, all three displayed reductions in numbers in comparison to the water only baseline. The antimicrobial properties of this clay are due to Fe^{2+} ions overwhelming bacterial outer membranes, oxidising inside the cells to produce Fe^{3+} ions and lethal hydroxyl (OH^-) radicals (Williams et al., 2011).

The laboratory strain, #25922, proved to be particularly vulnerable to this type of attack. Previous articles have highlighted the weakness of relying on strains that have been in the laboratory environment for years, even decades for research (Hobman et al., 2007). This laboratory strain was isolated by the FDA in Seattle during 1946 and since then has only been exposed to laboratory conditions, typically those optimal for growth. This has to encourage adaptation to these optimal conditions and thus various protective

mechanisms these organisms have evolved in the natural environment are likely to degrade through lack of use, becoming less efficient.

The environmental isolate of *E. coli* tested fell between the #25922 strain and *E. coli* O157 in terms of survivability when exposed to Illite. This strain has spent much less time in the laboratory since it was first isolated therefore retaining functioning protective mechanisms. In addition, because this was an environmental isolate rather than a clinical isolate, there is a high likelihood that this strain had been exposed to similar conditions during this period, potentially increasing resistance against antimicrobial clay attack. Unfortunately, there has yet to be a study investigating the genotypic characteristics of this organism in detail so the genetic differences between the strains cannot be identified other than their overall effect.

E. coli O157 has proven to be one of the most hardy *E. coli* strains that has been investigated, particularly when it comes to resisting low pH (Leyer et al., 1995; Foster, 2004). The resistance of the organism against many potentially damaging processes has assisted survival outside its primary habitat, and thus becoming a prominent pathogenic organism and a high priority target for research. When exposed to Illite, *E. coli* O157 was the most resistant of the three strains tested, surviving for at least three weeks in comparison to two weeks for the environmental isolate and only 24 hours for the laboratory strain.

When exposed to Montmorillonite, the three strains each survived longer than in the water only baseline. With this improvement seeming equal across the strains, the likely cause is the binding of the cells to the clay particles within the system, protecting them against damage.

Past research (e.g. Davies et al., 1995) has proven the effectiveness that the binding to sediment has on bacterial survival. Rather than focussing on general stream sediment, by looking at several different clays it becomes possible to tease apart the relative characteristics of each test clay and the impacts they have on bacterial survival.

It is also possible that the high specific capacity of Montmorillonite enabled the clay to mop up atomic ions within the microcosm that could potentially cause damage to the cells; additionally this may even be a source for the bacteria for essential ions that are needed to survive. However, further research is needed to explore these ideas.

This knowledge adds to the picture of bacterial survival in surface waters. It is clear that clays are an important part of this process, with both passive (Montmorillonite) and active (Illite) roles. In the environment, Montmorillonite clays would provide a place for the *E. coli* cells to bind to and thus provide a small level of protection from antimicrobial processes. However, there would be much more competition for these places as indigenous flora are likely to have already become bound to the in-stream clays, therefore denying their use by *E. coli*. Strong shear forces experienced during high flow rates have the potential to remove the bound microflora from Montmorillonite and thus free up binding sites when the flow rate reduces. It is not completely understood how well *E. coli* can compete with other species for binding sites giving a potential avenue of further research.

The obvious range of survival ability between the three *E. coli* strains tested, highlights the problems of monitoring generic *E. coli* numbers, particularly when it comes to hardy pathogenic strains such as the *E. coli* O157 studied here. After a faecal contamination event in surface waters, there is a large spike in *E. coli* numbers (Figure

8.1). The primary environment for these *E. coli* is the mammalian gut (Yoon and Hovde, 2008), thus, most would die-off relatively quickly when exposed to the relatively harsh conditions outside, leaving the hardier organisms to persist in the environment. The problem is that despite the drop numbers of *E. coli* after the spike, the risk from the pathogenic organisms persists.

This difference is also a problem when it comes to modelling the natural environment in the laboratory, for laboratory strains of *E. coli* isolated decades ago are just not representative of the *E. coli* strains that are likely to be found in the environment, particularly ones that have become environmentally adapted. The result of this is that survival of organisms such as *E. coli* can be underestimated due to the use of inferior strains such as the *E. coli* #25922 used in this study, when in reality strains found in the natural environment are much more able to survive long periods.

To summarise, the antimicrobial Illite clay proved to retain its antimicrobial properties when suspended within the water column at 400 mg/L. Montmorillonite extended the life of the bacterial cells through providing protection to the cells bound to it. However, only a partial picture exists of how binding to the clay extends cell life. The concentration of Kaolinite that was able to remain in suspension was not sufficient to differ in survivability to the water only baseline.

When the different *E. coli* strains were compared, *E. coli* O157 proved to be the hardiest, surviving the longest under all experimental conditions. The least hardy was the laboratory strain, #25922, particularly when exposed to the antimicrobial clay. The environmental isolate fell between the two other strains in terms of survivability proving

that it had become adapted to the environment while failing to have the ability of *E. coli* O157 to survive the many environments it is exposed to as a pathogen.

Chapter 6. Investigation of the effect of *Escherichia coli* strain on survival and transport

6.1 Introduction

One of the most important organisms within the surface waters in regards to humans is *Escherichia coli.* This species contains strains that can cause serious disease such as well known *E. coli* O157 and the species as a whole can be used to monitor faecal contamination in surface waters.

The pathogenic strains of *E. coli* are a large public health problem with *E. coli* O157 causing over 1000 cases in the UK alone (DEFRA, 2008). This number is relatively low in comparison to other enteropathogens, the most common being *Campylobacter* that causes ~50,000 per year in the UK (HPA, 2008). However, the disease caused by pathogenic *E. coli* has a greater risk of severe disease meaning that this species is of high importance to human health.

To reduce the likelihood of outbreaks, water supplies and their sources need to be monitored for these pathogens. However, most pathogens are found in relatively low numbers in comparison to background bacteria and their presence can be hidden. Current practice relies on the detection of faecal events through the monitoring of indicator organisms, such as generic *E. coli* (EA, 2002; SEPA, 2007), from this the likelihood of there being pathogens present can be estimated. For example, a high number of *E. coli* present suggests a large faecal pollution event meaning that there is a higher likelihood of pathogens being present.

The *E. coli* found in environmental waters are planktonic within the water column and evidence suggests that these do not survive for long periods of time (Davies et al., 1995).

The other 40% are found bound to sediment, however this can increase during high flow events (Characklis et al., 2005; Krometis et al., 2007; Jamieson et al., 2005a).

These interactions between *E. coli* and sediment in surface waters have been investigated by a number of authors (e.g. Muirhead et al., 2004; Jamieson et al., 2005: Wu et al., 2009). When bound to sediment particles, bacteria have been shown to be physically shielded against some of the antimicrobial processes, such as damaging ultraviolet light, that might occur in the water. Thus, the bacteria-sediment interaction plays an important role in *E. coli* survival and has a knock-on effect on human health.

The bacteria-sediment interaction is not yet fully understood but it is thought that surface characteristics of both the bacterial cell and the sediment particle play a key role in determining the binding (e.g. (Zhuang and Yu, 2002; Soni et al., 2007). Chemical composition of the surrounding environment can also play a role (Chapter 4).

