Bacteriophage of *Enterococcus* species for microbial source tracking

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Abstract

Contamination of surface waters with faeces may lead to increased public risk of human exposure to pathogens through drinking water supply, aquaculture, and recreational activities. Determining the source(s) of contamination is important for assessing the degree of risk to public health, and for selecting appropriate mitigation measures. Phage-based microbial source tracking (MST) techniques have been promoted as effective, simple and low-cost. The intestinal enterococci are a faecal "indicator of choice" in many parts of the world for determining water quality, and recently, phages capable of infecting *Enterococcus faecalis* have been proposed as a potential alternative indicator of human faecal contamination. The primary aim of this study was to evaluate critically the suitability and efficacy of phages infecting host strains of *Enterococcus* species as a low-cost tool for MST.

In total, 390 potential *Enterococcus* hosts were screened for their ability to detect phage in reference faecal samples. Development and implementation of a tiered screening approach allowed the initial large number of enterococcal hosts to be reduced rapidly to a smaller subgroup suitable for phage enumeration and MST. Twenty-nine hosts were further tested using additional faecal samples of human and non-human origin. Their specificity and sensitivity were found to vary, ranging from 44 to 100% and from 17 to 83%, respectively. Most notably, seven strains exhibited 100% specificity to cattle, human, or pig samples. Twenty phages infecting a human-specific host strain (MW47) were viewed by transmission electron microscopy (TEM) to determine their morphological diversity. The TEM examination revealed that all phages were members of the *Siphoviridae* and *Myoviridae* families. Pilot inactivation experiments using three phages (two members of the *Siphoviridae* family with differing capsid structures, and one myovirus), indicated that their survival did not vary significantly ($P > 0.05$).

The findings of this study offer an insight into host-phage interactions, specificity, sensitivity, and the suitability for MST application of phages infecting different *Enterococcus* strains. The high host specificity demonstrated by strains in this study suggests that they have a potential future role in MST. Although TEM revealed a range of phage morphologies capable of infecting MW47, the pilot inactivation study suggests that the phages have similar survival characteristics. These findings offer other scientists the opportunity to isolate effective enterococcal hosts for source tracking for a variety of scenarios in other parts of the world, and as such, this work supports the application of MST as a global tool for human health protection.

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Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed.....

Dated.....

Chapter One: Introduction

1.1 Water-related disease burden

Contamination of surface waters with faeces of human and non-human origin leads to increased risk of human exposure to pathogens through drinking water supply, aquaculture, and recreational activities. Unsafe water, inadequate sanitation, and insufficient hygiene are estimated to result in over two million deaths every year, amounting to 4% of all deaths globally, and 5.7% of the total disease burden in disability-adjusted life years (DALY) (Prüss *et al*., 2002). In this estimate, diarrhoeal diseases, including cholera, typhoid and dysentery, account for an estimated 1.5 million deaths per year, and children, predominantly those in less economically developed countries (LEDC), bear the largest share of this disease burden.

In recognition of the burden of waterborne disease, Goal Seven, of the eight Millennium Development Goals (MDG) agreed at the United Nations (UN) Millennium Summit in 2000, aims to reduce by 50% the population without sustainable access to 'improved' drinking water and basic sanitation by 2015. Although this is the only target specifically related to providing access to water and sanitation, it can be argued that all eight MDG relate to water quality in some way. Goal Four in particular has the target of reducing child mortality (under fives) by two thirds from 1990 to 2015. Many child deaths are directly caused by diarrhoea, or indirectly caused by malnutrition as a result of repeated diarrhoea caused predominantly by waterborne disease. A recent MDG report indicated that the world was likely to meet and surpass the drinking water target by 2015, but that this would still leave 1 in 10 people without sustainable access to safe drinking water (UN, 2011).

Despite advances in wastewater technology and access to treated drinking water, outbreaks of waterborne diseases are still reported in more economically developed countries (MEDC) (Rangel *et al*., 2005; Smith *et al*., 2006; Craun *et al*., 2010). In the UK during the last twenty years, *Cryptosporidium*, *Campylobacter* and *Giardia* spp. have been the enteric pathogens most frequently associated with waterborne disease outbreaks (Meinhardt *et al*., 1996; Smith *et al*., 2006).

Environmental factors affect the transmission of waterborne disease and statistically significant relationships have been found between rainfall and outbreaks of disease (Curriero *et al*., 2001; Nichols *et al*., 2009). In the US, Curriero *et al*. (2001) analysed 548 reported outbreaks (from 1948 to 1994), and found that 68% of these were preceded by heavy rainfall events. The outbreaks related to surface waters were found to have the strongest relationship with heavy rainfall events. Similarly, in the UK, Nichols *et al*. (2009) considered 89 outbreaks associated with the consumption of drinking water (between 1910 and 1999), and found that there was a significant association between cumulative rainfall (>40mm in the seven antecedent days) and disease outbreaks. Climate change predictions for the UK describe warmer, drier summer months, and warmer, potentially wetter winter months (Murphy *et al*., 2009). Climate change may also lead to an increase in extreme weather events, including heavy rainfall (Anon, 2011). Increases in rainfall during winter months, and increased frequency and intensity of extreme heavy rainfall events, could therefore potentially lead to increased incidence of waterborne disease outbreaks.

In 2009 the UK Environment Agency used the 2002 medium high UK climate impacts scenario (UKCIP02) to calculate the potential impact of population growth on water

availability in England and Wales. They determined that a predicted population increase in England and Wales of 20 million during the next 40 years could lead to a 15% reduction in available water (Office of National Statistics, 2008). Population growth is likely to increase pressures on water resources, through increases in wastewater discharges into surface waters, potentially decreasing water quality and limiting available drinking water abstraction sites. Predicted warmer, drier summers may further increase pressures on existing water resources as a result of increased water-usage and reduced dilution of contaminants.

Poor water quality also has economic consequences. Failures to meet microbial water quality standards can cause loss of revenue through the closures of beaches, shellfish harvesting sites, and the associated costs of increased morbidity and loss in working days. In California, the most conservative cost estimates equate to a loss of between $US 21 million and $US 51 million each year, as a result of human gastroenteritis contracted from swimming in faecally contaminated surface waters (Given, 2006). Ralston *et al*. (2011) employed a cost-of-illness model to calculate estimates of the economic impact of water-borne disease in coastal environments across the entire USA. Their findings suggested that marine-borne pathogens were responsible for an annual loss of $US 900 million. Losses resulting from seafood-borne disease and gastrointestinal illness from beach recreation were estimated to be $300 million in each case, and a loss of $30 million was estimated as a result of direct human exposure to *Vibrio* species.

1.2 Waterborne disease and its transmission

Pathogenic organisms present in human and non-human animal faeces may enter the aquatic environment by way of point and non-point diffuse sources. Point sources of faecal pollution, such as wastewater discharges, enter surface waters at distinct sites and are readily identifiable. Non-point diffuse faecal pollution, such as agricultural run-off and the faeces of wild animals, may derive from a combination of sources over a larger area and is therefore less easily identified. Water provides an effective vehicle of pathogen transmission, enabling pathogenic organisms to come into contact with greater numbers of the public via contaminated drinking water, recreational water, and food sources (Moe, 1997).

Bradley (1977) was the first to develop an environmental classification for water-related disease (Table 1.1). Within this classification, water-related diseases were divided into four main categories, waterborne, water-washed, water-based and water-related insect vectors.

Table 1.1 Bradley's environmental classification of water-related diseases

Category	Description
Waterborne	Diseases resulting from physical contact with microbially contaminated water or ingestion of contaminated water, or food items which have come into contact with contaminated water.
Water-washed	Diseases whose transmission is facilitated by insufficient quantities of water.
Water-based	Caused by pathogenic worms which must spend part of their life cycle within intermediate vertebrate or invertebrate hosts that reside in aquatic environments. Diseases are acquired by contact with contaminated water or inadvertent ingestion of infested intermediate host animals.
Water-related insect vectors	Diseases transmitted by insects that breed or spend part of their life cycle in water.

(Bradley, 1977)

In 1975 Feachem modified Bradley's classification by combining waterborne and water-washed diseases, with the exception of skin and eye infections, into one category designated the faeco-oral diseases. This was an important distinction, reflecting the nature of waterborne and water-washed disease transportation via faecal-oral transmission pathways. The main faecal-oral transmission pathways are illustrated in Figure 1.1.

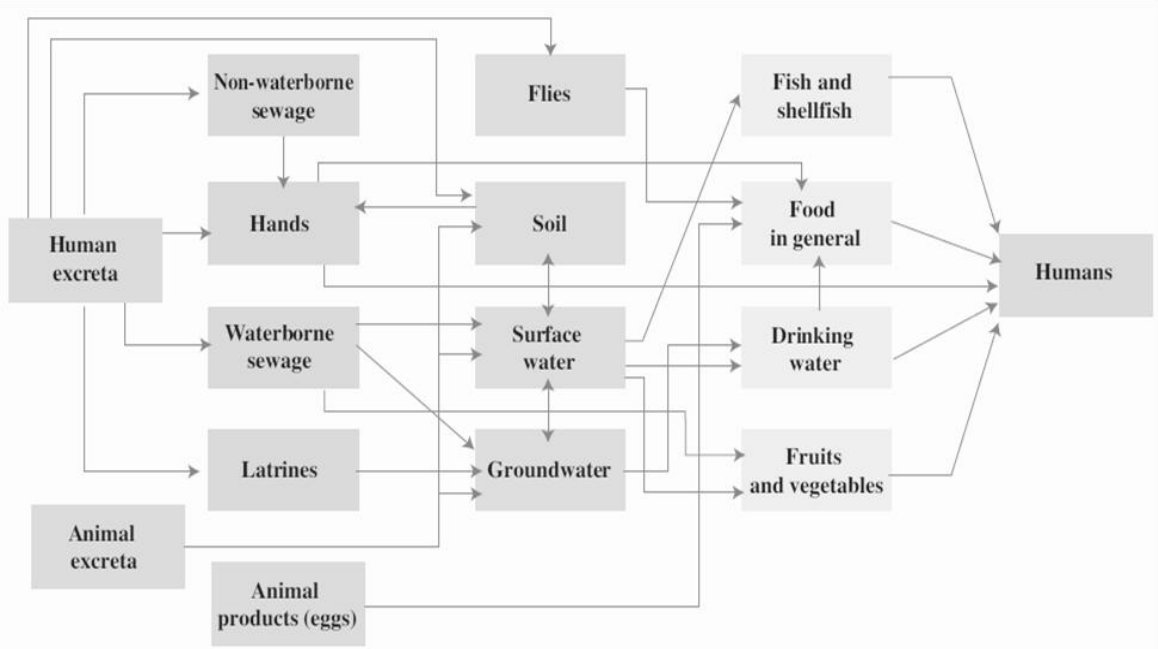

Figure 1.1 Transmission pathways of faecal-oral disease (Pruss *et al*., 2008).

1.2.1 Waterborne pathogens

Waterborne diseases are principally caused by three groups of organisms; bacteria, viruses and protozoa. The characteristics of major bacterial, viral and protozoal agents of waterborne disease are summarised in Table 1.2. Whilst associated severe diseases are also listed in Table 1.2, infection does not always result in disease. Disease refers to the symptomatic manifestation of an infection. Many pathogenic agents may be spread in low numbers producing asymptomatic infections (Leclerc *et al*., 2002). Although asymptomatic infections do not result in disease symptoms, infected persons may be sources of

continuous infection for the immediate population (Craun *et al*., 2006). The most common disease symptom of waterborne disease is diarrhoea. Global deaths from diarrhoea alone were calculated as 2.5 million by the World Health Organisation in 2008, of which 1.5 million can be attributed to waterborne disease (Prüss *et al*., 2002).

Table 1.2 Summary of the major bacterial, viral and protozoal agents of waterborne disease (Woodall, 2009)

Organism	Stability in water	Resistance to chlorine	Size (µm)	Associated disease
Bacterium				
Burkholderia pseudomallei	Can multiply	Low	1.0–4.0	Melioidosis (septicaemia)
Campylobacter jejuni, C. coli	Moderate	Low	1.5–4.0	Diarrhoea (frequently with blood in the faeces)
Escherichia coli (pathogenic and enterohaemorrhagic)	Moderate	Low	0.2–2.0	Diarrhoea (frequently with blood in the faeces); haemolytic uraemic syndrome
Legionella species	Can multiply	Low	0.5–3.0	Fatal pneumonia
Salmonella typhi	Moderate	Low	0.2–2.0	Typhoid fever
Shigella species	Short	Low	0.2–2.0	Bacillary dysentery
Vibrio cholera	Short	Low	1.0–3.0	Cholera
Yersinia enterocolitica	Long	Low	0.2–2.0	Diarrhoea (frequently with blood in the faeces)
Virus				
Adenovirus 40, 41	High	Moderate	0.07	Severe childhood gastroenteritis
Astroviruses	High	Moderate	0.03	Mild gastroenteritis
Enteroviruses	High	Moderate	0.03	Myalgia, meningitis, paralysis
Hepatitis A virus	High	Moderate	0.03	Hepatitis
Hepatitis E virus	High	Moderate	0.03	Hepatitis
Noroviruses	High	Moderate	0.03	Mild gastroenteritis
Sapoviruses	High	Moderate	0.03	Mild gastroenteritis
Rotaviruses	High	Moderate	0.06	Severe childhood gastroenteritis
Protozoan				
Cryptosporidium parvum	High	High	4.0–10.0	Cryptosporidiosis (diarrhoea)
Cyclospora cayetanensis	High	High	8.0–10.0	Cyclosporiasis (diarrhoea)
Entamoeba histolytica	Moderate	High	8.0–15.0	Amoebiasis (amoebic dysentery)
Giardia intestinalis	Moderate	High	7.0–12.0	Giardiasis (diarrhoea)
Toxoplasma gondii	High	High	2.0–70.0	Toxoplasmosis

The primary factors affecting the waterborne spread of infection by pathogenic organisms are: latency (the period after excretion when the pathogen is inactive or dormant); pathogen survival in the environment; the ability of the pathogen to multiply in the environment; and the dose required for infection to occur (infectivity) in a susceptible host (Leclerc *et al*., 2004). Different classes of waterborne pathogens have different transmission characteristics. Generally, viruses and protozoa have relatively low infectious doses (1 to 50 infectious units, plaque-forming units, cysts or oocysts), whereas bacterial pathogens require much larger infectious doses (in the range of 10^7 to 10^8 cells) to overcome the human immune response and cause infection (Moe, 1997; Mara and Feachem, 1999). The infectious dose will also vary according to the susceptibility of the host. Factors that have been observed to influence host susceptibility include immune status, behaviour, nutrition, age, occupation, education, income, and genetic traits (Craun *et al*., 2006).

Viruses are unable to multiply outside living cells in receiving waters, but generally have much greater resistance to natural inactivation and treatment than bacteria (Bonadonna *et al*., 2002[a]; Fujioka and Yoneyama, 2002; Leclerc *et al*., 2004). Some enteric bacterial pathogens are able to multiply outside the gut environment. For example, studies have shown that the bacterial pathogen *Legionella pneumophila* is able to multiply in free-living protozoa (Barbaree *et al*., 1986; Fields *et al*., 1989).

Waterborne pathogens can cause a variety of diseases and Table 1.2 lists the diseases associated with the major bacterial, viral and protozoal waterborne pathogens. However, the global burden of waterborne disease is difficult to quantify. Intermittent contamination of drinking water sources and recreational surface waters with pathogens (often in very low

concentrations), may lead to sporadic cases of illness and these cases may therefore not be recognised as waterborne outbreaks. It is particularly difficult to confirm the waterborne transmission of single disease cases, because pathogens may be transmitted by a number of pathways (Craun *et al*., 2006).

It is generally recognised that human faecal contamination of water poses a greater risk to human health, because it is more likely to contain human-specific pathogens that may cause infection (Fong and Lipp, 2005). However, faecal contamination from animals may also pose a risk to human health from zoonotic pathogens (organisms capable of causing disease in animal and human hosts), particularly in catchments where the ratio of grazing animals to humans is high (Sinton *et al*., 1998). Recent research has suggested that the risk to human health attributable to contamination by non-human faecal sources is varied and that the risk from fresh cattle and bird faeces in recreational waters is not substantially different than that from human faecal contamination (Colford *et al*., 2007; Soller *et al*., 2010). Contamination of water by non-human faeces poses a potential risk of human infection by zoonotic pathogens such as *Escherichia coli* (*E. coli*) O157, *Giardia* spp., *Campylobacter* spp., and *Cryptosporidium* spp. (Craun *et al*., 2004). A major concern is the emergence of zoonotic bacteria with new virulence factors, which may result in potent new pathogens (Till *et al*., 2004) and according to the WHO (2004), almost 75% of emerging waterborne pathogens may be of zoonotic origin.

1.3 Sources of faecal contamination in receiving waters

In many countries water pollution prevention has traditionally focused on the control of point sources of faecal contamination. Improvements to technology and the management of

wastewater treatment facilities have minimised faecal contributions from these sources in industrialised countries. Yet in some situations levels of faecal contamination have not been significantly reduced by these improvements (Connolly *et al*., 1999) and in a number of studies non-point diffuse sources of faecal pollution have been identified as dominant contributors to the problem of water pollution (Schnauder *et al*., 2007; Zhu *et al*., 2011). Importantly, the burden of water-borne disease is primarily felt in LEDC where uncontrolled point and non-point faecal pollution sources may be diverse and numerous. In many LEDC, the provision of potable water is often inadequate. Large proportions of the population may not be connected to water supplies let alone wastewater networks. Alternative water sources, such as springs, wells and streams, whose microbiological quality may be compromised by both point and diffuse sources of faecal pollution, therefore represent a significant public health concern in these areas (Dorice *et al*., 2010). However, most research into the contribution of different pollution sources and the survival of their microbial components derives from industrialised countries, which are often in temperate climates.

1.3.1 Point sources

Raw (untreated or partially treated) municipal wastewater, often comprised of both human and non-human faeces, commonly enters receiving waters through point sources. Where combined sewer systems are used, storm water and wastewater are collected in the same conduit. Combined sewer overflows (CSO) are flows that exceed the capacity of wastewater treatment works (WWTW) and sewerage systems and by-pass treatment, entering directly into receiving waters (Hammer and Hammer, 2008), constituting a significant source of faecal pollution.

Raw wastewater contains numerous pathogens and parasites and consequently poses a hazard to human health (Table 1.2). It is therefore important for wastewater to be treated prior to discharge into receiving waters. Physical, chemical and biological processes are involved in the treatment of wastewater. The degree of treatment is largely dependent on the type of receiving water and the stringency of wastewater standards, as set out in legislation (discussed further in section 1.5). Kay *et al*. (2008) obtained 1933 samples from 12 types of wastewater related discharge from the UK and Jersey. These included untreated sewage, primary, secondary and tertiary treated effluents. The results demonstrated a high variability in faecal bacteria concentrations in sewage related discharges. Faecal coliform concentrations ranged from <3 CFU/100ml^{-1} (tertiary-treated effluent) to 3.3 x 10^{9} CFU/100ml^{-1} (untreated sewage) and enterococci concentrations ranged from <3 CFU/100ml^{-1} (tertiary-treated effluent) to 4.8 x 10^{7} CFU/100ml^{-1} (secondary-treated effluent). Results suggested that there were statistically significant reductions in faecal bacteria concentrations following secondary and tertiary treatments. It is however important to note that the data should be regarded as 'indicative', because they were not based on comparisons of faecal bacteria concentrations in matched pairs of samples (Kay *et al*., 2008).

1.3.2 Diffuse sources

The composition of diffuse pollution can be highly variable. Diffuse pollution of water bodies may include contaminated urban run-off, leakage from septic tanks, and faeces from agricultural livestock, wild and domesticated animals. Many rural communities rely on septic systems, which store and partially treat wastewater onsite. Without regular and efficient maintenance these systems may fail and release faecal material (containing

pathogens) into the aquatic environment. The failure of septic systems is relatively common. Ahmed *et al*. (2005[a]) surveyed 48 septic systems in the Eudlo catchment, Australia, and found that 41 (85%) of the assessed systems were defective. In catchments where septic systems are common, they can be major sources of faecal contamination (Peed *et al*., 2011).

Agriculture and livestock farming can also be a significant contributor to faecal pollution loads in receiving waters. Diffuse agricultural inputs are associated with run-off from fields used for grazing, hard-standing areas, and sites where animals can access surface waters. In farms the presence of both stored and fresh faecal material therefore represents a source of microbiological contamination. Edwards *et al*. (2008) analysed the microbiological and chemical composition of roof and hard-standing run-off in four livestock farms and found variable but frequently high concentrations of numerous groups of contaminants (including faecal coliforms and intestinal enterococci). The results suggested that run-off arising from hard-standing areas represented a long-term source of contamination as faecal material is renewed frequently.

In many countries manure application onto land is common practice to increase nutrients and to promote the growth of crops. Application of manure reduces the costs associated with applying artificial fertilisers. Cattle manure (from fresh, stored and composted sources) has been shown to harbour high levels of faecal bacteria (10^5 to 10^8 CFU g^{-1}) and a diverse range of zoonotic pathogens (Klein *et al*., 2010). Pathogens such as *E. coli* O157:H7 may survive for long periods (over a year) in stored manure, emphasising its importance as a potential reservoir of zoonotic pathogens (Kudva *et al*., 1998). The

application of slurry (liquid form of manure) and manures also affects soil erosion processes. Ramos *et al*. (2006) noted that when manure was applied to soil surfaces, the quantity of material eroded during rainfall events decreased as the soil was protected, but run-off increased by up to 30%. They found the highest levels of microorganisms in run-off when rainfall occurred shortly after application.

Faecal material deposited directly onto land by grazing animals has been shown to be a significant source of microorganisms, including pathogens (Weaver *et al*., 2005; Donnison *et al*., 2008). Indeed, grazing areas of critical importance are those with direct connections to receiving waters (Heathwaite *et al*., 2005). Extensive research has been conducted into the survival of *E. coli* and intestinal enterococci in cattle pats (Meays *et al*., 2005; Sinton *et al*., 2007; Soupir *et al*., 2008; Texier *et al*., 2008; Muirhead *et al*., 2009; Oliver *et al*., 2010) and in New Zealand Sinton *et al*. (2007) demonstrated that, provided that pat water content remained above 80%, (7-30 days after deposition depending on the season and weather), there was growth of faecal bacteria (intestinal enterococci and *E. coli*). Results indicated that sunlight initially assisted replication of bacteria, enabling a crust to form, thus helping to retain moisture and providing optimum growth temperatures. Research has suggested that cattle pats may remain substantial sources of *E. coli* for at least 30 days and that concentrations in soil (grazed land) may remain elevated for up to six months after the removal of animals (Muirhead *et al*., 2005; Van Kessel *et al*., 2007; Muirhead *et al*., 2009).

