DOCTOR OF PHILOSOPHY

Persistence of escherichia coli O157 in contrasting environments and its implications for human health

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PERSISTENCE OF *ESCHERICHIA COLI* O157 IN CONTRASTING ENVIRONMENTS AND ITS IMPLICATIONS FOR HUMAN HEALTH

DYFALWCH *ESCHERICHIA COLI* O157 MEWN AMGYLCHEDDAU CYFERBYNIOL A'R GOBLYGIADAU I IECHYD DYNOL

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Thesis wedi'i gyflwyno i Brifysgol Cymru gan

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SUMMARY

Human ingestion of a very small number of *Escherichia coli* O157 cells may be sufficient to cause serious illness, or even death. Although most human *E. coli* O157 infections are food-borne, contact with the organism in the environment is an important vector of initiating outbreaks. Consequently, persistence of the organism in different environments needs to be elucidated. This thesis details the observed persistence of *E. coli* O157 under a range of environments and environmental conditions simulated in various laboratory experiments. The first experimental chapter (*Article I*) investigated the survival of *E. coli* O157:H7 in cattle faeces on the surfaces of materials commonly found on farms. Persistence was greatest on moist wood samples under cooler temperatures with large numbers remaining after 28 days. It was established that substantial numbers of cells may also be transferred to human hands from such surfaces during brief contact. In chapter 4 (*Article II*), the effect that the traditional practice of applying sawdust to floor tiles in meat-processing areas has on pathogen persistence was examined. Viable *E. coli* O157:H7 persisted on all tiles over 3 days, although desiccation of surfaces resulted in a more rapid decline in numbers, while cleaning of the tiles with bleach prior to contamination with meat juice enhanced recovery of the pathogen. The presence of sawdust was only found to reduce survival of *E. coli* O157:H7 on tiles under dry conditions. Chapter 5 (*Article III*) investigated the effect of earthworm digestion on the survival and dissipation of *E. coli* O157:H7 in compost and soil. Deep-burrowing earthworms (*Lumbricus terrestris*) significantly aided vertical movement of *E. coli* O157 in soil, whilst litter-dwelling earthworms (*Dendrobaena veneta*) significantly aided lateral movement within compost. Although the presence of earthworms in soil and compost may aid proliferation of *E. coli* O157:H7 in early stages of contamination, long-term persistence of the pathogen appears to be unaffected. In chapter 6 (*Article IV*), cattle slurry and ovine stomach content waste inoculated with *E. coli* O157:H7 were applied to soil cores ± established maize plants. The pathogen survived in soil for over 5 weeks, though at significantly greater numbers in soil receiving stomach content waste in comparison to cattle slurry. In contrast to published work, persistence of the pathogen in soil was unaffected by the presence of a rhizosphere. In chapter 7 (*Article V*), cow and sheep faeces were inoculated with either a high (*ca.* 10^8 CFU ml^{-1}) or low (*ca.* 10^5 CFU ml^{-1}) load of a *lux*-marked strain of *E. coli* O157:H7, and subjected to four simulated heavy rainfall events. Greater densities of pathogenic and generic *E. coli* cells were recovered in the leachates from sheep faeces compared to cattle faeces. Pathogen activity (luminescence) was also greater in sheep faeces. The technique employed may be useful to depict realistic pathogen dispersal from animal waste for use in microcosm studies. In chapter 8 (*Article VI*), we inoculated microcosms of lake, puddle, river, and animal-drinking trough waters with *E. coli* O157:H7 and incubated them at 10°C. Cells were still detected in 45% of non-sterile samples after 2 months. Persistence of the organism was enhanced by water aeration and by prior sterilisation; however there was no correlation between water chemistry and mean *E. coli* O157:H7 die-off times/rates in any water type. Lastly, we applied slurry inoculated with *E. coli* O157:H7 to sands; which were then maintained in a dry state (non-tidal), or subjected to a repeated seawater tidal simulation (*Article VII*). Pathogen activity declined with increasing seawater concentration; however, cells remained viable in all sand and water treatments over the 5 d period. In all treatments, cell activity rapidly increased upon addition of available nutrients to the water. In summary, this work shows that *E. coli* O157 is a highly resilient bacterium, capable of persisting in a range of matrices under varied environmental stresses.

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Diolch yn fawr i chwi i gyd.

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CHAPTER 1: INTRODUCTION

1. Overview of thesis

1.1. General introduction and the need for research

This study was funded by a Biotechnology and Biological Sciences Research Council (BBSRC) Agri-Food competitive studentship grant. One particular aim of the Agri-Food programme is to fund research into the '*Control of Food-borne Pathogens*' and to develop "*fundamental scientific understanding of transmissible agents that contaminate food, to improve our understanding of the mechanisms of pathogenesis, organism survival during food processing, storage and distribution, and to investigate antibiotic resistance in food-borne pathogens and microbial inactivation*" (BBSRC 2006). The aim of the work undertaken in this thesis was to shed light on some of these issues with regards to one particular human pathogen, *Escherichia coli* O157.

In recent years, *E. coli* O157 has contributed to numerous disease outbreaks, causing loss of human lives and extensive economic repercussions. Despite efforts to improve hygiene across the whole spectrum of food preparation and processing practices, human *E. coli* O157 infections remain a serious problem, with numerous serious incidences arising in the UK over the last few years. *E. coli* O157 is an intestine-inhabiting bacterium, and is generally carried asymptomatically in animals. However, human infection may lead to a range of symptoms, including haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS), Thrombotic thrombocytopenic purpura (TTP), and occasionally death (Chart 2000).

Due to the seriousness of human *E. coli* O157 infection, scores of studies have investigated numerous aspects of the organism's life-cycle and behaviour. Consequently, we know substantially more about this bacterium now than when outbreaks of human infections first arose in the early 80's. Nevertheless, numbers of outbreaks show no obvious sign of decreasing (Health Protection Agency 2006) and the pathogen remains a serious concern.

Whilst the majority of human *E. coli* O157 infections are food-borne, contact with the organism in the environment is also a notable pathway of illness. The threat posed to human health from the presence of *E. coli* O157 in different surroundings is augmented due to its resilience to wide-ranging environmental conditions and its notably low human infectious dose (Jones 1999; Chart 2000). Most studies on *E. coli* O157 transmission to humans have revolved around the role of food as vectors of the bacterium. However, there is a clear need to elucidate the importance of environmental contact with the bacterium and its significance as a pathway for human infection. Notably, studies are needed that reveal

the persistence of *E. coli* O157 in contrasting environments which may serve as a pathway for implementing disease. Such work is of great importance in the formulation and implementation of strategies to reduce the threat posed by the bacterium, and to help us understand its methods of survival.

1.2. Plan of thesis

Hereon, the thesis is divided into nine chapters, starting with a review of existing research performed on *E. coli* O157. In particular, this chapter chronicles what is known about its epidemiology, its pathogenicity and pathway of infection, sources, survival in different environments and conditions, recovery methods, and finally, possible methods of controlling or intervening the bacterium.

The objectives of this research were decided with adherence to the funding body's outline theme and purpose of the grant (described above).The experimental work is presented as seven separate scientific papers; therefore some repetition of introductory material, methodology, and references occurs but is unavoidable when preparing a thesis of this type.

The first two experimental chapters detail the findings of studies that monitored the survival of *E. coli* O157:H7 on different surfaces. Chapter 3 describes an investigation where the persistence of the organism was monitored on surfaces found on a typical farm (wood and galvanised steel) under contrasting environmental conditions (different temperature and moisture regimes). Chapter 4 reports on an investigation where the effects of moisture, sawdust, and bleach on the persistence of *E. coli* O157:H7 on floor surfaces in meat-processing environments were monitored.

Chapters 5, 6, and 7 encompass the findings of studies that monitored *E. coli* O157:H7 in three potentially important reservoirs of the bacterium; namely compost, soil, and ruminant faeces. Chapter 5 details the findings of a collaborative study where the persistence and dispersal of *E. coli* O157:H7 were monitored in compost and soil subject to earthworm digestion. In chapter 6, the persistence of the pathogen in fallow, relative to rhizosphere soil is reported. Chapter 7 details the findings of an experiment where leaching of bioluminescent *E. coli* O157:H7 from sheep and cattle faeces was traced under heavy rainfall conditions. Bacteria numbers and activity were then quantified and compared to those of non-toxigenic *E. coli*.

For the last two experimental chapters, persistence of *E. coli* O157:H7 in different waters of importance in the environmental cycling of the organism are reported. In chapter 8, the observed survival patterns of *E. coli* O157:H7 in contrasting surface waters (lake, puddle, river, and animal drinking trough) is reported. The reasons for such survival trends are then explained in the context of water chemistry and quality indicators. The effect of water aeration on pathogen numbers is also reported in this chapter. Chapter 9 describes the results of an experiment designed to elucidate the survival and dissipation of *E. coli* O157:H7 in a marine environment (sand and seawater). Sands of different origins were gathered and inoculated with the bacterium to simulate contamination of a beach with runoff from agricultural land. Sands were then maintained in a dry (non-tidal) state or subjected to periodic wetting (tidal) cycles. The activity of bioluminescent *E. coli* O157:H7 in varying concentrations of sea and fresh water was also monitored, as well as the response of bacteria to nutrient addition in these waters.

Chapter 10 includes a general discussion of results from all previous experimental chapters. Conclusions are drawn and areas of further work identified. Lastly, appendices include brief photographic illustrations of all the experimental work performed.

1.3. References

BBSRC (Biotechnology and Biological Sciences Research Council). (2006) http://www.bbsrc.ac.uk/science/areas/af/themes/cfp.html (accessed on 28-06-2006).

Chart, H. (2000) VTEC enteropathogeicity. *Journal of Applied Microbiology Symposium Supplement* **88**, 12S-23S.

Health Protection Agency, 2006. Food poisoning numbers and vero cytotoxin-producing *E. coli* O157 isolations from humans examined by LEP England and Wales, 1992-2003 quarterly. Health Protection Agency, London, UK.

Jones, D.L. (1999) Potential health risks associated with the persistence of *Escherichia coli* O157 in agricultural environments. *Soil Use and Management* **15**, 76-83.

CHAPTER 2: LITERATURE REVIEW

Escherichia coli O157: genetic characteristics, its prevalence, persistence and detection in different environments, and potential methods for control

2.1. Introduction to food-borne diseases

In recent years, bacteria, viruses, and parasites that have emerged as food-borne pathogens have contributed to numerous disease outbreaks. Indeed, food-borne illnesses remain a common problem, with a noteworthy increase in incidences occurring over the last two decades (Fig. 2.1) (Jones 1999; Dundas and Todd 2000).

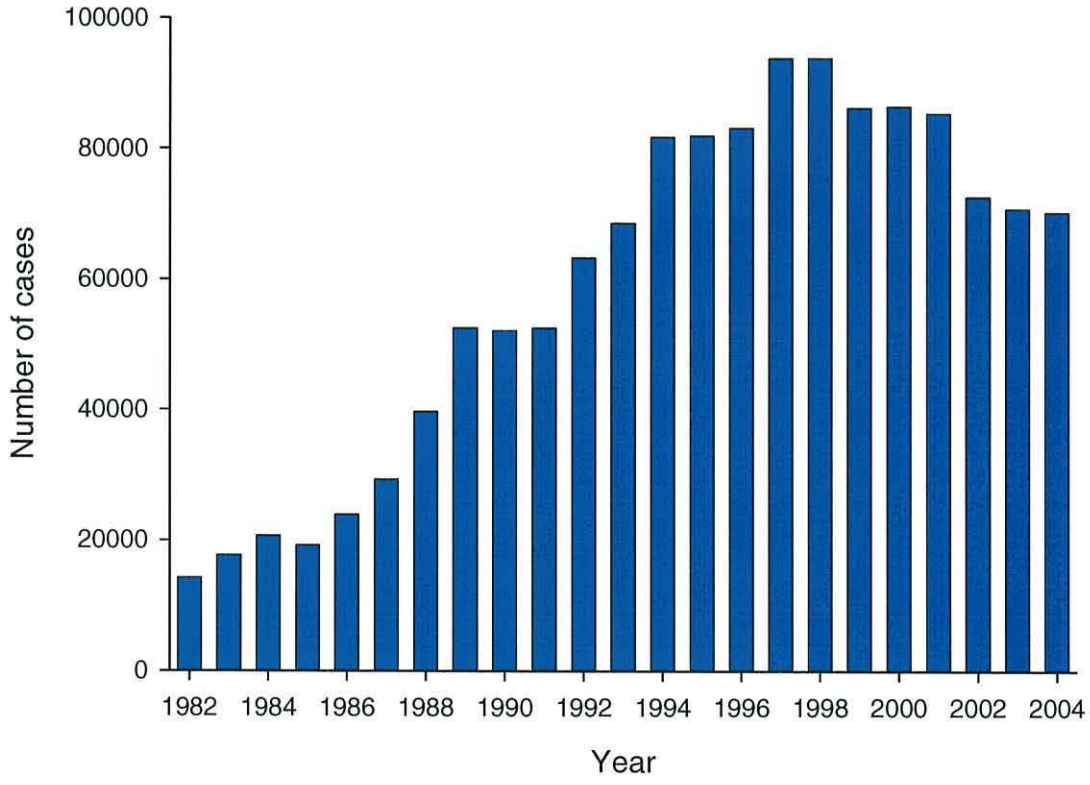

Figure 2.1. Numbers of food poisoning cases in England and Wales, 1982-2004 (Health Protection Agency 2006).

Globally, it is estimated that 76 million people annually fall ill due to food infection, with 325,000 people admitted to hospital, leading to many thousand deaths (CDC 2003). Nonetheless, it is evident that the actual number of food poisoning cases and cases misdiagnosed as other conditions would give a total far greater (by up to ten times) than the official reported number per year (Jones 1999; Dundas and Todd 2000; Parry and Palmer 2000).

The increase observed in the number of infections may be due to a range of factors, including:

- Emergence of new/new strains of food pathogens
- Genetic changes in micro-organisms resulting in increased virulence
- Increasing number of susceptible individuals i.e. those with depressed immunity (such as the growth seen in the elderly population)
- Increasingly global food market (variation in standards of food safety between countries could allow micro-organisms to spread quickly across the globe)
- Exposure to 'new' or 'different' pathogens from the greater range of foods available compared to previous years
- Changes in food manufacturing, retail, food distribution, and storage practices
- Intensification of agricultural systems
- Improved pathogen-detection methods
- Alternative cooking methods
- More dining out/takeaway meals
- Improved GP reporting and laboratory confirmation of cases

(Smith and Fratamico 1995; Cameron *et al.* 1997; Jones 1999; Chart 2000; Nicholson *et al.* 2000; Parry and Palmer 2000).

Health economists acknowledge the difficulty in estimating the full cost of food-borne disease (Cameron *et al.* 1997); but it is clear severe economic implications arise from food poisoning (Table 2.1) through the loss of working hours, reduced productivity, and health care costs.

Table 2.1. Estimates of the economic costs of human food-borne diseases in different countries (Sockett 1991; Cameron *et al.* 1997; Jones 1999).

Country	Estimated total annual cost
United States of America	Up to US$23 billion
Australia	$487 million - $1900 million
New Zealand	NZ$100 million
United Kingdom	> £1 billion

2.2. *Escherichia coli* and *Escherichia coli* O157

Phylogenetic trees formed following studies on the sequences of 16S rRNA and 5S rRNA suggest *Escherichia coli* diverged from an ancestor 120 to 160 million years ago (Park *et al.* 1999). Although DNA sequencing has shown that they still possess over 5,000 common genes (Anon 2001), it is thought that harmless and pathogenic strains of *E. coli* deviated

around 5 million years ago. The formulation of *E. coli* O157 as a pathogen probably occurred following the acquisition of virulence factors from other bacteria (Boerlin 1999; Vanselow *et al.* 2005). Generic *E. coli* is widespread throughout the animal kingdom, comprising 90% to 100% of all coliforms in faeces of eight domestic animal species, and approximately 97% of the total coliforms in human faeces (Health and Welfare, Canada 1992; Neill 2004). Over 250 different serotypes of *E. coli* have been isolated from man, with up to 100 associated with causing serious illness (Johnson *et al.* 1996; Lindqvist *et al.* 1998; Bettelheim 2000). Past cases of *E. coli* infections have been attributed to many different strains of the bacterium (Scotland *et al.* 1988; Pryor *et al.* 1990; Johnson *et al.* 1996; Tarr and Neill 1996). Indeed, an abundant number of pathogenic *E. coli* strains (6783) have been identified (National Institute of Health and Infectious Diseases Control Division, Japan 1996; Tosa and Hirata 1999). A paradigm of one pathogenic strain is *E. coli* O157. Although some have highlighted the dangers of solely focusing on only one *E. coli* serotype (Bettelheim 2000, 2001; Bettelheim and Beutin 2003), only strains of *E. coli* serotype O157 have been rigorously well characterised and studied. Because of the plethora of other *E. coli* strains, these have generally been poorly characterised and the incidence of virulence properties in these strains remains largely unknown (Law 2000). However, it is accepted that serotype O157 poses the greatest danger should infection occur, and is the serotype most prevalent in infection outbreaks (National Institute of Health and Infectious Diseases Control Division, Japan 1996; Bolton *et al.* 2000; Chart 2000; Law 2000; Rogerie *et al.* 2001; Vanselow *et al.* 2005).

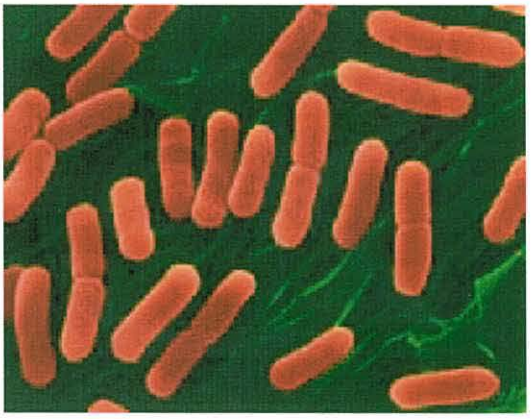

Figure 2.2. Rod-shaped *E. coli* O157 bacteria as viewed under an electron microscope (www.news.wisc.edu/ packages/biotech/5722.html).

The number of reported cases of *E. coli* O157 food poisoning is low in comparison with other pathogenic bacteria such as *Salmonella* spp. and *Campylobacter* spp. (Todd and Dundas 2001). However, the relentless rise seen in the number of cases in recent years (Fig. 2.3) and the severity of infection symptoms means it is of continuous concern (Parry and Palmer 2000; Health Protection Agency 2006).

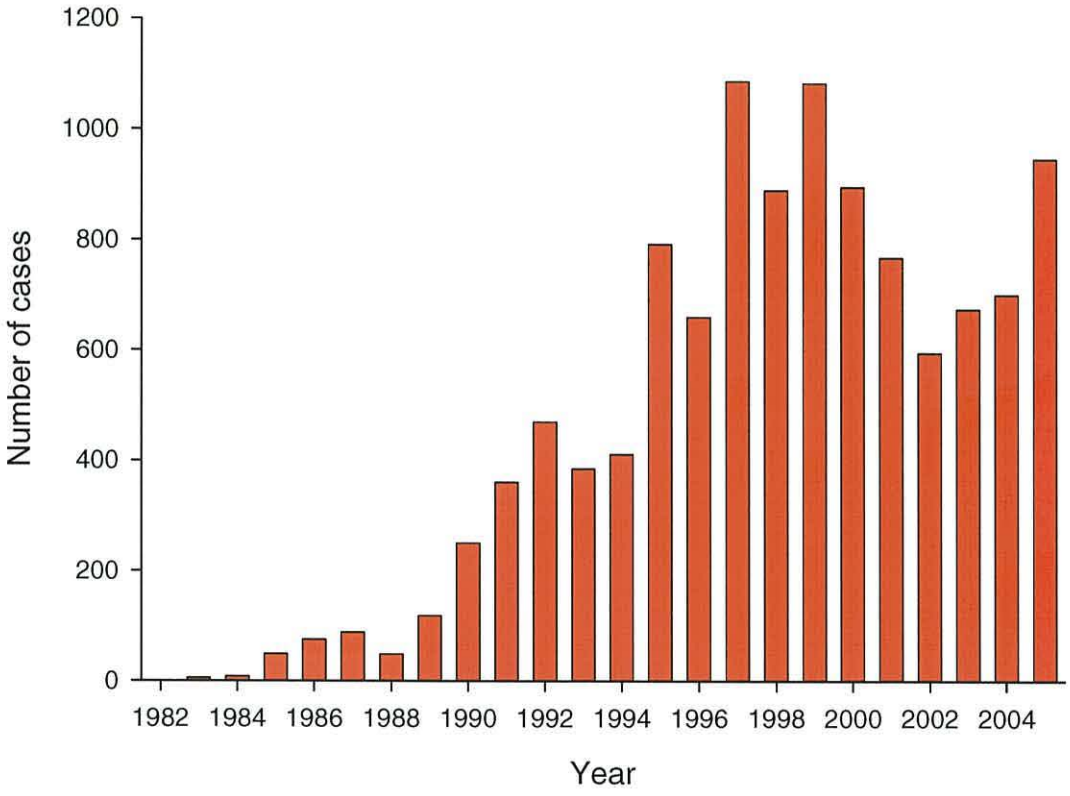

Figure 2.3. Numbers of *E. coli* O157 human infection cases in England and Wales, 1982-2005 (Health Protection Agency 2006).

A proportion of environmental isolates of this bacterium are actually classified as *E coli* O157:H7; so-named due to expression of the 157th somatic (O) antigen identified, and the 7th flagellar (H) antigen identified (Hancock *et al.* 1997b; Mead and Griffin 1998). For the majority of this review, the organism will be referred to as *E. coli* O157, comprising both +H and -H *E. coli* O157.

E. coli O157 was first recognised in 1977 in Canada (Chart 2000), but it wasn't until 1982 that its public health significance of was first acknowledged following a large infection outbreak at a fast-food restaurant in America (Riley *et al.* 1983). The largest ever

outbreak affected 9451 school children in Japan, causing 12 deaths (Michino *et al.* 1999; Terajima *et al.* 2000); whilst the most severe was in Lanarkshire, central Scotland, in 1996, which affected 503 people and resulted in 21 deaths (Pennington 2000; Cowden *et al.* 2001). Indeed, serious outbreaks of *E. coli* O157 infection have occurred in many countries around the world (Doyle 1991).

2.3. The pathogenicity of *E. coli* O157

2.3.1. Mode of gut adhesion and cell disruption

The pathogenicity of *E. coli* O157 is attributed to many factors. *E. coli* live in the intestines of humans and animals, and are excreted in faeces (Jones 1999; Chart 2000; Dundas and Todd 2000; Nicholson *et al.* 2000). Serotype O157 behaves differently to other serotypes, producing 'fimbrae' that facilitate attachment to the intestinal wall. In contrast to harmless strains of *E. coli*, serotype O157 possesses eight fimbrial gene sets, the structure of which resembles those found on another widespread instigator of food poisoning, *Salmonella* (Anon 2001). In humans, orally ingested *E. coli* O157 pass through the acid environment of the stomach and colonise the intestine by adhering to the intestinal wall, principally to the follicle-associated epithelium (FAE) in the distal small intestine, and the colon (Moxley 2004; Vanselow *et al.* 2005).

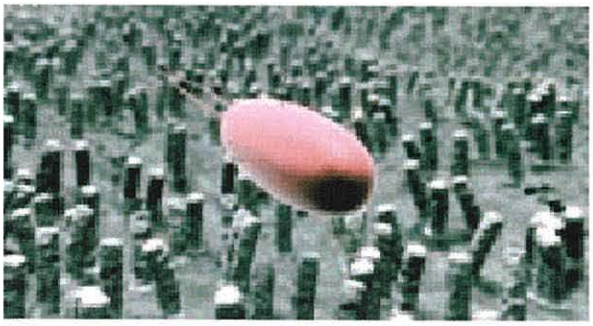

Figure 2.4. *E. coli* O157:H7 responds to host environmental signals in the intestine and expresses adhesins on its outer membrane (Quantrell *et al.* 2004).

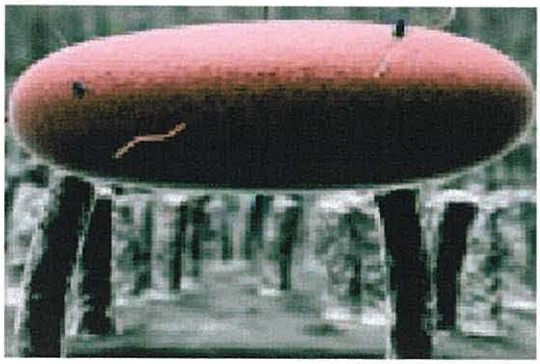

Figure 2.5. *E. coli* O157 binds to microvilli on the host-cell surface (Quantrell *et al.* 2004). Once bound, protein secretion commences.

Proteins secreted by the type III secretion apparatus unique to pathogenic bacteria play a fundamental role in colonisation of the intestine by *E. coli* O157 (DeVinney *et al.* 1999; Potter *et al.* 2003). One such protein is intimin, a 94-97 kDa integral outer membrane bacterial protein needed for adherence to host cells (Park *et al.* 1999). The receptor for intimin on the host cell is a 90 kDa bacterial protein called Tir (Translocated intimin receptor) (Chart 2000). Together, both proteins are fundamental in determining the virulence of *E. coli* O157. Secreted proteins must pass through the inner membrane surrounding the cytoplasm, and the outer membrane enclosing the periplasm. Tir is transported directly from the cytoplasm to the host cell surface from the bacterial cytoplasm, while the general secretory pathway transports proteins to the periplasm (Park *et al.* 1999). Bacterial motility is driven by the presence of flagellae that may enhance penetration of host defences, including mucus, thereby aiding initial attachment to host tissue (Quantrell *et al.* 2004). EspA and EspB (filamentous proteins) are secreted, forming further linkage between the bacterium-host cells and creating pores in host cells, respectively (Li *et al.* 2000). Once integrated into the host cell membrane, it mediates bacteria attachment to gut epithelial cells by serving as the intimin receptor; forming the Tir-intimin binding system (Chart 2000; Law 2000; Potter *et al.* 2003) (Fig. 2.6, 2.7).

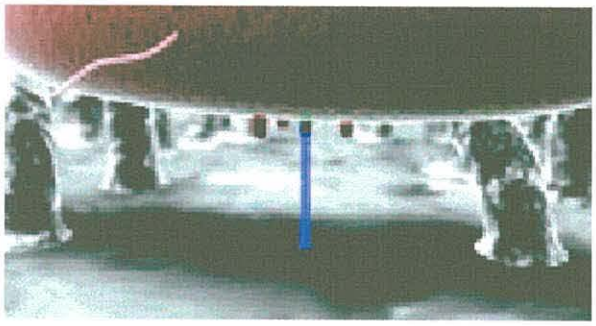

Figure 2.6. *E. coli* O157 produces a long EspA filament that allows the injection of the translocated intimin receptor (Tir) into the host cell where it inserts into the membrane. The pathogen then binds tightly to the host cell via the Tir-intimin interaction (Quantrell *et al.* 2004).

In this form, Tir acts as a focus for actin accumulation, and transmits signals to the host cell. This causes a variety of physiological changes, including calcium release from intracellular stores, activation of numerous enzymes (phospholipase C, protein kinase C, and myosin light chain kinase), and changes in membrane potential (Frankel *et al.* 1998; Law 2000), causing widespread changes in cytoskeletal structures and ion fluxes (Law 2000).

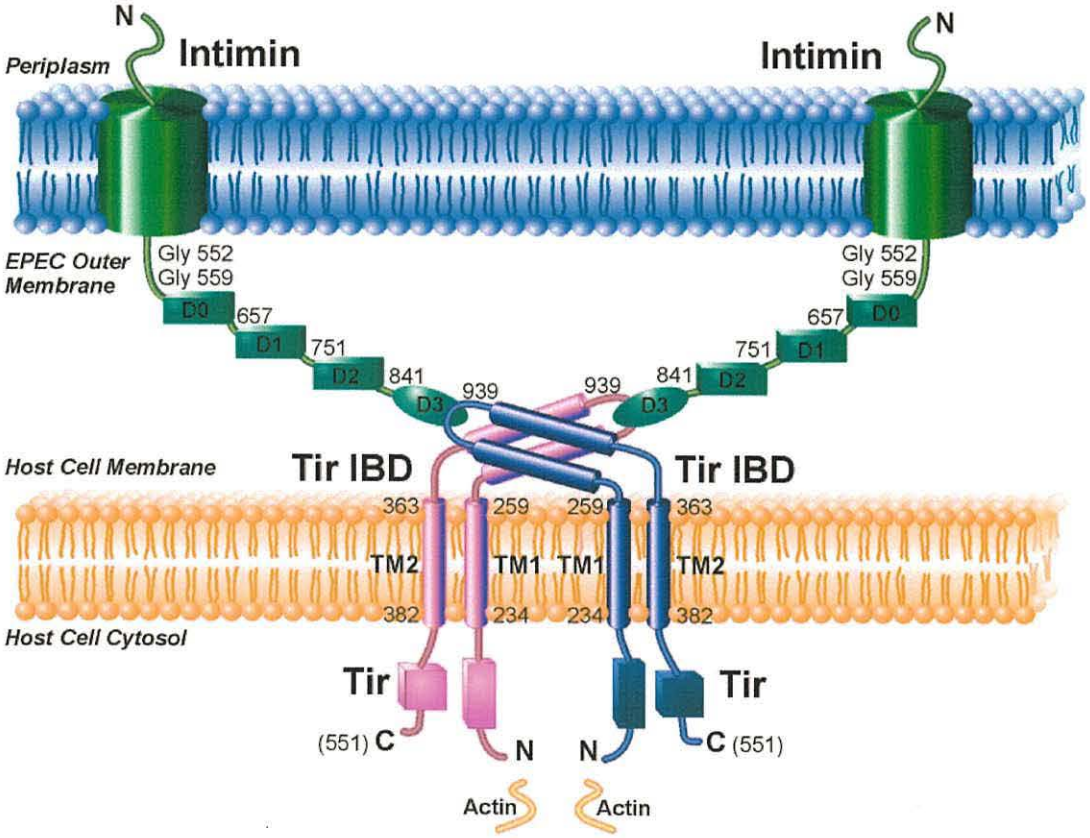

Figure 2.7. Tir-Intimin binding mediating bacteria attachment to gut epithelial cells (http://byron.biochem.ubc.ca/research.adhesion.htm).

Such bacterial adherence to epithelial cells allows progression of infection (Dean-Nystrom *et al.* 1998; DeVinney *et al.* 1999; Potter *et al.* 2003); and is the first example of a bacterial pathogen inserting its own receptor into mammalian cell surfaces to which it then adheres (Finlay and Haynes, undated). Following adhesion, cytoskeletal disruption occurs, leading to 'attaching and effacing' (A/E) lesions (Park *et al.* 1999; Law 2000) and pedestals (protrusions beneath adherent bacteria) in ileal tissue (Dean-Nystrom *et al.* 1998).

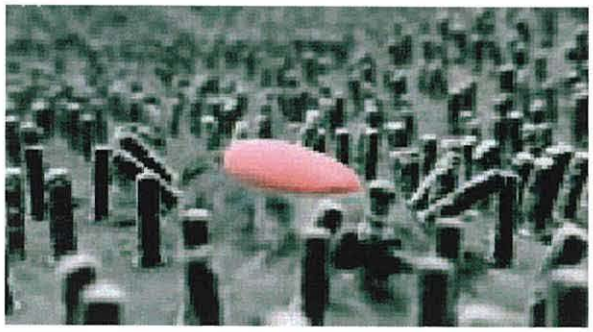

Figure 2.8. Host-cell cytoskeletal rearrangements result in *E. coli* O157 embedding in the host-cell membrane as part of the formation of an A/E lesion (Quantrell *et al.* 2004).

Attached bacteria may inject a variety of proteins into the cells, including virulent haemolysin (Potter *et al.* 2003; Mainil and Daube 2005). The genes of haemolysin and intimin synthesis are both encoded; the former on the *ehx* gene located on a large plasmid (90 kb), and the latter by the *eae* gene located on a 35 kb segment of DNA termed the locus of enterocyte effacement (LEE) region (Nataro and Kaper 1998; Park *et al.* 1999). Two types of haemolysin (α- and β-haemolysin) have been associated with *E. coli* O157 (Chart 2000; Law 2000). Research has shown that production of these (thus the virulence) is iron-dependent, depending on the genetic make-up of the *E. coli* strain (Chart *et al.* 1998; Chart 2000). On lysis, *E. coli* O157 may release lipopolysaccharide complexes (endotoxins) from their cell walls. These cause fluid secretion from the intestine, resulting in diarrhoea and salt loss from the body (Park *et al.* 1999).

2.3.2. Shigella*-type toxin production*

Early work on *E. coli* O157 genetics proved it carried two different shiga-toxin genes (*stx* 1 and *stx* 2) that were homologous to the ones borne by *Shigella dysenteriae* (Nataro and

Kaper 1998; Mainil and Daube 2005). Indeed, shiga-toxin 1 from *E. coli* O157 differs in sequence from shiga-toxin produced by *Shigella dysenteriae* type 1 by only one amino acid and is antigenically indistinguishable (Vanselow *et al.* 2005). Both toxins are composed of five (7.5 kDa) B subunits which binds to the specific-cell receptor, and a single (33 kDa), biologically active A subunit; and are encoded on a temperate lambdoid bacteriophage inserted into the *E coli* O157 chromosome (Law 2000; Rogerie *et al.* 2001; Mainil and Daube 2005). The B subunit in shiga toxins target and bind to tissues expressing the appropriate glycolipid, globotriaosylceramide (Gb3); - a receptor found to varying degrees within membranes of eukaryotic cells (Mead and Griffin 1998). Vascular endothelial cells of human kidney glomerulus and brain are considered major targets (Zoja *et al.* 2002; Vanselow *et al.* 2005). The ability to produce shiga toxins means serotype O157 is grouped as STEC (shiga-toxin-producing) *E. coli*. These toxins also affect Vero cells, hence *E. coli* O157 is also known as VTEC *E. coli* (verocytotoxigenic-producing *E. coli*) (Bettelheim and Beutin 2003). Whilst *stx* 1 is more lethal to Vero cells, *stx* 2 is much more lethal than *stx* 1 to human renal microvascular endothelia cells and has been shown to be most virulent of both toxins (Park *et al.* 1999; Vanselow *et al.* 2005). Incidentally, it is the toxin produced by most O157 strains (Law 2000). It is thought that the toxin-producing ability of *E. coli* O157 evolved from the enteropathogenic *E. coli* (EPEC) strain, O55:H7 (Feng *et al.* 1998). Although indubitably a crucial factor, the ability of an organism for toxin production alone is insufficient for that organism to cause disease. Rather, the pathogenicity of an organism relies upon other complementary virulence factors afore mentioned working in conjunction with toxins to cause cell disruption (Tarr and Neill 1996; Law 2000). Nonetheless, production of one or more shiga-like toxins by *E. coli* O157 in humans causes damage to the bowel mucosa and underlying blood vessels; leading to bleeding in the bowel [haemorrhagic colitis (HC)], in addition to lysis of red blood cells (Law 2000). This renders haemoglobin freely available, which may stimulate the growth of *E. coli* O157 thus enhance toxin production (Law 2000). Life-threatening kidney failure of the form haemolytic uraemic syndrome (HUS) may develop if toxins migrate from the gut to the blood (Dundas and Todd 2000; Law 2000; Russell *et al.* 2000). As *E. coli* O157 infection may result in HC or HUS, the serotype is also classified as enterohaemorrhagic *E. coli* (EHEC; Mainil and Daube 2005). Indeed, *E. coli* serotype O157 is responsible for 85-95% of North American cases of HUS (Armstrong *et al.* 1996). Ironically, having entered the blood stream, the

detrimental effects of these toxins are amplified by the body's immune system, with white blood cells transferring them to the kidney for disposal. The shiga toxins bind to human polymorphonuclear leucocytes in circulation, and are carried to target tissues where the toxin can transfer to microvascular endothelial cells (Vanselow *et al.* 2005). Furthermore, after endocytosis in targeted host cells, the toxin is transported to the endoplasmic reticulum, nuclear membrane, and nucleus; where the A subunit in shiga toxins may also enzymatically inactivate and catalytically cleave the 60*S* and 28*S* ribosomal subunits, effectively shutting down protein synthesis thus initiating cell death (Mead and Griffin 1998; Anon 2001; Vanselow *et al.* 2005). Moxley (2004) reported how the H7 flagellum induces production of chemokines such as interleukin 8, and neutrophilic infiltration of the intestinal mucosa, which in turn may enhance shiga toxin uptake across the intestinal epithelium. Further possible effects of toxin production include inhibition of pro-inflammatory responses, and apoptosis (Quantrell *et al.* 2004).

2.3.3. Symptoms and consequences of infection

The infectious dose of *E. coli* O157 is low, with ingestion of as few as 10 cells possibly sufficient to cause infection (Willshaw *et al.* 1994; Bolton *et al.* 1996; Chart 2000). Following ingestion, the incubation period may be as short as three hours (Fortin *et al.* 2001), or as long as five (Chart 2000) or nine (Doyle 1991) days. Typically, there is little or no fever (Doyle 1991); and even though human *E. coli* O157 infections may be asymptomatic (Griffin *et al.* 1988) and most patients fully recover after > 10 days, infection can result in a wide spectrum of clinical symptoms (Fig. 2.9).

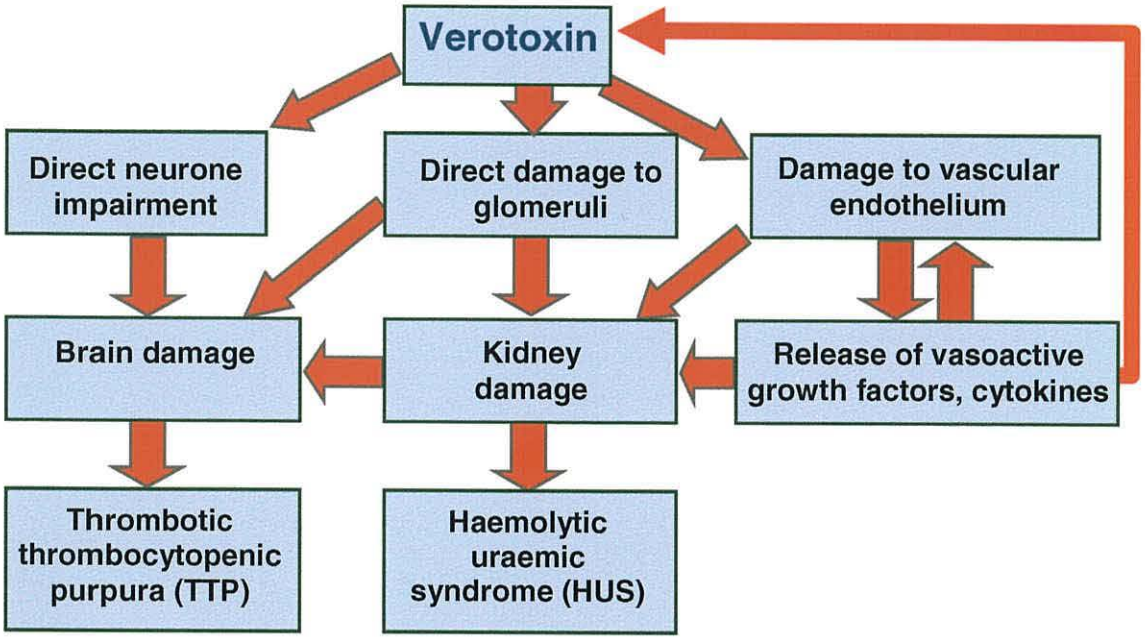

Figure 2.9. The role of verotoxins in disease caused by *E. coli* O157 (Jones 1999).

The sudden onset of severe abdominal pain and cramps signals the initial stages of HC, followed within 24 hours by watery diarrhoea which later becomes severely bloody (all blood and no stool) (Doyle 1991). HUS occurs in 2-15% of human infection cases (Fig. 2.10), consisting of haemolytic anaemia and acute renal failure; with fatality in roughly 3-17% of these patients (Bolton *et al.* 2000; Chart 2000; Todd and Dundas 2001); although it has been reported as high as 87% in the elderly (Carter *et al.* 1987).

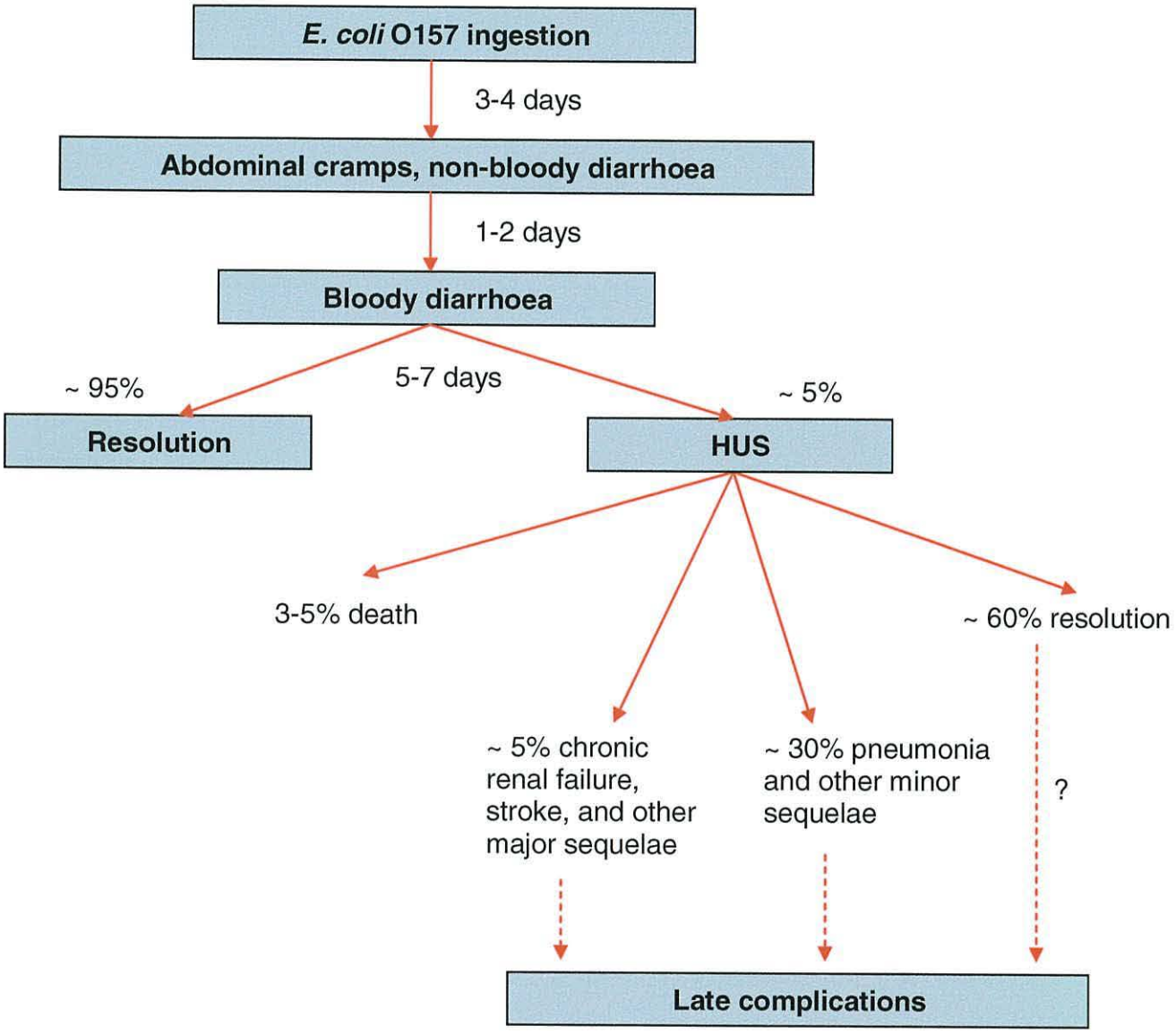

Figure 2.10. The common pathway of symptoms following *E. coli* O157 infection (adapted from Mead and Griffin 1998).

The percentage of patients that develop the various symptoms varies with each outbreak as it is a function the patients ages etc.; however, observations from outbreaks has allowed for documentation of the clinical features of this condition (Fig. 2.10; Table 2.2).

Table 2.2. Clinical features of *E. coli* O157:H7 infection (Todd and Dundas 2001).

Clinical feature	Percentage incidence (%)
Vomiting	30-60
Fever	< 30
Abdominal pain	70-80
Diarrhoea	95
Bloody diarrhoea	> 70
Complication rate (HUS/TTP)	2-15 in sporadic, 20 in some outbreaks
Asymptomatic infection	10-15

Production of bloody diarrhoea typically signifies the inception of HUS (Doyle 1991). Patients generally have a combination of complications, including microangiopathic haemolytic anaemia (intravascular coagulation of erythrocytes resulting in mechanical damage of erythrocytes as they squeeze through abnormally narrow channels), thrombocytopenia (low circulating platelets), and acute nephropathy (kidney disorder) (Doyle 1991). As a result, individuals who develop HUS may require dialysis and blood transfusions; and may develop central nervous system (CNS) damage characterised by recurrent seizures and coma. Whereas HUS mainly occurs in children, Thrombotic thrombocytopenic purpura (TTP) principally occurs in adult cases. Patients with TTP exhibit clinical and pathologic characteristics similar to HUS, with possibly severe implications on the CNS; in addition to brain blood-clot formation which often results in death (Park *et al.* 1999; Bolton *et al.* 2000). Some may exhibit neurological symptoms and can suffer recurrent strokes (Vanselow *et al.* 2005). Many patients who regain renal function suffer chronic proteinuria, and end-stage renal disease may develop years or even decades later. Other long-term sequelae include cholelithiasis, colonic stricture, chronic pancreatitis, glucose intolerance, and cognitive impairment (Mead and Griffin 1998).

2.3.4. Individuals most at risk

Some reports claim that individuals frequently in contact with farm animals are at increased risk of *E. coli* O157 infection (Parry *et al.* 1998), although contradictory evidence exists. For instance, Johnson *et al.* (1999) and Silvestro *et al.* (2004) concluded that stool samples from individuals often exposed to *E. coli* O157 (farmers and their families, abattoir workers) yielded more positive results for the bacterium. However, infection was more likely to be asymptomatic in these individuals as they possessed high levels of antibodies to verotoxins,

and hence held a greater degree of immunity to the pathogen (Johnson *et al.* 1999). Olsen *et al.* (2002) report of a large outbreak of human *E. coli* O157:H7 infection following drinking water from a (contaminated) rural system. In this instance, fewer of the resident population acquired infection relative to visitors. Acquisition of partial immunity may emerge in those subjected to recurrent exposure to the pathogen (e.g. farm and abattoir workers), possibly by exposure to the more common and less virulent non-O157 (Olsen *et al.* 2002; Silvestro *et al.* 2004). Consequently, persons (especially children) of urban background may be of greater vulnerability to infection following exposure to the pathogen following farm or countryside visits. Although some reports state females are more likely to develop clinical symptoms arising from *E. coli* O157 ingestion (Mead and Griffin 1998), recent data (Smith and Salmon 2003) does not support this (Table 2.3). It appears that immuno-suppressed individuals (children, the elderly, and pregnant women) tend to be at greatest risk from *E. coli* O157 infection (Cameron *et al.* 1997; Jones 1999), with the highest UK incidences (Table 2.3) occurring in young children (Parry and Palmer 2000; Smith and Salmon 2003).

Table 2.3. Incidence of VTEC O157 infections in Wales by age group and sex, 1990-2002 (adapted from Smith and Salmon 2003).

Age group (years)	Males (per 100,000 population)	Females (per 100,000 population)	Total (per 100,000 population)
< 1	11.4	9.0	10.2
1-4	10.7	6.9	8.8
5-14	2.0	1.5	1.8
15-24	1.2	1.7	1.5
25-34	1.1	1.6	1.4
35-44	0.9	0.8	0.9
45-54	0.5	1.2	0.9
55-64	0.6	1.2	0.9
65+	0.7	0.9	0.8
Total	**3.2**	**2.8**	**3.0**

Globally, extremely serious infection outbreaks have occurred in young children (Terajima *et al.* 2000) and the elderly (Carter *et al.* 1987; Cowden *et al.* 2001). To date, no current therapies for EHEC human infections exist other than general supportive measures and specific treatments for consequences of infection e.g. dialysis (Besser *et al.* 1999). However, the use of therapeutic plasma exchange (Dundas *et al.* 1999) and synthetic toxin receptor blockade (Takeda *et al.* 1999) as acute disease modifying interventions may prevent the

development of HUS; although they rely upon early recognition of infection and careful monitoring for any indicators of complication development (Todd and Dundas 2001). Other 'markers' may be used as indicators of HUS development (Table 2.4).

Table 2.4. Clinical markers which may be used to monitor HUS development in *E. coli* O157 -infected patients (Todd and Dundas 2001).

Clinical markers
Falling haemoglobin
Rising lactate dehydrogenase (LDH)
Falling platelet count
Rising urea/creatinine

The mainstay of therapy includes correcting and maintaining fluid and electrolyte balance, along with monitoring patients for the development of HUS and other serious consequences (Mead and Griffin 1998; Bolton *et al.* 2000). Monitoring white cell counts may prove relatively effective in predicting HUS development in patients, with those of numbers greater than 20 $\times 10^9 l^{-1}$ blood and an absolute neutrophil count greater than 15 $\times 10^9 l^{-1}$ blood being at particular risk (Todd and Dundas 2001). Even though *in vitro* experimentation have found *E. coli* O157 isolates to be susceptible to some prescribed antimicrobials, no convincing data currently indicate that antimicrobial agents alter the pathway of *E. coli* O157 infection or the duration of faecal excretion of the organism (Riley *et al.* 1983; Griffin *et al.* 1988; Proulx *et al.* 1992). Furthermore, selection pressure imposed by the use of some antimicrobials, whether therapeutically in human and veterinary medicine or as prophylaxis in animal production, may be a key driving force in the selection of antimicrobial resistance in *E. coli* O157 (Schroeder *et al.* 2002). However, some argue this may owe to substandard experimental procedures/design and the use of wrong drugs (Bolton *et al.* 2000). Nevertheless, results of studies of large outbreaks of VTEC O157 infection in Japan have suggested that two drugs, fosfomycin and fluoroquinolones, may reduce the duration and severity of disease and the risk of progression to HUS or TTP (Takeda *et al.* 1998; Bolton *et al.* 2000; Panos *et al.* 2006). Yet, antibiotic treatment of children with *E. coli* O157 infection may increase the risk of progressing VTEC diarrhoea to HUS (Wong *et al.* 2000; Todd and Dundas 2001). Also of concern is that sub-inhibitory concentrations of some antimicrobial agents *in vitro* may cause lysis or sub-lethal damage to VTEC cells with subsequent liberation of verocytotoxins (Walterspiel *et al.* 1992; Bolton *et al.* 2000). Oral preparations

of synthetic verocytotoxin receptors linked to silicon dioxide (Synsorb-Pk) which can bind to verocytotoxin in faeces may present a method of reducing faecal shedding in infected patients, although this warrants considerably greater investigation (Armstrong *et al.* 1998). Meanwhile, the role of conventional therapies and experimental approaches in established HUS has also been considered (Neild 1994; Bolton *et al.* 2000; Panos *et al.* 2006). Although treatment of patients is further complicated by the diverse symptoms and conditions which may arise, development of effective antimicrobial agents in treating and/or preventing patients with VTEC infections may provide some hope for limiting the impact of future outbreaks.

2.4. Limitations for research

Although numerous studies in identifying the factors and mode of *E. coli* O157 pathogenesis have been completed, several obstacles limit research. Research into the epidemiological characteristics of *E. coli* O157 may have been limited by the use of artificial inocula. Under these conditions, the pathogen may become lab-adapted and therefore not accurately mirror natural *E. coli* O157 characteristics (Jones 1999). Care should therefore be taken in extrapolating many experimental results to 'field' conditions. As *E. coli* O157 infection seldom affects animals, research into the pathogenesis of the bacteria on humans using an animal-based model is rendered unfeasible (Law 2000). This particularly limits studying the pathways of serious illnesses arising from infection (principally HC, HUS, and TTP), due to the high risk of potentially lethal complications following *E. coli* O157 infections using human-volunteer studies. Research on the virulence of the pathogen must therefore predominantly occur during, and following, natural infection outbreaks (Law 2000). Research is further restricted as the bacterium is a CAT III organism (ACDP) and hence requires specialised and expensive laboratory apparatus. However, it appears that this issue may be counteracted via employment of non-toxigenic *E. coli* O157 (absence of toxin activity and toxin genes) for lab-research. Non-toxigenic *E. coli* O157 are classified as CAT II (ACDP) organisms hence are not controlled by such rigid rules and regulations. Furthermore, such strains have been shown to possess very similar or identical survival patterns to toxigenic (wild-type) strains (Kudva *et al.* 1998; Campbell *et al.* 2001; Ritchie *et al.* 2003). This may prove especially valuable as laboratory-acquired *E. coli* O157 infections have occurred on some occasions (Coia 1998; Bolton *et al.* 2000). As the

virulence of the pathogen is multi-factorial and the relative contribution of the various pathogenic components is unknown, the effects of certain virulence properties may vary significantly with different cases and host factors (Law 2000). This makes accurate predictions of how future outbreaks may affect populations difficult.

As *E. coli* seems to possess intrinsically high rates of DNA repair mechanisms, this renders *E coli* O157 particularly adept at incorporating foreign DNA (LeClerc *et al.* 1996; Mead and Griffin 1998). Indeed, the O157 antigen is believed to have been acquired through horizontal transfer and recombination (Mead and Griffin 1998). Furthermore, some strains of *E. coli* O157 have high rates of spontaneous mutation, with more than 1% of O157:H7 strains having rates 1000-fold higher than those of typical *E. coli* (Vanselow *et al.* 2005). Akabi *et al.* (2002) reported on the ability of some *E. coli* O157:H7 strains to mutate in cattle. This may complicate research and enhance the threat posed by *E. coli* O157 as new (unknown) genotypes may possess different or enhanced virulence. Furthermore, the possibility of acquisition of certain genes into *E. coli* O157 from other pathogens (e.g. *Campylobacter* spp.) by horizontal gene transfer may impact on the emergence of new epidemic strains.

2.5. Sources of *E. coli* O157

2.5.1. Animal manure

As generic and serotype O157 *E. coli* are harmless to the majority of animals (Karmali 1989; Chapman *et al.* 1993a; Zhao *et al.* 1995; Kudva *et al.* 1998; Fukushima *et al.* 1999), and are sporadically harboured and excreted by healthy animals (Synge 2000; Omisakin *et al.* 2003), they are classified as zoonotics (Patriquin 2000). The difference in infectivity between humans and cattle primarily lies with the differing receptor distribution for the verocytotoxin, but is also likely to reflect their differences in colonisation patterns, gene expression, and signalling pathways (Quantrell *et al.* 2004). Furthermore, infection is asymptomatic in cattle due to their lack of vascular receptors, therefore systemic disease such as HUS may not develop; hence cattle become tolerant reservoirs of *E. coli* O157:H7 (Pruimboom-Brees *et al.* 2000). However, some evidence suggests that colonisation of the gut by some *E. coli* O157 strains with specific adhesins may affect young lambs, calves, or piglets (Synge 2000), with the associated A/E lesions causing elevated scouring or disease in some animals (Aktan *et al.* 2004). VTEC *E. coli* infection may also cause mastitis

(Thomas *et al.* 1983), tubeculous lesions (Park *et al.* 1999; Matlova *et al.* 2004), and bowel oedema in different animal species (Synge 2000; Mainil and Daube 2005). A comprehensive veterinary view of VTEC *E. coli* effects on animals can be seen in Synge (2000).

Cattle are considered to be the main environmental reservoir for *E. coli* O157, and a great deal of work has been done on measuring the prevalence rates within these animals. Although prevalence values between 0.1% and 62% have been reported (Duffy 2003), it is accepted that approximately 20% shed the bacterium in their faeces (Elder *et al.* 2000; Nicholson *et al.* 2000; Potter *et al.* 2003). Alarmingly, the prevalence of the pathogen within UK cattle herds may be as high as 63-75% (Hancock *et al.* 1997ab). In cattle, the primary colonisation site appears to be in the large intestine, especially in the follicle-associated epithelium mucosal of the terminal rectum in a region within 5 cm to the recto-anal junction (Rice *et al.* 2003b; Moxley 2004). In cattle, *stx* may suppress mucosal immunity, yet enhance other effects that promote intestinal colonisation (Moxley 2004). The distribution of *E. coli* O157 was surveyed in the digestive tract of cattle by Laven *et al.* (2003). Samples taken from the dorsal and ventral rumen wall and rumen contents, the colon wall and colon contents, and from faeces or caudal rectal contents showed *E. coli* O157 was detected more frequently in the colon than in the rumen, but the majority of detections (87.5%) were in samples of rumen wall. However, Grauke *et al.* (2002) found that the predominant location of *E. coli* O157:H7 persistence in cattle was the lower gastrointestinal tract; with only rare culturing from the rumen or duodenum after the first week post-inoculation; - suggesting the colon as the site for *E. coli* O157:H7 persistence and proliferation in mature ruminant animals. Others have stated the bacterium's tropism for the bovine anal tonsils (Wallis 2004). In calves, the rumen appears to be the primary site for *E. coli* O157; however, young calves are physiologically different from fully functional adult ruminants and have not acquired the full assortment of ruminal micro-organisms (Brown *et al.* 1997). In artificially-inoculated sheep, Grauke *et al.* (2002) found *E. coli* O157:H7 most prevalent in the lower gastrointestinal tract digesta, specifically the cecum, colon, and faeces.

The number of human *E. coli* O157 infection cases show seasonality, with incidences peaking towards mid-to late summer (Fig. 2.11).

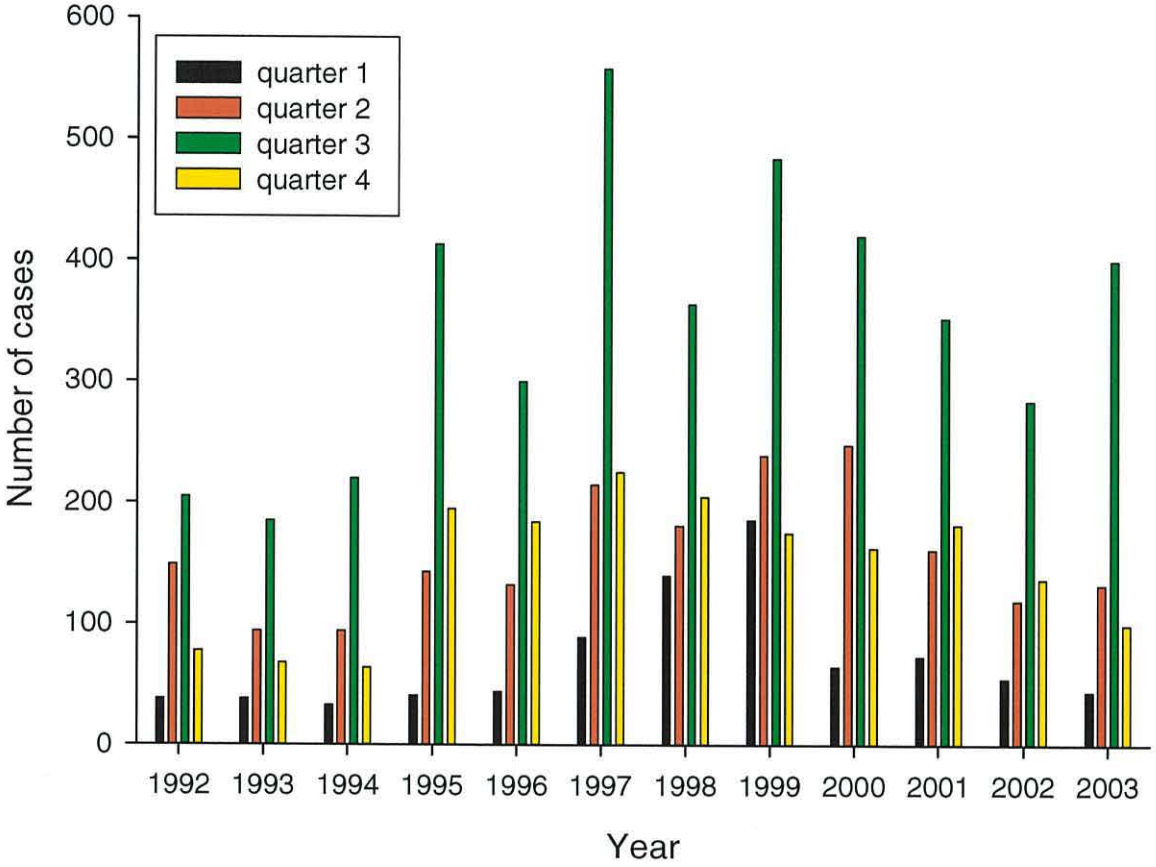

Figure 2.11. Quarterly-incidence rate (quarter 1: January-March; quarter 2: April-June; quarter 3: July-September; quarter 4: October-December) of human *E. coli* O157 infections in England and Wales, 1992-2003 (Health Protection Agency 2006).

This annual pattern may owe to a number of reasons. Higher temperatures in summer may enhance bacteria growth in foodstuffs (Bolton *et al.* 2000; Parry and Palmer 2000), or it may correlate with the barbecue season, where consumption of undercooked meat and contaminated raw salad vegetables is prominent (Chapman 1995). Cattle shedding rates also peak in spring and late summer (Chapman *et al.* 1997a; Mechie *et al.* 1997; Laven *et al.* 2003; Ogden *et al.* 2004); which might reflect changes in diet, or stress levels (from calving, or movement to and from housing). It has been proposed that the increase in human *E. coli* O157 infection cases may correlate (with a slight lag) to the peaks in shedding within animal reserves (Jones 1999; Ogden *et al.* 2004) (Fig. 2.12).