The *E. coli* species can be genotypically and thus phenotypically very diverse, indeed a study by Welch et al. (2002) found that only 40% of proteins were conserved across three separate strains of *E. coli.* This variability within the *E. coli* species gives rise to the possibility of different behaviours within the same environment. For example, evidence shows variability in competitive ability between *E. coli* O157 strains when exposed to background organisms despite equal survival ability when alone (Watterworth et al., 2006).

The differences in behaviour between *E. coli* strains is particularly important in relation to the pathogenic strains mentioned previously, for there exists the possibility that a pathogenic strain may behave atypically of the *E. coli* species and thus cause a risk to human health where none is thought to be present. The converse is also true with the

pathogenic strain causing no risk where the behaviour of the generic *E. coli* suggests there should be and as a result resources are wasted. This means that it is very important to investigate the differences (genotypic, phenotypic and behavioural) between *E. coli* strains.

This study investigates the sediment binding behaviours of several strains of *E. coli* and further investigates the binding mechanism.

6.2 Materials and Methods

6.2.1 Experimental Samples

Environmental samples were taken from the Tarland catchment, River Dee, north east Scotland. The surrounding area is well characterised with ongoing environmental monitoring. The stream runs through farmland, with grazing for cattle on both banks, and is regularly impacted by diffuse pollution. Specifically, both sediment and water samples were collected from the stream bed at various points along the impacted length that were then kept at 5 °C until use. In addition, several cow manure samples were taken from nearby and pooled together.

6.2.2 *E. coli* Strain Isolation and cultivation

Five g of each sediment and manure samples were subjected to vortexing in 5 mL sterile Maximum Recovery Diluent (MRD) for 5 minutes. After which the resulting suspension was submitted to a 10-fold dilution series using sterile MRD and plated on ES Chromocult agar that allows *E. coli* identification from colony colour. After 24 hour incubation at 37 °C, plates were examined for the presence of *E. coli*. The experimental strains (five manure and four sediment) were selected randomly from these isolated

strains with one from each environmental sample and, with *E. coli* O157 #3704, were cultured overnight at 37 °C in Luria-Bettani (LB) broth and further plated out on ES Chromocult agar.

6.2.3 Microcosm Preparation

Stream water was collected from the Afon Goch that runs past the Bangor University Henfaes Research Centre in North Wales, UK. This water was subsequently filtered through a sterile 0.45 μm filter in order to remove any microorganisms without damaging the nutritional constituents.

Stream sediment was collected from the same site as the *E. coli* samples (Section 6.2.1) and sterilised by autoclaving at 121 °C for 20 minutes. The sediment was plated out on various agars to make certain that sterilisation was achieved.

Experimental microcosms were prepared by adding 5 g sediment to 45 ml sterile streamwater in 250 ml sterile bottles. For each of the 10 experimental *E. coli* strains, 5 concentrations were used starting at 1 x 10^8 cfu mL^{-1} and decreasing by a factor of 10 for each step with the lowest concentration being 1 x 10^4 cfu mL^{-1}. Each concentration was replicated in triplicate giving a total of 50 experimental microcosms.

6.2.4 Zeta Potential

All experimental strains were grown for 18 hours in LB incubated at 37 °C. 1 mL of the culture was subsequently washed twice in sterile 0.1 mM NaCl. The zeta potential of the *E. coli* strains was measured (Malvern Zetasizer nano ZS; Malvern Instruments, UK) in 0.1 mM NaCl solution. Measurements were performed at 15 °C using a refractive index

of 1.5 within the general purpose methodology and applying the Smoluchowski equation. Where conductivity was over 10 mS cm^{-1}, monomodal analysis was applied. The Malvern Zeta Potential transfer standard was run at the start and end of analyses for quality control (Malvern Instruments, UK).

6.2.5 Binding Constant

The binding contant for each strain was calculated using Equation 1 derived from the Langmuir equation (Equation 2).

Equation 1

$$\alpha = \frac{\theta}{(1-\theta)p}$$

Where α = binding constant

θ = Filled surface sites

p = Bacterial concentration

Equation 2

$$\theta = \frac{\alpha \cdot p}{1 + \alpha \cdot p}$$

Where α = binding constant

θ = Filled surface sites

p = Bacterial concentration

6.2.5 Survival Experiment

A survival experiment was conducted using the same environmental conditions and the same *E. coli* strains as an initial investigation into the potential differences between the *E. coli* strains.

6.3. Results

6.3.1 Zeta Potential

The zeta potentials of the manure-isolated *E. coli* proved to be very similar with the most negative being isolate M1 with a mean value of -38.0 mV and the least negative M3 with a mean value of -33.9 mV (Figure 6.1). The sediment isolated strains were much more variable with two strains, S3 and S4, giving a value similar to that of the manure isolated strains (means -38.1 mV and -37.8, respectively), with the other two sediment isolates, S1 and S2, had significantly less negative zeta potentials (-28.5 mV and -25.5 mV, respectively). *E. coli* O157 (mean -6.73 mV) has the least negative zeta potential of any of the *E. coli* strains tested by a very large margin of nearly 20 mV (Figure 6.1).

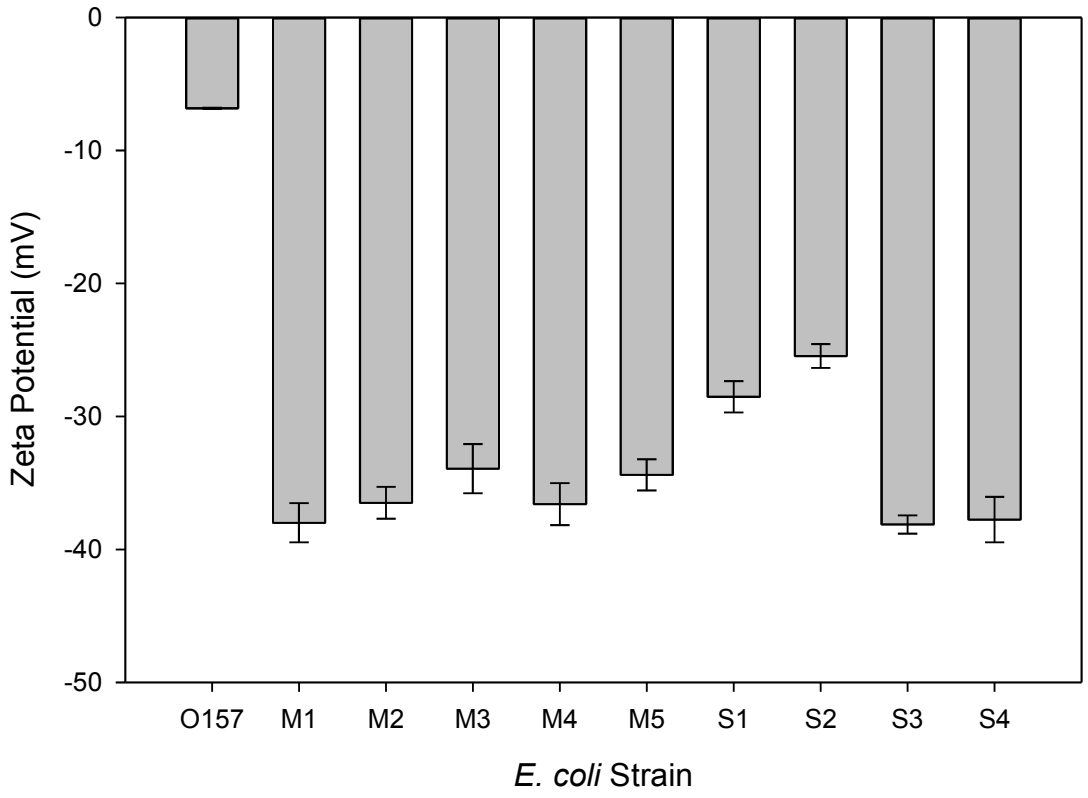


Figure 6.1. Zeta Potential of each Experimental *E. coli* Strain in 0.1 mM NaCl. ±SEM (n=3)