Diffuse pollution from wildlife sources can be harder to ascertain and quantify, but it is recognised that large populations of birds can have a large impact on water quality (Wither *et al*., 2005; Muirhead *et al*., 2011). The faeces of gull (*Laridae*), geese (*Branta*

canadensis) and duck (*Anas platyrhinchos*) are reported to contain average faecal coliform concentrations of 3.68×10^8, 1.53×10^4 and 7.83×10^{10} per gramme, respectively (Alderisio and DeLuca, 1999; Obiri-Danso and Jones, 1999). Pathogenic bacteria have also been identified in the faeces of gulls and wildfowl, implying there may be a public health risk from these faecal sources (Samadpour *et al*., 2002; Albarnaz *et al*., 2007). Evidence of waterborne human disease from exposure to bird faeces is limited, but in 1999 *E. coli* O157:H7 (associated with a waterborne outbreak) was identified in duck faeces and water samples from Battle Ground Lake, Vancouver (Samadpour *et al*., 2002). However, it was not clear if resident ducks from the lake were the source of contamination or if they were transiently infected by water contaminated from other sources. In coastal areas of the UK, gulls (*Laridae*), pigeons (*Columbidae*) and starlings (*Sturnidae*) are all prominent, whilst wildfowl such as swans, ducks and geese (from the family *Anatidae*) are common in inland and transitional surface waters.

Numerous worldwide studies have shown wildlife, including deer, fish, muskrats, racoons, rats, turtles and voles to be important contributors of faecal pollution to receiving waters (Ram *et al*., 2007; Ruecker *et al*., 2007; Somarelli *et al*., 2007; Habersack *et al*., 2011; Viau *et al*., 2011). In urbanised catchments companion animals such as cats and dogs can contribute to diffuse faecal pollution (Dabritz *et al*., 2006; Ram *et al*., 2007). Results from a water quality modelling study in South Florida, looking into non-point sources of faecal bacteria at recreational marine beaches, demonstrated that dog faeces had a large transient impact on concentrations of faecal bacteria within a few hours of deposition, contributing to the high (>300 CFU/100ml) concentrations of faecal bacteria observed in this study (Zhu *et al*., 2011).

1.4 Monitoring microbiological water quality

The ultimate aim of monitoring the microbiological quality of water is to protect public health. Bench marking the results against standards set out in national, European or international legislation (section 1.5) allows an estimate of the risk to human health. Water quality is often assessed using faecal indicator organisms (FIO), such as total and faecal coliforms, *Clostridium perfringens, Escherichia coli,* and intestinal enterococci. They are an index of faecal pollution because they inhabit the gastrointestinal tracts of human and non-human animals and are present in high numbers in faeces. Ideally, pathogenic organisms of concern would be monitored directly. However, it is often prohibitively expensive and complex to do so, because pathogens may be present in low numbers, have non-homogenous distributions, be difficult to culture and be highly infectious at low doses. Ideal faecal indicators should be non-pathogenic, unable to reproduce in the environment, correlated with the presence of pathogens, be rapidly detected and easily enumerated and have a survival profile similar to the pathogen they indicate (Cimenti *et al*., 2007). In reality however, it is unlikely that any current or future FIO will satisfy all these criteria and compromises will need to be met.

Epidemiological studies have demonstrated the relationship between FIO and illnesses (Cabelli *et al*., 1979, 1982; Cheung *et al*., 1990; Corbett *et al*., 1993; Kay *et al*., 1994; Haile *et al*., 1999; Wyer *et al*., 1999; Lipp *et al*., 2001). Reported symptoms after exposure to contaminated bathing waters include respiratory illness, gastroenteritis, skin complaints, and eye, ear and nose infections. Research has shown that rates of these symptoms increase among swimmers compared with non-swimmers (Prüss, 1998). Some authors have argued that symptoms are often minor and can be the result of contact with water itself or

prolonged periods in the sun (Prüss, 1998, Turbow *et al*., 2008). However, even minor symptoms can have a large cumulative impact on economies (Fleisher *et al*., 1998).

The validity of using bacterial faecal indicators to predict the presence of pathogens has been questioned (Harwood *et al*., 2005). Research has consistently failed to demonstrate a strong correlation between current bacterial faecal indicator levels and the presence of viral and protozoan pathogens, largely because bacterial indicators are more susceptible to natural inactivation and treatment processes (Bonadonna *et al*., 2002[b]; Lipp *et al*., 2001). There are also concerns over the possible re-growth of bacterial indicators in environmental matrices (Ferguson and Signoretto, 2001). Alternative indicators of faecal pollution have therefore been investigated (Savichtcheva and Okabe, 2006). Even though internationally there are concerns over the interpretation of faecal indicator bacteria data, legislation currently prioritises the monitoring and reduction of *E.coli* and intestinal enterococci.

1.5 European water quality legislation

In order to protect public health in Europe there are a number of EU Directives in place to monitor and regulate water quality. Important Directives include the Water Framework Directive (2000/60/EC), Bathing Water Directive (76/160/EEC) and revised Directive (2006/7/EC), the Urban Waste Water Treatment Directive (91/271/EEC), Freshwater Fish Directive (2006/44/EC), Shellfish Waters Directive (2006/113/EC), Groundwater Directive (2006/118/EC) Nitrates Directive (91/676/EEC), Dangerous Substances Directive (2006/11/EC) and the Drinking Water Directive (98/83/EC) (Commission of the European Union (CEU), 1976, 1991 [a, b], 1998, 2000, 2006 [a, b, c, d, e]).

1.5.1 The EU Bathing Water Directive

During the 1970s concern about environmental degradation and the health risks of bathing led to calls for water quality to be monitored and tested in order to protect bathers and the environment. In 1976, one of the first pieces of European environmental legislation was created in the form of the Council Directive 76/160/EEC on Bathing Water Quality (CEU, 1976). The Directive required Member States to identify popular bathing waters and to monitor these throughout the bathing season. The Bathing Water Directive set standards for different microbiological and physico-chemical parameters. Water quality had to comply with 'mandatory' standards and the achievement of the higher 'guideline' standards was encouraged (CEU, 1976). Mandatory standards were provided for ten parameters, namely total coliforms, faecal coliforms, salmonella, enteroviruses, pH level, colour, mineral oils, surface active substances (detergents), phenols and transparency (CEU, 1976). The Commission of the European Communities set mandatory standards for total and faecal coliforms (Tables 1.3 and 1.4), and three physico-chemical parameters (surface active substances, mineral oils and phenols) to determine compliance of bathing waters.

Table 1.3 EU mandatory standards for bathing waters (CEU, 1976)

	CFU/100ml	**Percentage of samples**
Total coliforms	10,000	95%
Faecal coliforms	2,000	95%

CFU= Colony Forming Units

Table 1.4 EU guideline standards for bathing waters (CEU, 1976)

	CFU/100ml	**Percentage of samples**
Total Coliforms	500	80%
Faecal coliforms	100	80%
Faecal streptococci	100	90%

CFU= Colony Forming Units

It was widely accepted that the Bathing Water Directive (76/160/EEC) required revision to take into account advances in science and knowledge regarding the risks associated with bathing. Changes in environmental protection offered in more recent EU legislation, such as the Water Framework Directive, also highlighted the need for an updated and simplified Bathing Water Directive (76/160/EEC) (CEU, 2000). The revised Bathing Water Directive (2006/7/EC) came into force on the 24th of March 2006. The revised Bathing Water Directive (2006/7/EC) focuses on the protection of public health at bathing water sites, but not all waters used for recreational activity are designated bathing waters under this piece of legislation. Some recreational waters are used all year round for a number of activities, including surfing, kayaking, canoeing, diving, wind surfing, kite surfing and sailing. These recreational pursuits can result in considerable contact with water, and may be undertaken hundreds of metres from the coast. Sports such as windsurfing are permitted on waters that have been judged unsafe for swimming, although research suggests these activities pose the same or greater level of risk to human health (Dewailly *et al*., 1986; Turbow *et al*., 2008). Water sports such as surfing and diving that involve full-body immersion have become more popular in recent years and wet/dry suits allow these sports to continue all year round in temperate climates (Bradley and Hancock, 2003). This may be a cause for concern as water quality monitoring is restricted to the identified bathing season and near-shore areas.

The revised Directive has simplified the way in which water quality is measured by focusing on fewer microbiological indicators, namely intestinal enterococci and *Escherichia coli*. From 2012, bathing waters will be classified as ‘excellent’, ‘good’, ‘sufficient’ or ‘poor’. Tables 1.5 and 1.6 show the revised EU standards for inland, coastal and transitional waters.

Table 1.5 EU standard values for intestinal enterococci and *E.coli* in inland waters (CEU, 2006)

Parameter	Excellent quality	Good quality	Sufficient
Intestinal enterococci (cfu/100ml)	200 (*)	400 (*)	330 (**)
Escherichia coli (cfu/100ml)	500 (*)	1000 (*)	900 (**)

(*) Based upon a 95-percentile evaluation
(**) Based upon a 90-percentile evaluation

Table 1.6 EU standard values for intestinal enterococci and *E.coli* in coastal and transitional waters (CEU, 2006)

Parameter	Excellent quality	Good quality	Sufficient
Intestinal enterococci (cfu/100ml)	100 (*)	200 (*)	185 (**)
Escherichia coli (cfu/100ml)	250 (*)	500 (*)	500 (**)

(*) Based upon a 95-percentile evaluation
(**) Based upon a 90-percentile evaluation

The selection of faecal indicator organisms (*E. coli* and intestinal enterococci) for use in the revised EU Bathing Water Directive (2006/7/EC) was influenced by a World Health Organization review of epidemiological evidence (Prüss, 1998). The review conducted by Prüss (1998) suggested that intestinal enterococci were the most appropriate currently available indicator of health risk in fresh and marine waters, whereas *E. coli* concentrations were predictive of illness in freshwater. Standard concentrations of *E.coli* and intestinal enterococci set out in the Directive are based on studies demonstrating dose-response relationships relating faecal indicator concentrations to rates of illness (Kay *et al*., 1994; Fleisher *et al*., 1998).

1.5.2 Water Framework Directive

The Water Framework Directive (WFD) is a major piece of European legislation, which came into force in December 2000 and was transposed into UK law in 2003 (CEU, 2000).

The WFD and its related Directives repeal twelve Directives that had previously created a fragmented regulatory system, consequently streamlining and improving current regulation. The Directive's purpose is to establish a framework for the protection of inland surface waters, transitional waters, coastal waters and groundwater, with the aim of preventing further deterioration, whilst protecting and enhancing the status of aquatic ecosystems, wetlands and the water needs of terrestrial ecosystems (CEU, 2000). The Directive also aims to promote the sustainable use of water, ensuring the continual reduction of pollution and prevention of pollution of groundwater.

The WFD sets objectives that aim to ensure that all waters meet a 'good status' by 2015. The Directive ensures that there is active participation in water management activities from all stakeholders, including non-governmental organisations (NGO) and local communities. The WFD sets objectives for ecological and chemical aspects of water quality. There are no direct objectives related to levels of FIO and waterborne pathogens, but the Directive does aim to reduce and control pollution from all sources, not just from point sources. The absence of microbial objectives is a potential shortcoming of the Directive, because improvements in ecological and chemical status may not automatically lead to reductions in levels of microbial pathogens. A key feature of the WFD is the introduction of River Basin Districts. Member States are required to identify river basins within their territory and assign them to districts. River basin districts are not restricted to territorial borders and follow the natural geographical and hydrological units of river basins. If basins cross member state borders then they are assigned to an international river basin district. For every district a River Basin Management Plan is required (CEU, 2000).

1.6 Microbial source tracking

As mentioned previously, FIO are currently used to assess the hygienic quality of water and are present in both the faeces of human and non-human animals. Their detection therefore offers no insight into the potential source(s) of contamination. Distinguishing the source(s) of contamination can potentially support the achievement of water quality standards set out in legislation through the selection, implementation and evaluation of appropriate mitigation measures, and can also help in assigning legal responsibility for such measures.

Recent international legislation in the form of the WFD (EU) and the Clean Water Act (USA) require the identification and management of point and diffuse sources of microbial pollution that lead to 'non-compliance' (EU) and 'impairment' (USA) in surface waters (CEU, 2000; USEPA, 2002). The WFD requires the establishment of a 'programme of measures' for identified noncompliant river basin districts and in the USA, impaired waters are investigated and actions set out by the principal of 'total maximum daily loads' (TDML). Determining the source(s) of faecal pollution can also give valuable information regarding the potential risk to human health as it is acknowledged that risks may vary according to animal host sources (Soller *et al*., 2010). MST can therefore support risk assessments, providing information on which reference pathogens to look for in samples.

Legislative requirements, the need to predict risk to human health accurately, and the implications of source on the selection of remediation measures, have led to the recent development of the field of microbial source tracking (MST). The field of MST encompasses a broad range of techniques that aim to distinguish source(s) of faecal contamination in surface waters utilising microbial populations with specificity to particular

animal hosts. MST is a rapidly advancing field but there is currently no single standardised method available to distinguish sources of faecal contamination in surface waters in all situations (Meays *et al*., 2004; Domingo *et al*., 2007). Many researchers have concluded that no single technique will be able to satisfy all scenarios and instead a 'toolbox' approach using multiple methods in conjunction is frequently recommended (Vogel *et al*., 2007; Plummer *et al*., 2009; Gourmelon *et al*., 2010[a]). MST methods should be chosen to suit the particular catchment scenario (problem-orientated), time and financial constraints of the region concerned.

MST methods may be either 'library-dependent' or 'library-independent' and can be further classified as either 'culture-dependent' or 'culture–independent'. Culture-dependent methods require the growth of particular microorganisms from water samples and library-dependent methods (LDM) require the construction of a library of bacterial isolate characteristics from known sources. Environmental isolates of unknown origin can then be compared to those from the library to determine the source(s) of faecal pollution. Libraries can contain phenotypic or genotypic bacterial characteristics (profiles), and the success of these methods relies heavily on the construction of a representative library (Field and Samadpour, 2007). Sufficient library size and diversity depends largely on the technique and organisms used. The two fundamental problems when using *E. coli* and enterococci in LDM are their high diversity and limited host adaptation. Cosmopolitan strains (strains that reside in more than one host type) hinder the identification of faecal sources and can cause inaccurate source identifications (Harwood, 2007). Phenotypic libraries tend to be much larger than those constructed for genotypic methods (Albert *et al*., 2003; Scott *et al*., 2003; Moore *et al*., 2005; Sayah *et al*., 2005). Small, unrepresentative libraries can result in

misclassifications (Olivas and Faulkner, 2008) and the development of comprehensive libraries, even with relatively low-cost resources is expensive and time-consuming. Importantly, the geographical stability of libraries (applicability of libraries across different geographical regions) has been shown to vary (Ebdon and Taylor, 2006; Ahmed and Katouli, 2008). Consequently separate libraries are recommended for larger geographical regions (Wiggins, 2003; Ebdon and Taylor, 2006). Many authors have also found temporal instability in genotypic and phenotypic characteristics when using *E. coli* and enterococci (Molina, 2005; Hansen *et al*., 2009) and this necessitates regular library updates to account for these changes. Additionally, the choice of statistical methods used to classify isolates in LDM has been shown to influence results (Hassan *et al*., 2005; Lasalde *et al*., 2005).

Because of the limitations of LDM, in recent years research into new MST tools has increasingly moved towards library-independent methods (LIM). These methods are either based on the cultivation of source-specific bacteria, or on the detection of source-specific genetic markers. LIM markers require validation against a range of faecal sources to ensure their specificity, but as the name suggests, they do not require libraries or databases of isolates from known sources. With culture independent LIM, multiple assays with multiple microbial targets may be performed with a single DNA extract relatively quickly (within a few hours). However, the major limitations of LIM relate to targeting a single gene, as the target may be present in the sample at low numbers (Stewart *et al*., 2007).

Cultivation-independent, library-independent PCR methods have emerged in recent years. They are often rapid, sensitive and can be less expensive than library-dependent comparisons. These techniques are based on the detection of molecular markers. Molecular

approaches can be used to detect markers that are difficult or impossible to culture, as is the case with many anaerobic bacteria present in the intestinal gut of humans and non-human animals. The majority of library-independent molecular methods target the 16S rRNA gene in sequences obtained from metagenomic fragments. Methods usually involve enrichment of target genes, extraction of nucleic acids, and then the amplification of target genes by PCR and quantitative PCR (qPCR) (Wuertz *et al*., 2011). There are two main differences between conventional PCR and real-time qPCR: 1) in qPCR the amplified target is quantified using a fluorescent reporter rather than traditional use of gel electrophoresis; and 2) in qPCR the amplified target is measured during each PCR cycle (McPherson and Møller, 2006).

The main advantage of using qPCR over conventional PCR is the ability to directly determine the concentration of a target gene. Whilst the quantification of faecal source contributions cannot be accomplished with qPCR-based MST data alone, recent studies have shown that it is possible to quantify faecal source contributions by relating MST results to other measures of water quality, including the level of total faecal pollution (Reischer *et al*., 2008; 2011). A probabilistic model has also been developed recently by Wang *et al*. (2010) that aims to account for the uncertainties with qPCR measurements, making quantitative MST feasible by connecting measured concentrations of host-specific faecal markers with a statistical model. There is concern that qPCR methods may overestimate concentrations because they are not capable of differentiating between viable and dead or dying cells (Byappanahalli *et al*., 2010; Noble *et al*., 2010). However, qPCR methods that can distinguish dead cells from viable cells have been developed in an attempt to determine recent pollution events (Bae and Wuertz, 2009). Recent advances in PCR

technology also mean it is now possible to produce PCR results for thousands of samples in a single day (Harwood *et al*., 2011).

In addition to MST markers, chemical tracers have been investigated as faecal source indicators. Caffeine, faecal sterols, pharmaceutical and personal care products, bile acids, laundry brighteners, surfactants, fragrances, pesticides, and polycyclic aromatic hydrocarbons have all been used to differentiate human from non-human faecal pollution in surface waters (Field and Samadpour, 2007).

MST has developed rapidly during the past fifteen years and the available literature is extensive. The following Tables (1.7-1.10) therefore give an overview of the main techniques developed for MST in recent years. Descriptions of MST techniques, including their advantages and disadvantages, are divided according to the following classification: library-dependent, phenotypic methods (Table 1.7), library-dependent, genotypic methods (Table 1.8), library-independent, culture-dependent methods (Table 1.9), and library-independent, culture-independent methods (Table 1.10). Those MST methods with direct relevance to the programme of research presented here are discussed in greater detail in Chapter Two.

Table 1.7 Library-dependent, phenotypic MST methods

Method	Brief outline	Advantages	Disadvantages	Investigators
Antibiotic resistance	Strains of faecal bacteria (*E.coli* and intestinal enterococci) can become resistant when exposed to antibiotics for prolonged periods of time. Antibiotic resistance profiles can therefore be developed by testing the resistance of faecal bacteria to a selection of antibiotics. A library of known resistance profiles from potential sources may be created and used to identify the source of unknown isolates.	High average rates of correct classification (ARCC) have been reported (≥95% for human vs. non-human classification).	The method requires a library of antibiotic resistance patterns from isolates of known origin. These libraries have been shown to temporally and geographically unstable. There is also evidence that resistance genes may transfer between bacteria.	Wiggins, 1996; Hagedorn *et al*., 1999; Wiggins *et al*., 1999; Harwood *et al*., 2000; Whitlock *et al*., 2002; Wiggins *et al*., 2003; Harwood *et al*., 2003; Ebdon *et al*., 2004; Carroll *et al*., 2005; Ebdon and Taylor, 2006; Vantarakis *et al*., 2006; Graves *et al*., 2007
Carbon-source utilization	Carbon-source (CSU) utilization profiling is a phenotypic MST method in which microplate systems containing substrates for bacterial growth are used to produce substrate utilization patterns of isolates of faecal indicator bacteria. Substrate utilisation patterns are then compared to a known source library.	Microplate systems are commercially available (Biolog and PhPlate). CSU is a relatively simple and rapid MST technique that can be used with large numbers of isolates. The commercially available Biolog system has been reported to achieve ARCC as high as 92.7% for a human vs. non-human classification.	In MST comparison studies CSU has performed poorly, In one such study CSU was only able to identify the dominant source of faecal pollution correctly in 50% of samples.	Parveen *et al*., 2001; Griffith *et al*., 2003; Hagedorn *et al*., 2003; Wallis *et al*., 2003; Ahmed *et al*., 2005[b]; Blanch *et al*., 2006; Ahmed and Katouli, 2008
Fatty acid methyl ester profiling	Fatty acid methyl ester (FAME) profiling is phenotypic MST method. Bacteria have distinct FAME profiles, which have been used to distinguish faecal sources in water.	Studies have shown that FAME profiles of faecal indicator bacteria have both quantitative and qualitative host species distributions. They present evidence that FAME profiles have statistically significant host specificity and are therefore useful in determining sources of faecal pollution.	Although initial results have been very positive, more recent studies have found that FAME profiling achieves much lower ARCC when classifying isolates into individual host groups (≤ 66%).	Parveen *et al*., 2001; Genthner *et al*., 2005; Haznedaroglu *et al*., 2005; Duran *et al*., 2006; Seurinck *et al*., 2006; Haznedaroglu *et al*., 2007; Duran *et al*., 2009