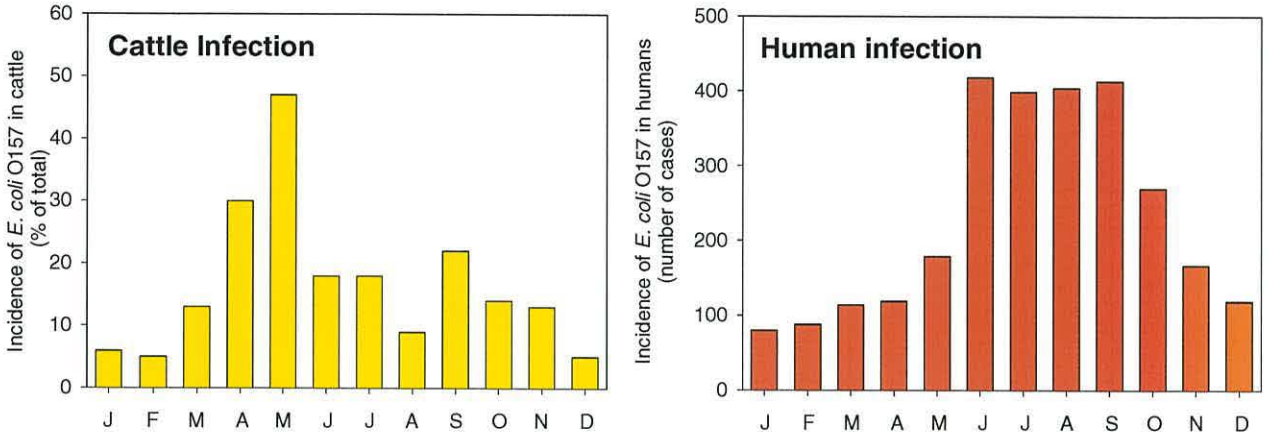

Figure 2.12. Seasonality incidences of human and cattle *E. coli* O157 infection rates (Jones 1999).

Inevitably, many papers report incidences of human *E. coli* O157 infection arising from contact with cattle and their faeces (Chapman *et al.* 1989, 1993a; Cieslak *et al.* 1993; Trevena *et al.* 1996; Nielsen and Scheutz 2002).

Although a small number of animals in a herd may initially excrete the bacterium, it may be rapidly spread to other animals within the herd leading to epidemic transmission and excretion (Besser *et al.* 2001; McGee *et al.* 2004). Buchko *et al.* (2000) fed cattle grain inoculated with a particular strain of *E. coli* O157, and found the strain could be detected in animal drinking water samples, water trough biofilm swabs, feed samples, and mouth swabs of the cattle for the ten-week experimental period. Similarly, McGee *et al.* (2004) surveyed cattle housed in pens where one animal was inoculated with (and was proved to be shedding) a marked strain of *E. coli* O157. Faecal (rectal swabs) and hide samples, water, feed, and gate or partition samples from each pen were examined for the strain, and within 24 h of introducing the inoculated animals into the pens, samples collected from the drinking water, pen barriers, and animal hides were positive for the pathogen. Within 48 h, the hides of 66% of cohort animals from the six pens were contaminated with the marker strain of *E. coli* O157. Three days after the introduction of the inoculated steers, faecal samples from the non-inoculated cohort animals detected positive for the pathogen. The high prevalence of the bacterium in cattle feedlot pens and water tanks observed by Sargeant *et al.* (2003, 2004) suggests such widespread contamination may affect huge numbers of cattle. Furthermore, spreading of the organism between animals has been found to be significantly greater and

prolonged when animals are housed indoors rather than kept on pasture (Jonsson *et al.* 2001), and the problem is exacerbated as only a small number of cells (< 260 CFU) may be sufficient to infect animals (Besser *et al.* 2001). Faeces from *E. coli* O157-shedding animals on pen floors present a significant source of infection to other cattle, more so than contaminated feed or drinking water (Bach *et al.* 2005), highlighting the importance of regular muck-clearing. Periodic excretion events tend to last 2 to 4 months (Heuvelink *et al.* 1998b; Jones 1999), although strains may persist in a herd for periods lasting over two years (Shere *et al.* 1998). In most animals, faecal shedding occurs in sharp bursts, followed by long periods of low prevalence (Hancock *et al.* 2001). As mature cattle may produce up to 40 kg day^{-1} of faeces (Hoar *et al.* 2001; DEFRA 2005), and that *E. coli* O157:H7 excretion levels in infected cattle typically range between 10^2-10^5 CFU g^{-1} faeces (Omisakin *et al.* 2003; Ogden *et al.* 2004), cattle present a considerable source of pathogen loading into the environment. Furthermore, some have recovered 10^7 CFU *E. coli* O157:H7 g^{-1} faeces in cattle (Fukushima and Seki 2004). Young calves tend to shed higher (10^7 CFU g^{-1}) levels of the bacterium than older animals, and are particularly susceptible to infection, especially post-weaning (Synge 2000; Besser *et al.* 2001; Rugbjerg *et al.* 2003). It is unclear whether the prevalence or shedding rates are affected by the sex of cattle, although some studies indicate a higher prevalence within males at some age groups (Nielsen *et al.* 2002). The interaction of different herd characteristics (herd size and type (greater in dairy compared to beef herds), animal weights, weaning method, protein level of calf starter, feeding of ionophores in heifer rations, distribution of animal groups, breed, and number of animals not born into the herd) also collectively affect the prevalence of the pathogen within cattle (Dargatz *et al.* 1997; Herriott *et al.* 1998; Nielsen *et al.* 2002). Alteration of feeding regimes or diets may also have profound effects on the carriage of *E. coli* O157 in animals (Chapman *et al.* 1997a; Kudva *et al.* 1997; Herriott *et al.* 1998; Duncan *et al.* 2000; Lema *et al.* 2002; section 2.10.1).

Sheep are known to excrete the pathogen, and probably represent the second greatest pool of the bacterium in the environment (Kudva *et al.* 1996, 1997, 1998; Chapman *et al.* 1997a; Heuvelink *et al.* 1998a; Nicholson *et al.* 2000; Johnsen *et al.* 2001; Rey *et al.* 2003). Even though 31% of sheep were found positive for *E. coli* O157 in one report (Kudva *et al.* 1996), a review of scientific literature generally concur that incidence levels in sheep are less than in cattle, although the excretion periods in sheep tend to be longer (> 1 year;

Kudva *et al.* 1996). Regarding shedding rates, Strachan *et al.* (2001) witnessed notably high concentrations of the pathogen in some sheep (10^6 CFU g^{-1} faeces). Equal figures were reported by a recent study which also found an overall incidence of the pathogen within 6.5% of sheep faecal samples tested (Ogden *et al.* 2005). Sheep therefore play a significant part in the introduction and dispersal of *E. coli* O157 in the farming environment, and human infections have arising following contact with sheep faeces (Strachan *et al.* 2002).

The organism is also carried (to a lesser degree) by other farm and domestic animals e.g. pigs (Chapman *et al.* 1997a, 2000; Nicholson *et al.* 2000; Johnsen *et al.* 2001), horses, dogs, and geese (Bolton *et al.* 2000; Chapman *et al.* 2000); and has also been isolated in wild birds (Benton *et al.* 1983; Dell'Omo *et al.* 1998; Wallace *et al.* 1997) and animals (Chapman 2000; Hartel *et al.* 2003). Flies have also been found to spread the bacterium on numerous occasions (Rahn *et al.* 1997; Hancock *et al.* 1998; Heuvelink *et al.* 1998b; Janisiewicz *et al.* 1999; Rice *et al.* 2003a). The carriage and dissipation of the pathogen into the wider environment via wild birds and flies is of concern due to the widespread areas they may contaminate en-route.

Farming intensification, increased farm housing, and the collection of faeces mixed with liquid waste (slurry) has led to production of greater volumes of animal waste (Jones 1999). In the UK, up to 170 million tonnes of animal manure is annually produced, accounting for 90% of organic waste production (DEFRA 2002a). Consequently, 80 million tonnes of animal waste (manure and slurry) is spread onto land annually (DEFRA 2005), in addition to the volume excreted by animals. The presence of organic matter and plant nutrients makes land application of waste the most favourable method of disposal (Gilbertson *et al.* 1979). However, this form of waste management may introduce *E. coli* O157 to previously uninfected herds and grazing areas. Furthermore, intestinal micro-organisms such as *E. coli* O157 may develop resistance to antibiotics used in animal husbandry (Jones 1999; Teuber 1999; Cools *et al.* 2001), or mutate to form new strains. Spreading animal wastes may thus lead to transmission of bacteria which are even more pathogenic and dangerous to man (Cools *et al.* 2001).

2.5.2. Abattoir waste

Abattoir waste is characterised by high concentrations of blood and suspended particles of semi-digested and undigested feeds from within the stomach and intestine of slaughtered

animals (Buncic *et al.* 2002). Akin to animal manure, it contains nutrients which enhance soil fertility, and it is routinely applied to land. Indeed, SEPA (1998) estimate 26,000 tonnes of abattoir waste are applied to land annually in Scotland; and Buncic *et al.* (2002) reported that 68% of the abattoirs studied applied effluent-based wastes to land.

Due to the varied nature of abattoir wastes, pathogen occurrence may be sporadic, and routine examination of such waste may provide a false depiction of contamination patterns. Virtually no published data is available about the prevalence of pathogens in abattoir waste, the variability between different waste types, when fresh, after storage, and after application to land (Buncic *et al.* 2002). Howeve, some wastes, notably gut contents, are likely to contain high numbers of enteric bacteria, including *E. coli* O157 (Buncic *et al.* 2002; Laven *et al.* 2003).

Figure 2.13. Laboratory sample of (ovine) stomach content abattoir waste.

Conversely, the initial contamination level of blood waste is generally low. In truth, blood may even kill or impede bacteria growth due to the presence of complement factors and/or serum proteins, particularly iron-binding proteins (Buncic *et al.* 2002; Luzzaro *et al.* 2002). However, abattoir blood waste is a rich protein medium, and mixing blood with other waste types may provide conditions for pathogen growth. The energy for growth may be obtained by the breakdown of amino acids and/or peptides, which serve as sources of nitrogen and carbon (Kauppi *et al.* 1998). Consequently, intestinal bacterial fauna of slaughtered animals

(and possibly other organisms from pathological lesions on slaughtered animal tissues) suspended in abattoir waste may multiply. The pathogen loads of abattoir wastes vary according to different factors, such as the animal species killed [waste generated from cattle and sheep abattoirs may contain significantly higher *E. coli* levels than poultry-only abattoirs (Richards *et al.* 1998)]. Abattoirs slaughtering more than one species may handle wastes separately or (usually) as an amalgamation (Buncic *et al.* 2002). Such mixing may lead to cross-contamination of wastes along with growth of pathogens in wastes initially devoid of them, a factor which may become exacerbated by poor handling of different wastes (Hudson *et al.* 1996). Ammonia production by the activity of other indigenous bacteria may affect *E. coli* O157 numbers. Likewise, the age of blood waste may affect neutrophil, antibody, and complement activity (Hepburn *et al.* 2002). Aged abattoir waste (especially blood) might therefore support pathogen growth (Fig. 2.14) due to lesser antimicrobial properties (Hepburn *et al.* 2002).

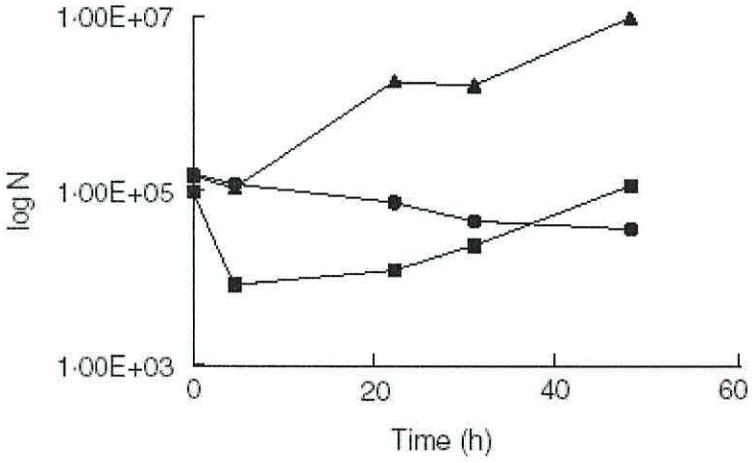

Figure 2.14. The fate of *E. coli* O157 in aged bovine blood waste during aerophilic storage at: 5°C (●), 10°C (▲), 15°C (■) (Hepburn *et al.* 2002).

In addition to the spread of pathogens, land application of abattoir wastes may possibly spread antibiotic-resistant bacteria into the environment and between animals (Haapapuro *et al.* 1997; Teuber 1999). From an abattoir hygiene perspective, wastes should be stored for the minimum duration of time possible; whereas from an agricultural point of view, waste storage times should be maximised to lower pathogen levels (Smith 1996; Buncic *et al.*

2002). A trade-off between the two opposing interests is therefore needed to minimise pathogen dissemination in each environment (Buncic *et al.* 2002). However, if waste is to be stored at abattoir premises for substantial periods of time, more research is needed to learn about pathogen survival rates under different conditions (Hudson *et al.* 1996).

Current European legislation requires that all animals slaughtered in abattoirs should be in healthy condition (Jones 1999; Collis *et al.* 2003); but occasionally, diseased animals may enter the system. These animals may carry notifiable and zoonotic disease agents, particularly in their faeces (Hoar *et al.* 2001). With regards to *E. coli* O157, the problem is exacerbated as there are no visible symptoms, rendering identification and isolation of infected animals particularly difficult. Although legislation has been introduced to enforce treatment of some organic waste materials prior to land disposal, animal gut waste and farm slurries are unaffected (DEFRA 2003). Consequent to agricultural intensification, the costs and impracticality and regulations of other disposal methods (e.g. large-scale composting), and government pressure for reduced landfill, it is anticipated that future volumes of organic waste applied to land will increase, hastening the need for apposite monitoring of waste pathogen loads.

2.5.3. Person-to-person spread of infection

Even though most infections are food-borne, the faecal-oral spread of VTEC is also a frequent instigator in outbreaks (Mainil and Daube 2005). In Wales, 1982-1998, 17% of recorded human infections were traced back to person-to-person spread (Table 2.5; Parry and Palmer 2000). Secondary transmission rates of up to 22% reported in young children (Dundas and Todd 2000) emphasises the significance of this transmission route.

Table 2.5. Probable sources of *E. coli* O157 human infections in USA outbreaks, 1982-1998 (adapted from Parry and Palmer 2000).

Probable source	1982-1989	1990-1992	1993	1994	1995	1996	1997	1998	**Total**
Food/milk	7	6	11	14	17	10	6	20	**91**
Water (consumption and recreational use)	1	1	0	1	6	3	2	4	**18**
Animal contact	0	0	0	0	0	0	0	1	**1**
Person-to-person	3	1	1	4	3	9	7	6	**34**
Person-to-person and food	0	1	0	0	0	0	0	1	**2**
Unknown	1	1	5	13	6	7	7	10	**50**
Total	**12**	**10**	**17**	**32**	**32**	**29**	**22**	**42**	

Person-to-person spread of the organism has been responsible for a second wave of illnesses in serious outbreaks at nursing homes (Carter *et al.* 1987; Griffin *et al.* 1988). It also appears that the duration of outbreaks arising from person-to-person spread may be greater than infections from other sources (Fig. 2.15; Wall *et al.* 1996).

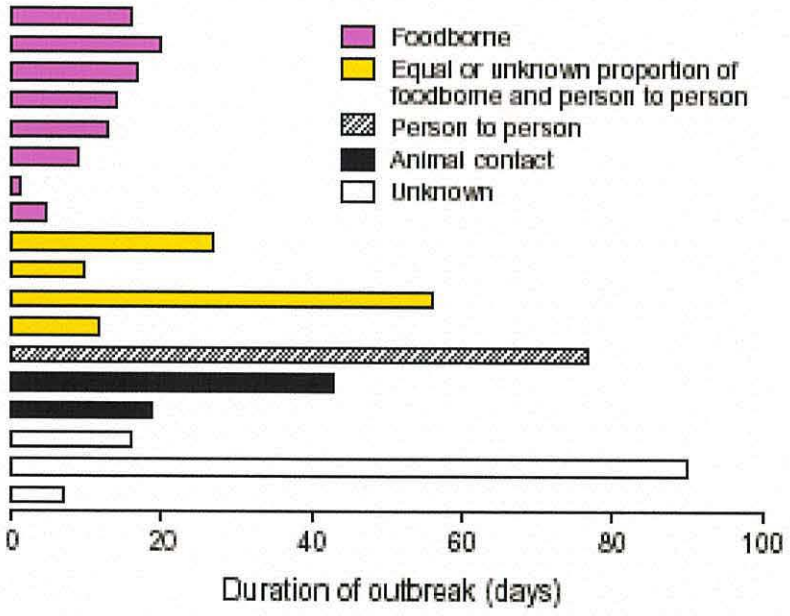

Figure 2.15. Duration and attributed mode of transmission of outbreaks of *E. coli* O157 infection in England and Wales, 1992-1994 (Wall *et al.* 1996).

Hence, *E. coli* O157 can spread within families or institutionalised patients through modes alternative to food-borne routes (Doyle 1991). Secondary transmission is of particular

concern in schools, nurseries, and elderly nursing homes (Bolton *et al.* 2000) as individuals in such establishments are at the age groups of greatest vulnerability to *E. coli* O157 infection. Other additional factors in such areas may propagate infection:

- These environments are typically warm (which might facilitate bacteria growth)
- Large numbers of individuals are in close contact (facilitating transmission)
- Cooking utensils, toilets, and other facilities are conceivably shared by a number of individuals
- Shedding of the organism in stools may continue well after clinical symptoms of infection have cleared [particularly in young children (Mead and Griffin 1998)].

The large variation of values recorded for human VTEC excretion periods following inception of infection (< 10-120 days; Pai *et al.* 1988; Belongia *et al.* 1993; Karch *et al.* 1995; Chapman *et al.* 1997b) shows the distinct variation between outbreaks. In addition, different policies or methods for follow-up testing might influence the values obtained (Bolton *et al.* 2000). Nevertheless, it is clear that excretion may be prolonged (Swerdlow and Griffin 2000), generating concern of further infections.

2.6. Tolerability of *E. coli* O157 to environmental conditions

A fundamental factor in *E. coli* O157 survival is its ability to tolerate and adapt to a range of environmental conditions. Research concludes the bacterium to be highly adaptive and tolerable to diverse stresses. Furthermore, *E. coli* O157 cells subjected to any form of stress or those originating from nutrient-limiting conditions may also be more pathogenic as they produce increased amounts of verotoxin (Buncic and Avery 1998).

2.6.1. pH

Working with synthetic rumen fluid, Cheng and Kaspar (1998) showed *E. coli* O157 acid tolerance to be a function of numerous variables; principally anaerobiosis, bacteria growth phase, NaCl concentration, and temperature. Although even closely related strains may show wide variability in acidity-sensitivity (Rowbury 1995), it is agreed that *E. coli* O157 is tolerable to a wide pH range. This owes to two primary response systems: a pH-dependent system (acid-induced), and a pH independent system (general stress protection system) involving complex proteins and enzymes (Rowbury 1995; Cheng and Kasper 1998). Although growth is reduced at low pH (Jones 1999), the ability of *E. coli* O157 to survive acidic conditions in the stomach of carriers and vectors contributes to its pathogenesis.

Indeed, growth at acidic pH may enhance the organisms' acid tolerance (Rowbury 1995). Arnold and Kaspar (1995) showed the pathogen survived for 3 hours in synthetic gastric juice of pH 1.5, sufficient for passage along the gut into faeces. Furthermore, incidences of *E. coli* O157 infections arising from drinking contaminated apple juice (pH < 3.7) are well documented (Zhao *et al.* 1993; Miller and Kaspar 1994). Some evidence suggests heat stress may increase the acid resistance of *E. coli* O157 (Buncic and Avery 1998; Wang and Doyle 1998a). This may raise problems as subjecting the bacterium to acidic conditions in a bid to eliminate it from food may prove inadequate if the food is also heated (to a point where *E. coli* O157 isn't killed). Furthermore, the acid resistance of the pathogen may be enhanced when the organism forms a biofilm (Rowbury 1995). This has implications in the food industry where cleaning procedures may rely upon acid-based disinfectants or detergents to sterilise surfaces. Acid-adapted cells are more tolerant than un-adapted cells when subsequently exposed to reduced pH and other stresses e.g. heat; although not to all stresses e.g. osmotic stress (Ryu *et al.* 1999). Organisms grown in nutrient-limited mediums or under anaerobic conditions may also display enhanced acid resistance (Rowbury 1995). Conversely, it appears that pathogenic bacteria grown in high NaCl concentrations become acid-sensitive due to damage to cell proteins (Rowbury 1995).

The pathogens' penchant for alkaline conditions is less well understood; however, it appears that survival is reduced at high pH. Exposure to acidic conditions may also induce sensitivity to alkaline conditions, and vice-versa (Rowbury 1995). Avery *et al.* (2005) could recover no cells from creamery waste (pH 12.4) two hours following inoculation with *E. coli* O157, and lime treatment of sewage sludge has been implemented to kill a range of enteric pathogens, including *E. coli* (Boost and Poon 1998; Water Industry Research Ltd. 2002). Raising and maintaining slurry pH to 12 for two hours by the addition of lime yields significant pathogen reduction (Duffy 2003).

2.6.2. O_2 concentration

E. coli O157 is a facultative anaerobe and may survive both aerobic and anaerobic conditions (Jones 1999). However, evidence suggests it to be additionally prolific under anaerobic conditions. For instance, Bromberg *et al.* (1998) showed sub-lethally heat-damaged cells grew at a greater rate in anaerobic conditions. It should be noted in experiments utilising aerobic plate counting that this might cause discrepancies when

enumerating numbers of cells (Jones 1999); especially as the source of tryptone in growth media affect *E. coli* oxidative stress resistance (Spiegeleer *et al.* 2004). Aeration of organic wastes (e.g. slurry) may induce a reduction in pathogen levels (Kudva *et al.* 1998). However, Avery *et al.* (2006) showed the bacterium survived better in aerated (relative to non-aerated) surface water; although this may owe to differences in background protozoa levels causing differential *E. coli* O157 grazing rates with varying oxygen levels. This relationship was displayed by Vold *et al.* (2000), where inhibition of *E. coli* O157 by indigenous bacteria in meat samples was more pronounced under anaerobic conditions. Anaerobic digestion has long been used as a method of reducing pathogen levels in organic wastes; with effectiveness largely depending upon the temperatures achieved in the waste (Bromberg *et al.* 1998; Nicholson *et al.* 2000). As it seems *E. coli* O157 may survive in atmospheres exhibiting normal and anaerobic oxygen levels, this has implications for food packaged in modified environments (Bromberg *et al.* 1998). For instance, enhanced *E. coli* O157 survival may occur in vacuum-packed (i.e. an anaerobic environment) meat (Cosansu and Ayhan 2000; Nissen *et al.* 2001). This is of concern as vacuum packing is one of the commonest measures undertaken to preserve meat from microbial degradation.

2.6.3. Temperature

The heat resistance of *E. coli* O157 is affected by several intrinsic and extrinsic factors, especially the growth phase and water activity (Kaur *et al.* 1998). Subjecting *E. coli* O157 to high temperatures induces stress due to large increases in unfolding/misfolding of molecular bonds. Ultimately, 'heat-shock response' may occur; - a protective cellular response to deal with heat-induced protein damage. This involves the building and dispatching of heat-shock proteins such as chaperones and proteases (Juneja *et al.* 1998). The effectiveness of heat-shock response systems in pathogens such as *E. coli* is not fully understood; however, it is of significance in governing the effects of heat-treatment on pathogen survival and recovery (Murano and Pierson 1993; Stringer *et al.* 2000). *E. coli* O157 cells exposed to heat-shock may also show enhance thermo-tolerance (Juneja *et al.* 1998). Although non-O157 *E. coli* has been reported to grow at a temperature range of 7.5°C to 49°C (Kauppi *et al.* 1998), Stringer *et al.* (2000) achieved a 6-decimal reduction in cell numbers when *E. coli* O157-contaminated food was held at 70°C for a period of > 2 minutes. However, in one study, *E. coli* O157 was detected in all sprout samples (n = 32) subjected to 30 seconds at 100°C (i.e.

blanching) (Fratamico and Bagi 2001). Slow heating of food, together with exposing bacteria to temperatures slightly above that of their optimum growth (37°C for *E. coli* O157) may increase the heat resistance of bacteria (Kaur *et al.* 1998); emphasising the need for thorough heating/cooking of food for a sufficient length of time. Duffy *et al.* (1999) reported the significant differences in thermo-tolerance between various strains of *E. coli* O157, and that significant differences occurred depending on the method of heat treatment. The potential for sub-lethally damaging cells (hence increasing their virulence) was also noted. Ironically, cold shocking of *E. coli* O157 may also significantly enhance survival (Bollman *et al.* 2001). The persistence of the bacterium in frozen soil was clearly emphasised by Gagliardi and Karns (2002), who obtained viable cells from soil samples frozen to -20°C for over 500 days. Likewise, the bacterium has been shown to survive well in foods at low temperatures (McClure and Hall 2000). Even though excessive solute levels may decrease *E. coli* O157 heat resistance, the presence of sugars and/or salt in foods (subsequently reduced water content) may increase bacteria survival during cooking due to bacteria dispersal into fatty components (Kaur *et al.* 1998). Along with its resistance to low NaCl, moisture, and pH conditions, this may explain the frequency of *E. coli* O157 infections from consumption of processed meat products such as salami (Duffy *et al.* 2000; Moore 2004). A comprehensive review on the thermal inactivation kinetics of *E. coli* O157 by Stringer *et al.* (2000) discovered thermal resistance to be affected by cell growth conditions, bacterial strain, pH, heating system/rate, the medium in which cells exist, and water activity. The model developed by Buchanan *et al.* (1993) suggests that temperature has a profound effect on *E. coli* O157 sensitivity to other variables, and vice versa.

2.7. Survival of *E. coli* O157 in the environment

The fate of pathogens that have entered the environment varies considerably. Whilst some enter soil, water, or crop systems, others may be passed along the food web by different vectors, only to eventually be re-deposited into aquatic or terrestrial systems (Fig. 2.16). The survival of *E. coli* O157 at various points in the environment may determine the risk of human infection.

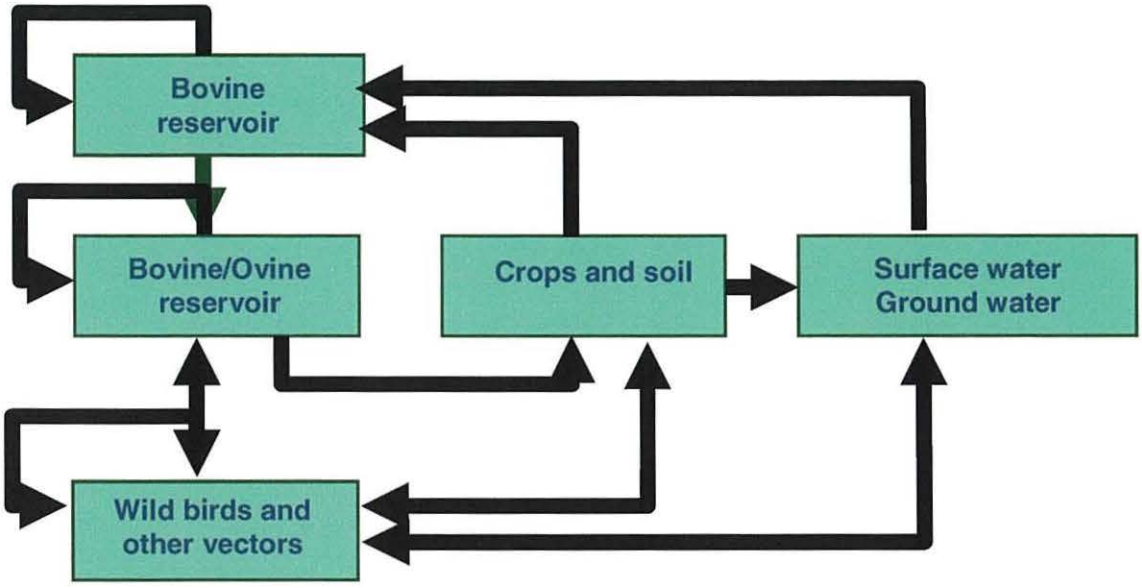

Figure 2.16. A schematic diagram of the possible ecological cycle of pathogens in the environment (Jones 1999).

2.7.1. Animal waste

2.7.1.1. Manure and faeces

Pathogen persistence in manure (and slurry) essentially depends on management and storage conditions. Survival in manure appears to be strain-dependent (Kudva *et al.* 1998); with decline rates varying with aeration (Heinonen-Tanski *et al.* 1998), pH (Himathongkham and Riemann 1999), dry matter content (Jones 1976), and temperature (Jones 1976; Zhao *et al.* 1995; Wang *et al.* 1997; Kudva *et al.* 1998; Fukushima *et al.* 1999; Himathongkham *et al.* 1999) (Fig. 2.17).

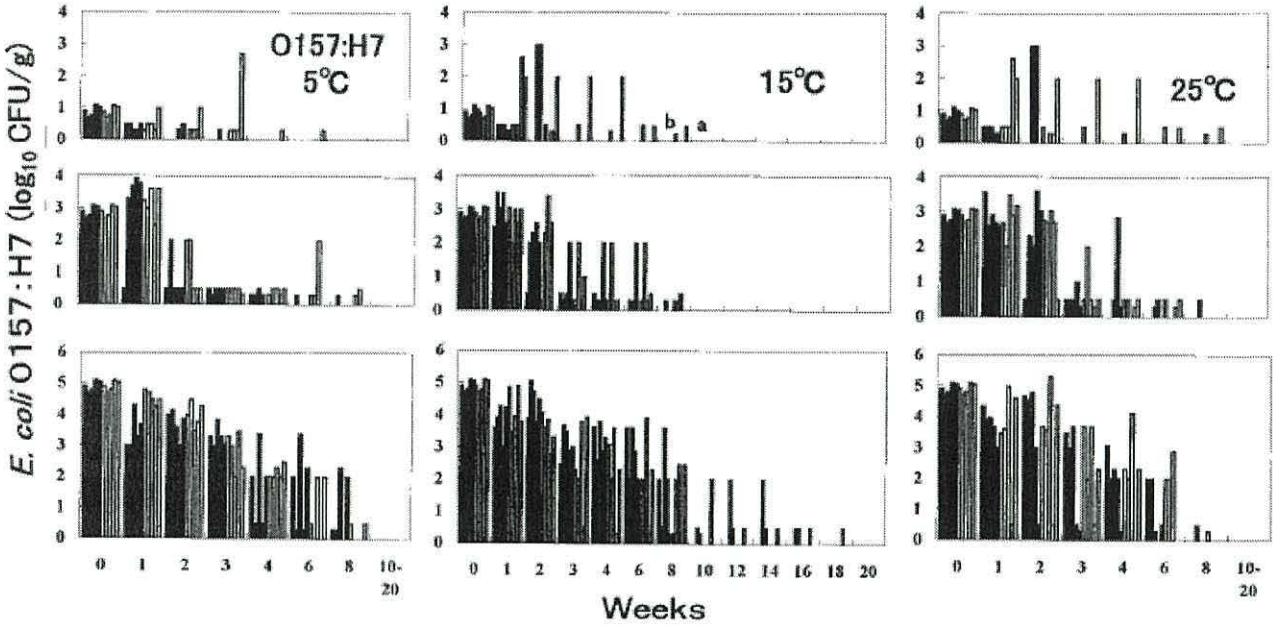

Figure 2.17. Survival of a five-strain mixture of *E. coli* O157:H7 in bovine faeces at 5°C, 15°C, and 25°C (Fukushima *et al.* 1999).

Many studies conclude that *E. coli* O157 can survive weeks or months in manure (Maule 1997; Kudva *et al.* 1998; Fukushima *et al.* 1999). Kudva *et al.* (1998) showed an ephemeral increase in numbers in drying faeces for a week followed by a gradual decline, but viable levels were detectable for seven weeks. The same study found *E. coli* O157 remained viable in aerated manure for approximately 2 months and for 21 months in non-aerated manure. It was believed that aeration accelerated the drying of manure, leading to bacteria desiccation and death (Kudva *et al.* 1998). Wang *et al.* (1996) and Fukushima *et al.* (1999) demonstrated that incubation temperature and inoculation levels had significant affects on survival on five different *E. coli* O157 strains. Their study showed the organism survived for 70 days at 5°C, 56 days at 22°C, and 49 days at 37°C. Furthermore, *E. coli* O157 has been shown to survive over 100 days in manure frozen at -20°C (Kudva *et al.* 1998), illustrating the pathogens' penchant for cooler conditions. A comprehensive synopsis of the issue of pathogens in manure and slurries is given in Nicholson *et al.* (2000).

In the UK, most cattle are housed indoors over winter, and return outdoors in spring. Traditionally, their manure is collected from farm buildings, and then stored outside in heaps prior to land spreading (Fig. 2.18).

Figure 2.18. Traditional heap where manure generated over winter is stored prior to spreading.

No enforced regulations exist for storage conditions of manure heaps, and all guidelines for farmers are entirely voluntary. Adequately high temperatures for a sufficient length of time to guarantee sanitation of manure are not always achieved in heaps, especially in cooler outer regions (Himathongkham *et al.* 1999; Patriquin 2000; Nicholson *et al.* 2005). The following time (minutes)/temperature (°C) combinations have been shown to successfully eradicate *E. coli* from manure/compost heaps: 60/55, 20/60, and 5/70 (Jones and Martin 2003). If manures are composted correctly, temperatures of > 55°C are achieved and last for days to weeks in the thermophilic stage (Patriquin 2000), hence destroying mesophilic organisms such as *E. coli* O157. 'Maturing times' of two to four months have been recommended to rid backyard composts of *E. coli* O157 (Patriquin 2000); even though solid manure storage for at least one month was reported sufficient to eliminate most pathogens by Himathongkham *et al.* (1999). A nation-wide survey by Hutchison *et al.* (2004) testing the prevalence and levels of the most common zoonotic agents linked to gastroenteritis and food-borne illnesses (*E. coli* O157, pathogenic *Listeria*, *Salmonella*, *Campylobacter*, *Giardia* and *Cryptosporidium*) in livestock wastes found overall higher levels of pathogens in fresh wastes (Table 2.6). However, these differences weren't apparent with *E. coli* O157; possibly as wastes are added to stores on a continual basis, thus manure is a mixture of old material with numerous additions of newly deposited waste.

Table 2.6. Summary of the levels and prevalence of *E. coli* O157 observed in British livestock manures. Data shown are calculated as percentage; and as both arithmetic (A) and geometric (G) means. The highest levels of *E. coli* O157 observed in each manure type (M) and the number of positive isolations are shown (n) (Hutchison *et al.* 2004).

	Levels of *E. coli* O157 (CFU g^{-1}) found in livestock waste types					
	Cattle		Pig		Sheep	
Measurement	Fresh ($n = 810$)	Stored ($n = 429$)	Fresh ($n = 126$)	Stored ($n = 58$)	Fresh ($n = 24$)	Stored ($n = 9$)
%	13.2	9.1	11.9	15.5	20.8	22.2
A	2.9×10^6	8.6×10^3	6.9×10^4	4.5×10^3	1.1×10^4	2.5×10^3
G	1.2×10^3	2.6×10^2	3.9×10^3	1.3×10^3	7.8×10^2	2.5×10^2
M	2.6×10^8	7.5×10^4	7.5×10^5	1.8×10^4	4.9×10^4	5.0×10^3
n	107	39	15	9	5	2

By using the following key factors, Nicholson *et al.* (2000) attempted to assess the potential risk of pathogens in manures transferring into the food chain following land-spreading of manure:

- The relative quantities of manure produced by different livestock types
- The prevalence and levels of pathogens in different manure types
- Pathogen survival during manure storage
- Pathogen survival in soils and on crops.

Although they highlight it is unattainable to produce a numerical risk assessment of pathogen transfer to the human food chain from the land application of farm manures, tables of comparative risks (Table 2.7 and Table 2.8) have been drawn using the above factors. Risks are represented by a number of asterisks according to the following scale:

***** Very high risk; **** High risk; *** Medium risk; ** Low risk; * Very low risk

Table 2.7. Risk of *E. coli* O157 transfer into crops grown in contact with the soil where manures have been spread previously or excreta deposited in the field (Nicholson *et al.* 2000).

Manure/livestock type		No storage **or** harvest restriction	Storage > 3 months	Harvest > 6 months	Storage > 3 months **and** harvest > 6 months
Slurry	- cattle	*****	***	**	*
	- pig	**	*	*	*
FYM	- cattle	*****	***	**	*
	- pig	**	*	*	*
	- sheep	***	**	*	*
Poultry	- layer	*	*	*	*
	- litter	*	*	*	*
Dirty water	- cattle	***	**	**	*
	- pig	*	*	*	*
	- poultry	*	*	*	*
Grazing	- cattle	*****	NA	**	NA
	- sheep	****	NA	*	NA
	- outdoor pigs	**	NA	*	NA

NA = Not applicable; FYM = Farm yard manure

Table 2.8. Risk of *E. coli* O157 transfer to grazing or foraging livestock (hence meat and dairy products) following manure spreading (Nicholson *et al.* 2000).

Manure type		No storage **or** grazing restriction	Storage > 3 months	No graze period		Forage land (grass silage/hay, maize)
				4 weeks	6 months	
Slurry	- cattle	*****	***	***	**	**
	- pig	**	*	*	*	*
FYM	- cattle	*****	***	***	**	**
	- pig	**	*	*	*	*
	- sheep	***	**	**	*	**
Poultry	- layer	*	*	*	*	*
	- litter	*	*	*	*	*
Dirty water	- cattle	****	**	***	*	*
	- pig	*	*	*	*	*
	- poultry	*	*	*	*	*

Illustrating the significance of ample manure composting times, Mukherjee *et al.* (2004) found the prevalence of non-O157 *E. coli* were 19 times higher in vegetables grown on farms which had used manure aged < 12 months; although no details of the composting process were given. Efficient turning of the manure heap (Fig. 2.19) ensures uniformly high temperature and further UV exposure to manure; both harmful to pathogens. In truth, on-

farm time and cost restrictions mean heaps are often inadequately turned, exacerbating the potential for pathogen survival.

Figure 2.19. Turning a manure pile with a tractor bucket loader to ensure efficient composting.

Current guidelines from UK regulatory authorities (DEFRA) propose animals should be kept off land for 42 days following application of manure or slurry (Avery *et al.* 2005). As research has shown *E. coli* O157 to survive for longer than this in manure and slurry (Wang *et al.* 1996; Maule 1997; Kudva *et al.* 1998; Avery *et al.* 2005), this may be insufficient to curb possible livestock re-infection (Table 2.8). The lack of enforced laws and intensive agriculture systems mean farmers may graze animals on land only recently applied manure or slurry. Additionally, storage limitations often compel farmers to spread raw (from farm buildings) or partially digested faeces/manure at periods of suitable weather; - usually frosty periods when hard ground permits the use of heavy machinery. As *E. coli* O157 survives well at low temperatures and fresh/raw manure supports greater numbers of the bacterium,

this may lead to propagation of *E. coli* O157 in the environment. Wild birds, animals, and pets may also be attracted to manure piles or spread manure, thus becoming vectors of *E. coli* O157 within the environment (Wallace 1997; Patriquin 2000; Duffy 2003). Pathogens may also be reintroduced into mature, composted manure by mixing it with raw or partially composted manure or from animals housed in different buildings. Separate storage allocation for manure of different age and origin is therefore preferable, although impractical and unfeasible on most farms.

Typical manure surface-spreading equipment used on farms render uniform spreading virtually impossible; leaving some areas of fields with a much higher load of manure, hence of bacteria. This may have substantial effects on pathogen movement through soil and subsequent pollution (McGechan and Vinten 2003). All solid manures are surface applied in the UK (Nicholson *et al.* 2005), which raises the possibility of vegetation and soil contamination. Consumption of faecal-contaminated vegetables such as potatoes (Chapman *et al.* 1997b), lettuce (Abdul-Raouf *et al.* 1993a; Armstrong *et al.* 1996; Solomon *et al.* 2002), and sprouted seeds (Park *et al.* 1999; Terajima *et al.* 2000) has led to sporadic infection outbreaks. Indeed, consumption of faecal-contaminated fruit and vegetables may be responsible for > 10% of human *E. coli* O157 infections (Maule 1999).

2.7.1.2. Slurry

E. coli O157 survival in slurry is reduced relative to that in manure and faeces (Kudva *et al.* 1998; Himathongkham *et al.* 1999; Jones 1999; Guan and Holley 2003). This trend is evident on comparison of *E. coli* O157 persistence data in both substrates e.g. Avery *et al.* (2005) and Bolton *et al.* (1999). As slurry contains a high proportion of liquid waste (urine, wash-water), it is inevitable that the chemistry differs to that of predominantly solid manure; affecting bacteria survival. Higher levels of organic acid and volatile fatty acids (VFA), coupled with reduced O_2 and available C in slurry may formulate slurry less hospitable than manure for bacteria such as *E. coli* O157 (Jones 1999; Harris *et al.* 2001; Avery *et al.* 2005). Avery *et al.* (2005) observed significantly different *E. coli* O157 survival characteristics in five chemically different slurries. They reported differences might owe to substrates and nutrient supply, namely NH_4^+; which is the preferential nutrient supply of VTEC bacteria (Kauppi *et al.* 1998). Likewise, the differences may be attributable to differing nitrate/nitrite levels; high levels of which can be toxic to *E. coli* O157 (Tamási and Lantos 1983; Casey and Condon 2000). Sufficient ammonia emissions have also been shown to kill a range of

pathogens, including *E. coli* O157 (Himathongkham and Riemann 1999). Although ammonia emissions weren't measured directly, Nicholson *et al.* (2005) reported *E. coli* O157 survival was reduced in broiler litter compared to a range of other manures, which may owe to the high ammonia levels associated with such waste. Enhanced predation from grazing protozoa in slurry may also be an underlying factor (Heinonen-Tanski *et al.* 1998; Jones 1999). All the while, laboratory-stored slurry has been shown to support *E. coli* O157 for up to three months (Maule 1997; Nicholson *et al.* 2005). Indeed, results by Nicholson *et al.* (2005) contrast with the general trend of decreased survival in slurry relative to manure. However, the temperature pathogens were subjected to in the manure was (at many instances) over three times greater that of pathogens in slurry. Due to its high proportion of liquid (Fig. 2.20), slurry does not undergo a composting process during storage and may therefore host higher levels of enteric bacteria (Cools *et al.* 2001). Slurry stored in lagoons from cattle herds is more likely than individual faecal samples to contain *E. coli* O157; albeit at reduced levels due to dilution from non-shedding stock and rain. As lagoons typically contain large volumes of slurry accumulated from large numbers of cows over an extended period of time (Fenlon *et al.* 2000), adequate storage times for pathogen demise prior to land application is clearly desirable.

Figure 2.20. Slurry storage in a typical lagoon.

Although pressures to reduce ammonia and odour emissions are moving the industry towards deep-injecting disposal (Fig. 2.21 (bottom); Nicholson *et al.* 2005), over 90% of slurry is disposed via surface-spreading (Fig. 2.21 (top); Nicholson *et al.* 2000).

Figure 2.21. Conventional surface spreading (top) and contemporary deep-injection (bottom) of slurry.

Nicholson *et al.* (2005) claim that survival of *E. coli* O157 may be enhanced by deep injection owing to a reduced slurry drying rate and reduced exposure of bacteria to UV radiation. However, Avery *et al.* (2004) compared survival of *E. coli* O157 in soil and on vegetation following surface spreading and deep-injection (25 cm) of organic (slurry and abattoir) wastes to soil cores and found contradictory evidence. Whereas no *E. coli* O157 cells were detected on the surface vegetation when the waste was deep injected into soil cores; viable *E. coli* O157 cells persisted on surface vegetation for 6 weeks when waste was surface-applied. Quinton *et al.* (2003) also reported that runoff of faecal coliforms was greater when waste was surface applied rather than incorporated into soil. The weight of evidence therefore suggests that, in the interest of minimising pathogen contamination of

crops and waterways, organic wastes should preferably be applied to land via deep-injection. Although this may incur additional costs to the farmer, deep-injecting slurry may still prove to be an economically-viable option due to the supplementary levels of nitrogenous compounds attained (needed for plant growth) relative to levels attained following surface-spreading (DEFRA 2005).

2.7.2. Water

Pathogens present in animal faecal deposits excreted to land undergo a process of dispersion, transport or attenuation, and inactivation. However, significant knowledge gaps exist in the precise mechanisms of pathogen transport (Ferguson *et al.* 2003). Undoubtedly though, the role of water as an environmental dispersing agent of the bacterium is crucial. Preserving the microbiological quality of waters is important due to its demand from households, for industry, and for recreational activities. The presence of *E. coli* in water has long been used as an indicator of faecal pollution (van Donsel *et al.* 1967; McMath and Holt 2000; Ling *et al.* 2002). Contamination of water supplies by *E. coli* O157 is still a noticeable problem in developing countries (Ashbolt 2004). For instance, thousands of cases of HC occurred in South Africa in one area following consumption of waters of which 18.4% tested positive for *E. coli* O157 (Isaacson *et al.* 1993). The cause of such contamination levels was eventually traced to the washing of cattle carcasses and manure into rivers. However, the frequency of water pollution by agricultural waste is also an issue of concern in the UK, accounting for 27% of all serious water pollution incidents in 2000 (DEFRA 2002a). The presence of waterborne enteric pathogens (such as *E. coli* O157) in domestic water supplies represents a potentially significant risk to human health (Theron and Cloete 2002). In the UK, stringent procedures and treatments by water companies aim to eliminate such micro-organisms from water supplies. Water disinfection processes (predominantly based on chlorination and ozonation) occur in the final stages of water treatment (Chalmers *et al.* 2000; McMath and Holt 2000) at an annual cost estimated at £23,000,000 (DEFRA 2002a). Chlorination has been shown to be proficient and rapid at *E. coli* O157 destruction (Kaneko 1998; Park *et al.* 1999). Whilst European microbiological standards for drinking water include the absence of faecal coliforms in 100 ml of water (Chalmers *et al.* 2000; McMath and Holt 2000), repeated and continuous sampling from UK water companies typically yields *E. coli* incidence rates of 0.02% (McMath and Holt 2000). As their water is not

subjected to stern cleansing treatments, those with a private water supply might be at higher risk of *E. coli* O157 infection. Jackson *et al.* (1998) reported on an outbreak of *E. coli* O157 infection arising from drinking well water contaminated by faeces of infected cattle. The illness of 268 children and two mortalities at a nursery in Japan was eventually linked to the consumption of *E. coli* O157-contaminated tap and well water (Akashi *et al.* 1991). As < 70% of water supplies are sanitised in certain eastern European countries and parts of Africa and Asia (WHO, 2000), this raises concern of significant infection outbreaks. Numerous human *E. coli* O157 infection cases associated with consumption of contaminated water are highlighted by Chalmers *et al.* (2000).

Microbial contamination of ground and surface water supplies may occur due to runoff from agricultural land, surface and lateral flow through contaminated soil, excretion of contaminated faeces by animals, industrial pollution (sewage works and abattoirs), flood events, drainage systems, and burst pipes (Benkacoker and Ojior 1995; Marsalek *et al.* 1996; Sibille 1998; Chalmers *et al.* 2000; McMath and Holt 2000; Heinonen-Tanski and Uusi-Kamppa 2001; Collins and Rutherford 2004; Muirhead *et al.* 2004). As 58% of water used for irrigation in England is surface water, contaminated sources may become important vectors in crop contamination (Solomon *et al.* 2002; Avery *et al.* 2006). Unscrupulous waste spreading near watercourses poses a notable threat of water contamination. Wild birds may also contaminate major water supplies (Benton *et al.* 1983). Overland pathogen transport via surface runoff is undoubtedly responsible for increases in the concentrations of waterborne pathogens in many watersheds, with higher levels of pathogens at periods of heavy rainfall (McMath and Holt 2000). However, work based on an agriculture *E. coli*-input model by Collins and Rutherford (2004) suggested riparian buffer strips might improve bacterial water quality by eliminating livestock defecation in and around streams, and by the trapping of bacteria by vegetation. Other measures to reduce microbial contamination of water supplies include cautious application of animal-derived waste in conjunction with a code of practice and restricting animal grazing near waters used for recreational or drinking purposes (DEFRA 1998, 2002a).

Occurrences of groundwater contamination leading to sporadic cases of human infection have been reported (Wang and Doyle 1998b; Licence *et al.* 2001). Furthermore, indirect ingestion of contaminated water during recreational activity has also led to cases of human *E. coli* O157 infection e.g. Ackman *et al.* (1997) reported cases arising in campers

following swimming in a lake. Preventing individuals suffering from diarrhoea from swimming or bathing in communal water used for recreational activity (e.g. pools, lakes) is imperative as this risks secondary spread of the pathogen (Chalmers *et al.* 2000). *E. coli* bacteria embedded within lake sediments over time may be re-suspended following boating activity, thereby increasing the possibility of human contact (An *et al.* 2002). Similarly, pathogenic bacteria in river sediments may be re-suspended within the water column at flood events (Muirhead *et al.* 2004).

The survival of the bacterium in water is a function of protozoal grazing (Wang and Doyle 1998b; Artz and Killham 2002), water source (Jones 1999), temperature (Wang and Doyle 1998b), UV radiation (Davies-Colley *et al.* 1997; Sun *et al.* 2003), and nutrient availability (Flint 1987). Non-O157 *E. coli* has been shown to survive in filtered, sterile river water for up to 260 days, with no loss of viability (Flint 1987), whilst Wang and Doyle (1998b) demonstrated serotype O157 to survive in river water for up to 90 days. Warburton *et al.* (1998) showed the bacterium could persist for over 300 days in bottled water, and Kerr *et al.* (1999) showed the bacterium persisted for over 70 days in mineral water. The extended survival of the pathogen in these conditions may partially owe to the formation of a biofilm on the polyethylene bottles; enabling it to metabolise nutrients released by lysis of other bacteria (Warburton *et al.* 1998). As with other matrices, persistence of *E. coli* O157 in water is enhanced at lower temperatures (Chalmers *et al.* 2000); as shown in waters of municipal, reservoir, and lake origin by Wang and Doyle (1998b). Working with 31 widely contrasting range of surface waters, Avery *et al.* (2006) showed *E. coli* O157 to persist for over 2 months in 45% of samples, including cattle trough water. This reflects work by LeJeune *et al.* (2001) whom showed *E. coli* O157 survived for 245 days in trough-derived water. As approximately 10-15% of cattle troughs probably harbour *E. coli* O157 (LeJeune *et al.* 2001; Sargeant *et al.* 2003), this highlights the potential for re-infection of cattle herds and spread of the pathogen through large numbers of animals.

Less research has occurred into the persistence of *E. coli* O157 in marine water compared to other water types; however, it is still of interest due to the popularity of marine-based recreational activity (e.g. water skiing, diving) and the fishing industry. Although new laws forbid sea disposal of sewage sludge (DEFRA 2002b), *E. coli* O157 may still reside in marine environments from past disposal, or from storms and pollution events transporting animal waste from land to sea. Miyagi *et al.* (2001) observed the

survival of *E. coli* O157 in marine water, and found the pathogen could survive, and multiply for at least 15 days. The *stx* gene was also isolated from an estuary port, raising concerns of fish and shellfish contamination. Shellfish are filter-feeders which concentrate and retain micro-organisms (MacRae *et al.* unpublished; Marino *et al.* 2005) therefore may pose particular risk; whilst consumption of raw fish as 'sushi' may also pose elevated threat. The handling of bacteria-contaminated fish at markets and ports may pose a health risk owing to cross-contaminating other fish (Ayulo *et al.* 1994). In addition, the presence of clay-rich sediments typically found in estuaries may also favour survival of faecal bacteria (Roper and Marshall 1978).

The ability of the bacterium to survive long-distance transportation in river water also raises possibilities of infecting animals, humans, or other water sources down-stream (Marsalek *et al.* 1996; Avery *et al.* 2006). As river water may be nutrient-limited, *E. coli* O157 bacteria may to enter a viable but non-culturable (VBNC) or 'dormant' state (Jones 1999). Measurement of the degree of bacteria that enter this state and knowledge of their pathogenesis is limited, but it is perceived that they may still be a source of human infection (Jackson *et al.* 1998). Furthermore, nutrient-limiting conditions may initiate chlorine-resistant phenotypes in *E. coli* O157 (Chalmers *et al.* 2000; Maule 2000), rendering chlorination inefficient in water purification.

As water is central to such a diverse range of use, an obvious need exists for assessing and monitoring the microbiological qualification of rivers, streams, and lakes. However, this may be complicated by difficulties arising in culturing the desired organism, in addition to the wide fluctuations and irregular nature of bacteria abundance in these environments (Hadas *et al.* 2004). Furthermore, *E. coli* O157 survival in different waters may be strain-dependent (Chalmers *et al.* 2000), causing discrepancy between experimental results.

2.7.3. Soil

The majority of work undertaken with *E. coli* survival in soil is associated with generic *E. coli*; and it is well acknowledged that a void of information exists with regards to the survival of serotype O157 in this environment. At first, it would seem that the survival of gut-inhabiting bacteria such as *E. coli* O157 would be limited in soils due to the inhospitable conditions which prevail. However, *E. coli* O157 is a resilient bacterium, tolerant to a wide

range of environmental conditions; therefore persistence may be greater than anticipated. Furthermore, the survival of pathogens in soils may be extended as they are protected from exposure to UV radiation and desiccation (Nicholson *et al.* 2005). The persistence of *E. coli* O157 in soil raises concern about pathogen transmission as it increases the likelihood of on-farm livestock re-infection cycles or infecting previously unaffected herds. Indeed, *E. coli* O157 have been found to remain viable in soil for extended periods of time after manure spreading or excretion onto grassland (Maule 1999; Fenlon *et al.* 2000; Avery *et al.* 2004). Bolton *et al.* (1999) showed there to be an immediate, initial die-off of *E. coli* O157 following application of inoculated faeces to grassland, but of long-term persistence at lower levels. Indeed, colonies could still be recovered from soil for up to 99 days without the need for enrichment procedures (Bolton *et al.* 1999). However, times quoted in studies for its persistence in soil conjure conflicting values (Maule 2000). Such variability may be explained as the pathogens' persistence in soil is dependent on a considerable variety of factors (Table 2.9).

Table 2.9. Factors affecting the survival of *E. coli* O157 in soil (Smith 1995; Barker *et al.* 1999; Jones 1999; Gagliardi and Karns 2002).

Factor	Remarks
Moisture content	Greater survival in moist soils and during wet weather
Permeability	Survival times less in sandy soils
Temperature	Longer survival times at low temperatures
pH value	Shorter survival times at soil surface
Organic matter	Increased survival and possible re-growth when sufficient amounts of organic matter are present
Antagonism by soil microflora	Increased survival in sterile soil
Soil clay content	Greater persistence in higher clay-content soil

Persistence of bacteria in soil is further convoluted due to interacting factors. For instance, soil temperature and moisture content affect growth of the intrinsic microbial community, which in turn may affect predation rates on a specific organism. Soil texture affects soil water content, which affects soil temperature etc. It is therefore palpable that parameters are inter-twined and consequently may affect bacteria populations both directly and indirectly.

Maule (1997, 1999) investigated the survival of *E. coli* O157 in microcosms containing soil and tuftgrass. Following incubation at 18°C and under continuous illumination, cells were still detectable in soil for 130 days (Fig. 2.22). Survival of the pathogen was also greater in cores containing rooted grass relative to those with only sieved, grass-free soils; as experienced by Gagliardi and Karns (2002).

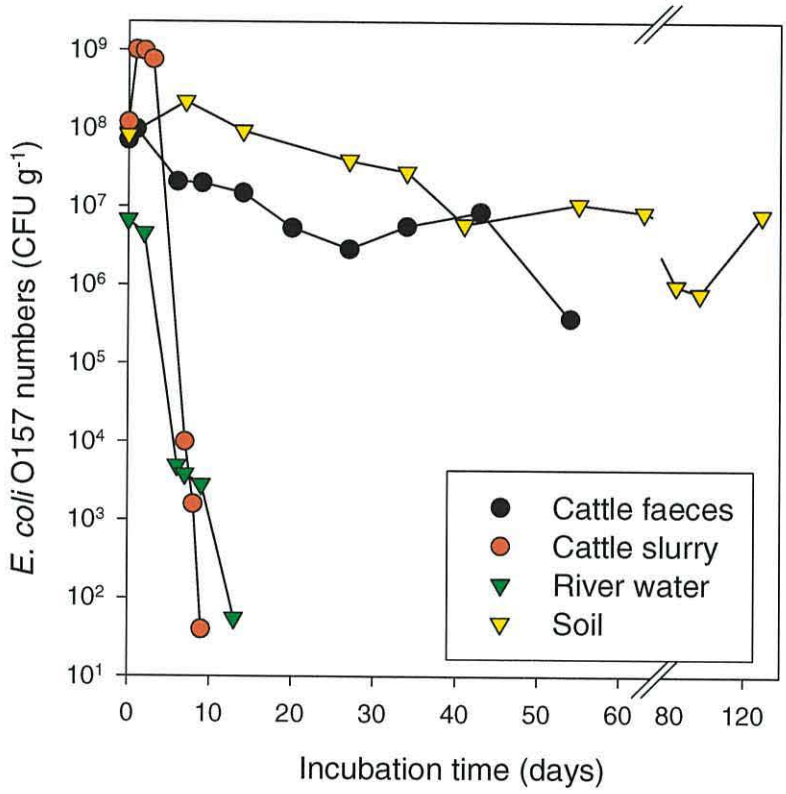

Figure 2.22. Survival of *E. coli* O157 in artificially-inoculated cattle faeces, cattle slurry, river water, and soil [Maule 1997, 1999 (from Jones 1999)].

Particulate micro-organisms such as *E. coli* O157 appear to move readily and quickly through soil macropores (McGechan and Lewis 2002; McGechan and Vinten 2003). The degree of micro-organism movement through soil is highly dependent on soil colloid and pore sizes (McGechan and Lewis 2002), therefore varies with soil texture. The degree of vertical and horizontal pathogen movement (Table 2.10) in soil affects the risk of pathogen transfer into aquifers or surface waters (Mawdsley 1995; Nicholson *et al.* 2000). Removal of pathogens from water percolating through soil is due to both mechanical action and adsorption due to soil particles (Smith 1996). However, groundwater contamination may

occur with pathogen movement through surface runoff, and via lateral/vertical flow from and through pathogen-contaminated soil (Gagliardi and Karns 2000; Heinonen-Tanski and Uusi-Kamppa 2001; Ferguson *et al.* 2003).

Table 2.10. Factors known to influence pathogen movement in soil (Mawdsley *et al.* 1995; Nicholson *et al.* 2000).

Movement type	
Vertical	Horizontal
Soil type	
Soil water content	
Rainfall intensity/volume	Rainfall intensity/volume
Temperature	Pollutant source proximity
Mesofaunal activity	Agricultural practice
Surface charge and micro-organism size	Weather/season of application
Presence of plant roots	Land topography
Soil pH	Rate of micro-organism partitioning into runoff

The importance of pathogen leaching rates was emphasised by McGechan and Vinten (2003), whom simulated slurry-derived *E. coli* movement through soil. They showed *E. coli* losses were strongly influenced by soil wetness at the instance of spreading; and also (more weakly) by rainfall occurring soon after spreading. Spreading on wet soil led to high, short-lasting leaching of micro-organisms; whilst spreading on drier soil avoided such loads; except occasionally after heavy rain soon following spreading. As much of UK permanent grassland is on impermeable soils, the most likely route of pollution is as surface or sub-surface runoff. On more permeable soils, the most likely route is down through the soil profile to land drains or ground water (Mawdsley *et al.* 1995). Rapid transportation of faecal coliforms such as *E. coli* O157 to field drains during rain events threatens the microbiological quality of water systems and could increase colony numbers (McGechan and Vinten 2003). Fenlon *et al.* (2000) noted that heavy rain preceding waste application may lead to substantial flushing of *E. coli* O157 from soil into drainage waters, depending upon soil permeability. Pathogen leaching rates from waste-amended soil will also vary with the type of waste applied as bacterial binding within the waste matrix (hence bacteria mobility) is waste-dependent (Rees 1990).

It should be noted that experiments designed to simulate pathogen leaching rates using soil cores may not always accurately reflect field conditions, as soil mixed or sieved prior to use bears little structure resemblance to undisturbed soil in the field. Sieving soil

removes natural macropores formed by plant roots, earthworms, and soil micro/mesofauna, in addition to cracks caused by freeze-thaw cycles and fissures caused by soil drying. As a result, laboratory studies using such soil may demonstrate significantly less microbial movement than intact soil cores (Mawdsley *et al.* 1995).

In addition to enhanced pathogen leaching when waste is spread in wet conditions, it may also lead to greater pathogen persistence. Cools *et al.* (2001) showed significantly enhanced generic *E. coli* survival in soil when field water capacity was at 100%. High soil-water levels may also decrease soil oxygen levels, initiating growth of facultative anaerobes such as *E. coli* O157 on the soil-liquid interface (Gagliardi and Karns 2000). Applying wastes to vegetated, rather than bare land may reduce pathogen persistence as soil water loss through plant evapo-transpiration may greatly reduce faecal bacteria survival in soil (Chandler and Craven 1978; Mubiru *et al.* 2000). *E. coli* survival is enhanced with increasing soil organic matter; mainly due to increased soil water-holding capacity and enhanced nutrient availability (Cools *et al.* 2001). Furthermore, higher organic matter content provides greater opportunities for bacteria binding with negatively charged sites (Mawdsley *et al.* 1995). As with other environments, persistence of *E. coli* O157 in soil is enhanced at lower temperatures; possibly as predation rates is reduced due to a decrease in activity of background bacteria (Cools *et al.* 2001).