6.3.2 Adsorption Isotherm

Adsorption to sediment was measured through the reduction in bacterial numbers seen in the water, and thus the proportion bound can be calculated. At the lowest *E. coli* concentration of 10^4 CFU mL^{-1}, the strain that showed the highest proportion bound was *E. coli* O157 (over 45%) significantly more than the other experimental strains (Figure 6.2). The strain with the lowest proportion bound were strains M1, M2, S3 and S4 that all had values in the region of 0.15. The other manure strains tested (M3, M4 and M5) did not vary significantly from this group (0.30, 0.25 and 0.30 respectively) (Figure 6.2a).

The final two experimental strains (S1 and S2) were significantly different to these strains and that of *E. coli* O157 with proportions bound 0.32 and 0.39 (Figure 6.2b).

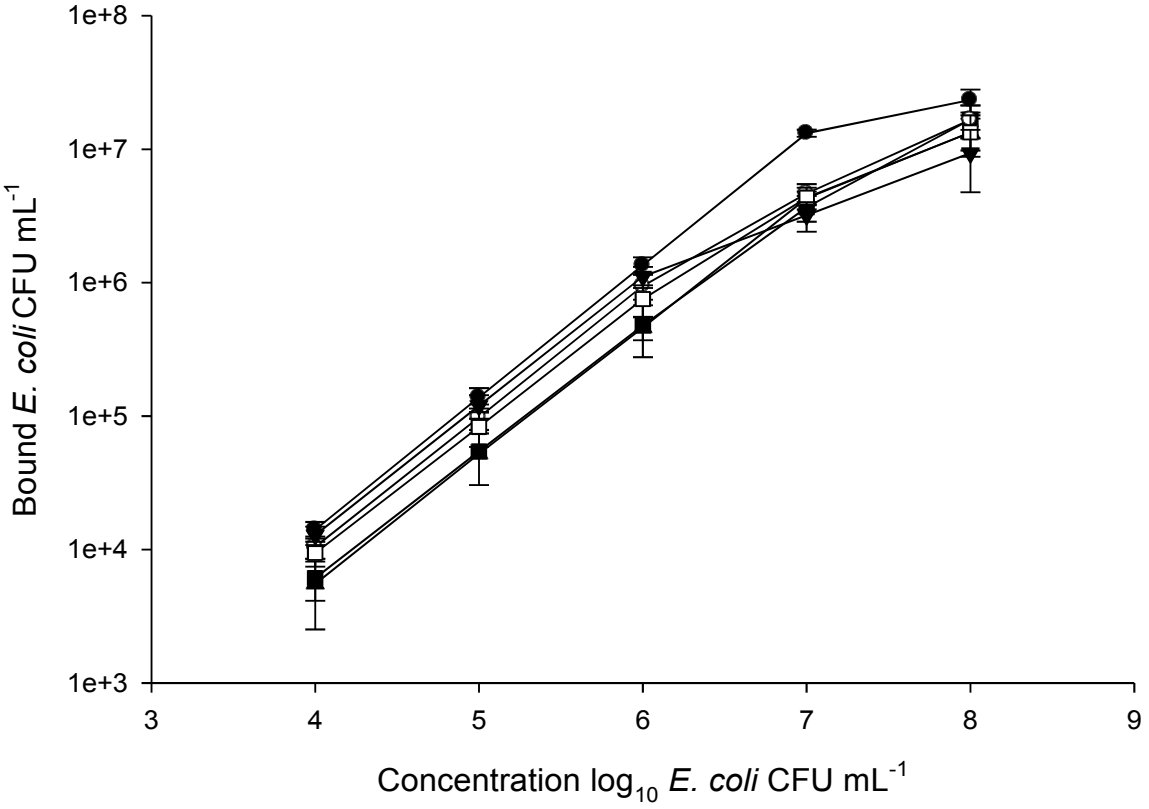


Figure 6.2a. The number of bound *E. coli* after shaking for 60 minutes at 10 °C; 100 rev min^{-1}. *E. coli* O157 (●); *E. coli* M1 (■); *E. coli* M2 (▲); *E. coli* M3 (○); *E. coli* M4 (□); *E. coli* M5 (▼). ±SEM (n=3).

This pattern continues at concentrations 10^5, 10^6 and 10^7 CFU $mL^{-1}$with proportion bound seemingly decreasing very slightly as concentrations increase for all experimental strains. At these concentrations, the binding process is not limited by the number of binding sites available on the sediment. However, at 10^8 the proportion bound for most of the strains becomes much less particularly those that had higher values at the lower concentrations,

indicating that there is a limit to the number of sites on the sediment where the *E. coli* can bind. The maximum number of bound seems to be approximately 2 x 10^9 CFU g^{-1} sediment for all the *E. coli* strains as calculated from those that had reached that value, indicating that it is likely that there were no differences in the areas of the sediment particles that the *E. coli* cells bound to.