Table 1.8 Library-dependent, genotypic MST methods

Method	Brief outline	Advantages	Disadvantages	Investigators
Ribotyping	Ribotyping refers to methods that create a banding pattern from 16S ribosomal DNA extracted from bacterial isolates. The banding pattern is used to distinguish between isolates from different faecal sources.	Ribotyping has excellent reproducibility and discriminatory power. Studies have reported high ARCC (≥ 95%) when distinguishing between human and non-human faecal sources.	The method is slow, expensive and labour intensive. Studies have shown geographic and temporal instability of libraries. Cosmopolitan subspecies can result in misclassifications of isolates.	Parveen *et al*., 1999; Carson *et al*., 2001; Hartel *et al*., 2002; Jenkins *et al*., 2003; Scott *et al*., 2003; Scott *et al*., 2004; Kelsey, 2008
Repetitive element-based polymerase chain reaction	In repetitive element-based polymerase chain reaction (rep-PCR), palindromic sequences occurring in multiple copies within bacterial genomes are amplified. Four types of rep-PCR have been applied in MST studies; repetitive extragenic palindromic PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR), PCR with extragenic repeating elements (BOX-PCR), and the polytrinucleotide sequences $(GTG)_5$.	High rates of ARCC (≥96%) have been demonstrated, particularly with rep-PCR (BOX A1R primers) which has been shown to produce significantly superior ARCC to ribotyping. Less expensive than other genotypic methods.	Whilst rep-PCR methods are less technically demanding than other genotypic LDM, traditional PCR methods are dependent on cultivation and the construction of a known host source library. As is the case with other LDM, geographical and temporal instability is a major limitation of rep-PCR.	Dombek *et al*., 2000; Carson *et al*., 2003; McLellan *et al*., 2003; Myoda *et al*. 2003; Johnson *et al*., 2004; Leung *et al*., 2004; Seurinck *et al*., 2005; Mohapatra *et al*., 2007
Pulsed-Field Gel Electrophoresis	In pulsed-field gel electrophoresis (PFGE) rare-cutting restriction enzymes are used on the whole DNA genome. Genomic fragments are then separated by alternatively pulsed, perpendicularly orientated electrical fields. This is followed by electrophoretic analysis to produce banding patterns. Banding patterns are compared to a known source library in order to determine the host source.	The technique has been widely used in clinical microbiology and the DNA fingerprints created are considered to be the 'gold standard' in this field. PFGE is a highly sensitive and discriminatory method.	Due to the high sensitivity of the method, an extensive library is often required. Consequently the method can be labour-intensive and expensive. PFGE also requires a series of time-consuming steps and therefore may not be appropriate for complex studies where larger known host source libraries are required.	Tynkkynen *et al*., 1999; Hager *et al*., 2001; Simpson *et al*., 2002; Myoda *et al*., 2003; Lu *et al*., 2004; Meays *et al*., 2004

Table 1.8 Library-dependent, genotypic MST methods (continued)

Method	Brief outline	Advantages	Disadvantages	Investigators
Amplified Fragment Length Polymorphism	Amplified fragment length polymorphism involves the selective PCR amplification of restriction fragments from a total digest of genome DNA.	The method has good reproducibility, resolution and sensitivity. Studies using AFLP have reported high ARCC for *E. coli* isolates from human and non-human sources (90-98%).	AFLP shares the drawbacks of many DNA fingerprinting methods, as it requires highly skilled labour, is time-consuming and relatively expensive.	Vos *et al*., 1995; Gaun *et al*., 2002; Leung *et al*., 2004
Random amplified polymorphic DNA analysis	In Random amplified polymorphic DNA (RAPD) analysis, DNA fragments from PCR amplification of random segments of genomic DNA are used to create fingerprints. RAPD has not been used extensively in MST.	The method has relatively high specificity and sensitivity. RAPD has been shown to have higher discriminatory ability than ribotyping when subtyping *E.coli.*	The main limitation of the method is the random relationship between the primers and target sites, making the method sensitive to alterations in reaction conditions.	Olive and Bean, 1999; Vogel *et al*., 2000; Ting *et al*., 2003; Venieri *et al*., 2004
Denaturing Gradient Gel Electrophoresis	Denaturing gradient gel electrophoresis (DGGE) produces 'fingerprints' by separating closely related PCR generated DNA fragments, based on their different DNA sequences. The band created on the gel, results from a specific sequence of a gene.	The method has relatively high sensitivity. Unique distributions have been found in human and non-human animal *E. coli* isolates. Species-specific community fingerprints can be generated.	The technique has been described as being comparable to antibiotic resistance analysis and analysis of repetitive DNA fingerprints, but has lower reported ARCC then ribotyping and AFLP, by which rates of >90% have been observed.	Farnleitner *et al*., 2000; Buchan *et al*., 2001; Chee-Sanford *et al*., 2001; Madigan *et al*., 2003; Sigler and Pasutti, 2006; D'Elia *et al*., 2007
Matrix-assisted laser desorption/ ionization time of flight mass spectroscopy	Matrix-assisted laser desorption/ ionization time of flight mass spectroscopy (MALDI-TOF-MS) is a soft ionization technique that enables spectral fingerprints to be produced rapidly. The method has been applied in very few MST studies.	A high level of correct classification was achieved when assigning *E. coli* isolates to human, bovine, canine and avian source groups (73%)	Lower reproducibility in comparison to techniques such as rep-PCR. Reproducibility can be increased by treating samples with lysozyme for 20 hours, but this makes the method time-consuming.	Siegrist *et al*., 2007; Giebel *et al*., 2008

Table 1.9 Library-independent, culture-dependent MST methods

Method	Brief outline	Advantages	Disadvantages	Investigators
Detection of *Bifidobacterium* species	*Bifidobacterium* spp. are anaerobic bacteria that are found in high numbers in the human intestine and have been investigated as possible indicators of human faecal pollution.	Proposed as a robust method for differentiating between human and animal faeces. Where sorbitol-fermenting bifidobacteria have been found in animal faeces, they have been isolated in different frequencies for different animals. Library-independent.	Rapid die-off of the bacterium at higher temperatures limits the use of bifidobacteria in summer months in temperate climates and in tropical and subtropical climates.	Mara and Oragui, 1983; Gavini *et al*., 1991; Jagals and Grabow, 1996; Rhodes and Kator, 1999; Bonjoch *et al*., 2005; Blanch *et al*., 2006; Bonjoch *et al*., 2009; Ottoson *et al*., 2009; Mushi *et al*., 2010
Detection of *Clostridium perfringens*	*Clostridium perfringens* is a species of anaerobic, spore-forming bacteria. The species has been proposed as a conservative marker of anthropogenic faecal pollution because it has been found in human, livestock, and carnivore animal faeces but has been rarely detected in herbivore wildlife faeces	They have been proposed as an alternative indicator of faecal pollution in tropical waters because it is unlikely to multiply in the environment. Library-independent.	The species has high resistance to natural inactivation and wastewater treatment. It may, therefore persist for long periods in the environment. Its detection might not always represent recent contamination.	Bisson and Cabelli, 1980; Roll and Fujioka, 1997; Byamukama *et al*., 2005; Cimenti *et al*., 2005; Farnleitner *et al*., 2010
Detection of *Rhodococcus coprophilus*	*Rhodococcus coprophilus* is a species of an aerobic actinomycete. The bacterium has been proposed as an indicator of non-human faecal pollution.	The bacterium is highly restricted to non-human animal faecal pollution. Library-independent.	The method is limited by the need for a lengthy incubation period (18 days). *Rhodococcus coprophilus* is able to persist for long periods in the environment (120 days) and therefore its presence may not indicate recent faecal contamination	Rowbotham and Cross, 1977; Mara and Oragui, 1981; Oragui and Mara, 1983; Jagals *et al*., 1995
Bacteriophage methods	The assessment of sewage pollution of sea water by analysis of bacteriophages infecting *E.coli* was first proposed by Kott (1966). Since then a number of phage detection methods have been developed for a variety of bacterial host strains. Bacteriophage methods are discussed in greater detail in Chapter Two (section 2.3).			

Table 1.10 Library-independent, culture-independent MST methods

Method	Brief outline	Advantages	Disadvantages	Investigators
Host-specific 16S rRNA gene markers	Host-specific 16S rRNA gene markers have been used to differentiate a range of faecal source groups including human, herbivore, ruminant, bovine, cattle, pig, elk, horse, gull, and dog.	*Bacteroidales* markers appear to be geographically stable, having been used in many geographical regions including the US, Canada, Europe, Hawaii, Japan, and New Zealand. Correlations have been found between *Bacteroidales* markers and faecal indicator organisms. Library-independent.	A limitation of targeting host specific 16S rRNA gene markers is the possible horizontal transfer of faecal bacteria among animal species in close contact. This may affect the specificity of the method and its ability to discriminate between sources.	Bernhard and Field, 2000; Savill *et al*., 2001; Gilpin *et al*., 2003; Simpson *et al*., 2004; Dick *et al*., 2005; Seurinck *et al*., 2005; Betancourt and Fujioka, 2006; Layton *et al*., 2006; Reischer *et al*., 2006, 2007; Sosiak and Dixon, 2006; Kildare *et al*., 2007; Okabe *et al*., 2007; Lu *et al*., 2008; Mieszkin *et al*., 2009; Zheng *et al*., 2009
Toxin/ virulence gene markers	Among *Enterococcus faecium* the enterococcal surface protein (*esp*) gene (a putative virulence factor) has been targeted, as a possible indicator of human faecal pollution. Assays have also been developed for *E.coli* toxin genes showing specificity to cattle, human, rabbit and bird faeces.	The methods are rapid in comparison to LDM. *E.coli* toxin genes appear to be highly host specific. Library-independent.	The Scott *et al*. (2005) *esp* gene method is only partly library-independent. Inconsistency of detection of the *esp* gene in human faecal samples. Detection of the gene in non-human animal faeces. The prevalence of *E.coli* toxin genes is low. Horizontal gene transfer may affect the specificity of these markers.	Ochman *et al*., 2000; Khatib *et al*., 2002, 2003; Field *et al*., 2003; Chern *et al*., 2004; Harada *et al*., 2005; Scott *et al*., 2005; Jiang *et al*., 2007; Ahmed *et al*., 2008; Byappanahalli *et al*., 2008; Layton *et al*., 2009; Balleste *et al*., 2010
Viral markers	Human-specific viruses proposed as MST markers include: enteroviruses, adenoviruses, noroviruses and polyomaviruses. Bovine-specific adenoviruses and enteroviruses, and porcine- and ovine-specific adenoviruses have also been proposed as markers of non-human faecal pollution	Highly host-specific. Directly related to human health risk and disease. Can be monitored directly without the need for culturing.	Viral pathogens are present in low numbers in environmental waters. Viral pathogens are likely to be highly infectious at low doses. Traditionally time-consuming.	Jiang *et al*., 2001; De Motes *et al*., 2004; Fong *et al*., 2005; Fong and Lipp, 2005; He and Jiang, 2005; Xagoraraki *et al*., 2007; Ahmed *et al*., 2010; Tong *et al*., 2011

Chapter Two: Introduction to the ecology of the genus *Enterococcus* and its phages

2.1 The genus *Enterococcus*

2.1.1 Historical background

The term “entérocoque” was first coined by Thiercelin in 1899, when he used it to describe a newly isolated Gram-positive diplococcus of intestinal origin. The genus *Enterococcus* was later officially named by Thiercelin and Jouhaud (1903). In 1906 Andrewes and Horder named an organism, which had been isolated from a patient with endocarditis, *Streptococcus faecalis.* A second organism within this genus was later described as *Streptococcus faecium* (Orla-Jensen, 1919). A review by Sherman (1937) proposed a classification scheme that divided streptococci into four separate divisions: pyogenic, viridans, lactic, and enterococcus. Organisms within the enterococcus division were those that grew at 10 and 45^{o}C, in 6.5% sodium chloride (NaCl), and at pH 9.6, and which survived at 60^{o}C for 30 minutes (Sherman, 1937). In 1970, the establishment of a genus for the enterococcal streptococci was proposed, with *S. faecalis* and *S. faecium* and the subspecies of these two taxons being classified as *Enterococcus* (Kalina, 1970). However, the use of the genus name *Streptococcus* continued until 1984, when DNA-DNA and DNA-RNA hybridisation studies demonstrated that *Streptococcus faecalis* and *Streptococcus faecium* were sufficiently different from non-enterococcal streptococci (*Streptococcus bovis* and *Streptococcus equinus*), to warrant their transfer to a separate genus, *Enterococcus* (Schleifer and Kilpper-Balz, 1984).

2.1.2 Identification and taxonomy of enterococcal species

A large variety of phenotypic and genotypic methods exist for the identification of enterococcal species. Phenotypic typing methods that have been applied to differentiate these organisms include bio-typing (Devriese *et al*., 1994, 1995, 1996; Manafi, 1996; Kühn *et al*., 1995; Manero and Blanch, 1999; Reed *et al*., 1999; Day *et al*., 2001) protein fingerprinting by standardised sodium dodecyl sulfate polyacrylamide gel electrophoresis (Andrighetto *et al*., 2001), multilocus enzyme electrophoresis (Tsakalidou *et al*., 1993), antimicrobial susceptibility testing (Willey *et al*., 1999), serotyping (Petts, 1995), long-chain fatty acid analysis (Tyrrell *et al*., 2002), fatty acid methyl esters (FAME) analysis (Lang *et al*., 2001), enterocin typing (Pompei *et al*., 1992), pyrolysis mass spectrometry (Morrison *et al*., 1999), vibrational spectroscopic methods (Kirschner *et al*., 2001) and proton magnetic resonance spectroscopy (Bourne *et al*., 2001).

Bio-typing is considered the traditional method for identifying *Enterococcus* species. Identification relies on results from an array of biochemical tests detecting carbohydrate fermentation and enzyme activity, demonstrated by a change in colour of indicator dyes. Identification using biochemical tests can be complicated as it requires a large number of tests, making routine application difficult. Manero and Blanch (1999) developed a six step biochemical key based on biochemical data from the analysis of over 1,600 isolates. The key identifies 19 *Enterococcus* species, including all of the most commonly isolated species. Twelve tests are presented, but only six are required for the identification of a single isolate. The key has an identification threshold of 99%, with only a few exceptions (87, 91.5, and 97% for *E. avium*, *E. hirae*, and *E. durans*, respectively). The system may be considered practicable, reliable and easy to perform, producing rapid results.

Commercial test kits have also been designed to simplify and reduce the time-consuming nature of bio-typing methods. The most commonly used test kits have included API 20 Strep (BioMérieux, UK), Rapid ID32 Strep (BioMérieux, UK), API 50 CH (BioMérieux, UK), *Api zym* (BioMérieux, UK), the PhenePlate PhP plate system (PhPlate Microplate Techniques, Sweden), and the automated system Vitek 2 (BioMérieux, UK). The commercial test kits offer a rapid identification of *Enterococcus* species. Many authors have raised concerns over the misclassification of isolates when using commercial kits. Facklam and Texeira (2003) questioned the accuracy of the tests, suggesting that they could only reliably identify *Enterococcus faecalis*. Phenotypic methods may certainly fail to identify *Enterococcus* isolates that display resistance to antibiotics (Reed *et al*., 1999; Eisner *et al*., 2005; Winston *et al*., 2004), although the reliability of the methods have improved in recent years (Garcia-Garrote *et al*., 2000; Van den Braack *et al*., 2001). The main advantage of using commercial test kits is their ability to identify large numbers of isolates with relative ease. For example, the PhenePlate PhP plate system was successfully used for the preliminary identification of species in a large pan-European study in which more than 20,000 isolates from 2868 samples were analysed (Kühn *et al*., 2003). In response to concerns about the accuracy of commercial kits, it has been recommended that they be used in combination with more traditional biochemical tests (Fontan *et al*., 2002).

The genotypic methods that have been used in the identification of *Enterococcus* species include restriction endonuclease analysis (REA) of total chromosomal DNA (Stosor *et al*., 1999), plasmid profiles (Morrison *et al*., 1999), pulsed-field gel electrophoresis (Gelsomino *et al*., 2002), ribosomal RNA gene restriction analysis (Švec *et al*., 2001), polymerase chain reaction (PCR)- based typing systems (Table 2.1), nucleic acid hybridisation (Lewis *et al*.,

2002), PCR identification [genus specific (Ke *et al*., 1999), species specific (Knijff *et al*., 2001[a]), and detection of van genes and multiplex assays (Kariyama *et al*., 2000)], reverse transcription-PCR (Privitera *et al*., 1999), partial sequence analysis (Poyart *et al*., 2000), multilocus sequence typing (Naser *et al*., 2005), and whole-cell protein (WCP) analysis (Tyrrell *et al*., 2002).

Table 2.1 Polymerase chain reaction (PCR) - based typing systems used to identify species of the genus *Enterococcus*

Method	**Reference**
Randomly amplified polymorphic DNA (RAPD)-PCR	Quednau *et al*., 1998
Specific and random amplification (SARA)-PCR	Knijff *et al*., 2001[b]
Amplified fragment length polymorphism (AFLP)	Antonishyn *et al*., 2000
Rep-PCR	Del Vecchio, 1998
PCR-ribotyping	Sechi *et al*., 1998
PCR amplification of intergenic rRNA spacer regions (ITS-PCR)	Nelson *et al*., 2000
Amplified ribosomal DNA restriction analysis (ARDRA)	Moschetti *et al*., 1995
Restriction fragment length polymorphism (RFLP) of PCR-amplified 16 S rDNA	Müller *et al*., 2001
Broad range PCR-RFLP	Teng *et al*., 2001
Temporal temperature gradient gel electrophoresis (TGGE)	Monstein *et al*., 2001
Denaturing gradient gel electrophoresis (DGGE)	Ercolini *et al*., 2001
intergenic length polymorphism analysis (tRNA-PCR)	Devriese *et al*., 2002

Novel species are currently included in the genus *Enterococcus* on the basis of a combination of results from DNA-DNA re-association studies, 16S rRNA gene sequencing, whole-cell protein (WCP) analysis and conventional phenotypic tests.

Phenotypic methods, such as conventional biochemical tests and commercially available test systems, were traditionally used to identify enterococcal species in taxonomic studies. Many recently described species included in the genus *Enterococcus* (based on phylogenetic evidence from 16S rRNA sequencing) do not show typical phenotypic characteristics classically associated with the genus, thus making identification of species using phenotypic methods increasingly difficult (Devriese *et al*., 1993, 2002). Misclassifications are also more common with phenotypic methods, as some enterococcal species differ by just a single phenotypic trait. Studies have also shown a lack of correlation between identification by phenotypic methods such as API 20 Strep, the Vitek-2 system and PCR-based methods (Eisner *et al*., 2005; Velasco *et al*., 2004; Gomes *et al*., 2007). It has therefore been suggested that phenotypic tests should, where feasible, be used in conjunction with genotypic methods to increase accuracy (Scheidegger *et al*., 2009).

Table 2.2 lists the forty-one currently recognised *Enterococcus* species, as of 6th October 2011 (Euzéby, 2011). However it is noted that further investigations of taxonomic relatedness suggest that *E. porcinus* is a later synonym of *E. villorum* (De Graef *et al*., 2003), and that *E. flavescens* and *E. saccharominimus* are later synonyms of *E. casseliflavus* and *E. italicus*, respectively (Naser *et al*., 2006). Genotypic and phenotypic evidence also supports the transfer of *E. solitarius* to the genus *Tetragenococcus* (Ennahar and Cai, 2005), and high levels of DNA relatedness between strains of *Lactococcus garvieae* and *E. seriolicida* also suggest that *E. seriolicida* is a later synonym of *Lactococcus garvieae* (Teixeira *et al*., 1996).

Table 2.2 Recognised species of the genus *Enterococcus* (as of 2011)

***Enterococcus* species**	**Reference**
E. aquimarinus	Švec *et al*., 2005
E. asini	De Vaux *et al*., 1998
E. avium	Collins *et al*., 1984
E. caccae	Carvalho *et al*., 2006
E. camelliae	Sukontasing *et al*., 2007
E. canintestini	Naser *et al*., 2005
E. canis	De Graef., *et al* 2003
E. casseliflavus	Collins *et al*., 1984
E. cecorum	Devriese *et al*., 1983
E. columbae	Devriese *et al*., 1993
E. devriesei	Švec *et al*., 2005
E. dispar	Collins *et al*., 1991
E. durans	Collins *et al*., 1984
E. faecalis	Schleifer and Kilpperbalz, 1984
E. faecium	Schleifer and Kilpperbalz, 1984
E. flavescens	Pompei *et al*., 1992
E. gallinarum	Collins *et al*., 1984
E. gilvus	Tyrrell *et al*., 2002
E. haemoperoxidus	Švec *et al*., 2001
E. hermanniensis	Koort *et al*., 2004
E. hirae	Farrow and Collins, 1985
E. italicus	Fortina *et al*., 2004
E. malodoratus	Collins *et al*., 1984
E. moraviensis	Švec *et al*., 2001
E. mundtii	Collins *et al*., 1986
E. pallens	Tyrrell *et al*., 2002
E. phoeniculicola	Law-Brown and Meyers, 2003
E. porcinus	Texeira *et al*., 2001
E. pseudoavium	Collins *et al*., 1989
E. raffinosus	Collins *et al*., 1989
E. ratti	Texeira *et al*., 2001
E. saccharolyticus	Rodrigues and Collins, 1990
E. saccharominimus	Vancanneyt *et al*., 2004
E. seriolicida	Kusada *et al*., 1991
E. silesiacus	Švec *et al*., 2006
E. solitaries	Collins *et al*., 1989
E. sulfurous	Martinez-Murcia and Collins, 1991
E. termitis	Švec *et al*., 2006
E. thailandicus	Tanasupawat *et al*., 2008
E. viikkiensis	Rahkila *et al*., 2011
E. villorum	Vancanneyt *et al*., 2001

2.1.3 Habitat of *Enterococcus* spp.

Enterococci are ubiquitous in the gastrointestinal tracts of human and non-human animal sources (Martin and Mundt, 1972; Devreise *et al*., 1987; Kuhn *et al*., 2003; Farnleitner *et al*., 2010; Layton *et al*., 2010). Enterococcal species are also commonly isolated from the surface of plants, from water and sediment (Müller *et al*., 2001; Badgley *et al*., 2011). A number of enterococci have also been isolated from processed foods (Collins *et al*., 1984; Fortina *et al*., 2004; Vancanneyt *et al*., 2004; Tanasupawat *et al*., 2008). A list of enterococci species associated with different human and non-human host sources is given in Table 2.3.

Enterococcus densities between 10^5 and 10^8 CFU/g have been observed in the human intestine (Tannock and Cook, 2002) and the most abundant species detected in human faeces and wastewaters are *E. faecalis* and *E. faecium* (Ruoff *et al*., 1990; Manero *et al*., 2002; Gelsomino *et al*., 2003). *Enterococcus avium, E. casseliflavus, E. durans*, *E. gallinarum*, and *E. hirae* are also commonly isolated from human faecal sources. A diverse range of enterococcal species have been associated with the non-human mammalian gut and in some cases species have displayed a degree of host specificity. Among the species showing higher specificity, *E. asini* has been found exclusively in donkeys, and *E. columbae* appears to be specific to pigeons. A number of the newly recognised species of *Enterococcus* currently have only been found in the faeces of the mammalian host from which they were originally isolated (Table 2.3). Species of *Enterococcus* have also been found in the guts of a number of invertebrates, including snails and insects such as beetles, cockroaches, houseflies, horseflies and weevils (Martin and Mundt, 1972; Švec *et al*., 2002; Ahmad *et al*., 2011).