Studies confirm that survival of *E. coli* O157 varies with soil type. For instance, Fenlon *et al.* (2000) applied inoculated slurry to clay, loam and sandy soils, which were subsequently stored in plastic lined bins and exposed to external ambient temperatures for six months. The bacterium survived for up to approximately 16 weeks in the loam and clay soils compared with approximately eight weeks in the sandy soil. Lau and Ingham (2001) found the rate of (non-O157) *E. coli* decline to be greater in sandy soil compared to clay loam soil; and recent work by Nicholson *et al.* (2005) also showed the pathogen survived longer in clay loam grassland soil than in sandy arable soil. In addition to the greater water-holding capacity of the former soil, higher soil clay-content facilitates colony survival by providing sites for formation of microhabitats (Mawdsley *et al.* 1995) which provide adequate moisture and nutrients for bacteria, along with supplementary protected sites against predation (Mubiru *et al.* 2000; Cools *et al.* 2001). As with soil organic matter, micro-organisms may also be adsorbed onto negatively charged surfaces of clay particles, particularly in the presence of cations such as Ca^{2+} or Na^{+} which neutralise repulsive forces

between the organisms and soil particles (Bitton 1975; Ling *et al.* 2002). Owing to their small size (< 0.002 mm), clay particles have a high surface area, resulting in high cation exchange capacity. This facilitates bacterial adhesion thus reduces bacteria leaching loads.

2.8. Survival of *E. coli* O157 in food

Many projects have studied the growth and persistence *E. coli* O157 in various foods; or have endeavoured to identify possible measures to reduce prevalence of *E. coli* O157 in food. The survival of the bacterium in food depends upon hygiene factors employed during processing, storage, retailing, and preparation. It is unambiguous that only vigilant hygiene and hygiene procedures interfacing along all levels of food production may significantly reduce the occurrence of *E. coli* O157 infections. Most human infections derive from consuming contaminated food such as undercooked beef products (Doyle 1991; Willshaw *et al.* 1994; Bolton *et al.* 1996; Chapman 2000; McClure and Hall 2000; Parry and Palmer 2000). Indeed, the two infection outbreaks with the highest mortalities to date have occurred following consumption of adulterated meat (Canada, Carter *et al.* 1987; Scotland, Cowden *et al.* 2001). Cattle hides appear to be the major source for carcass contamination. Presumably, faeces are the major source of *E. coli* O157 for hide contamination. Despite stringent rules and regulations regarding slaughterhouse procedures, research concludes microbial contamination of carcasses in abattoirs (from faeces) is comparatively common (Bell 1997; Richards *et al.* 1998; Guyon *et al.* 2001; Heuvelink *et al.* 2001; Omisakin *et al.* 2003; Schlegelová *et al.* 2004). Indeed, cross-contamination of carcasses with intestinal content or faeces during slaughter and processing is a fundamental problem in spreading pathogens such as *E. coli* O157 (Bell 1997; Bacon *et al.* 2000; Bouvet *et al.* 2002). Studies by Guyon *et al.* (2001) and Chapman *et al.* (2001a) isolated *E. coli* O157 from carcasses at slaughterhouses, and the latter study also investigated the prevalence in raw meat samples from small retail butchers. The bacterium was isolated in 1.4% of carcasses, and 0.8% of raw meat from butchers' shops (Chapman *et al.* 2001a). Another study found almost 6% of lamb products yielding *E. coli* O157 (Chapman *et al.* 1996). In all cases, the pattern showed clear seasonality, with levels peaking in the summer months. The degree of carcass and meat contamination primarily owes to the high prevalence of the bacterium in animal faeces; nevertheless, unnecessary microbial contamination of products may occur as a result of inadequately cleaned equipment or hygiene practices (Hudson *et al.* 1996; Gill and

McGinnis 2000; Heuvelink *et al.* 2001). The degree of microbiological loads carried by incoming cattle to abattoirs is important as the external hide is a primary source of faecal contamination likely to eventually be transferred to the underlying sterile carcass tissue (Bacon *et al.* 2000). In response to the recommendations of the Pennington Report (1996), the Meat Hygiene Service introduced the Clean Livestock Policy (CLP) which assesses the cleanliness of animals presented for slaughter. This assists in achieving safe levels of pathogenic bacteria in dressed carcasses. With regards to the safety of raw red meat, the regulation of animals accepted for slaughter is regarded as a Critical Control Point (CCP) in the production chain, where control can limit the microbiological hazards to a safe level. If accepting a particular animal into the slaughter-hall would compromise hygienic dressing operations, the Official Veterinary Surgeon (OVS) or Meat Hygiene Inspector (MHI) has legislative powers to prohibit the entry of that animal. Each animal is assessed against the cleanliness scoring scale. Animals in categories 1 and 2 are considered safe for slaughter with no further precautions; animals in category 3 are accepted subject to the adherence of subsequent cleaning processes; and animals classified in category 4 and 5 are rejected for slaughter outright (Food Standards Agency (FSA) 2002).

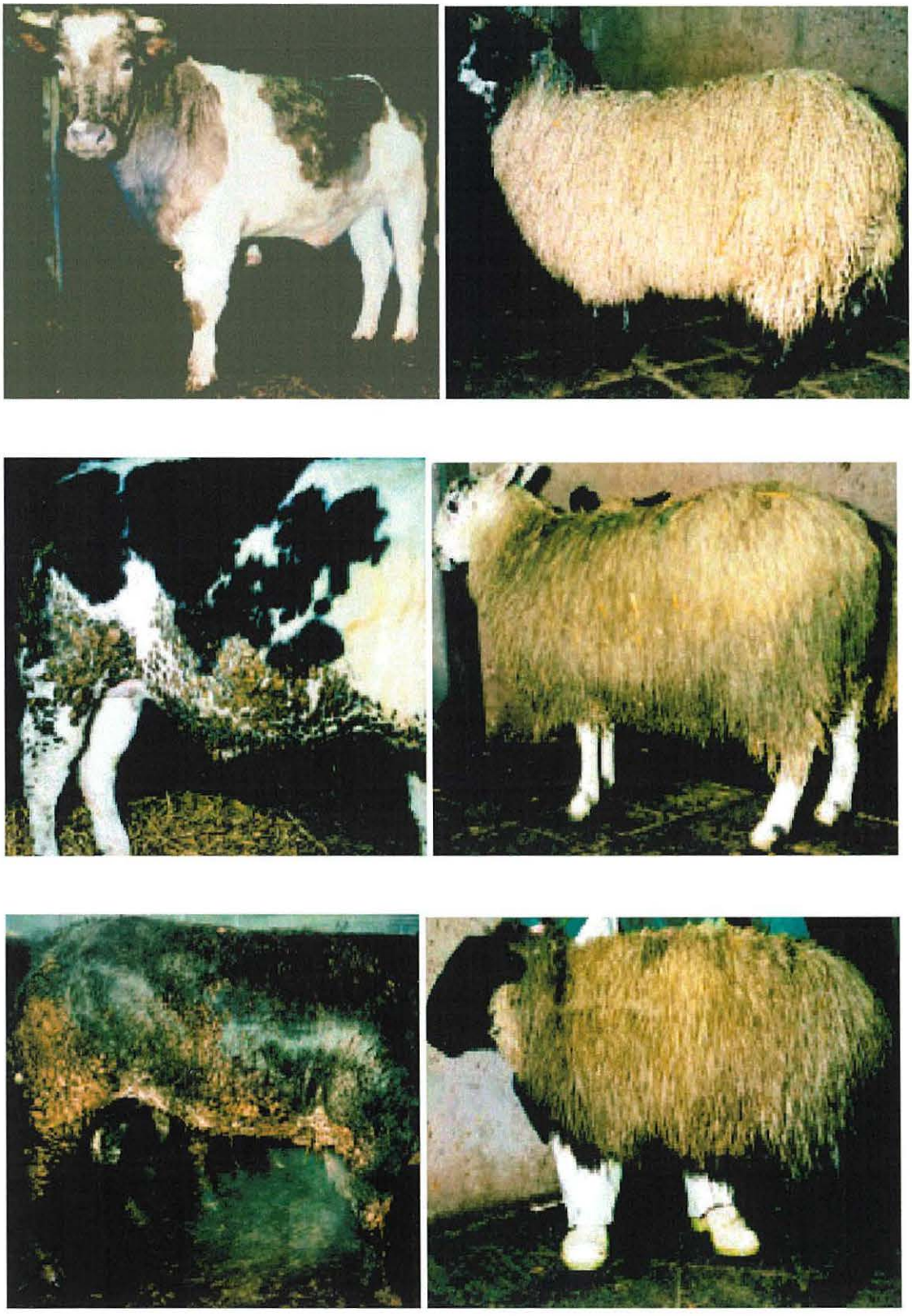

Figure 2.23 CLP categories for animal cleanliness at slaughter: 'Category 1' (top), '3' (middle) and '5' (bottom) cattle and sheep (FSA 2002)

A reduction of the microbial load carried into abattoirs on animal skin and hides may be achieved via implementing the following measures individually, or as a combination:

- Adequate and sufficient bedding for animals
- Good ventilation in sheds/holding areas
- Control of mineral/salt uptake
- No sudden alterations to animal diet
- Appropriate stocking densities when housing animals
- Washing or clipping animals pre-slaughter
- 'Chemical de-hairing' animals, which removes the hair, faeces and microbiological contamination from the hides of beef carcasses prior to hide opening
- Reducing transport and lairage times

(Bacon *et al.* 2000; FSA 2002).

In addition to causing food-borne disease, carcass contamination during processing is of concern in that it may represent the potential cause for the drug resistance displayed by human pathogens such as *E. coli* O157 (Schlegelová *et al.* 2004). Awareness of the prevalence of *E. coli* O157 on carcasses and meat is an important factor in alimentary risk assessment of meat and complementary infection rates (Guyon *et al.* 2001).

E. coli O157 has been shown to survive over 18 months in frozen meat, although inactivation may be accelerated by the presence of NaCl (Conner and Hall 1994) or freeze-thawing procedures (Sage and Ingham 1998). As previously stated (section 2.6.2), the pathogen also survives well in meat stored in modified atmospheres (Cosansu and Ayhan 2000; McClure and Hall 2000). Processed meat products, such as salami, may be an additional cause of concern due to the reliance of fermentation, drying, or high sodium levels for preservation, rather than heat treatment. Although processes such as fermentation have been shown to successfully decrease *E. coli* O157 levels within meats (Cosansu and Ayhan 2000; Duffy *et al.* 2000), fermented meat products are still frequent vectors of infection (Moore 2004). Survival of *E. coli* O157 after preservation processes is largely dependent on storage conditions, with 'mild' heat-treating of meat (e.g. 54°C for 30 minutes) being effective in ensuring the safety of fermented meat products (McClure and Hall 2000). Abdul-Raouf *et al.* (1993b) found the survival of the bacterium in ground, roasted beef was affected by the pH, acidulant levels, and temperature. Recent work has shown that the

addition of mustard flour may reduce persistence of the organism in raw, ground beef, owing to the presence of natural antimicrobial compounds (allyl isothiocyanate, AIT), without effecting palatability (Nadarajah *et al.* 2005ab).

Consumption of raw milk is known as the second most important vehicle for the transmission of *E. coli* O157 (Reitsma and Henning 1996); and identified as the cause of many infections (Chapman *et al.* 1993b; Neaves *et al.* 1994; Wang *et al.* 1997; Chapman 2000). Wang *et al.* (1997) reported that *E. coli* O157 could survive well in milk, with a decrease of only 2 $\log_{10}$ in 28 days when the milk was kept at 5°C. Interestingly, growth of the pathogen was found to be slower in unpasteurised milk, probably due to competition and/or predation from other micro-organisms. This might explain the absence of the bacterium in a large sample number of raw milk and cheese tested by Coia *et al.* (2001). Although outbreaks of human infection have arisen following consumption of pasteurised milk (Upton and Coia 1994), pasteurisation (72°C, 16.2 s) will kill over 10^4 *E. coli* O157 ml^{-1} (D'Aoust *et al.* 1988). Rahn *et al.* (1997) found the organism survived on filters used for milking; which is of concern as it might lead to contamination of substantial volumes of milk. McClure and Hall (2000) found that storage of milk or milk products at a temperature < 5°C may prevent growth of *E. coli* O157; however, Ramsaran *et al.* (1998) could detect the bacterium 75 days after storage at 2°C in the brine of Feta cheese. The organism has been shown to survive for 158 days during the manufacturing and curing of Cheddar cheese (Reitsma and Henning 1996); and growth of the bacterium during soft cheese and yoghurt production has also been demonstrated (Kasrazadeh and Genigeorgis 1995; Ogwaro *et al.* 2002). As with meat, growth during fermentation of milk products is of concern as this is the basic technique for preserving some products. Studies have also shown that *E. coli* O157 may survive on salad dressings e.g. mayonnaise, although it seems that they do not pose a significant risk of causing infection (Raghubeer *et al.* 1995). Further, it appears that the temperature during fermentation and subsequent storage is critical to the growth and survival of *E. coli* O157 in fermented milk-based products (Raghubeer *et al.* 1995; Ogwaro *et al.* 2002).

Although most human *E. coli* O157 infections derive from consumption of animal products, incidences arising from consumption of fruit, fruit drinks, and vegetables have also occurred, as previously highlighted. Gale (2005) showed that consumption of root crops grown in soil applied treated sewage sludge in accordance with current regulations

poses only a minute risks of *E. coli* O157 infection to humans. However, as disposal of farm waste is not as tightly regulated (section 2.5.1), contamination of vegetables may occasionally occur. Furthermore, produce may be indirectly contaminated by *E. coli* O157 residing in soil or water used for vegetable production. Under processing and storage conditions frequently used in commercial practice, Abdul-Raouf *et al.* (1993a) showed that *E. coli* O157 may grow for two weeks on raw salad vegetables. However, the authors reported that previous concerns about pathogen numbers on fruit or vegetables increasing following packaging in a modified environment were unfounded. Contaminated water may lead to internalisation of *E. coli* O157 (endophytes) into lettuce tissue following migration from the root system (Solomon *et al.* 2002). Thorough washing and peeling of produce reduces the threat posed by green products in transmitting food-borne infections (McClure and Hall 2000); however, these processes are ineffective in eliminating endophytic pathogens. Salad vegetables such as lettuce may present particular risk for transmitting *E. coli* O157 infection as they are usually consumed raw. Similar internalisation of the pathogen from soil to plant tissue was observed by Warriner *et al.* (2003); - when 20-day-old spinach seedlings were transferred to soil inoculated with *E. coli*, the bacterium became established on the plant surface. Furthermore, when inoculated seeds were sown in soil microcosms and cultivated for 42 days, *E. coli* was recovered from the external surfaces of spinach roots and leaves, as well as from surface-sterilised roots. Vegetable-seed contamination may thus serve as an important vector for large-scale outbreaks of *E. coli* O157 infections. Indeed, the fact that contaminated radish sprouts seeds were the source of a huge outbreak in Japan in 1996 [which led to 6309 cases of HC, > 100 cases of HUS, and 12 deaths (Michino *et al.* 1999; Vanselow *et al.* 2005)] corroborates this notion.

The ability of *E. coli* O157 to withstand acidic conditions is a fundamental factor in its ability to transmit infection via apple juice and cider (Cody *et al.* 1999). Zhao *et al.* (1993) claimed that rapid decreases in numbers of the organism could be achieved in cider by incorporating the use of a common cider preservative. However, evaluation of later studies reveals that numbers of the organism decreased only slightly over three weeks in the presence of the same preservative (Miller and Kaspar 1994).

Organic farming is a rapidly expanding sector of the food market; with the notion that its produce is perceived as being 'safer' being one of the underlying reasons (Blaine and Powell 2004). However, as organic farming uses manure as its primary source of

fertiliser, some debate as to whether enhanced risks of contracting *E. coli* O157 infection occur from consuming organically-grown produce (Patriquin 2000; Mukherjee *et al.* 2004). The use of several drugs developed to reduce levels of bacteria and viruses within farm animals is prohibited in organic systems. This may generate manure harbouring greater numbers of *E. coli* O157 (Nicholson *et al.* 2000). In some countries, food safety watchdogs have voiced their concern over the stricter guidelines regarding the use of manure as fertiliser in non-organic, relative to organic, systems (Blaine and Powell 2004). Cases of *E. coli* O157 infections have occurred via consumption of organic products contaminated with manure (Nelson 1997). However, recent studies propose that no greater risk of contracting *E. coli* O157 infection exists from consumption of organic, relative to non-organic produce (Duffy 2003; Blaine and Powell 2004; Mukherjee *et al.* 2004).

2.9. Recovery methods for *E. coli* O157

In recent years, numerous methods for detecting *E. coli* O157 cells in different matrices and environments have evolved from conventional, culture-based methods. Numerous factors affect the prevalence findings of *E. coli* O157 in the environment, - the isolation method being central. In the past, this has led to difficulty in acquisition of reliable records as the ranges of sensitivity between different methods create inconsistent data (Chapman *et al.* 1997a; Bolton *et al.* 2000; Chapman 2000). As the infectious dose for *E. coli* O157 is so low, there is a requirement for sensitive, reliable, and accurate methods of recovery to reduce danger to public health. However, isolating the bacterium can be notably difficult from substrates containing high background flora numbers (e.g. sewage, slurry, manure, and soil) as the population of *E. coli* O157 usually represents only a small fraction of the total bacteria load. Furthermore, *E. coli* O157 may closely resemble other *E. coli* serotypes in many aspects, raising problems in characterising the pathogen (Bettelheim and Beutin 2003).

The diversity of available *E. coli* O157 detection methods provides versatility in microbiological work. Still, the validity of even newly developed techniques has occasionally been called into question due to false-positive/negative results, or the method has been found to isolate only non-verocytotoxin producing *E. coli* O157 strains (Veronzy-Rozand *et al.* 1998). The merit of each method is a function of the required sensitivity, speed of results, availability, costs, required expertise, and its efficiency for the specific matrix tested. Ultimately, combining techniques may be the most comprehensive means of

detecting and quantifying *E. coli* O157. However, whatever method/technique is employed, the results must be interpreted with care, in particular, with regards to calculating pathogen densities from samples. Such calculations are based on the assumption that the organism is evenly distributed throughout a sample; when it is manifest that this may not be true. As a result, samples may be falsely classed as containing no *E. coli* O157, or, conversely, erroneously portrayed as being highly contaminated (Allen *et al.* 2000).

2.9.1. Culture-based methods

Cultural methods (those that grow bacteria on a prepared medium), have been used for bacteria detection and enumeration for over a century (Pyle *et al.* 1995) The most widely used culture for *E. coli* O157 isolation is SMAC (Sorbitol MacConkey) agar. Due to it's simplicity of use and low cost, it has been the mainstay for *E. coli* O157 recovery for a number of years. SMAC was developed on knowledge of the biochemical and nutritional differences of the O157 serotype relative to other *E. coli* varieties (Park *et al.* 1999). Principally, the ability of SMAC to isolate *E. coli* O157 from other serotypes is based upon the inability of serotype O157 to ferment sorbitol, - whilst most *E. coli* isolates ferment sorbitol within 24 hours (March and Ratnam 1986; Doyle 1991; Vernozy-Rozand 1997). As a result, *E. coli* O157 colonies appear colourless, and are therefore readily distinguishable from other bright pink colonies on the agar (Fig. 2.24). Once dilutions have been plated, plates are incubated at approximately 37°C for 18 hours, and the number of colonies calculated from the mean colony forming units (CFU) counted on the respective dilution (Oxoid products website 2006).

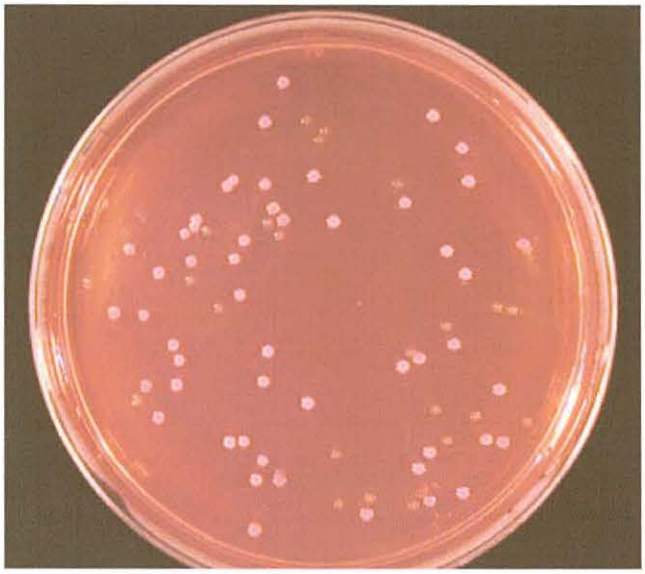

Figure 2.24. *E. coli* O157 (colourless/clear) and *E. coli* colonies (pink) on SMAC agar.

March and Rantam (1986) reported *E. coli* O157 detection on this medium had a sensitivity of 100% and a specificity of 85%. Nevertheless, discrepancy occurs in the validity of this recovery method, mainly stemming from potentially false-positive counts. This may occur due to the inability of some other Gram-negative organisms in addition to *E. coli* O157 e.g. *Pseudomonas* and *Proteus* (widely distributed in nature) to ferment sorbitol (Chapman *et al.* 1991; Park *et al.* 1999, Fujisawa *et al.* 2002). Other *E. coli* serotypes, such as types O111 and O26 may also show inability to ferment sorbitol (Bettelheim 2000). As a result, colonies of these species may be mistaken to be those of *E. coli* O157. Significant growth of sorbitol-fermenting organisms on plates following recovery from some substrates may render *E. coli* O157 detection impossible, as experienced by Chapman *et al.* (2001b). Paradoxically, there is also evidence to suggest that recovery methods using culture techniques may underestimate numbers due to clumping, particle association, cell injury, and especially at times of cell stress when cells may enter the VBNC state (Abdul-Raouf *et al.* 1993b). Although cells maintain viability and metabolic activity in the VBNC state, colonies fail to grow and multiply on culture plates, leading to undervalue quotations.

The desire for more reliable and accurate isolation methods led to the development and inclusion of numerous additives to conventional SMAC agar. These permit easier detection of the desired organisms as they encourage colony growth, whilst limiting the growth of other (non-desired) organisms. Chapman *et al.* (1991) added two such compounds,

rhamnose, and cefixime (CR-SMAC agar). They concluded that whilst rhamnose is not fermented by *E. coli* O157, it is by most non-sorbitol-fermenting *E. coli* of other serogroups. Furthermore, cefixime is a cephalosporin antibiotic with greater activity against *Proteus* species than against *E. coli*. It was found that the selectivity of this isolation method was enhanced when these compounds were included. Zadik *et al.* (1993) further developed the idea, with inclusion of potassium tellurite with cefixime to SMAC agar (CT-SMAC). They found that whilst the agar permitted growth of *E. coli* O157 and *Shigella sonnei*, it partially or completely inhibited growth of 67% of other *E. coli* strains and all or most strains of other non-sorbitol-fermenting species tested. A synopsis of research papers suggests that CT-SMAC agar is the preferred agar for isolation of *E. coli* O157 today, although controversy still arises on the aptness of such isolation method. MacRae *et al.* (1997) and Taormina *et al.* (1998) claim stressed or damaged cells in unfavourable matrices can be highly susceptible to cefixime and tellurite, hence leading to the poor isolation of cells. However, these fears may be overcome as it's been shown that using reduced tellurite concentrations permits growth of *E. coli* O157 strains sensitive to the compound, whilst inhibiting background flora as successful (Hiramatsu *et al.* 2002; Wasteson 2002). Blackburn and McCarthy (2000) found that the incorporation of a non-selective pre-enrichment medium improved *E. coli* O157 detection rates by up to ten-fold when used in conjunction with other recovery methods.

Agars other than SMAC have also been formulated to isolate *E. coli* O157. Wallace and Jones (1996) employed selective and differential agars for the isolation of the bacterium from the faeces of dairy herds, but reported the problem of widespread false-positive counts with many. Thippareddi *et al.* (1995) developed a universal pre-enrichment (UP) medium containing oxyrase which enhanced growth of *E. coli* O157, allowing recovery of injured bacteria from a range of different foods. Silk and Donnelly (1997) reported that using trypticase soya agar allowed detection of acid-injured *E. coli* O157:H7 at higher sensitivities than other media; permitting identification of viable organisms after 72 hours in cider of pH 3.2. Chapman (1989) concluded that the latex slide agglutination test offered a rapid and economical assay for identifying *E. coli* O157. The development of commercially available antiserum or latex agglutination kits to assay for the O157 antigen permits rapid, easy confirmation of suspect colonies on all agars (Mead and Griffin 1998; Oxoid products website 2006). However, a foremost shortcoming of the culturing method for *E. coli* O157

detection is that isolation and positive identification of the organism is a multi-step procedure, requiring a day as a minimum, which is of disadvantage when rapid elucidation and detection of the organism is required (Campbell *et al.* 2001).

2.9.2. Immuno-magnetic methods

In addition to being time-laborious, separation of organisms such as *E. coli* O157 from a mixed medium (e.g. food, slurry) via culture-based methods may be ineffective without pre-enrichment steps (Safařik *et al.* 1995). The development of immuno-magnetic separation (IMS) techniques eliminates this problem by using a specific magnetic to separate the target organism directly from the sample or pre-enrichment medium (Deisingh and Thompson 2004).

Particles displaying paramagnetism permit easy removal by a magnetic separator (Safařik *et al.* 1995). Dynabeads^{R} (Dynal, Oslo, Norway) are the most common magnetic carriers used. These are small polystyrene-based particles coated with covalently immobilised streptavidin or secondary antibodies against selective primary antibodies (Deisingh and Thompson 2004). *E. coli* O157 isolation using IMS may occur in two ways. The method may be employed directly, whereby immunomagnetic particles specific for the target organism are suspended in the mixed cell suspension. Bacteria presenting the O157 surface antigen on their surface adhere to the magnetic beads via reacting with their antibody coating. This enables subsequent separation of bound target cells from the suspension with a magnetic particle separator, and the removal of remaining suspension (Vernozy-Rozand 1997). Subsequently, the magnetic particles are washed, and cells are habitually cultured onto CT-SMAC agar, which further separates the desired organism (Safařik *et al.* 1995; Wasteson 2002). Indirectly, IMS may be used to isolate *E. coli* O157 via adding primary antibodies to the suspension, whereby it again binds to form a bacteria-bead complex. Thereafter, magnetic particles with immobilised secondary antibodies are added, which interact with primary antibodies. This permits removal of the entire complex from the suspension using a magnetic separator (Safařik *et al.* 1995; Wasteson 2002).

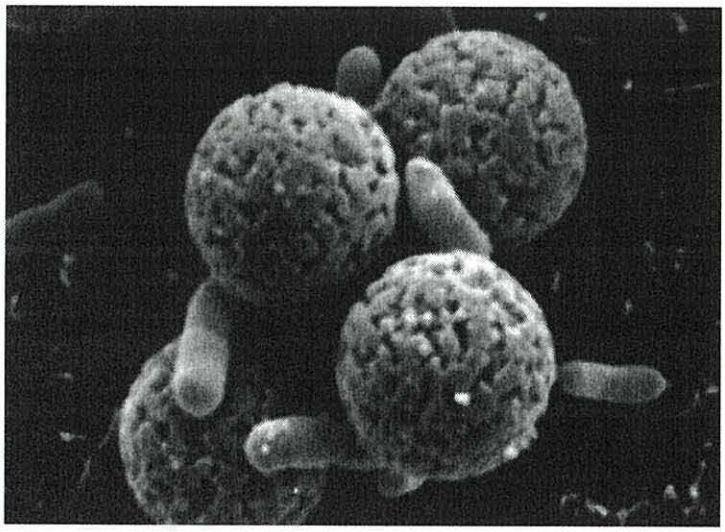

Figure 2.25. *E. coli* O157 bacteria (rod-shaped) immobilisation onto magnetic beads (D.L. Jones, personal communication).

IMS beads can also be altered to isolate other *E. coli* serotypes linked to human infection via changing the surface antigens for the specific target (Bettelheim and Beutin 2003). This greatly increases the technique's expediency. Šafaříková and Safařik (2001) demonstrated this by using IMS to isolate serotype O26 and O111 from inoculated vegetables, yielding recovery values of 93-100%. Urdahl *et al.* (2002) also illustrated the versatility of the method when isolating *stx*-producing serotype O103.

The applicability of IMS techniques for *E. coli* O157 research has received a good deal of interest. Chapman *et al.* (1994) and Chapman and Siddons (1996) examined IMS for isolation of *E. coli* O157 from bovine faeces and human faecal samples, respectively. For both experiments, samples were enriched in modified buffered peptone water, and cells were subsequently concentrated by IMS. This recovery method was compared with direct culture onto CR-SMAC and CT-SMAC. Distinctly superior recovery rates were acquired using the former method, regardless of sample type. In fact, when examining bovine faecal material inoculated with 12 different strains of *E. coli* O157, IMS was approximately 100-fold more sensitive for detection than direct culture on either medium (Chapman *et al.* 1994). Karch *et al.* (1996) also reported 100-fold increased sensitivity of IMS compared to other methods when recovering *E. coli* O157 from stool and serum samples from HUS patients. The usefulness of IMS for sensitive detection of *E. coli* O157 in infection cases was clearly illustrated by Chapman *et al.* (1997a). In this instance, four families were infected via cross-contamination and secondary transmission from the same initial source

(manure-contaminated potatoes). Whereas direct culture of faecal samples onto CR-SMAC and CT-SMAC isolated *E. coli* O157 from only one patient, a combination of IMS and detection of *E. coli* O157-specific secretory IgA suggested *E. coli* O157 infection in all eight symptomatic patients, but not in any family member who wasn't ill. Working with meat samples inoculated with various strains of *E. coli* O157, Bolton *et al.* (1996) and Chapman and Ashton (2003) reported the benefits of IMS followed by culture of magnetic beads onto CT-SMAC agar for detection of the organism on beef carcasses. Likewise, Onoue *et al.* (1999) evaluated a range of methods for *E. coli* O157 detection from contrasting substrates (ground beef and radish). They too found greater exclusivity when IMS coupled with CT-SMAC plating was used for isolation of the organism. By combining IMS and sub-culturing onto CT-SMAC, Voitoux *et al.* (2002) detected *E. coli* O157 cells up to 1-2 CFU 25 g^{-1} from dairy products. Wright *et al.* (1994) demonstrated the sensitivity of IMS when isolating *E. coli* O157 from food samples. After comparison of a range of isolation techniques following a milk-borne outbreak of *E. coli* O157 infection, Cubbon *et al.* (1996) also recommended IMS for isolation of O157 VTEC. The conclusion from many of these experiments was that IMS is a straightforward, useful tool for elucidating numbers of *E. coli* O157 from a range of contrasting samples.

However, the validity of *E. coli* O157 recovery using IMS has also been questioned. Hara-Kudo *et al.* (2000) suggested antibody-based approaches may result in false negative detection due to stress/starvation inducing loss of surface antigen properties. Conversely, non-target organism carry-over may occur due to bacterial adhesion on the test tube walls (Vernozy-Rozand 1997). Campbell *et al.* 2001 acknowledged that the time required for a positive identification to be a major limitation of using IMS. Parham *et al.* (2003) described the limitations of IMS as they deemed it as being employable as a qualitative (not quantitative) detection strategy only.

2.9.3. Nucleic acid-based methods

Although the development of immunological methods was a great innovation in detecting *E. coli* O157, these methods can't always differentiate serotype O157:H7 from other (less virulent) EHEC; whilst some shiga toxins may not be detected at all (Karmali 1989). Similarly, other methods based on detecting the O157 somatic and H7 flagellar antigens displayed obvious shortcomings in specificity (Paton and Paton 1998). However, isolation

methods based on the biomolecular properties of pathogens such as *E. coli* O157 may present a notable progress.

The biomolecular make-up of generic *E. coli* and *E. coli* O157 is complex but well studied; a comprehensive and definitive account of which is given by Park *et al.* (1999). Polymerase chain reaction (PCR) analysis is a nucleic acid-based method used for the detection of numerous bacteria, including *E. coli* O157. The exact methodology of PCR is given in Alvarez and Toranzos (1997). In summary, PCR uses a thermostable polymerase enzyme (e.g. the *Taq* enzyme) to create multiple copies of a target section of a micro-organism's DNA. Detection of target DNA is achieved through the use of short sections of synthetic, single-stranded DNA known as oligonucleotide primers; which can be designed to be specific for an individual organism, or for a group of organisms. PCR works by using a cycle of (usually three) distinct, different temperatures. Firstly, a high temperature (commonly > 90°C) separates the double-stranded DNA into individual strands (Alvarez and Toranzos 1997; Toze 1999). Thereafter, a lower temperature is used to anneal the primers to the target section of DNA. At an intermediate temperature between the previous two temperatures, the polymerase enzyme produces a mirror copy of the target DNA, and the temperature cycle is repeated 25-30 times. Due to the exponential nature of the PCR method, more than 10^9 copies of the target DNA can be theoretically produced (Toze 1999). This large number of a target DNA segment can then be detected using gene probes or agarose gel electrophoresis. Gel electrophoresis is a process used to impart an electric current to DNA fragments in a gel of specific density. Different sized fragments move at different rates and can be visualised as a series of 'bands' in the gel (Toze 1999).

The effectiveness of PCR assay is not dependent on large amounts of target DNA, and it rapidly detects pathogens to high specificity and sensitivity (Meng *et al.* 1994, 1996). As DNA from a single bacterial cell can be amplified very rapidly (about 1 hour; Deisingh and Thompson 2004), this presents obvious benefits for PCR-based isolation techniques. The sensitivity of PCR has been reported in many papers e.g. Chapman *et al.* (2001b) found PCR was up to 4 $\log_{10}$ more sensitive than culture for detecting three strains of *E. coli* O157 from minced beef samples following enrichment. Furthermore, whereas the applicability of other *E. coli* O157 detection methods may be limited when cells are stressed, PCR sensitivity appears to be unaffected (Campbell *et al.* 2001). This was shown when cells previously exposed to very high temperatures (121°C) were still detectable using PCR

(Vernozy-Rozand 1997). Several variations of PCR have evolved from standard PCR, creating a versatile, automated, and sensitive *E. coli* O157 detection method. Of these methods, multiplex PCR and real-time PCR are the most implemented (Deisingh and Thompson 2004).

With multiplex PCR, several targets are co-amplified in one PCR by 'multiplexing' primer pairs. With regards to detecting *E. coli* O157, this permits the presence of numerous important 'markers' to be obtained e.g. O157, H7, intimin and *stx* genes (Campbell *et al.* 2001; Cagney *et al.* 2004; Deisingh and Thompson 2004). Crucially, this differentiates between non-O157 serotypes in one step (Hu *et al.* 1999). In addition, determination of the *stx* and O-antigen gene profile in clinical cases enables identification of related cases and the exact serotype in question (Perelle *et al.* 2004). Cebula *et al.* (1995) displayed the value of multiplex PCR by simultaneously amplifying two alleles to identify the O157:H7 serotype and the shiga toxin for which it encoded. The ability of multiplex PCR to co-amplify many genes renders it especially useful to distinguish a specific *E. coli* serotype from other serotypes; especially in substrates with high numbers of background bacteria e.g. slurry. In short, multiplex PCR appears to offer a sensitive and specific method of detecting *E. coli* O157, whilst being capable to detect phenotypic variants of serotype O157:H7 (Park *et al.* 1999).

Real-time PCR allows characterisation of reactions by the time when amplification of the PCR product is first detected (via the use of a fluorogenic probe). Increased speed is one of many advantages of real-time PCR in relation to conventional PCR (Fitzmaurice *et al.* 2004). The detection of amplified products occurs during the amplification step in real-time PCR, eliminating the need for post-PCR manipulations such as gel electrophoresis. This reduces the potential of cross-contamination and thus increases sensitivity (Fortin *et al.* 2001; Yoshitomi *et al.* 2003). Furthermore, real-time quantitative PCR assays measure the amount of PCR product generated in the exponential phase of PCR, providing more accurate and reproducible quantification data (Wenli and Drake 2001; Fitzmaurice *et al.* 2004). The quantification range of between four and five logarithmic decades greater than conventional PCR ensures a wider quantification range when using real-time PCR (Klein 2002). In addition, recent advances in real-time PCR technology allow quicker amplification and detection of gene targets (Bettelheim and Beutin 2003). One of the greatest assets of real-time PCR is its ability to quickly detect minor changes in genetic

sequences of related bacteria, aiding the battle against new serotypes or strains of *E. coli* (Yoshitomi *et al.* 2003). Usefully, Bhagwat (2003) demonstrated the ability of real-time PCR to simultaneously detect *E. coli* O157 with two other common food-borne pathogens, *Salmonella typhimurium* and *Listeria monocytogenes*. This may inexorably offer substantial time savings in identifying the instigator of infection outbreaks.

Other variations of PCR such as reverse transcriptase PCR (RT-PCR) have been developed in recent years. RT is an enzyme which synthesises single-stranded DNA from RNA, and is of high sensitivity. As this method does not require pre-enrichment steps, this saves time in analysis (McIngvale *et al.* 2002; Deisingh and Thompson 2004). A PCR system which amplifies DNA, followed by gel electrophoresis to determine the presence or absence of a specific gene has been developed, called BAX® automated PCR (Fritschel 2001; Deisingh and Thompson 2004). This PCR system has proved swift and effective for *E. coli* O157 identification from a range of foods and environmental samples (Johnson *et al.* 1998); however, as yet it is too early to pronounce whether it is a valuable method for all-round *E. coli* O157 quantification (Deisingh and Thompson 2004).

PCR necessitates the use of skilled technicians and specialised equipment which may be expensive (Toze 1999). Experimental design must take into account the potential of PCR to amplify the DNA of dead bacteria cells which may over-estimate the numbers of bacteria in a sample (Deisingh and Thompson 2004). Consistency in the mode of sample preparation is also paramount as this may have a profound effect on the isolation of *E. coli* O157 (Gioffé *et al.* 2002). Some soil properties (e.g. humic content) have been shown to affect or inhibit successful PCR (Tebbe and Vahjen 1993), and factors inhibitory to PCR may also be present in stool cultures (Beutin *et al.* 1998). Together with samples where *E. coli* O157 numbers are low in relation to other bacteria, PCR-inhibitory effects might result in false-negative counts of the target bacterium (Beutin *et al.* 1998; Bettelheim and Beutin 2003). Conversely, false-positive results may occur in PCR analysis when alternate DNA gives PCR amplification products, or when cryptic target gene sequences are present in a sample (Karch *et al.* 1992; Bettelheim and Beutin 2003). Past instances using only PCR methods for *E. coli* O157 detection has led to errors in tracing infection sources, as highlighted by Bettelheim and Beutin (2003). This underlines the need for a secondary *E. coli* O157 detection system to be used for confirmation of samples. All the while, PCR alone only indicates the presence or absence of a target genetic material, and does not

directly provide information about the viability or infectiousness of *E. coli* O157; which would be desirable as DNA may persist in the environment (Sobsey *et al.* 1998). For routine analysis of food or clinical specimens, the complexity and costs of multiplex PCR assays may also limit their use (Park *et al.* 1999). Conventional PCR assays require less expensive and less sophisticated equipment than real-time PCR methods (Perelle *et al.* 2004). However, combining a multiplex real-time PCR strategy would be the best approach for future large time-savings (Perelle *et al.* 2004).

2.9.4. Additional methods

Biosensors are included in a new wave of emerging technologies developed to isolate *E. coli* O157. Briefly, a laser diode is used to direct light into optical fibres, exciting fluorescent molecules within the evanescent field to create an evanescent-wave. A part of the emission re-couples into the fibre probe, which is then quantified by a photodiode (Deisingh and Thompson 2004). Whilst it appears that discrepancy exists in the sensitivity of biosensors at this early stage of development, it is clear that they provide unprecedented time savings when detecting *E. coli* O157, as results from samples are often retrieved in seconds (Deisingh and Thompson 2004).

Johnson *et al.* (2001) reported on the use of laser-induced fluorescence coupled with flow cytometry for detecting *E. coli* O157. This method possesses notable benefits, including the ability to detect single organisms, it is an automated system, it displays high specificity, and large volumes of a sample material may be examined in real-time. Bono *et al.* (2002) used one such system, called RAPID (Ruggedized advanced pathogen identification device) for detecting *E. coli* O157 from bovine, food, and human samples. In addition to being accurate, employment of this kit (which is based on an air thermocycler with fluorescence monitoring) enabled identification of the bacterium within 30 minutes.

Other attempts to differentiate *E. coli* O157 cells to other bacteria include staining cells with a green fluorescent protein (GFP). Fratamico *et al.* (1997) explored the potential of tracking *E. coli* O157 in apple juice and cider by introducing green fluorescent protein genes from the firefly (*Photinus pyralis*) to the pathogen. A similar project was later undertaken by Burnett and Beuchat (2002). Here, cells were primarily treated with chemicals, and changes in fluorescence observed through an electron microscope. The authors reported that different substances were required to determine the proportions of

living and dead cells. In addition to studies monitoring *E. coli* O157 survival and behaviour, these readily identifiable and stable markers could be useful as positive controls in microbial assays (Fratamico *et al.* 1997). Siragusa *et al.* (1999) used a bioluminescent reporter to study bacteria attached to carcass surfaces. Tissue on the carcass surface was inoculated with the bacterial strain, and visualisation of adherent bacteria was completed using a photon-counting camera. As this generated images in-situ, it allowed valuable real-time quantification of carcass contamination levels and eliminated the need for exogenous sampling (Siragusa *et al.* 1999). Bioluminescence has also been used to study the survival of *E. coli* O157 in cheese (Ramsaran *et al.* 1998), water (Artz and Killham 2002; Ritchie *et al.* 2003), and soil (Ritchie *et al.* 2003). Kourkine *et al.* (2003) combined immunofluorescent staining and capillary electrophoresis for the successful detection of the bacterium. Pulse-field gel electrophoresis (PFGE) of genomic DNA is a method which has been used to differentiate between *E. coli* O157 bacteria of different strains, and is recommended to be used in conjunction with other typing methods in epidemiological studies of VTEC infections (Vernozy-Rozand 1997).

Many other immunoassay-based methods, too numerous to list, are commercially available for detection of *E. coli* O157. Blackburn and McCarthy (2000) evaluated six such methods, including: Reveal 8 *E. coli* O157-H7 screening test and VIP EHEC (based on immunoprecipitation technology), VIDAS *E. coli* O157, EHEC-Tek, and Tecra *E. coli* O157 visual immunoassay (based on the 'ELISA' technique, see afterward). Vernozy-Rozand (1997) also reports on the success of the VIDAS method and Chapman *et al.* (2001b) evaluated two visual immunoassays, BioSign™ and Path-Stik™. Both studies conclude immunoassays to be extremely rapid and easy to perform, and Chapman *et al.* (2001b) witnessed favourable sensitivity compared to culturing techniques. Nevertheless, the specific pre-enrichment required adds to the total time required for detection, and may lead to underestimations of *E. coli* O157 numbers as stressed or injured cells may die in the enrichment process (Blackburn and McCarthy 2000; Chapman and Ashton 2003). However, such problems may be overcome by incorporating a pre-enrichment stage which provides optimal conditions for the recovery of injured cells, prior to the application of selective agents (Chapman and Ashton 2003). Not all immunoassay-based methods show the same degree of success (Chapman and Ashton 2003); therefore evaluation of different methods is advisable prior to opting for a specific type.

Deisingh and Thompson (2004) report of three technologies which deserve noteworthy attention in *E. coli* O157 detection. These are: microarrays, molecular beacons, and integrated (lab-on-a-chip) systems. Due to their ability to simultaneously detect thousands of specific DNA or RNA sequences without the need for pre-enrichment, microarrays have been reported to yield extremely sensitive results in a short time period (Wu *et al.* 2003; Deisingh and Thompson 2004). Microarrays have also been shown to successfully distinguish different *E. coli* O157 genotypes, and to detect antibiotic resistant markers (Wu *et al.* 2003). It has also been found to work well in combination with IMS when detecting *E. coli* O157 in poultry wash-water (Chandler *et al.* 2001). However, the costs involved, their limited availability, and the expertise needed to operate the technology and analyse microarray data may suppress its applicability, at least when the method is still in its infancy (Deisingh and Thompson 2004). Molecular beacons (MBs) are oligonucleotide probes that fluoresce upon hybridisation to their complementary DNA target (Fortin *et al.* 2001). The stem-loop structure of the probes consists of two short differently-labelled oligodeoxynucleotide arms. Hybridisation of the loop causes the stems to open and separate, leading to emission of fluorescence (Deisingh and Thompson 2004). Fortin *et al.* (2001) developed a real-time PCR assay in conjunction with MB to detect the presence of *E. coli* O157:H7 in milk and apple juice. MBs may be carried out entirely in sealed PCR tubes, thus preventing sample contamination. The probes may be designed to hybridise different regions of *E. coli* O157 genes; ensuring rapid, semi-automated differentiation between even very closely related strains in food and environmental samples. DNA probe-based tests specific for the genes of *stx* production, or those responsible for the attaching and effacing of the microvilli of entterocytes during infection have been developed. Probes may also be designed to differentiate between the two types of *stx* produced by bacteria, or the several variants of *stx* 2 (Vernozy-Rozand 1997). Feng (1993) used a probe which was specifically directed to a gene region only expressed by *E. coli* serotype O157. Although useful, the use of such DNA probes is limited from the fact that large amounts of DNA, along with thousands of cells are required from a sample to produce a detectable signal (Feng 1993). Although the use of nucleic acid-based approach in *E. coli* O157 detection is undoubtedly useful, most require the use of an initial recovery method e.g. IMS (Perelle *et al.* 2004). However, a brief enrichment period does significantly improve their sensitivity (Fortin *et al.* 2001). Lastly, Deisingh and Thompson (2004) address the

promising field of lab-on-a-chip systems for *E. coli* O157 detection. Although work using such systems with other bacteria has proved successful, this method is in its infancy regarding research with *E. coli* O157. Essentially, it involves merging different biomolecular-based systems to identify genotypic features typical of *E. coli* O157. Evidently, this may present a potentially valuable method of pathogen detection in the future.

Enzyme-linked immunosorbent assay (ELISA) has also been developed to detect *E. coli* O157 via tagging an antibody with an enzyme. Following incubation, an enzyme substrate is added, and the formation of a pigmented product is indicative of the amount of enzyme (and therefore the amount of micro-organism) present in the sample. From this, many other different methods have developed e.g. automated enzyme-linked fluorescence immunoassay (ELFA) to detect *E. coli* O157 from a range of matrices; including food, and stools (Dylla *et al.* 1995; Vernozy-Rozand *et al.* 1998; Urdahl *et al.* 2002). Furthermore, positive samples according to ELFA may be confirmed by incorporation of an automated immunoconcentration system e.g. VIDAS which allows selective capture and release of target organisms (Vernozy-Rozand *et al.* 1998). From this work, it is gathered that ELISA techniques offer a sensitive, accurate, robust, versatile, and easily readable screening method for detecting *E. coli* O157. However, as with any immunoassay, limitations are related to the specificity of the antibodies used. The labour-intensive nature and expense of these methods, along with a tendency to overestimate bacteria numbers (which can be difficult to identify) may also limit their use (Chapman *et al.* 2001b).

2.10. Control and reduction of *E. coli* O157

2.10.1. Animal diet/feed alterations

Due to the ubiquitous nature of the pathogen, its short infection period, and its ability to survive in such diverse environments, it seems that traditional means of controlling infectious agents (such as eradication or test and removal of carrier animals), are unfeasible for *E. coli* O157 (Hancock *et al.* 2001). Nevertheless, amending certain farm management practices (especially those which influence the incidence of *E. coli* O157 in animal feed and water) may provide practical means to reduce the prevalence of this organism on farms, abattoirs, and meat (Jordan *et al.* 1999; Hancock *et al.* 2001); hence the number of human infections.

Reducing *E. coli* O157 survival, proliferation, and viability in the ruminant gut should efficiently lessen *E. coli* O157 prevalence in the environment. Many studies have reviewed the value of altering ruminant feeds or diets in a bid to reduce levels of pathogenic *E. coli* in the animal gut. Supplying contaminated feed to animals could clearly be a pathway for herd infection. Herriott *et al.* (1998) found a tentative association between the prevalence of *E. coli* O157 within cattle herds and the feeding of animal by-products to cows; however, this practice is now forbidden in the UK. As previously discussed (section 2.7.1.1; Table 2.8), the persistence of *E. coli* O157 on vegetation may lead to contamination of cattle-feed crops such as silage. Inefficient ensiling methods may not generate the heat and acidic conditions (from fermentation) needed to destroy pathogens such as *E. coli* O157 (Fenlon *et al.* 2000). Indeed, poor ensiling and delayed fermentation (e.g. via inletting air to the system) may provide conditions suitable for the growth of the bacterium (Fenlon *et al.* 2000; Fenlon and Wilson 2000). This may explain why a significantly higher prevalence of *E. coli* O157 has been noted in cattle fed silage compared to those not (Herriott *et al.* 1998); and highlights the need for efficient ensiling of potentially manure-contaminated crops.

Recent work indicates that rumen diets may be changed to decrease fermentation acid accumulation in the colon. When fermentation acids accumulate in the colon and the pH decreases, the numbers of acid-resistant *E. coli* O157 increase; which are more likely to survive in the gastric stomach of humans (Russell *et al.* 2000). Feeding calves high-concentrate diets may rapidly induce development of acid-tolerant *E. coli* O157 in the rumen fluid. This permits larger populations to survive passage through the acidic abomasum and to proliferate in the colon hence influence faecal shedding (Tkalcic *et al.* 2000). Feeding varying concentrate diets to lambs formulate similar outcomes (Lema *et al.* 2002). Grain feeding also seems to promote the growth of acid resistant *E. coli* O157 in fattening beef, as highlighted by Buchko *et al.* (2000) and Russell *et al.* (2000). In the former study, steers were fed one of three different grain diets inoculated with *E. coli* O157; and the presence of the inoculated strain was traced in the rumen fluid and faeces for 10 weeks. Significantly higher numbers of animals fed barley were culture positive for the strain as opposed to those fed corn. Feeding cattle with hay for a brief period (< 7 d) may dramatically reduce both levels of acid-resistant *E. coli* O157 and the number of cattle shedding the bacterium (Russell *et al.* 2000). Kudva *et al.* (1995, 1997) showed that stress induced by a sudden change in sheep diet initiated every animal to carry the bacterium

within seven days (or within 24 hours in some cases) of the dietary change. However, it is unclear whether a sudden change of diet to hay may reduce carriage of VTEC in cattle as witnessed by Russell *et al.* (2000), or that the dietary change may invoke an increase; as witnessed in sheep by Kudva *et al.* (1995, 1997). Anticipating both possible events, it seems that careful timing of such dietary change would yield the greatest reduction in animal carriage of *E. coli* O157; although this warrants further investigation. Rasmussen *et al.* (1993) and Duncan *et al.* (2000) report how feed withdrawal prior to animal slaughter reduces the volume of volatile fatty acids (weak acids with bactericidal properties) produced by anaerobic microbes in the rumen; leading to increased *E. coli* O157 levels in the intestinal contents (Fig. 2.26). Feed withdrawal is common practice employed at abattoirs prior to slaughter (to reduce the volume of waste generated hence probability of cross-contamination), but may therefore prove counter-productive with regards to hygiene. It is also apparent that recent colonisation of the gut by this bacterium does not prevent re-colonisation (Kudva *et al.* 1997).

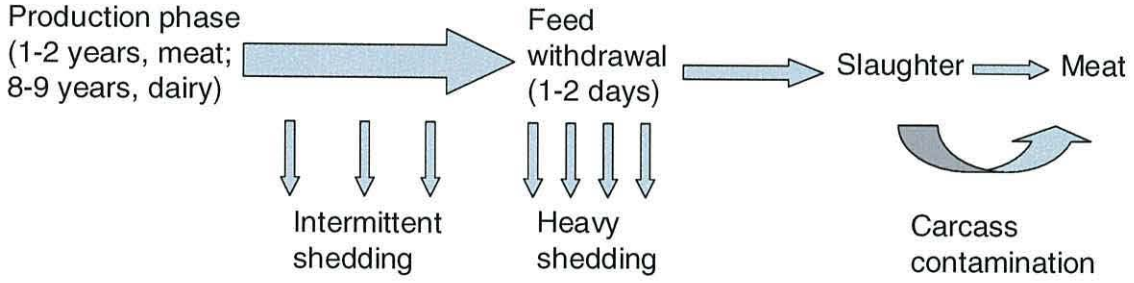

Figure 2.26. Shedding of *E. coli* O157 during production and processing of meat. Intermittent shedding may occur during the production phase. Numbers of the bacterium in the gut may increase when the feed is withdrawn shortly before slaughter. Meat may then be contaminated by transfer of faeces or gut contents in the slaughterhouse (Duncan *et al.* 2000).

The efficacy of feeding probiotic supplements to reduce *E. coli* O157 proliferation within rumens is well studied, with encouraging results from animal of different species and ages. The benefits of feeding a bacterial mélange (of either *Lactobacillus acidophilus*, *Streptococcus faecium*, a mixture of the two, and a mixture of *L. acidophilus*, *S. faecium*, *Lactobacillus casei*, *L. fermentum* and *L. plantarum*) to lambs was evaluated by Lema *et al.* (2001). It was shown to reduce both the total number of *E. coli* O157 shed in faeces, and to improve animal meat production performance. Supplementing cattle feed with a high dose

mixture of *Lactobacillus* spp. and *Propionibacterium* spp. has also been shown to reduce *E. coli* O157 prevalence in faeces and on cattle hides (Younts-Dahl *et al.* 2004). A probiotic product containing lactic acid-producing *S. bovis* and *L. gallinarum* was isolated from cattle by Ohya *et al.* (2000). Oral dosage of this completely inhibited faecal shedding of *E. coli* O157 in calves; and no animal re-started shedding thereafter. The attenuation of *E. coli* O157 in faeces correlated with the notable increase of VFAs in faeces subsequent to the administration of probiotic bacteria (Ohya *et al.* 2000). Although only in preliminary stages of research, feeding of *Enterococcus* or non-pathogenic *E. coli* to animals may notably reduce the occurrence of *E. coli* O157 in animal faeces (Loneragan and Brashears 2005). Studies have also shown supplementary feeding of *Saccharomyces cerevisiae* to heifers may initiate production of substances which inhibit *E. coli* O157 growth and survival (Bach *et al.* 2003). Zhao *et al.* (1998) isolated bacteria inhibitory to *E. coli* O157 from cattle; and from these, administered a selected probiotic mix to calves. After 30 days, *E. coli* O157 was recovered from the faeces of one animal; however, the organism was not recovered from the rumen of any animal treated with probiotic bacteria. The authors concluded that administration of selected probiotic bacteria to cattle prior to exposure to *E. coli* O157 may reduce the level of carriage of *E. coli* O157 in most animals. However, whilst oral administration of probiotics to calves may yield successfully reduce carriage of *E. coli* O157, such results may not be extrapolated to cattle. This is because higher levels of native bacteria inhabit the gut in older ruminants, hence the probiotic species may be out-competed (Patriquin 2000). When applying a direct-fed microbial product to reduce carriage of VTEC in animals, two apparent salient points are that both the dose level and selection of particular bacterial strains are important. In fact, administration of the wrong microbial strain may lead to a consequent increase in *E. coli* O157 shedding and resultant hide contamination (Loneragan and Brashears 2005). Whilst a low dose level of the correct microbial strain will reduce carriage, higher dose levels will yield superior results (Younts-Dahl *et al.* 2004; Loneragan and Brashears 2005).

Both Smith *et al.* (1987) and Kudva *et al.* (1999) have explored the use of strain-specific bacteriophages to control *E. coli*. The former study showed phages may possess major potential for the control of *E. coli* infections in cattle; - perhaps curing or preventing diarrhoea in infected calves when applied orally or sprayed on the litter (Smith *et al.* 1987). However, phage administration to infected animals was only effective when administered

before or together with the infective bacteria. In an effort to find phages lethal to *E. coli* O157 and not to background flora, Kudva *et al.* (1999) successfully employed phages that binded to the O157 antigen and to other common *E. coli* receptors, such as pili, fimbriae, flagella, and other outer membrane proteins. The study proved that naturally prevalent O157-specific phages (or mutants of these phages) could be used to eliminate *E. coli* O157 from the gastrointestinal tracts of carrier ruminants (Kudva *et al.* 1999). Incorporation of such phages into ruminant diets may thus provide an effective bio-control mechanism against *E. coli* O157.

Recent studies have highlighted the effectiveness of adding chlorate supplements to cattle feed and water sources in reducing numbers of both generic and pathogenic *E. coli* from the animal gut and faeces (Callaway *et al.* 2002; Anderson *et al.* 2005). Of significance was that animals exhibited no symptoms of chlorate toxicity and negative effects on feed or water intake or animal performance were not observed (Anderson *et al.* 2005). However, Callaway *et al.* (2002) fed potassium nitrate to animals to up-regulate the nitrate reductase enzyme which aids in eliminating pathogenic cells. Extrapolation of these results to naturally infected feedlot cattle not consuming supplemental potassium nitrate must therefore occur with caution. Nevertheless, supplementing animal diets with sodium chlorate may still be an efficacious pre-harvest intervention; with the additional benefit that it may be used for a variety of food-borne pathogens that are facultative anaerobic e.g. *Salmonella* (Callaway *et al.* 2002; Loneragan and Brashears 2005).

Loneragan and Brashears (2005) reported how *E. coli* O157 isolates from naturally infected animals were susceptible to neomycin sulphate, an antimicrobial drug. Faecal shedding and hide contamination were significantly reduced in harvest-ready cattle. As this drug is approved for in-feed or in-water administration to cattle for the control or treatment of colibacillosis and because of its route of administration, it can be applied to entire groups of animals. Canadian Scientists explored the possibility of expressing an *E. coli* O157-specific antibody in plants and then incorporating the plant-made antibody into animal feed. In theory, cattle feeding on this plant would possess a natural antibody that would lead to the elimination of *E. coli* O157:H7 in their digestive tracts. When coming into contact with the bacteria, it is anticipated that the antibody would prevent *E. coli* O157 from attaching to the bovine intestinal wall and therefore inhibit its growth (Webb 2003). The findings of such research are of interest.

Collective observations indicate that animal feeding practices may be manipulated to decrease the risk of food-borne illness from *E. coli* O157; and remain practical options in confined animal feeding operations. However, the knowledge of how diet impacts the faecal shedding of *E. coli* O157 is still vague, and may depend on dietary effects on fermentation in the colon and on diet-induced changes in the resident microflora (Tkalcic *et al.* 2000). Numerous dietary supplements initially considered of potential use in reducing proliferation of *E. coli* O157 have proved futile (Bach *et al.* 2005). Furthermore, application of feed supplements has largely been restricted to artificially infected animals, and more research with naturally infected animals is needed. Frequent cleaning of water troughs, treating water with UV irradiation or ozone, or the addition of probiotic bacteria and bacteriophages may also be effective in reducing the prevalence of *E. coli* O157 in animal water sources (Vanselow *et al.* 2005). However, as with many methods investigated to lessen on-farm carriage and occurrence of pathogenic bacteria, it is highly unlikely that systems such as irradiation or ozonation will be employed on a commercial scale under current agricultural systems.

2.10.2. Vaccine/drug development

Development of an efficient vaccine would hold great practical application in reducing the prevalence of *E. coli* O157 within the beef industry. This is because cattle producers are familiar with administration of vaccines, vaccination could easily be incorporated into existing cattle management systems, and vaccines could be used in all sectors of the industry, - not just confined animal feeding operations (Loneragan and Brashears 2005). However, the fact that animals sporadically harbour the bacterium without displaying any obvious symptoms renders treating infected herds additionally difficult. Furthermore, early work investigating the susceptibility of *E. coli* O157 to antibiotics found that this serotype possessed particularly resistant characteristics, as summarised by Stephan and Schumacher (2001). However, this does not discount vaccination treatment of animals as a possible method of reducing *E. coli* O157 proliferation in the future. Potter *et al.* (2003) reports interesting progress regarding the development of a vaccine to reduce carriage of *E. coli* O157 within animals. They targeted several proteins (e.g. Tir and EspA) produced by the bacterium to colonise the intestine to reduce levels of the organism in cattle. Vaccinating cattle with such proteins significantly reduced the numbers of bacteria shed in faeces, the

numbers of animals that shed, and the duration of shedding in an experimental challenge model and in a clinical trial conducted in a typical feedlot setting (Fig. 2.27).

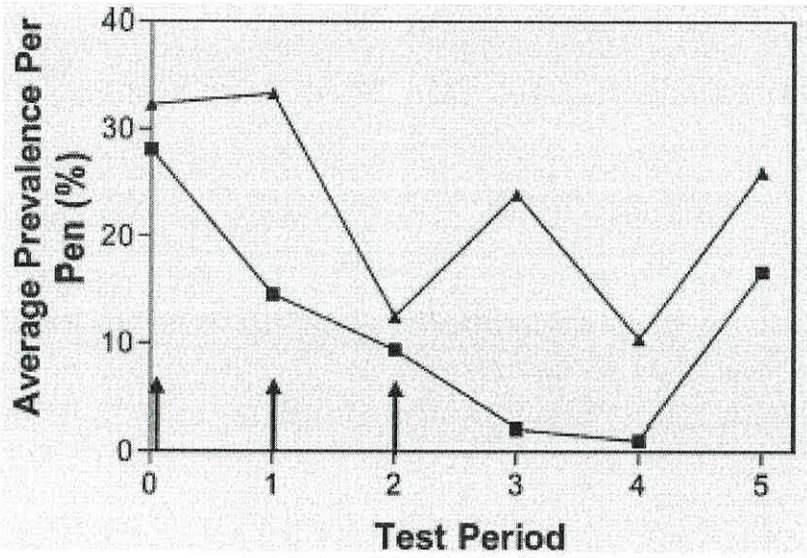

Figure 2.27. The proportion of cattle shedding *E. coli* O157:H7 within pens of vaccinated and unvaccinated cattle under natural conditions. Arrows indicate the time of vaccination. Test-periods represent the day of sampling at 3 week intervals (treatment days 0, 21, 42, 63, 84, and the day of marketing), with 0 representing sampling prior to the first vaccination. Unvaccinated controls (▲), vaccinated animals (■) (Potter *et al.* 2003).