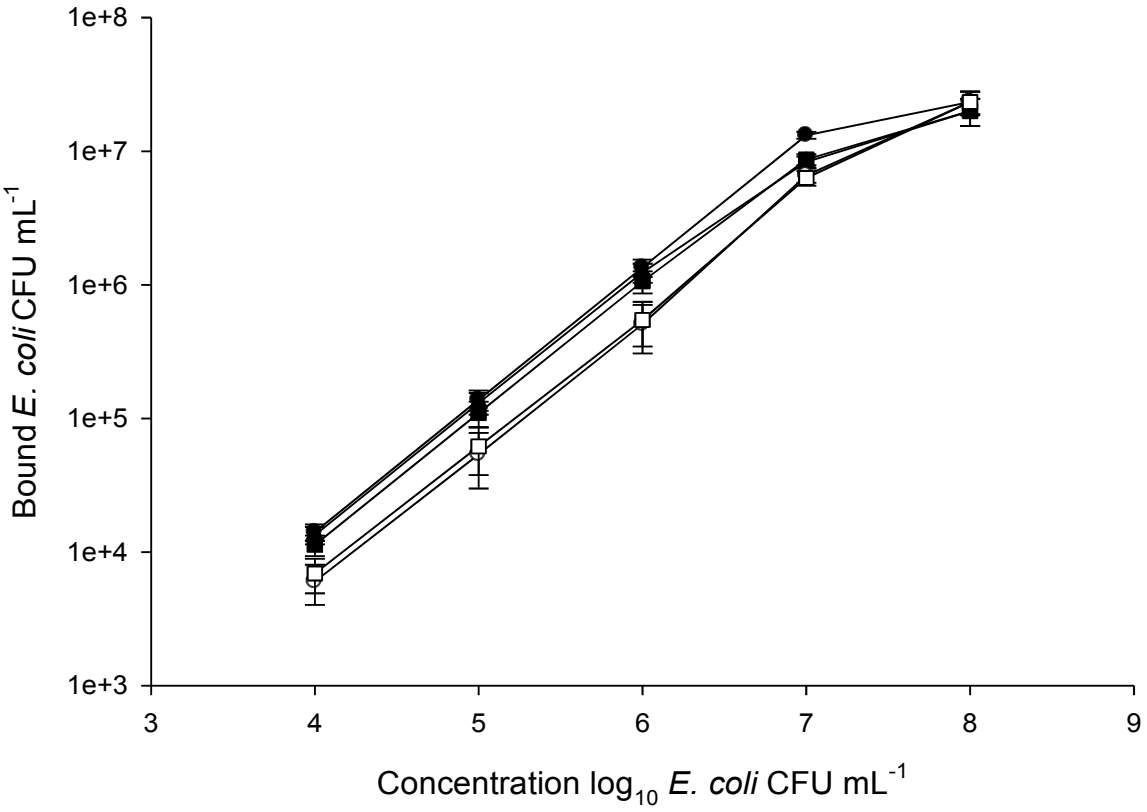


Figure 6.2b. The number of bound *E. coli* after shaking for 60 minutes at 10 °C; 100 rev min^{-1}. *E. coli* O157 (●); *E. coli* S1 (■); *E. coli* S2 (▲); *E. coli* S3 (○); *E. coli* S4 (□). ±SEM (n=3)

Table 6.1 shows the binding constant for each strain calculated as using these data described in 2.5. All of the manure strains (M1-5) and two sediment strains (S3 and S4)

proved to have very similar values for this constant, indicating similar levels of binding ability. Sediment isolates S1 and S2 had a higher value with the *E. coli* O157 having the highest of all of the experimental strains. This indicates that these three *E. coli* strains have higher sediment binding ability than strains typically found in manure.

TABLE 6.1: Binding Constant at 1 x 10^7 CFU mL^{-1} as calculated using Equation 1

Experimental *E. coli* Strain	Binding constant at 1 x 10^7 CFU mL^{-1}
O157	4.13 x 10^{-8}
Manure 1	1.11 x 10^{-8}
Manure 2	1.13 x 10^{-8}
Manure 3	1.12 x 10^{-8}
Manure 4	1.28 x 10^{-8}
Manure 5	1.22 x 10^{-8}
Sediment 1	2.16 x 10^{-8}
Sediment 2	2.07 x 10^{-8}
Sediment 3	1.25 x 10^{-8}
Sediment 4	1.02 x 10^{-8}

6.3.3 Zeta Potential verses Binding Constant

The data from the previous sections were analysed to investigate if zeta potential had any influence over the binding process (Figure 6.3). The R-squared value from this analysis was 0.96 indicating that there is a statistical significance between the two. However, the data set is limited with most of the strains clustering around the more negative zeta potential, meaning that some caution needs to be taken when interpreting this result.

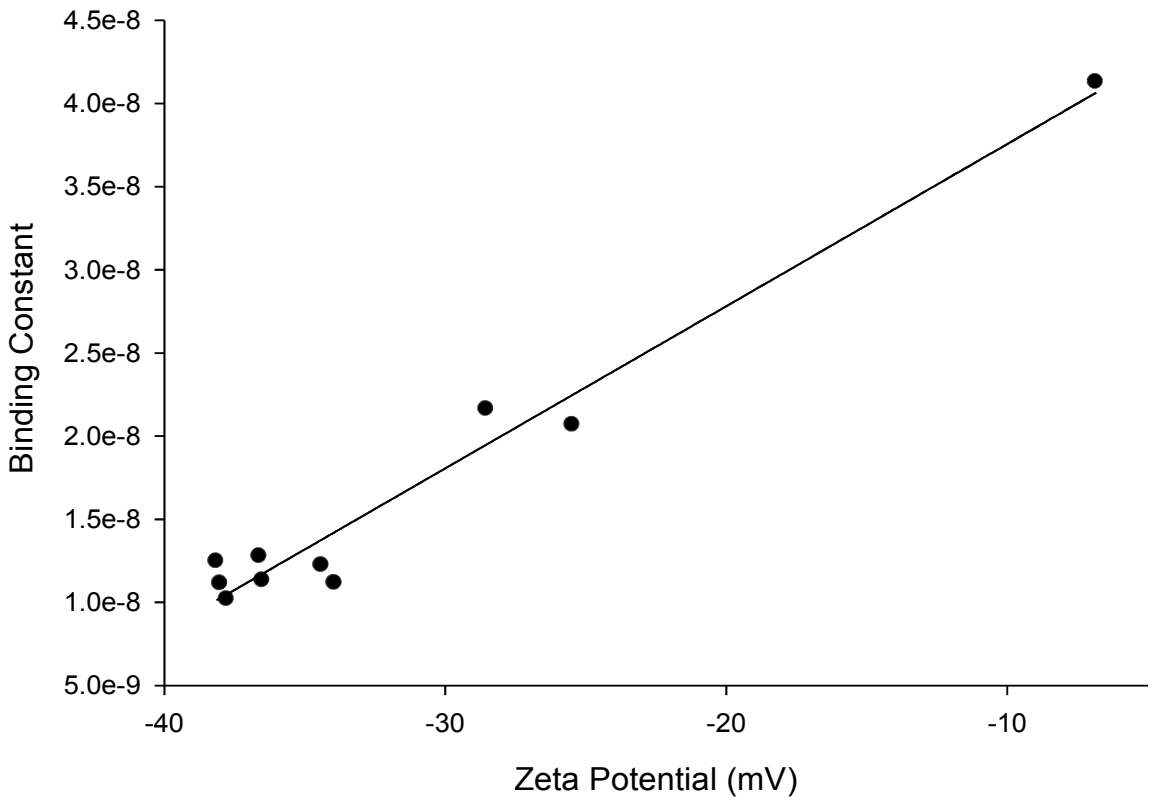


Figure 6.3. Binding Constant at 1 x 10^{7} CFU mL^{-1} verses the corresponding zeta potential. Trend line is linear with equation: y = 1 x 10^{-9} x + 4.73 x 10^{-8}. R^2 = 0.967

6.3.4 Survival Experiment

The short survival experiment again showed up the variabilities between *E. coli* strains. The least resilient strains proved to be the manure isolates surviving 2-3 days under these conditions. Two of the sediment isolates again displayed similar qualities to those isolated from manure both surviving for three days. *E. coli* O157 survived for seven days under these conditions giving further credence to its perception as environmentally resilient. However, the final two sediment isolates, S1 and S2, proved to survive the longest, lasting for a minimum of two weeks.