Table 2.3 Enterococcal species and their association with human and non-human hosts

***Enterococcus* spp.**	**Host**
E. asini	Donkey
E. avium	Birds, broiler processing plant air, cats, cattle, dogs, fish, gulls, horses, humans, pigs, poultry, sea lions, seals, sewage
E. caccae	Humans
E. canintestini	Dogs, and humans (clinical infection)
E. canis	Dogs
E. casseliflavus	Birds, cattle, chickens, dogs, geese, gulls, horses, humans, insects, poultry, pigeons, seals, sewage, sheep, and snails
E. cecorum	Cattle, cats, chickens, dogs, humans (clinical infection), pigs, pigeons and poultry
E. columbae	Pigeons
E. devriesei	Bovine
E. dispar	Human sources
E. durans	Broiler processing plant air, cattle, dogs, gulls, horses, humans, pigs, poultry, sea lions, seals, sewage, and sheep
E. faecalis	Birds, cattle, dogs, goats, gulls, horses, humans, insects, pigs, poultry, pigeons, rabbits, sea lions, seals, sewage, and sheep
E. faecium	Birds, cats, cattle, dogs, fish, gulls, goats, horses, humans, insects, pigs, pigeons, poultry, rabbits, sea lions, seals, sewage, and sheep
E. flavescens	Humans, cattle, horses and sheep
E. gallinarum	Birds, cattle, cats, dogs, domestic fowls, gulls, horses, humans, insects, pigs, pigeons, sea lions, seals, sewage, sheep
E. gilvus	Broiler processing plant air and human clinical specimens
E. hermanniensis	Broiler meat and canine tonsils
E. hirae	Birds, cattle, cats, chickens, dogs, geese, gulls, horses, humans, insects, pigs, poultry, sea lions, sewage, horses and sheep
E. malodoratus	Broiler processing plant air, humans, pigs and poultry

Table 2.3 Enterococcal species and their association with human and non-human hosts (continued)

***Enterococcus* spp.**	**Host**
E. mundtii	Cattle, horses, pigs, poultry, sheep, and silkworms
E. pallens	Human clinical specimens
E. phoeniculicola	Birds
E. porcinus	Pigs intestines and faeces
E. pseudoavium	Cattle and sheep
E. raffinosus	Cats, dogs, and humans
E. ratti	Intestines and faeces of rats
E. saccharolyticus	Cattle, sewage
E. seriolicida	Fish
E. solitarius	Humans
E. termitis	Termites
E. viikkiensis	Broiler processing plant air
E. villorum	Chickens, pigeons and piglets

References: Devriese *et al*., 1983, 1987, 1991, 1992[a], 1992[b], 1993, 1994; Collins *et al*., 1984; Schleifer and Kilpperbalz, 1984; Farrow and Collins, 1985; Collins *et al*., 1989; Rodrigues and Collins, 1990; Collins *et al*., 1991; Devriese *et al*., 1991; Kusada *et al*., 1991; Pompei *et al*., 1992; De Vaux *et al*., 1998; Texeira *et al*., 2001; Vancanneyt *et al*., 2001; Baele *et al*., 2002; Murase *et al*., 2002; Rodrigues *et al*., 2002; Švec *et al*., 2002; Tyrrell *et al*., 2002; De Graef., *et al* 2003; Law-Brown and Meyers, 2003; Koort *et al*., 2004; Naser *et al*., 2005; Švec *et al*., 2005; Carvalho *et al*., 2006; Fei *et al*., 2006; Švec *et al*., 2006; Larson *et al*., 2008; Graves *et al*., 2009; Marrow *et al*., 2009; Moneoang and Bezuidenhout, 2009; Bulushi *et al*., 2010; Layton *et al*., 2010; Ahmad *et al*., 2011; Han *et al*., 2011; Jackson *et al*., 2011; Rahkila *et al*., 2011

E. faecium, E. faecalis, E. casseliflavus, *E. hirae*, *E. mundtii*, and *E. sulfureus* have all been associated with plant habitats (Martinez-Murcia and Collins, 1991; Cai, 1999; Ulrich and Müller, 1998; Müller *et al*., 2001). Whilst many of these species have also been isolated from human and non-human animal faeces, evidence presented by Ulrich and Müller (1998) and Müller *et al*. (2001) suggests that the phenotypic characteristics of some plant-associated enterococci may be dissimilar to species commonly isolated in the guts of human and non-human animals. The presence of plant associated enterococci in human and non-human animal faeces may be the result of incorporation into the digestive tract through the host animals' diet (Layton *et al*., 2010).

Once enterococci are excreted from their primary hosts, they are often able to survive in secondary habitats in the aquatic environment (Ferguson *et al*., 2005; Badgley *et al*., 2011; Halliday *et al*., 2011). Badgley *et al*. (2010) investigated enterococcal concentrations in water, sediment and submerged aquatic vegetation, and discovered high densities of enterococci in all three matrices. Three enterococcal strains (*E. faecium, E. mundtii and E. hirae*) were present at high levels in all three sample types and may have represented naturalised environmental strains. Populations of enterococci have also been found in beach sand at consistently higher levels than in the overlying waters, and on material deposited on beaches, such as algae, seaweed, and debris (Bonilla *et al*., 2007; Imamura *et al*., 2011). The survival and potential multiplication of enterococci in secondary habitats is discussed further in section 2.1.4.

2.1.4 Survival of enterococci in the environment

The survival of enterococci in the environment is influenced by a variety of factors, including sunlight, temperature, salinity, predation, turbidity (including particle size), nutrient availability, organic carbon, pH level, dissolved oxygen concentration, and the presence of chemicals used in disinfection, such as chlorine (Tyrrell *et al*., 1995; Burkhardt *et al*., 2000; Davies and Bavor, 2000; Sinton *et al*., 2002; Kay *et al*., 2005; Fries *et al*., 2006; Haller *et al*., 2009; Bolton *et al*., 2010; Chandran *et al*., 2011). Of these factors, solar radiation from sunlight has repeatedly been shown to be the dominant influence on the survival of faecal indicator bacteria in surface waters (Davies-Colley *et al*., 1994; Sinton *et al*., 1994; Sinton *et al*., 1999). Sinton *et al*. (2002) found that the degree of inactivation of faecal indicators in sunlight including that of enterococci was ten times greater than that observed in the dark. Their results suggested that, although enterococci are initially more resistant to solar radiation than faecal coliforms, once damage to enterococcal cells occurs, it appears to be irreversible. Damage in enterococcal cells may be irreversible because unlike faecal coliforms, enterococci lack repair mechanisms and are unable to photo-reactivate (Harris *et al*., 1987; Locas *et al*., 2008).These findings were supported by Noble *et al*. (2004), who demonstrated that solar irradiation and temperature had significant effects on rates of inactivation ($P<0.001$), and recorded faster inactivation of enterococci in sunlight than *Escherichia coli* (a member of the faecal coliform group). Higher levels of turbidity appear to decrease inactivation rates. Indeed, results from a study by Kay *et al*. (2005) suggested that the decay of enterococci in waters with turbidity of >200 NTU was similar to that observed under dark conditions. These results indicate that sediments in the water column may protect bacteria from irradiation.

It is generally recognised that enterococci survive longer in marine environments in comparison to members of the coliform group (Vasconcelos and Swartz, 1976; Evison and Tosti, 1981). The longer survival of enterococci in marine environments may be due to their greater tolerance of higher concentrations of NaCl (Sherman, 1937). Imamura *et al*. (2011) demonstrated that beach wrack, such a kelp and seaweeds, appears to increase the survival of enterococci in marine waters and sediments. Research has shown that attachment to secondary habitats can provide favourable environments for bacterial survival, largely because of reductions in sunlight inactivation and predation, and the increased availability of nutrients and organic carbon (Sinton *et al*., 1999; Davies and Bavor, 2000; Haller *et al*., 2009; Chandran *et al*., 2011). Authors have suggested that secondary habitats, such as sediments, seaweeds and algae, can provide reservoirs for faecal indicator bacteria and act as suitable substrates for multiplication (Anderson *et al*., 1997; Byappanahalli *et al*., 2003; Ferguson *et al*., 2005; Pote *et al*., 2009).

2.1.5 Isolation and enumeration

A variety of techniques and media for the isolation and enumeration of enterococci in food, water, environmental, faecal and clinical samples has been described and critically reviewed (Barnes, 1976; Levin *et al*., 1975; Sinton *et al*., 1993[a]; Domig *et al*., 2003). Enterococci are difficult to grow on synthetic media as they require several vitamins and amino acids. Rapid growth is achievable with rich complex media such as Tryptone Soya (TS) and Brain Heart Infusion (BHI). These media sustain the growth of a number of bacteria, not just enterococci. The use of intestinal enterococci as faecal indicator organisms was prevented until the advent of selective media, which allowed for isolation of enterococci from environmental samples (Mallman and Seligmann, 1950). Initially

concentrations of intestinal enterococci were determined using the most probable number (MPN) method, but a membrane filtration method, first reported by Slanetz and Bartley (1957), soon followed. Membrane filtration has the advantage of producing faster results, enabling direct colony counts and allowing large sample volumes to be tested (Sinton *et al*., 1993[a]). Slanetz and Bartley (SB), otherwise known as m-*Enterococcus* agar, KF streptococcus agar and Kanamycin Aesculin agar (KAA) are the media that have been most commonly used with the membrane filtration method.

The influence of different incubation temperatures and duration times on the isolation of enterococci has been investigated. Elevated temperatures (between 42^{o}C and 44^{o}C), and shorter incubation duration can increase selectivity and reduce the growth of background microflora, but ultimately higher sensitivity results in lower counts (Brodsky and Schiemann, 1976; Dutka and Kwan, 1978; Reuter, 1985). There is therefore a trade-off between selectivity and recovery rates. The current ISO European and British standard for the isolation of enterococci from surface waters and wastewaters recommends membrane filtration using Slanetz and Bartley medium (m- *Enterococcus* agar) with incubation at 37^{o}C and a confirmation step using bile aesculin-azide agar (Anon, 2000). For this medium, a confirmation step is necessary to differentiate enterococci from Lancefield group D streptococci species such as *Streptococcus bovis* (Chuard and Reller, 1998).

For further confirmation of isolates belonging to the genus *Enterococcus*, additional tests may be performed. The genus *Enterococcus* is described in Bergey's Manual of Determinative Bacteriology (1994) as being comprised of Gram positive, facultatively anaerobic, spherical or ovoid cells occurring in pairs or short chains. They are catalase

negative and usually grow at both 10^{o}C and 45 ^{o}C, at pH 9.6, with 6.5% NaCl, and with 40% bile. By testing an isolate for a combination of these characteristics, most members of the enterococcal genus can be differentiated from those of other Gram-positive, facultatively anaerobic genera. This differentiation is often necessary before an isolate can be identified to the species level. Although these characteristics are true for most enterococci, as noted in section 2.1.2, some recently described species included in the genus *Enterococcus* do not share all of the these conventional traits.

2.1.6 The use of species of *Enterococcus* in MST

Enterococcal species are good candidate organisms for MST because they are present in high numbers in faeces, and several species although found in a number of host animals, can be divided into host-specific groups on the basis of phenotypic and genotypic characteristics (Devriese *et al*., 1993; Quednau *et al*., 1999). Enterococci were first used in MST in 1969 when Geldreich and Kenner proposed the faecal coliform to faecal streptococci (enterococci) ratio as a tool for discerning human from non-human sources of faecal pollution after Litsky *et al*. (1955) found a 1:7 *E.coli*: enterococci ratio in surface water, but a 13:1 ratio in raw wastewater (Litsky *et al*., 1953). Geldreich and Kenner (1969) determined that a high faecal coliform to faecal streptococci ratio (>4.0) indicated human faecal pollution, whereas lower ratios were representative of mixed faecal sources. In 1976 Geldreich further suggested that a ratio of <0.7 should be considered indicative of animal faecal sources. However, later research showed the ratio to be unreliable. The ratios were found to be inconsistent in human and animal faeces and further research suggested that the two bacterial groups displayed unequal survival rates in the environment (Doran and Linn, 1979; Mara and Oragui, 1981; Pourcher *et al*., 1991). Subsequently, enterococci have been

the focus of considerable numbers of MST studies involving numerous phenotypic, genotypic, library-dependent and independent methodologies (Bahirathan *et al*., 1998; Wheeler *et al*., 2002; Wiggins *et al*., 2003; Moore *et al*., 2005; Scott *et al*., 2005; Soule *et al*., 2006; Ahmed *et al*., 2007; Brownell *et al*., 2007; Dikerson *et al*., 2007; Jiang *et al*., 2007; Whitman *et al*., 2007; Korajkic *et al*., 2009).

Some authors have suggested that specific *Enterococcus* species have potential application as MST markers. Bahirathan *et al*. (1998) explored the potential of using a ratio of *E.coli* to yellow pigmented enterococci, combined with vancomycin susceptibility profiles, as an indication of human and non-human sources of faecal pollution. Their results showed yellow-pigmented enterococci were more prevalent and abundant in non-human faecal sources. Wheeler *et al*. (2002) targeted *E. faecalis* species and used ribotyping to distinguish isolates from human and chicken sources. Soule *et al.* (2006) used DNA microarrays to identify library-independent, host-specific *Enterococcus* markers. They identified fifteen markers originating from human, cattle, and cervid (elk and deer) isolates. Two markers were cattle specific, five were human specific, one was associated with elk and deer, and the remaining markers were found to be present in a number of other non-human hosts. Interestingly, cervid markers were only present in *E. mundtii* and *E. casseliflavus*, while *E. faecalis* harboured only human markers. Many other authors have focused their efforts on a human-associated *esp* gene marker for *E. faecium*, although there is contradictory evidence regarding its specificity, and therefore its suitability as an indicator of human faecal pollution is in doubt (Scott *et al*., 2005; Ahmed *et al*., 2008; Byappanahalli *et al*., 2008; Layton *et al*., 2009).

2.2 The basis of phage lysis as an MST tool

In this section the biology of bacteriophages is briefly described before the current use of phages of enteric bacteria (including the enterococci) as faecal indicator organisms, microbial source tracking markers and potential pathogen surrogates is critically reviewed**.**

2.2.1 Basic phage biology

Bacteriophages are viruses capable of infecting, and multiplying within, prokaryotes (Abedon, 2008). Phages can be either 'virulent' or 'temperate'. Temperate phages are able to multiply by either a lytic cycle, ultimately resulting in the lysis of the host cell (Figure 2.1), or alternatively by means of a lysogenic cycle. Virulent phages can only replicate by means of a lytic cycle. There are variations in the life cycles of various phages. A basic lytic cycle is illustrated in Figure 2.1, and involves the stages of adsorption, infection, and the eventual lysis of the host cell and release of progeny phages.

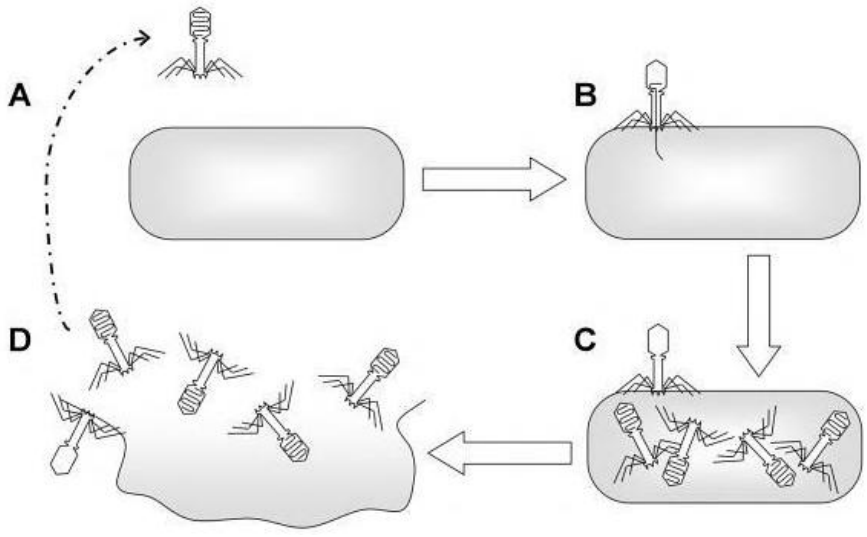

Figure 2.1 The life cycle of a typical lytic bacteriophage. (A) A single infective phage particle with its bacterial host cell. (B) The phage binds to receptors on the surface of its host (adsorption) and injects its genome into the bacterial cytoplasm. (C) Within the bacterial cell the phage genome is copied, phage structural proteins are synthesised, and the genome is packaged. (D) At the end of the latent period, the host cell lyses, releasing the new progeny phages. The cycle may then start again (Kerr *et al*., 2008)

Adsorption is the process by which tailed phages with specialised structures (fibres or spikes), bind to specific receptors on the host bacterium. Receptor sites may be proteins, oligosaccharides, or lipopolysaccharides. Several phages require concentrated clusters of these sites in order to position the tail for penetration, however some are capable of utilising receptors that are only present in a few copies per cell (Guttman *et al*., 2005). Successful adsorption is followed by the onset of the latent period and subsequent infection of the bacterial cell. At this stage genetic material is transferred from the phage into the host through the tail (in most cases). The host converts into a phage-producing cell (redirecting biosynthetic pathways to make copies of nucleic acid and to synthesise phage proteins) by transcribing and translating phage genes (Malacinski, 2005). Phage particles are assembled by a process referred to as morphogenesis. During morphogenesis DNA is packaged into icosahedral protein shells (procapsids). Phages are then assembled via complex interactions between scaffolding proteins and head structural proteins. The separately assembled tail is combined once the head is complete (Guttman *et al*., 2005). Phage lysis then allows for the release of new progeny phage. In the case of filamentous phage (discussed later in this chapter), release does not always result in the destruction of the host cell (Abedon, 2006).

Within a lysogenic cycle, the phage does not actively replicate. Instead the phage genome adopts a dormant state (known as a prophage), integrated into the host genome or maintained as a plasmid (Guttman *et al*., 2005). In this state replication occurs as the host cell reproduces to make a clone of cells, all containing prophages. It has been postulated that lysogeny may be an adaption for phages to maintain their populations during 'hard times', when bacterial densities fall below what is necessary for phage populations to be maintained through lytic infection alone (Stewart and Levin, 1984).

It is thought that there are approximately 10^{30} to 10^{32} phages within the earth's diverse environments, making them ten or more times greater in number than bacteria (Whitman *et al*., 1998; Summers, 2005). Phages are known to infect over 140 bacterial genera (Ackermann, 2001) and whereas many have very restrictive host ranges, others display broader host specificity (Kutter, 2009).

2.2.2 Early research into phage ecology

The activity of phages was independently observed and reported by Frederick W. Twort in 1915 and by Felix d'Herrelle in 1917. Twort (1915) reported a "glassy transformation" of micrococci cultures, whilst d'Herrelle noted lysis in liquid culture by a microbe "antagonistic" to bacteria. D'Herrelle (1917) also observed killing in discrete patches (he named these plaques) on the surface of an agar seeded with the bacteria (Calender, 2006). D'Herrelle conceived the concept of "ultraviruses" that invaded bacteria and multiplied at their expense. He named these microbes bacteriophages, from the Latin for 'bacteria eater' (Summers, 2005). For many years the phage phenomenon discovered by Twort and d'Herrelle caused substantial controversy and debate, directly challenging the views of many bacteriologists at the time. It is now however understood that bacteriophages are viruses with prokaryotic host ranges (Summers, 2005; Abedon, 2008).

2.2.3 Phage classification

Electron microscopy allows simple, rapid and cost-effective phage identification by morphological criteria. Over 5500 phages have been examined under the electron microscope since 1959. They are primarily identified by defining the nature of their nucleic acid and virion morphology (Ackermann, 2007). The International Committee of

Taxonomy of Viruses (ICTV), founded in 1966, currently recognises one order, fourteen families (with at least five other potential families), and 37 genera of phages (Ackermann, 2009). The ICTV adopts the 'polythetic species concept' that defines species by a set of properties, some of which may be absent from any given member (Van Regenmortel, 2000). As many as 70 phage properties have been used to characterise phage, but two of the most useful of these properties are the nature of the nucleic acid and morphology. Figure 2.2 illustrates the major phage groups.

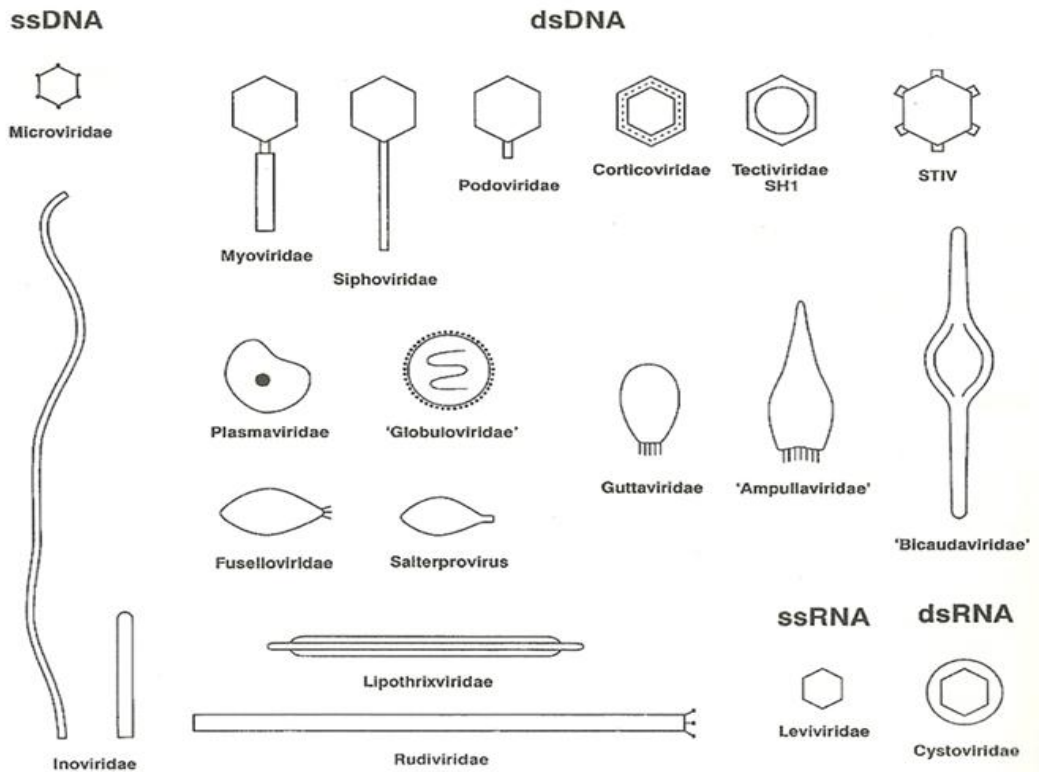

Figure 2.2 Schematic representations of prokaryote virus morphotypes. Descriptions are given in Table 2.4 (Ackermann, 2009)

The groups include phage with double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA). Over 96% of all characterised phage contain dsDNA and are tailed (Ackermann, 2009). All tailed phages are members of the order *Caudovirales*. They contain dsDNA and have icosahedral

or elongated heads, helical tails and are of binary symmetry (Ackermann, 2005). Although members of the *Caudovirales* share common features, they are an extremely diverse group of viruses (Casjens, 2005). The order *Caudovirales* is divided into three families that may be differentiated primarily by tail structure:

1. ***Myoviridae***- Myoviruses tend to be larger than other phage groups, and include some of the largest and most highly evolved tailed phages. Their tails are contractile and consist of a neck, a contractile sheath, and a central tube. Myoviruses constitute 25% of known tailed phages (Ackermann, 2006; 2009).
2. ***Siphoviridae***- Siphoviruses have long simple tails that are non-contractile, and have flexible or rigid tubes. Sixty percent of characterised tailed phages are members of the *Siphoviridae* family (Ackermann, 2009).
3. ***Podoviridae***- Podoviruses have short non-contractile tails. They constitute 14% of known tailed phages (Ackermann, 2006).