Some studies have used mice as models to investigate the efficacy of different vaccinating process to predict or reduce colonisation of the gut by *E. coli* O157. Conlan *et al.* (1999a) parenterally inoculated two groups of mice with a horse serum albumin-*E. coli* O157 antigen conjugate vaccine. Whereas both mice groups developed systemic, specific antibodies to the carrier protein, only one group routinely developed antibodies to the O157 section. In general, the authors found little convincing evidence to show that antibodies transuded into the intestinal tract either naturally or in response to an oral inoculum of *E. coli* O157. The vaccination procedure employed also failed to protect mice against intestinal colonisation following oral challenge with *E. coli* O157. The results of their study suggest that parenteral vaccination might be an unsuitable strategy for combating *E. coli* O157 organisms located in the gut. Conlan *et al.* (2000) orally immunised mice with a glycoconjugate vaccine consisting of horse serum albumin and an O157 polysaccharide mixed with a mucosal adjuvant, cholera toxin. Although mice consistently developed robust local and systemic immune responses to the cholera toxin adjuvant, their reaction to the test

vaccine was irregular. Moreover, following challenge with an isolate of *E. coli* O157, no difference occurred in the degree of intestinal colonisation between vaccinated and unvaccinated mice (Conlan *et al.* 2000). However, Conlan *et al.* (1999b) achieved some degree of success after orally applying an inoculum of *Salmonella landau* (a *Salmonella* strain that naturally expresses the O157 antigen) to mice. Although *Salmonella landau* was avirulent for mice, those mice exposed to *S. landau* developed high titres of serum and coproantibodies against the O157 antigen. Compared to controls, these mice demonstrated some ability to resist transient intestinal colonisation by an oral inoculum of an isolate of *E. coli* O157. This suggested that a local immune response directed against the O157 antigen might increase host resistance to this pathogen (Conlan *et al.* 1999b). Dean-Nystrom *et al.* (2002) developed parenteral vaccines (based on the intimin protein) against *E. coli* O157. The vaccine was tested in pigs, and vaccination of pregnant dams induced protection against *E. coli* O157 colonisation and intestinal damage in suckling piglets, through colostrum-derived maternal antibodies against intimin.

Currently, antimicrobial drugs are employed in animal production purely as a means to improve animal health, or the efficiency of production. Employing vaccination (or other antimicrobial methods) as a pre-harvest intervention to reduce intestinal carriage of *E. coli* O157 will likely generate much debate amongst farmers and consumer bodies (Loneragan and Brashears 2005). From a farming aspect, administrating a vaccine would have little (if any) benefit to animal health or production, and be administered solely to improve public health. It is contentious that farmers would (and perhaps, should) therefore have to pay for vaccination. From a consumer (and perchance from a long-term animal health) aspect, the use of antimicrobial drugs in animal agriculture is becoming increasingly litigious. Other, more natural avenues to reduce carriage of VTEC in animals must be thoroughly investigated prior to widespread adoption of drug application.

2.10.3. Waste

Bicudo and Goyal (2003) review a range of management and treatment options to reduce pathogen levels in manure being used on-farm; including vegetative filter strips, ozonation, electrolytic treatment, anaerobic lagoon, anaerobic digester, and aeration. It would be no surprise that future laws may enforce pre-treatment of all organic wastes of animal origin prior to land disposal e.g. in the form of aeration or liming. However, other, more intensive

treatment methods (- similar to those currently used for sewage processing e.g. ozonation, radiation) would be unfeasible due to the massive economic implications. Minimum waste storage times, composting regimes, or maximum applications may also become obligatory, rather than voluntary to farmers. In addition, extensions may be enforced to the animal 'clear' period and harvest times after waste application; plus stricter rules on appropriate times waste may be spread with regards to weather conditions etc. Waste may also have to be incorporated into soil, rather than surface spread, a practice shown to lessen proliferation of *E. coli* O157 (Avery *et al.* 2004).

2.10.4. Food and drinking water

If reductions are to be gained in occurrences of food-borne illnesses, appropriate strategies to minimise pathogen survival that interface along the whole spectrum of food production must occur. Measures to reduce the carriage of *E. coli* O157 on the hides of incoming cattle to abattoirs have already been discussed (section 2.8). As soiled hides are the primary vector for meat contamination, adherence to such measures may significantly aid in reducing the prevalence of this organism in meat. With regards to fruit, vegetables, and other produce, greater use of irradiation techniques may reduce pathogen loads; although any side-effects on food quality and safety must be clarified for public acceptance. New packaging and storage procedures may also be developed to decrease pathogen survival (Nadarajah *et al.* 2005b).

Reducing the persistence of *E. coli* O157 on surfaces in contact with food may yield benefits in the battle to reduce proliferation of the bacterium. Although chemical treatments are undoubtedly most widely used (and probably most effective) for disinfecting surfaces, it appears that biological control may warrant further investigation. Fratamico and Whiting (1995) and Fratamico and Cooke (1996) explored the potential for using predatory *Bdellovibrio* for the biological control of a range of food-borne pathogenic organisms on stainless steel surfaces used in the food industry. They found that *Bdellovibrio* reduced *E. coli* O157 numbers by 3.6 $\log_{10}$ CFU cm^{-2} within 24 h of contact when present at a 10-fold higher population. Although effective, the feasibility of employing such a control method on a large scale is questionable. The findings of a recent study showed that the use of a surface material with antibacterial properties could aid in preventing cross-contamination events in food processing and domestic environments, if standard hygiene measures fail (Wilks *et al.*

2005). It appears that some metals may possess greater antibacterial properties than others (Wilks *et al.* 2005) hence may be especially valuable in high risk areas such as abattoirs. Along with the existing and proposed methods to reduce carcass contamination previously discussed (section 2.8), washing carcasses with water, disinfectants, or with organic acids may yield noteworthy reductions in bacterial carriage (Park *et al.* 1999).

Those with private water supplies and bottled-water companies may be enforced to efficiently sterilise and filter water prior to human consumption; although this would undoubtedly face objection. Furthermore, although cases of human *E. coli* O157 infections have arisen following consumption of water from private supplies (as highlighted previously), there are clearly cheaper, more effective, and easier ways to reduce the overall number of human infections from this pathogen.

2.10.5. Genetic manipulation

Although such developments are clearly in their infancy, it may be possible to genetically manipulate *E. coli* O157 to interfere with its ability to adhere to the mucosal border (Todd and Dundas 2001), hence impede the onset of infection. Dundas *et al.* (1999) and Todd and Dundas (2001) suggest that further genetic work may allow manipulation of toxin levels and/or toxin-cytokine interactions to either prevent toxin adherence to receptors, or to selectively bind toxin to prevent binding to endothelial receptors. However, in view of the rapid evolution and development of new strains of VTEC, the feasibility of adopting such methods as a way of lessening the cases of human infection is highly questionable.

2.11. Conclusions

Human VTEC infections have severe consequences from both economic and health perspectives. The recent emergence of *E. coli* O157 as a major human pathogen has had a significant impact on the outlook and policies on all aspect of food production and food safety; from 'farm to fork'.

Although it appears that numbers of human VTEC infections in the UK have steadied, it is ambiguous if this is the case globally. Indeed, it is likely that the lack of surveillance systems in poorer countries leads to under-reporting of the numbers of human infections.

Improved interdisciplinary cooperation between geneticists, microbiologists, ecologists, and virologists has increased our understanding of the interaction between genetic changes in pathogens, their environment, their host, and outbreaks of infection. Nonetheless, it is clear that many voids remain in our understanding of the ecology of *E. coli* O157 and possible control measures. Future research must be directed towards filling some of these knowledge gaps, with the ultimate goal of reducing human exposure to this harmful bacterium.

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CHAPTER 3: ARTICLE I

Persistence of *Escherichia coli* O157 on farm surfaces under different environmental conditions

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3.1. Abstract

Escherichia coli O157:H7 is a prevalent pathogen, common in ruminant faeces. Contact with contaminated faeces may lead to human infection, resulting in possible severe illness. Although the pathogen is known to persist on surfaces, its ability to survive on farmyard surfaces (gates, stiles etc.) is unclear. As humans may come into contact with contaminated faeces on such surfaces, persistence of *E. coli* O157:H7 may lead to infections. Samples of field-conditioned farmyard materials (galvanised steel and wood) were cut into pieces and contaminated with fresh cattle faeces inoculated with non-toxigenic *E. coli* O157:H7 (strain #3704). Thereafter, they were stored under four different environmental conditions; with temperature (5°C and 20°C) and moisture (moist or dry) as variables. Transfer of the pathogen to hands from the surfaces was also evaluated. *E. coli* O157:H7 numbers declined over time on all surfaces albeit at different rates according to the sample material and environmental conditions. Persistence was greatest on moist wood samples under cooler temperatures, with large population numbers remaining after 28 days. Desiccation of surfaces resulted in a more rapid decline in *E. coli* O157:H7 populations under both temperature regimes. Our findings show that substantial numbers of cells may potentially be transferred to human hands from the surfaces during brief contact. The potential of *E. coli* O157:H7 to persist for extended periods on gates, stiles, and other farmyard surfaces under a range of environmental conditions poses a potential infection pathway, particularly where there is a high risk of direct human contact (e.g. child petting zoos, open farms).

Keywords: animal faeces, cattle, *Escherichia coli* O157:H7, farm, gates, public health, stiles, survival.

3.2. Introduction

Escherichia coli O157:H7 is recognised as the most prevalent of all pathogenic *E. coli* serotypes, with infection often leading to a range of clinical symptoms including haemorrhagic colitis or life-threatening haemolytic uraemic syndrome (HUS) (Chart 2000; Dundas and Todd 2000; Rogerie *et al.* 2001). The occurrence of this bacterium in gastro-intestinal illness in humans has been observed world-wide, and worryingly, the UK incidence rate shows no obvious signs of decreasing (Jones 1999; Parry and Palmer 2000).

Although the pathogen can be carried by a range of wild and domesticated animals (Chapman *et al.* 1997; Johnsen *et al.* 2001; Rey *et al.* 2003), the foremost pool of *E. coli* O157:H7 in the environment is cattle (Cobbold and Desmarchelier 2000; Ogden *et al.* 2004), and to a lesser extent, sheep (Kudva *et al.* 1997). Current evidence suggests that *E. coli* O157:H7 may be prevalent within over 10% of cattle herds (Chapman *et al.* 1997; Mechie *et al.* 1997; Sargeant *et al.* 2003; Ogden *et al.* 2004) with excretion levels in infected cattle typically ranging between 10^2-10^5 CFU g^{-1} (Wang *et al.* 1996). *E. coli* O157:H7 can survive a considerable time in manure and slurry (Kudva *et al.* 1998; Bolton *et al.* 1999; Lau and Ingham 2001; Avery *et al.* 2004). Contact with ruminant faeces may thus provide a primary vector for cattle and human *E. coli* O157:H7 infection and direct transmission from infected animal faeces to humans has been recognised on numerous occasions (Cieslak *et al.* 1993; Strachan *et al.* 2002).

Within agricultural environments, it is common for farm gates and fencing to become contaminated to some degree by animal wastes. This might occur via conventional agricultural practices such as manure-spreading, via direct excretion onto surfaces by animals, or passively via carriage on human footwear from grazing land when climbing over gates or stiles. Subsequently, this may act as a vector of *E. coli* O157:H7 transmission to humans through ingestion of cells following direct hand contact with faecally contaminated surfaces (Payne *et al.* 1999).

Commonly, UK farm gates are constructed of zinc-galvanised steel or wood (softwood or hardwood). Galvanised steels are widely used because of their resistance to corrosion along with low production costs (Tamura 2003). Wood also represents a popular material due to its wide availability, its greater aesthetic value, and promotion within agri-environment schemes such as 'Tir Gofal' (Countryside Council for Wales 2004). Previous research on *E. coli* O157:H7 survival on surfaces has been mainly associated with food

environments (Ak *et al.* 1994a, 1994b; Hood and Zottola 1997; Foschino *et al.* 2003; Speranza *et al.* 2004); however, little is known about the factors regulating pathogen persistence on agricultural surfaces. The degree of microbial adhesion and survival on a surface is influenced by many factors, including material geometry, porosity, roughness, composition, and hydrophobicity (Bower *et al.* 1996; Cunliffe *et al.* 1999; Foschino *et al.* 2003). If there are differences in *E. coli* O157:H7 persistence on gates and stiles constructed from different materials this may have implications for reducing risk to humans.

The aim of this study was to assess the variation in *E. coli* O157:H7 persistence on common farm gate and fence materials (galvanised steel and wood), under different moisture content and temperature regimes. It was hypothesised that survival would be enhanced under cooler temperatures and higher moisture content, and that persistence would differ between materials.

3.3. Materials and Methods

3.3.1. Preparation of materials

Two wooden fence, one wooden gate, and two zinc-galvanised gate steel samples were collected from a farm in Abergwyngregyn, North Wales, UK (Table 3.1). From these, smaller pieces of wood (3.5 × 2.0 × 0.4 cm) were cut using an electric Wadkin Bursgreen band saw, and metal (3.5 × 2.0 × 0.1 cm) with a guillotine (Wadkin UltraCare Ltd., Leicester, UK). The pieces were then laid out with the weathered face uppermost in a randomised-block design in metal trays containing filter paper. Half of the material pieces were kept moist via spraying with artificial rainwater every two days and covering the trays with plastic film to maintain a relative humidity close to 100%. The artificial rainwater contained 96 μmol l^{-1} NaCl, 10 μmol l^{-1} K_2SO_4, 5 μmol l^{-1} $CaCl_2$, 6 μmol l^{-1} $MgCl_2$, 15 μmol l^{-1} NH_4NO_3 and 0.1 μmol l^{-1} KH_2PO_4 (Jones and Edwards, 1993). No water was applied to the other half of materials and these samples were not covered with a plastic film, allowing them to gradually dry. The trays were maintained in cooled incubators (LMS Ltd., Sevenoaks, UK) at either 5.0 ± 0.1°C or 20.0 ± 0.1°C.

3.3.2. Characterisation of materials

The moisture content of each material was determined by drying overnight at 80°C. A relative estimate of background microbial counts on each material was undertaken by

shaking the material pieces at 250 rev min^{-1} for 15 min at room temperature in ¼-strength Ringers solution (Oxoid, Basingstoke, UK), followed by 4 × 5-second bursts on a vortex mixer. Solutions were then plated out onto R2A agar (a low-nutrient medium used for enumerating aerobic heterotrophic organisms; Oxoid) and colony counts were made following incubation for 48 h at 20°C. The same solutions were also plated onto Sorbitol MacConkey agar (Oxoid) supplemented with cefixime (0.05 mg l^{-1}) and potassium tellurite (0.05 mg l^{-1}; CT-SMAC) to test for the presence of *E. coli* O157:H7.

Three replicate pieces of each material were shaken for 1 h at 250 rev min^{-1} in 10 ml of distilled water, centrifuged (12,000 *g*, 5 min) and the supernatant recovered for analysis. K, Na and Ca were determined using a PFP7 flame photometer (Jenway Ltd., Dunmow, UK), electrical conductivity with a 4010 EC meter (Jenway Ltd.) and pH with an Orion 410A pH meter (Thermo Ltd., Runcorn, UK). Total soluble phenols were determined by the method of Swain and Hillis (1959), phosphate by the method of Murphy and Riley (1962), total dissolved nitrogen and dissolved organic carbon with a TC-TNV analyzer (Shimadzu Corp., Kyoto, Japan) and Zn with a Quantofix® assay kit (Camlab Ltd., Cambridge, UK).

3.3.3. Characterisation of cattle faeces

Electrical conductivity and pH were measured after a 1:1 (v/v) dilution of the cattle faeces with distilled water. Waste moisture content was determined after drying at 80°C overnight, whilst total C and N were quantified using a CHN2000 elemental analyser (Leco Corp., St Joseph, MI). To determine waste chemical characteristics, 10 g of the faeces were shaken with 30 ml of deionised water for 1 h on a reciprocating shaker (200 rev min^{-1}), centrifuged (4000 *g*, 10 min) and the supernatant retained for analysis. Thereafter, the supernatant solutions were used to determine P as described above, and NO_3^- and NH_4^+ via a segmented flow San plus analyser (Skalar, York, UK). All data for cattle faeces are presented on a wet weight basis. Five ml of faeces were placed in 15 ml modified Tryptone Soya broth (mTSB) containing VCC supplement (Oxoid), and shaken at 150 rev min^{-1} for 6 h at 37°C before streaking the broth onto CT-SMAC agar plates. This enrichment procedure tested for the presence of *E. coli* O157:H7.

3.3.4. Preparation of E. coli *O157:H7 inoculum*

An inoculum was prepared by culturing a non-toxigenic strain (#3704) of *E. coli* O157:H7 in Luria Bertani (LB) broth (Difco, Oxford, UK) for 18 h at 37°C (150 rev min^{-1}). The strain was originally isolated from a farm drain and has been proven to be non-toxigenic due to the absence of toxin activity and toxin genes (Campbell *et al.* 2001). Cells were washed three times in sterile ¼-strength Ringers solution, and concentrated by centrifugation as described in Avery *et al.* (2005).

3.3.5. Preparation and application of spiked cattle faeces

Bovine faeces were collected from a local commercial dairy farm in North Wales, macerated to remove large particles using a food blender, and stored at 4°C for 24 hours. Ten ml *E. coli* O157:H7 inoculum was added to 190 ml of faeces and the suspension was thoroughly mixed. Serial dilutions (10^{-1} to 10^{-6}) of this were plated in duplicate onto CT-SMAC agar, and colonies exhibiting typical *E. coli* O157:H7 morphology were counted following incubation at 37°C for 18 h. This revealed a final *E. coli* O157 concentration of approximately 2.5×10^8 CFU ml^{-1} faeces. On the uppermost surface of each material (area = 7 cm^2), 500 µl of *E. coli* O157:H7 spiked cattle faeces was evenly spread to a depth of 0.7 mm, avoiding runoff (1.8×10^7 CFU cm^{-2}). Four treatment combinations were tested in triplicate for each material: 20°C + artificial rainwater applied; 20°C + no artificial rainwater applied; 5°C + artificial rainwater applied; 5°C + no artificial rainwater applied.

3.3.6. Harvesting of materials

Harvesting was carried out at day 0, 3, 7, 14, and 28 following the application of waste to material surfaces. Material pieces from each treatment combination were placed into 20 ml of ¼-strength Ringers solution in 50 ml sterile centrifuge tubes (a sufficient volume to immerse the material pieces). Tubes were subsequently shaken at 250 rev min^{-1} at 20°C for 15 min, followed by 4 × 5-second bursts on a vortex mixer. Visual inspection indicated that all faecal material on surfaces was suspended in solution by this mixing process. Thereafter, plating of serial dilutions of the suspension (10^{-1} to 10^{-6}) onto CT-SMAC agar as described previously allowed quantification of *E. coli* O157:H7 numbers. When counts were approaching the detection limit by this method (22 CFU cm^{-2}), an enrichment technique as

previously was utilised; this time adding 5 ml of the Ringers solution from the 50 ml tubes to 15 ml of mTSB. This was used to confirm the presence/absence of *E. coli* O157:H7. Where *E. coli* O157:H7 was detectable only following enrichment, the result was assigned an arbitrary value equal to half of the detection limit of plate counts (i.e. 11 CFU cm^{-2}). At each harvest, the moisture content of the faecally-contaminated surfaces was also determined.

3.3.7. Transfer of faeces from gate to hand

A simple side-experiment was designed to attempt an estimation of the amount of cattle faeces transferred from a gate to a human hand on brief contact. For this, a 30 cm, round, galvanised pipe from a farm gate was weighed. Subsequently, fresh cattle faeces (not inoculated with *E. coli* O157:H7) were applied to the surface as described previously and the pipe weighed again. It was then grasped by a human hand for a duration of 3 seconds. All four participants grasped the pipe twice i.e. once with each hand independently. Each participant was right-handed, and the group displayed a range of hand sizes. The pipe was re-weighed prior to each hand grasping it to work out the mass of faeces transferred to the hand during each contact event and thus the potential CFU of *E. coli* O157:H7 transferred to a hand if faeces were contaminated.

3.3.8. Data analysis

Values of $\log_{10}$ CFU cm^{-2} were calculated for each sample using Microsoft Excel. All experiments were performed in triplicate. Data gathered displayed normal distribution and equal variances. All data was analysed using a multi-factorial analysis of variance (ANOVA), using Genstat 6 (Statistics Department, Rothamsted Experimental Station, UK). Significant differences between treatments were determined to be those with a 0.1% level of significance ($P < 0.001$), and were identified using Fisher's LSD output on Genstat.

3.4. Results

3.4.1. Characterisation of material surfaces

A summary of the physical, chemical and microbiological characteristics of the 5 material surfaces is shown in Tables 3.1 and 3.2. Statistical analysis showed that each of the three wood surfaces were chemically different (ANOVA, $P < 0.05$), with large differences

apparent between the two wood types (hard and softwood). In particular, the oak was acidic in nature and contained significantly more soluble phenols. Although statistical differences existed between the wood types, the levels of Na, K, Ca and P were generally similar. The two galvanised steels possessed similar properties to each other but in comparison to the wood samples had significantly lower nutrient concentrations and higher soluble Zn^{2+} levels ($P < 0.05$). The background microbiological counts on the three wood surfaces were similar and about an order of magnitude higher than those present on the galvanised steel. Prior to addition of faeces, no *E. coli* O157:H7 cells were detected on any of the materials examined.

Table 3.1. Characterisation of materials used in the study.

Material	Reference code	Origin	Approximate age spent in field	Surface	Treated with preservative
Spruce wood	Wood A (WA)	Farm gate	< 3 years	Quite smooth	Yes, clearly visible
Spruce wood	Wood B (WB)	Fence	< 7 years	Reasonably smooth	Yes, visible
Oak wood	Wood C (WC)	Fence	> 10 years	Quite rough	Not visible
Galvanised steel	Metal A (MA)	Gate	< 1 years	Smooth	None
Galvanised steel	Metal B (MB)	Gate	< 3 years	Smooth	None

Table 3.2. Intrinsic chemical and biological characteristics of the materials used in the study. All values are the means of three replicates.

	Wood			Metal	
	WA	WB	WC	MA	MB
pH	7.11	6.20	4.18	6.54	6.57
Electrical conductivity ($\mu S\ cm^{-1}$)	93	46	95	16	29
Dissolved organic carbon ($mg\ cm^{-2}$)	2.7	4.0	25.7	0.3	0.1
Dissolved total nitrogen ($mg\ cm^{-2}$)	0.23	0.23	0.44	0.16	0.04
Ca ($mg\ cm^{-2}$)	1.36	0.90	1.79	0.31	0.62
Na ($mg\ cm^{-2}$)	1.43	1.06	0.99	0.20	0.32
K ($mg\ cm^{-2}$)	6.77	1.69	8.42	0.53	1.26
P ($mg\ cm^{-2}$)	1.01	0.06	0.89	< 0.01	< 0.01
Zn ($mg\ cm^{-2}$)	< 0.01	< 0.01	< 0.01	2.22	3.39
Soluble phenols ($mg\ cm^{-2}$)	1.4	0.7	13.2	< 0.1	< 0.01
Moisture content ($g\ kg^{-1}$)	106	102	98	< 0.01	< 0.01
Total aerobic bacteria ($CFU\ cm^{-2}$)	28	31	35	2	2
E. coli O157:H7 ($CFU\ cm^{-2}$)	0	0	0	0	0

3.4.2. Characterisation of faeces

A summary of the physical, chemical and microbiological characteristics of the faeces used is shown in Table 3.3. Both faecal moisture content and electrical conductivity were expectantly high. Tests showed that most available nitrogen was in the form of ammonium rather than nitrate, leading to slightly alkaline conditions. Although the faeces used contained high levels of aerobic background bacteria, no *E. coli* O157:H7 cells were present prior to inoculation.

Table 3.3. Intrinsic chemical and biological characteristics of cattle faeces used in the study. All values are the means of three replicates.

Parameter	
Moisture content (g kg^{-1})	841
Total C (g kg^{-1})	34.2
Total N (g kg^{-1})	1.9
pH	8.04
Electrical conductivity (µS cm^{-1})	6790
Available P (mg kg^{-1})	94
NO_3^- (mg N kg^{-1})	1.8
NH_4^+ (mg N kg^{-1})	44.7
Total aerobic bacteria (CFU ml^{-1})	1.4×10^7
E. coli O157:H7 (CFU ml^{-1})	0

3.4.3. Survival of E. coli *O157:H7 on material surfaces*

Overall, numbers of *E. coli* O157:H7 significantly declined over time on all of the faecally-contaminated surfaces ($P < 0.001$; Fig. 3.1). Only when materials were kept at 5°C under moist conditions was any increase in *E. coli* O157:H7 recovery observed between harvests with all but one material supporting a higher level of bacteria at day 7 compared to day 3. In general, die-off occurred at a rate of approximately 1 log unit per harvest with the most rapid decline typically occurring in the initial stages of the experiment [mean $\log_{10}$ CFU cm^{-2} ± standard error of the mean (SEM): first harvest 5.2 ± 0.1; final harvest 0.46 ± 0.07]. *E. coli* O157:H7 survival was influenced by a significant interaction between all variables (harvest × material type × moisture × temperature interaction, $P < 0.001$). Overall, *E. coli* O157:H7 persistence was greater on hardwood > softwood > galvanised steel (Fig. 3.1). The same pattern was generally reflected under all environmental conditions, although the differences weren't always statistically significant.

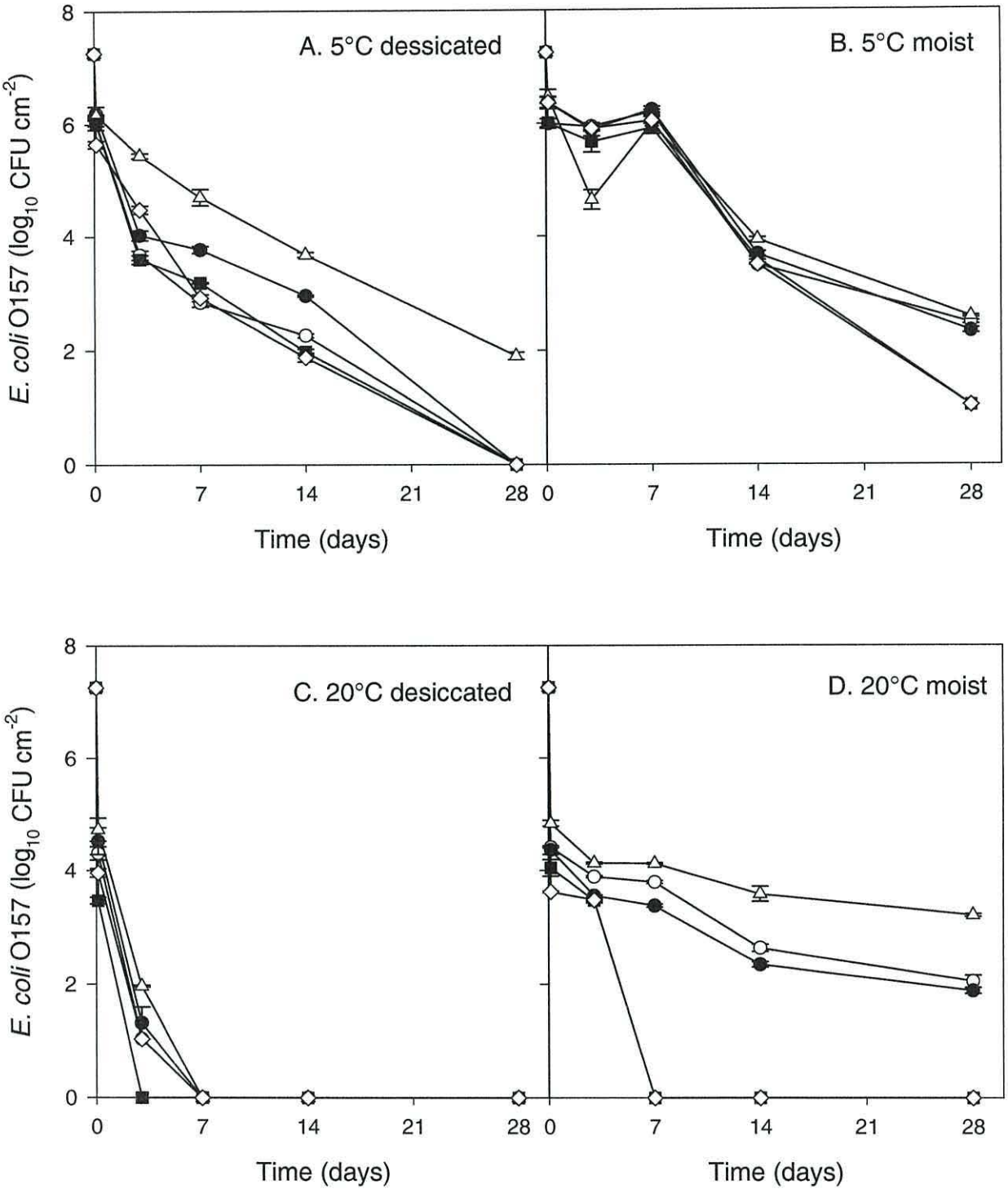

Figure 3.1. Survival of *E. coli* O157:H7 on WA (○), WB (●), WC (Δ), MA (■), and MB (◊) surfaces under different environmental conditions. Curves are representative of mean $\log_{10}(y + 1)$ *E. coli* O157:H7 $cm^{-2} \pm$ SEM of three replicate material pieces.

3.4.4 Impact of temperature on E. coli *O157:H7 survival*

Our results indicate that persistence of *E. coli* O157:H7 was notably decreased when materials were incubated at 20°C relative to those held at 5°C; however, this response was significantly affected by moisture regime (moisture × temperature interaction, $P < 0.001$; Fig. 3.1). The influence of moisture content on the temperature effect was especially notable for contaminated faeces applied to wood rather than metal surfaces (Fig. 3.1). In conjunction with the moisture regime, it appears that the temperature at which the materials were stored was a primary factor in determining *E. coli* O157:H7 survival on all material surfaces.

3.4.5. Impact of moisture availability on E. coli *O157:H7 survival*

The application of artificial rainwater maintained the faeces and underlying wood or metal surface in a moist state throughout the experiment (Fig. 3.2). In contrast, the materials which did not receive artificial rainwater rapidly dried out, returning within 16 hours to the initial moisture content prior to the application of faeces for both temperature regimes (Fig. 3.2).

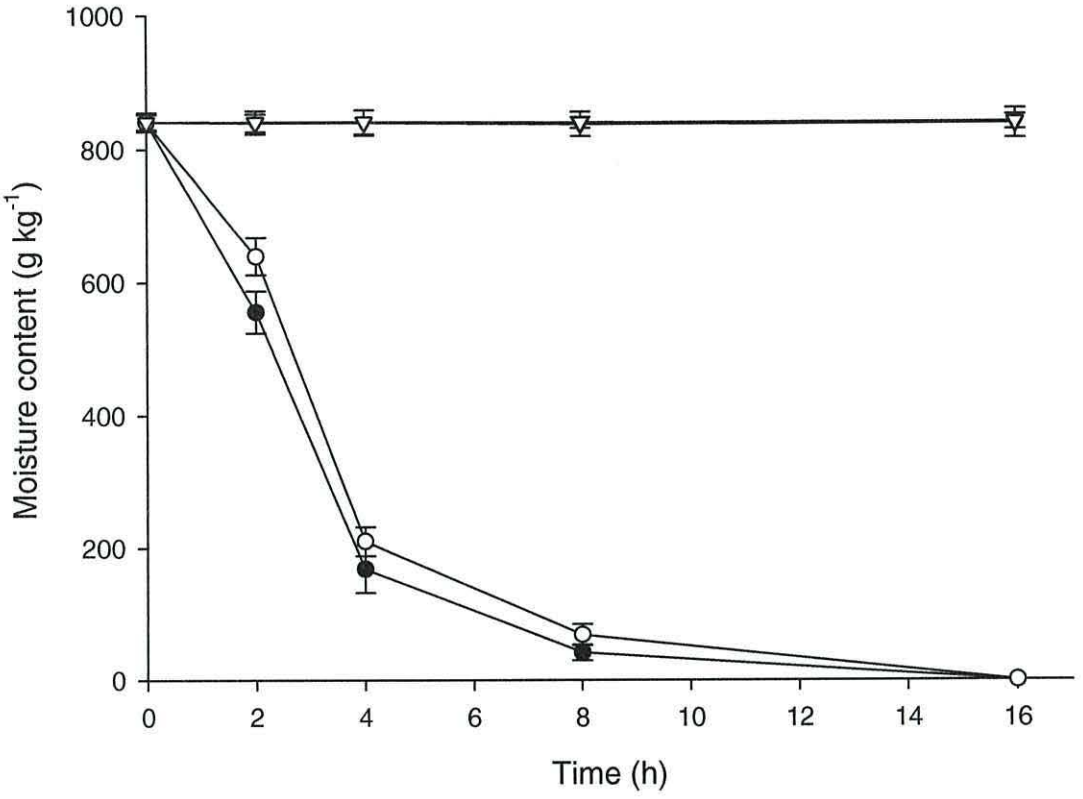

Figure 3.2. Changes in faeces moisture content with time under different environmental conditions [metal 20°C + desiccated (●), wood 20°C + desiccated (○), metal 20°C + moisture applied (▼), and wood 20°C + moisture applied (▽)]. Curves are representative of mean moisture content (g kg^{-1}) ± SEM of three replicate pieces for both metal and wood surfaces.

The addition of artificial rainfall significantly increased pathogen survival ($P < 0.001$; Fig. 3.1). In most of the samples which were allowed to dry out by withholding water, the pathogen could not be detected by enrichment after 28 d (Fig. 3.1). In contrast, significant numbers of pathogen were still present after 28 d on the samples to which artificial rainwater was added (mean $\log_{10}$ CFU cm^{-2} ± SEM: 3.58 ± 0.14), particularly on the three wooden surfaces. Generally, the moisture content of the samples had a much greater effect than the type of material surface although survival under both moisture regimes was greater on wood than on galvanised steel.

3.4.6. Transfer of faeces from gate to hand

On average, 6.4% of the total mass of faeces on the gate was transferred to hands upon contact (Table 3.4). More faeces were consistently transferred to participants' right hands, however, this wasn't significantly greater (t-test left vs right hand, $P > 0.05$).

Table 3.4. The amount of faeces (mass; % of total mass) and potential CFU of *E. coli* O157:H7 transferred from gate to hand following brief contact. Values are mean ± SEM ($n = 4$).

	Hand	
	Left	Right
Amount of faeces transferred to hand (% that on surface)	4.8 ± 2.30	8.0 ± 1.90
E. coli O157:H7 (CFU hand^{-1})	$2.4 \times 10^3 \pm 1.15 \times 10^3$	$4.0 \times 10^3 \pm 0.95 \times 10^3$

3.5. Discussion

Faecal contamination of hard surfaces in agricultural environments is a common phenomenon both in fields and around the farmyard and the experiments performed here aimed to reflect typical conditions. Our results clearly show that *E. coli* O157:H7 survival on such surfaces is dependent upon a range of interacting environmental factors. Firstly, we have established that the material type significantly affects *E. coli* O157:H7 persistence. Cells were shown to survive for significantly greater time on wood than galvanised steel. Secondly, we have shown how temperature and moisture influence persistence on surfaces; with cooler temperatures (5°C) and moist conditions enhancing survival. Finally, from a human health perspective, our study shows that *E. coli* O157:H7 can persist for a substantial time (> 28 days) on common farmyard materials under a variety of environmental conditions, thus present a potential source of infection. Our findings, alongside others, highlight the persistence of *E. coli* O157:H7 in farm environments and emphasise the year-round risk of infection (Jones 1999).

One reason for the reduced persistence on galvanised steel relative to wood may be the high levels of Zn (Johnston and Ryan 2000). During exposure to air and water, Zn^{2+}, ZnO and $ZnCO_3$ can be released from galvanised surfaces as a result of corrosion (Oesch and Heimgartner 1996; Yadav *et al.* 2004). Smolders *et al.* (2003) showed that elevated Zn in soil close to galvanised electric pylons can significantly affect microbial activity, while Momba and Notshe (2003) showed that bacterial survival in water held in galvanised steel

(< 48 h) was much poorer than in plastic containers. In this study, soluble Zn levels were much higher on galvanised steel materials in comparison to wood (Table 3.2). Assuming that the Zn levels presented in Table 3.2 are dissolved in a thin moisture film, it can be expected that the levels of Zn will be toxic to most bacteria, particularly if the pH is low (*ca.* 5 to 50 mM Zn; Dehri *et al.* 1994). The metabolic activity of *E. coli* O157:H7 is greater at temperatures of 20°C compared to 5°C and our results show that pathogen die-off on galvanised steel was much more pronounced at 20°C compared to 5°C. Interestingly, a similar survival pattern was not evident for *E. coli* O157:H7 inoculated onto wood samples, where at day 28 similar pathogen numbers were present at both 5 and 20°C. We speculate that increased activity of cells at 20°C may have led to greater metabolic exposure to Zn, resulting in a more rapid death. Although no data exists for *E. coli* O157:H7 and Zn, Grey and Steck (2001) determined that Cu concentrations initially considered toxic could induce *E. coli* to enter a viable but non-cultureable (VBNC) condition, rendering them unrecoverable by plating techniques. As we only used cultureable enumeration techniques, the values reported here represent minimum estimates of pathogen survival. Incorporation of immunomagnetic separation techniques towards the end of the experimental period may have allowed detection of viable cells over a longer time-period.

Pathogen survival was significantly greater on the hardwood (WC) relative to the other materials tested, with viable numbers detectable on all samples at day 28 (except those stored at 20°C with no water applied). Greater survival of *E. coli* O157:H7 bacteria on this wood may reflect the treatment of softwoods with preservative. In this instance, the nature of any preservative applied was not known. However, softwoods are usually treated with copper-chromium-arsenic (CCA) or creosote on farms. Sakagami *et al.* (1999) found that creosote and its main phenolic components inhibited *E. coli* O157:H7 verotoxin production. It must be noted that the use of both CCA and creosote are being phased out due to environmental and health concerns and little information exists on how new preservatives may affect *E. coli* O157:H7 survival. In addition to the impact of preservatives, pathogen persistence on softwoods may be inhibited by polyphenolic substances (Miller *et al.* 2003). However, no correlation was apparent between survival and soluble phenols in this study. We conclude that the environmental conditions rather than the chemical, physical and biological properties of the wood exert the greatest effect on *E. coli* O157:H7 persistence.

Pathogen persistence was significantly enhanced ($P < 0.001$) when the materials were kept moist. This supports work by Ak *et al.* (1994b) who also reported that the persistence and degree of overnight multiplication of bacteria on plastic and wooden surfaces was regulated by humidity. In our study, desiccation induced a rapid decline in pathogen numbers with no *E. coli* O157:H7 recovered after 7 d at 20°C. Indeed, persistence on all materials was notably less when the temperature was 20°C and no moisture was applied. This contrasts with a study by Maule (1999) who showed that a range of *E. coli* O157:H7 strains could survive in a desiccated state on stainless steel in excess of 60 days. However, as in our study, Maule (1999) did show that survival was enhanced at lower temperatures (4°C) relative to higher temperatures (18°C). Our results suggest that higher temperatures exacerbated die-off of *E. coli* O157:H7 caused by lack of moisture leading to a rapid extinction within 7 days. This suggests that under harsh environmental conditions, survival is limited to a short time period, regardless of underlying material type.

The experiments undertaken here were performed under laboratory conditions and therefore no consideration was given to the impact of exposure to solar UV radiation; however, this factor can also be expected to reduce pathogen persistence, particularly on exposed surfaces. Although a non-toxigenic *E. coli* O157:H7 strain was used in this experiment, past research reveal identical or very similar survival patterns for toxigenic and non-toxigenic strains (Kudva *et al.* 1998). Albeit *E. coli* O157:H7 strain #3704 possesses the same survival characteristics as wild-type *E. coli* O157:H7 (Ritchie *et al.* 2003), incorporation of other strains to similar experiments would further enhance our knowledge of *E. coli* O157:H7 survival on farm surfaces.

Arguably, the possibility of humans ingesting substantial volumes of faecal material following hand contact with faeces is low. However, if faeces were contaminated to levels of 1×10^5 CFU g^{-1}, our work shows that 3.15×10^3 CFU *E. coli* O157:H7 may be transferred onto hands following contact with soiled gates or stiles (Table 3.4). Infection risk is greatest in young children (1-4 years; Parry and Palmer 2000), and ingestion of a very small number of cells (< 10 CFU; < 1 mg of faeces) is sufficient to cause human infection (Chart 2000). Furthermore, children are likely to practice poorer hygiene (e.g. hand washing), and their height often means they're more likely to come into contact with the lower portions of gates, stiles, and fences, where faecal contamination is more apparent. Our work therefore highlights the dangers of contact with cattle faeces present on farm fences and gates, and is

particularly relevant in considering the dangers posed to children at open farms and petting zoos.

The effectiveness of common hypochlorite disinfectants in reducing pathogen survival on farm surfaces remains unknown. However, it is effective on food surfaces (Maule 2000), and could probably be employed to kill *E. coli* O157:H7 in potentially high-risk agricultural environments. Nevertheless, the financial and time implications of regular disinfectant applications to farmyard surfaces reduce the practicality of this method. Fratamico and Whiting (1995) and Fratamico and Cooke (1996) explored the potential for using predatory *Bdellovibrio* for the biological control of a range of food-borne pathogenic organisms on stainless steel surfaces used in the food industry. They found that *Bdellovibrio* reduced *E. coli* O157:H7 numbers by 3.6 $\log_{10}$ CFU cm^{-2} within 24 h of contact when present at a 10-fold higher population. Although effective, the feasibility of employing such a control method on a large scale to farmyard surfaces is questionable. Regarding *E. coli* O157 prevalence in farm environments, the proverb 'prevention is better than cure' is particularly apposite i.e. reducing *E. coli* O157:H7 primary proliferation in ruminant farm animals is optimal (Duncan *et al.* 2000).

In conclusion, *E. coli* O157:H7 persisted for a considerable length of time on a range of common farmyard surfaces, under a variety of environmental conditions (temperature and moisture). However, persistence was significantly affected by the type of surface material, with survival greater on wood than on galvanised steel. Employment of steel gates, rather than wood, should therefore lessen the threat of *E. coli* O157:H7 infection to humans. Furthermore, persistence was also notably different under varying environmental conditions, with significantly greater pathogen recovery from surfaces under cool temperatures (5°C) and humid conditions. As farmers might posses a degree of immunity to *E. coli* O157:H7 (Silvestro *et al.* 2004), it is likely that the greatest risk will be to 'day' visitors to farm environments. Based upon UK climatic conditions, our results suggest that pathogen persistence on surfaces and the subsequent risk of infection will be greatest in winter, and reduced in the summer when visitor numbers are highest. As cattle are primarily housed indoors in winter, it is likely that the risk of contamination in winter will be largely associated with housing areas or where animal waste spreading on fields has occurred.

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CHAPTER 4: ARTICLE II

Moisture, sawdust, and bleach regulate the persistence of *Escherichia coli* O157:H7 on floor surfaces in meat-processing environments

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4.1. Abstract

Escherichia coli O157:H7 has been widely associated with uncooked meat products through which introduction of the organism into butcher's premises can occur. Persistence of the bacterium on floor tiles of butcher shops is likely to increase the risk of food-borne human infection through cross-contamination of meat or other surfaces. Survival of *E. coli* O157:H7 on typical butcher shop ceramic floor tiles contaminated with meat juice was compared in the presence and absence of sawdust, and under different moisture and cleaning regimes. Floor tiles from a butcher shop were cut into 5 × 5 cm pieces, and half were cleaned with commercial bleach diluted with water at 60°C to simulate mopping. A coating of commercial sawdust collected from a butcher shop was applied to half of the tiles, while the other half were left bare. Meat juice collected from beef joints was inoculated with *E. coli* O157:H7 (strain #3704), and subsequently applied onto tiles at a density of 3×10^6 CFU cm^{-2}. Thereafter, tiles were stored at room temperature (20 ± 2°C), with half maintained under moist conditions [relative humidity (RH) close to 100%] and the other half gradually air-dried (RH 70 ± 5%). Viable *E. coli* O157:H7 persisted on all tiles over 3 days, although the rate of die-off varied with environmental conditions. Desiccation of surfaces resulted in a more rapid decline in *E. coli* O157:H7 numbers, while cleaning of the tiles with bleach prior to contamination with meat juice resulted in significantly greater recovery of the pathogen. Overall, greater numbers of cells were recovered from tiles when no sawdust had been applied; however, the presence of sawdust was only found to reduce survival of *E. coli* O157:H7 on tiles under dry conditions, and damp sawdust actually increased survival. This highlights the importance of regular cleaning and removal of sawdust to reduce pathogen persistence.

Keywords: bacteria; beef; coliform; contamination; disinfection; hypochlorite; meat products; public health; slaughterhouse; survival; tiles; VTEC.

4.2. Introduction

The inevitable spillage of fluids (e.g. water, blood) and fat-based solids onto tiled floors of meat-processing environments (e.g. butcher shops, slaughterhouses) presents both cleaning problems and dangerous working conditions from slippery surfaces. Due to its absorbent nature, a coating of sawdust is frequently applied to floors to reduce such problems in butcher shops; hence sawdust may possibly come into contact with meat.

Cross-contamination of carcasses with intestinal content or faeces occasionally arises during slaughter and processing (Borch and Arinder 2002; Bouvet *et al.* 2002), and this contamination can remain on the carcass during subsequent processing (McEvoy *et al.* 2003). Raw meat may therefore harbour a number of intestinal-derived bacteria associated with human food-poisoning, including *Escherichia coli* O157:H7 (Little and de Louvois 1998; Borch and Arinder 2002; Nel *et al.* 2004). Studies have shown that the prevalence of *E. coli* O157 in raw meat products may be as high as 9% (Caprioli *et al.* 1997); and consumption of contaminated meat is one of the main vectors of *E. coli* O157:H7 transmission to humans (Bolton *et al.* 1996; Chapman 2000; Coia *et al.* 2001).

E. coli O157:H7 infection symptoms may include haemorrhagic colitis (HC) or life-threatening haemolytic uraemic syndrome (HUS) (Chart 2000). As ingestion of a very small number of cells (< 10 CFU) may be sufficient to initiate human infection (Chart 2000), this accentuates the importance of minimising survival and contamination routes in meat-processing environments.

As *E. coli* O157 is known to persist on a range of different surfaces under varying environmental conditions (Ak *et al.* 1994; Maule 2000; Mattick *et al.* 2003; Williams *et al.* 2005), the organism is likely to survive on floor tiles within a meat-processing environment. Although there are a number of reports on survival of non-O157 *E. coli* in sawdust incorporated into animal bedding (Nodar *et al.* 1990; Hogan and Smith 1997; Hogan *et al.* 1999), no studies to date have addressed the use of sawdust on floors in meat-processing and retail environments and whether this traditional practice is beneficial in terms of hygiene. As *E. coli* O157:H7 infections have occurred following contact with sawdust (Varma *et al.* 2003), and the organism has been shown to survive, and grow, for substantial periods in soiled sawdust (LeJeune and Kauffman 2005), this raises concern over the implications of its use in meat preparation areas. The aim of this study was to compare *E.*

coli O157:H7 survival on bare floor tiles, in the presence and absence of sawdust, and under different moisture and cleaning regimes.

4.3. Materials and methods

4.3.1. Preparation of materials

A representative sample of commercial sawdust was collected from a butcher shop in North Wales, and stored at room temperature (20 ± 2°C) in a sealed plastic bag. Ceramic 'quarry' floor tiles were collected from the same shop, and were subsequently cut to smaller samples (5 × 5 cm) using a diamond-blade saw (Herbert Arnold GmbH & Co., KG Weilburg an der Lahn, Germany). The pieces were then laid out surface uppermost in a randomised-block design in metal trays containing filter paper. Prior to the commencement of the experiment, half of the tile pieces were swabbed rigorously (60 s, vertically and horizontally) with sterile cotton wool immersed in commercial bleach (10% sodium hypochlorite; Arco Ltd., Hull, UK) diluted with heated (60°C) water according to the manufacturer's instructions (1:100 v/v). These were left to gradually dry overnight so as to simulate standard cleaning procedures ('mopping') at a butcher shop. The following morning, 0.5 g of sawdust was applied as a representative dose to half the tiles of the same treatment (i.e. half of bleached and unbleached tiles; sawdust density of 0.02 g cm^{-2}). Throughout the experimental period, half of the tile pieces of each sawdust/bleach treatment combination were kept moist by misting with sterile deionised water twice, daily (approximately 0.5 ml cm^{-2} per application); whilst no water was applied to the other half, allowing them to gradually dry (RH 70 ± 5%). All trays were stored at room temperature (20 ± 2°C).

4.3.2. Characterisation of sawdust

Sawdust moisture content and organic matter were determined after drying overnight at 80°C and ashing at 450°C, respectively. Total C and N were quantified using a CHN2000 elemental analyser (Leco Corp., St Joseph, MI). The water-holding capacity was determined from weighing the sawdust after immersing it in water overnight, and particle size was determined by sieving. Electrical conductivity and pH were measured on a 1:1 (v/v) dilution of the sawdust with distilled water using a 4010 EC meter (Jenway Ltd., Dunmow, UK) and an Orion 410A pH meter (Thermo Ltd., Runcorn, UK). To determine sawdust chemical characteristics, 10 g of the sawdust was shaken with 100 ml of deionised water for 1 h on a

reciprocating shaker (250 rev min^{-1}), centrifuged (12,000 *g*, 15 min), and the supernatant solution retained for analysis. Thereafter, this solution was used to determine Ca, Na, and K via a PFP7 flame photometer (Jenway Ltd.), total dissolved nitrogen and dissolved organic carbon via a TC-TNV analyser (Shimadzu Corp., Kyoto, Japan), NO_3^- and NH_4^+ via a segmented flow San plus analyser (Skalar, York, UK), and available P via a CE 343 Spectrophotometer (Cecil Instruments Ltd., Cambridge, UK).

A relative estimate of background heterotrophic bacteria counts of sawdust were determined by shaking 0.5 g (250 rev min^{-1}, 45 min, room temperature) in 10 ml ¼-strength Ringers solution (Oxoid, Basingstoke, UK), followed by 4 × 5-second bursts on a vortex mixer. Solutions were then plated in duplicate onto R2A agar, and colonies counted following incubation for 48 h at 20°C. To confirm absence of *E. coli* O157:H7 on clean sawdust, an enrichment procedure was carried out. Specifically, 5 g of sawdust was shaken (150 rev min^{-1}, 6 h, 37°C) in 15 ml modified Tryptone Soya broth (mTSB; Oxoid) and streaked onto Sorbitol MacConkey agar plates supplemented with 0.05 mg l^{-1} cefixime and 2.5 mg^{-1} potassium tellurite (CT-SMAC; Oxoid). After incubation (18 h, 37°C), plates were examined for colonies exhibiting typical *E. coli* O157:H7 morphology. Non-sorbitol-fermenting colonies were confirmed as *E. coli* O157:H7 colonies or not by latex agglutination (Oxoid DR620).

4.3.3. Characterisation of meat juice

Meat juice (approximately 150 ml) was collected from 3 bovine meat joints that had been hung in sterile plastic bags in the butchers' cold-room (4 ± 1°C) for 10-12 d. The juice was pooled together, and stored at 4°C for 24 h prior to experimental work. All chemical characteristics were measured directly without dilution by the methods described previously.

A relative estimate of background heterotrophic bacteria counts were determined by plating serial dilutions (10^{-1} to 10^{-5}) of the juice in duplicate onto R2A plates, as described above. Enrichment (as for sawdust with the exception that 5 ml meat juice was added to 15 ml mTSB) and latex agglutination was used to confirm absence of *E. coli* O157:H7.

4.3.4. Characterisation of bleach

Electrical conductivity and pH were measured directly without dilution by the methods described previously, whilst chloride was measured using a Sherwood MK II Chloride Analyser 926 (Sherwood Scientific Ltd., Cambridge, UK).

4.3.5. Preparation of E. coli *O157:H7 inoculum*

An inoculum was prepared from a fresh overnight culture (LB broth; Difco Ltd., Surrey, UK; 18 h, 37°C, 150 rev min^{-1}) of an environmental isolate of *E. coli* O157:H7 (strain #3704) (Campbell *et al.* 2001). The strain has been proven to be non-toxigenic due to the absence of toxin activity and toxin genes (Campbell *et al.* 2001). Cells were washed and concentrated as described in Avery *et al.* (2005).

4.3.6. Preparation and application of inoculated meat juice

A 10 ml aliquot of *E. coli* O157:H7 inoculum was added to 90 ml of meat juice and the suspension was thoroughly mixed. To determine the concentration of the inoculum, serial dilutions (10^{-1} to 10^{-6}) were performed and plated in duplicate onto CT-SMAC agar, and colonies exhibiting typical *E. coli* O157:H7 morphology and agglutination following the latex test were counted following incubation (18 h, 37°C). While *E. coli* O157 numbers in infected cattle faeces may reach up to 10^{7} CFU g^{-1} faeces (Fukushima and Seki 2004), the inoculation density aimed to imitate potential numbers in faecally-contaminated meat, and similar densities found in previous studies (Nel *et al.* 2004). Furthermore, *E. coli* O157:H7 numbers on contaminated meat may increase considerably in a short period of time under a range of temperatures (Nissen *et al.* 2001; Borch and Arinder 2002; Li and Logue 2005). A 1 ml sample of the inoculated juice was evenly spread with a sterile spreader on the surface of each tile piece; taking care to avoid runoff, especially on bare tiles (i.e. sawdust not applied). The following 4 factorial tile-treatment combinations were finally prepared for both bleached and unbleached tile pieces: water + meat juice, sawdust + water + meat juice, meat juice only, sawdust + meat juice. Identical tile-treatment combinations were applied the same volume of unspiked meat juice ('controls').

4.3.7. Sampling of tiles for E. coli *O157:H7*

Three randomly selected replicate tiles of each treatment combination were sampled for *E. coli* O157:H7 at day 0 (within 3 h), and days 1, 2, and 3 following the application of inoculated juice onto tiles. Sawdust from tiles was collected in 50 ml sterile centrifuge tubes, and the tile surface was subsequently swabbed rigorously (60 s, vertically and horizontally) with sterile cotton wool dampened with ¼-strength Ringers solution; which was placed in the same centrifuge tube. Similarly, bare tile pieces were swabbed with sterile cotton wool and the cotton wool placed in sterile centrifuge tubes. Each tube was applied 20 ml of ¼-strength Ringers solution, and subsequently shaken at 250 rev min^{-1} for 45 min at 20°C, followed by 4 × 5-second bursts on a vortex mixer. *E. coli* O157:H7 were enumerated by plating serial dilutions of the suspension onto CT-SMAC agar, and incubating and latex agglutination as described previously.

4.3.8. Determination of meat juice moisture content and sawdust pH

Using 'control' tiles and the methods described previously, the moisture content of the meat juice on the tile surfaces was determined at each sampling date, whilst the pH of all sawdust from tiles (± bleach) was determined at the final sampling (day 3).

4.3.9. Data analysis

All experiments were performed in triplicate, unless otherwise stated. Plate count data from *E. coli* O157:H7 were log_{10} transformed and analysed using a multi-factorial analysis of variance (ANOVA) on Genstat 7 (VSN International Ltd., Hemel Hempstead, UK), with significant differences between treatments identified using Fisher's LSD test.

4.4. Results

4.4.1. Characterisation of sawdust and meat juice

A summary of the physical, chemical, and biological characteristics of the sawdust and meat juice is shown in Table 4.1. The bulk of sawdust particles were between 0.6-2 mm in size; and the solution extracted from the sawdust was moderately acidic (pH 4.56). Although it exhibited substantial moisture-holding capacity (451 g kg^{-1}), the moisture content of the sawdust was low (144 g kg^{-1}). The electrical conductivity (EC) of sawdust was low (80 µS cm^{-1}), with very low free ion concentrations. The sawdust possessed a high C-to-N ratio

(350). Only a relatively low number of bacteria were harboured within sawdust (2.8×10^2 CFU cm^{-3}), with no *E. coli* O157:H7 isolated. The meat juice was only slightly acidic (pH 6.35); and it possessed a high free ion concentration (such as Na, K, and P) and EC. The meat juice was relatively N rich (C-to-N ratio = 3.3). Although it supported a large microbial population (4.7×10^4 CFU cm^{-3}), no *E. coli* O157:H7 was isolated from meat juice.

Table 4.1. Intrinsic physical, chemical, and biological characteristics of sawdust and meat juice used in the study (units given in respective order). All values represent the means of three replicate samples. Units of physical values are displayed; while chemical values are in mg g^{-1} and mg l^{-1} for sawdust and meat juice, respectively (unless otherwise stated); and bacteria counts are in CFU cm^{-3}. The detection limit for *E. coli* O157:H7 counts was 5 CFU cm^{-3}. NA = not applicable.

Parameter	Sawdust	Meat juice
pH	4.56	6.35
Electrical conductivity (μS cm^{-1})	80	11400
Moisture content (g kg^{-1})	144	940
Organic matter (g kg^{-1})	855	898
Water-holding capacity (g kg^{-1})	451	NA
Particle size (%)	74.9 > 0.6 mm 25.1 > 2 mm	NA
Total C (%)	45.5	3.85
Total N (%)	0.13	1.42
Dissolved organic carbon	6.32	48240
Dissolved total nitrogen	0.18	14420
Available P	0.05	2100
NO_3^-	0.002	0.71
NH_4^+	0.027	87.42
Ca	0.44	280
Na	0.52	500
K	1.2	4600
Background heterotrophic bacteria	2.8×10^2	4.7×10^4
E. coli O157:H7	0	0

4.4.2. Survival of E. coli *O157:H7 on tile pieces*

The *E. coli* O157 density on each tile at the onset of the experiment was approximately 3×10^6 CFU cm^{-2}. Data gathered displayed normal distribution and equal variances following log transformation. In general, numbers of *E. coli* O157:H7 declined significantly ($P < 0.001$) over time on all of the tile pieces, however, numbers remained notably high on day 3 under many treatments (Fig. 4.1). Whilst the greatest drop in counts for the majority of treatment combinations typically occurred between day 0 and day 1, counts increased

slightly or stabilised in all but two (bleached, sawdust + meat juice; unbleached, sawdust + meat juice) treatments between days 1-2 (Fig. 4.1). By day 3, numbers varied markedly between different treatments, having further increased from the previous sampling point in most 'bleached' treatments, but declined to their lowest number in other (mainly 'unbleached') treatments (Fig. 4.1). *E. coli* O157:H7 numbers did not decline sufficiently within the 3 d period to allow calculation of D-values.

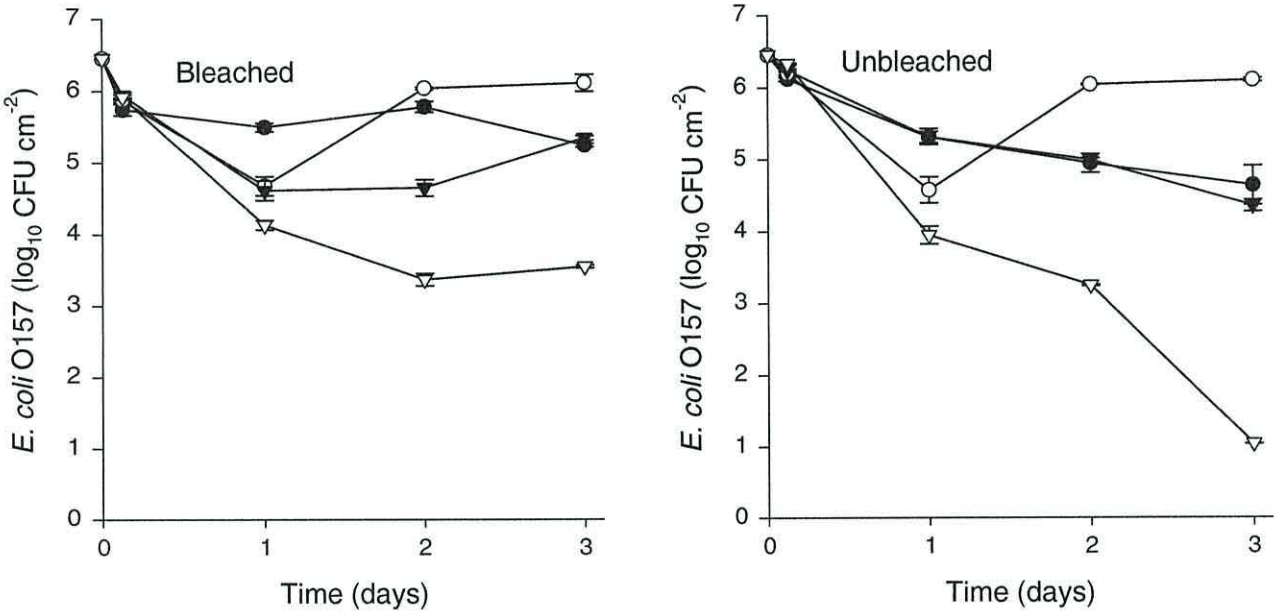

Figure 4.1. Survival of *E. coli* O157:H7 on bleached and unbleached tiles under different conditions: water + meat juice (●), sawdust + water + meat juice (○), meat juice only (▼), sawdust + meat juice (▽). Curves represent the mean $\log_{10}$ (y + 1) *E. coli* O157:H7 cm^{-2} ± SEM of three replicate tile pieces.

4.4.3. Impact of sawdust on E. coli *O157:H7 survival*

On visual inspection, it appeared that a considerable portion of the meat juice was absorbed by the sawdust when tiles were maintained in a dry state, owing to the sawdusts' high moisture-holding capacity (Table 4.1). Meat juice on tiles with neither water nor sawdust became completely desiccated after 36 h; whilst this occurred after 42 h on tiles with sawdust alone (Fig. 4.2). However, drying rates in the presence or absence of sawdust were not significantly different ($P > 0.05$). On the whole, survival of *E. coli* O157:H7 on tiles was decreased in the presence of sawdust [overall mean ± SEM ($\log_{10}$ CFU cm^{-2}): sawdust present, 4.82 ± 0.08; sawdust absent, 5.29 ± 0.21; $P < 0.001$]. However, it was observed that

the presence of sawdust only reduced survival of *E. coli* O157:H7 on tiles under dry conditions, and damp sawdust actually significantly increased survival, regardless of tiles' bleaching status [overall mean ± SEM (log_{10} CFU cm^{-2}): sawdust + water + meat juice, 5.70 ± 0.19; sawdust + meat juice, 3.94 ± 0.43; $P < 0.001$; Fig. 4.1].