On comparison with binding ability the manure strains, and two sediment strains (S3 and S4), have the lowest survival and lowest binding ability and the lowest survival in this environment. *E. coli* O157 proved to be the best of the experimental strains at binding to the sediment but falls behind two environmental strains (S1 and S2) in relation to survival time, indicating other factors controlling survival.

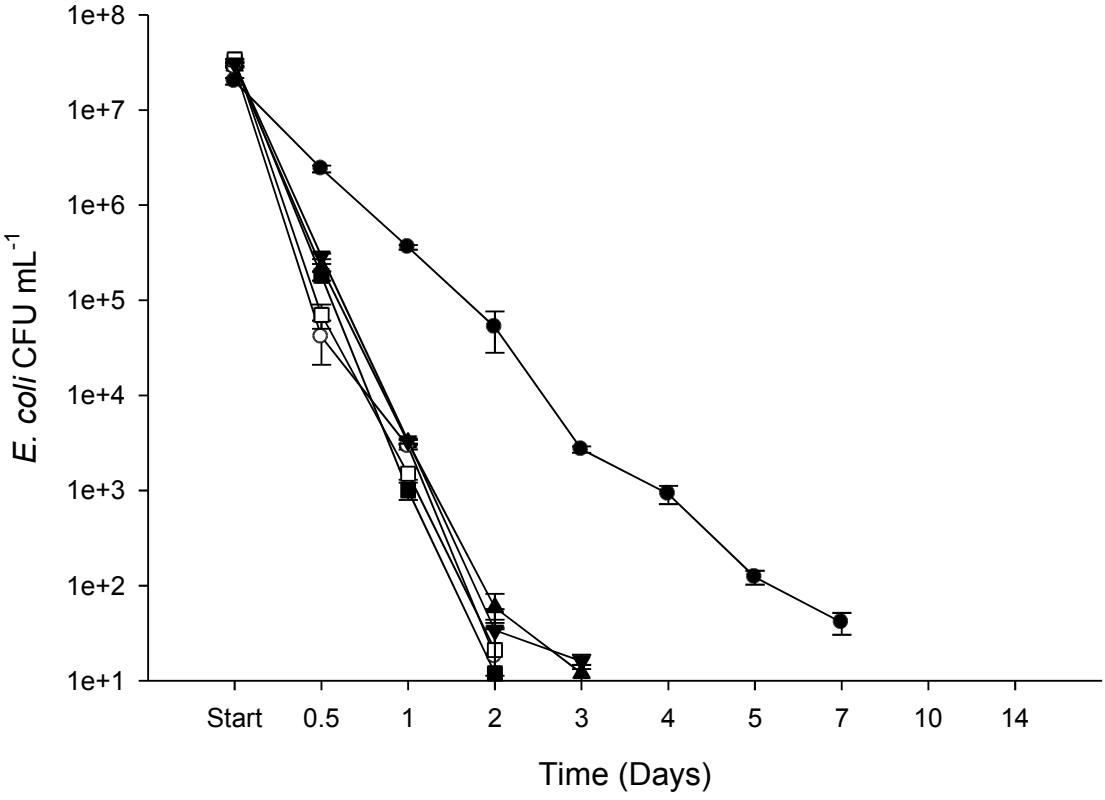


Figure 6.4a. The change in *E. coli* number within the microcosm over time. *E. coli* O157 (●); *E. coli* M1 (■); *E. coli* M2 (▲); *E. coli* M3 (○); *E. coli* M4 (□); *E. coli* M5 (▼). +SEM (n=3)

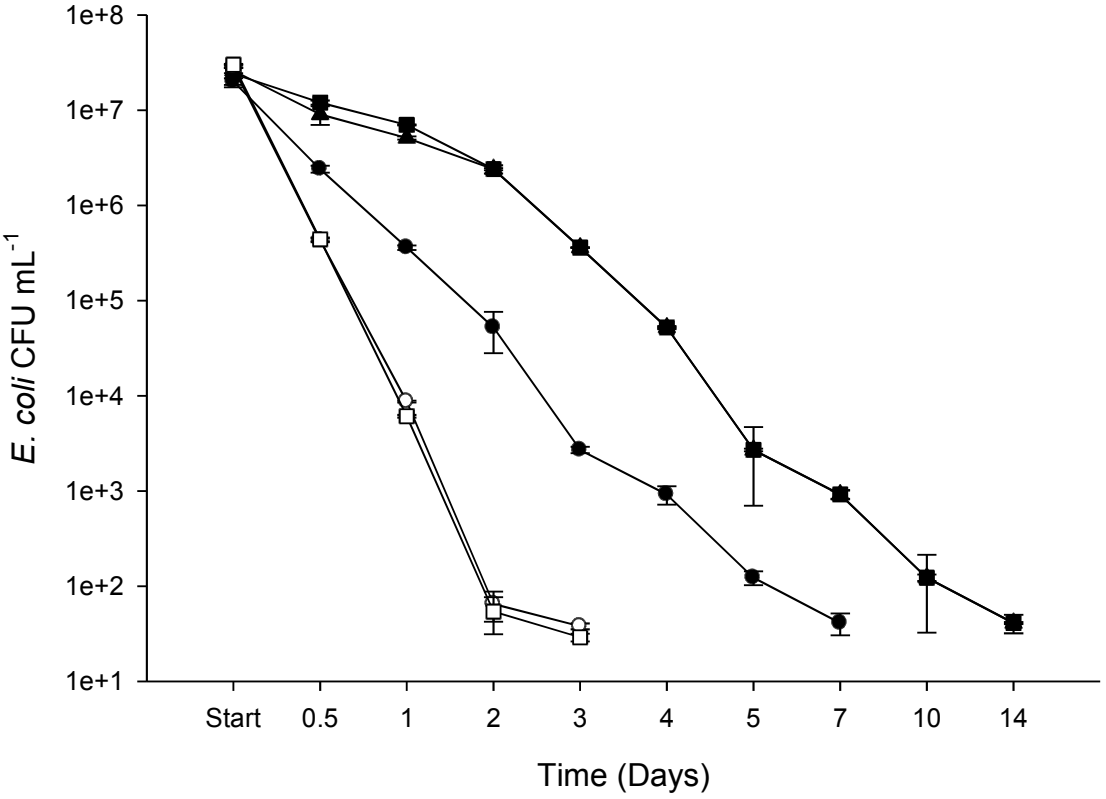


Figure 6.4b. The change in *E. coli* number in the microcosm over time. *E. coli* O157 (●); *E. coli* S1 (■); *E. coli* S2 (▲); *E. coli* S3 (○); *E. coli* S4 (□). +SEM (n=3)

6.4 Discussion

The *E. coli* strains proved to have a broad range of zeta potentials under the same conditions indicating variation on the bacterial cell surface. This property is a result of the construction of the bacteria cell membrane particularly the outer envelope. Typically the surface charge, and thus the zeta potential, is negative by virtue of ionised phosphoryl and carboxylate substituents present (Wilson et al., 2000). However, this property is subject to the conditions of the surrounding medium. For example, in Chapter 4, the bacterial zeta potential measured increased gradually when exposed to an increasing gradient of sodium chloride, from -24 mV at 0 mM NaCl to -4 mV at 100 mM NaCl (data not shown). Change in zeta potential due to changing environmental conditions is not confined to the bacterial cell, as mentioned previously in Chapter 3. However, within this system the electrostatic forces, while important, did not seem to be the controlling factor.