Polyhedral, filamentous, and pleomorphic (tail-less) phages account for the remaining 3.6% of all phages observed to date (190 viruses). There are currently eleven accepted families, with a further six families awaiting classification (Figure 2.2). The polyhedral or 'cubic' phages, as they have recently been termed by Ackermann (2009), include *Microviridae*, *Corticoviridae*, *Tectiviridae*, *SH1* (from "Serpentine-lake-Hispanica'), *STIV* (Sulfolobus-Icosahedral-Turreted-Virus), *Leviviridae*, and *Cystoviridae*. The filamentous phage families are *Inoviridae*, *Lipothrixviridae* and *Rudiviridae*, and the pleomorphic phages include the families *Plasmaviridae*, *Fuselloviridae*, *Salterprovirus*, *Guttaviridae*, *Ampullaviridae*, *Bicaudaviridae* and *Globuloviridae*.

2.2.4 Classification of phages infecting *Enterococcus* species

Ideally bacterial host strains capable of detecting homogenous groups of phages are recommended for use in MST-phage based methods (Queralt *et al*., 2003). Studies have shown that phages of different morphologies may have different survival characteristics, which can lead to inaccurate interpretation of results (Muniesa *et al*., 1999; Muniesa *et al*., 2009). It is therefore important to assess the morphology of phages capable of infecting *Enterococcus* host strains with potential MST application.

A relatively limited number of enterococcal phages have been examined by electron microscopy. The majority of these have belonged to tailed phage families, of which most are members of the *Siphoviridae* family (Ackermann, 2007). Much of the published research into enterococcal phage has focused on phages that are infectious against antibiotic resistant bacteria and therefore have potential therapeutic use. These phages have shown varying host ranges. Some phage appear only to be able to infect their original host, whilst others are able to infect almost all *Enterococcus* species (Paisano *et al*., 2004; Ramírez *et al*., 2006; Son *et al*., 2010; Vinodkumar *et al*., 2011). Although the majority of enterococcal phages observed have been members of the *Siphoviridae* family, authors have reported tail-less phages capable of infecting enterococcal host strains. Bachrach *et al*. (2003) reported tail-less phages that were uniform, spherical, enveloped, spiked structures with a diameter of roughly 70 nm. Fard *et al*. (2010) recently reported the isolation of novel polyhedral, filamentous and pleomorphic phages capable of infecting *Enterococcus* species (Figure 2.3). Filamentous phages were isolated from *E. faecalis*, and *E. gallinarum* host strains. Polyhedral phages were isolated from an *E. faecium* strain and pleomorphic-shaped phages

were isolated from an *E. faecalis* host strain. The study demonstrated the large diversity of phages capable of infecting enterococcal host strains of animal origin.

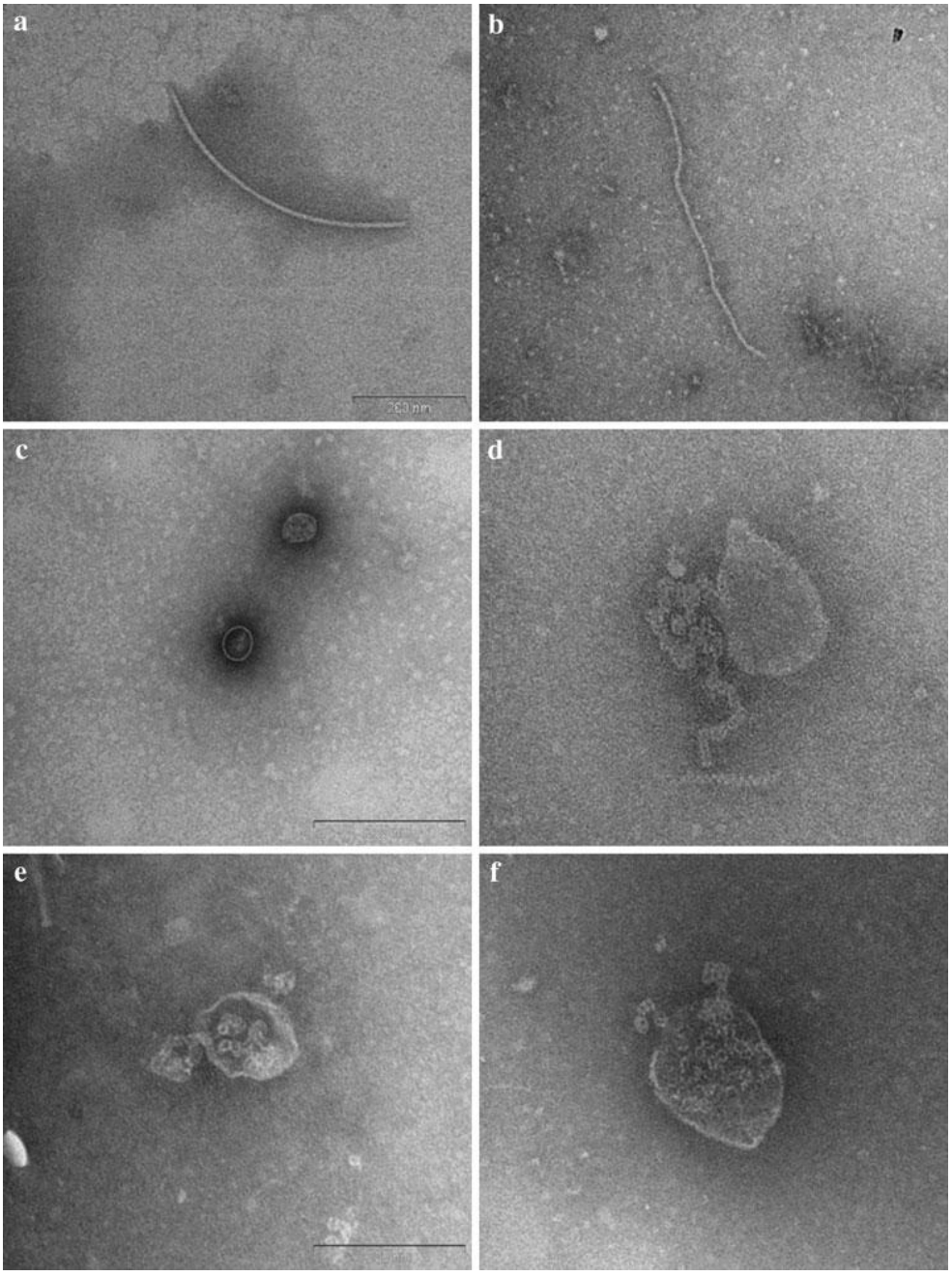

Figure 2.3 Enterococcal polyhedral, filamentous and pleomorphic phages. (a) A filamentous phage. (b) A filamentous phage. (c) Spherical (polyhedral) phages. (d) A droplet-shaped (pleomorphic) phage. (e) A lemon-shaped (pleomorphic) phage. (f) A lemon-shaped (pleomorphic) phage (Fard *et al*., 2010)

2.3 Application of phages as water quality indicators and markers for MST

Studies have shown that enteric viruses survive longer in the environment then traditional bacterial faecal indicators such as *E.coli* (Nasser and Oman, 1999; Moce-Llivina *et al*., 2005). Bacteriophages therefore have been suggested as alternative indicators that may better predict health risks associated with enteric viruses. Three phage types have been investigated for potential use as faecal indicator and index organisms - the somatic coliphage, the F-specific RNA phage, and the phages of *Bacteroides fragilis* (Tartera *et al*., 1989; Scott *et al*., 2002; Moce-Llivina *et al*., 2005; Ebdon *et al*., 2007). All are considered potentially useful for determining water quality because they share with some important groups of human viruses, similar resistance to environmental stressors and water treatment processes (IAWPRC, 1991).

2.3.1 Phages infecting *Bacteroides* species

Phages capable of infecting host strains of *Bacteroides fragilis* (*B. fragilis*), an obligately anaerobic, Gram negative, rod-shaped bacterium, have been used as water quality indicators and as MST markers (Tartera *et al*., 1989; Grabow *et al*., 1994; Puig *et al*., 1999; Blanch *et al*., 2004; Payan *et al*., 2005; Ebdon *et al*., 2007; Gomez-Donate *et al*., 2011). *B. fragilis* is one of the most numerous bacterial species in the human intestine, and experiences rapid inactivation in the environment under oxic conditions (Ballesté and Blanch, 2010). Importantly, the presence of *B. fragilis* phages in the environment has been correlated with the presence of human enteric viruses (Jofre *et al*., 1989; Ebdon *et al*., 2011).

During the 1980s a University of Barcelona research group experimented with 12 strains of *B. fragilis* before focusing on HSP40 (Jofre *et al*., 1986), a strain only found in human faeces. No phage of HSP40 were found in the faeces of non-human animals, including cows, pigs, rabbits, mice, hens, quail, seabirds, or even larger primates, such as gorillas, orangutans and chimpanzees. Their findings also suggested that phages active against *B. fragilis* species were unable to replicate in the environment, potentially making them an ideal faecal indicator and MST marker. Unfortunately, in areas of northern Europe and the United States the percentage of the human population that excrete HSP40 appears to be low and consequently recorded phage counts have been lower in these geographic locations (Puig *et al*., 1999). The detection of low phage numbers in some wastewaters prompted Puig *et al.* (1999) to look for additional host strains of *Bacteroides* in an attempt to isolate hosts that would have greater potential as an effective MST marker than HSP40. This led to the isolation of alternative host strains including a strain known as RYC2056, which detected larger numbers of phages in waters known to be affected by human faecal pollution. RYC2056 demonstrated higher sensitivity than HSP40, but lower specificity. RYC2056 detected phage in low numbers in some non-human faeces.

To overcome the problem of geographical instability, Payan *et al.* (2005) presented a method for isolating new *Bacteroides* host strains that could be used for different geographic areas. They undertook four trials (in Spain, Colombia and the UK) in which they isolated *Bacteroides* host strains from raw municipal wastewater. Useful strains were identified in both Spain and the UK (where strains GA-17 and GB-124 were isolated respectively) (Payan *et al*., 2005). A further study conducted at the University of Brighton by Ebdon *et al*. (2007) into the potential of GB-124 as a host strain, demonstrated that high

levels of GB-124 phages were present in municipal wastewater and river samples impacted by human faecal pollution. It was also noted that GB-124 phages were absent in the faeces of non-human animals. Isolating local host strains for MST use in specific regions has successfully countered the geographical stability question associated with this approach. Vijayavel *et al*. (2010) recently isolated and applied host strain HB-73 (specific to human wastewater) to Hawaii beaches, the results suggesting that raised enterococcal levels may not be a result of human faecal contamination.

More recently, research has focused on isolating *Bacteroides* host strains for the detection of animal faecal contamination in surface waters. In 2011, both Gomez-Donate *et al*. and Wicki *et al*. isolated host strains with potential use as indicators and markers of animal faecal contamination. Gomez-Donate *et al*. (2011) isolated host strains that showed some specificity to individual animal sources (cow, pig, and poultry). Although the host strains were not 100% host specific they detected fewer phages in non-target samples.

2.3.2 Coliphages

Coliphages are viruses that infect *E.coli* and other closely related species (Gerba, 2006). They can be divided into two groups: Somatic coliphages and F-specific RNA coliphages. Somatic coliphage attach to the lipopolysaccharide of *E.coli* and F-specific RNA coliphages only infect bacteria that have an F plasmid, which codes for an F pilus that serves as the attachment site for the phage (Scott *et al*., 2002). Somatic coliphage are present in higher numbers in the environment than phage infecting *Bacteroides* species, largely because *E. coli* are facultative anaerobes (Mocé-Llivina *et al*., 2005), but they cannot be used to determine sources of pollution as they do not demonstrate host specificity

(Skraber *et al*., 2002). F-specific coliphage have been correlated with the presence of enteric viruses in the environment (IAWPRC, 1991; Havelaar *et al*., 1993).

There are four subgroups into which F-specific RNA coliphage can be classified: I, II, III and IV. Group I contains phages present in both human and animal faeces, as well as wastewater. Those in groups II and III have strong associations with human faecal pollution and domestic wastewater, and group IV coliphages are more commonly associated with the faeces of non-human animals including livestock (Sinton *et al*., 1998; Scott *et al*., 2002). In order to classify faecal sources using these phages, the subgroup must be identified by either serotyping or genotyping. Serotyping has often produced unclear results and this has led to greater use of genotyping (Hsu *et al*., 1995; Beekwilder *et al*., 1996). The varying incidence of these four groups in the faeces of human and non-human animal hosts has been used to determine likely sources of faecal contamination (Griffin *et al*., 1999; Gourmelon *et al*., 2010 [a], [b]; Lee *et al*., 2011), but the additional step of genotyping makes the method more expensive and laborious than methods based on phage lysis alone. The use of F-specific RNA coliphage has also been questioned, because survival studies have shown differing persistence rates between the four subgroups (Brion *et al*., 2002; Muniesa *et al*., 2009). The limitations of differential phage survival are discussed in greater detail in section 2.4.

2.3.3 Phages infecting *Enterococcus* species

In 2010 Bonilla *et al*. described a group of phage (which they referred to as ‘enterophage’), that infected an *E. faecalis* host strain and appeared to be specific to human faecal pollution (absent in non-human animals including birds, dogs, cats and sand flies). They were able to

detect phages infecting the *E. faecalis* host strain in wastewater and environmental samples, including surface water and beach sand and their results suggested that these phages may have potential for MST application. The data also indicated that there were different phage populations able to infect the *E. faecalis* host strain, but initial survival experiments showed that the phages all showed similar survival. The potential use of 'enterophage' as an indicator of human faecal pollution was further supported in 2010, when Santiago-Rodríguez *et al*. proposed 'enterophage' as a potential surrogate of enteric viruses in recreational waters, as they were more resistant to primary and tertiary wastewater treatment then somatic coliphage, and their survival in fresh and marine waters was comparable. The specificity of the *E. faecalis* host strain has been tested against a relatively small range of animal faecal sources (cattle, birds, dogs, cats and sand flies) and the numbers of samples tested for all host animals apart from cattle is unclear. The geographical stability of 'enterophages' also needs further elucidation.

2.4 Phage survival

Somatic coliphage represents a heterogeneous group of phages encompassing *Myoviridae, Siphoviridae, Podoviridae,* and *Microviridae* (Francki *et al*., 1991) and the heterogeneity of these organisms raises questions as to their suitability as a faecal indicator. Studies have shown that resistance to environmental stressors and water treatment processes differ between somatic coliphage of different families, and even between phage of the same family (Muniesa *et al*., 1999; Muniesa *et al*., 2009; Lee and Sobsey, 2011). Muniesa *et al.* (1999) found *Siphoviridae* and *Myoviridae* to be the most abundant coliphage groups in raw wastewater, treated wastewater and faecally contaminated river water. *Siphoviridae* demonstrated greater resistance to natural inactivation in fresh water environments (*in situ*

inactivation experiment in the River Llobregat). Many authors state that a major limitation of using somatic coliphage as a faecal indicator is their ability to replicate in the water environment (Seeley and Primrose, 1980; Borrego *et al*., 1990). These authors propose that coliphages are capable of replicating once they have left the host in *E. coli* or in other enterobacteria occurring naturally in the environment. More recent research however suggests that naturally occurring densities of bacterial hosts and phages are much less than would be required for significant phage replication in environmental matrixes (Jofre, 2009). Contributions from replication of somatic coliphage are therefore likely to have a negligible influence on the numbers of somatic coliphage detected in surface waters.

F-specific RNA coliphages represent a more homogenous group than somatic coliphage, belonging to the families *Leviviridae* and *Inoviridae*. However, numerous studies have shown differential survival of the four subgroups of F-specific RNA phage in aquatic environments (Brion *et al*., 2002; Long and Sobsey, 2004; Schaper *et al*., 2002; Muniesa *et al*., 2009). The most recent of these studies conducted by Muniesa *et al*. (2009) found that the distribution of the percentages of each subgroup changed following inactivation by natural stressors (including sunlight, pH level and temperature), and after wastewater treatment processes such as activated sludge, and tertiary disinfection. These findings weaken the case for the use of F-specific RNA coliphage as an MST tool, because differential persistence may lead to incorrect source identification.

Phages infecting *B. fragilis* are predominantly from the *Siphoviridae* family, showing a greater degree of homogeneity than both types of coliphages described above (Queralt *et al*., 2003). Inactivation studies have shown phages of *B. fragilis* to be more resistant to

water and wastewater treatment than either somatic coliphages or F-specific RNA coliphages (Jofre *et al*., 1995; Duran *et al*., 2003). Duran *et al*. (2003) found that the *Siphoviridae* group demonstrate significantly lower inactivation rates than phages of the *Myoviridae* and *Microviridae* families.

2.5 Rationale for use of phages infecting *Enterococcus* in MST

As demonstrated above, research has shown that phages have potential to be effective indicator and index organisms in water quality assessment. All the phage groups discussed in this chapter have, to some degree, co-presented in polluted aquatic environments with enteric viruses, and they share similar or greater resistance to inactivation by natural stressors and treatment processes. F-specific RNA phages and phages infecting *Bacteroides* species have been used to distinguish sources of faecal pollution in water (Gourmelon *et al*., 2010[a]; Vijayavel *et al*., 2010; Gómez-Doñate *et al*., 2011). In this chapter the limitations of these phages as faecal indicators and potential MST markers have been discussed. In brief, the subgroups of F-specific RNA phages require identification by serotyping or genotyping, making the method more expensive and time-consuming (Scott *et al*., 2002). Differential survival of the sub-groups also calls into question the use of these indicators for MST (Muniesa *et al*., 2009). The use of phages of *Bacteroides* species in MST appears more promising. A key advantage of using an anaerobic host is that it is unlikely to replicate outside the gut environment. However, recent research into the ecology of somatic coliphage suggests that any replication of their bacterial host in the natural environment is unlikely to have a significant impact on the numbers of phages detected (Jofre, 2009). *Bacteroides* species require strictly anaerobic growth conditions, necessitating overnight growth of the host before phage assays can be performed. The

relatively slower growth of the *Bacteroides* hosts, results in slower plaque formation (18-24h incubation being typical). Recreational water quality can vary significantly over short periods (minutes to hours), and within 24 hours the risk to public health may have changed considerably, putting into question the validity of decisions based on tests requiring 18 to 24hours incubation prior to enumeration (Rabinovici *et al*., 2004). With the above points in mind, it could be highly beneficial to use an alternative bacterial host genus for phage detection, one which has simpler growth requirements and more rapid growth, and which ideally facilitates same-day assessment of water quality.

At a time of climate uncertainty, environmental and public health legislation is developing rapidly. In Europe attempts to implement the 'river basin management plans' required by the EU Water Framework Directive, and the 'bathing water profiles' required by the 2006 revision of the EU Bathing Water Directive have already demonstrated a need for better tools to monitor and predict effectively the dynamics of faecal pollution entering recipient waters during dry weather and storm events (CEU, 2000; CEU 2006). These European legislative developments coincide with a growing public concern about the impact on human health of emerging waterborne zoonotic diseases. More than 75% of emerging or re-emerging human diseases are caused by pathogens originating from animal reservoirs (Cotruvo *et al*., 2004) and the identification of both human and non-human (point and diffuse) sources of faecal contamination in waters, would support effective river basin management plans and consequently protect human health.

The European environmental and public health agenda may therefore be considered to support the need for cost-effective pollution source tracking methods. More specifically, in

the area of MST it is timely to investigate how a group of enteric bacteria (namely the intestinal enterococci), which are used extensively to quantify levels of faecal pollution in water, may also form the basis of a relatively simple MST approach (namely, phage lysis). Strains of various *Enterococcus* species were considered as potential alternative hosts because some species are more abundant in human faeces (*E. faecalis and E. faecium*) (Wheeler *et al.*, 2002), whilst others are more restricted to the faeces of non-human animals (e.g., yellow-pigmented *E. casseliflavus*) (Bahirathan *et al.*, 1998). The intestinal enterococci are also considered to be a 'faecal indicator of choice' for water quality testing in many parts of the world, as they survive longer in marine environments and a correlation with gastro-enteric disease has been demonstrated (Noble *et al.*, 2003). Enterococci are easily cultured and can produce confluent lawns within 4h, allowing for same-day assessment. Species of the genus *Enterococcus* were therefore selected for further investigation as potential alternative host strains for MST and water quality assessment.

2.6 Aim and objectives

The aim of this programme of research was to evaluate the suitability and effectiveness of bacteriophages (phages) infecting host strains of *Enterococcus* species as a low-cost tool for MST. To achieve this overall aim, four primary objectives were set, namely;

1) To develop an effective protocol for isolating *Enterococcus* spp. host strains suitable for MST;

2) To evaluate critically the ability of phages infecting host strains of *Enterococcus* spp. to detect both human and non-human sources of faecal pollution;

3) To assess the diversity and survival of phages capable of infecting potential *Enterococcus* host strains.