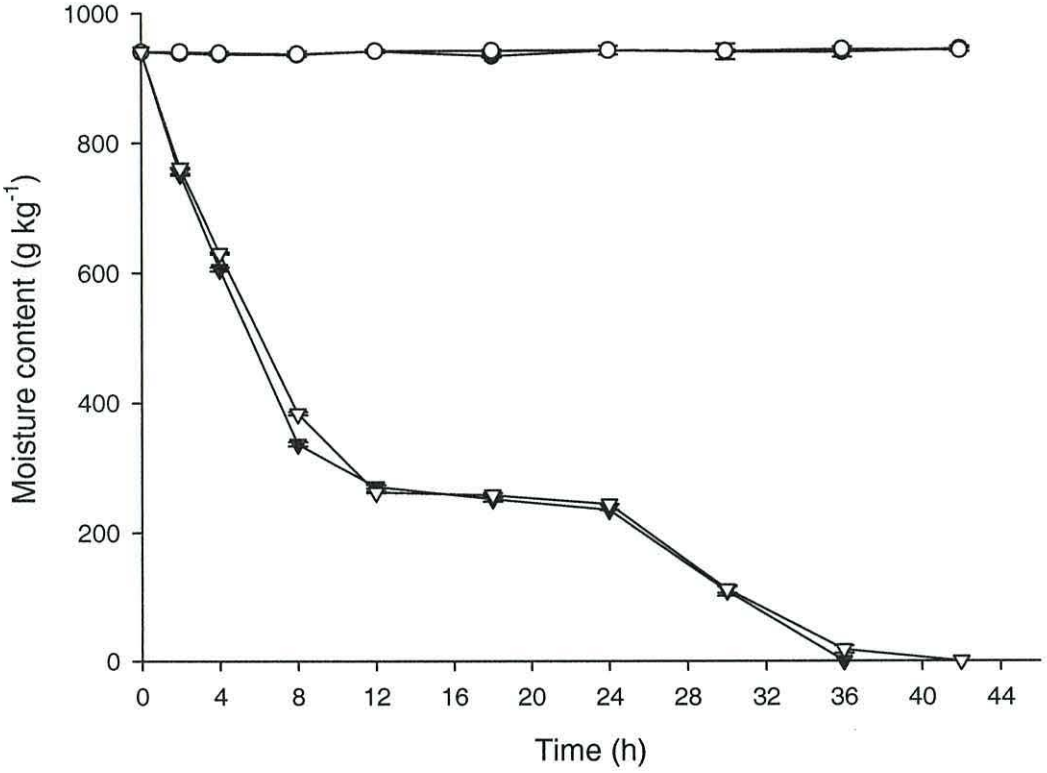

Figure 4.2. Changes in meat juice moisture content on tiles with time under different environmental conditions [water + meat juice (●), sawdust + water + meat juice (○), meat juice only (▼), sawdust + meat juice (▽)]. Curves represent the mean moisture content (g kg^{-1}) ± SEM of four replicate tile pieces for all treatments.

4.4.4. Impact of moisture on E. coli *O157:H7 survival*

Where sawdust and water were applied, the damp sawdust absorbed less of the juice therefore more was partitioned on the tile surface. Water application maintained the juice, the underlying tile, and sawdust (where present) in a moist state throughout the experiment; whilst in contrast, meat juice on tiles which received no water dried out (Fig. 4.2). The application of moisture to tile surfaces significantly increased survival of *E. coli* O157:H7 ($P < 0.001$); with the overall mean greater by 1.00 log_{10} CFU cm^{-2} on tiles maintained in a moist state compared to counterpart treatment combinations maintained in a desiccated state.

4.4.5. Impact of bleach on E. coli *O157:H7 survival*

Bleach pH was moderately high (9.32); whilst it displayed reasonably high electrical conductivity (1740 μS cm^{-1}) and chloride concentration (487 mg l^{-1}) values. In initial stages of the experiment, numbers of *E. coli* O157:H7 were somewhat greater on tiles to which bleach had not been applied (Fig. 4.1). However, this trend reversed with time, and overall significantly greater numbers of cells were recovered from pre-bleached tiles [overall mean ± SEM (log_{10} CFU cm^{-2}): bleached, 5.15 ± 0.04; unbleached, 4.96 ± 0.09; $P < 0.001$]. Applying water to tiles did not significantly affect the impact of bleach on *E. coli* O157:H7 numbers (bleach × moisture interaction, $P = 0.059$). However, the presence of sawdust on tiles affected *E. coli* O157:H7 numbers depending on their bleaching status (bleach × sawdust interaction, $P = 0.016$); with greatest numbers recovered from tiles applied both bleach and sawdust, whilst lowest numbers were recovered from tiles applied neither of the two.

On day 3, the mean pH of sawdust from tiles applied bleach was greater than from tiles not applied bleach (5.97 and 5.29, respectively), however, this difference wasn't significant (t-test, $P > 0.05$).

4.5. Discussion

As carcasses may be contaminated with *E. coli* O157 (Little and de Louvois 1998; Borch and Arinder 2002), spillage of blood/meat juices onto the floors of butcher shops could potentially lead to cross-contamination of meat or preparation surfaces. Consumption of contaminated meat has been responsible for numerous human *E. coli* O157 infections (Bolton *et al.* 1996; Chapman 2000; Coia *et al.* 2001); and cross-contamination poses particular risk in butchers trading both raw and cooked meats (The Pennington Group 1997; Little and de Louvois 1998; Pennington 2000). Adhesion of pathogenic micro-organisms in food-processing environments is a major public health concern (Ryu *et al.* 2004b). Our study shows that *E. coli* O157:H7 is likely to persist for a substantial time (> 3 d) on tiles commonly found on butcher shop floors if left uncleaned, although survival may be affected by a range of interacting environmental factors.

Following a report by The Pennington Group (1997), UK butchers are subject to a licensing scheme based upon numerous requirements to ensure food safety; including food

hygiene training of staff, and documentation of hazard analysis and critical control points (HACCP) (The Pennington Group 1997; Little and de Louvois 1998). However, appraisals of butcher premises and cleaning regimes reveal considerable differences in levels of staff training in food safety procedures/activities, leading to inadequate cleaning frequency and improper use of cleaning chemicals (Little and de Louvois 1998; Gillespie *et al.* 2000; Pennington 2000; Worsfold 2001). This is of concern as work routines provide many opportunities for contamination of surfaces and floors (Worsfold 2001). Thereafter, pathogenic bacteria can survive extensive periods if inefficient cleaning procedures are in place (Scott and Bloomfield 1990; Mattick *et al.* 2003).

As the majority of raw meat requires some form of preparation (e.g. boning, cutting) prior to being sold, this greatly increases the likelihood of blood/meat juice spillage onto floors and equipment in processing areas (O.E. Metcalfe, personal communication) and thus of contamination with pathogens such as *E. coli* O157 (McEvoy *et al.* 2003; Nel *et al.* 2004). Floors in processing areas therefore warrant extra frequent cleaning; although in reality this is difficult to regulate. Studies to establish the prevalence of *E. coli* O157:H7 on butcher shops' floors would be of interest. We aimed to simulate floor tiles in this environment and a standard cleaning procedure with bleach, a common hypochlorite sanitizer widely used in the food industry to clean surfaces. Surprisingly, greater numbers of *E. coli* O157:H7 were recovered from tiles cleaned with bleach prior to application of meat juice. The disinfection procedure may have reduced background microbial populations which could otherwise potentially inhibit *E. coli* O157:H7 survival; or may have altered tile surface characteristics - reducing the adhesion force with which cells bound, thus rendering them easier to remove by swab-sampling. Common hypochlorite disinfectants are generally regarded effective in reducing survival of pathogens on food surfaces (Maule 2000), including a range of *E. coli* strains (Vijayakumar and Wolf-Hall 2002). However, serotype O157:H7 may possess extraordinary tolerance to a wide range of environmental stresses (Park *et al.* 1999), and may therefore exhibit greater resistance to hypochlorite than other strains. Furthermore, the sanitising ability of bleach largely depends upon its chlorine concentration (Brackett 1987; Vijayakumar and Wolf-Hall, 2002), and nutrient-limiting conditions such as tile surfaces may initiate chlorine-resistant phenotypes in *E. coli* O157 (Chalmers *et al.* 2000; Maule 2000) and enhance biofilm formation (Ryu *et al.* 2004a). This may reduce the effectiveness of removing or inactivating cells by conventional cleaning and sanitising procedures used

during food-processing (Stopforth *et al.* 2003; Ryu *et al.* 2004ab). Other studies have shown the bacterium to survive a simulated washing-up process using detergent and warm water (Mattick *et al.* 2003). Further studies are therefore needed to evaluate the efficacy of conventional and novel (e.g. commercial sodium hydroxide) cleaning chemicals used for sanitisation in the food industry.

Divergence in *E. coli* O157:H7 recovery increased with time between desiccated and moist tiles, indicating that prolonged desiccation augmented cell-stress. However, the resilience of *E. coli* O157:H7 in a desiccated state on surfaces has also been demonstrated (Maule 1999; Mattick *et al.* 2003; Williams *et al.* 2005), and it was recovered from dry tiles throughout the three-day experimental period of the current study. The effect of different temperature regimes was not investigated in this study, as it was designed to simulate conditions reflecting a typical butcher shop. However, as higher temperatures exacerbate desiccation effects on *E. coli* O157:H7 cells (Maule 1999; Williams *et al.* 2005), survival may thus be extended under cooler conditions such as in butchers' cold-rooms.

As the sawdust gathered for this experiment originated from pine-wood, bacteria persistence may be reduced due to polyphenolic substances naturally present in the wood (Miller *et al.* 2003; Milling *et al.* 2005), or those derived from wood preservatives. Although the sawdust used in this trial was a representative sample of that habitually used by the butcher shop where it was sourced, sawdust of different origin and age may possess different polyphenol contents. This, along with other physico-chemical variations may influence the persistence of *E. coli* O157:H7. When maintained in a dry state, the absorbent nature of sawdust may further reduce the moisture availability to bacterial cells, which may also induce a decline in their numbers. However, the actual hygiene benefits from applying sawdust to butcher shop floors are unclear since survival of *E. coli* O157:H7 on sawdust-covered tiles under moist conditions was greater than in any other treatment. Furthermore, studies show that *E. coli* O157:H7 is readily aerosolised when contaminated sawdust is disturbed on floors and may subsequently be swallowed by humans (Varma *et al.* 2003; LeJeune and Kauffman 2005). Collectively, it therefore appears that sawdust may be useful in reducing O157:H7 persistence on floors if kept dry and frequently changed when soiled; but, when moist, sawdust appears to provide a favourable environment for harbouring the pathogen, or even allowing growth. It does, however, remain unclear whether employees tend to reduce the frequency of mopping due to additional work required to remove and

replace sawdust. This and other cleaning-regime issues may be clarified with a survey of butchers' cleaning practices.

Along with rapid die-off of less resistant cells, the initial drop in *E. coli* O157:H7 counts observed within 3 h of inoculating the meat juice and applying onto tiles may have been due in part to the presence of background bacteria, neutrophils, antibodies, and complement present in the juice (Nissen *et al.* 2001; Hepburn *et al.* 2002). However, the age of the meat juice when collected (10-12 d) meant that the activity of some, or all, of these components is likely to have been reduced; and together with bacteria acclimatisation, possibly facilitated the growth of *E. coli* O157:H7 observed thereafter. Enumerating total microbial counts over time in further investigations would elucidate the interaction between the meat juice natural fauna and the pathogen.

Swabbing may have led to underestimations of the total bacterial numbers on surfaces due to the formation of biofilms, although bacteria counts were similar to those quoted in the literature for sawdust (Nodar *et al.* 1990; Hogan and Smith 1997; Hogan *et al.* 1999), and meat juice (Gill and McGinnis 2004; Gill *et al.* 2005). Recovery of stressed *E. coli* O157:H7 cells may have also been reduced on the selective medium employed (Taormina *et al.* 1998). Nevertheless, the aim of this study was to provide a comparative insight into the impact of environmental conditions on *E. coli* O157:H7 survival on floor tiles. While it is not possible to extrapolate the behaviour under laboratory conditions of a single non-toxigenic strain of *E. coli* O157:H7 to that of all strains under environmental conditions (Duffy *et al.* 1999; Chalmers *et al.* 2000), such data can provide valuable information relating to the factors likely to influence survival patterns of toxigenic strains (Kudva *et al.* 1998; Ritchie *et al.* 2003).

In conclusion, persistence of *E. coli* O157:H7 was significantly reduced by the presence of sawdust on tiles under dry conditions. Spreading sawdust onto floors therefore has both practical and hygiene benefits for the meat retailer as it reduces persistence of *E. coli* O157:H7. However, moist or soiled sawdust should be disposed of and replaced frequently to minimise risk of contaminated, aerosolised sawdust coming into contact with meat or surfaces. Our findings emphasise the need for frequent cleaning in meat preparation environments.

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CHAPTER 5: ARTICLE III

Earthworms as vectors of *Escherichia coli* O157:H7 in soil and vermicomposts

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5.2. Abstract

Survival and movement of *Escherichia coli* O157:H7 in both soil and vermicompost is of concern with regards to human health. Whilst it is accepted that *E. coli* O157:H7 can persist for considerable periods in soils, it is not expected to survive thermophilic composting processes. However, the natural behaviour of earthworms is increasingly utilised for composting (vermicomposting), and the extent to which earthworms promote the survival and dispersal of the bacterium within such systems is unknown. The faecal material produced by earthworms provides a ready supply of labile organic substrates to surrounding microbes within soil and compost, thus promoting microbial activity. Earthworms can also cause significant movement of organisms through the channels they form. Survival and dispersal of *E. coli* O157:H7 were monitored in contaminated soil and farmyard manure subjected to earthworm digestion over 21 d. Our findings conclude that anecic earthworms such as *Lumbricus terrestris* may significantly aid vertical movement of *E. coli* O157 in soil, whilst epigeic earthworms such as *Dendrobaena veneta* significantly aid lateral movement within compost. Although the presence of earthworms in soil and compost may aid proliferation of *E. coli* O157 in early stages of contamination, long-term persistence of the pathogen appears to be unaffected.

Keywords: contamination; earthworms; *Escherichia coli* O157:H7; soil; survival; vermicompost.

5.3. Introduction

There is increasing awareness that waste management needs to be an integral part of a sustainable society, hence necessitating diversion of biodegradable fractions from landfill into alternative management processes such as composting. This generates substantial volumes of 'green waste'-derived composts for commercial markets. Such composts may include animal-derived wastes, which frequently harbour pathogenic bacteria such as *Escherichia coli* O157:H7 (Hutchison *et al.* 2004; Nicholson *et al.* 2005).

E. coli O157:H7 is an intestine-inhabiting bacterium associated with many severe disease outbreaks worldwide. While typically asymptomatic in animals, human infection may lead to haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS), or even death (Chart 2000). Although *E. coli* O157:H7 is harboured by a range of different animals, cattle represent the main environmental reservoir. Infected animals typically excrete between 10^2-10^5 CFU *E. coli* O157:H7 g^{-1} faeces (Hutchison *et al.* 2004); however, recent studies have recovered up to 10^7 CFU *E. coli* O157:H7 g^{-1} faeces (Besser *et al.* 2001; Fukushima and Seki 2004). The human infectious dose is very low, and ingestion of as few as 10 cells is thought to be sufficient to cause illness (Chart 2000).

The high temperatures generated within thermophilic compost (50-70°C) destroy mesophilic organisms such as *E. coli* O157:H7 (Ndegwa and Thompson 2001; Jones and Martin 2003). However, the presence of mutant thermophilic strains and/or failure to maintain high temperatures for a sufficient length of time may lead to survival and growth of the bacterium (Droffner *et al.* 1995; Elorrieta *et al.* 2003; Nicholson *et al.* 2005). In addition, the observed long-term persistence of this organism in manure-amended soil (Bolton *et al.* 1999; Jiang *et al.* 2002) raises concerns about the use of compost for soil fertilisation. Growing salad vegetables in *E. coli* O157:H7-contaminated soil or compost may pose a health risk as such vegetables are frequently consumed raw, and the bacterium may survive or even grow on their surface (Abdul-Raouf *et al.* 1993), or become internalised within tissue (Solomon *et al.* 2002; Jablasone *et al.* 2005). Furthermore, pre-harvest contamination of vegetables with *E. coli* O157:H7-infected compost is known to be responsible for enterohemorrhagic food poisoning outbreaks (Islam *et al.* 2005).

Using earthworms as a substitute to thermophilic composting or as a secondary waste treatment method (vermicomposting) is becoming increasingly common as several

studies have shown increased plant growth and yield when grown in the presence of vermicomposts (Atiyeh *et al.* 2000; Arancon *et al.* 2004ab; Lee *et al.* 2004).

Earthworms can exert a considerable influence on the surrounding microbial community, and may promote microbial activity within soil and composts due to the faecal material or 'casts' they produce, which provide a rich carbon source (Ndegwa and Thompson 2001; Li *et al.* 2002). Earthworms may also indirectly induce significant movement of faecal indicator organisms and pathogens via mass water flow through abandoned channels (Joergensen *et al.* 1998; Artz *et al.* 2005). Different earthworm species inhabit different soil regions according to whether they are 'anecic' (inhabiting deep soil layers, e.g. *Lumbricus terrestris*) or epigeic (inhabiting surface organic layers e.g. *Dendrobaena veneta*) species (James and Hendrix 2004; Parkinson *et al.* 2004). However, it is unclear whether the behaviour of earthworm species affects movement of soil bacteria. As earthworms form a central part of the biological community in most agricultural soils, their presence may thus enhance persistence and dissemination of pathogens such as *E. coli* O157:H7 within this environment. Although a few studies have reported significant reductions of faecal coliforms and *Salmonella* spp. during vermicomposting (Murry and Hinckley 1992; Eastman *et al.* 2002), the fate and movement of *E. coli* O157:H7 in compost remains unclear. Furthermore, vermicomposting is not yet considered by the US Environmental Protection Agency as an alternative method for pathogen reduction for 'class A' products [biosolids than can be land-applied without any pathogen-related restrictions at the site and can be sold bagged to the public (EPA 1999; Tognetti *et al.* 2005)]. Since earthworms are often commercially bred in a matrix containing cattle manure and other waste materials, this may potentially serve as a vector for generating large volumes of *E. coli* O157:H7-contaminated compost.

The aim of this current study was to assess the impact of earthworm activity on *E. coli* O157:H7 movement and persistence in soil and vermicomposts.

5.4. Materials and methods

5.4.1. Soil, compost, and manure collection and characterisation

Soil (Eutric cambisol of the 'Denbigh' series, 0-15 cm, Table 5.1) and earthworms (*Lumbricus terrestris*) were collected from a sheep-grazed pasture at Abergwyngregyn, North Wales, UK (53° 13.9' N, 4° 0.9' W). Earthworm bedding material (digested paper pulp and green waste (Roberts *et al.* 2006) and earthworms (*Dendrobaena veneta*) were

collected from commercial composting beds at the same site. Aged (> 1 month old) cattle manure was collected from a commercial farm in North Wales. After collection, all samples were stored in a climate-controlled room (Hemsec Ltd., Kirkby, UK) at 20°C, 70% relative humidity for the duration of the experimental period. This temperature aimed to reflect summertime soil and compost temperatures.

Nutrients were extracted using 1 M KCl at a 1:5 w/v ratio of soil, compost, and manure-to-1 M KCl. The samples were extracted by shaking (250 rev min^{-1}, 1 h, room temperature), centrifuged for 10 min (14,000 *g*), filtered (Whatman no. 42), and the supernatant recovered for analysis. NO_3^- and NH_4^+ were determined colorimetrically (Downes, 1978; Mulvaney, 1996) with a Skalar SAN^+ segmented flow analyser (Skalar Analytical, Breda, The Netherlands). Phosphate was measured colorimetrically (Murphy and Riley 1962), while K, Na and Ca were measured using a Sherwood Scientific 410 flame photometer (Sherwood Scientific, Cambridge, UK). Electrical conductivity (EC; Jenway 4010 EC meter) and pH (Orion 410A pH meter) were determined after a 1:1 (v/v) dilution of the soil, compost, and manure with distilled water. Moisture content was determined by drying for 24 h at 105°C and water-holding capacity gravimetrically (Rowell 1994). Total organic carbon and nitrogen were measured using a CHN2000 elemental analyser (Leco Corp., St Joseph, MI), whilst dissolved organic carbon (DOC) and dissolved nitrogen (DN) were measured using a TC-TNV analyser (Shimadzu Corp., Kyoto, Japan).

5.4.2. Background microbiology of samples

An enrichment technique was utilised to check for the presence or absence of background *E. coli* O157:H7 in the soil, compost, and manure. This was achieved by placing 5 g of each sample into 15 ml modified Tryptone Soya Broth (mTSB; Oxoid Ltd., Basingstoke, UK), and shaking (150 rev min^{-1}, 6 h, 37°C), before plating onto Sorbitol MacConkey agar plates supplemented with 0.05 mg l^{-1} cefixime and 2.5 mg l^{-1} potassium tellurite (CT-SMAC; Oxoid). Plates were then incubated for 18 h at 37°C, then examined and scored for presence or absence of colonies with the characteristic appearance of *E. coli* O157:H7. The detection limit of the enrichment technique was 5 CFU g^{-1} of matrix.

An estimate of background heterotrophic bacterial counts was undertaken by shaking 5 g of each sample (200 rev min^{-1}, 15 min, room temperature) in 15 ml of sterile ¼-strength Ringers solution (Oxoid), followed by 4 × 10-second bursts on a vortex

mixer. Serial dilutions of the solutions were subsequently plated in duplicate onto R2A agar (Oxoid), and colonies were counted following incubation for 48 h at 20°C.

5.4.3. Preparation of boxes and cores

To mimic field bulk density (data not presented), 10 kg of soil and 5 kg of compost (at field moisture contents) were manually sieved to pass 5 mm, and spread evenly in wooden boxes (550 × 550 × 150 mm) in triplicate. To assess lateral movement of *E. coli* O157:H7, 22 g of *L. terrestris* (all earthworm weights expressed as 'live weights') were added to soil [representing measured field density, data not presented); and 500 g of *D. veneta* were added to compost (representing typical vermicomposting densities (Williams 2004)]. Other boxes without the addition of worms were also prepared and stored in triplicate under the same conditions (controls). Vertically-held PVC cores (66 × 66 × 500 mm) were also prepared, containing 1.5 kg of soil and 5 g of earthworms (*L. terrestris*). These were to assess vertical movement of *E. coli* O157:H7 by earthworms in soil. Cores without the addition of worms were also prepared and stored in triplicate under the same conditions (controls). All boxes and cores were left for 72 h prior to commencing the experiment to allow worms to acclimatise and distribute themselves evenly throughout the soil or compost. Soil in cores were maintained at their original moisture contents by establishing an individual 'water table' around each core by placing the bottom of the core into a plastic sleeve and topping up the water level daily to a maximum height of 10 cm with an artificial rainwater solution (Jones and Edwards 1993). Soil and compost in boxes were maintained at their original weights by daily watering to their original weights and misting the surfaces with artificial rainwater. This watering method was employed in order to prevent leaching/movement of bacteria though matrices via water.

5.4.4. Preparation of E. coli *O157:H7 inoculum*

An inoculum was prepared from a fresh overnight culture (LB broth; Difco Ltd., Teddington, Surrey, UK; 150 rev min^{-1}, 18 h, 37°C) of an environmental isolate of *E. coli* O157:H7 [strain #3704 (Campbell *et al.* 2001)]. Although the strain has been proven to be non-toxigenic due to the lack of toxin gene expression (Campbell *et al.* 2001), it (and similar strains) still accurately reflects survival patterns of toxigenic *E. coli* O157:H7 strains (Kudva *et al.* 1998; Bolton *et al.* 1999; Ritchie *et al.* 2003). Cells were washed and concentrated by centrifugation as described in Avery *et al.* (2005).

5.4.5. Preparation and application of spiked manure

A 120 ml aliquot of the *E. coli* O157:H7 inoculum was added to 2.3 kg of manure and thoroughly mixed to give a final concentration of approximately 3.0×10^8 CFU g^{-1} manure [to imitate a 'worst case' scenario where initial contamination levels are similar to the highest naturally encountered (Besser *et al.* 2001; Fukushima and Seki 2004)]. This was determined by enumeration on CT-SMAC agar as described previously. Spiked manure (200 g) was then applied in a linear band at one end of each box. Correspondingly, 5 g of the manure was applied to the surface of the soil cores. This gave triplicates of six treatment combinations as follows: soil boxes ± *L. terrestris* earthworms; compost boxes ± *D. veneta* earthworms; soil cores ± *L. terrestris* earthworms.

5.4.6. Harvests

Harvests were performed 1, 3, 7, 14, and 21 days following application of inoculated manure to the compost or soil. At each harvest, 5 g of soil or compost was gathered from three linear points in the boxes (2 × 5 cm and one central point at 25 cm from box edge) and cores (2 × 4 cm and one central point at 6 cm from core edge) at distances of 0, 10, 20, 30, and 40 cm from the manure band (Fig. 5.1A, 5.1B), and placed into individual 31 ml sterile plastic bottles.

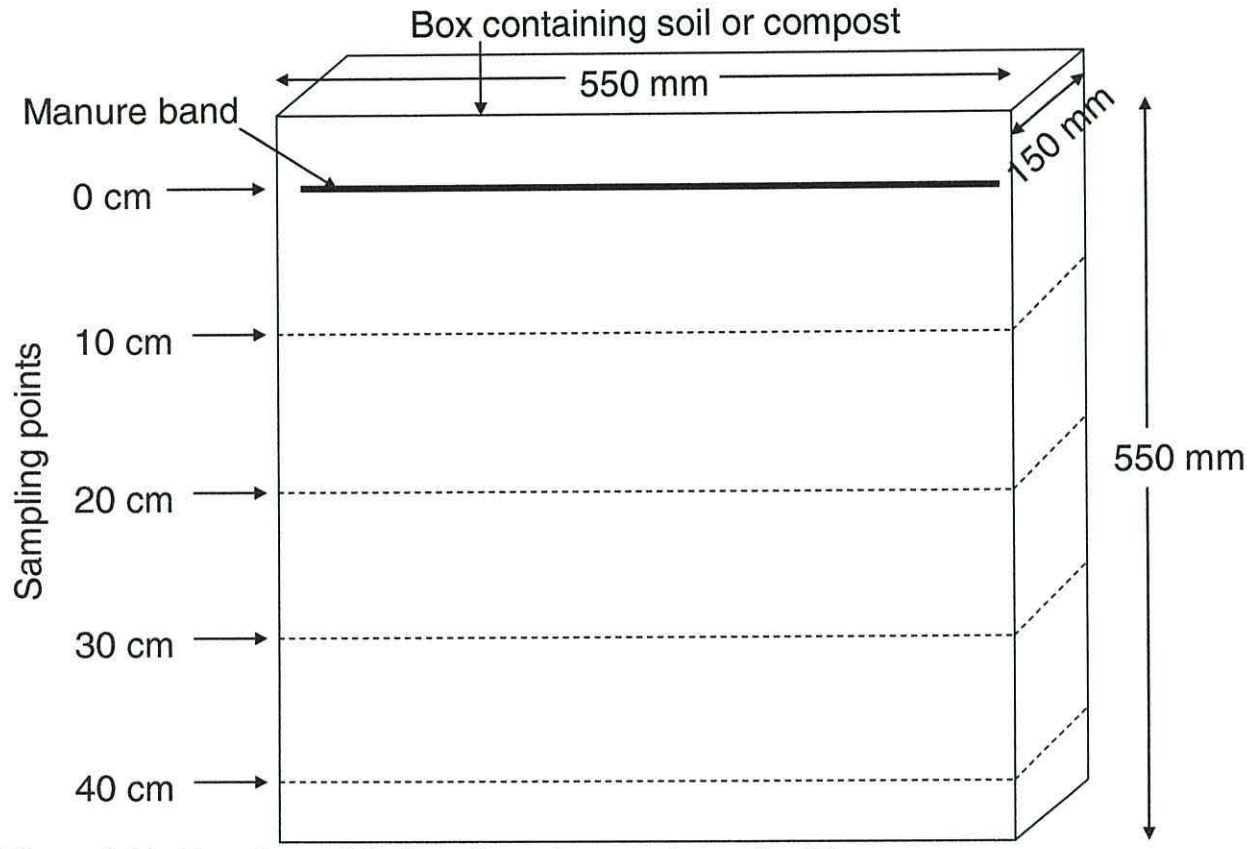

Figure 5.1A. Experimental design of vermicomposting and soil boxes.

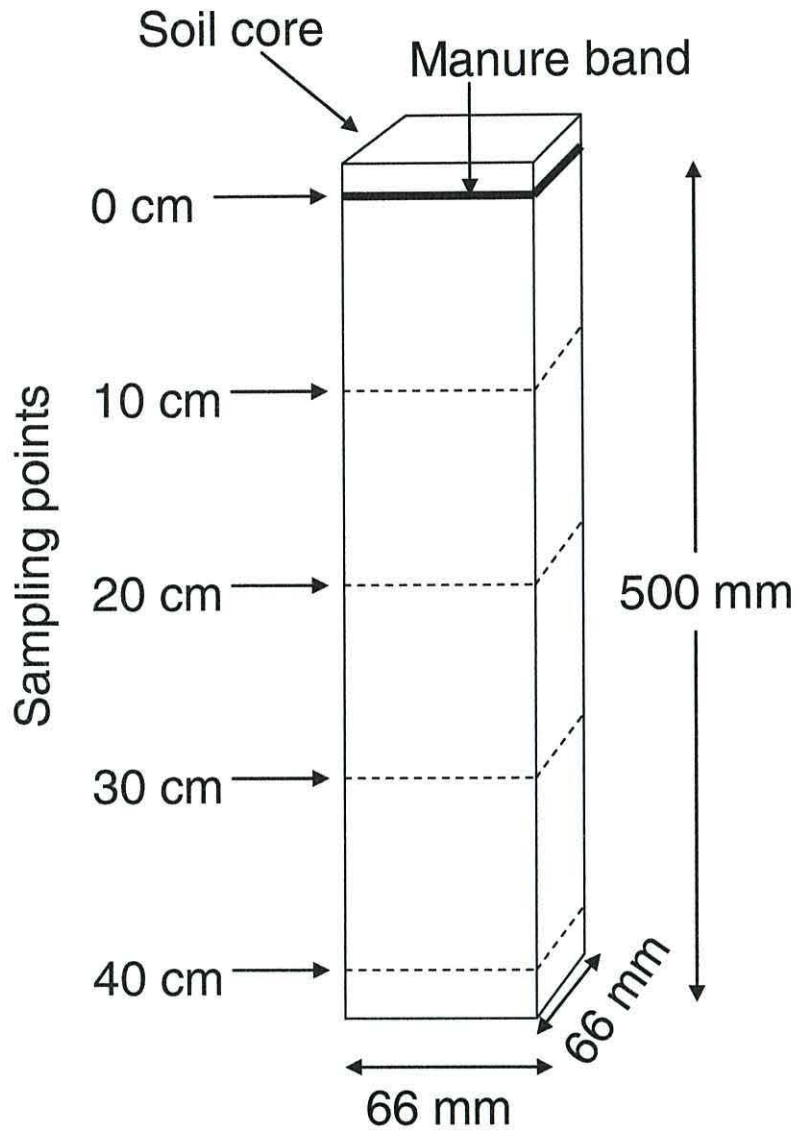

Figure 5.1B. Experimental design of soil cores.

Due to their narrow dimension, harvesting of cores was destructive (i.e. 3 cores per harvest selected randomly on each date and discarded after sampling) to avoid unrealistic results due to disturbance of soil structure.

Bottles were subsequently shaken at 200 rev min^{-1} for 15 min at room temperature in 15 ml of sterile ¼-strength Ringers solution, followed by 4 × 10-second bursts on a vortex mixer. Dilutions were plated out in duplicate onto CT-SMAC agar, then incubated and colonies enumerated as described previously. When CT-SMAC plate counts were approaching their theoretical detection limit (20 CFU g^{-1}), enrichment was carried out as described previously to determine whether low numbers of culturable cells were still present. Where *E. coli* O157:H7 was detectable only following enrichment, the

sample was assigned an arbitrary value equal to half of the detection limit of plate counts (i.e. 10 CFU g^{-1}). Boxes and cores were re-randomised within the growth room at each harvest.

5.4.7. Determination of E. coli *O157:H7 numbers on and within earthworm tissue*

Due to time constraints, this experiment was performed using only one species of earthworm. Five g of *D. veneta* were starved for 12 h in Petri dishes, and then fed 5 g of manure spiked with *E. coli* O157:H7 inoculum (prepared as described previously) to a final concentration of approximately 5.0×10^5 CFU g^{-1} manure. This was performed in triplicate. After overnight feeding (12 h), worms were subsequently transferred to clean Petri dishes containing damp filter paper until all the cast contained within the earthworm had been excreted (4 h, adapted from Toyota and Kimura 2000), and their intestines were empty (as verified by visual inspection under an illuminated microscope). Subsequently, excess cast was removed from the surface of the worms with sterile tweezers. Casts and washings were collected, weighed, and placed in sterile plastic bottles containing 15 ml of sterile ¼-strength Ringers solution as previously. To determine the number of *E. coli* O157:H7 bacteria on their surface, the worms themselves were placed in separate plastic bottles containing 15 ml of sterile ¼-strength Ringers solution. Thereafter, all bottles were shaken (250 rev min^{-1}, 20 min, room temperature), and the solutions diluted, plated, incubated, and enumerated on CT-SMAC agar as described previously. In order to determine the number of *E. coli* O157:H7 bacteria held within worms' intestine, the same worms were then ground in a sterile pestle and mortar, shaken in 15 ml of sterile ¼-strength Ringers (200 rev min^{-1}, 15 min, room temperature), and colonies plated and enumerated as described previously.

5.4.8. Total microbial activity

Microbial activity can be evaluated by studying the rate at which simple sugars, such as glucose, are mineralised by the microbial population (Jones *et al.* 2004). The total microbial activity of soil, compost, manure, along with 'wormcasted' soil and compost was determined according to Palomo *et al.* (2006). Briefly, 500 µl of ^{14}C-labelled glucose (50 mM) was added to 5 g of field-moist soil, compost, and manure contained in a 60 ml polypropylene tube; and $^{14}CO_2$ evolution measured of over a 360 h period using a Wallac 1409 Liquid Scintillation Counter (Wallac Oy, Turke, Finland).

5.4.9. Data analysis

Plate count data from *E. coli* O157:H7 were $\log_{10}$ transformed and analysed using a multi-factorial analysis of variance (ANOVA) on Genstat 7 (VSN International Ltd., Hemel Hempstead, UK), with significant differences between treatments identified using Fisher's LSD test. To estimate glucose half-life ($t_{1/2}$) in soil, a double first-order exponential decay equation was fitted by a least squares optimisation routine to the glucose mineralisation data (Kemmitt *et al.* 2006) using Sigmaplot 8.0 (Systat Software UK Ltd., London, UK):

$$y = Y_r \exp^{(-At)} + Y_b \exp^{(-Bt)}$$

Where y is the amount of ^{14}C remaining in the soil, t is time, Y_r and Y_b represent the amount of ^{14}C-glucose partitioned into microbial respiration and biomass production, respectively, and A and B represent the rate constants for these two components. Based upon the assumption that the time-dependent mineralisation of glucose matches their removal from soil solution (Jones *et al.* 2004), the $t_{1/2}$ of the soil solution glucose pool can thus be defined as:

$$t_{1/2} = \ln 2/A.$$

5.5. Results

5.5.1. Characterisation of soil, compost and manure

A summary of chemical and microbiological characteristics of substrates prior to earthworm digestion are presented in Table 5.1. The pH of the soil used was slightly acidic (pH 5.96), and the original moisture content was relatively high, at 68.9% of its water-holding capacity. Even though it possessed only low levels of nitrogenous compounds, the C-to-N ratio was 10.2. The soil harboured high numbers of indigenous bacteria (7.85 $\log_{10}$ CFU g^{-1} soil); however, enrichment yielded no *E. coli* O157:H7 prior to inoculation. Compost and manure were moderately basic (pH 8.09 and 8.59, respectively). The moisture content of compost was moderate, at 48.5% of its water-holding capacity. Although their C-to-N ratios were similar, manure possessed higher levels of both soluble C and N compounds, the latter mostly in the form of NH_4^+ (Table 5.1). Available phosphate values were much higher in manure, while K and Na levels were similar for both substrates. Ca levels were notably higher in compost. Background heterotrophic bacteria counts were somewhat greater in cattle manure than in compost (8.93 and 8.41 $\log_{10}$ CFU g^{-1}, respectively). No *E. coli* O157:H7 was detected in either compost or manure prior to inoculation.

Table 5.1. Chemical and microbiological properties of soil, compost and manure. Values represent means ± standard error of the mean (SEM) (n = 3). NM = not measured.

	Sample		
Parameter	Soil	Compost	Manure
pH	5.96 ± 0.11	8.09 ± 0.09	8.59 ± 0.14
Electrical conductivity (mS cm^{-1})	0.33 ± 0.04	0.18 ± 0.02	3.05 ± 0.08
Moisture content (g kg^{-1})	249 ± 4	535 ± 3	864 ± 5
Water-holding capacity (g kg^{-1})	361 ± 4	1103 ± 13	NM
DOC (mg g dry $matter^{-1}$)	0.13 ± 0.03	0.60 ± 0.24	15.4 ± 7.91
DN (mg g dry $matter^{-1}$)	0.04 ± 0.01	0.07 ± 0.03	1.89 ± 1.07
Total C (g kg^{-1})	31 ± 1	181 ± 3	299 ± 6
Total N (g kg^{-1})	3.0 ± 0.3	8.7 ± 0.7	14.2 ± 1.5
C-to-N ratio	10.2	20.9	21.1
NO_3^- (mg g dry $matter^{-1}$)	< 0.1	< 0.1	< 0.1
NH_4^+ (mg g dry $matter^{-1}$)	< 0.1	< 0.1	1.0 ± 0.7
P (mg g dry $matter^{-1}$)	< 0.1	< 0.1	2.2 ± 1.6
K (mg g dry $matter^{-1}$)	0.9 ± 0.2	1.3 ± 0.1	2.1 ± 0.1
Ca (mg g dry $matter^{-1}$)	10.5 ± 3.4	26.3 ± 2.2	0.3 ± 0.1
Na (mg g dry $matter^{-1}$)	0.05 ± 0.05	0.21 ± 0.04	0.42 ± 0.02
Background heterotrophic bacteria (log_{10} CFU g^{-1})	7.85	8.41	8.93
E. coli O157:H7 (log_{10} CFU g^{-1})	0.00	0.00	0.00

5.5.2. Survival and lateral movement of E. coli *O157:H7*

5.5.2.1. Compost

Overall, numbers of *E. coli* O157:H7 declined over the course of the experiment; however, following the sharp drop in numbers in the initial 24 h, the rate of decline reduced considerably (mean log_{10} CFU g^{-1} compost ± SEM: day 21, 0.49 ± 0.21; Fig. 5.2). Horizontal movement of *E. coli* O157:H7 was evident at early stages in the experiment, with the pathogen being detected 30 cm and 40 cm away from the inoculation area from day 7 onwards (mean log_{10} CFU g^{-1} compost ± SEM: day 21, 40 cm, 0.95 ± 0.47; Fig. 5.3; Table 5.2). By the end of experimental period, *E. coli* O157:H7 was measured throughout experimental worm beds but had reduced by approximately 5 log_{10} units. Although both followed a notably similar survival pattern, the number of *E. coli* O157:H7 recovered were consistently higher in vermicomposts than those in control compost (mean log_{10} CFU g^{-1} compost ± SEM: 1.79 ± 0.35 and 0.87 ± 0.19, respectively; Fig. 5.2, 5.3; Table 5.2). However, no lateral movement was detected in control boxes containing no earthworms, with all *E. coli* O157:H7 cells recovered on the inoculation point.

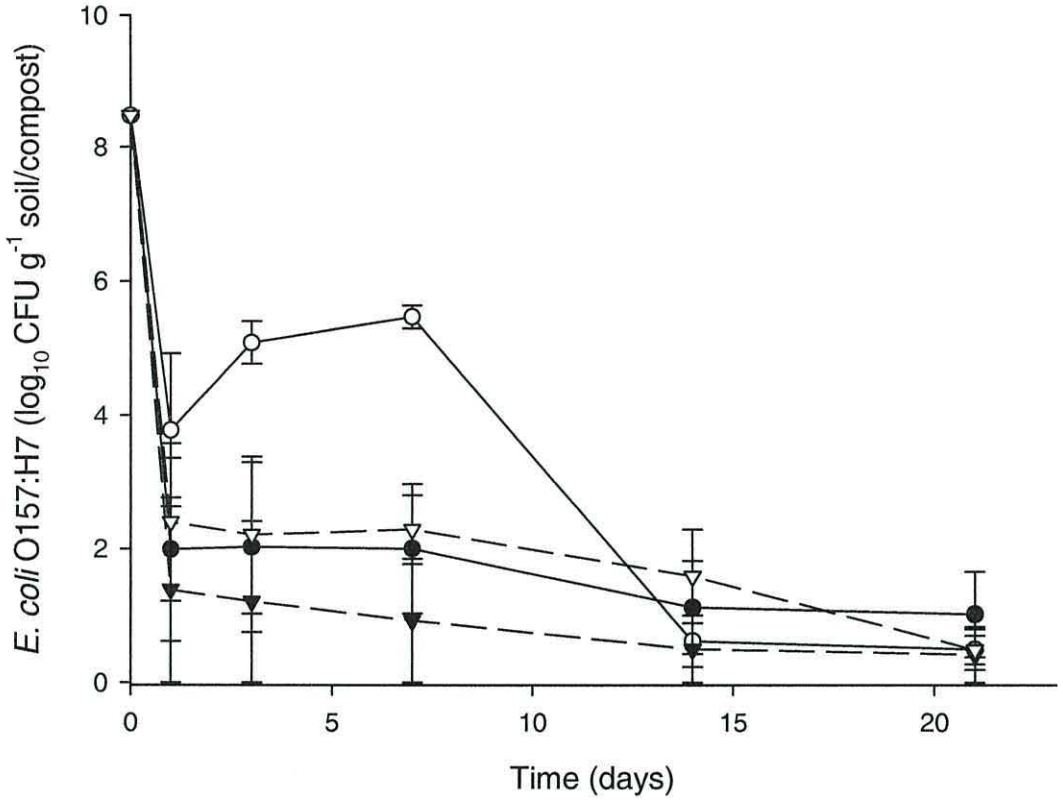

Figure 5.2. Mean survival of *E. coli* O157:H7 over 21 d in soil cores including *L. terrestris* (–○–), and excluding *L. terrestris* (–●–); and in compost boxes including *D. veneta* (– –▽– –), and excluding *D. veneta* (– –▼– –). Curves are representative of mean $\log_{10}$ (y + 1) *E. coli* O157 g^{-1} ± SEM of three replicate cores (soil), and boxes (compost).

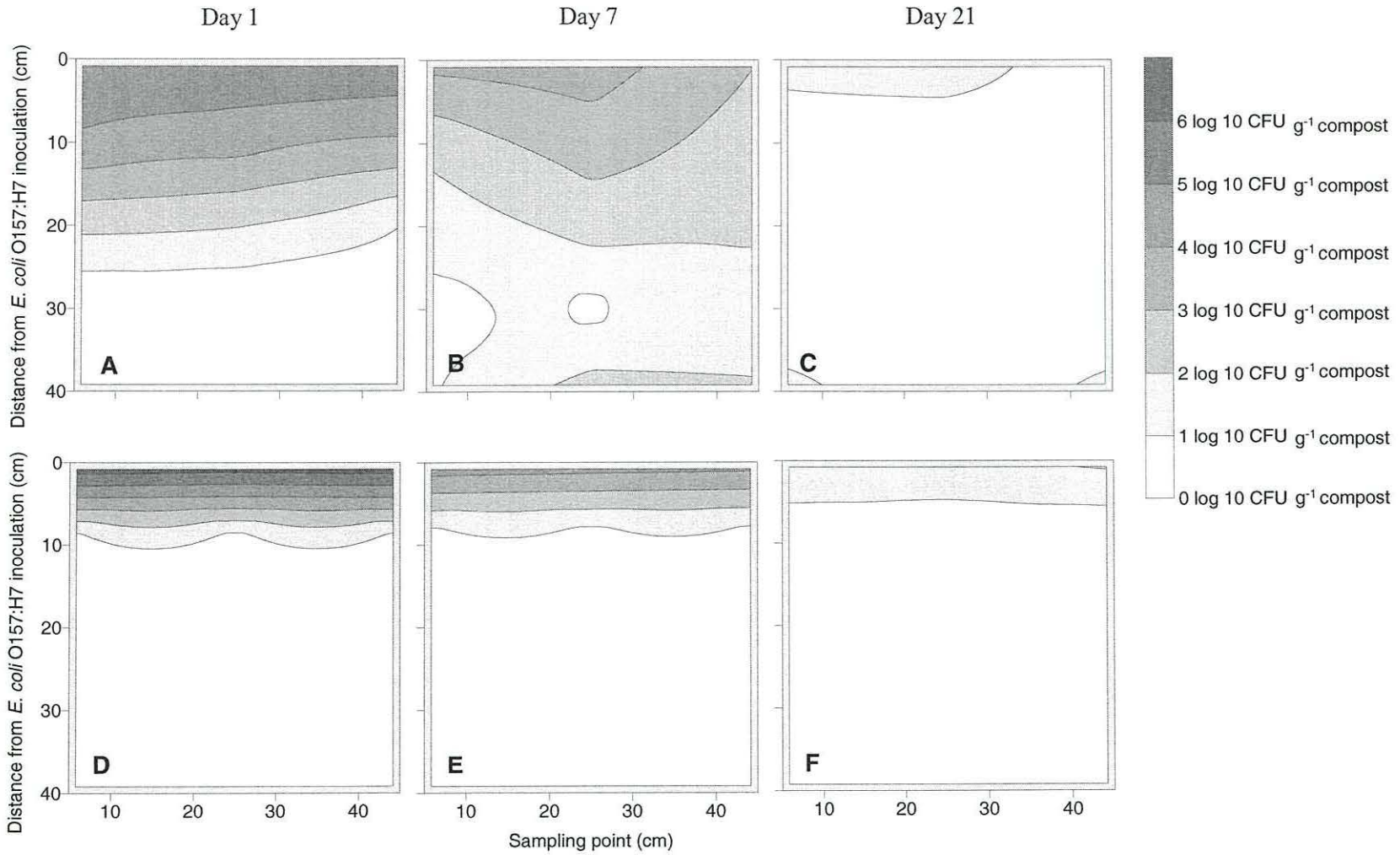

Figure 5.3. Lateral movement of *E. coli* O157:H7 by the earthworm *D. veneta* in actively vermicomposting cattle manure (panels A, B, and C) in comparison with control manure containing no earthworms (panels D, E, and F). Values represent mean $\log_{10}$ CFU g^{-1} compost ($n = 9$).

Table 5.2. Statistical comparisons of *E. coli* O157:H7 numbers (log_{10} CFU g^{-1}) in earthworm-digested compost (bold font) with control (-undigested) compost; t-test, where * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ ($n = 9$); Sig. = significance level; NS = not significant.

	Distance from *E. coli* O157:H7 inoculation (cm)														
	0			10			20			30			40		
Day	Mean log_{10} CFU g^{-1}		Sig.	Mean log_{10} CFU g^{-1}		Sig.	Mean log_{10} CFU g^{-1}		Sig.	Mean log_{10} CFU g^{-1}		Sig.	Mean log_{10} CFU g^{-1}		Sig.
1	**5.87±0.04**	6.92±0.03	***	**4.36±0.27**	0.00	***	**1.76±0.40**	0.00	***	**0.00**	0.00	NS	**0.00**	0.00	NS
3	**6.29±0.12**	6.03±0.03	NS	**1.61±0.96**	0.00	NS	**3.12±0.40**	0.00	***	**0.00**	0.00	NS	**0.00**	0.00	NS
7	**3.96±0.48**	4.63±0.43	*	**2.74±0.38**	0.00	***	**2.04±0.32**	0.00	***	**0.85±0.16**	0.00	*	**1.84±0.49**	0.00	***
14	**3.86±0.07**	2.51±0.06	***	**1.51±0.49**	0.00	***	**0.31±0.31**	0.00	NS	**0.00**	0.00	NS	**2.24±0.23**	0.00	***
21	**1.04±0.54**	2.50±0.06	NS	**0.32±0.16**	0.00	NS	**0.00**	0.00	NS	**0.14±0.14**	0.00	NS	**0.94±0.46**	0.00	NS

5.5.2.2. Soil

Overall numbers of *E. coli* O157:H7 declined considerably over the course of the experiment in boxes containing earthworms; however, low numbers (mean $\log_{10}$ CFU g^{-1} soil ± SEM: 0.08 ± 0.07) were still recovered at the last harvest (21 d post soil inoculation; data not presented). While a small number of *E. coli* O157:H7 cells (mean $\log_{10}$ CFU g^{-1} soil ± SEM: 0.45 ± 0.22) were recovered 20 cm laterally away from the point of inoculation at the first harvest, overall lateral movement was limited; with the majority of the *E. coli* O157:H7 recovered at the inoculation point. Moreover, numbers declined so that *E. coli* O157:H7 could only be detected at one point in the centre of the inoculation zone and 10 cm beyond that by the end of the experiment. No lateral movement of *E. coli* O157:H7 was detected in control boxes.

5.5.3. Survival and vertical movement of E. coli *O157:H7 in soil*

Numbers of the pathogen increased from day 1 to day 7 (mean $\log_{10}$ CFU g^{-1} soil ± SEM: day 1, 3.78 ± 1.14, day 7, 5.46 ± 0.17), but decreased thereafter towards the last harvest (mean $\log_{10}$ CFU g^{-1} soil ± SEM: day 21, 0.49 ± 0.30; Fig. 5.2). *E. coli* O157:H7 movement was much more pronounced in soil cores rather than boxes, with the bacterium being recovered at all sampling points (0-40 cm from inoculation point) on day 1, 3, and 7 (Fig. 5.4; Table 5.3). Furthermore, numbers were markedly similar at all sampling points at day 3 and 7, varying by only approximately 1.5 $\log_{10}$ CFU *E. coli* O157:H7 g^{-1} soil. The absence of *E. coli* O157:H7 at distances greater than 10 cm from the inoculation point towards the latter stages of the experiment coincided with the overall decrease in numbers observed at other sampling points (Table 5.3). Following a sharp drop in the first 24 h post-inoculation, the mean number recovered from control cores gradually decreased at all subsequent harvests (Fig. 5.4; Table 5.3). *E. coli* O157:H7 numbers in control cores varied by only 1.0 $\log_{10}$ CFU units over the whole experimental period, in contrast to 5.0 $\log_{10}$ CFU units in soil cores with earthworms (Fig. 5.4; Table 5.3). Over the course of the experiment, *E. coli* O157:H7 numbers were higher in soil cores containing earthworms than control cores (mean $\log_{10}$ CFU g^{-1} soil ± SEM: 3.09 ± 0.17 and 1.63 ± 0.23, respectively). However, whereas numbers of the bacterium were much higher in initial stages of the experiment in soil containing earthworms, a drastic decline was observed following day 7, whereby fewer of the organisms were recovered than from control cores (Fig. 5.4; Table 5.3).

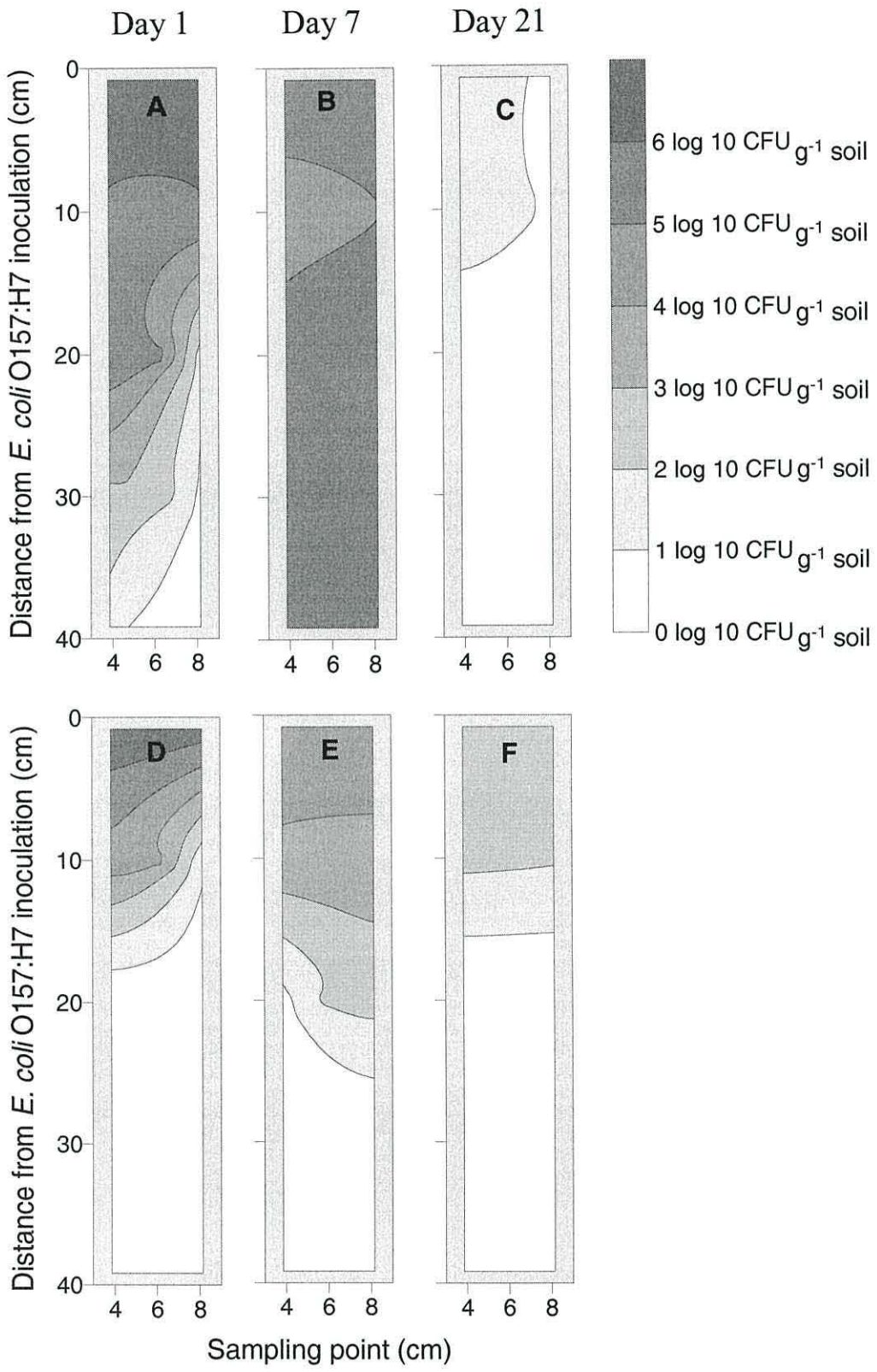

Figure 5.4. Vertical movement of *E. coli* O157:H7 by the earthworm *L. terrestris* in soil (panels A, B, and C) in comparison with control soil containing no earthworms (panels D, E, and F). Values represent mean $\log_{10}$ CFU g^{-1} soil ($n = 9$).

Table 5.3. Statistical comparisons of *E. coli* O157:H7 numbers (log_{10} CFU g^{-1}) in earthworm-digested soil (bold font) with control (-undigested) soil; t-test, where * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ ($n = 9$); Sig. = significance level; NS = not significant.

	Distance from *E. coli* O157:H7 inoculation (cm)														
	0			10			20			30			40		
Day	Mean log_{10} CFU g^{-1}		Sig.	Mean log_{10} CFU g^{-1}		Sig.	Mean log_{10} CFU g^{-1}		Sig.	Mean log_{10} CFU g^{-1}		Sig.	Mean log_{10} CFU g^{-1}		Sig.
1	**6.66±0.05**	6.98±0.02	**	**5.85±0.17**	2.98±1.49	NS	**3.90±1.94**	0.00	NS	**1.81±0.90**	0.00	NS	**0.67±0.67**	0.00	NS
3	**5.87±0.07**	5.94±0.00	NS	**5.31±0.25**	4.18±0.04	NS	**5.54±0.50**	0.00	**	**4.32±0.12**	0.00	***	**4.35±0.15**	0.00	***
7	**5.61±0.18**	4.77±0.02	***	**4.78±0.15**	3.69±0.06	**	**5.63±0.23**	1.53±0.76	**	**5.72±0.09**	0.00	***	**5.56±0.17**	0.00	***
14	**1.75±0.88**	3.12±0.01	NS	**1.35±0.67**	2.48±0.14	***	**0.00**	0.00	NS	**0.00**	0.00	NS	**0.00**	0.00	NS
21	**1.25±0.64**	2.90±0.02	NS	**1.23±0.64**	2.18±0.05	NS	**0.00**	0.00	NS	**0.00**	0.00	NS	**0.00**	0.00	NS

5.5.4. E. coli *O157:H7 numbers on and within earthworm tissue*

E. coli O157:H7 was detected in earthworm cast, earthworm epidermis and from earthworm intestine following starvation (Fig. 5.5). *E. coli* O157:H7 numbers in excreted cast exceeded the initial inoculation concentration (by 0.5 ± 0.1 $\log_{10}$ CFU g^{-1} manure). The greatest numbers were recovered from earthworm cast, with numbers significantly higher than from earthworm epidermis (mean $\log_{10}$ CFU g^{-1} earthworm ± SEM: 4.9 ± 0.1; $P < 0.001$), which were in turn significantly higher than those recovered from the intestine (mean $\log_{10}$ CFU g^{-1} earthworm ± SEM: 3.3 ± 0.1; $P < 0.001$; Fig. 5.5).

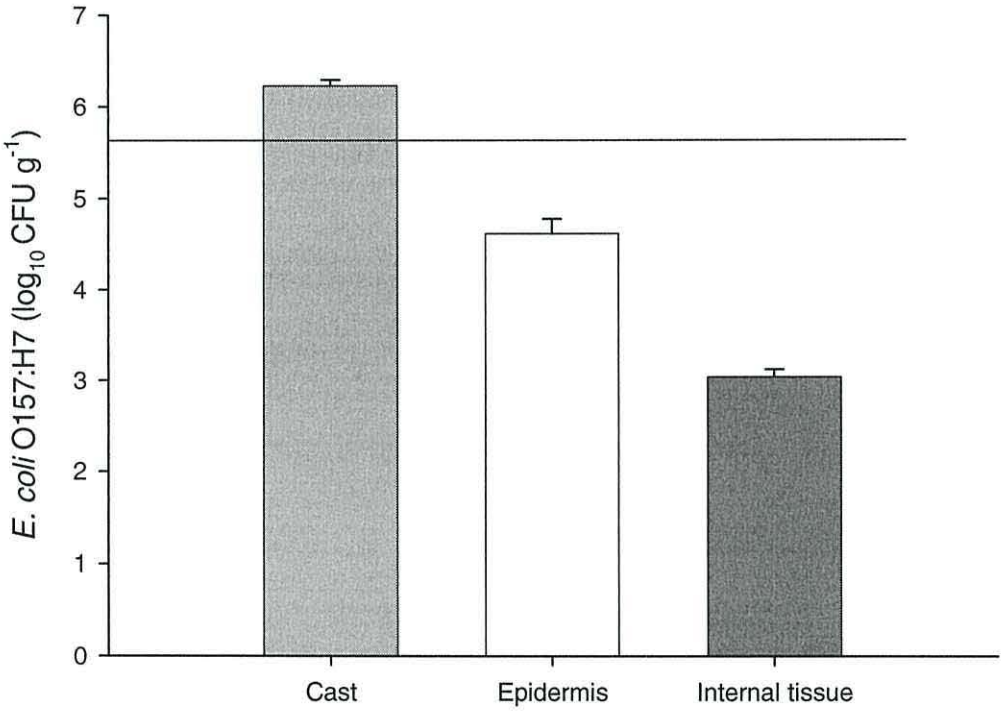

Figure 5.5. Distribution of *E. coli* O157:H7 in the earthworm *D. veneta* after feeding on contaminated cattle manure for 12 h. The initial *E. coli* O157:H7 inoculation level was 5.7 $\log_{10}$ CFU g^{-1} manure and is represented by the solid line. Values represent means ± SEM (n = 3).

5.5.5. Total microbial activity

The initial rates of mineralisation of glucose by indigenous micro-organisms were rapid in all substrates. In the initial 48h, the presence of earthworms did not reduce $t_{1/2}$ of ^{14}C-

glucose mineralisation in substrates (Table 5.4); however; the final mineralisation rates in substrates containing earthworms was higher than in the controls (Fig. 5.6). In soil, the presence of earthworms significantly increased microbial activity and therefore, final concentration of glucose mineralised ($P < 0.05$). Conversely, the difference in microbial activity between earthworm-digested and -undigested compost was not significant ($P > 0.05$; Fig. 5.6). Two weeks after the addition of glucose, a comparative assessment of microbial activity showed that total ^{14}C-glucose mineralisation by substrate micro-organisms was as follows (Fig. 5.6):

earthworm-digested compost > earthworm-digested soil > compost > manure > soil.

Table 5.4. Variability of microbial mineralisation of ^{14}C-Glucose expressed as half-life ($t_{1/2}$) values in the initial 48h following glucose addition. r^2 denotes the variability of each data point compared to that predicted by the double-exponential decay equation.