In this study, the range of experimental organisms was much greater as they were more recently isolated and are likely to be representative of those *E. coli* strains found within those particular environments. In this case, we found that there was variation in zeta potential between the manure isolates and two of the environmental isolates. This suggests differences in the construction of the cell surface, however, these have not yet been characterised. It is possible that these alterations may be a result of exposure to the environment for an extended period of time and thus may be adaptations to survival in the aquatic environment. However, the changes in zeta potential are likely incidental to other adaptations and not the primary selective pressure.

The similarity in zeta potential between the sediment isolates S3 and S4 and the manure isolates suggests that they have similar cell surface structure and therefore live in a similar environment. This further suggests that these two strains were recent additions to the sediment ecosystem probably introduced through surface run-off from the surrounding fields.

The interaction between the *E. coli* and the stream sediment is very important as this impacts both the survival of the organism in the environment and subsequent transport once introduced. The survival of *E. coli* in the aquatic environment is a very complex issue with no one controlling factor (Avery et al., 2008). Planktonic *E. coli* suspended in the water column have been proven to suffer within the stream environment (Davies et al., 1995) thus becoming bound to sediment enables *E. coli* to survive much longer. For when bound to sediment particles, bacteria are physically shielded against some of the antimicrobial processes, such as damaging ultraviolet light (Sinton et al., 2002) that might occur in the water. Thus, the bacteria-sediment interaction is important in understanding survival behaviour.

The *E. coli* experimental strains were examined for binding ability as described thoroughout and Langmuir absorption isotherm typically used to calculate the adsorption of molecules onto a solid surface. The Langmuir adsorption constant was subsequently calculated and used to give a value to the binding ability of each strain. *E. coli* O157 proved to have the best binding ability with the manure isolated strains, plus the two sediment isolates previously hypothesised to be recent additions, had the worst. This suggests that if introduced together into a stream environment *E. coli* O157 is most likely to bind to the sediment present. This in turn would improve its survival chances and

therefore allow it to remain in that environment. The manure strains would be more likely than *E. coli* O157 to remain unbound in the water column and thus be exposed to antimicrobial processes killing them quickly. The two sediment isolates of *E. coli* S1 and S2 fell between these two extremes having a better binding ability than the manure isolates but not as good as *E. coli* O157. This suggests that these *E. coli* have become adapted to that environment and become part of a community of naturalised *E. coli* (Ishii et al., 2006; Badgley et al., 2011).

Unbound *E. coli* and those bound to small particles are more likely to travel further due to the lower mass when compared to a *E. coli*-sediment complex (Muirhead et al., 2006; Goldscheider et al., 2010). This potentially risks public health by impacting sites at relatively great distances from the point of contamination. However, the survival of these organisms is much lower than those bound to sediment (Davies et al., 1995) and thus there is only a short-term risk when compared to areas nearer the source that have the naturalised *E. coli* populations.

Interestingly, when zeta potential and binding ability (as measured through the Langmuir adsorption constant) were examined together a strong correlation was observed, linking a more negative zeta potential to lower binding ability. This is in contrast to results in Chapter 3, where only a weak correlation was found. This is likely due to the differing environmental conditions and not having *E. coli* strains covering part of the zeta potential range. Potentially, the sediment might have an unusually strong negative charge thus the differences between the strains are exaggerated and under conditions where the sediment has a positive charge the binding abilities would be reversed.

In order to investigate if the binding abilities of these experimental strains were related to the survival of *E. coli* a survival experiment was established under the same conditions. As expected the manure strains proved to be the weakest in these conditions. Sediment isolates S3 and S4 again proved to have similar behaviour to the manure isolates again suggesting that these are recently introduced.

The non-toxigenic *E. coli* O157 managed to survive much longer despite this not being its primary environment (Yoon and Hovde, 2008), adding further weight to its perception as a hardy organism.

However, the two other sediment isolates managed to survive for a minimum of two weeks further suggesting adaptation to this environment. The fact that these two strains survive much longer than the *E. coli* O157 with the better binding ability indicate that while binding of *E. coli* to sediment plays an important role in survival, it is part of a much more complex picture.

This study highlights the differences between strains of *E. coli* and the importance of understanding the behaviour of different strains within a species as a whole.

7. Conclusions

Escherichia coli is widely used as an indicator for pathogenic organisms and some strains are themselves pathogenic. It is therefore essential to understand the behaviour of this organism within the environment as there can be large knock-on effects to public health.

7.1 Key Findings of the study

7.1.1 Influence of native biofilm on the behaviour of *E. coli* within an aquatic system

The key finding from this study is that an established native biofilm on sediment does not seem to assist in the binding of *E. coli* or coliforms to sediment when the organisms are introduced to the water column from short-term faecal pollution incidents. However, over longer periods of time, *E. coli* are incorporated within these native biofilms as they develop. In addition, clean sediment again proved to be a strong sink for planktonic *E. coli,* highlighting their importance within these systems. Both the sediment and the biofilm seem to provide good areas of refuge within the stream environment, protecting bacteria from any antimicrobial processes present. Thus biofilms have the potential to be both a source and sink of *E. coli*.

7.1.2 Electrolyte Type and Concentration Regulates the Binding of an Environmental *E. coli* Isolate onto Kaolinite and Montmorillonite Clays

The findings from this study indicate that electrostatic forces within the freshwater environment do influence the binding behaviour of *E. coli* to colloidal clays but are not the most important factor controlling these interactions. This effect is principally seen within the more highly charged particles, with no effect observed with monovalent cations. However, in the environment, apart from a few select cases such as divalent cation concentrations spiking after heavy rainfall, this effect is likely to be hidden behind much stronger physical factors.

7.1.3 Influence of clay type on survival of *E. coli* in freshwater

The clays proved to have a varying influence on the survival time, dependent of the *E. coli* strain tested. The antimicrobial Illite clay proved to have the strongest effect on the laboratory strain #25922, killing it within a few hours. The non-toxigenic *E. coli* O157 survived much longer, seemingly much better at resisting the oxidation caused by the hydroxyl radicals produced by the clay.

For all strains tested, Montmorillonite clay increased survival over that of the others and over the water only (control) baseline. The mechanism is currently unknown but the most likely possibility is that the clay provides a haven from any ongoing antimicrobial processes.