4) To evaluate critically the practical and economic case for implementing phage lysis of *Enterococcus* as a future MST tool.

These objectives were designed as a rational and tiered approach to providing the evidence needed to support a new MST protocol.

Chapter Three: Materials and methods

3.1 Sample collection

Samples of animal faeces, livestock run-off, municipal wastewater and surface water were collected for the isolation of potential *Enterococcus* host strains and for subsequent bacteriophage (phage) detection. Faecal material, from either pooled faecal samples (at least twenty individuals) or agricultural run-off was collected from cattle, ducks, geese, goats, horses, pigs, rabbits, and sheep from eight farms, all located in South East England, UK. Gull (*Larus* spp.) faeces were collected on fishing boat jetties, located in the estuary of the River Ouse, Newhaven, UK. Samples were collected using sterile swabs and 100ml sampling containers (Fisher) as appropriate, between October 2008 and December 2010. Faecally contaminated run-off water was collected from cattle housing and from the drainage channels of pig housing at Wales Farm, Plumpton Agricultural College, in East Sussex, UK. Samples of municipal wastewater (raw and treated) were collected from seven wastewater treatment works (WWTW) situated in South East England, UK. Population equivalents of the sites ranged from 258 to 53,425 (Table 3.1).

Table 3.1 Population equivalent data for WWTW from which municipal wastewater samples were taken

Site	Population equivalent [a]
Ditchling	1,621
Fittleworth	723
Goddards Green	49,410
Poynings	258
Scaynes Hill	37,327
Shoreham	53,425
Steyning	10,037

[a] Population equivalent data provided by Southern Water Ltd (UK).

Surface water samples were collected downstream of two livestock farms (Wales Farm and Pellingbridge Farm) and downstream of a medium-sized wastewater treatment works (WWTW) (population equivalent, 55,955). All surface water samples were collected in 1 litre sterile polypropylene sample bottles (Nalgene) using an extendable sampling pole, from approximately 30 cm below the surface of the water. Wastewater samples were collected by lowering a steel can into the flow of water, and decanting the sample into 1 litre sterile sample bottles, in accordance with Southern Water's sample collection policy. Following collection, samples were transported to the laboratory in the dark, at 4 ^{o}C, within 4 h. In the laboratory individual stool samples were pooled, mixed and homogenized using a Seward Stomacher 400 (Lab System, UK) in sterile one quarter strength Ringer's solution (Fisher Scientific, UK). Samples collected for the isolation of enterococci were processed immediately, whereas samples for phage enumeration were preserved with 10% glycerol at -20 ^{o}C (Mendez *et al*., 2002) for analysis within four weeks.

3.2 Phage enumeration

Phages were detected and enumerated for the purpose of host screening, host specificity and sensitivity analysis, and in order to isolate phage for characterisation (discussed in sections 3.5, and 3.7, respectively). The double agar-layer and spot test plaque assays as described below were used during this study (Adams, 1959). A negative control was used for both methods and for all assays performed. Tryptone soya broth (TSB) (Oxoid, Fisher Scientific, UK) was used as the growth medium for enterococcal hosts. TSB is a general medium used for culturing and maintenance of enterococci and a number of other aerobic and facultative bacteria.

3.2.1 Double agar-layer plaque assay

Phages infecting *Enterococcus* hosts were enumerated using the double-agar-layer method as described elsewhere (Adams, 1959; Jofre *et al*., 1986; Tartera *et al*., 1992) and clearly visible circular 'zones of lysis' in a confluent lawn of enterococcal host were expressed as plaque-forming units (PFU) (Figure 3.1) per 100 ml of sample.

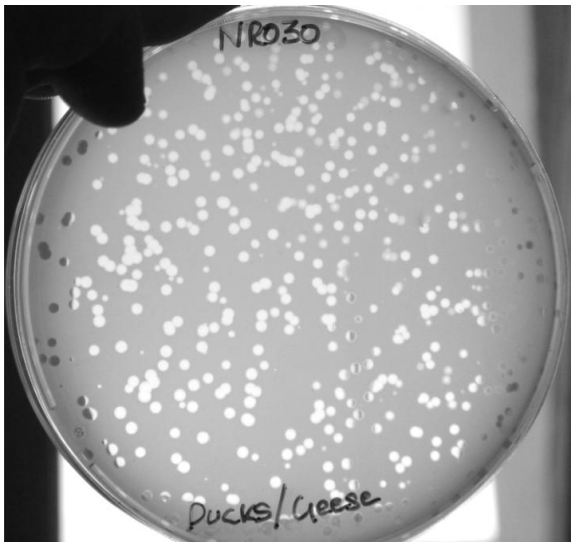

Figure 3.1 Enterococcal host strain demonstrating zones of lysis (plaques)

Homogenised faecal samples were diluted (1:10 w/v) with one quarter strength Ringer's solution and centrifuged at 3000 x g for 10 minutes. The resulting supernatants were filtered through 0.22 µm polyvinylidene difluoride membrane syringe filter units (Millipore, US), to remove bacteria and organic debris, whilst retaining phages in the sample. 1 ml of each sample was added to 1 ml of exponentially growing host strain (approximately 2 x 10^8 bacterial cells per ml, measured as 0.3 OD at 600 nm on a spectrophotometer), and 2.5 ml of tryptone semi-solid agar (TSAss). The resulting suspension was mixed briefly using a Whirlimixer™ (Fisher Scientific, UK), and poured onto previously prepared tryptone soya agar (TSA) (Oxoid, Fisher Scientific, UK) in 90

mm diameter Petri-plates. Once the top layer had solidified, plates were inverted and incubated at 37 ^{o}C (±2 ^{o}C) for 18-24 h. The concentrations of agar in top and bottom layers used were the same as those reported elsewhere (ISO 10705/2) (Anon, 2001[a]).

3.2.2 Spot test assay

The spot test assay has the advantage of being simpler, faster and more efficient than a double agar-layer assay, particularly when large numbers of samples are processed (Mazzocco *et al.*, 2009). The disadvantage of the assay is that only small volumes of sample (5-20 µl) can be assessed. The spot test was therefore used for determining the titres of highly concentrated phage lysates, but not for testing environmental samples.

Double agar-layer plates were produced (as described above), with 1 ml of exponentially growing *Enterococcus* host and 2.5 ml of tryptone soya semi-solid agar (TSAss), without the addition of sample and were poured onto previously prepared 90 mm TSA bottom agar -layer Petri-plates. Once solidified, 10 µl phage lysates and dilutions thereof were spotted onto the top agar, being careful to label each spot, so that they could be identified after incubation (Figure 3.2). The drops were left to air dry before plates were inverted and incubated at 37 ^{o}C (±2 ^{o}C) for 18-24 h. Following incubation, as with the double agar-layer assay method, circular ’zones of lysis’ in the confluent lawn were expressed as PFU per ml of sample.

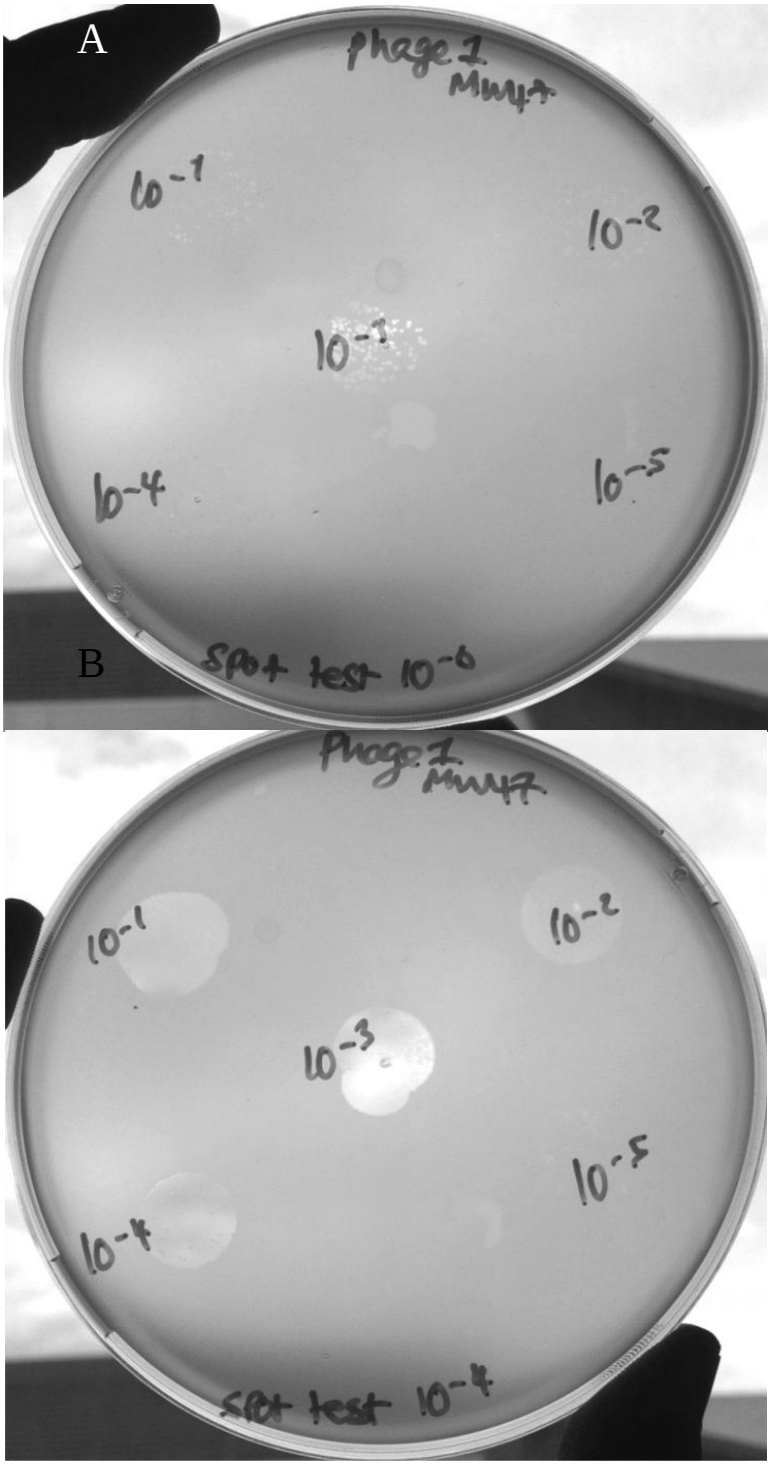

Figure 3.2 Spot test assays showing different degrees of lysis, (A) well-defined plaques and (B) complete clearance as a result of a high phage titre

3.3 Isolation of presumptive *Enterococcus* spp.

Potential host strains were isolated from faecal material from pooled cattle samples, cattle and pig run-off, municipal wastewater, and surface waters impacted by human and non-human livestock sources of faecal contamination in Southeast England, UK. The reference strain *Enterococcus faecalis* (ATCC 19433) was used as a quality control for all media used in the isolation of presumptive *Enterococcus* spp.

Grossly contaminated samples were serially diluted prior to isolation of presumptive enterococci. Samples were passed through 0.45 µm nitrocellulose membrane filters for isolation of presumptive enterococci in accordance with ISO 7899/2 (Anon, 2000). M-*Enterococcus* agar (Difco, BDMS, UK) was used as a selective medium for the isolation of presumptive enterococci, and was incubated at 37 ^{o}C (±2 ^{o}C) for 44 h (±2 h). M-*Enterococcus* agar contains sodium azide to suppress the growth of Gram negative organisms and triphenyl tetrazolium chloride, a dye, which is reduced to red formazan by intestinal enterococci, resulting in red colonies (Anon, 2000) (Figure 3.3).

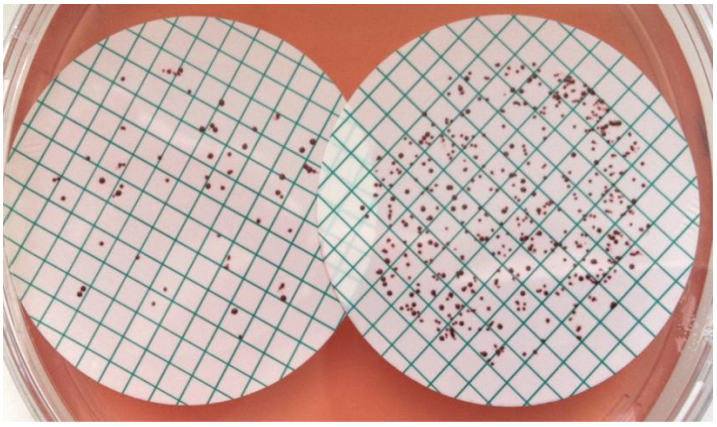

Figure 3.3 Presumptive intestinal enterococci colony forming units (CFU) on m-*Enterococcus* agar (Difco, BDMS, UK) after incubation for 44 hours

Original in Colour

Following incubation, 0.45 µm filter membranes (Thermo Scientific Nalgene, UK) with between 10-60 colony forming units (CFU) were transferred to pre-warmed bile aesculin agar (BEA) (Oxoid, UK) plates and incubated for a further 4 h at 44 ^{o}C. BEA (Oxoid, Fisher Scientific, UK) is used to differentiate enterococci and to distinguish the *Streptococcus bovis* group from other streptococci. Intestinal enterococci are able to hydrolyse aesculin, (6, 7-dihydroxycoumarin, combines with iron (III) ions) giving a tan-coloured to black compound which diffuses into the medium around the colony (Anon, 2000). The appearance of a tan to black colour around the colony therefore acts as a confirmation for presumptive enterococci. Aesculin positive colonies were picked and streaked onto m-*Enterococcus* agar (Difco, BDMS, UK) in order to obtain pure cultures.

Further identification was achieved by undertaking catalase and Gram stain tests (in which morphology was also recorded). Catalase activity was assessed by adding one drop of 3% hydrogen peroxide to a single colony of host on a glass microscope slide. The slide was examined immediately for the generation of gas (bubble) formation. The reference strains *Staphylococcus aureus* (ATCC 6538) and *Enterococcus faecalis* (ATCC 19433) were used as positive and negative controls for catalase tests, respectively. Gram staining was performed with a Gram stain kit (Pro-Lab Diagnostics, UK) and assessed under a conventional light microscope. Presumptive *Enterococcus* host strains (Gram positive, coccoid, catalase negative, and aesculin positive) were then grown at 37 ^{o}C (±2 ^{o}C) for 24 h (±2 h) in tryptone soya broth (TSB). Those host strains demonstrating good growth after 24h (±2 h) were mixed with 50% glycerol (Fisher Scientific, UK) and preserved at -80 ^{o}C in cryogenic vials (Fisher Scientific, UK) for up to six months, prior to further testing. The hosts were named according to their origin. For example, hosts isolated from municipal

wastewater were named MW followed by the number of their isolation, MW1 being the first host isolated from municipal wastewater, and so on.

3.4 Calibration of absorbance measurements for counts of viable host bacteria

Standard methodologies for the enumeration of phage (Anon, 2001[a]; Anon, 2001[b]; Anon, 2001[c]), recommend that inoculum host cultures used in double agar-layer assays should be used once they reach an exponential (log) growth phase, when the suspension contains approximately 2 x 10^8 to 5 x 10^8 cells/ml. Previous research has also shown that host culture cell numbers of between 2 x 10^8 and 5 x 10^8 cells/ml produce the largest and most visible plaques (Tartera *et al*., 1992). It was therefore important to determine the absorbance measurements that corresponded to these cell densities for the *Enterococcus* host strains, in order to optimise plaque visualisation. Initial growth curves were performed using *Enterococcus* host strain CR1, to calibrate absorbance measurements for subsequent phage assays.

Growth curves were performed by growing the host strain (CR1) in TSB at 37 ^oC. The optical density (OD) was measured at 30 minute intervals using a spectrophotometer at 600nm and zeroed using a pre-warmed TSB blank, and 1 ml of the host (and dilutions thereof) were taken and passed through 0.45 µm membrane filters in duplicate. The filters were placed onto m-*Enterococcus* agar and incubated at 37 ^oC (±2 ^oC) for 44 h (±2 h) (Anon, 2000). Resultant colonies growing on the surface of the agar were enumerated and expressed as mean CFU per ml.

3.5 Screening for potential host strains

In order to facilitate the isolation of potential *Enterococcus* host strains a tiered approach was designed (Purnell *et al*., 2011 and discussed in detail in Chapter Five). This was implemented in order to reduce, in a rational manner, the initial large number of enterococcal hosts to a smaller sub-group that would be suitable for phage enumeration and MST (Figure 3.4). This approach provided a protocol that could rapidly eliminate enterococcal strains that would not be effective hosts (due to a lack of specificity and low phage detection), and focus efforts on those host strains warranting further investigation.

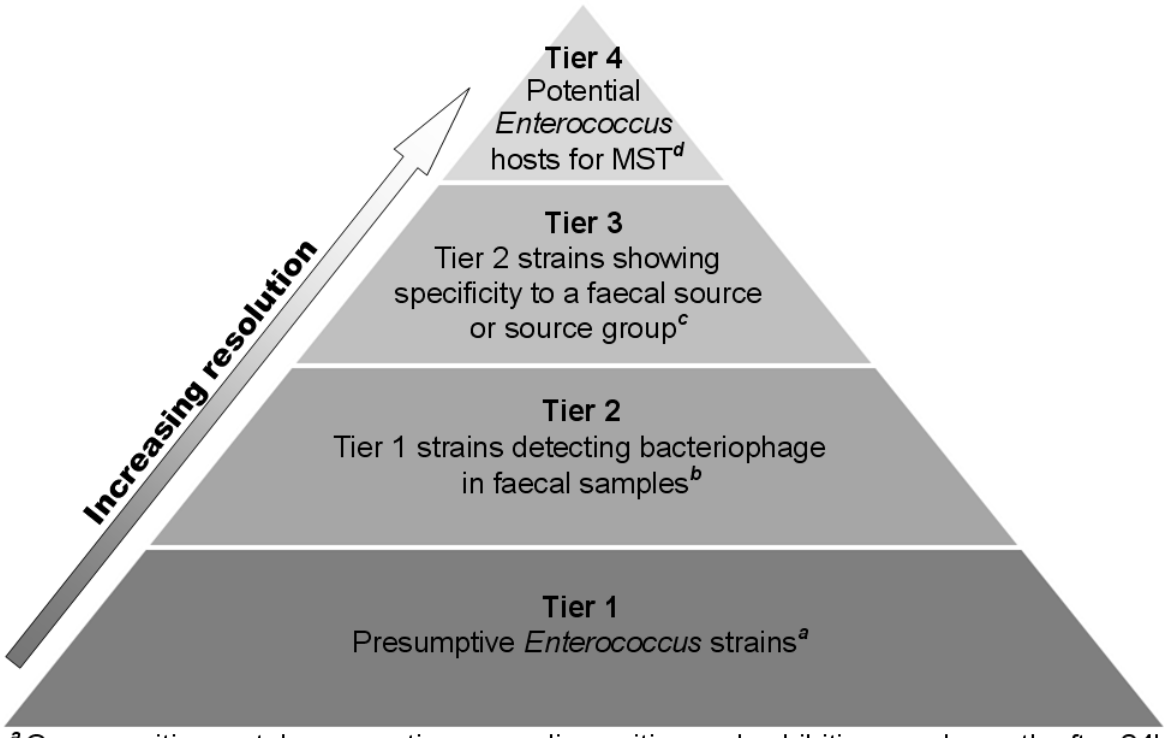

Figure 3.4 Tiered approach to the isolation of *Enterococcus* host strains

Tier 1 hosts were those strains confirmed as being presumptive *Enterococcus* spp. that grew well at 37 ^{o}C (±2 ^{o}C) for 24 h (±2 h) in TSB. All tier 1 host strains were screened against a battery of reference samples containing phages from municipal wastewater, and cattle, sheep and pig faeces. Reference samples were comprised of pooled faecal or wastewater samples. This allowed the initial specificity of host strains (to a particular source) to be determined rapidly from a small number of assays, keeping labour and consumable costs to a minimum. It is important to note that the use of pooled samples can inflate the sensitivity (to a particular source) of host strains. For example, if one sample positive for phages is included into a pooled sample, the sample will be positive even if all other samples included are negative. For this reason the sensitivity of the host strains was not calculated at this stage. Reference samples were prepared in bulk in advance of screening and preserved in multiple containers with 10% glycerol at -20^{o}C. For every new batch of host strains tested, fresh reference samples were defrosted and used within 24h. Enterococcal strains that detected one or more PFU per 100 ml (tier 2 hosts) were ranked according to their specificity and to the numbers of phages that they detected (PFU/100 ml). It was also important that phages infecting the hosts produced clear well-defined plaques (Figure 3.1). Hosts that demonstrated plaques that were unclear, and therefore difficult to identify and enumerate, were not assessed further. Tier 2 hosts that showed specificity to a particular faecal source or source group (tier 3 hosts), and which also detected phage in greater numbers than 100 PFU per 100 ml (tier 4 hosts) were considered to be the host strains with the greatest potential for MST application.

To assess further the performance of the hosts for MST application, all tier 4 hosts (Figure 3.4), as well as tier 3 hosts demonstrating 100% specificity and clear plaque production,

were subjected to additional testing using a broad range of inputs representative of those present across the study catchment (i.e. municipal wastewater and faeces from cattle, ducks, geese, goats, horses, pigs, rabbits, and *Larus* spp. gulls) in triplicate.

3.6 Identification of potential host strains

Identification of presumptive *Enterococcus* host strains to species level was carried out using the API 20 Strep identification system (BioMérieux, UK) according to the manufacturer's instructions, and a six step biochemical key described by Manero and Blanch (1999). Initially, only API 20 Strep identification was used. The biochemical key was introduced after API 20 Strep failed to identify successfully three of the isolates. All host strains were then subsequently re-tested and reconfirmed using the six step biochemical key (Manero and Blanch, 1999). A negative control was used for both identification methods

API 20 Strep strips combine twenty biochemical tests (enzymatic and carbohydrate fermentation tests) and a haemolysis test, enabling the identification of the majority of streptococci and enterococci. To test for haemolysis, a well-isolated host colony was suspended in 300 µl of sterile water and homogenised. This suspension was used to flood a Columbia sheep blood agar plate (BioMérieux, UK), which was then incubated for 24 h (±2 h) at 36 ^{o}C (±2 ^{o}C) under anaerobic conditions. Following incubation, a reaction was recorded as either ß- haemolysis positive (complete lysis of red cells in the media), or negative. API strip tests were performed in accordance with the manufacturer's instructions. For enzymatic tests, a positive reaction resulted in a colour change, either spontaneously, or after the addition of reagents (Figure 3.5), signalling metabolism.