Substrate	$t_{1/2}$ (h)	r^2
Soil	23 ± 7	0.967
Compost	30 ± 6	0.988
Manure	14 ± 3	0.987
Earthworm-digested soil	40 ± 4	0.999
Earthworm-digested compost	61 ± 9	0.998

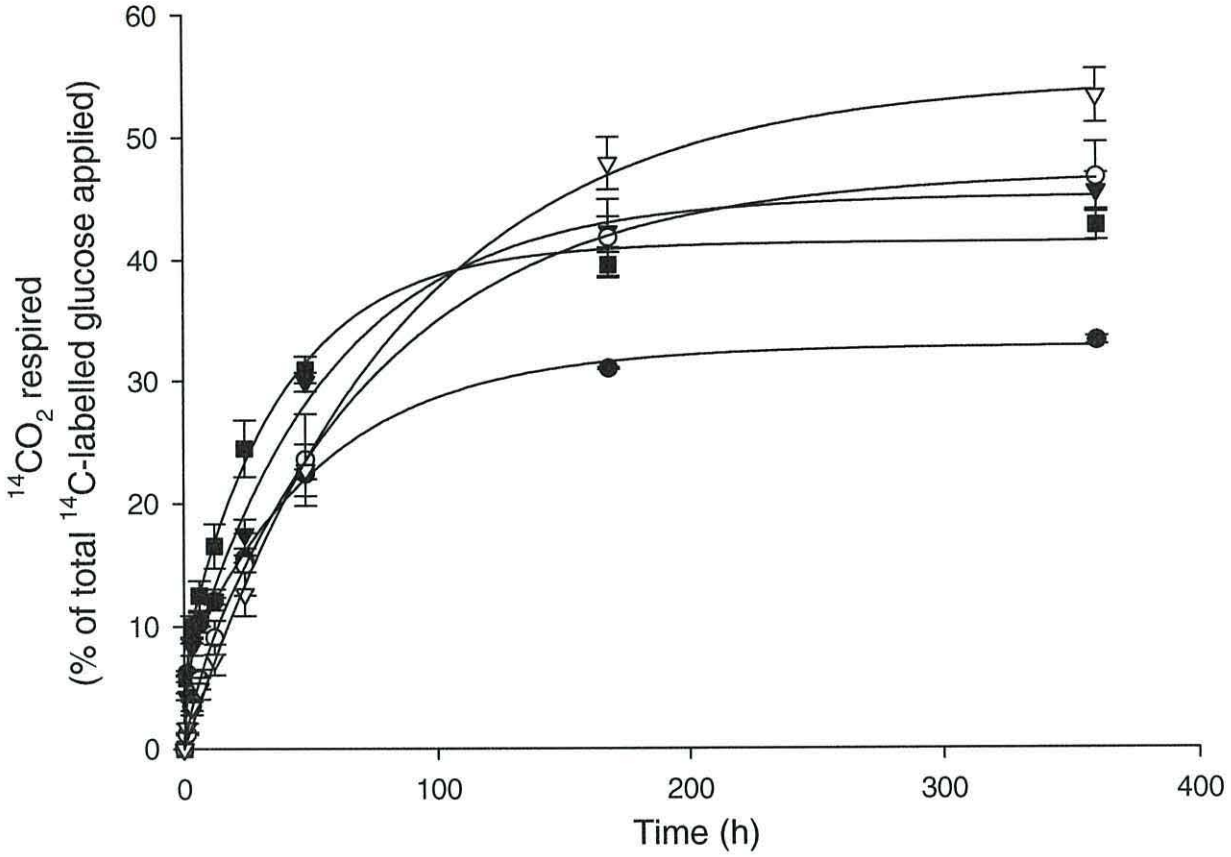

Figure 5.6. Time-dependent mineralisation of ^{14}C-labelled glucose to $^{14}CO_2$ in soil (●), compost (▼), manure (■), *L. terrestris* earthworm-digested soil (○), and *D. veneta* earthworm-digested compost (▽) (as a % of total ^{14}C added). The substrate concentration initially added was 50 mM for glucose. Values represent means ± SEM (n = 3).

5.6. Discussion

The reduction of human pathogens resulting from earthworm digestion has previously been reported (Eastman *et al.* 2002; Dominguez 2004; Edwards and Arancon 2004). However, several studies have shown that Gram-negative soil bacteria can survive passage through the earthworm gut (Thorpe *et al.* 1993; Hendriksen 1995).

Digestion of organic matter by earthworms imposes significant changes on a range of chemical, physical, and biological characteristics (Ndegwa and Thompson 2001; Li *et al.* 2001). Of particular interest in this case is the reported increase in soil microbial activity resulting from an increased availability of easily-catabolised compounds expelled in earthworm casts (Tiunov and Scheu 2000; Li *et al.* 2002), competition by earthworm

intestinal flora (Thorpe *et al.* 1993), and the secretion of immuno-protective and antimicrobial compounds (Cho *et al.* 1998; Cooper *et al.* 2002; Wang *et al.* 2003).

Although it is known that earthworms aid movement of micro-organisms through soil (Brown 1995; Joergensen *et al.* 1998), the exact method of transportation is unclear. In this current study, little or no movement of *E. coli* O157:H7 was observed where earthworms were absent; hence the movement observed in populated boxes or cores can be attributed directly to earthworm activity. At present, the prevalence of *E. coli* O157:H7 in earthworms resulting from ingestion of infected wastes is unknown. The earthworm species in soil and compost were chosen to represent the typical species found in each respective substrate. It was anticipated that the direction of pathogen movement via worms would be species-specific as earthworms species differ in the ecological niches which they inhabit. Anecic earthworms such as *Lumbricus terrestris* maintain deep vertical burrows (James and Hendrix 2004), whereas epigeic species such as *Dendrobaena veneta* inhabit surface organic layers (Parkinson *et al.* 2004). In this study, *E. coli* O157:H7 movement by *L. terrestris* was limited to a vertical plane, whereas movement by *D. veneta* was observed in the horizontal plane. Although we evaluated the carriage of bacteria on only one species, our findings that *E. coli* O157:H7 may be present on the epidermis and within the digestive tract of earthworms (Fig. 5.5) implies that bacterial movement may be attributed to both worm excretion and to carriage on worm exterior; although the relative proportions attributable to each were not determined. Selective disinfection of their surfaces prior to grounding worms would reduce the potential of over-estimating intestinal bacteria numbers (due to 'carry over' from bacteria present on worm epidermis).

Along with rapid die-off of less resistant cells, the initial drop in *E. coli* O157:H7 counts observed within 24 h of inoculating the manure and applying to soil and compost may have been in part due to bacteria numbers falling to the substrate carrying capacity (Byappanahalli *et al.* 2003), competition and antagonistic effects from background bacteria, and from the shock induced by a sudden change in environmental conditions. The stabilisation in numbers observed in most treatments and the increase of 1 $\log_{10}$ unit *E. coli* O157:H7 in soil between day 3 and day 7 (Fig. 5.2) could reflect bacteria acclimatisation to environmental conditions, and utilisation of available food sources. It has been shown that survival of *E. coli* O157:H7 varies in compost and soils of different properties (Jones and Martin 2003; Franz *et al.* 2005). Although we chose specific temperatures and relative

humidity to reflect a particular time of year, these factors are also likely to affect the longevity of bacteria in different substrates. Similarly, use of a culture-based technique can lead to an underestimate of bacterial numbers as *E. coli* O157:H7 can enter into a 'dormant' viable but non-culturable (VBNC) under stress conditions such as starvation; hence are unable to grow in nutrient-rich media. It is therefore important to note that data obtained in the current experiment should not be directly extrapolated to predict persistence in all soil and vermicompost types, but rather provides a comparative study of the influence of earthworms in these matrices under specific conditions.

It is accepted that earthworm casts accelerate total microbial activity by increasing labile C (Tiunov and Scheu 2000). However, in initial stages of this study, analysis of wormcasted soil and compost revealed significantly lower ($P < 0.001$) microbial activity than undigested soil/compost (Fig. 5.6; Table 5.4); but significantly higher ($P < 0.001$) numbers of *E. coli* O157:H7 (Fig. 5.2; Tables 5.2, 5.3). Furthermore, where wormcasted substrates were analysed for microbial activity, *E. coli* O157:H7 numbers were higher in those substrates with the longest glucose half-life (Table 5.4). The presence of earthworms in substrates and the corresponding decrease in numbers of antagonistic microbes may thus have lead to an increase in numbers of *E. coli* O157:H7 as observed in the initial stages of the experiment. Protozoa are known predators of *E. coli* O157:H7 in a range of environments, and several studies have reported on the selective predation of protozoa by earthworms (Bonkowski and Schaefer 1997; Brown and Doube 2004). Passage through the earthworm intestine may also lead to further reductions in protozoa numbers (Brown and Doube 2004). The external structures (casts, burrows, middens) created by earthworm activity may also produce a 'barrier-effect', where microbial populations within may be 'shielded' due to changes in soil physical properties restricting movement of other microbes (Brown *et al.* 2000). Collectively, reduced antagonism due to earthworm digestion may have facilitated the persistence of significantly higher numbers of *E. coli* O157:H7 observed in worm-populated soil and compost in the initial stages of the current study. Nevertheless, such effects appear to have abated with time, as the long-term persistence of *E. coli* O157:H7 in soil or compost was unaffected by the presence of earthworms. Longer-term analysis of substrate microbial respiration suggests that activity increased in wormcasted materials (Fig. 5.6), coinciding with a reduction in *E. coli* O157:H7 numbers; particularly in soil (Fig. 5.2). At the end of the experiment, numbers of *E. coli* O157:H7 in earthworm-

digested substrates were statistically the same as in -undigested material (Fig. 5.2; Tables 5.2, 5.3).

Manure from *D. veneta* was purged prior to exposure to the spiked manure to increase the probability of isolating *E. coli* O157:H7. It is possible that this could have led to reduced inter-bacterial competition and thus higher pathogen numbers than would normally be encountered. Nevertheless, our results support the findings of previous studies on the survival of Gram-negative bacteria through earthworm gut and in casts (Thorpe *et al.* 1993; Hendriksen 1995). Moreover, our work suggests that earthworm digestion (Fig. 5.5) and presence may lead to temporarily higher numbers of *E. coli* O157:H7 in some substrates, especially soil (Fig. 5.2). Although the gut transit time in most earthworms is approximately 1-5 hours, this may prove sufficient to allow partial bacteria growth or for the resuscitation of VBNC bacteria; especially where worms feed upon organic-rich materials (Brown and Doube 2004) as in the current study.

Earthworms are known to synthesise and secrete a variety of immuno-protective proteins which mediate lytic reactions against several micro-organisms (Cooper *et al.* 2002). In addition, one antimicrobial peptide, Lumbricin I, isolated from adult *L. rubellus*, has been reported to display antimicrobial activity against one serotype of *E. coli* (Cho *et al.* 1998). Our work suggests that this is not effective against *E. coli* O157:H7 over short time periods and at high contamination levels, or that these antimicrobial peptides are present in insufficient amounts to be effective against elevated numbers/densities of *E. coli* O157:H7. A recent study identified a similar antimicrobial peptide produced on the epidermal layer of the earthworm *Pheretima tschiliensis* (Wang *et al.* 2003). In this study, numbers of *E. coli* O157:H7 were somewhat reduced on earthworm epidermis relative to numbers in the initial inoculum (Fig. 5.5), and the presence of a similar peptide might explain this; however, this phenomenon has not been reported for *D. veneta* to date.

We conclude that the earthworms *L. terrestris* are not significant vectors for lateral movement of *E. coli* O157:H7 in soil; however, they may significantly aid vertical movement. Litter-dwelling earthworms such as *D. veneta* can significantly aid lateral movement of *E. coli* O157:H7 within compost. Our results imply that whilst long-term persistence of *E. coli* O157:H7 in soil and compost may be unaffected by the presence of earthworms, digestion from worms may aid proliferation of the pathogen during initial stages of soil or compost contamination.

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CHAPTER 6: ARTICLE IV

Survival of *Escherichia coli* O157:H7 in the rhizosphere of maize grown in waste-amended soil

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6. 1. Abstract

Escherichia coli O157:H7 can be present within animal-derived organic wastes which are routinely spread on land. Introduced measures with regards to such waste disposal may decrease exposure to the organism; however, the persistence of *E. coli* O157:H7 for considerable periods in waste-amended soil may still pose some risk for both human and animal infection. In this study, cattle slurry and ovine stomach content waste were inoculated with *E. coli* O157:H7. Wastes were then applied to soil cores with and without established maize plants. The pathogen survived in soil for over 5 weeks, though at significantly greater numbers in soil receiving stomach content waste in comparison to cattle slurry. Persistence of the pathogen in soil was unaffected by the presence of a rhizosphere. This study has shown that whilst survival of *E. coli* O157:H7 in waste-amended soil isn't significantly affected by the presence or absence of a maize rhizosphere; it may vary significantly with waste type. This may have implications for land and waste management.

Keywords: cattle, *Escherichia coli* O157:H7, maize, manure, ovine, rhizosphere, stomach contents, survival.

6.2. Introduction

Escherichia coli O157:H7 is recognised as a causative agent of severe intestinal illness in man, with rare cases leading to haemorrhagic colitis or life-threatening haemolytic uraemic syndrome (Chart 2000). The infectious dose is low and ingestion of as few as 10 cells may be sufficient to cause infection (Chart 2000). Cattle are considered to be the main environmental reservoir for *E. coli* O157:H7, with infected animals typically excreting between 10^2-10^5 CFU of *E. coli* O157:H7 g^{-1} faeces (Nicholson *et al.* 2000). However, a recent study recovered up to 10^7 CFU *E. coli* O157:H7 g^{-1} faeces (Fukushima and Seki 2004). Sheep may also extensively carry and shed the bacterium, with cell concentrations occasionally as high as 10^6 CFU g^{-1} faeces (Ogden *et al.* 2005). Although defecation on pasture-land is a significant source of the bacterium, the widespread application of animal-derived wastes such as slurry and abattoir waste (stomach and rumen contents) to land is likely to introduce additional *E. coli* O157:H7 to the agricultural environment (Jones 1999; Laven *et al.* 2003; Buncic *et al.* 2004). This is of concern as the pathogen has been shown to persist for extensive periods of time in waste and waste-amended soil (Kudva *et al.* 1998; Bolton *et al.* 1999; Avery *et al.* 2005).

Whilst the survival of *E. coli* O157:H7 in soil is affected by numerous biotic and abiotic factors, the presence of plant roots may help support a substantially greater population of micro-organisms as root exudates provide a supply of readily available labile organic substrates, forming a substrate- and nutrient-rich niche for bacteria in comparison with bulk soil (Lugtenberg and Dekkers 1999; Pinton *et al.* 2001). Although legislation and guidelines have been introduced in the UK regarding treatment of some organic wastes prior to, or when implementing, land disposal (e.g. anaerobic digestion and sub-surface injection, respectively), raw animal gut waste and farm slurries/manure may be applied to grazing or arable land (DEFRA 2006). Thus, the influence of a rhizosphere on persistence of *E. coli* O157:H7 in waste-amended soil may have implications for land management. Indeed, Gagliardi and Karns (2002) added *E. coli* O157:H7 immediately after sowing seeds and observed enhanced persistence of this organism in soil in the presence of roots of two plant species. However, there are many factors which may have influenced their results. Firstly, they added *E. coli* O157:H7 at the initial stages of rhizosphere development, and plant maturity has been shown to affect the composition and abundance of root exudates (Ibekwe and Grieve 2004; Jones *et al.* 2004). Different soil types may also facilitate or suppress the occurrence of a 'rhizosphere effect' (Gagliardi and Karns 2002). The waste application rate may also

influence the relative impact of inputs by root exudates to the soil nutrient profile and hence on soil microbial biomass.

The aim of this current study was to further develop the ideas of Gagliardi and Karns (2002) and assess the impact of a developed maize rhizosphere upon the survival of *E. coli* O157:H7 in soils to which pathogen-contaminated organic wastes had been applied. It was hypothesised that the enhanced supply of organic carbon from rhizodeposits would mediate greater persistence of *E. coli* O157:H7 in soil.

6.3. Materials and methods

6.3.1. Preparation and maintenance of cores

A factorial experiment was established with 60 plastic (PVC) cores (15 cm height, 5 cm diameter) packed with soil (Eutric Cambisol of the 'Denbigh' series which has a clay-loam texture, Table 6.1) collected from a sheep-grazed pasture at Abergwyngregyn, N. Wales (53° 13.9' N, 4° 0.9' W), to a bulk density of 1 g cm^{-3}. Maize seeds (*Zea mays* L. cv 'Merit') were soaked overnight in aerated distilled water and subsequently allowed to germinate over three days on dampened filter paper. Three seedlings were then sown into each of 30 cores, and the remaining cores left unplanted. All cores were subsequently placed in a Fi-totron PG 660 cabinet (Sanyo Gallenkamp Ltd., Loughborough, UK) at 15°C, 70% relative humidity, with an 18-hour photoperiod and light intensity of 600 μmol m^{-2} s^{-1} (Powertone 250W MHN/TD; Phillips, Eindhoven, Holland). The moisture content of soil in cores was maintained at its original value (214 g kg^{-1}) by watering (surface applying) cores with artificial rainwater solution (Jones and Edwards 1993) to their original weights, daily. Following watering, cores were re-randomised within the plant growth cabinet. All cores were placed on a wire mesh base with drip trays beneath to prevent cross-contamination.

6.3.2. Preparation of E. coli *O157:H7 inoculum*

An inoculum was prepared from a fresh overnight culture (LB broth; Difco Ltd., Teddington, Surrey, UK; 18 h, 37°C, 150 rev min^{-1}) of *E. coli* O157:H7 (strain #3704) (Campbell *et al.* 2001). Although this strain is non-toxigenic (Campbell *et al.* 2001), it (and similar strains) have exhibited survival patterns reflecting those of toxigenic strains (Kudva *et al.* 1998; Bolton *et al.* 1999; Ritchie *et al.* 2003). Cattle slurry (CS), and ovine stomach contents (StC) (Table 6.1) were collected from a farm and large commercial abattoir in N. Wales, respectively. The wastes were macerated using a food blender to

destroy large (> 1 cm) particles. *E. coli* O157:H7 cells were washed three times in sterile ¼-strength Ringers solution (Oxoid Ltd., Basingstoke, UK), concentrated by centrifugation, and added to wastes to give a final concentration of approximately 4.8×10^8 and 2.5×10^8 CFU ml^{-1} in cattle slurry and ovine stomach contents, respectively.

6.3.3. Preparation and application of spiked wastes

Aliquots (10 ml) of *E. coli* O157:H7 inoculated organic wastes were applied to the soil surface ten days after the plants became established in the cores. The waste addition rate was equivalent to 50 m^3 ha^{-1} which is typical of field application rates in the UK. Wastes were applied to the cores in a factorial design to give four treatment combinations as follows: unplanted soil + stomach contents; unplanted soil + slurry; planted soil + stomach contents; planted soil + slurry. In addition to the main experiment, unspiked organic wastes were also applied to a further twelve cores to determine the impact of the maize plants on the properties of the soil under all the above treatment conditions. All cores were stored at 15°C in a bid to represent spring air temperatures, when most wastes are spread.

6.3.4. Characterisation of soil and wastes

Physical and chemical analysis of soil and wastes was performed as described by Avery *et al.* (2005). Electrical conductivity (EC) and pH of stomach contents were measured directly and following a 1:1 (v/v) dilution with distilled water for both slurry and soil. Soil texture was assessed by hand and cation exchange capacity was determined according to Page *et al.* (1982). Soil water-holding capacity was determined according to Rowell (1994). Heterotrophic bacterial counts were estimated by standard plate counts on R2A agar (Oxoid); and 5 g of soil or 5 ml waste was enriched in TSB broth (6 h; 37°C; 150 rev min^{-1}) before plating onto CT-SMAC agar (Oxoid) to confirm absence of *E. coli* O157:H7.

6.3.5. Harvests

Three replicate cores from each treatment were harvested at 1, 2, 3, 4 and 5 weeks following application of wastes. Total root biomass, dry weight (following drying at 80°C) and organic matter content (after ashing at 450°C) were recorded. Soil (5 g) from 0-5, 5-10 and 10-15 cm depths of cores from all treatment combinations was homogenised manually in sterile sample bags. Soil was extracted and CFU of *E. coli*

O157:H7 were quantified on CT-SMAC agar by methods described in Avery *et al.* (2004). Numbers of non-O157 *E. coli* on the same plates (colonies of pink appearance; March and Rantam 1986; Anon 2006) were also recorded. Further sub-samples of soil were retained for determination of soil moisture content at each harvest. Harvesting of unspiked cores took place 4 weeks after waste addition. Soil pH, EC, and phosphate were measured using the methods described previously. Exchangeable cations were extracted from soil by shaking 5 g of soil with 25 ml of 0.5 M acetic acid for 60 min and K, Na and Ca concentrations determined using a flame photometer (Sherwood 410; Sherwood Scientific Ltd., Cambridge, UK). Dissolved organic carbon (DOC) was measured in the top section of cores using a TC-TNV analyzer (Shimadzu Corp., Kyoto, Japan).

6.3.6. Data analysis

All experiments were performed in triplicate. Plate count data for *E. coli* O157:H7 were converted to (soil) dry weight equivalents and $\log_{10}(y + 1)$ transformed, and subjected to a multi-factorial analysis of variance (Genstat 8.1, Rothamsted Experimental Station, Hertfordshire, UK). Significant differences between treatments were identified using Fisher's LSD test within the same software.

6.4. Results

6.4.1. Soil characteristics and moisture content

The Cambisol (clay loam) soil used here represents a dominant agricultural soil type in the UK therefore typifies a soil type that frequently receives agricultural organic waste (DEFRA 2005). Due to its free-draining nature, moderate pH, regular fertilisation and consequent high nutrient levels (Table 6.1), the soil supports high levels of plant and livestock productivity. The cation exchange capacity was 17.6 mmol $100g^{-1}$ soil. Organic matter content was 89 g kg^{-1} and the sand, silt, and clay contents were 47.7, 30.0, and 22.3%, respectively. Background microbial counts were relatively high (5.30 $\log_{10}$ CFU g^{-1} dry soil), supporting previous studies made at the site. Enrichment techniques revealed no *E. coli* O157:H7 present in the soil prior to inoculation with the organic wastes.

The original (field) soil moisture content (214 g kg^{-1}) was at 59.3% of the water-holding capacity (361 g kg^{-1}) and there were no significant difference in soil moisture content between any treatments throughout the experiment ($P > 0.05$).

Table 6.1. Physical, chemical, and microbiological properties of the soil and wastes used in the experiments. CS = cattle slurry, StC = ovine stomach content. All values are the means of 3 replicates.

	Sample		
Parameter	Soil	CS	StC
pH	6.80	8.17	7.21
Electrical conductivity (mS cm^{-1})	32	882	955
Moisture content (g kg^{-1})	214	680	890
Total C (g kg^{-1})	34.2	31.3	14.1
Total N (g kg^{-1})	3.0	2.20	9.0
C-to-N ratio	11.4	14.2	1.56
NO_3^- (mg l^{-1})	4.77	1.5	0.1
NH_4^+ (mg l^{-1})	3.86	42	569
Available P (mg l^{-1})	9.9	97	1372
Background heterotrophic bacteria (log_{10} CFU g^{-1})	5.30	7.20	7.47
E. coli O157:H7 (log_{10} CFU g^{-1})	0.00	0.00	0.00

6.4.2. Waste characteristics

The ovine stomach content was notably more liquid than the cattle slurry, although both had high moisture contents (Table 6.1). Both wastes were slightly basic, whilst stomach content waste had the highest concentrations of dissolved salts. The stomach content waste was also associated with a considerably greater P and NH_4^+ concentration than the slurry, although the latter contained a greater concentration of NO_3^-. Background heterotrophic bacteria counts were somewhat greater in ovine stomach content than in cattle slurry (7.47 log_{10} and 7.20 log_{10} CFU g^{-1} dry waste, respectively); but no *E. coli* O157:H7 cells were detected in either waste prior to spiking.

6.4.3. Plant biomass production

At the time of the final harvest, the plants were over 30 cm tall and at the V3 developmental stage. The mean plant above-ground biomass increased significantly with each successive harvest (biomass × time interaction, $P < 0.001$) up to week 4 (means: week one 0.08 ± 0.02 g; week four 0.53 ± 0.05 g). Thereafter, a general trend of increasing above-ground biomass was evident in all treatments, but was no longer significant.

Root biomass decreased significantly with soil depth (waste × depth interaction, $P < 0.001$; Table 6.2). Waste type had no significant effect on plant root density ($P > 0.05$; Table 6.2). At the final harvest, the soil possessed a great abundance of primary, secondary and tertiary roots as well as root hairs. The root length density in the soil was

approximately 3 cm root cm^{-3} soil in the surface layer (0-5 cm) decreasing to 0.6 cm root cm^{-3} soil in the lower layer (10-15 cm). All the soil in the cores could therefore be effectively termed rhizosphere soil.

Table 6.2. Chemical properties in different sections of the soil columns (top, middle and bottom) in which maize plants were (+ rhizosphere) or were not grown (- rhizosphere) for 4 weeks after the addition of either cattle slurry waste (CS) or ovine stomach content (StC) to the surface of soil cores. EC = electrical conductivity. All values represent means ± SEM (n = 3).

Rhizosphere	Waste	Core section	pH	EC ($\mu S\ cm^{-1}$)	K	Na	Ca	DOC	P	NO_3^-	NH_4^+	Root dry mass (g)
								($mg\ kg^{-1}$)				
+	CS	Top	6.43 ± 0.04	27 ± 4	47 ± 3	29 ± 2	586 ± 16	9 ± 1	0.14 ± 0.01	0.45±0.04	0.05±0.01	0.29 ± 0.01
+	CS	Middle	6.52 ± 0.07	17 ± 2	57 ± 6	35 ± 4	573 ± 16		0.15 ± 0.01	0.27±0.06	0.11±0.04	0.15 ± 0.04
+	CS	Bottom	6.40 ± 0.04	29 ± 3	56 ± 1	32 ± 2	606 ±14		0.15 ± 0.01	0.76±0.08	0.06±0.01	0.06 ± 0.01
+	StC	Top	7.00 ± 0.14	57 ± 9	56 ± 1	80 ± 4	596 ± 14	15 ± 4	0.23 ± 0.01	0.70±0.13	0.14±0.03	0.32 ± 0.01
+	StC	Middle	6.76 ± 0.11	23 ± 1	56 ± 6	36 ± 2	583 ± 16		0.16 ± 0.01	0.24±0.02	0.20±0.10	0.11 ± 0.04
+	StC	Bottom	6.53 ± 0.03	25 ± 3	56 ± 1	31 ± 4	590 ± 15		0.15 ± 0.01	0.77±0.11	0.13±0.08	0.07 ± 0.01
-	CS	Top	6.32 ± 0.14	48 ± 9	62 ± 5	33 ± 2	550 ± 17	13 ± 2	0.15 ± 0.01	0.74±0.08	0.09±0.01	
-	CS	Middle	6.42 ± 0.17	40 ± 2	55 ± 2	34 ± 1	523 ± 12		0.15 ± 0.01	0.49± 0.01	0.03±0.03	
-	CS	Bottom	6.44 ± 0.09	30 ± 4	57 ± 1	37 ± 6	556 ± 21		0.14 ± 0.01	0.52±0.01	0.05±0.01	
-	StC	Top	6.74 ± 0.21	62 ±22	60 ± 1	61 ± 5	566 ± 12	10 ± 2	0.18 ± 0.01	0.75±0.03	0.16±0.06	
-	StC	Middle	6.60 ± 0.01	25 ± 1	60 ± 4	31 ± 1	560 ± 14		0.15 ± 0.01	0.59±0.01	0.12±0.01	
-	StC	Bottom	6.61 ± 0.11	33 ± 7	57 ± 1	32 ± 2	573 ± 8		0.15 ± 0.01	0.60±0.03	0.11±0.01	

6.4.4. E. coli *O157:H7 colony counts*

Although *E. coli* O157:H7 numbers declined significantly ($P < 0.001$) between the first and second harvest dates (mean $\log_{10}$ CFU g^{-1} dry soil ± SEM: first harvest 4.29 ± 0.21; second harvest 3.43 ± 0.22), they remained relatively stable in the soil thereafter. Indeed, only a 9% decrease in counts occurred between the first and last harvests. The organism persisted in sufficient numbers to be detectable by the plate count technique throughout the experiment.

Mean viable counts of *E. coli* O157:H7 were greater in the upper layers of soil, decreasing significantly ($P < 0.001$) with soil depth at all sampling dates (overall mean $\log_{10}$ CFU g^{-1} dry soil ± SEM: top 4.61 ± 0.13, middle 3.65 ± 0.10, bottom 3.09 ± 0.11).

Mean *E. coli* O157:H7 colony counts were similar in rhizosphere and non-rhizosphere soil. Thus, the presence (Rh) or absence (NRh) of a rhizosphere did not significantly affect overall persistence of *E. coli* O157:H7 in soil (mean $\log_{10}$ CFU g^{-1} dry soil: Rh 3.80 ± 0.11, NRh 3.77 ± 0.10; Fig. 6.1A; $P > 0.05$).

Overall counts were significantly greater from cores treated with stomach contents compared with those treated with cattle slurry regardless of rhizosphere treatment (single factor effect of waste type, $P < 0.001$; mean $\log_{10}$ CFU g^{-1} dry soil: CS 3.46 ± 0.09, StC 4.11 ± 0.12); although the difference between the waste treatments became less apparent over time (Fig. 6.1B).

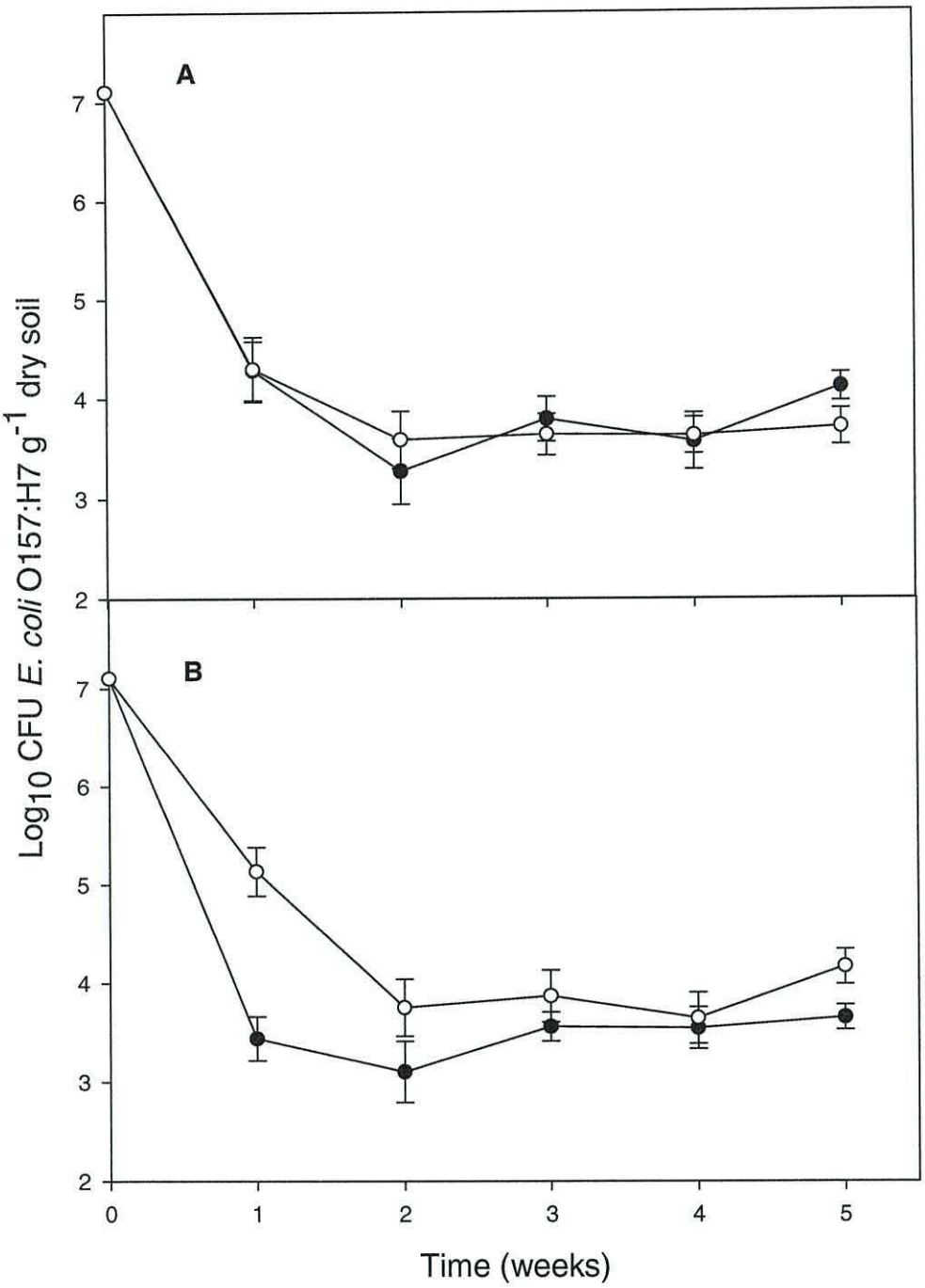

Figure 6.1. Panel A: Survival of *E. coli* O157:H7 in rhizosphere and non-rhizosphere (unplanted) soil cores following the application of pathogen-contaminated waste to the soil surface, irrespective of waste type. Rhizosphere soil (●), non-rhizosphere soil (○). Data points represent means ± SEM (n = 12).
Panel B: Survival of *E. coli* O157:H7 in soil cores following the application of pathogen-contaminated cattle slurry or ovine stomach content waste to the soil surface, irrespective of the presence of plants. Cattle slurry (●), ovine stomach content (○). Data points represent means ± SEM (n = 12).

6.5. Discussion

E. coli O157:H7 counts were similar in unplanted soil cores and cores planted with maize seedlings, demonstrating that in this case, the presence or absence of a rhizosphere did not significantly influence pathogen survival. We propose that waste characteristics, competitive interactions with intrinsic biota, and abiotic factors such as soil temperature, type, and moisture availability are likely to be more influential in regulating persistence of this pathogen in soil than rhizosphere-associated effects. Whilst the relative significance of each parameter is unclear, other authors (Chandler and Craven 1980; Jones 1999; Mubiru *et al.* 2000; Lau and Ingham 2001) have highlighted their influence on the longevity of *E. coli* and *E. coli* O157 in soil.

This study did demonstrate that survival of *E. coli* O157:H7 in soil differed significantly with the type of waste applied. There is evidence that persistence of *E. coli* O157 varies in different wastes (Kudva *et al.* 1998; Nicholson *et al.* 2000, 2005; Avery *et al.* 2005); however, the post-application effects of organic wastes on longevity of the organism are less clear. Interestingly, our study corroborates the findings of a previous study in which greater counts of *E. coli* O157:H7 were recorded in soil cores receiving spiked ovine stomach contents compared with those receiving cattle slurry (Avery *et al.* 2004). Survival of bacterial pathogens in soil has been shown to be influenced by soil characteristics such as water and nutrient content, C-to-N ratio, and pH (Jones 1999; Maule 2000; Mubiru *et al.* 2000; Cools *et al.* 2001; Eiland *et al.* 2001). Thus, the altered physico-chemical characteristics of the soils following addition of the two wastes may have led to the difference in numbers of *E. coli* O157:H7 recovered. However, during a 2-month laboratory microcosm study in which *E. coli* O157:H7-spiked organic wastes were not combined with soil (Avery *et al.* 2005), the bacterium also survived better in ovine stomach contents than in cattle slurry. This suggests that the intrinsic waste characteristics may be particularly important with regards to survival of *E. coli* O157:H7.

Gagliardi and Karns (2002) reported that *E. coli* O157 cells persisted longer in soil in the presence of roots of Rye (*Secale cereale* L.) and Alfalfa (*Medicago sativa* ROTH). However, as in the current study, they found no changes in the survival of *E. coli* O157:H7 in soil owing to the presence of roots of other plant species [Clover (*Trifolium incarnatum* L.) and Hairy Vetch (*Vicia sativa* L.)]. Collectively, these findings indicate that the 'rhizosphere effect' phenomenon on soil bacteria populations is plant species-specific.

Variation in the quality and quantity of rhizodeposition with plant species is widely reported (e.g. Baudoin *et al.* 2001; Pinton *et al.* 2001). Maize was chosen as a model plant due to its extensive root structure and the findings of previous studies which show that maize rhizodeposits affect soil microbial community structure. For example, Baudoin *et al.* (2001) and Benizri *et al.* (2002) reported a decrease in microbial diversity with dominance of just a few species within the maize rhizosphere when compared with the equivalent unplanted soil.

The two studies were performed under quite different environmental conditions. In particular, Gagliardi and Karns (2002) employed a higher incubation temperature (25°C, compared with 15°C for the maize cores). Higher temperatures may stimulate plant photosynthetic activity, leading to increased rhizodeposition (Franzluebbers *et al.* 1996; Pramanik *et al.* 2000); which in turn may generate greater contrast between rhizosphere and non-rhizosphere soils. Gagliardi and Karns (2002) utilised dairy manure solids with notably different characteristics to the stomach contents and cattle slurry described in this current study. Survival of *E. coli* O157:H7 tends to be greater in livestock manures as opposed to slurries (Kudva *et al.* 1998; Himathongkham *et al.* 1999; Jones 1999; Guan and Holley 2003). Longevity may also be expected to be lower in abattoir waste due to the presence or accumulation of toxic compounds such as ammonium ions (Buncic *et al.* 2004). The waste application rate in the current study was over three times greater than that employed by Gagliardi and Karns (2002). Thus, the importance of root exudates as a nutrient source was proportionally less; with consequences for microbial activity and community structure. This is supported by the fact that Gagliardi and Karns (2002) observed little effect on the microbial community on addition of manure. In this study, spiked wastes were added to a progressing rhizosphere. Numbers of bacterial populations such as *E. coli* O157:H7 may be activated, inhibited or out-competed at different stages of plant/rhizosphere development as different compounds are secreted into the rhizosphere (Ibekwe and Grieve 2004). For example, Ibekwe and Grieve (2004) showed that as lettuce plant roots aged, there was a corresponding decrease in the concentration of inoculated *E. coli* O157:H7 in the rhizosphere.

Although we anticipated that rhizodeposits would facilitate *E. coli* O157:H7 survival in soil due to the enhanced supply of organic carbon, this could also induce the opposite effect due to enhanced competition and/or predation from antagonistic microbes with superior carbon-utilisation abilities (Benizri *et al.* 2002). Nevertheless, considerably greater

numbers of non-O157 *E. coli* bacteria were recovered on CT-SMAC agar from rhizosphere soil in comparison to unplanted soil (data not presented); indicating that a classic 'rhizosphere effect' was apparent for some bacterial species. Similarly, Gagliardi and Karns (2002) reported enhanced coliform and other microbial populations in soil in the presence of plant roots.

This study is unlikely to have fully accounted for *E. coli* O157:H7 trapped in the endorhizosphere due to bacteria-root adherence (van Peer *et al.* 1990), although these numbers are expected to be low. Recovery of stressed cells may also have been reduced on the selective medium utilised (MacRae *et al.* 1997). Albeit *E. coli* O157:H7 strain #3704 possesses the same survival characteristics as wild-type *E. coli* O157 (Ritchie *et al.* 2003), other strains of *E. coli* O157:H7 may respond differently to various treatments (Kudva *et al.* 1998; Duffy *et al.* 1999). Similar experiments where a range of different strains are incorporated would further elucidate the survival characteristics of *E. coli* O157:H7 in vegetated and fallow soil. Research has shown that if *E. coli* O157:H7 is present in the rhizosphere, it may become internalised within tissues in a range of vegetables (Jablasone *et al.* 2005). Ascertaining if any cells became internalised within the maize plants (hence affect numbers recovered from soil) may therefore have been worthwhile. Finally, re-packing soil into cores inevitably led to disruption of soil structure, with a resulting change in soil porosity. Ultimately, this may have affected the water matric potential and therefore bacterial distribution, survival, and activity (Rattray *et al.* 1992). Such points should be considered when extrapolating any such laboratory-based findings to environmental situations. However, the nature of the study was intended to be comparative rather than to give absolute die-off times for the organism under the conditions tested.

Although the majority of the pathogen population remained in the upper 5 cm of soil, *E. coli* O157:H7 did migrate to lower depths with time, as reported in other studies (Fenlon *et al.* 2000; Gagliardi and Karns 2000). Pathogen leaching rates from waste-amended soil is governed by the degree of bacteria-binding within the waste matrix, which is associated with waste physical properties (Rees 1990). The comparable physical properties of both waste types may have led to similar pathogen leaching rates and may be partly responsible for the lack of effect of treatment on depth profiles of the organism.

Throughout the experiment, the maize plants appeared healthy upon visual inspection; however, growth in confined cores may have induced plant stress responses,

especially towards latter stages of the experiment. Stress can alter maize rhizodeposition (Singh and Pandey 2003; Liu *et al.* 2004), which in turn may affect soil bacteria populations (Jones *et al.* 1996). Such effects warrant consideration, and employing larger cores or plants with a smaller root system in future experiments may resolve this issue.

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6.6. References

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CHAPTER 7: ARTICLE V

Leaching of bioluminescent *Escherichia coli* O157:H7 from sheep and cattle faeces during rainstorm events

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7.1. Abstract

Rural outbreaks of the human pathogen *Escherichia coli* O157:H7 are frequently associated with close contact with contaminated soil and recreational waters in agricultural environments. As ruminants comprise the primary reservoirs of *Escherichia coli* O157:H7 in agricultural areas, we developed a novel inoculation technique to investigate the dispersal of the pathogen from animal faeces during rain events. Cow and sheep faeces were inoculated with either a high (*ca.* 10^6 CFU g^{-1}) or low (*ca.* 10^3 CFU g^{-1}) load of a *lux*-marked strain of *E. coli* O157:H7 and subjected to four simulated heavy rainfall events. The population density and metabolic activity of *E. coli* O157:H7 recovered within the faecal leachate was determined following each rain event. Similarly, non-O157 *E. coli* numbers were also assessed. Typically, the concentration of *E. coli* O157:H7 in the leachates was high (10^5 to 10^6 CFU ml^{-1}) and followed a similar trend to that of non-pathogenic *E. coli*. Greater densities of pathogenic and generic *E. coli* cells were recovered in the leachates generated from sheep faeces compared to cattle faeces. Pathogen metabolic activity was also greater in sheep faeces. We conclude that it is likely that *E. coli* O157 will be readily leached from faeces in the field during rain events leading to the subsequent contamination of underlying soil and adjacent watercourses.

Keywords: activity; animal waste; pathogen; ruminants; runoff; VTEC.

7.2. Introduction

Human infection with *Escherichia coli* O157:H7 may lead to a number of severe sequelae, including haemorrhagic colitis, haemolytic uraemic syndrome, or even death (Chart 2000). The pathogen is of particular concern as the infective dose is very low with ingestion of as few as 10 cells possibly sufficient to cause illness (Chart 2000). Furthermore, incidence numbers show no obvious signs of decreasing (Health Protection Agency 2006).

The gastrointestinal tract of ruminants, particularly cattle and sheep, represents the main natural reservoir of *E. coli* O157:H7; and infection in animals is typically asymptomatic. Approximately 10% of cattle typically excrete between 10^2-10^5 CFU *E. coli* O157:H7 g^{-1} faeces (Omisakin *et al.* 2003; Ogden *et al.* 2004); although shedding rates of up to 10^7 CFU g^{-1} have been reported (Fukushima and Seik 2004). A recent study showed that approximately 6.5% of sheep sporadically excrete *E. coli* O157:H7 at concentrations occasionally as high as 10^6 CFU g^{-1} faeces (Ogden *et al.* 2005). Following excretion, the longevity of *E. coli* O157:H7 is likely to be extended as the pathogen is known to survive for considerable periods in soil (Bolton *et al.* 1999), faeces (Kudva *et al.* 1998; Avery *et al.* 2005), on vegetation (Jones 1999; Avery *et al.* 2004; Ibekwe and Grieve 2004), and in water (Wang and Doyle 1998; Avery *et al.* 2006).

Once introduced into the environment, hydrological pathways such as overland flow and preferential flow through soil readily facilitate the dispersal of *E. coli* O157:H7 and other pathogens, while leaching can lead to groundwater contamination (Chalmers *et al.* 2000; McMath and Holt 2000; Heinonen-Tanski and Uusi-Kamppa 2001; Collins and Rutherford 2004). Pathogen transport following periods of heavy rainfall is especially important in increasing the concentrations of pathogens in many watersheds (Fenlon *et al.* 2000; McMath and Holt 2000). This is of concern as human *E. coli* O157 infections have occurred as a result of groundwater contamination (Wang and Doyle 1998; Licence *et al.* 2001; Artz and Killham 2002).

The physical action of rain dropping onto faeces will extricate bacteria such as *E. coli* O157:H7 from within the faeces into soil water and, potentially into leachate. It is unclear to what degree pathogen cells leach from faeces into the surrounding environment during rain events. However, it is expected that numbers will depend upon a range of parameters, including the initial bacterial population in the faeces, rainfall intensity and duration, and the physical structure of the faeces.

Our aim was to improve existing models that endeavour to predict or quantify pathogen dispersal following rain events with a view to developing risk assessment strategies. During this study, a novel inoculation technique was developed which could be used in conjunction with soil microcosms to improve on current experimental strategies where bacteria are introduced directly into the soil.

7.3. Materials and methods

7.3.1. Faeces collection

All samples were collected in sterile plastic bags (one day prior to commencement of experimental work) from a commercial sheep and beef farm in Kingswells, Aberdeen, North Scotland, in September, 2005. Samples were transported to the laboratory within 30 min and stored at 4 ± 0.1°C prior to use.

7.3.2. Faeces and leachate characterisation

7.3.2.1. Chemical characterisation

A portion of the faeces used for the main experiment was retained so that the moisture content and organic matter could be determined after drying overnight at 105°C and ashing at 405°C, respectively. All other analyses were performed on leachates generated from faeces after the simulation of one 'rainfall event' as in the main experiment (see below). Electrical conductivity (EC) and pH were determined using standard probes (Jenway 4010 EC meter and Jenway 3020 pH meter, respectively; Jenway Ltd., Dunmow, UK). NO_3^- and NH_4^+ concentrations were determined colorimetrically (Downes 1978; Mulvaney 1996) with a Skalar SAN^+ segmented flow analyser (Skalar Analytical, Breda, The Netherlands). Phosphate concentration was determined according to the method of Murphy and Riley (1962), while K, Na and Ca concentrations were determined using a Sherwood Scientific 410 flame photometer (Sherwood Scientific, Cambridge, UK). Total organic carbon and nitrogen concentrations were determined using a CHN2000 elemental analyser (Leco Corp., St Joseph, MI), and dissolved organic carbon (DOC) and dissolved nitrogen (DN) concentrations were determined using a TC-TNV analyser (Shimadzu Corp., Kyoto, Japan).

7.3.2.2. Microbiological characterisation

For determination of the background heterotrophic bacterial population, serial dilutions of the leachates were plated in triplicate onto R2A agar (Oxoid Ltd., Basingstoke, UK), and colonies enumerated following incubation at 20°C for 48 h. Generic *E. coli* numbers were determined by plating leachates in triplicate onto Tryptone bile X-glucuronide agar (TBX; Merck, Darmstaldt, Germany), and enumerating colonies of characteristic *E. coli* appearance following incubation at 37°C for 24 h. To test for the presence of *E. coli* O157:H7 in faeces, solutions were plated onto sorbitol MacConkey agar plates supplemented with 0.05 mg l^{-1} cefixime and 2.5 mg l^{-1} potassium tellurite (CT-SMAC; Oxoid), and onto Harlequin™ SMAC BCIG (LabM, IDG, Bury, UK) agar plates also supplemented with the same amounts of cefixime and potassium tellurite (CT-BCIG; Harlequin™). Plates were incubated at 37°C for 24 h, then examined and scored for colonies with the characteristic appearance of *E. coli* O157:H7. Presumptive (non-sorbitol fermenting) *E. coli* O157:H7 colonies were confirmed by latex agglutination (Oxoid DR620).

7.3.3. Preparation of E. coli *O157:H7 inoculum*

Two inocula of differing densities [one low (*ca.* 10^5 CFU ml^{-1}), the other high (*ca.* 10^8 CFU ml^{-1})] were prepared from a fresh overnight culture (18 h, 37°C, 150 rev min^{-1}) of *E. coli* O157:H7 (strain Tn*5 luxCDABE*; Ritchie *et al.* 2003) grown in LB broth (Difco Ltd., Surrey, UK). Although this strain is non-toxigenic (Ritchie *et al.* 2003), it (and similar strains) exhibits survival patterns reflecting those of toxigenic strains (Fratamico *et al.* 1997; Bolton *et al.* 1999; Kudva *et al.* 1998; Ritchie *et al.* 2003). Cell numbers in both inocula were determined from a calibration curve using optical density (CE373 spectrophotometer; Cecil Instruments, Ltd., Cambridge, UK). Cells were concentrated by centrifugation (5 min, 20°C, 10,000 *g*), before being re-suspended and serially diluted in sterile ¼-strength Ringers solution to achieve the cell concentration needed for faeces inoculation.

7.3.4. Preparation of funnels and inoculated faeces

All treatment combinations were performed with 5 replicates (n = 5) at 20°C. This temperature was chosen to reflect spring/early summer air temperatures, when most animals are out on pasture and animal waste of faecal origin is most frequently spread on land.

Plastic funnels (5 cm diameter) were placed in 40 ml glass bottles. Filter paper (Whatman no. 42) was then folded twice in a half-circle, and placed inside each funnel. Ten g of sheep faeces was placed in half of the funnels, and 10 g of cattle faeces in the other half. To prepare faeces with a high bacteria load (HL), 2 ml of the high-density inoculum was applied to 5 replicates of each faeces. To prepare faeces with a low bacteria load (LL), 2 ml of the low-density inoculum was applied to 5 replicates of each faeces. The final population density of *E. coli* O157:H7 in the HL and LL faeces was approximately 10^6 and 10^3 CFU g^{-1} faeces, respectively; verified by plating and incubation on CT-SMAC and CT-BCIG agar plates, as previously. For 'controls', 5 replicates of each faeces were amended with 2 ml of sterile ¼-strength Ringers solution, and 5 replicates of each faeces were left unamended. The inocula and Ringers solution were injected evenly into the faeces with a hypodermic needle to ensure uniform distribution of cells without disrupting faeces matrix and structure. The 4 final treatment combinations for sheep and cattle faeces were thus as follows: faeces + HL *E. coli* O157:H7, faeces + LL *E. coli* O157:H7, faeces + Ringers, faeces only (unamended).

7.3.5. Water application

Following inoculation of the faeces, samples were left for 1 h, and funnels were subsequently placed in clean, glass universal bottles. Thereafter, to mimic a storm/high rainfall event, 10 ml of artificial rainwater solution (Jones and Edwards 1993) was applied over 1 h to all replicates (using a 1 ml Gilson pipette to drop water across the faeces from a height of 5 cm). The drop volume 310 ± 3 µl and the drop diameter was approximately 8 mm. This was performed twice over the first day (within 1 h of inoculating the faeces, and 5 h thereafter). All funnels were then covered with a porous plastic film (Parafilm) and left overnight. Water application on the second day followed the same procedure as day 1 (i.e. 24 h after the very first application and 5 h thereafter). Clean glass universal bottles were placed under each funnel prior to each rain event so that all leachate collected could be associated with a particular rain event.

7.3.6. Harvests

Samples were left for 1 h following each water application, and the volume of leachate generated was then recorded. A 1 ml aliquot of leachate from each sample containing

luminescent *E. coli* O157:H7 was pipetted into a plastic luminometer cuvette, and its luminescence (relative light units [RLU]) determined using a Jade bench-top luminometer (Labtech International Ltd., Sussex, UK). Serial dilutions of the leachate from control faeces were then plated onto TBX as previously, and onto LB agar supplemented with 2.5 mg l^{-1} ampicillin and 5 mg l^{-1} kanamycin (Oxoid). Serial dilutions of the leachate from faeces with added *E. coli* O157:H7 (high- and low-load) were plated onto LB agar (supplemented with the same amounts of ampicillin and kanamycin). *E. coli* and *E. coli* O157:H7 colonies were enumerated from respective TBX and LB plates following incubation at 37°C for 24 h. Presumptive *E. coli* O157:H7 colonies were confirmed via latex agglutination as previously.

7.3.7. Filter paper effects on bacterial leaching

A calibration experiment was performed to determine the number of *E. coli* O157:H7 cells retained by the filter paper. This was carried out by applying the inoculum to funnels containing only the paper (5 replicates). Filter paper was placed into funnels that were, in turn, placed into glass universal bottles as previously. Using a Gilson pipette, 10 ml of two separate *E. coli* O157:H7 inocula (concentrations of 10^6 CFU ml^{-1} and 10^3 CFU ml^{-1}) was applied to separate filter papers over 1 h (to represent a high and low bacteria load, as previously). One hour after this application, rainfall was applied using artificial rainwater as described previously. Leachate collected following the initial inoculation was sampled after 2 h, and leachate from the additional rainwater applications was also sampled 2 h after watering, as previously. In both cases, serial dilutions of leachate were plated onto LB agar, and the number of *E. coli* O157:H7 colonies counted following incubation as above.

7.3.8. Data analysis

Bioluminescence and plate count data were $\log_{10}(y + 1)$ transformed, and analysed by a multi-factorial analysis of variance (ANOVA) using Genstat 8.1 (Rothamsted Research, Hertfordshire, UK). Significant differences between treatments were identified using Fisher's LSD test within the same software.

7.4. Results

7.4.1. Characterisation of faeces and leachates

A summary of the chemical and microbiological characteristics of the faeces and their respective leachates is presented in Table 7.1. As expected, the moisture content of the cattle faeces was greater than that of sheep faeces, whilst their organic matter content and pH were similar. Generally, levels of nitrogenous compounds were higher in the sheep faeces leachate and consequently the C-to-N ratio was notably less than in the cow faeces leachate. Levels of NH_4^+ were notably greater than of NO_3^- in both leachates. Overall, the cation concentration and electrical conductivity of the sheep faeces leachate was greater than that of the cattle faeces. Numbers of indigenous bacteria and generic *E. coli* were greatest in leachate from the sheep faeces in comparison to the cattle faeces. Nevertheless, no *E. coli* O157:H7 were detected in either leachate prior to inoculation.

Table 7.1. Intrinsic physical, chemical, and microbiological properties of sheep and cattle faeces used in the experiments. Values represent means ± SEM (n = 5).

Parameter	Sheep faeces	Cattle faeces
pH	8.7 ± 0.1	8.4 ± 0.1
Electrical conductivity (mS cm^{-1})	8.4 ± 0.3	7.1 ± 0.4
Moisture content (g kg^{-1})	677 ± 22	815 ± 2
Organic matter (g kg^{-1})	173 ± 7	187 ± 22
Dissolved organic carbon (mg l^{-1})	2157 ± 200	1197 ± 49
Dissolved total nitrogen (mg l^{-1})	131 ± 15	71 ± 5
Total C (g kg^{-1})	296 ± 16	438 ± 2
Total N (g kg^{-1})	24 ± 1	20 ± 0.4
C-to-N ratio	12.3	21.9
Ca (mg l^{-1})	40.3 ± 11.6	7.1 ± 2.9
Na (mg l^{-1})	335 ± 112	139 ± 22
K (mg l^{-1})	81 ± 19	531 ± 53
PO_4^{3-} (mg l^{-1})	196 ± 68	99 ± 3
NO_3^- (mg N l^{-1})	4.4 ± 0.8	6.2 ± 1.0
NH_4^+ (mg N l^{-1})	48.6 ± 11.8	33.2 ± 13.0
Background heterotrophic bacteria (log_{10} CFU ml^{-1})	10.4 ± 0.5	8.2 ± 0.2
Generic *E. coli* (log_{10} CFU ml^{-1})	9.0 ± 0.2	7.3 ± 0.3
E. coli O157:H7 (log_{10} CFU ml^{-1})	0	0

7.4.2. Filter paper and faeces effects on leachate volumes and bacterial density

When no faeces were present, approximately 70% of the applied rainwater was recovered as leachate; - the remainder being lost by evaporation or absorbed by the filter paper (Fig. 7.1).

However, the addition of sheep and cow faeces reduced the recovery of rainwater to approximately 63% and 40% respectively (Fig. 7.1).

Enumeration of pathogen cells within the leachates on LB agar showed that a high proportion of the bacteria passed freely through the paper (84 ± 2 and 90 ± 3% of the total amount of bacteria applied in the HL and LL treatments, respectively; data not presented).

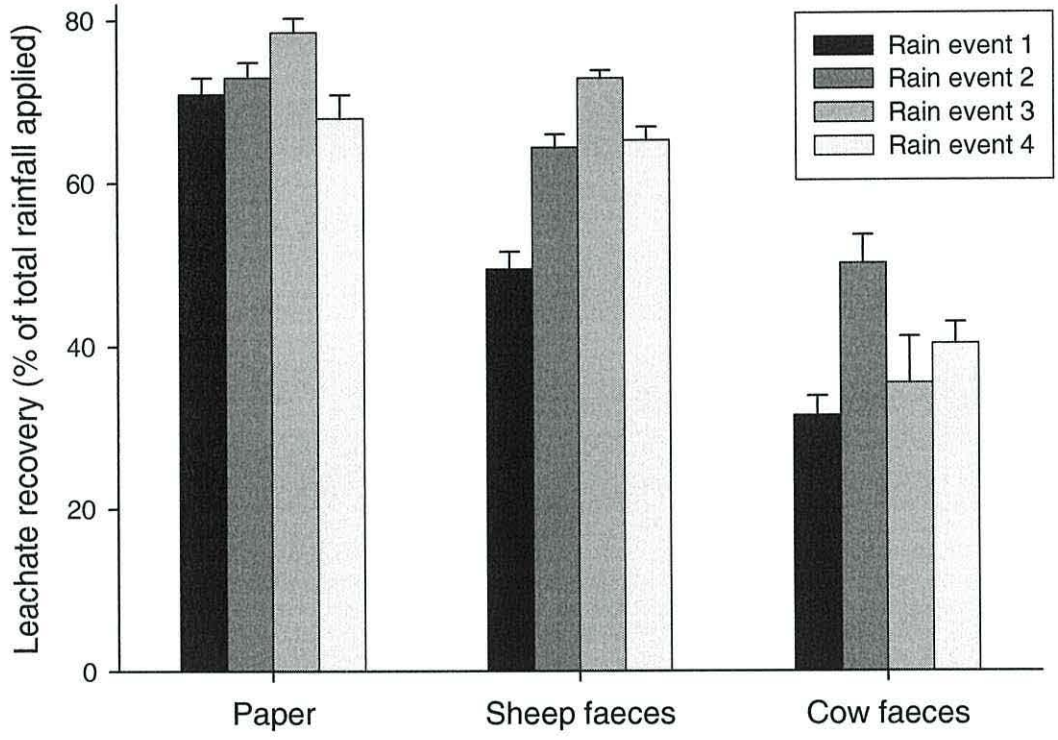

Figure 7.1. Percentage volumes of 'rain' recovered as leachate after the addition of artificial rain to filter paper ± sheep/cattle faeces. Values represent means ± SEM (n = 5).

7.4.3. Recovery of lux-*marked* E. coli *O157:H7 in leachates*

Overall, significantly greater population densities of *E. coli* O157:H7 ($P < 0.001$) were associated with leachates from sheep faeces compared to cattle (overall mean $\log_{10}$ CFU ml^{-1} leachate ± SEM: sheep, 6.55 ± 0.12; cattle, 4.78 ± 0.39; Fig. 7.2). Calculation of the total number of *E. coli* O157:H7 leached from the faeces indicated a notable growth of pathogen between rain events, especially with the sheep faeces. This growth was particularly evident in the LL treatments relative to the HL treatment. Indeed, the notable growth of *E. coli* O157:H7 witnessed in the LL cattle faeces treatment in the early stages of the experiment meant that the population densities from leachates of HL and LL treatments were

statistically similar for the first two rainfall events ($P > 0.05$). However, population densities of the pathogen declined at a much greater rate in leachates from the LL cattle treatment following the third rainfall event, and by the fourth rainfall event no pathogen could be detected in the LL leachate. The temporal changes in *E. coli* O157:H7 population densities recovered in leachate from the LL sheep treatment were notably different from the HL sheep treatment. Although the pathogen densities increased in the LL sheep samples, they were significantly ($P < 0.001$) lower than in leachates from the HL treatment following each rain event (Fig. 7.2A). With the exception of the second rain event, the population density in the sheep LL leachates remained statistically similar throughout the experimental period ($P > 0.05$). However, the densities of *E. coli* O157:H7 recovered in leachates from both faeces type were most stable when a high load of bacteria had been applied.