7.1.4 Investigation of the effect of *Escherichia coli* strain on survival and transport

The experimental *E. coli* strains proved to have much different zeta potentials to each other, with the non-toxigenic laboratory strain of *E. coli* O157 having a zeta potential an

order of magnitude less negative than the other strains. The strains isolated for the manure samples had similar zeta potentials within the group, in contrast to those isolated for the sediment that were much more variable. The subsequent binding behaviour observed for these strains also proved to vary between the strains, with some environmental strains being significantly better at binding to the stream sediments than the manure strains but with the *E. coli* O157 strain being better again. Further investigation found a strong link between the zeta potential and the binding efficiency of the *E. coli* strain. While this may be a result of the combination of conditions, the sediment may have atypically strong negative charge. The study shows that there is great variation between different strains of *E. coli* when exposed to the same conditions and thus it is important to take this into account when describing the species as a whole.

7.2 Further Work

Each aspect of this thesis has provided or highlighted areas in need of further study.

7.2.1 Influence of native biofilm on the behaviour of *E. coli* within an aquatic system

Further investigation is needed into the *E. coli* contained within biofilms, particularly investigating their source. It is unknown how the *E. coli* replenish their numbers in the biofilm; it is either through cell division within the biofilm or through replenishment from the water column. The implication of the latter would be that if it is possible to reduce faecal pollution of streams, the population of *E. coli* would fall thus reducing the store within the stream. If the former is true, there is the potential of stores of pathogens replicating and being released back into the water column, causing a risk to human health. How this varies with strain type is also worthy of investigation.

7.2.2 Electrolyte Type and Concentration Regulates the Binding of an Environmental *E. coli* Isolate onto Kaolinite and Montmorillonite Clays

The small investigation into the effect of humic acid on the binding process suggests a path to follow for how the substance increases the binding of *E. coli* to the clays. This suggests that future studies should address the impact of organic materials and environmental modifications to clay colloid surfaces.

7.2.3 Influence of clay type on survival of *E. coli* in freshwater

Again, this study proved the importance of taking strain effect into account and future studies must account for this. In addition, the processes that control survival are as complex as those that govern binding. In this instance, the influence that clays have is known but the mechanisms through which they work are still uncertain. Further research needs to investigate what happens to an *E. coli* cell when it binds to clays that allow it to survive longer than unbound cells. Also the weakness of *E. coli* strains that have lived in the laboratory environment is highlighted in this study; further research could investigate what these changes are and thus ensure that laboratory strains are actually representative of those found in nature.

7.2.4 Investigation of the effect of *Escherichia coli* strain on survival and transport

Throughout this thesis the effect of *E. coli* strain is one of increasing importance particularly as most environmental studies focus on one strain of *E. coli* as representative of the whole species. While this can be a valid investigative technique, the limitations must be recognised in future research as the variation within the species can be massive. Further research needs to investigate binding process, for there are many factors that may influence it the primary drivers behind it are not yet characterised.

Additionally, much could be learnt by investigating the changes and adaptations an *E. coli* strain undergoes when introduced into an aquatic environment after living in the mammalian gut. This would help us understand how the *E. coli* survive in the environment and potentially help identify the sources of faecal contamination.

8. Appendix

8.1. Total and Faecal coliform levels during Storm events

For several storm events at the Cessnock catchment in western Scotland, water samples were taken from a stream adjacent to farmland. These samples were taken by an Autosampler that started when the stream flow reached predetermined levels and took samples every hour until the flow returned to normal. Some of these samples were retained for other work while the others were transported to the laboratory under refrigeration.

Each sample was subjected to the following protocol:

- Bottle containing sample was gently inverted three times by hand to resuspend material settled during transport (as per Characklis et al., 2005).
- 50 mL was poured into a 50 ml centrifuge tube (volume determined by weight) and 12 ml placed into a 15 ml centrifuge tube.
- 50 mL volume was centrifuged on the Beckman coulter J6-MI centrifuge at 1350 rpm = 500 g for 14 minutes.
- 14 ml supernatant was carefully removed with snipped off 5ml pipettes to minimise shear force, & placed into fresh 15 ml universals. This equated to removal of supernatant to a depth of 2.4 cm.
- 10 mL volumes of centrifuged samples were filtered using a Whatman 45 μm gridded filter.
- These were then placed on ES Chromocult Agar, made up using manufacturer's instructions.

- The remaining volume of the centrifuged samples were subjected to a 10-fold dilution series so that 1 mL, 0.1 mL and 0.01 mL were in the final solution to be plated out.
- These were plated out on ES Chromocult agar as above.
- A small drop (approx. 2.5 µL) of Tween 80 was added to each of the 14ml subsamples of the undisturbed samples.
- Each tube was then hand shaken fairly vigorously 4 times and then vortexed for 10 seconds.
- This process was repeated for the uncentrifuged samples.
- Once dry, all plates were incubated at 37 °C for 18 hours.

The stream flow data from these times were also recorded and allowed some insight into how bacterial numbers respond to stream flow.

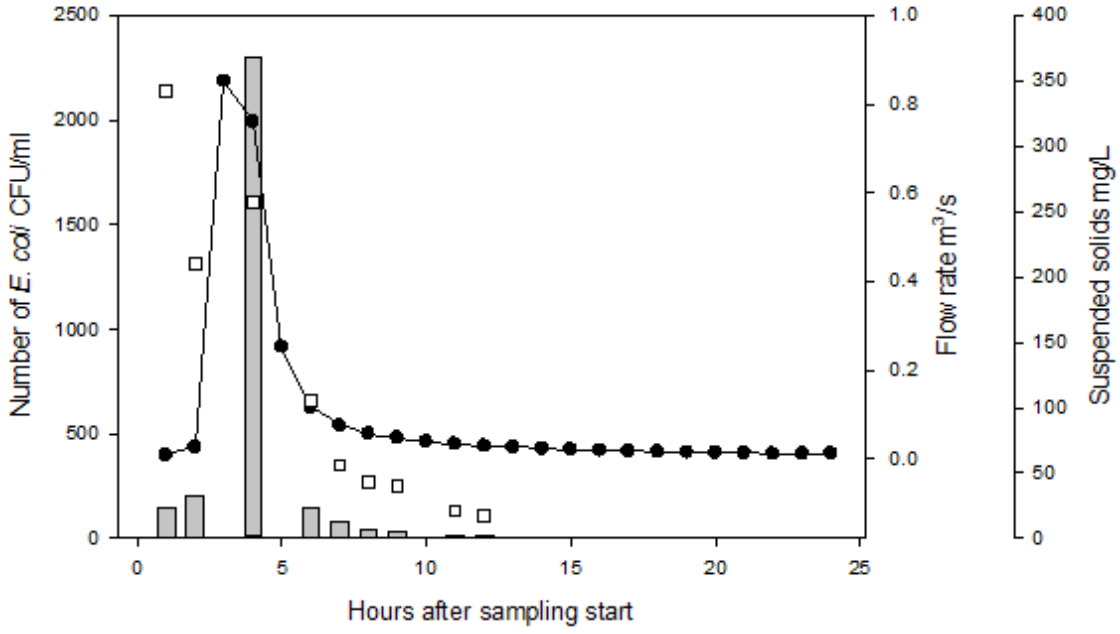


Figure 8.1. Storm Event 4/07/09. Bound *E. coli* (Grey Bars), Free *E. coli* (Black Bars), Stream flow rate (●) and Suspended Solids (□).

8.2. Photographs of the stream channels as described in Chapter 3

Figure 8.2. Examples of stream channels with biofilm growth (left (6) and right (4)) and without biofilm growth (centre (5)).