Positive fermentation of carbohydrates resulted in a shift in pH level and a colour change from red to yellow (Figure 3.5).

Figure 3.5 Inoculated API 20 Strep test strip before incubation (A) and after incubation (B), with resultant colour changes. This isolate was consequently identified as *Enterococcus* gallinarum

To aid identification using API 20 Strep, tests for yellow pigmentation were also performed. Production of yellow pigmentation was demonstrated by growing the enterococcal hosts on nutrient agar and incubating for 24 h at 37 ^{o}C. Following incubation, host strains were checked for yellow pigmentation against a white filter paper.

The biochemical key described by Manero and Blanch (1999) consists of a total of twelve different tests, with only six tests required for the identification of an isolate. The key identifies nineteen species of *Enterococcus* (Figure 3.6).

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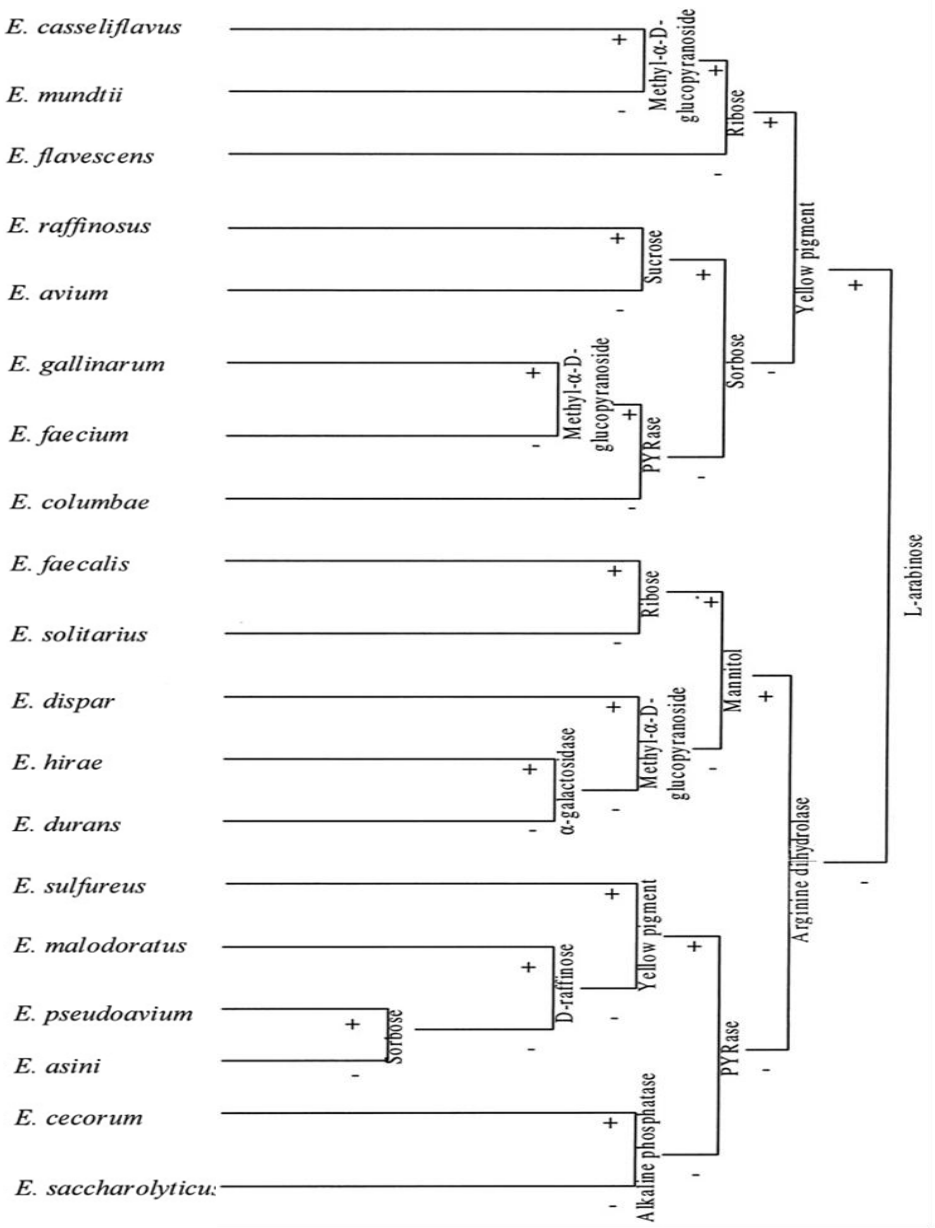

Figure 3.6 Identification key for *Enterococcus* spp. (Manero and Blanch, 1999)

Only nine tests were required for the identification of isolates in this study. Six carbohydrate fermentation tests (L-arabinose, mannitol, methyl-α-D glucopyranoside, ribose, sorbose, and sucrose) were performed along with the yellow pigmentation, pyrrolidonyl aminopeptidase (PYRase) (Oxoid, Fisher Scientific UK) and arginine dihydrolase tests.

To determine carbohydrate fermentation, one percent concentrations of L-arabinose, mannitol, methyl-α-D glucopyranoside, ribose, sorbose, and sucrose were added to phenol red broth, with the pH adjusted to between 7.4 and 7.5 with sodium hydroxide. The phenol red broth (200 µl), with carbohydrate added, was pipetted into the wells of 96-well sterile microplates (Fisher Scientific, UK). A small quantity of isolated host was picked using a toothpick from a streaked plate and inoculated into each well containing carbohydrate. The 96-well plates containing the inoculated carbohydrate broths were then incubated at 37 ^{o}C (±2 ^{o}C) for 24 h (±2 h). A positive reaction was only recorded if the broth turned yellow. PYRase activity was determined using the Oxoid Biochemical Identification System (O.B.I.S) PYR test kit. The test was performed according to the manufacturer's instructions. Development of a vivid purple colour on and around the colonies within 20 seconds confirmed PYRase activity. Finally, an arginine dihydrolase test was performed according to standard methods (MacFaddin, 2000) using Møller decarboxylase base medium. The medium, which had a thin mineral oil layer added to the surface, was inoculated with host and incubated at 37 ^{o}C (±2 ^{o}C) for 24 h (±2 h). A purple to violet colour change after 24 h (±2 h) indicated a positive result.

3.7 Phage isolation, purification and concentration

Well-distributed plaques enumerated by the double agar-layer method (section 3.2.1) were picked at random for isolation to avoid plaque morphology bias, with the intention of characterising twenty bacteriophages, from a single host bacterium. Twenty bacteriophages were isolated, as it has been previously suggested that this is the minimum number necessary to estimate the diversity of a microbial population (Bianchi and Bianchi, 1982).

The *Enterococcus* host strain analysed for bacteriophage isolation was MW47 (Municipal Wastewater 47), an *Enterococcus faecium* host that showed specificity to wastewater in this study (Chapter Six). This host strain was chosen because it has high potential for future application in MST investigations. Phage were purified and concentrated by a plate propagation method, modified from previously described methods used by Carey-Smith *et al*. (2006) and Fard *et al*. (2010). Cores of agar containing a distinct single plaque were picked using sterile glass Pasteur pipettes (Figure 3.7) and suspended in 200 µl of phage buffer (19.5 mM Na_2HPO_4, 22 mM KH_2PO_4, 85.5 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) in microcentrifuge tubes (Fisher Scientific, UK). The phage suspensions were left at 4^oC overnight to allow diffusion of phage into the buffer. The phage suspensions and dilutions were retested with the double agar-layer method (section 3.2.1), to purify and confirm the presence of phage. This was repeated three times to obtain purified phage.

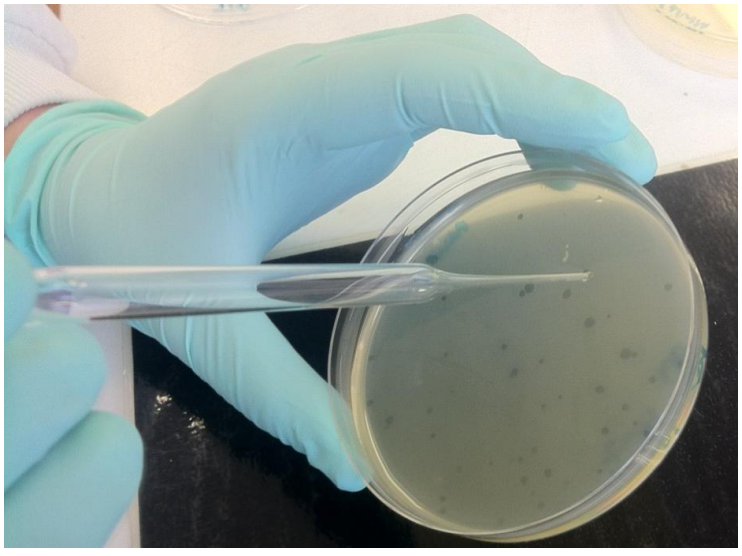

Figure 3.7 A core of agar containing a single plaque, picked using a sterile glass Pasteur pipette

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Once purified, 5 ml of phage buffer were added to plates with near complete lysis of the host bacterium, and left at room temperature for 1 h, the plates being 'swirled' regularly. The liquid and top agar-layer were then scraped into a 50 ml centrifuge tube (Fisher Scientific, UK), and mixed briefly using a Whirlimixer™, and left at room temperature for a further thirty minutes. Bacterial debris and top agar-layer were removed from the suspension by centrifugation at 3000 *g* for 20 min. The supernatant was then filtered through 0.22 µm polyvinylidene difluoride membrane syringe filter units, and stored in light tight glass bottles at 4 ^{o}C in the dark. The titre of the suspension was determined by testing ten-fold dilutions (10^{-1}-10^{-8}) using the spot test assay (section 3.2.2). The process was repeated until a minimum titre of 1 x 10^{8} PFU/ml was achieved with all phage suspensions.

3.8 Phage characterisation

3.8.1 Transmission electron microscopy

All twenty propagated phages were examined by transmission electron microscopy (TEM), so that phage morphology could be determined. Novel phages are no longer characterised using TEM alone, but TEM was chosen for this study because the technique has been widely applied in virology and allows instant comparison, classification, and identification of viruses. Further characterisation of phages was not required for this research. It was important to determine the level of morphological diversity between the phages, because phages with differing morphology may have dissimilar resistance to inactivation, limiting their use as MST markers. This is discussed further in section 3.9. In order to view phage under the TEM, the phage suspensions were negatively stained. This is achieved by mixing the phage particles with an electron-dense solution of a metal salt of high molecular weight

and small molecular size, into which the particles are embedded, appearing white on a dark background (Ackermann, 2009). Uranyl acetate (UA) stain (pH 4-4.5) was used to stain the phage suspensions. UA produces good contrast but does have the disadvantage of producing unpredictable results, as it can produce both negative and positive staining, often on the same grid (Ackermann, 2009). One drop (10 µl) of previously prepared high-titre phage suspension (section 3.7) was applied to 200 mesh Formvar/Carbon copper electron microscope grids (Agar Scientific, UK). After two minutes, any excess suspension was removed with Whatman No.1 filter paper (Whatman, UK). One drop (10 µl) of UA stain (1 % w/v, previously filtered through a 0.22 µm filter unit) was then applied to the grid for one minute. Excess stain was removed again with Whatman No. 1 filter paper, and the grids were then left to dry. Grids were kept in labelled Petri-plates (55mm) prior to viewing under the TEM (Hitachi-7100) at 100 kV.

3.8.2 Assessment of phage host range

The host range of a bacteriophage is defined by what bacterial genera, species and strains it can lyse (Kutter, 2009). The phages isolated from the *Enterococcus faecium* host strain MW47 were tested for their ability to infect other species of the genus *Enterococcus* (including, *E. asini, E. casseliflavus, E. durans, E. faecalis, E. gallinarum, E. hirae,* and *E. mundti, E. pseudoavium, E. saccharolyticus,* and *E. sulfureus*), and their ability to infect two other bacterial host strains from genetically very different genera, GB-124 (*Bacteroides*) and WG5 (*E. coli*). Host strains used in MST, require phage with narrow host ranges, to ensure the accurate detection of faecal contamination from the target source. Host range also provides further information on phage diversity and was determined by

spot tests of dilutions (10^{-1}-10^{-8}) of each high titre phage stock suspension on lawns of each host bacterium. Spot tests were performed in triplicate and reported as mean PFU/ml.

3.9 Phage inactivation experiments

In vitro inactivation experiments were performed to gain a greater understanding of the inactivation characteristics of phages capable of infecting host strain MW47. It was important to perform these initial investigations into phage survival at this point because an observed limitation of some phage-lysis methods has been the different resistance of phages (infecting certain host strains) to inactivation from natural stressors and wastewater treatment (Muniesa *et al*., 1999; Brion *et al*., 2002; Long and Sobsey, 2004; Schaper *et al*., 2002; Muniesa *et al*., 2009). These limitations are discussed in detail, in section 2.4. The three phages (from the 22 isolated in this study active against host strain MW47) with the most distinct morphologies (MW47-1, MW47-5 and MW47-15) were chosen for *in vitro* inactivation experiments in fresh- and sea-water.

Two litre surface water samples (sea water and fresh water) were taken from Newhaven beach (adjacent to the mouth of the River Ouse), UK, and from the Bevern Stream, a tributary of the River Ouse, UK. The samples were analysed for temperature, pH level, electrical conductivity, salinity, dissolved oxygen, and turbidity, *in situ* using an Aquaread probe (Aquaread Ltd, UK). All three phages were spiked into 500 ml of both fresh water and sea water in stoppered glass bottles (bottles were pre-sterilised by autoclaving at 121 ^{o}C for 15 minutes) at concentrations greater than 1 x 10^{4} PFU/ml. High titre phage concentrations were used to avoid interference from background levels of phages cable of infecting host strain MW47. To limit the variables affecting phage survival, the stoppered

glass bottles were wrapped in foil to exclude light and placed in the dark at 4 ^{o}C. All three phage were enumerated by the double agar-layer method (section 3.2.1) for both fresh water and sea water in duplicate, immediately after spiking (T_0), and after two (T_{48}), seven (T_{168}), 14 (T_{336}), 21 (T_{504}) and 28 (T_{672}) days. Results were reported as mean PFU per ml of sample.

3.10 Statistical analysis

Statistical tests were performed using the statistical package SPSS 18.0, with the significance level set at 5%. The Spearman's correlation coefficient, Wilcoxon signed-rank, Friedman's ANOVA, Kruskal-Wallis and Mann Whitney tests are all non-parametric and were used where the data failed to meet parametric assumptions (not normally distributed), even after log-transformation. Parametric statistical test ANOVA and the independent *t-test* were used when analysing phage inactivation data, as these data were normally distributed and therefore did meet parametric assumptions.

3.10.1 Specificity and sensitivity

Performance of the enterococcal strains as potential hosts for MST was evaluated in relation to their specificity and sensitivity to a particular faecal source. Wilbur and Whitlock (2007) define the sensitivity and specificity with respect to a particular source as the "probability that a sample from that source will be correctly identified as originating from that source", and the "probability that a sample which is not from that source is correctly identified as not originating from that source" respectively. Table 3.2 shows the possible outcomes when testing a sample.

Table 3.2 Four possible outcomes of source identification (Wilbur and Whitlock, 2007)

Actual status	Test positive	Test negative
Positive	True Positive (TP)	False negative (FN)
Negative	False positive (FP)	True negative (TN)

A result was considered a true positive when phages were correctly detected in a target faecal sample (e.g., a human associated host strain detected phages in a human wastewater sample). A result was considered a true negative when phages were not detected in a non-target faecal sample (e.g., a human associated host strain did not detect phages in a cattle faecal sample). False positive results were recorded where phages were detected in non-target samples (e.g., a human associated host strain detected phages in a cattle faecal sample) and false negative results were recorded when phages were not detected in target samples (e.g., a human associated host strain did not detect phage in a human wastewater sample). Once the outcomes of source identification were recorded the sensitivity and specificity percentages of *Enterococcus* host strains could be calculated as follows:

$$\text{Sensitivity (Se)} = \frac{TP}{(TP+ FN)} \text{ x100}$$

$$\text{Specificity (Sp)} = \frac{TN}{(TN+FP)} \text{ x100}$$

Qualitative approaches to calculating specificity and sensitivity such as this are only one part of determining methodological performance, but approaches like this are an important first step. It is important to note however that this approach does not harness the potential

of quantitative measurements, which are becoming increasingly important (Wuertz *et al*., 2011).

3.10.2 Spearman's rank correlation coefficient

The Spearman's rank correlation coefficient was used to test the hypothesis that a negative relationship between specificity and sensitivity existed. The test operates by first ranking the data and then applying Pearson's equation, shown below:

$$r = \frac{cov_{xy}}{s_x s_y} = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{(N-1)s_x s_y}$$

Where x is the first variable, y is the second variable, cov is the covariance, s_x and s_y are the standard deviations of the first variable and second variable, $\bar{x}$ and $\bar{y}$ are the sample means of x and y, x_i and y_i are particular sample points, and N is the total sample size (Field, 2009).

3.10.3 Wilcoxon signed-rank test

The Wilcoxon signed-rank test was used to determine the significance of differences between plaque sizes and phage numbers detected by MW47 at two optical densities (OD). The test is based on the differences calculated between the two conditions. Positive and negative ranks are calculated and the smaller of the two numbers becomes the test statistic (T). To calculate the significance of T, the mean $(\bar{T})$ and standard error $(\mathrm{SE}_{\bar{T}})$ are determined. The test statistic is then converted to a z-score using the following equation:

$$z = \frac{x - \bar{x}}{s} = \frac{T - \bar{T}}{SE_{\bar{T}}}$$

If the values are larger than 1.96 then the test is significant ($p < 0.05$) (Field, 2009).

3.10.4 Friedman's ANOVA

Friedman's ANOVA was used to determine whether host strain MW47 detected statistically greater phage numbers and plaque sizes (using the reference phage MW47-1) at different optical densities. It is designed to test the differences between several related groups and is based on ranked data. Each of the data points for all optical densities were ranked. Once the sum of ranks was calculated for each of the optical densities, the test statistic, F_r, is calculated as:

$$F_r = \left[\frac{12}{N\hbar\,(\hbar + 1)} \sum_{i=1}^{\hbar} R_i^2 \right] - 3N\,(\hbar + 1)$$

R_i is the sum of ranks for each group, N is the total sample size and k is the number of conditions (Field, 2009).

3.10.5 The Kruskal-Wallis test

The Kruskal-Wallis test was used to determine variation of specificity and phage numbers detected on host strains obtained from different source groups. First the data are ranked, ignoring the groups to which the data belong. The scores are then collected back into groups and added together. The sum of ranks for each group is denoted by R_i (where i is

used to denote the particular group). After the sum of ranks is calculated for each group, the test statistic, *H*, is calculated as:

$$H = \frac{12}{N\,(N-1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3\,(N+1)$$

Where R_i represents the sum of ranks for each group, N is the total sample size and n_i represents the sample size of the particular group (Field, 2009). The Kruskal-Wallis test helped to determine whether variation of specificity and phage numbers detected, between the host source groups occurred. The test however, does not demonstrate between which groups this variation took place. *Post hoc* Mann Whitney tests were therefore used to assess where the variation occurred.

3.10.6 *Post hoc* Mann-Whitney tests

Six Mann-Whitney tests were used as a follow up to the Kruskal-Wallis test to determine variation between each of the host strain source groups for both specificity and phage numbers detected. By performing multiple Mann-Whitney tests, the Type I error rate is inflated, which is why Mann-Whitney is not used instead of the Kruskal-Wallis test. The Bonferroni correction (Sullivan *et al*., 2011) was used to prevent type I errors from exceeding 0.05. Therefore, instead of using 0.05 as the critical value for significance for each test, the critical value of 0.05 divided by the number of tests conducted was used. Six tests were performed, and so the critical value of significance used was 0.008.

3.10.7 Independent *t-test*

An independent *t-test* was used to test the significance of differences in inactivation between two phage families isolated in this study. The test produces a t-statistic which is calculated using the equation below (assuming the sample sizes are equal):

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\left(\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}\right)}}$$

Where $\bar{x}_1$ and $\bar{x}_2$ are the overall means for the two sample groups, s_1 and s_2 are the standard deviations of the two sample groups and N_1 and N_2 are the total sample size of the two groups (Field, 2009).

3.10.8 Independent ANOVA

Independent ANOVA was used to determine the significance of differences between the inactivation rates of phages. ANOVA tests the null hypothesis that all group means (differences) are equal. This is accomplished by fitting a regression model to the data and using the *F-statistic* to determine how well the data fit the model (Field, 2009). *F* is the ratio of the model to its error and is calculated using the equation below:

$$F = \frac{MS_M}{MS_R}$$

Where MS_M represents the mean squares for the model and MS_R represents the residual mean squares.

3.10.9 Inactivation rate coefficient

In accordance with the literature, the inactivation rate coefficient (k_D) was calculated for phages MW47-1, MW47-5 and MW47-15 in freshwater and seawater so that comparisons between the phages could be made. Linear regression was applied to log-transformed values of phage enumerations to determine k_D values in log units per day (Noble *et al*., 2004). For further comparison with results from other studies, the time to reach a 90% reduction in phage concentrations (T_{90}) was calculated. T_{90} values were taken from the k_D value as 2.303/ k_D (given that 1n (0.1) = -2.303) (Noble *et al*., 2004; Santiago-Rodríguez *et al*., 2010).

Chapter Four: Development of a tiered screening approach

A tiered screening approach was created in order to maximise the chance of successfully identifying an *Enterococcus* host strain suitable for MST application (Purnell *et al*., 2011), and reducing the effort required to screen for effective new host strains (Figure 3.4). The aim was therefore to create a system that rapidly eliminated enterococcal host strains that would not be suitable for MST application, allowing effort to be focussed on those host strains worthy of further investigation. Four screening tiers were designed, each of which considered characteristics previously demonstrated to be important for effective MST.

4.1 Tier 1

The target host was the genus *Enterococcus*, so the first screening tier was primarily designed to eliminate other genera. At this stage, host strains that did not demonstrate good growth overnight in TSB were also eliminated from further investigation. The ability of a host strain to grow relatively rapidly was considered important, largely because, as highlighted above, rapid methods have the potential to produce more reliable information for health risk management and as such may ultimately lead to a reduction in costly and unnecessary closures of recreational waters.