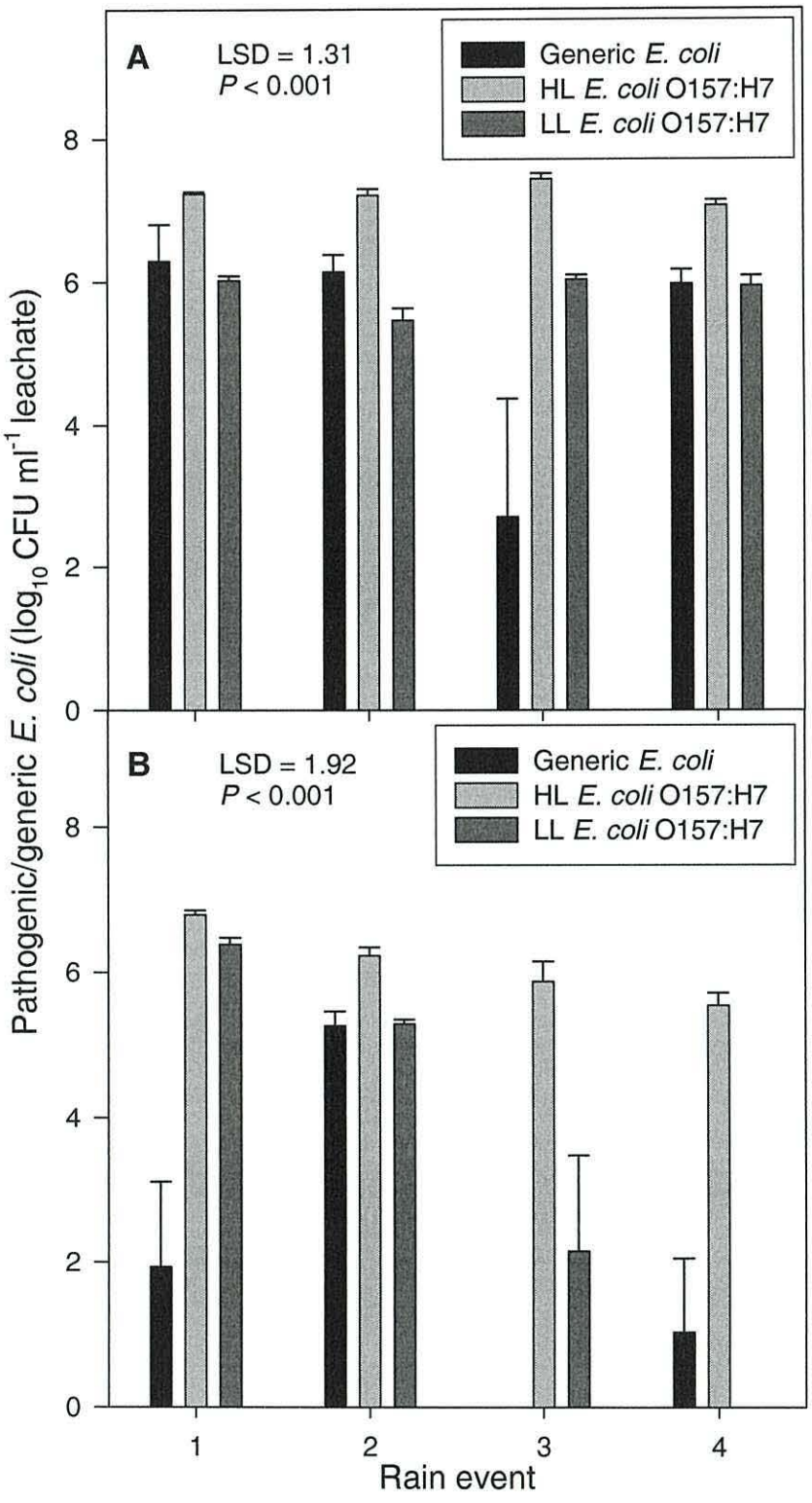

Figure 7.2. Population densities of generic and pathogenic (serotype O157:H7) *E. coli* recovered in leachates of sheep (panel A) and cattle (panel B) faeces inoculated with a high (HL) or low (LL) bacterial load (10^6 and 10^3 CFU ml^{-1} leachate, respectively) following repeated rain events. Values represent means ± SEM (n = 5).

7.4.4. Recovery of generic non-O157 E. coli *in leachates*

Numbers of generic non-O157 *E. coli* were statistically similar ($P > 0.05$) in leachates from both unamended faecal types (data not presented). As with pathogenic *E. coli*, significantly greater ($P < 0.001$) densities of generic *E. coli* were found in leachates from sheep faeces compared to cattle over the experimental period (mean $\log_{10}$ CFU ml^{-1} leachate ± SEM: sheep, 5.27 ± 0.52; cattle, 2.05 ± 0.57; Fig. 7.2). The population densities of generic *E. coli* also displayed less variance in leachates recovered from sheep faeces relative to cattle faeces. Indeed, although statistical analysis revealed that leachate population densities of *E. coli* recovered from sheep faeces significantly varied over the course of the experiment ($P < 0.05$), this was only due to the abrupt drop after the third rain event. Population densities of *E. coli* recovered in leachates from cattle faeces, however, statistically varied after each rain event (single factor effect of time, $P > 0.001$). The densities of indigenous *E. coli* in leachate from sheep faeces were similar or slightly less than those for *E. coli* O157:H7 (Fig. 7.2A). However, this was not the case in leachates generated from cattle faeces, where population densities showed high variability (Fig. 7.2B).

7.4.5. Monitoring of cell activity using bioluminescence

Several observations were made with respect to cellular activity: Firstly, the RLU per CFU ml^{-1} leachate was significantly greater in sheep faeces than in cow faeces ($P < 0.001$; mean RLU per CFU ml^{-1} leachate: sheep, 1.23×10^{-7}; cattle, 2.04×10^{-8}; Fig. 7.3). Secondly, although pathogen bioluminescence decreased over time for both the high and low load treatments, greater activity was seen for faeces with a lower load of bacteria. The biggest decrease in bioluminescence was seen in the final two treatments for both faecal types. Analysis of variance showed that luminescence per cell was significantly different between faeces type ($P < 0.001$) and bacterial load ($P < 0.001$). However, cells recovered in the leachate from cattle faeces showed an increase in RLU per CFU ml^{-1}, indicating recovery or increased activity of cells. This was particularly evident for the cattle faeces with the low pathogen load.

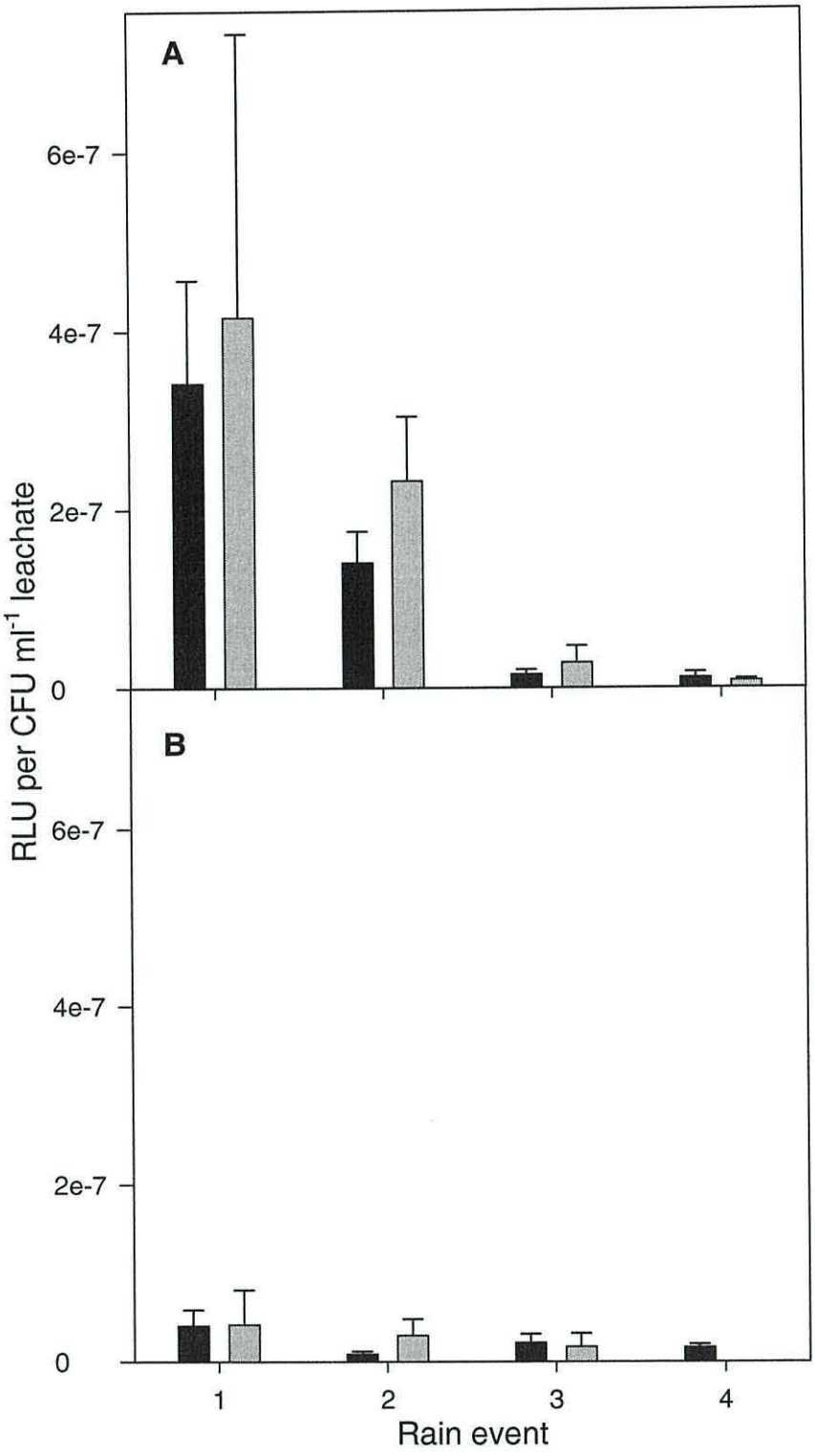

Figure 7.3. Relative light units (RLU) output per CFU ml^{-1} of leachate gathered from sheep (panel A) and cattle (panel B) faeces inoculated with luminescent *E. coli* O157:H7 following repeated rain events. Faeces were inoculated with a high (HL) or low (LL) bacterial load (10^6 and 10^3 CFU ml^{-1} leachate, respectively). Values represent means ± SEM ($n = 5$).

7.5. Discussion

Rain events have been shown to promote the leaching of faecal bacteria within soil environments, leading to direct contamination of the wider environment (McMath and Holt 2000; McGechan and Vinten 2003). This study has shown that raindrops readily disperse *E. coli* O157:H7 from both sheep and cattle faeces. Rain events can be expected to result in both lateral and vertical water flow in soil leading to pathogen contamination of adjacent soils, surface, and ground waters. This dispersal, coupled with the long term persistence of *E. coli* O157:H7 in soil, suggests that the pathogen may be transported considerable distances from the initial point of faecal contamination.

The inoculation technique used in the current study, coupled to simple soil core microcosms, may provide a straightforward method to ensure realistic *E. coli* O157 dispersal models and inputs from animal wastes into soil. This is critical in determining the fate of pathogens, as cells released from such wastes may behave very differently from cells prepared and added to soil/wastes in a laboratory culture liquid media (Gagliardi and Karns 2000; Recorbet *et al.* 1995; Bolton *et al.* 1999; Lau *et al.* 2001). The technique reported here should provide a more realistic method for quantifying microbial dispersal and metabolic state in soil environments. The results showed that quantifying cells in leachate from faeces injected with inoculum offers a relevant and highly controlled means of characterising the input signatures of pathogens released by rainfall into soil. The reproducibility of the assay was greater for sheep than for cattle faeces, probably because of the more favourable hydraulic characteristics of the former.

The growth of *E. coli* O157:H7 observed in the current study concurs with that previously reported for animal faeces or manure incubated at a range of temperatures (Kudva *et al.* 1998; Gagliardi and Karns 2000; Jiang *et al.* 2002; Avery *et al.* 2005). The considerably greater growth of pathogen observed in the LL faecal treatment relative to the HL faecal treatment implies that bacterial population densities increased to the substrate carrying capacity (Byappanahalli *et al.* 2003), and stabilised thereafter. The population densities of *E. coli* O157:H7 supported by faeces in the current experiment concurs with previous data for animal faeces (Fukushima and Seki 2004; Ogden *et al.* 2005); and illustrates the potential of pathogen populations to increase in environmental samples. Interestingly, the total population of generic, non-O157 *E. coli* recovered in the leachates

from all samples was lower than originally found in the faeces. This may indicate that generic *E. coli* may bind more strongly to faeces than their pathogenic counterparts and hence are less susceptible to being leached out during rainfall. This may also be due to differences in the degree of bacterial binding within the matrix (or conversely bacteria mobility) (Rees 1990). The finding of the close affiliation between numbers of indigenous and pathogenic *E. coli* in leachate from sheep faeces may prove valuable (e.g. for estimating numbers of *E. coli* O157:H7 leached via monitoring numbers of generic *E. coli*).

The extent of bacteria leaching from waste is known to be waste-dependent (Rees 1990). Indeed, the experimental data showed that the type of faeces significantly affected the density and temporal dynamics of pathogen release from the faeces into the leachate. The overall significantly greater population densities of *E. coli* O157:H7 ($P < 0.05$) and generic *E. coli* ($P < 0.001$) in leachate from sheep faeces compared to cattle may therefore be due to greater leaching rather than die-off within the cattle faeces. Enumerating the total number of bacteria within both leachates and respective faeces would elucidate this. The pathogen population densities in leachate from cattle faeces were also notably more variable than from sheep faeces (Fig. 7.2). This may be due to the 'ponding' of water on top of some samples of cattle faeces with progressive rain events, due to its relatively high moisture content and lower hydraulic conductivity. Inevitably, this reduced the volume of leachate generated, and hence bacterial carriage. Furthermore, these hydraulic factors may have affected the bacterial habitat through changes in matric potential (Rattray *et al.* 1992), redox (George *et al.* 1998), and nutrient availability (Dickson and Craig 1990), although none of these were directly evaluated. The structure of faeces is likely to alter with the age and breed of animal (larger animals will produce larger stools hence less surface area in comparison to smaller animals), and with animal diet. These may have implications for the mechanics of pathogen release from faeces. Although we simulated a typical rain event, greater variability in pathogen leaching from cattle faeces would be expected in the field. We showed that the filter paper retained approximately 10-15% of the initial number of cells applied, and around 30% of the volume of rainwater. The population densities and total counts of *E. coli* O157:H7 in the leachates reported here may therefore slightly underestimate those occurring in natural environments. .

The bioluminescence measurements provided an insight into the metabolic state of the pathogen cells in the different leachates. It is clear that the *E. coli* O157:H7 leached

from the faeces were still active and presumably capable of causing human and animal infection. Our results also showed that cells in the sheep faeces-derived leachate were more active than those present in the leachate derived from the cow faeces (Fig. 7.3). We speculate that this reflects the greater availability of soluble carbon and nutrients in the sheep faeces leachate in comparison to the cow faeces leachate (Table 7.1). In addition, the waterlogging of the cattle faeces may have reduced the O_2 concentration causing a depression in expression of the luciferase enzyme (Kricka 1988). Another important observation was that bioluminescence per cell decreased over time. This was particularly evident with sheep faeces. This was expected, as key nutrients for bacterial growth will be gradually leached out of the faeces with each rainfall event. This gives an important insight for risk assessment as faeces left on a field in various weather conditions may show different levels of activity and pathogenicity. Finally, the bioluminescence results also indicated that greater levels of metabolic activity were seen in faeces with a lower bacterial load. This effect could be due to either reduced levels of bacterial competition or increased O_2 supply. This is also important for risk assessment as most animals infected with *E. coli* O157 shed a relatively small amount of cells (Ogden *et al.* 2005). However, due to the low infective dose, more active cells from lower shedding animals could pose a greater problem than those from high shedding animals.

In conclusion, the reported technique provides a rapid and flexible method for determining the input signature of a microbe from a slow release matrix such as faeces to soil. This is a vital parameter when considering the effects of microbial dispersal from animal faeces/wastes on a field scale. Although this study focussed on *E. coli* O157, the flexibility of the technique may readily allow variation in bacterial species and load without compromising the substrate release characteristics and natural behaviour/fate of bacterial cells. It therefore offers a potentially powerful research tool for dispersal of the many other micro-organisms, which are introduced into the environment from animals.

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7.6. References

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CHAPTER 8: ARTICLE VI

Survival of *Escherichia coli* O157:H7 in surface waters

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8.1. Abstract

Survival of *Escherichia coli* O157 in surface waters may lead to human infections, facilitate cycles of livestock re-infection, and increase the potential for dissipation of the organism. Persistence of *E. coli* O157:H7 was compared in a variety of surface and farmyard waters to assess the influence of water quality on pathogen survival. Microcosms of four different surface water types (n = 31) from the UK were inoculated with *E. coli* O157:H7 and incubated at 10°C. The water types studied were: lake, puddle, river, and animal-drinking trough waters. *E. coli* O157:H7 numbers declined over time in all waters, although cells were still detected in 45% of non-sterile samples after 2 months. Persistence of *E. coli* O157:H7 was enhanced by water aeration and by prior sterilisation; however, there was no correlation between water chemistry and mean *E. coli* O157:H7 die-off times/rates in any water type. Survival of the pathogen was better in lake and puddle waters than in river or drinking trough waters. However, our study demonstrates that drainage ditches, streams, and rivers may still be major routes of pathogen dispersal into terrestrial and marine environments. Further studies are needed to establish the key water quality factors that regulate pathogen survival.

Keywords: animal waste, freshwater, manure, recreation, streams, VTEC.

8.2. Introduction

Escherichia coli O157 is a major enteropathogen responsible for causing outbreaks of haemorrhagic colitis and haemolytic uraemic syndrome (Chart 2000). Although *E. coli* O157:H7 is harboured by a range of different animals, cattle represent the main environmental reservoir (Jones 1999; Chapman 2000). Infected cattle typically excrete between 10^2-10^5 CFU *E. coli* O157:H7 g^{-1} faeces, however, studies have recovered up to 10^7 CFU *E. coli* O157:H7 g^{-1} faeces (Besser *et al.* 2001; Fukushima and Seki, 2004). The human infectious dose is very low, and ingestion of as few as 10 cells is thought to be sufficient to cause illness (Chart 2000).

The microbiological quality of surface waters is extremely important. They not only provide a basis for numerous recreational activities, but are also essential sources of irrigation water (Weatherhead and Danert 2002). However, surface waters may be subject to microbial contamination through run-off from organic wastes applied to agricultural land and occasionally from direct faecal deposition. Freshwater close to livestock farming systems may therefore represent a potential reservoir for enteric pathogens (McGee *et al.* 2002). In addition, UK rivers receive a large proportion of treated sewage. There is usually limited regulation on microbiological quality of discharge from many sewage treatment works (Tyrrel 1999). Consequently, there is a strong possibility that irrigation water sourced from rivers could act as a vector for contamination of crops by faecal pathogens (Tyrrel 1999).

On-farm water sources (e.g. drinking troughs) may also become reservoirs of *E. coli* O157:H7, allowing cycles of re-infection of livestock and increasing the potential for the organism to spread. Cattle are liable to pick up the bacterium during grazing with subsequent oral transmission to drinking troughs (Rice and Johnson 2000; Lejeune *et al.* 2001b). Faecal contamination of drinking troughs is also common (Lejeune *et al.* 2001a; Sargeant *et al.* 2003). *E. coli* O157:H7 may also be present in manure heaps and surrounding run-off puddles which provide another opportunity for the pathogen to be washed into surface waters.

Although *E. coli* O157 infections are primarily food-borne, there have been a number of water-associated outbreaks following contamination of water by *E. coli* O157:H7 (Chalmers *et al.* 2000). Swimming-associated outbreaks (Ackman *et al.* 1997; Paunio *et al.* 1999) and outbreaks linked with contamination of private water supplies have been

described (Artz and Killham 2002). A few studies have addressed the viability or survival of *E. coli* O157:H7 in surface waters including lakes (Wang and Doyle 1998), rivers (Tanaka *et al.* 2000), marine water (Miyagi *et al.* 2001), animal trough water (Rice and Johnson 2000) and farm waters (McGee *et al.* 2002). However, studies to date have not specifically addressed the influence of physico-chemical characteristics of water on survival of *E. coli* O157:H7. The primary aim of this study was to directly compare the survival of the organism in a wide variety of surface waters grouped into four types: lake, trough, and river waters, and faecally-contaminated water from puddles around livestock troughs, manure heaps, or farm yards. The aim was then to draw conclusions about the influence of chemical and biological water characteristics on survival of *E. coli* O157:H7.

8.3. Materials and methods

8.3.1. Preparation of E. coli *O157:H7 inoculum*

A non-toxigenic environmental isolate of *E. coli* O157:H7 originating from a farm drain (#3704 - Scottish *E. coli* O157 Reference Laboratory; Campbell *et al.* 2001) was shaken (150 rev min^{-1}; 37°C) in LB broth (Difco Ltd., Oxford, UK) for 18 h. A ten-fold dilution of the culture was centrifuged for 2 min (10,000 *g*), and washed three times in the same volume of sterile deionised water. The final inoculum was prepared by performing a further ten-fold dilution of the cell suspension. Deionised water was used as a diluent so as not to introduce additional nutrients or minerals to the river water microcosms.

8.3.2. Water samples

Water for the survival experiment was collected in acid-washed polypropylene bottles from 31 different locations in North Wales and central England. A total of eight samples of lake, faecally-contaminated puddle water (from farm yards, at the foot of animal-drinking troughs and leachate from manure heaps), and animal-drinking trough waters, and seven samples of river water were collected over two separate periods during 2003. This allowed the experiment to be performed over two runs, thus reducing the potential for laboratory-associated bias. The strain preparation was identical for each run. Details of the sampling sites are provided in Table 8.1. Locations were chosen to provide a diverse range of water quality characteristics. After collection, samples were transported at 4°C to the laboratory where they were refrigerated at 4°C prior to establishing the experiment. At each location,

three independent samples of water were taken which subsequently represented the experimental replicates. Sub-samples of water from ten locations (7, 12, 17, 18, 20-25; Table 8.1) were autoclaved immediately upon return to the laboratory and stored as described for the other samples.

Table 8.1. Sampling locations for collection of waters. FCP = faecally-contaminated puddle water.

Water source	Description	Location	Latitude and longitude
1	Lake (urban boating)	Eirias Park, Colwyn Bay, Conwy	53:17:28N, 3:43:09W
2	Lake (reservoir)	Llyn Cowlyd, Conwy	53:08:40N, 3:54:28W
3	Lake (water sports)	Llyn Crafnant, Conwy	53:07:54N, 3:52:17W
4	Lake (agricultural)	Llyn Geirionydd, Conwy	53:07:52N, 3:51:00W
5	Lake (agricultural)	Llyn Idwal, Gwynedd	53:06:39N, 4:02:00W
6	Lake (water sports)	Llynnau Mymbyr, Gwynedd	53:05:52N, 3:56:01W
7	Lake (agricultural)	Llyn Ogwen, Gwynedd	53:07:27N, 4:00:16W
8	Lake (wooded)	Llyn y Sarnau, Conwy	53:06:55N, 3:49:31W
9	FCP (sheep)	Abergwyngregyn, Gwynedd	53:14:16N, 4:01:05W
10	FCP (sheep)	Abergwyngregyn, Gwynedd	53:14:16N, 4:01:05W
11	FCP (sheep)	Abergwyngregyn, Gwynedd	53:14:16N, 4:01:05W
12	FCP (equine)	East Leake, Leicestershire	52:49:45N, 1:10:59W
13	FCP (cattle)	Llanddaniel Fab, Anglesey	53:12:24N, 4:15:05W
14	FCP (cattle)	Llanrwst, Conwy	53:07:59N, 3:47:47W
15	FCP (cattle)	Near Llyn Geirionydd, Conwy	53:07:51N, 3:51:00W
16	FCP (cattle)	Plas Newydd, Anglesey	53:12:10N, 4:12:57W
17	River (agricultural)	Afon Aber, Gwynedd	53:13:52N, 4:00:32W
18	River (agricultural stream)	Fairham Brook, Nottinghamshire	52:51:36N, 1:08:08W
19	River (stream)	Near Llyn Cowlyd Resr, Conwy	53:08:40N, 3:54:29W
20	River (urban)	River Leen, Nottinghamshire	52:56:21N, 1:08:01W
21	River (agricultural)	River Soar, Leicestershire	52:46:28N, 1:10:33W
22	River (agricultural)	River Trent, Nottinghamshire	52:57:45N, 1:01:57W
23	River (agricultural)	River Wye, Derbyshire	53:11:17N, 1:37:53W
24	Trough (sheep)	Abergwyngregyn, Gwynedd	53:14:16N, 4:01:05W
25	Trough (sheep)	Abergwyngregyn, Gwynedd	53:14:16N, 4:01:05W
26	Trough (sheep)	Abergwyngregyn, Gwynedd	53:14:16N, 4:01:05W
27	Trough (sheep)	Abergwyngregyn, Gwynedd	53:14:16N, 4:01:05W
28	Trough (sheep)	Abergwyngregyn, Gwynedd	53:14:16N, 4:01:05W
29	Trough (cattle)	Llanddaniel Fab, Anglesey	53:12:24N, 4:15:05W
30	Trough (cattle)	Llanddaniel Fab, Anglesey	53:12:24N, 4:15:05W
31	Trough (cattle)	Plas Newydd, Anglesey	53:12:10N, 4:12:57W

8.3.3. Characterisation of water samples

Sub-samples from each location were subjected to the following analyses:

Electrical conductivity (Jenway 4010 EC meter; Jenway Ltd., Dunmow, UK) and pH (Orion 410A pH meter; Thermo Ltd., Runcorn, UK) were measured directly after sample collection. For soluble elemental analysis, samples were centrifuged for 5 min (12,000 *g*) to remove particulate matter and the supernatant subsequently used for analyses. Phosphate in solution was determined colorimetrically using the method described by Anderson and Ing (1993) while concentrations of NO_3^- and NH_4^+ were determined using a segmented flow San-plus autoanalyser (Skalar Ltd., York, UK). Dissolved organic carbon was determined using a TOC-V analyser (Shimadzu Corp., Kyoto, Japan). Total Al, Ca, Cu, Fe, K, Mg, Mn, Na and Zn concentrations were determined using a JY-Ultrace ICP-OES (Jobin-Yvon, Longjumeau Cedex, France).

Indigenous microflora of the water samples were quantified by performing serial dilutions in ¼-strength Ringers solution (Oxoid Ltd., Basingstoke, UK) and standard (duplicate) plate counts on R2A agar (Oxoid) for background heterotrophic counts; and onto Sorbitol MacConkey agar (Oxoid) containing 0.05 mg l^{-1} cefixime and 0.05 mg l^{-1} potassium tellurite (Dynal Biotech, Wirral, UK) (CT-SMAC) for presumptive *E. coli* O157 colonies. Plates were incubated at 20°C for 48 h and 37°C for 24 h, respectively, before enumeration.

8.3.4. Preparation and inoculation of microcosms

Twenty-four microcosms of each water (4.5 ml) were spiked with *E. coli* O157:H7 inoculum to give a starting concentration of 8×10^4 CFU ml^{-1} (determined by plating serial dilutions in ¼-strength Ringers solution onto CT-SMAC agar), and placed randomly into a temperature controlled chamber at 10°C (the mean annual temperature of UK freshwaters). Both autoclaved and non-autoclaved waters were used.

8.3.5. Quantification of E. coli *O157:H7 survival*

Three replicate microcosms of each water were randomly selected and harvested within two hours of inoculation and at intervals of 1, 2, 4, 8, 16, 32 and 64 d thereafter. At each harvest, microcosms were vortexed for 15 seconds and *E. coli* O157:H7 remaining in each were quantified on CT-SMAC plates as described previously.

When CT-SMAC plate counts were approaching their theoretical detection limit (5 CFU ml^{-1}), enrichment was carried out to determine whether low numbers of culturable cells were still present. This was achieved by adding 1 ml of water from each of three replicate microcosms to 15 ml modified tryptone soya broth containing VCC supplement (Oxoid). Broths were then shaken (150 rev min^{-1}, 37°C, 6 h), and aliquots plated in duplicate onto CT-SMAC agar and incubated at 37°C for 24 h. Plates were then examined and scored for presence or absence of colonies with the characteristic appearance of *E. coli* O157:H7. Where *E. coli* O157:H7 was detectable only following enrichment, samples were assigned an arbitrary value equal to half of the detection limit of plate counts (i.e. 2.5 CFU ml^{-1}).

8.3.6. Impact of aeration status on E. coli *O157:H7 survival*

An extra experiment was performed in parallel with the main survival experiment to assess the impact of water aeration on survival of *E. coli* O157:H7. Additional aliquots of 35 ml of water from each of three sites (7, 17 and 26) were placed into six replicate (sterile) polypropylene tubes. Dissolved oxygen concentration of each water was measured at source (HQ10 Portable Oxygen Meter; Hach Lange Ltd., Salford, UK). Each microcosm was spiked with *E. coli* O157:H7 inoculum (prepared and quantified as described previously) to a starting concentration of 7×10^4 CFU ml^{-1}. A factorial design was established in which three replicate microcosms per site were continuously shaken at 300 rev min^{-1} for the duration of the experiment (aerated treatment), while a further three were tightly capped and purged with N_2 gas at a flow rate of 3.2 cm^3 $microcosm^{-1}$ s^{-1} (anaerobic treatment) for 45 min daily. All microcosms were incubated at 10°C. *E. coli* O157:H7 surviving in the microcosms was quantified within 1 hour of inoculation, and then after 1, 3, and 7 d. On each occasion, 0.1 ml aliquots were removed from each of the 18 microcosms, diluted, and plated on CT-SMAC agar as described above. At each sampling time, dissolved oxygen concentration in each microcosm was measured as above, using a sterile probe between each sampling.

8.3.7. Data analysis

The means of triplicate plate count data for *E. coli* O157 survival in water samples (CFU ml^{-1}) were gathered, then were $\log_{10}(y + 1)$ transformed prior to statistical analyses. For the

survival experiment, two-way analysis of variance (ANOVA) was carried out upon data from the 31 non-sterile waters incorporating water type (i.e. lake, faecally-contaminated puddle water, river or trough) and time (harvest date, 0 to 64 d) as factors. Individual two-way ANOVA were also performed to identify differences in survival within each water type (time and water source as factors). Where survival was monitored in both sterile and non-sterile water from the same location, these data were subjected to a further two way ANOVA with autoclave treatment (i.e. autoclaved or not), time, and water source as factors.

Plate count data from the aeration experiment (CFU ml^{-1}) were $\log_{10}(y + 1)$ transformed and subjected to a repeated measures ANOVA incorporating water source and aeration treatment as factors.

Time taken (days) for *E. coli* O157:H7 CFU ml^{-1} to undergo a two-log drop from that recovered within 2 h of inoculation (T_{99}; Flint, 1987) was calculated for each water (SigmaPlot 5.0, SPSS Inc., USA) and was subjected to one-way ANOVA for water type. Regression analysis was used to identify correlations between T_{99} values or mean CFU ml^{-1} and water characteristics.

Fisher's LSD was used to identify significant differences arising from ANOVA. All statistical tests were carried out using Genstat 5, release 4.1, third edition (Lawes Agricultural Trust, IACR-Rothamsted, UK).

8.4. Results

8.4.1. Characterisation of water samples

8.4.1.1. Chemical characteristics

Chemical characteristics of the water samples used in the study are displayed in Table 8.2. Water sample pH ranged from 6.2 to 8.9, tending to be lowest in lake waters and highest in river waters. Electrical conductivity (EC), dissolved organic carbon (DOC), NH_4^+, K and Na also tended to be lower in lake waters and were higher in puddle waters, with concentrations in most river and trough waters being intermediate. Nitrate concentrations varied among the water types but tended to be higher in river water. Phosphate concentration was generally lower in river and lake waters and highest in trough and puddle waters. Calcium and Mg concentrations tended to be greater in river and puddle waters, least in lake waters, and intermediate in trough waters. Concentrations of copper were lowest in rivers and variable within the other water types. Iron concentration was generally greatest in puddle waters but

varied widely within the other water types, as did concentrations of Al and Zn. Both pH and EC generally increased following autoclaving, but followed a similar pattern to those in non-autoclaved water (data not shown).

The initial oxygen saturation levels of water samples 7, 17, and 26 used for the aeration experiment were 89, 96, and 46%, respectively.

8.4.1.2. Microbiological characteristics

Background heterotrophic bacteria and presumptive *E. coli* O157:H7 counts from water samples used in the survival experiment are shown in Table 8.2. Background heterotrophic bacteria counts ranged from 66 (water 2) to 7.6×10^6 CFU ml^{-1} (water 13); tending to be greatest in puddle waters, lowest in lake waters, and intermediate in river waters. Trough water contained the most variable numbers of bacteria (4.8×10^2 to 1.4×10^6 CFU ml^{-1}). Colonies of characteristic *E. coli* O157:H7 morphology were present in 7 of the 31 water samples. The greatest number was observed in sample 12 (faecally-contaminated puddle water). However, the organism was most prevalent in river water, being present in 5 of the 7 samples.

Table 8.2. Water characteristics. All values are in mg l^{-1} except pH, electrical conductivity (µS cm^{-1}) and bacterial counts (log_{10} CFU ml^{-1}). DOC = dissolved organic carbon. FCP = faecally-contaminated puddle water. ND = non-detectable (< 5 CFU ml^{-1}).

Water type	Water source	pH	Electrical conductivity	DOC	PO_4^{3-}	NO_3^-	NH_4^+	Al	Ca	Cu	Fe	K	Mg	Na	Zn	Heterotrophic bacteria	Presumptive *E. coli* O157
Lake	1	8.8	209	20.3	< 0.1	< 0.1	< 0.1	0.1	12.4	< 0.1	< 0.1	0.3	0.7	18.6	0.1	2.70	ND
	2	6.6	43	19.1	< 0.1	0.1	< 0.1	0.1	1.9	< 0.1	< 0.1	0.5	0.1	5.5	< 0.1	1.82	ND
	3	6.6	54	3.7	< 0.1	0.2	< 0.1	< 0.1	2.5	< 0.1	< 0.1	2.3	1.0	1.8	< 0.1	2.18	ND
	4	6.8	92	22.8	< 0.1	0.3	< 0.1	0.1	6.1	< 0.1	< 0.1	0.7	0.2	7.4	0.7	2.64	ND
	5	6.4	31	2.0	< 0.1	0.1	< 0.1	< 0.1	1.5	< 0.1	< 0.1	2.2	0.5	0.4	0.1	2.80	ND
	6	6.2	34	3.8	< 0.1	< 0.1	< 0.1	< 0.1	0.9	< 0.1	< 0.1	2.2	0.4	0.8	< 0.1	2.50	ND
	7	6.7	49	12.4	< 0.1	0.3	< 0.1	0.5	1.6	< 0.1	< 0.1	1.7	0.4	5.7	0.4	3.37	ND
	8	6.4	67	7.8	21.7	< 0.1	0.1	< 0.1	3.4	< 0.1	< 0.1	2.3	1.2	2.5	0.6	2.52	ND
FCP	9	8.6	6803	2199.8	15.0	3.3	46.0	0.5	36.3	0.1	13.0	1497.7	3.1	253.5	0.3	5.45	ND
	10	7.9	20163	8115.0	13.2	0.3	25.1	4.2	73.9	0.2	83.1	3133.6	14.7	366.6	0.8	6.86	ND
	11	7.6	2036	334.7	5.5	1.4	135.2	0.2	16.6	< 0.1	1.4	210.4	1.0	23.9	< 0.1	6.30	ND
	12	8.1	5410	1376.8	4.0	0.2	6.8	1.1	76.8	0.1	2.8	818.3	46.8	240.9	0.1	4.06	3.91
	13	7.3	1694	316.6	0.1	< 0.1	28.2	0.1	54.2	< 0.1	2.5	93.2	12.2	77.0	< 0.1	6.88	ND
	14	7.4	1872	554.1	5.7	< 0.1	0.6	0.1	45.5	< 0.1	0.7	234.8	18.1	81.8	< 0.1	6.29	ND
	15	7.5	5243	1410.5	2.2	0.6	37.0	1.2	168.5	< 0.1	9.6	571.6	60.3	287.8	0.2	5.78	ND
	16	6.9	332	30.3	0.1	5.8	2.1	< 0.1	54.2	< 0.1	< 0.1	8.0	6.5	10.6	< 0.1	5.70	2.62
River	17	7.4	73	36.1	< 0.1	0.4	< 0.1	< 0.1	4.2	< 0.1	< 0.1	1.9	1.3	6.2	< 0.1	4.12	ND
	18	8.7	739	36.6	< 0.1	6.4	0.2	0.3	111.7	< 0.1	< 0.1	11.1	25.0	30.5	< 0.1	4.31	2.92
	19	7.3	170	13.3	< 0.1	< 0.1	0.3	0.1	19.1	< 0.1	< 0.1	1.0	0.2	6.0	< 0.1	4.15	ND
	20	8.7	846	32.5	< 0.1	10.4	0.2	0.9	57.0	< 0.1	< 0.1	11.5	51.7	79.9	< 0.1	3.82	2.66
	21	8.4	990	34.7	< 0.1	9.0	0.2	0.3	94.2	< 0.1	< 0.1	13.5	22.3	63.5	< 0.1	3.87	2.04
	22	8.9	840	34.2	0.4	8.9	1.1	0.3	72.9	< 0.1	< 0.1	15.7	23.4	68.3	< 0.1	3.70	1.30
	23	8.2	372	25.0	< 0.1	4.0	< 0.1	< 0.1	63.3	< 0.1	< 0.1	3.9	6.1	15.4	< 0.1	3.77	1.65
Trough	24	6.9	84	19.2	< 0.1	< 0.1	< 0.1	2.7	4.0	< 0.1	< 0.1	4.4	2.8	6.1	0.6	5.30	ND
	25	6.9	104	41.1	< 0.1	< 0.1	< 0.1	5.5	4.9	< 0.1	0.1	5.6	3.0	6.9	2.5	5.26	ND
	26	7.2	100	7.2	1.5	0.1	< 0.1	0.1	10.8	< 0.1	< 0.1	0.7	0.1	6.6	< 0.1	5.87	ND
	27	7.6	93	6.6	1.7	0.1	< 0.1	0.1	11.5	< 0.1	< 0.1	0.5	0.1	6.4	< 0.1	6.13	ND
	28	7.2	302	42.3	6.0	0.4	14.2	0.1	18.5	< 0.1	0.1	5.9	0.2	9.3	8.1	4.40	ND
	29	7.8	226	13.9	< 0.1	0.8	< 0.1	< 0.1	29.2	< 0.1	< 0.1	4.6	4.3	8.8	< 0.1	2.68	ND
	30	7.8	226	16.3	< 0.1	0.3	0.1	< 0.1	31.3	< 0.1	< 0.1	4.6	4.3	9.0	< 0.1	2.68	ND
	31	7.4	277	16.9	< 0.1	6.2	< 0.1	< 0.1	56.0	< 0.1	< 0.1	5.7	5.8	8.7	< 0.1	2.78	ND

8.4.2. Survival of E. coli *O157:H7*

Figure 8.1 shows the decline in mean viable counts of *E. coli* O157:H7 in each water type during the course of the experiment. Water type significantly influenced survival of *E. coli* O157:H7 (time × water type interaction, $P < 0.001$). In general, the number of cultureable *E. coli* O157:H7 recovered from the microcosms declined over time, but tended to be greater in lake and faecally-contaminated puddle waters than in river and trough waters (single factor effect of water type, $P < 0.001$). Mean $\log_{10}$ CFU of *E. coli* O157 recovered over the course of the experiments were 2.93, 2.83, 2.68 and 2.23 (pooled SEM = 0.05) for lake, puddle, river and trough waters, respectively. There were also significant differences in survival patterns between waters from individual sources within each of the four water types (time × water source interaction, $P < 0.001$ for each water type; data not shown).

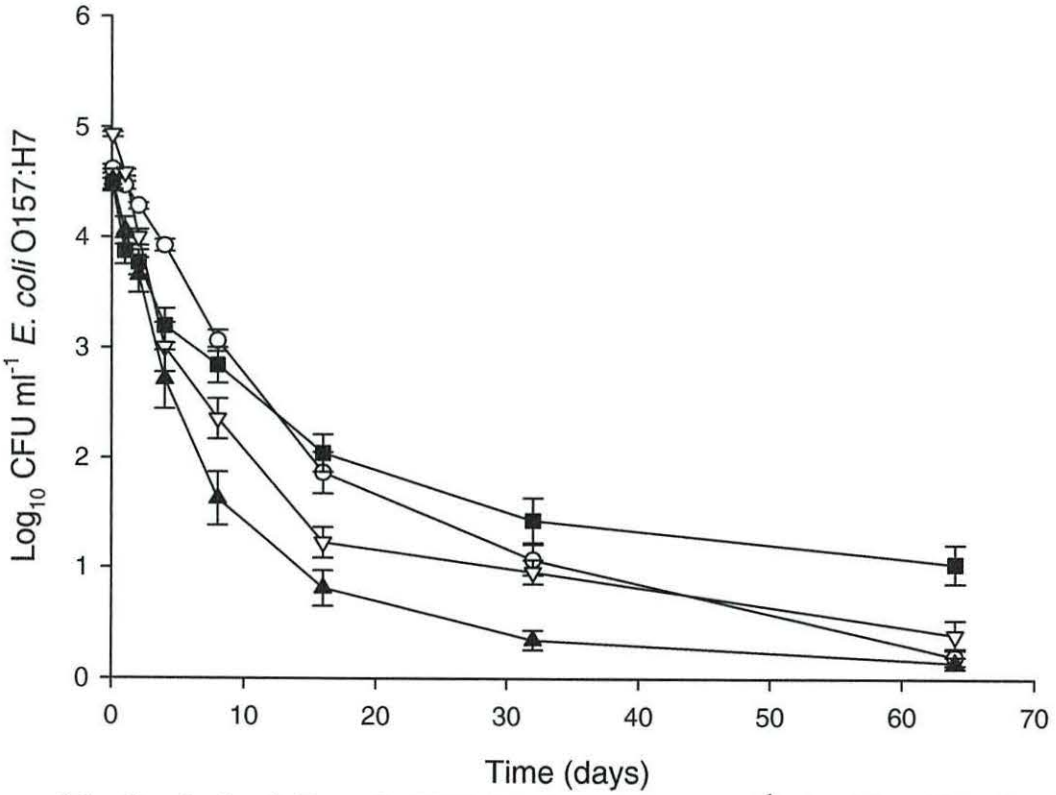

Figure 8.1. Survival of *E. coli* O157:H7 (Log_{10} CFU ml^{-1}) in lake (○), faecally-contaminated puddle (■), river (▽), and animal-drinking trough (▲) waters. Data are $\log_{10}$ ($y + 1$) transformed means of three replicate microcosms per individual water source pooled to give overall means for each water type ± pooled SEM (time × water type interaction, $P < 0.001$).

T_{99} values for pathogen survival are shown in Table 8.3. Log_{10} transformed T_{99} values were greater in lake and puddle waters (means 1.09 and 1.02 d, respectively) than in river and trough waters (means 0.72 and 0.73 d, respectively; $P < 0.05$, pooled SEM = 0.31), thus following a similar trend to that of actual viable counts of *E. coli* O157:H7. Overall, T_{99} values ranged from 2.3 d (water 24) to over 64 d (water 14).

Table 8.3. T_{99} values for *E. coli* O157:H7 in 4 water types (lake, faecally-contaminated puddle, river, and animal-drinking trough). T_{99} values are defined as the time (d) taken for *E. coli* O157:H7 to undergo a two-log drop in CFU ml^{-1} from the Day 0 count. Numbers in brackets denote water sample number as described in Table 8.1. Means ± SEM are given at the foot of each column. FCP = faecally-contaminated puddle water.

	Water type			
	Lake	FCP	River	Trough
T_{99} value (water source)	9.2 (1)	6.5 (9)	10.6 (17)	2.3 (24)
	19.4 (2)	12.1 (10)	5.7 (18)	2.8 (25)
	12.8 (3)	32.1 (11)	10.4 (19)	5.0 (26)
	18.2 (4)	13.9 (12)	3.8 (20)	7.1 (27)
	13.6 (5)	3.8 (13)	2.8 (21)	6.2 (28)
	9.0 (6)	< 64 (14)	3.0 (22)	11.4 (29)
	12.1 (7)	7.5 (15)	5.5 (23)	3.9 (30)
	9.2 (8)	2.5 (16)		11.5 (31)
Mean ± SEM	12.9 ± 1.4	17.8 ± 7.4	6.0 ± 1.2	6.2 ± 1.3
One-way ANOVA	Single factor effect of water type, $P = 0.034$			

Regression analysis revealed no significant correlation between T_{99} values and any of the measured water quality characteristics presented in Table 8.2. However, significant negative correlations were evident between mean log_{10} viable counts and log_{10} [Zn] (r^2 = 0.34; $P < 0.001$, $n = 31$) and log_{10} [Al] ($r^2 = 0.15$; $P < 0.05$, $n = 31$).

There was an interaction between time, autoclaving treatment, and water source ($P <$ 0.001) influencing survival of *E. coli* O157:H7 in the ten waters which were either pre-sterilised by autoclaving or maintained in a non-sterile condition. Overall, survival was greater in autoclaved waters (mean 3.76 log_{10} CFU ml^{-1}) than in those not autoclaved (mean 2.54 log_{10} CFU ml^{-1}; single factor effect of autoclaving, $P < 0.001$; Fig. 8.2). In water samples 7 (lake water), 24 and 25 (trough waters), survival patterns were not significantly different between autoclaved and non-autoclaved treatments. In water samples 17, 18, and 20-23 (river waters), counts of *E. coli* O157:H7 declined less rapidly in autoclaved waters than that in the non-autoclaved equivalents. Counts of the organism in sample 24 (trough

water) exhibited no decline in the autoclaved treatment with counts at 64 d remaining similar to those on day 0 (means 5.28 and 5.43 $\log_{10}$ CFU ml^{-1}, respectively).

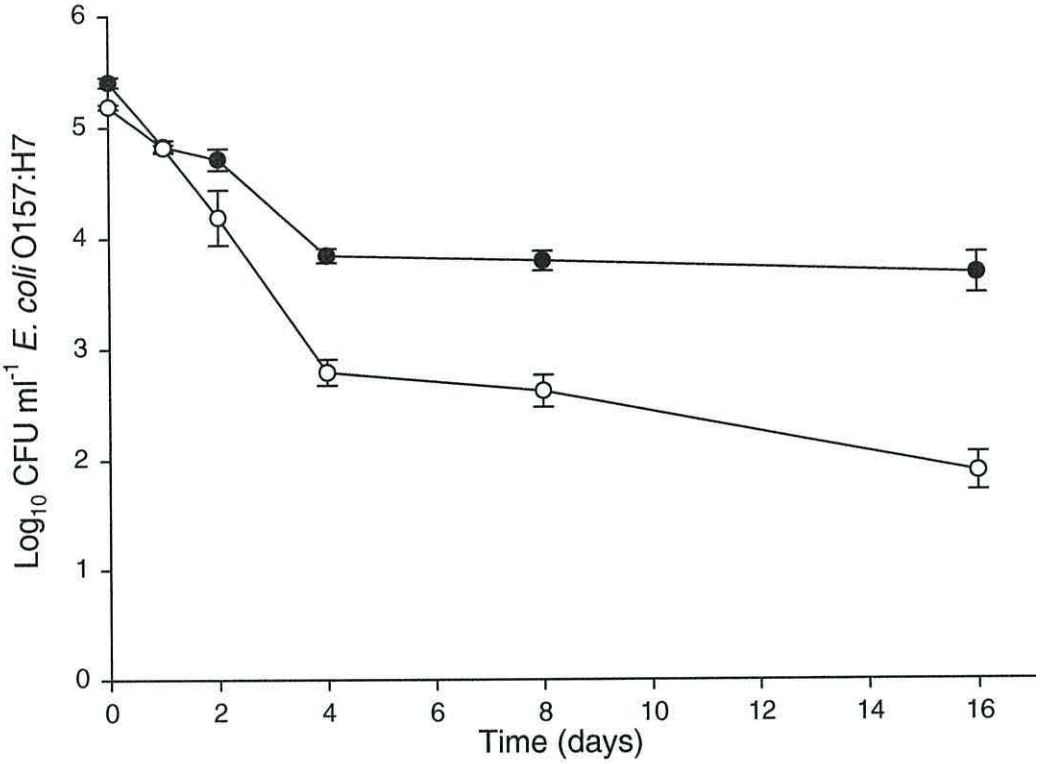

Figure 8.2. Survival of *E. coli* O157:H7 (Log_{10} CFU ml^{-1}) from a range of autoclaved (●) and non-autoclaved (○) waters. Data are $\log_{10}$ (y + 1) transformed means of 30 microcosms (3 per individual water source) pooled to give overall means for autoclaved and non-autoclaved treatments ± pooled SEM (single factor effect of autoclaving, $P < 0.001$).

8.4.3. Impact of aeration status on E. coli *O157:H7 survival*

Waters were maintained either in an O_2 saturated or anoxic condition throughout (98-100% or 2-5% O_2 saturation, respectively; data not presented). In this experiment, there was a significant interaction between harvest time and aeration treatment ($P < 0.001$) and between harvest time and water source ($P = 0.004$). Numbers of *E. coli* O157:H7 recovered from the waters decreased in each of the three waters over the duration of the experiment (Fig. 8.3). There was significantly greater recovery of *E. coli* O157:H7 from aerated waters ($P < 0.001$) (means: aerated 3.05; non-aerated 2.31 $\log_{10}$ CFU ml^{-1}). Recovery of *E. coli* O157:H7 was greatest in lake > river > trough water, regardless of aeration status; matching the pattern of results that occurred in the main survival experiment (Fig. 8.1).

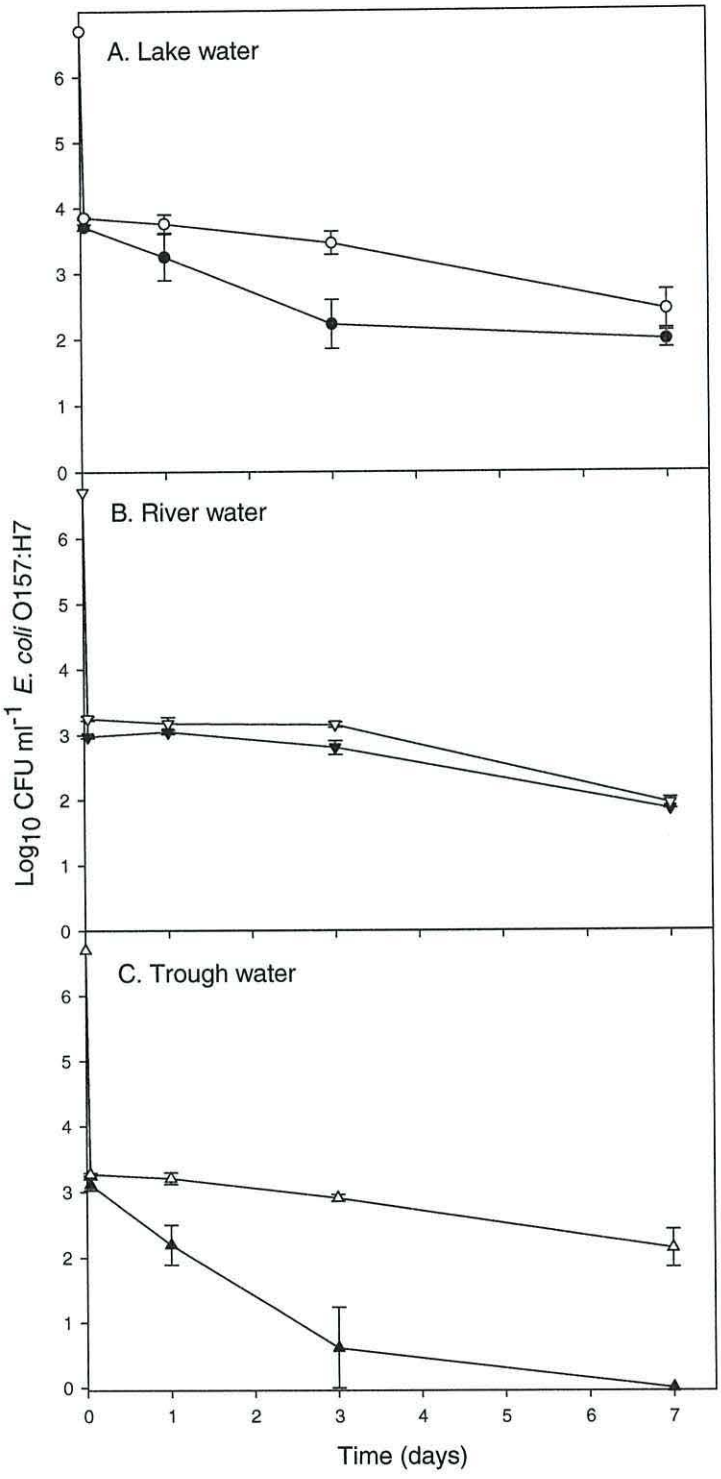

Figure 8.3. Survival of *E. coli* O157:H7 (Log_{10} CFU ml^{-1}) in non-aerated (●) and aerated (○) lake water (Panel A), non-aerated (▼) and aerated (▽) river water (Panel B), and non-aerated (▲) and aerated (Δ) animal-drinking trough water (Panel C). Data are $\log_{10}(y + 1)$ transformed means of three replicate microcosms ± SEM (aeration × time interaction, $P < 0.001$).

8.5. Discussion

From the 31 waters and particular bacterial strain studied in this investigation, it appears that there is substantial variability in persistence of *E. coli* O157:H7 in waters of different qualities. Although the bacterium was detected in all water types for 2 months after inoculation, it appeared to survive better in low-nutrient lake waters and high-nutrient faecally-contaminated puddle water than in livestock-drinking troughs and river waters. Our results, alongside others, suggest that *E. coli* O157:H7 may persist in drinking troughs and manure heaps for months or years, facilitating re-infection of animals (Bolton *et al.* 1999; Lejeune *et al.* 2001ab; Guan and Holley 2003). Its ability to persist in lake waters could have implications for recreational water use and public health risks associated with direct contact. Our results also suggest that survival during long-distance transportation of the organism via river water through to marine environments is also possible.

The longevity displayed by *E. coli* O157:H7 in the current study corroborates the findings of other investigations into the survival of the organism in aquatic environments. For example, Wang and Doyle (1998) observed the survival of *E. coli* O157:H7 in sterile reservoir water and two lake waters held at 10°C over a three-month period. McGee *et al.* (2002) observed survival of *E. coli* O157:H7 under fluctuating environmental temperatures (< 15°C) for 14 days in 'farm' water taken from an untreated river source, and 17 days in sterile municipal water. However, with the addition of faeces, survival in farm water was extended to 24 days. Similarly, in this current experiment, faecally-contaminated puddle water appeared to strongly support persistence of *E. coli* O157:H7. Lejeune *et al.* (2001a) detected a bovine faecal isolate of *E. coli* O157:H7 for 245 d in the sediments of microcosms designed to simulate cattle-drinking troughs. This contrasts with our findings where survival in trough water tended to be comparatively short-lived, but is likely to reflect the effect of sediment harbouring bacteria.

The mechanisms by which *E. coli* O157:H7 is able to persist in water are not clear. The survival of faecal bacteria in aquatic environments is dependent upon their ability to tolerate a range of biological, physical and chemical stresses. These include the influence of temperature, UV radiation, predation and nutrient availability (Flint 1987). Impacts of temperature and solar radiation have been addressed in other studies (e.g. Lejeune *et al.* 2001b). The waters examined here represent the main potential on-farm or environmental aquatic vectors or reservoirs. Water type significantly influenced survival of *E. coli*

O157:H7 whether measured as actual viable counts (CFU ml^{-1}) or as T_{99} values. It is interesting that persistence was greater in lake and faecally-contaminated puddle water than in river and trough waters. Out of the four water types, nutrient concentrations tended to be greatest in faecally-contaminated puddle waters, and least in lake waters. The water characteristics gave little indication as to why survival should be greater and indeed similar in these two very different water types. We hypothesise that the bacteria may be employing different survival strategies within these two water types e.g. the ability of bacteria to utilise nutrients may support populations for longer periods in high-nutrient faecally-contaminated puddle water; whilst low-nutrient conditions (e.g. lake waters) may induce metabolic dormancy. Additionally, a combination of biotic and abiotic factors may interact to further influence pathogen survival. Regression analysis revealed no significant correlation between water quality characteristics and survival of *E. coli* O157:H7, with the exception of zinc and aluminium concentrations. We acknowledge, however, that the dataset may not be large enough or provide a wide enough span of values for the influence of some of the measured characteristics to be detected. Furthermore, it is likely that characteristics which were not measured may also affect persistence of the organism (e.g. NO_2^-, Mn, etc). For example, nitrite is toxic to *E. coli* O157:H7 (Casey and Condon 2000) and is present in cattle saliva (Blum *et al.* 2001). Thus, nitrite may be present in cattle troughs at low concentrations, offering a possible explanation for the overall increased die-off rate in trough waters compared with lake and faecally-contaminated puddle waters. The interaction of individual water quality parameters also needs to be considered in the search for the key factors regulating pathogen persistence.

Predation and competition for nutrients may also influence survival. Lejeune *et al.* (2001b) and Scheuerman *et al.* (1988) found increased persistence of *E. coli* O157:H7.in trough water, and of *E. coli* in lake water, respectively, when protozoal numbers were reduced. Scheuerman *et al.* (1988) also found that competition from indigenous bacteria reduced proliferation of *Pseudomonas* response to nutrient addition. Artz and Killham (2002) found that protozoal grazing of *E. coli* O157:H7 may be responsible for die-off in well waters. In the current study, protozoal counts were not performed. Although there was no significant correlation between counts of indigenous heterotrophic bacteria and *E. coli* O157:H7 persistence, this did not account for variation in predominant species or functional groups of micro-organisms with water source. From the ten waters in which survival of *E.*

coli O157:H7 was monitored for both autoclaved and non-autoclaved waters, it appeared that biotic factors had a strong influence on survival in water from all but three sources. These data should, however, be interpreted with some caution because autoclaving can alter the speciation of some chemical components (Hage *et al.* 1998; Tilston *et al.* 2002).

Aluminium and Zn are known to be toxic to *E. coli* (Guida *et al.* 1991; Chaudri *et al.* 1999). In the current experiment, there were significant negative correlations between mean $\log_{10}$ CFU *E. coli* O157:H7 and both $\log_{10}$ [Zn] and $\log_{10}$ [Al]. Although non-significant, a similar trend was observed when T_{99} values were used in place of mean $\log_{10}$ CFU values. Artz and Killham (2002) reported that metal toxicity was particularly important in the die-off of *E. coli* O157:H7 in well water. The authors found that persistence of the bacterium in well waters with high Cu (3.9 and 0.6 mg l^{-1}) and Zn (0.1 and 0.04 mg l^{-1}) concentration was much lower than that in well waters with low Zn (< 0.015 mg l^{-1}) and Cu (< 0.01 mg l^{-1}) concentrations.

Water turbulence and consequential aeration may influence the survival of *E. coli* O157:H7 and thus the potential for transfer to the wider environment via rivers. Data from our aeration experiment suggest that survival of this strain of *E. coli* O157:H7 is enhanced in aerated water. Concomitant influences of aeration on the autochthonous microbial population may alter the response of *E. coli* O157:H7 to aeration as a consequence of the predation and competition effects already described. For example, microflora indigenous to turbulent river water are likely to be better adapted to higher oxygen saturation than those associated with stagnant trough water. Thus predators and competitive microflora may be reduced in the latter, giving rise to greater persistence of *E. coli* O157:H7, as observed in the current experiment.

While it is not possible to extrapolate the behaviour under laboratory conditions of a single non-toxigenic strain of *E. coli* O157:H7 to that of all strains under environmental conditions, such data can provide valuable information relating to the factors likely to influence survival patterns of toxigenic strains (Kudva *et al.* 1998; Bolton *et al.* 1999; Ritchie *et al.* 2003). It is important to note that data obtained under laboratory conditions should not be directly extrapolated to predict persistence under environmental conditions, but rather, this study has provided an insight into the impact of water type and characteristics on *E. coli* O157:H7 survival.

In conclusion, *E. coli* O157:H7 displayed notable persistence in a range of contrasting surface waters. However, it appears that the organism is better adapted to survive in low-nutrient lake waters and high-nutrient faecally-contaminated puddle waters than in livestock-drinking troughs and river waters. Further studies need to be undertaken to establish which water quality factors are the key regulators of pathogen survival.

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CHAPTER 9: ARTICLE VII

Persistence, dissipation, and activity of *Escherichia coli* O157:H7 within sand and seawater environments

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9.1. Abstract

Runoff from agricultural land into watercourses may transport and deposit animal-derived waste contaminated with *Escherichia coli* O157:H7 onto beaches; hence may lead to human infection. To simulate contamination, freshwater mixed with cattle slurry containing *E. coli* O157:H7 was added to sand from three recreational beaches. The sand was then maintained in a dry state (non-tidal) or subjected to a repeated seawater tidal simulation. The pathogen could still be recovered from all sands by day 5. Survival of the pathogen did not significantly vary with sands of different origin under non-tidal conditions; however, significantly greater numbers were recovered from some sands when subject to tidal simulation. In the tidal simulations, a considerable proportion of the *E. coli* O157:H7 dissipated rapidly from sand into the seawater. In a separate experiment, the activity of bioluminescent (*lux*-marked) *E. coli* O157:H7 cells was monitored in varying mixtures of contaminated runoff water and seawater over 5 days. Pathogen activity declined with increasing seawater concentration; however, cells remained viable in all treatments over the 5 d period. In all treatments, the activity of the pathogen rapidly increased upon addition of available nutrients to the water. Our findings highlight the resilience of *E. coli* O157:H7 in aquatic and marine environments.

Keywords: animal waste; bioluminescence; marine; pollution; survival; VTEC.

9.2. Introduction

Escherichia coli O157:H7 infections remain an issue of global concern owing to their severe health and economic implications, and due to incidence rates showing no obvious signs of decreasing (Health Protection Agency 2006). Infections may give rise to serious symptoms, including haemorrhagic colitis, haemolytic uraemic syndrome, and even death (Park 1999). The problem is exacerbated as no therapies currently exist, only general supportive measures and monitoring of patients (Besser *et al.* 1999).

Cattle are the primary reservoir of *E. coli* O157 in the environment, with approximately 10% typically excreting 10^2-10^5 CFU g^{-1} faeces (Chapman *et al.* 1997; Omisakin *et al.* 2003). However, recent studies have recovered up to 10^8 CFU *E. coli* O157:H7 g^{-1} in faeces and fresh manure (Besser *et al.* 2001; Fukushima and Seki 2004; Hutchison *et al.* 2004).

Approximately 80 million tonnes of animal manure are annually applied to land in the UK (DEFRA 2005). Storage of farm slurries and manures (in tanks and piles, respectively) prior to land disposal normally decreases their pathogen load significantly (Nicholson *et al.* 2005). Nevertheless, limitations in on-farm waste storage capacity frequently lead to only short-term storage of wastes, inadequate to guarantee sanitation, prior to waste spreading. This may lead to dissemination of pathogens such as *E. coli* O157:H7 in the environment, especially as the organism is known to persist in manure, slurry (Kudva *et al.* 1998; Avery *et al.* 2005), and soil (Bolton *et al.* 1999) for many months.