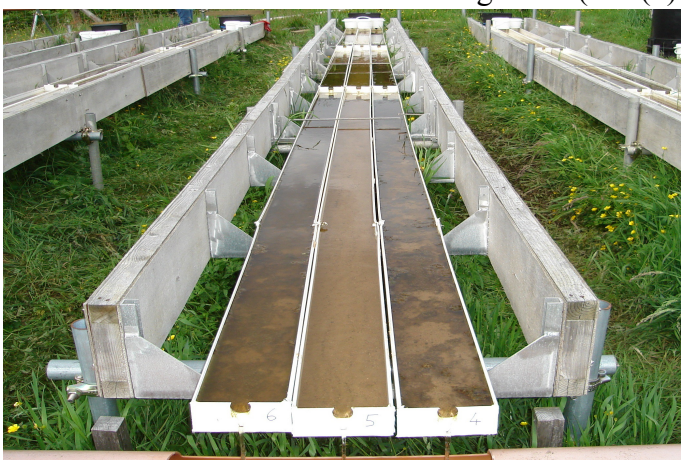


Figure 8.3. Buckets used for manure slurry addition in Run 1.

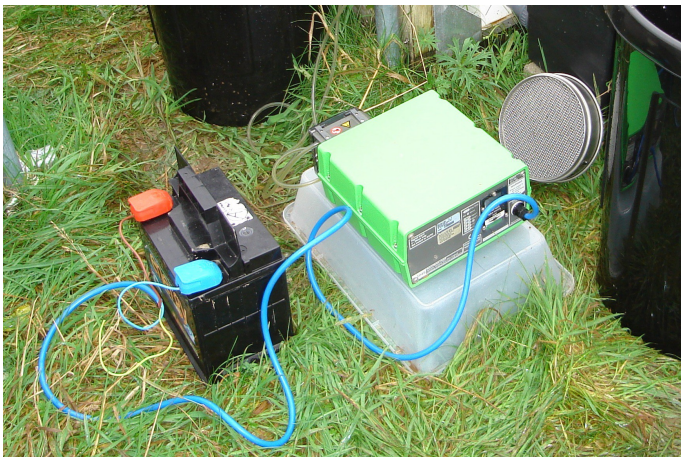


Figure 8.4. Peristaltic pump used for manure slurry addition in Run 2.

8.3. Small study of the influence of nutrients (cow manure) addition on survival of *E. coli*

Aim: To investigate the effect of nutrient addition to the survival of *E. coli* O157 in stream sediment.

A manure slurry was made by taking 75 g of fresh manure and mixing with 1 L water and leaving to settle for 30 min. The stock solution was then taken from the unsettled fraction and further dilution were made using sterile d.i. water.

5 g fresh stream sediment is added to a universal tube with 2.5 mL of manure solution at 25%, 50% or 75% concentration of the stock solution. Control micocosms were also made using sterile d.i. water replacing the manure solution.

To this 50 μm of an overnight culture of *E. coli* O157 is added at 1 x 10^7 CFU mL^{-1} (twice washed in Maximum Recovery Diluent) to give a start concentration of 1 x 10^5 CFU g^{-1}.

These microcosms were placed in a 10 °C incubator for 24 hours before the first sample group were taken for enumeration.

A small drop of Tween 25 (~2 μm) was added to each sample. This was then vortexed for 25 seconds and subjected to 10-fold serial dilution in Maximum Recovery Diluent before being plated out on ES Chromocult Agar plates. These were incubated overnight at 37 °C and the number of colonies were counted. Additional sampling occurred on days 4, 7, 11 and 18 after the experiment had begun.

The presence of nutrients, in this case cow manure, does make a big difference is terms of *E. coli* O157 survival (Figure 8.5). There does seem to be a critical nutrient level at which other factors become limiting and the second lowest addition amount (25 %) does not seem to be enough to overcome the limiting factors.

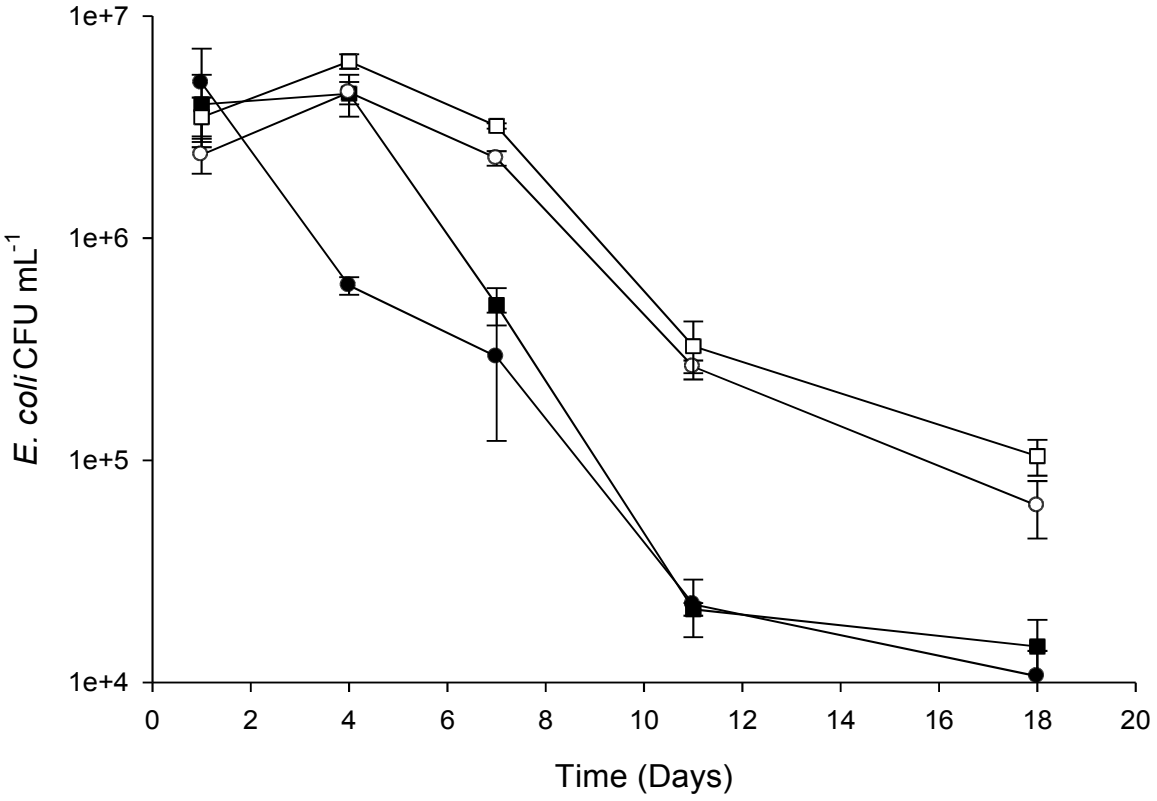


Figure 8.5. Change in *E. coli* number with time with manure slurry of 0 % (●), 25 % (■), 50 % (○) and 75 % (□) ±SEM (n=4) .

9. References

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