E. coli O157:H7 may also survive for up to a year in an extensive range of water types (Wang and Doyle 1998; Chalmers *et al.* 2000; Avery *et al.* 2006). Although serotype O157 has been isolated in marine environments (Lemarchand *et al.* 2001; Ramaiah *et al.* 2005), its persistence in such surroundings remains understudied. However, some evidence suggests that the pathogen may survive and multiply for at least 15 days in marine water (Miyagi *et al.* 2001). In the UK, it was likely that the greatest previous risk of introducing *E. coli* O157:H7 into marine systems came from disposal of sewage sludge at sea, a practice now forbidden (DEFRA 2002). However, runoff from agricultural land used for cattle grazing or for disposal of animal-derived waste may lead to transportation of *E. coli* O157:H7 onto beaches either directly (e.g. carriage in runoff and subsurface flow water) or indirectly (e.g. from secondary vectors such as dogs/birds or transport to the marine

environment from contaminated freshwaters). Storm events causing widespread runoff from agricultural land into watercourses may also lead to sudden influxes of bacteria at beaches.

Pathogenic bacteria have been isolated from beach sand (Alm *et al.* 2003; Whitman and Nevers 2003; Elmanama *et al.* 2005), and concern has been expressed that beach sand may act as reservoirs or vectors for human infection (WHO 2005). It is anticipated that the physical and chemical stresses a bacterium may be subject to (e.g. in the form of osmotic and desiccation stress) in addition to UV effects, may limit its survival on a beach environment; however, no conclusive research exists to substantiate this. The aim of this study was to assess the survival of *E. coli* O157:H7 in sand left to dry naturally (non-tidal) and in sand exposed to a simulated marine tidal cycle.

9.3. Materials and methods

9.3.1. Sample collection and preparation

Sand was collected from the surface (0-10 cm) of three recreational beaches located in Anglesey, north Wales (Table 9.1). Runoff water (from a small outlet running onto the beach from grazing land) and seawater samples were collected from site 'A'. All samples were gathered in acid-washed polypropylene bottles on the same morning in early January, 2005. On each occasion, three independent samples were taken from each location, subsequently representing the replicates in the experiments. Following collection, samples were transported at 10°C to the laboratory, and sand samples were spread onto trays for 2 h to simulate natural air-drying and to facilitate further mechanical manipulation. Samples were then stored in cooled incubators (LMS Ltd., Sevenoaks, UK) at 10.0 ± 0.1°C for 48 h prior to establishing the experiment. Samples (10 g) of each sand were then placed into 100 × 100 mm mesh bags (pore size 64 μm). Sand-filled bags were subsequently placed into sterile 50 ml polypropylene tubes containing either seawater (20 ml) for tidal simulation or no seawater (non-tidal). At this stage, the bags were suspended above the seawater.

Cattle slurry (approximately 4 weeks old) was collected from a local commercial farm in Conwy, North Wales and stored under the conditions described above.

Table 9.1. Sampling locations for collection of sands.

Sand sample	Origin description	Location	Grid reference
A	A small bay with surrounding cliffs. A small caravan park lays north-west of the beach; surrounding area consists of grazing land	Porth Dafarch, West Anglesey	53:17:12N, 4:39:07W
B	A large, open beach, mainly surrounded by sand dunes with six houses nearby (*ca.* 500 m)	Traeth Llydan, South-west Anglesey	53:13:21N, 4:30:54W
C	A large, rocky bay with surrounding cliffs. The bay is also surrounded by farmland with a farm to the south of the beach	Porth Trecastell, South-west Anglesey	53:12:27N, 4:29:49W

9.3.2. Characterisation of sands

Electrical conductivity (EC) and pH were measured after a 1:1 (v/v) dilution of the sands with distilled water using a 4010 EC meter (Jenway Ltd., Dunmow, UK), and an Orion 410A pH meter (Thermo Ltd., Runcorn, UK). Sand moisture contents were determined after drying at 105°C overnight, and water-holding capacity gravimetrically (Rowell 1994). To determine sands' chemical characteristics, 10 g of each sand were shaken with 30 ml of deionised water for 1 h on a reciprocating shaker (200 rev min^{-1}), centrifuged (12, 000 *g*, 10 min), and the supernatant retained. Thereafter, the supernatant solutions were used to determine the following chemical characteristics: Concentrations of K, Na and Ca were determined using a PFP7 flame photometer (Jenway Ltd.). Phosphate concentration in solution was determined by the method of Murphy and Riley (1962) and concentrations of total dissolved nitrogen and dissolved organic carbon with a TC-TNV analyser (Shimadzu Corp., Kyoto, Japan). Concentrations of NO_3^- and NH_4^+ were determined colorimetrically (Downes 1978; Mulvaney 1996) with a Skalar SAN^+ segmented flow San-plus autoanalyser (Skalar Analytical, Breda, The Netherlands). Total C and N were quantified using a CHN2000 elemental analyser (Leco Corp., St Joseph, MI). Chloride was measured using a Sherwood MK II Chloride Analyser 926 (Sherwood Scientific Ltd., Cambridge, UK).

Background heterotrophic bacteria numbers were estimated by shaking 5 g of each sand (250 rev min^{-1}, 15 min, room temperature) in 15 ml of sterile ¼-strength Ringers solution (Oxoid, Basingstoke, UK), followed by 4 × 5-second bursts on a vortex mixer.

Decimal dilutions (10^{-1} to 10^{-5}) of the extracts were then plated in duplicate onto R2A agar (Oxoid), and colonies counted following incubation for 48 h at 20°C.

An enrichment procedure as follows was employed to test for the presence of presumptive *E. coli* O157:H7 in the sand: a 5 g aliquot of each sand was placed in 15 ml modified Tryptone Soya broth (mTSB; Oxoid), and shaken at 150 rev min^{-1} for 6 h at 37°C before streaking the broth onto Sorbitol-MacConkey agar (Oxoid) supplemented with 0.05 mg l^{-1} cefixime and 2.5 mg^{-1} potassium tellurite (CT-SMAC; Oxoid). Plates were then incubated at 37°C for 18 h and presumptive (non-sorbitol-fermenting) *E. coli* O157:H7 colonies were confirmed by latex agglutination (Oxoid DR620).

9.3.3. Characterisation of waters

All physical and chemical analysis was performed directly on waters according to the methods described previously. An estimate of background heterotrophic bacteria numbers in the water was performed by serial dilution on R2A agar as described above. An enrichment procedure similar to that described above was used to test for the presence of presumptive *E. coli* O157:H7 (5 ml of each water placed in 15 ml mTSB).

9.3.4. Characterisation of cattle slurry

Chemical analysis was performed on supernatant obtained from the slurry by the same methods used for sand. Biological analysis was performed using the same methods as described for the waters.

9.3.5. Preparation of the E. coli *O157:H7 inoculum*

An inoculum was prepared from a fresh overnight culture (LB broth; Difco Ltd., Teddington, Surrey, UK; 18 h, 37°C, 150 rev min^{-1}) of an environmental isolate of *E. coli* O157:H7 (strain #3704) (Campbell *et al.* 2001) in stationary growth phase. Cells were washed and concentrated by centrifugation as described in Avery *et al.* (2005). A 10 ml aliquot of the inoculum was added to 190 ml of the cattle slurry and the suspension was thoroughly mixed. A 5 ml aliquot of the inoculated slurry was then placed in 245 ml of the runoff water, and the mix was thoroughly shaken by hand. The final number of *E. coli* O157 cells in the mix (approximately 7.00 log CFU ml^{-1}) was chosen to imitate a 'worst case' scenario where initial contamination levels are similar to the highest naturally encountered

(Besser *et al.* 2001; Fukushima and Seki 2004). Numbers were determined following duplicate plating of dilutions onto CT-SMAC agar, and incubation as described previously.

9.3.6. Inoculation of sand and simulation of tidal movements

A 2 ml aliquot of the runoff water/slurry mix was applied to the sand in each bag. Bags placed in tubes containing the seawater were subsequently lowered so that the sand was totally immersed in seawater; whilst all other bags were maintained in a non-tidal state. To simulate tidal movements, bags immersed in water were re-suspended out of the water, and then placed back in the water in a 6 h cycle for the duration of the experimental period. In addition, the tidal bags were shaken at 150 rev min^{-1} (on an orbital shaker) when immersed in water to simulate tidal dispersion of sand. All bags were stored at 10 ± 0.1°C (mean UK annual air and seawater temperature) in a glass-fronted incubator (LMS Ltd.) for the duration of the experiment. This gave two treatment combinations (tidal and non-tidal simulation) for each sand (A, B, and C).

9.3.7. Harvests

Destructive harvests of randomly-selected sand samples were performed in triplicate at 2, 24, 48, and 120 hours post-inoculation. Sand bags from the non-tidal treatments were placed in 15 ml of Ringers solution, and shaken at 250 rev min^{-1} for 15 min, followed by 4 × 5-second bursts on a vortex mixer. Serial dilutions of the solutions were then plated out in duplicate onto CT-SMAC agar and colonies were counted following incubation as previously. Sand bags from the tidal simulation treatment were taken out of the water, shaken, and serial dilutions plated onto CT-SMAC as for the non-tidal treatment. The number of *E. coli* O157:H7 cells in the remaining seawater in each tube were quantified by plating out dilutions onto CT-SMAC as previously. Presumptive (non-sorbitol fermenting) *E. coli* O157:H7 colonies were confirmed by latex agglutination from all sand and water samples as described previously. When CT-SMAC plate counts for water and sand were approaching their theoretical detection limits (5 CFU ml^{-1} and 20 CFU g^{-1}, respectively), enrichment procedures as previously were carried out to determine whether low numbers of culturable cells were still present. Where *E. coli* O157:H7 was detectable only following enrichment, samples were assigned an arbitrary value equal to half of the detection limit of plate counts (i.e. 2.5 CFU ml^{-1} for water, 10 CFU g^{-1} for sand).

9.3.8. Bioluminescence of E. coli *O157:H7 in different strengths of seawater*

A separate experiment was designed to assess how the relative mixing of runoff water with seawater may affect the activity of *E. coli* O157:H7. An inoculum was prepared to the same density of *E. coli* O157:H7 as previously with a bioluminescent strain of *E. coli* O157:H7 (strain Tn*5* *luxCDABE*; Ritchie *et al.* 2003). An aliquot of this inoculum was then applied to cattle slurry, thoroughly shaken, and a portion of this applied to runoff water, by the same volumes and methods as described previously. Nine ratios of runoff water-to-seawater were prepared in triplicate, with each progressive dilution containing 12.5% greater seawater. Thus, the final waters ranged from pure runoff water to pure seawater.

Bioluminescence (activity) of bacteria in waters was measured at numerous times over a 120 h period post-inoculation. At each point, a 1 ml aliquot from randomly-selected samples (destructive harvests) was placed into a plastic luminometer cuvette and its luminescence [relative light units (RLU)] determined using a SystemSURE 18172 luminometer (Hygiena Int., Watford, UK). Glucose and glutamate (10 mM) were applied to each treatment immediately after measuring luminescence at 19 h post-inoculation to assess the physiological state of the population. These concentrations were employed as they are within the typical range naturally encountered by microbes when plant cells autolyze (e.g. seaweed). Tubes were then capped and gently inverted numerous times to ensure mixing, and luminescence was monitored for a further 100 h.

9.3.9. Data analysis

Plate count data were $\log_{10}(y + 1)$ transformed prior to analysis. All data were analysed by a multi-factorial analysis of variance (ANOVA) using Genstat 8.1 (Rothamsted Experimental Station, Hertfordshire, UK). Significant differences between treatments were identified using Fisher's LSD test within the same software. Half-maximal bioluminescence values (EC_{50}) in the seawater dilution experiment were calculated using the logistic function:

$$RLU = RLU_{\min} + (RLU_{\max} - RLU_{\min})/(1 + 10^{((\log S - \log EC_{50}) \times B)})$$

Where $RLU_{\min}$ and $RLU_{\max}$ are the minimum and maximum bioluminescence readings (RLU), respectively, S is the salinity of the solution (% seawater), EC_{50} represents the salinity at which half maximal bioluminescence occurs, and B is a constant. The equation

was fitted to the data obtained at each time point with SigmaPlot 8.0 (SPSS Inc., Chicago, IL).

9.4. Results

9.4.1. Characterisation of sands

A summary of chemical and microbiological characteristics of the three sands are presented in Table 9.2. The moisture contents of sand A and B were very similar, at approximately 92% of their respective water-holding capacities. Sand C was noticeably drier, although still at 87% of its water-holding capacity. The solutions extracted from the sands were similar and all moderately basic, and samples showed comparable cation (Ca, Na, K) and chloride levels; however, the EC of samples were markedly different. Sand A had the highest C-to-N ratio; and levels of N were low in all sands. Phosphate levels were below the detection limit for sand A and C, and were low for sand B. Background heterotrophic bacteria counts were also similar for all sands, and enrichment confirmed no *E. coli* O157:H7 were present in any of the three samples.

Table 9.2. Intrinsic chemical and microbiological characteristics of runoff water, seawater, sands, and slurry used in the study. NM = not measured; ND = non-detectable (< 5 CFU ml^{-1} for water; < 20 CFU ml^{-1}/g^{-1} for slurry and sand, respectively); NA = not applicable. All chemical values are in mg l^{-1} for waters/slurry, and mg kg^{-1} for sand, unless otherwise stated. Bacterial counts are in log CFU ml^{-1} and log CFU g^{-1} for waters/slurry and sand, respectively. All values represent means ± SEM (n = 3).

	Sample					
Parameter	Sand A	Sand B	Sand C	Seawater	Runoff water	Slurry
pH	8.27 ± 0.1	8.93 ± 0.1	8.50 ± 0.1	8.12 ± 0.1	7.92 ± 0.1	8.02 ± 0.1
Electrical conductivity (mS cm^{-1})	0.6 ± 0.2	1.2 ± 0.3	1.8 ± 0.2	55.0 ± 0.4	0.7 ± 0.2	7.1 ± 0.4
Dissolved organic carbon	20 ± 1	22 ± 0	98 ± 13	32 ± 1	51 ± 1	32 ± 3
Dissolved organic nitrogen	2.5 ± 0	2.1 ± 0	2.6 ± 1	0.7 ± 0	2.2 ± 0	2.4 ± 1
Ca	4 ± 1	4 ± 0	4 ± 1	2130 ± 22	42 ± 6	NM
Na	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.2	8734 ± 186	68 ± 21	NM
K	49 ± 7	50 ± 5	50 ± 7	205 ± 18	1 ± 0	NM
P	< 0.01	0.07 ± 0.02	< 0.01	0.71 ± 0.1	< 0.01	94.00 ± 6.1
Cl	58 ± 3	56 ± 3	59 ± 4	18400 ± 497	120 ± 13	791 ± 22
NO_3^- (mg N kg^{-1})	< 0.01	< 0.01	< 0.01	2.17 ± 0.60	0.47 ± 0.14	1.50 ± 0.88
NH_4^+ (mg N kg^{-1})	< 0.01	< 0.01	< 0.01	0.6 ± 0.2	0.6 ± 0.1	49.0 ± 14.1
Total C (g kg^{-1})	34.0 ± 6.2	6.5 ± 1.6	3.3 ± 0.9	1.1 ± 0.7	0.1 ± 0.1	36.2 ± 3.8
Total N (g kg^{-1})	< 0.01	< 0.01	< 0.01	0.2 ± 0.1	< 0.01	1.8 ± 0.8
Water-holding capacity (g kg^{-1})	213 ± 41	211 ± 32	161 ± 24	NA	NA	NM
Moisture content (g kg^{-1})	197 ± 30	193 ± 22	140 ± 17	NA	NA	798 ± 39
Background heterotrophic bacteria	4.83 ± 0.01	4.77 ± 0.01	4.75 ± 0.01	9.65 ± 0.01	9.83 ± 0.03	7.36 ± 0.02
E. coli O157:H7	ND	ND	ND	ND	ND	ND

9.4.2. Characterisation of water samples and cattle slurry

A summary of chemical and microbiological characteristics of the waters and slurry are presented in Table 9.2. The seawater and runoff water were both moderately basic. Seawater displayed very high cation and chloride levels in comparison to runoff water, leading to a markedly greater EC value. Both waters' C-to-N ratio was low, with most N in seawater being present as nitrate and in runoff water as ammonium. Seawater and runoff water supported large microbial populations, but no *E. coli* O157:H7 were detected in either following enrichment.

Slurry moisture content and EC were high (Table 9.2). Most available nitrogen was in the form of ammonium rather than nitrate, leading to slightly alkaline conditions. Its C-to-N ratio and phosphate levels were also notably high. Although the slurry contained high levels of background heterotrophic bacteria, enrichment confirmed no *E. coli* O157:H7 cells were present prior to inoculation.

9.4.3. Survival of E. coli *O157:H7 in sands and water*

Approximately 6.30 log CFU g^{-1} sand of *E. coli* O157:H7 cells were present in each sand microcosm at the onset of the experiment. All data gathered displayed normal distribution and equal variances. In the non-tidal treatments, survival of *E. coli* O157:H7 did not significantly vary with sand origin (overall mean log CFU g^{-1} sand ± standard error of the mean (SEM): A 5.83 ± 0.26, B 6.10 ± 0.22, C 6.05 ± 0.24; $P > 0.05$). However, in sand subject to a simulated tidal environment, significantly greater numbers of *E. coli* O157:H7 were recovered from sands B and C than from sand A (overall mean log CFU g^{-1} sand ± SEM: A 3.55 ± 0.71, B 4.18 ± 0.49, C 4.18 ± 0.64; $P < 0.001$). Although overall numbers of *E. coli* O157:H7 declined significantly over time in all sand treatments (single factor effect of time, $P < 0.001$; Fig. 9.1), cells were still recovered from all microcosms (without enrichment) after five days. In the non-tidal treatment, pathogen numbers initially dropped, however, they subsequently increased to surpass the inoculation number at 24 h post-inoculation (mean log CFU g^{-1} sand ± SEM: first harvest, 5.73 ± 0.05, second harvest 6.40 ± 0.04; Fig. 9.1). Thereafter, numbers recovered at each sampling point remained relatively stable, being only slightly reduced at the last harvest (Fig. 9.1). This pattern contrasted with the numbers recovered from simulated tidal sand treatment, where numbers progressively declined with each successive harvest (Fig. 9.1). Over the course of the experiment,

significantly higher numbers of the organism were recovered from sand maintained in a non-tidal state than sand subject to simulated tidal cycles (single factor effect of moisture, $P < 0.001$).

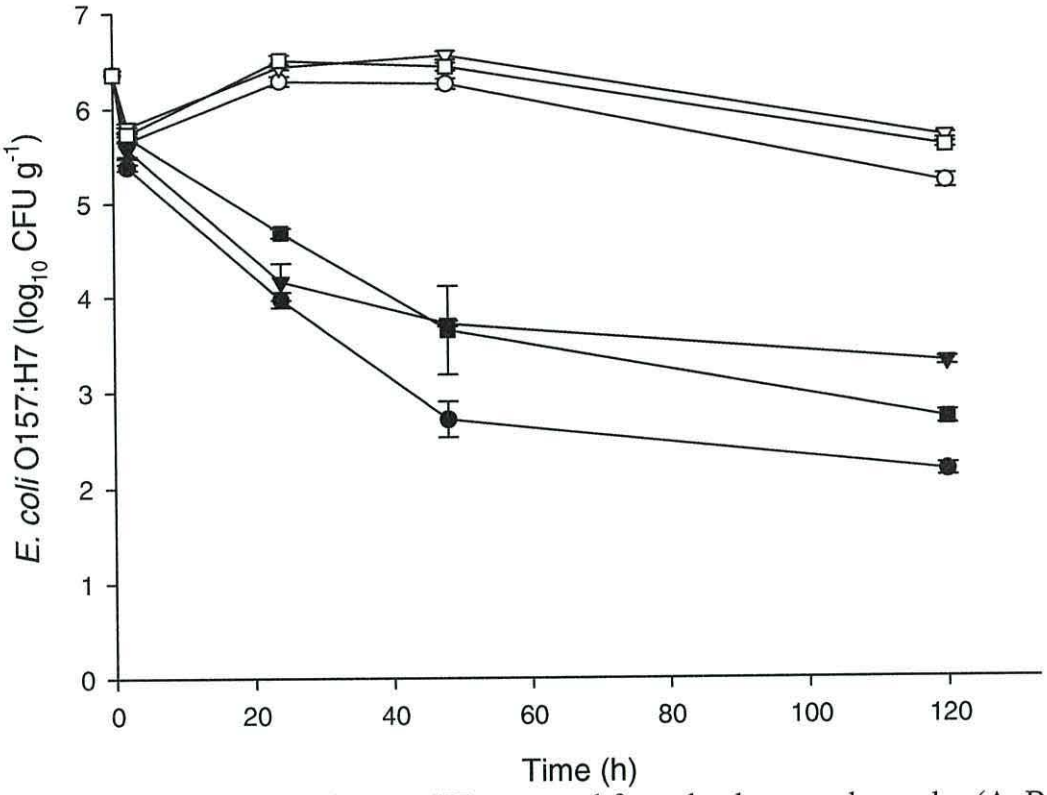

Figure 9.1. Numbers of *E. coli* O157:H7 recovered from the three sand samples (A, B, and C) under simulated tidal (_T) and non-tidal (_NT) conditions [AT (●), ANT (○), BT (▼), BNT (▽), CT (■), CNT (□)].Values represent means ± SEM ($n = 3$).

In the simulated tidal treatments, a notable proportion of the *E. coli* O157:H7 present in the sand became dissipated into the seawater within the first 2 h of the experiment. The average *E. coli* O157:H7 density in the seawater was 3.39 log ml^{-1}, representing approximately 0.5% of the total pathogen population (Fig. 9.2). Generally, the amount of pathogen washed out into the simulated tidal water reduced after 24 h (Fig. 9.2). Indeed, towards the latter part of the experiment, *E. coli* O157:H7 could only be recovered following enrichment from waters A and C. Overall, a significantly ($P < 0.001$) greater numbers of *E. coli* O157:H7 were recovered from water B and C than from water A.

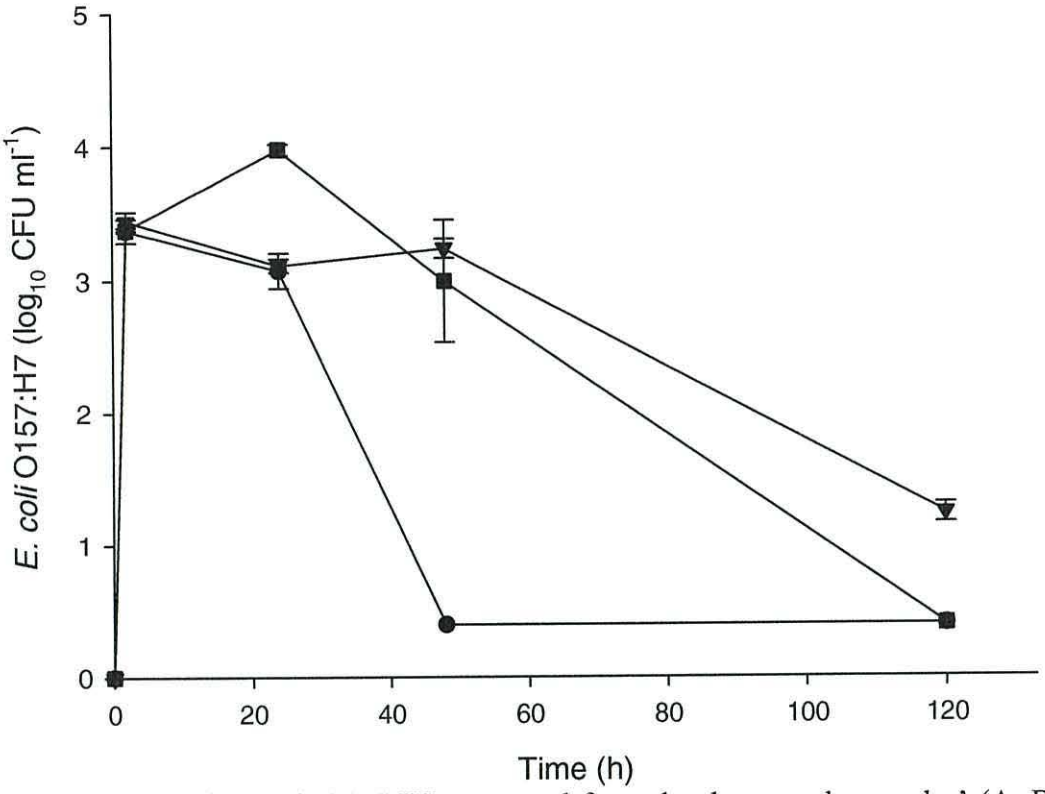

Figure 9.2. Numbers of *E. coli* O157:H7 recovered from the three sands samples' (A, B, and C) respective waters [A (●), B (▼), C (■)].Values represent means ± SEM (n = 3).

Combining the numbers of pathogen recovered from the water and sand in the simulated-tidal treatments showed that survival of *E. coli* O157:H7 over five days was initially greater in this treatment in comparison to the non-tidal treatments (mean log CFU cm^{-3} ± SEM: non-tidal 5.99 ± 0.13, simulated tidal 6.39 ± 0.34). This treatment difference was most clear in the initial 2 h of the experiment when *E. coli* O157:H7 numbers increased in all simulated-tidal treatments (data not presented). Subsequently, overall numbers declined; with pathogen numbers being significantly lower in all simulated-tidal treatments relative to their non-tidal counterparts after 120 h ($P < 0.001$).

9.4.4. Bioluminescence of E. coli *O157:H7 in different strengths of seawater*

When *lux*-marked *E. coli* O157:H7 were placed into water of different salinities in the absence of sand, their activity declined with increasing salinity (mean RLU ± SEM: 0% seawater 43 ± 3, 50% seawater 38 ± 4, 100% seawater 7 ± 1; $P < 0.001$; Fig. 9.3). In the initial 5 h following the addition of the inoculated slurry/water mix to the different waters, bacteria luminescence increased in all dilutions (mean RLU ± SEM; 22 ± 3 at 1 min, 43 ± 5

at 5 h; $P = 0.001$; Fig. 9.3). However, luminescence had decreased in all dilutions by the sampling taken at 19 h (mean RLU ± SEM at 19 h: 7.3 ± 1.1; Fig. 9.3).

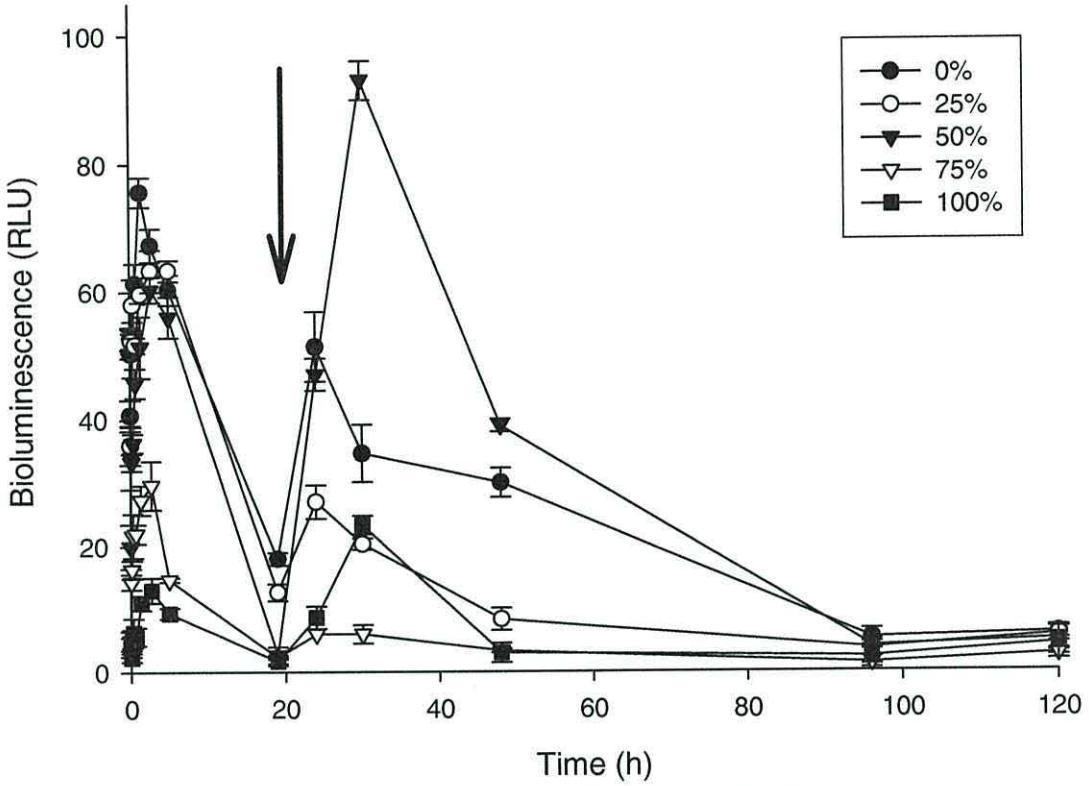

Figure 9.3. Mean bioluminescence (RLU) of *E. coli* O157:H7 over time in waters of varying ratios of runoff water-to-seawater ($n = 3$). Numbers in the legend refer to the percentage of water made up by seawater. The arrow indicates the point at which glucose and glutamate (10 mM) were applied to each dilution. Values represent means ± SEM ($n = 3$).

Generally, the logistic equation used to calculate EC_{50} values gave close fits to the experimental data for all the time points measured over a 19 h period (mean ± SEM $r^2 = 0.97 \pm 0.01$, $n = 9$). Overall, the EC_{50} value remained relatively constant over time with on average half maximal luminescence occurring at seawater concentrations of 58 ± 3% ($n = 9$; Fig. 9.4).

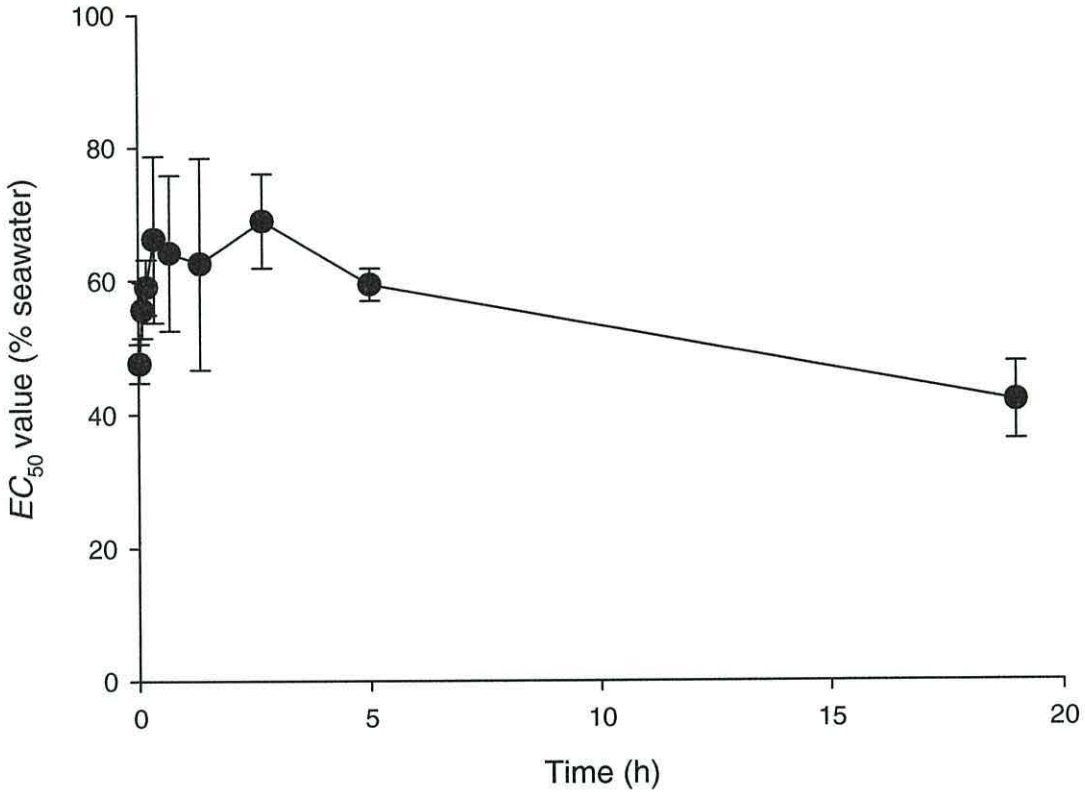

Figure 9.4. EC_{50} values for *E. coli* O157:H7 when exposed to a range of water salinities ranging from freshwater to pure seawater. Values represent means ± SEM ($n = 3$).

Following the addition of C and N in the form of glucose and glutamate (19 h post-inoculation), *E. coli* O157:H7 luminescence rapidly increased in all samples ($P < 0.001$; Fig. 9.3); although this increase was most apparent for the medium seawater dilution. This increase in bioluminescence, however, was short-lived and by 96 and 120 h post-inoculation, RLU values obtained from all dilutions were statistically similar (mean RLU ± SEM: time 120 h, 5.0 ± 0.5; $P > 0.05$; Fig. 9.3).

9.5. Discussion

As waterborne *E. coli* infections represent a major worldwide health problem (Kong *et al.* 1999) and past human *E. coli* O157:H7 infections have been associated with beaches (Proctor and Davis 2000; Bruneau *et al.* 2004; Harrison and Kinra 2004), survival of the organism in marine environments is of concern. Runoff from land receiving animal faeces or farm wastes (e.g. slurry and manure) into surrounding watercourses is a common phenomenon, particularly in agricultural environments (DEFRA 2005). As these wastes are

often contaminated with *E. coli* O157:H7 (Nicholson *et al.* 2005), freshwaters discharging into marine areas may readily transport and deposit *E. coli* O157 onto adjacent beaches. We believe that this may represent a significant pathway for potential human infection. The experiments performed here aimed to reflect typical beach conditions whereupon sand below the inter-tidal zone may be subject to periodic cycles of wetting and drying, whilst sand above the inter-tidal zone may remain in a drier state. Our results show that under typical UK seawater and air temperatures, *E. coli* O157:H7 may survive for at least five days in sand under both dry conditions and under regular wetting-drying tidal cycles. Although the force of tidal flow varies, we also showed that simulated tidal movements may cause rapid dissipation of the pathogen into seawater. Although *E. coli* O157:H7 may survive for at least five days in seawater, the dilution within the water body can be expected to be large; minimising the risk of human infection. However, it is possible that the pathogen may infect and multiply in shellfish causing an indirect risk to human health if consumed (Marino *et al.* 2005). We have also revealed that *E. coli* O157:H7 can remain metabolically active for days in waters of different salinities, and that activity may increase sharply in response to nutrient addition. Our findings, alongside others, highlight the resilience of *E. coli* O157:H7 in aquatic and marine environments.

Numbers of *E coli* O157:H7 in marine environments may vary greatly with distance from an estuary or freshwater source, being greater in density close to the freshwater discharge point due to greater deposition and more hospitable conditions (Neill 2004). Tidal movements and variations in freshwater flow (producing continual change in the freshwater/seawater mix) may also cause bacterial counts to vary greatly throughout an estuary (Neill 2004). From the results presented here, it is clear that although *E. coli* O157:H7 activity declines when it comes into contact with marine waters, it still remains capable of surviving for significant periods of time. Although pathogen activity was initially greatest in freshwaters (possibly due to a greater availability of nutrients), activity increased at all salinities upon addition of C and N. This, coupled with survival numbers gathered in the main experiment, implies that the pathogen population was simply less active (and not killed) by the presence of seawater. However, the lag phase and reduced bioluminescence response after the addition of nutrients in pure seawater may reflect lower numbers and greater stress under these conditions (Neill 2004). Further, the mean EC_{50} occurred in waters at medium salinities (58 ±3% seawater), though the greatest bioluminescence

response to nutrient addition occurred at similar seawater dilutions. This implies that *E. coli* O157:H7 cells become less efficient at utilising nutrients under increasing (medium-high) salinities (hence their metabolic activity is reduced); however, under medium salinity conditions, nutrient addition temporarily formulates notably active cells. Further, the greater response to nutrient addition at the medium seawater dilution rather than in freshwater may owe to reduced competition for nutrients as numbers of background heterotrophic bacteria were less. Although this study and others have demonstrated that *E. coli* O157:H7 possesses an extraordinary tolerance to a wide range of environmental stresses, including seawater (Jones 1999; Park *et al.* 1999; Miyagi *et al.* 2001), further work is needed to elucidate whether *E. coli* O157:H7 remains infective to humans and marine life after being exposed to mild and extreme stress. Indeed, exposure to seawater may increase *E. coli* O157:H7's general stress resistance characteristics as the bacterium may develop extensive structural, metabolic and genetic changes (e.g. increased expression of the *rpoS* gene which regulates stress response; repair and protection of DNA) as part of an adaptive process, termed 'cross-protection' (Gauthier *et al.* 1991; Martin *et al.* 1998; Troussellier *et al.* 1998). Such a process may be induced within less than 15 minutes (Rowe and Kirk 1999), and may therefore be a factor in the initial increase in *E. coli* O157:H7 numbers and activity observed under some conditions in the present study. Marine deposits may therefore act as reservoirs for resistant enteric pathogens (Gauthier *et al.* 1991; Troussellier *et al.* 1998). Furthermore, our work has shown that an influx of nutrients (in this case glucose and glutamate) to both fresh and marine waters results in the rapid increase of *E. coli* O157:H7 activity. This is of interest when considering the effects of repeated pollution events (hence nutrient inflows) on the threat posed by both inherent and introduced pathogenic bacteria. The low, but consistent activity displayed by cells in all water mixes at latter stages of the experiment (Fig. 9.3) indicates the pathogen's ability to persist at low metabolic activity for extended periods.

Due to the lack of measured differences in chemical composition, we could not identify why *E. coli* O157:H7 was more persistent in sands B and C relative to A; and similarly, that the population also decreased sharply in water A after 24 h. The most obvious reasons are that both numbers of background heterotrophic bacteria and total amount of carbon in sand A were the greatest of all three sands. This may have resulted in considerably greater predation and/or competition for nutrients between the introduced and

indigenous microbial populations. Our results exemplify the variability of pathogen survival in seemingly analogous substrates, and reiterate the danger of extrapolating the findings of a single experiment to predict persistence under particular environmental conditions. Although our experiments were carried out in daylight, UV-effects on bacteria cells were inevitably reduced relative to those occurring naturally *in situ*. Nevertheless, tidal movements may dissipate cells through sand or water to depths where UV-effects are minimal, hence UV may destroy only a relatively small proportion of the population (those dwelling on the surface layers). This was illustrated by Fujioka and Yoneyama (2002) who showed faecal indicator bacteria survived up to several days in surface water, but for months in sediments where they were protected from sunlight. Similarly, Ngadi *et al.* (2003) concluded UV-inactivation of *E. coli* O157:H7 in apple cider was inefficient when the fluid depth was greater than 10 mm. The assumption that sunlight alone will adequately reduce numbers of pathogenic bacteria on beaches following pollution incidences is therefore likely to be incorrect.

Although the non-toxigenic strain of *E. coli* O157:H7 used here provides an accurate representation of toxigenic strains (Kudva *et al.* 1998; Bolton *et al.* 1999; Ritchie *et al.* 2003), similar experiments with other strains would be of interest as their respective halotolerance may vary (Rowe and Kirk 1999). It must be noted, however, that variations in strain-survival were not apparent in aquatic environments by McGee *et al.* (2002). Despite the proven resilience displayed by *E. coli* O157:H7, the physiological stresses imposed on cells upon entry to a marine environment may induce some cells to enter a viable but non-cultureable (VBNC) condition (Martin *et al.* 1998; Troussellier *et al.* 1998). As recovery of stressed cells may have been reduced on the selective medium utilised (MacRae *et al.* 1997), the values reported here represent minimum estimates of pathogen survival. Nevertheless, the employment of *lux*-based technology offsets many issues regarding bacteria VBNC (Ritchie *et al.* 2003). Combining experiments investigating pathogen numbers and activity would therefore be of use in future experiments.

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CHAPTER 10: GENERAL DISCUSSION

10.1. Discussion of results

As explained in chapter 1, the work encompassed in this thesis attempted to achieve a number of specific results:

1. To perform a review of existing research on many aspects of *E. coli* O157; including its genetic characteristics, its prevalence, persistence, and detection in different environments, and to summarise potential methods for its control.
2. To further our knowledge of the longevity of *E. coli* O157 in a range of environmental matrices; all of which may serve as pathways for human infection. We aimed to correlate the pathogens' persistence with biotic and abiotic factors that may influence its survival pattern.
3. Finally, to identify particular areas related to *E. coli* O157 that warrant further research; discussed at the end of this chapter.

As is evident from chapter 2, *E. coli* O157 has generated great interest in the scientific world over the last two decades, producing a significant volume of research. This has greatly enhanced our understanding of the pathogen across many areas; from its genetic make-up, modes of implementing infection, prevalence in different environments, detection methods, and lastly, possible methods of control. By encompassing the findings from existing literature, we've obtained a fuller, clearer depiction of the interacting factors that characterise a pathogen of such significant concern.

The experimental work performed satisfied our initial aims and objectives (discussed in chapter 1). Here, we discuss the implications of our findings:

Although previous experiments have depicted the survival characteristics of *E. coli* O157 on surfaces employed in the food industry (Ak *et al.* 1994a; Hood and Zottola 1997), it seems that the danger posed by persistence of this pathogen on surfaces encountered within the agricultural environment has been overlooked. In chapter 3 (*Article I*), we witnessed the notable differences in survival of the bacterium on a range of materials commonly used on farms. We also explicated the number of *E. coli* O157 cells that may be transferred onto hands following brief contact with contaminated faeces. Whilst adhesion of pathogenic micro-organisms to surfaces in food-processing environments is a major public health concern (Ryu *et al.* 2004), we are not aware of any work which has investigated the survival of *E. coli* O157 on floors in such surroundings. In addition, although soiled sawdust

has been identified as instigating an outbreak of human *E. coli* O157 infection (Varma *et al.* 2003), there have been no evaluations of the implications that spreading sawdust on floors in meat-processing environments has on hygiene. The findings of chapter 4 (*Article II*) will therefore be of interest to the food and meat-processing industry. Importantly, it highlights the perils that inadequate cleaning procedures may pose with regards to reducing microbial contamination of surfaces. The findings of both chapters support each other in that survival of *E. coli* O157 was found to vary with prevailing environmental conditions. It is clear that cool, moist conditions formulate greater survival of the organism on surfaces, corroborating similar results found by others on different surfaces (Ak *et al.* 1994b; Maule 1999). It is unambiguous that *E. coli* O157 possesses the ability to survive on a range of surfaces and under differing environmental conditions for extended periods. However, we have also identified factors that may be amended to lessen its survival on surfaces found in both agricultural and food-processing environments. Such factors should therefore be taken into account with regards to farm management and food production.

Of the many aspects of research performed on *E. coli* O157, a considerable proportion of work has focussed on two important environmental reservoirs of the bacterium, namely animal (ruminant) faeces, and soil. Chapter 5 (*Article III*) examined its survival in another potentially significant source, vermicompost. Earthworms for vermicompost production are commercially bred in a matrix of paper pulp and cattle manure; and as the vermicompost market grows, the role of earthworms in dissipating and affecting numbers of the pathogen need to be elucidated. We concluded that the direction of *E. coli* O157 movement in vermicompost or soil is dependent upon earthworm species; whilst the long-term persistence of the pathogen appears to be unaffected by the presence of earthworms. In chapter 6 (*Article IV*), we demonstrated that survival of *E. coli* O157:H7 in waste-amended soil isn't significantly affected by the presence or absence of a maize rhizosphere. The findings of this study somewhat contradict previous work which noted enhanced survival around the root zones of some plants in soil due an input of labile nutrients to the soil (rhizodeposition) which the microbial community could utilise (Gagliardi and Karns 2002). Our work and others therefore strongly suggest that this phenomenon is plant-species specific. Furthermore, this reiterates that the survival of bacteria in soil is a multi-factorial variable of many biotic and abiotic factors. For instance, this study showed that pathogen survival in soil may vary significantly with waste type;

which may have implications for land and waste management. Chapter 7 (*Article V*) demonstrated the importance of heavy rain events in the leaching of pathogenic bacteria from faeces. Some have explored similar areas (e.g. Heinonen-Tanski and Uusi-Kamppa 2001; Collins and Rutherford 2004); although no work to date has compared leaching from different types of faeces with regards to the number of pathogenic *E. coli* leached in relation to generic *E. coli*, and the activity of cells once leached. Greater numbers of cells were leached from sheep faeces, and those cells displayed greater metabolic activity than cells leached from cattle faeces. Although cattle faeces are the main environmental reservoir of *E. coli* O157 (Jones 1999), we conclude that sheep faeces may also pose considerable 'risk' with regards to leaching and propagation of *E. coli* O157. However, as yet, we cannot specifically link *E. coli* O157 cells' metabolic activity to the threat or infectivity they pose to human health.

The last two experimental chapters monitored the survival of *E. coli* O157 in a range of contrasting waters, namely surface and sea water. The ability of this bacterium to persist in a range of waters is well established (Wang and Doyle 1998; McMath and Holt 2000; Tanaka *et al.* 2000; McGee *et al.* 2002). However, what is less clear is which water quality factors are the key regulators of pathogen survival. The work presented in chapter 8 (*Article VI*) sheds some light on this as it showed the non-chemical components of surface waters were of greater importance in regulating pathogen persistence; albeit we obviously cannot extrapolate our findings to all waters. Furthermore, survival of *E. coli* O157 in aerated water reported in this chapter reiterates the possibility of the transportation of the pathogen (via rivers) to wider environments; for example estuaries and beaches. It is known that such marine environments may harbour bacterial pathogens (Lemarchand *et al.* 2001; Ramaiah *et al.* 2005), however hardly any studies have investigated the endurance of *E. coli* O157 under such conditions. In chapter 9 (*Article VII*), we report that viable numbers of the pathogen were recovered for five days from sand under dry (non-tidal) and wet (tidal) conditions. Although cells rapidly dissipated from contaminated sand into the surrounding seawater, the pathogen may still pose threat to human health on an authentic beach as it also subsisted in seawater. We also showed that *lux*-marked *E. coli* O157 cells remained active in varying ratios of runoff water-to-seawater for five days, but that activity tended to be greatest under freshwater conditions. In all treatments, the activity of the pathogen rapidly increased upon addition of available nutrients to the water. This illustrates its ability to

reside in a state of low metabolic activity for extended periods, before rapid awakening under suitable conditions. The results of chapters 8 and 9 highlight the resilience of *E. coli* O157 in aquatic environments. As numerous different water types may become contaminated by various means with pathogenic bacteria such as *E. coli* O157, the role that water plays in the cycling of the bacterium in the environment shouldn't be underestimated.

The low infectious dose of *E. coli* O157 (Chart 2000) only serves to enhance the threat it provides from residing in the environment. Our experimental work emulated numerous scenarios where the presence of *E. coli* O157 may be of peril or have implications to human health, or lead to wider dissipation into the environment. From our results and others, it is clear that this organism possesses a notable amount of resilience to a wide range of environmental stresses. Reducing its general prevalence once introduced in the environment will thus be difficult. Nevertheless, our experiments have shown that some actions (e.g. selective choosing of surface materials, efficient cleaning regimes, and careful spreading of animal waste) may lessen its persistence and hence the overall threat it poses.

10.2. Further work

The extensive studies into the evolution and ecology of *E. coli* O157 have yielded major advances in our understanding of this pathogen. It is now known that many of the recognised virulence factors are on mobile genetic elements, and that its evolution is ongoing. Nevertheless, there is still a clear requirement for further research into many aspects of the life-cycle of this pathogen before significant reductions in human infections can occur. Many specific aspects in need of greater work are discussed in individual chapters; however the following is a more general overview of areas identified that may warrant greater exploration.

Clearly, most outbreaks of human *E. coli* O157 infections occur following ingestion of contaminated food. Nevertheless, notable numbers occur following contact with the organism within the wider environment. Henceforth, many areas concerning survival of this bacterium in non-food quarters require investigation. The degree to which wild animals, birds, and insects dissipate the bacterium into the wider environment requires clarifying. Research is needed to determine the persistence of this organism in trucks, lairage pens, and yards used for animal handling. Concurrently, there is a need for developing ways to limit

its survival in such areas. It is encouraging to see focussed research to establish how manure storage, handling, and treatment methods affect the persistence of *E. coli* O157. Research into the prevalence and persistence of this organism in animal feed and on pasture is needed as contamination of feed source is likely to be an important vector in spreading the bacterium. The frequency of human *E. coli* O157 infections in many countries relative to the near-absence in other countries is of interest, the reasons for which need to be elucidated. Disparities in hygiene at the farm and abattoir, differing food storage and preparation practices between different countries, along with animal management factors may explain this variance. Of course, it is always possible that differences in infectivity rates between countries is a result of, or lack of a reporting policy and not to actual infectivity rates; unless there is political will to report *E. coli* O157 infections it is unlikely that infection rates will be known and published.

As *E. coli* bacteria are commonly found as part of normal intestinal flora in cattle, sheep, and other animals, it is probable that human intervention may (at best) lessen levels of the bacterium; not lead to eradication. Because most *E. coli* O157 are associated with ruminant intestinal systems, more needs to be known on the primary reasons why these bacteria thrive in this environment. Nevertheless, whilst it is clear that most studies thus far have concentrated on cattle and (to a lesser extent) sheep, there is an obvious need for greater insight into the prevalence of the pathogen within other animal species.

Although improved abattoir hygiene in recent years has limited the spread of pathogens to meat, reducing the primary input of the organism into abattoirs would be a significant further development. Broadly speaking, pre-harvest control of *E. coli* O157 can be categorised in two ways. Firstly, existing animal production strategies may be modified e.g. via applying a feed ingredient or increasing the frequency with which water troughs are washed. Secondly, intervention technologies may be developed and employed. The former strategy is the most appealing method as implementing modifications into existing animal management practices would have less impact on farming operations. Conversely, the second approach would require significant investment in development and implementation; and it is doubtful that many farmers would invest in technological changes that have no real (economic) pay-back in terms of animal production. Based on studies previously described, it is clear that several interventions show considerable promise. Unfortunately, none to date have identified a single animal management practice that may both reduce the prevalence of

E. coli O157, and that can be readily modified. Although the employment of vaccines has shown encouraging results, it is unclear if the cost and ethic debates surrounding antimicrobial agents will limit their use. Modification of feeding regimes and the use of probiotics is an area that merits particular analysis in the effort to reduce levels of the organism within the animal gut. The water treatment methods discussed in chapter 2 warrant greater investigations due to the obvious role that contaminated drinking water plays in propagating *E. coli* O157 between, and within animal herds and wildlife.

One specific area which warrants greater examination is quantifying the infectivity of *E. coli* O157. At present, ingestion of high numbers of the organism is considered a 'greater risk' to humans than of low numbers; however, this notion may not necessarily be accurate. Low numbers of highly infective cells may indeed pose a greater risk than greater numbers of less active cells. Similarly, such differences in infectivity may arise between different strains of *E. coli* O157. Lastly, as haemorrhagic disease may occur following ingestion of other *E. coli* serotypes (Bettelheim 2000), it is imperative that future studies do not concentrate solely on *E. coli* O157.

10.3. References

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APPENDIX

Photographic illustrations of experimental set-up

Figure I. Cattle faeces contaminated with *E. coli* O157:H7 on wood and galvanised steel pieces maintained under desiccated conditions (*Article I*).

Figure II. Cattle faeces contaminated with *E. coli* O157:H7 on wood and galvanised steel material pieces maintained under moist conditions (*Article I*).

Figure III. Meat juice contaminated with *E. coli* O157:H7 on tile pieces ± sawdust, maintained under desiccated (left) or moist (right) conditions (*Article II*).

Figure IV. Experimental box containing the earthworm *Dendrobaena veneta*, compost, and cattle manure contaminated with *E. coli* O157:H7 (*Article III*). This was to assess lateral movement of the bacterium due to earthworm activity. Sampling points can be seen at the forefront of the photo.

Figure V. Close-up of experimental box containing the earthworm *Dendrobaena veneta*, compost, and cattle manure contaminated with *E. coli* O157:H7 (*Article III*). Earthworm activity was clearly visible in the manure soon after its application.

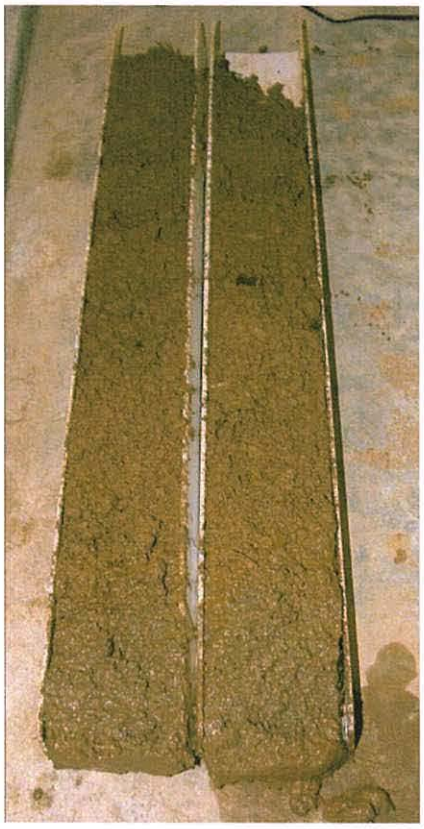

Figure VI. Experimental core containing the earthworm *Lumbricus terrestris*, soil, and cattle manure contaminated with *E. coli* O157:H7 (*Article III*). This was to assess vertical movement of the bacterium due to earthworm activity. Samples were taken from marked points along the core (note: core has been opened for sampling).

Figure VI. Close-up of experimental cores containing the earthworm *Lumbricus terrestris*, soil, and cattle manure contaminated with *E. coli* O157:H7 (*Article III*). Earthworm activity (channels, etc.) was clearly visible throughout the cores.

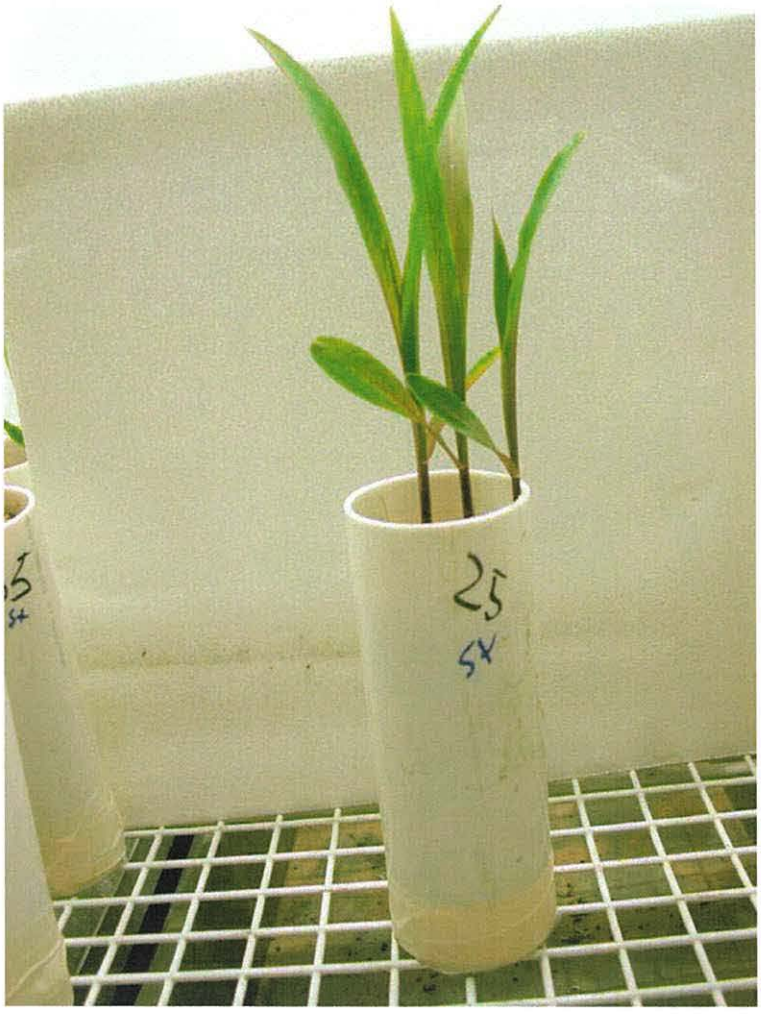

Figure VII. Experimental cores containing soil (Eutric Cambisol) planted with three maize seeds (*Zea mays* L. cv 'Merit') (*Article IV*). Ten days following planting, cores were applied 10 ml of cattle slurry or ovine stomach contents contaminated with *E. coli* O157:H7.

Figure VIII. Funnels containing 10 g of sheep faeces contaminated with *E. coli* O157:H7 (*Article V*). Leachate formation can be seen in the glass universals following the simulation of heavy rainfall.

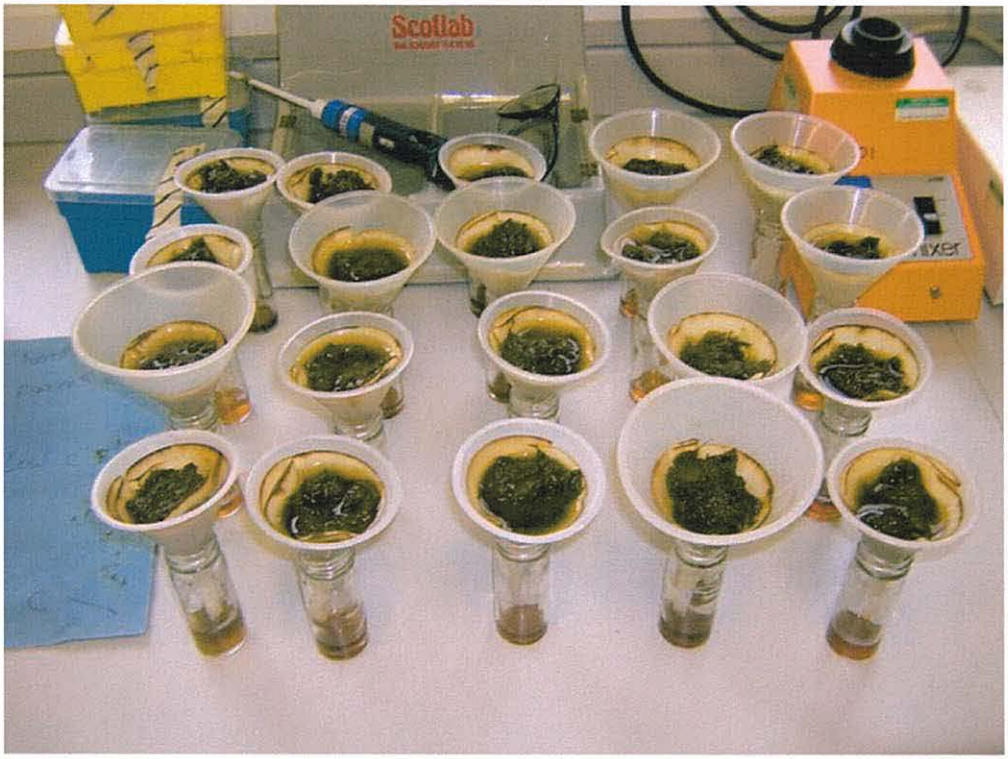

Figure VIII. Funnels containing 10 g of cattle faeces contaminated with *E. coli* O157:H7 (*Article V*). Leachate formation can be seen in the glass universals following the simulation of heavy rainfall.

Figure IX. Aliquots (35 ml) of lake, river, and trough water contaminated with *E. coli* O157:H7, purged with N_2 gas (anaerobic treatment) in the water aeration experiment (*Article VI*).

Figure X. Porth Dafarch beach sampling site for sand (*Article VII*).

Figure X. Traeth Llydan beach sampling site for sand (*Article VII*).

Figure XII. Porth Trecastell beach sampling site for sand (*Article VII*).

Figure XIII. Runoff water collection site (*Article VII*).

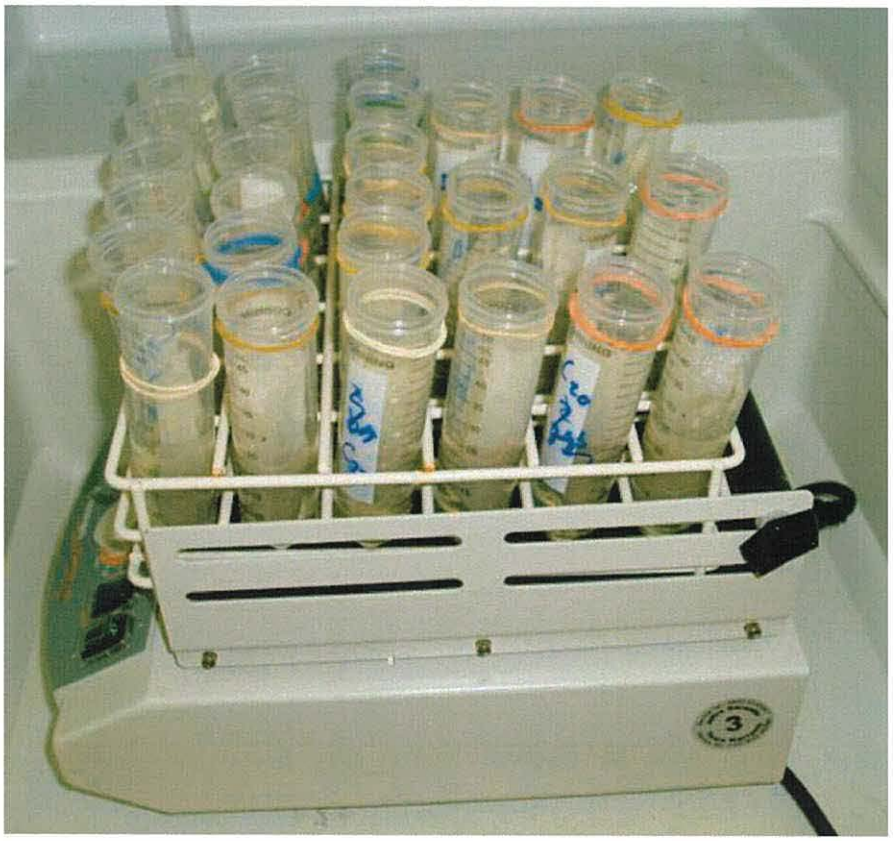

Figure XIV. Samples (10 g) of sands applied a water/slurry mix contaminated with *E. coli* O157:H7. Samples were placed in mesh bags, immersed in seawater, and shaken to simulate tidal movements (*Article VII*).