4.2 Tier 2

The second tier was designed to reduce further the potential number of host strains by screening them against a battery of reference samples containing phages from municipal wastewater, and cattle, sheep and pig faeces. Host strains that did not detect phages in these

reference samples were not investigated further. The four reference samples were selected to represent major sources of faecal contamination present in Southeast England. Therefore host strains demonstrating specificity to these faecal sources, may have applications as useful MST hosts within this geographical region. Host strains were not tested against a broader set of faecal samples at this stage, in order to minimise time and costs associated with labour and consumables during the screening process.

4.3 Tier 3

It has been proposed that emerging candidate MST approaches should be assessed using a unified set of performance criteria, which would allow techniques to be compared on a rational basis, and enable those that do not meet set standards to be eliminated at an early stage (Domingo *et al*., 2007; Field and Samadpour, 2007). The specificity of a marker to a faecal source, is considered one of the most important criteria for effective MST, and this characteristic is widely used to assess their performance as an MST marker (Gourmelon *et al*., 2007; Stoeckel and Harwood, 2007; Harwood *et al*., 2009). Specificity was chosen as the major criterion to assess the performance of the host strains at this stage of the screening process. Tier 2 host strains were ranked, and divided into three groups:

1) host strains demonstrating no specificity;

2) host strains demonstrating specificity to a broader source category (e.g., non-human or ruminants); and,

3) host strains with specificity to a single faecal source (e.g., cattle faeces).

Host strains that demonstrated specificity to a source category, or to an individual faecal source were considered to be useful for MST purposes, and were consequently elevated to the third tier.

4.4 Tier 4

Finally, the threshold level of faecal contamination above which MST markers can be detected (known as the detection limit) is a key consideration (Stoeckel and Harwood, 2007). The phage must be present at sufficiently high concentrations in faecal samples if they are likely to be present at detectable levels in faecally contaminated waters. Consequently, the fourth and final tier was designed to eliminate host strains that detected phage at very low numbers in faecal samples (<100 PFU/100ml). This tiered approach to selecting potentially effective MST markers resulted in a more manageable group of strains, which were subsequently challenged against faeces from a range of sources in the study region.

Chapter Five Results: Development of the method

5.1 Development of a double agar-layer assay for *Enterococcus* host strains

Although the double agar-layer assay has been standardised for the detection and enumeration of somatic coliphages infecting *Escherichia coli* host strains (Anon, 2001[a]), phages infecting *Bacteroides* spp. (Anon, 2001[b]), and F-specific RNA phages (Anon, 2001[c]), a number of modifications were required in order to optimise the assay for use with *Enterococcus* host strains. Ideally assay optimisation should be undertaken for every new host strain used. However it was not feasible to optimise the method for all the host strains isolated throughout this research programme. Therefore to keep labour and consumable costs to a minimum, the host strain 'Cattle Run-off 1' (CR1), and reference phage CR1-1, isolated from Wales Farm, Plumpton, UK, were used in all initial double agar-layer assay development experiments. Additional optimisation was performed for host strain 'Municipal Wastewater 47' (MW47) before use in survival experiments.

5.1.1 Development of assay growth medium

M-*Enterococcus* agar (Becton Dickinson Microbiology Systems, UK), nutrient agar (NA), KF streptococcus agar, brain heart infusion (BHI) agar and tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) were all tested as potential top and bottom agar-layers for phage enumeration. The concentrations of agar used in both agar-layers were the same as those reported in standard phage enumeration methodology elsewhere (ISO 10705/2) (Anon, 2001[a]). The two main considerations in choosing the medium were the growth of a confluent lawn of bacteria, which was required for the visualisation and clarity of plaques

on the agar surface. However, m-*Enterococcus* agar and KF streptococcus agar, both selective media for the isolation of *Enterococcus* spp., did not support sufficient growth to produce a confluent lawn of bacteria. NA, BHI agar and TSA, all successfully supported growth of the host strain to produce a confluent lawn of bacteria and when double agar-layer assays were performed, plaques were visible on all three media. The clearest plaques however, were obtained using TSA for both agar-layers. This observation was in agreement with results from a previous study that focused on phage of *E. faecalis* (Bonilla *et al.*, 2010). Therefore, TSA was selected for all future phage assays.

5.1.2 Calibration of absorbance measurements for counts of viable host bacteria

It was important to calibrate absorbance measures for counts of viable host bacteria because cell density affects the length of time available for productive phage infections. The majority of phages require the host to be in an exponential growth phase to sustain productive infections (Gallet *et al*., 2011). Plaque size and phage yield can therefore be maximised when cell density is optimised. A growth curve was performed for host strain CR1 (methodology discussed in Chapter Three), in order to calibrate the absorbance measurements for viable counts of enterococcal host bacteria. The growth curve was repeated in triplicate. As it was not feasible to perform growth curves for all potential host strains, the results for CR1 (Figure 5.1) were used as a guideline for future phage assays using isolated enterococcal host strains.

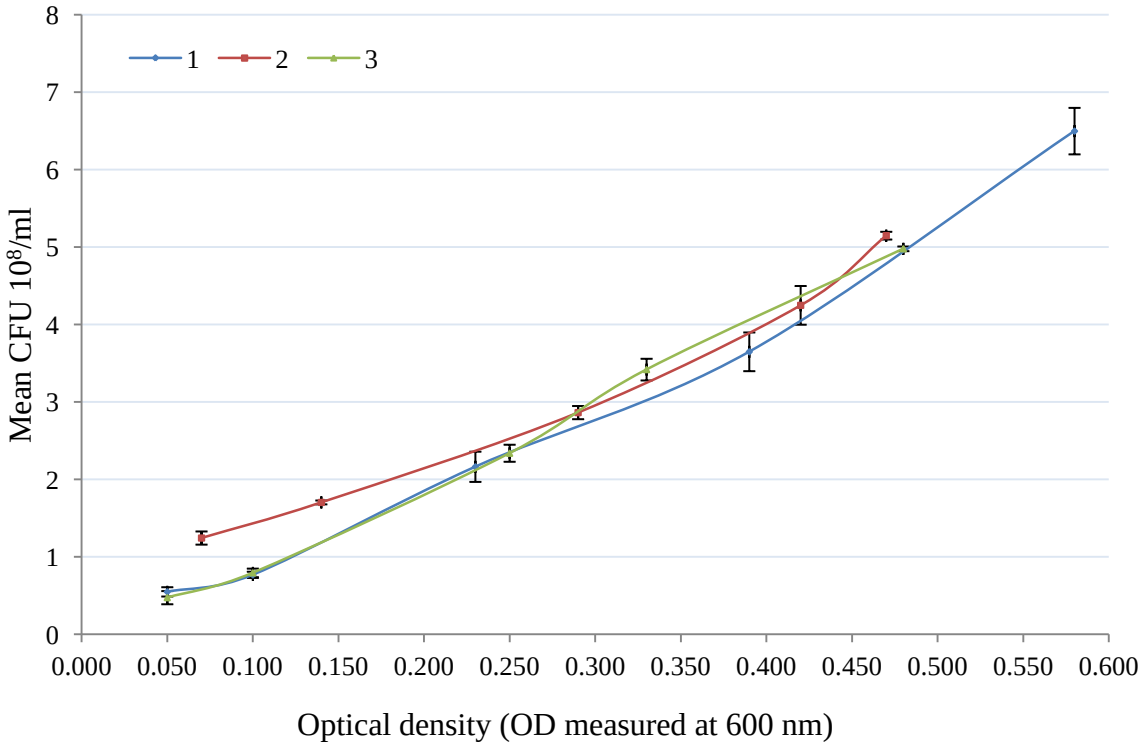

Figure 5.1 The results of three growth curves performed with *Enterococcus* host strain CR1 (Error bars represent the standard error of triplicate samples)

All three growth curves show an exponential growth phase from an optical density (OD) of 0.050. The recommended optimum cell density (2 x 10^8 cells/ml; Chapter Three) was reached between 0.180 and 0.220 OD and remained within the upper recommended limit (5 x 10^8 cells/ml) until an OD of at least 0.460 was reached. As the host strain consistently reached an optimum cell density at 0.250 OD and greater, all future phage assays were performed with host strains at 0.250 OD. To optimise the plating efficiency of phages infecting host strain 'Municipal Wastewater 47' (MW47) before use in survival experiments, additional growth curves were completed. The results of the three growth curves performed in triplicate are shown in Figure 5.2.

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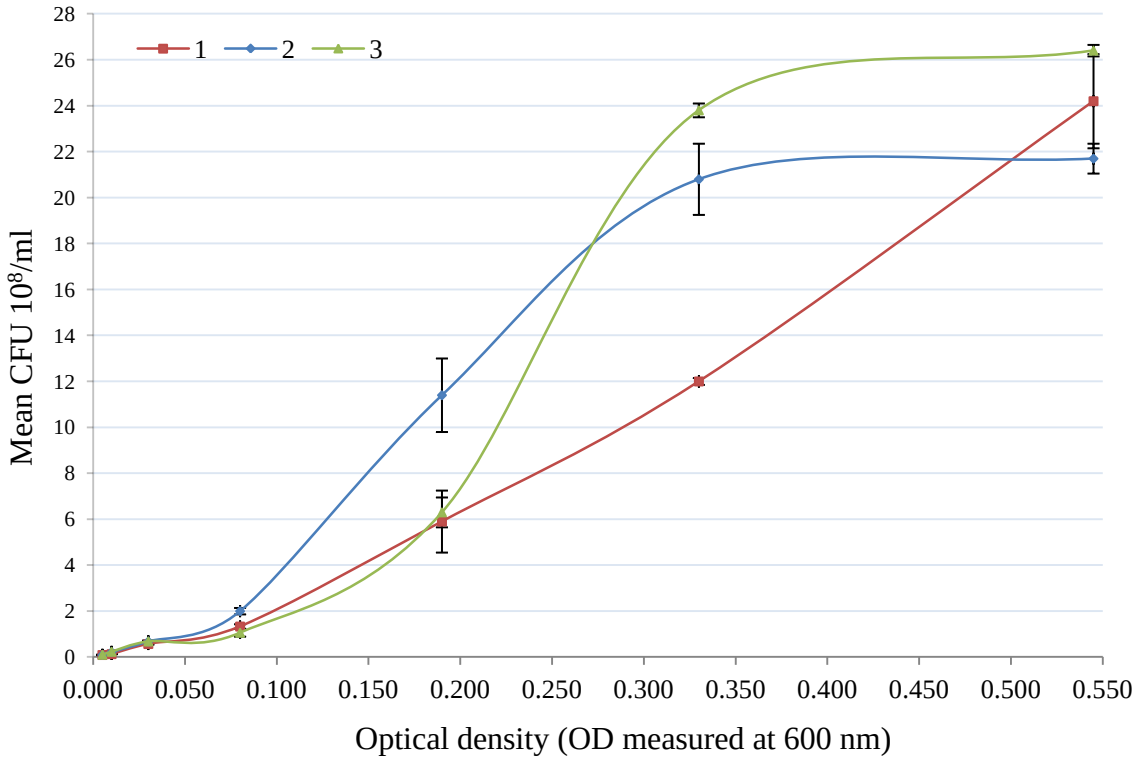

Figure 5.2 The results of three growth curves performed with *Enterococcus* host strain MW47 (Error bars represent the standard error of triplicate samples)

Host strain MW47 displayed faster growth than host strain CR1. Exponential growth was evident from between 0.060 and 0.080 OD. Unlike host strain CR1, two of the growth curves (2 and 3) show diminishing growth at approximately 0.280 OD, followed by a stationary phase from an OD of 0.400. An optimum cell density (2 x 10^8 cells/ml) was achieved at between 0.080 and 0.110 OD. The upper recommended limit (5 x 10^8 cells/ml) was reached at between 0.120 and 0.180 OD.

Double agar-layer assays were then performed, in quintuplicate, using a reference phage isolated from *Enterococcus* host strain MW47 (MW47-1). Assays were carried out with the

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host strain MW47 at optical densities of 0.050, 0.100, 0.150, 0.200, 0.250, 0.300, 0.350, 0.400, 0.450, 0.500 and 0.550. The purpose of the assays was to determine the cell density that provided the optimum phage detection and plaque clarity. Plaques with larger diameters are more desirable because they are easier to identify. However in this instance plaques were clearly visible at all optical densities tested and plaque size did not affect plaque clarity (Figure 5.3). The diameters of ten plaques from each assay were measured using a hand lens and a calliper to determine mean plaque size. The results of mean plaque size and mean phage numbers are shown in Figures 5.4 and 5.5.

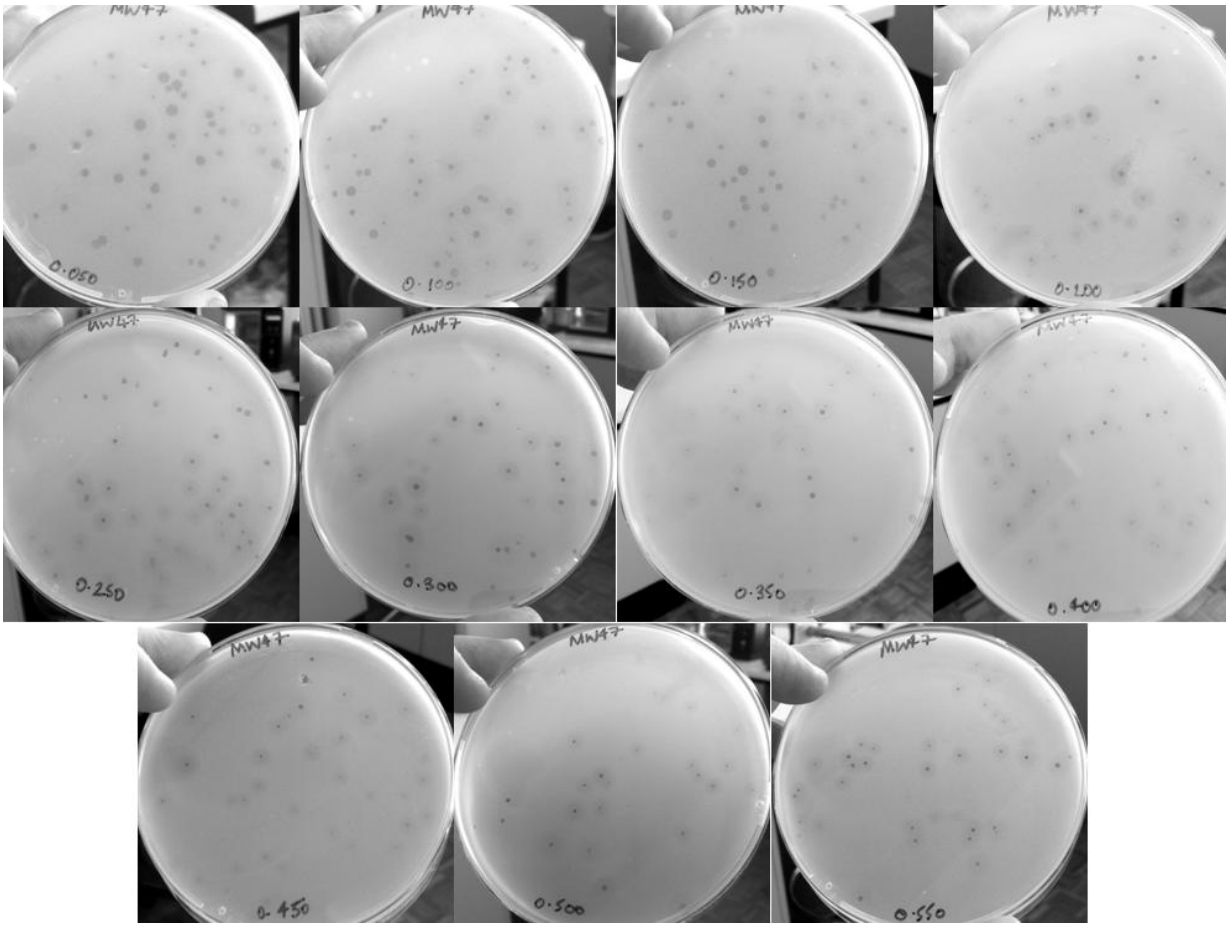

Figure 5.3 Photographic evidence of plaque clarity at different optical densities

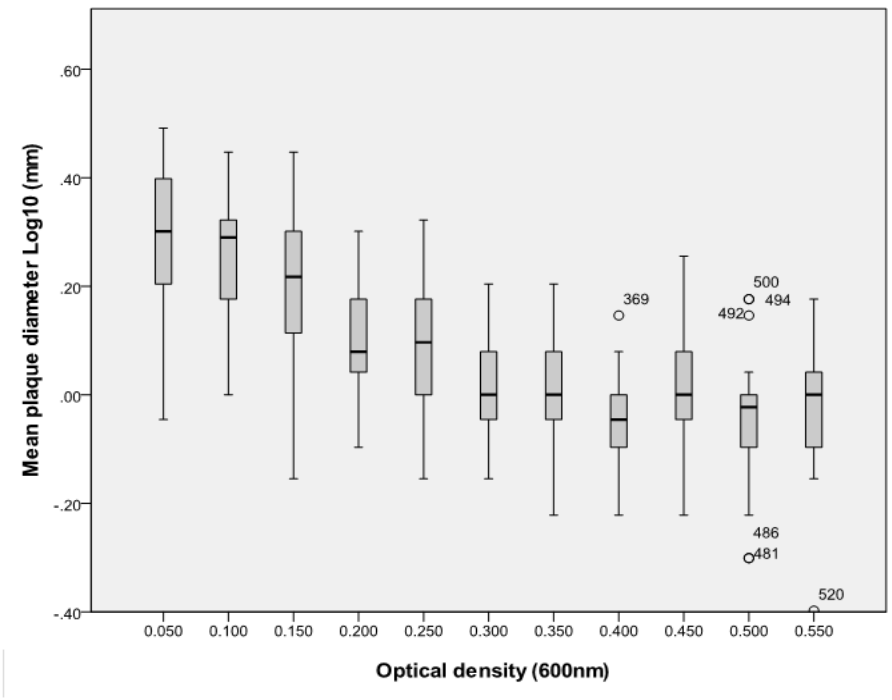

Figure 5.4 Mean plaque size at different optical densities

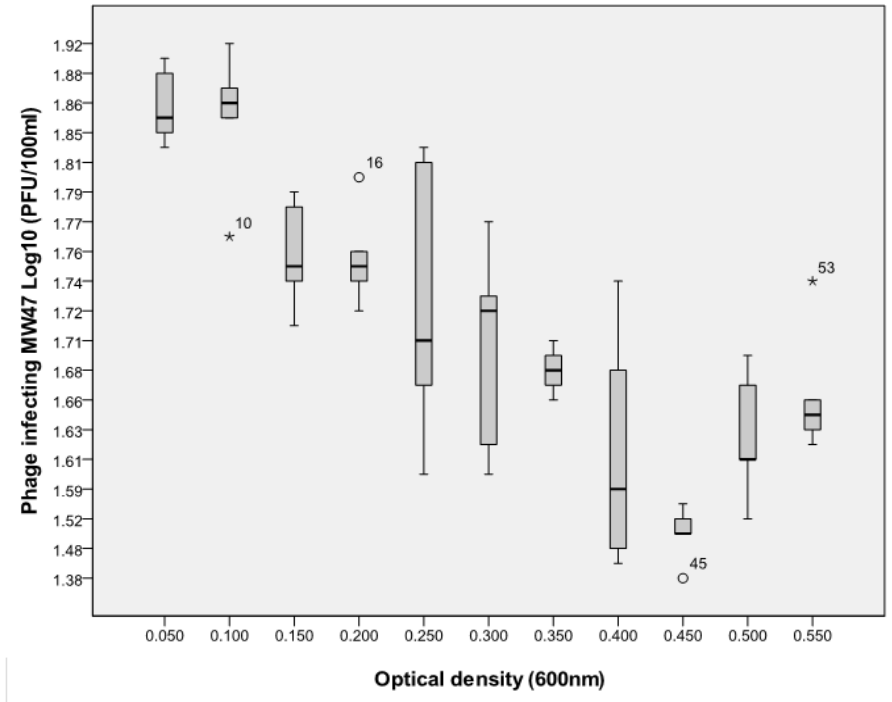

Figure 5.5 Mean plaque numbers at different optical densities

Mean plaque size and phage numbers at different optical densities were significantly varied (P=<0.01; Friedman's ANOVA). The largest mean plaque sizes were observed at 0.050 and 0.100 OD (2.0 and 1.9mm, respectively). The highest mean phage numbers were also observed at 0.050 and 0.100 OD (72 and 71 PFU/ml, respectively). Plaque size and phage numbers were not significantly different between an OD of 0.050 and 0.100 (P=>0.05; Wilcoxon signed- rank). It is likely that plaque diameter and phage yield were largest at an OD of between 0.050 and 0.100, because this OD corresponds to the early exponential growth phase of the host strain (Figure 5.2). All inactivation assays were performed with the host strain (MW47) at an OD of 0.100, because optimum cell density was achieved between 0.080 and 0.180 OD and significantly greater plaque size and phage numbers were observed at optical densities of 0.100 and lower (P=<0.01; Wilcoxon signed- rank).

Chapter Six Results: Isolation of *Enterococcus* host strains

6.1 Isolation and screening of potential host strains

In total, 554 potential enterococcal host strains were isolated and screened using the tiered screening approach (as previously discussed in Chapters Three and Five). Three hundred and ninety potential host strains were initially isolated and confirmed as tier 1 strains (Table 6.1).

Table 6.1 Assignment of potential *Enterococcus* host strains from various sources according to tier category

Host Origin	No. of samples	No. of strains in:			
		Tier 1[a]	Tier 2[b] (%)	Tier 3[c] (%)	Tier 4[d] (%)
Pooled faeces from cattle	1	76	1 (1)	1 (1)	0 (0)
Liquid run-off from cattle	2	83	56 (67)	52 (63)	17 (20)
Liquid run-off from Pig	1	31	21 (68)	12 (39)	1 (3)
Municipal wastewater	1	112	38 (34)	29 (26)	1 (1)
Impacted surface waters	3	88	31 (35)	23 (26)	6 (7)
Total	8	390	147 (38)	117 (30)	25 (6)

[a] Tier 1, Presumptive *Enterococcus* (Gram positive, catalase negative, aesculin positive and exhibiting good growth after 24h at 37°C in TSB). [b] Tier 2, tier 1 strains that detect bacteriophage >0 PFU/ml in human, cattle, pig or sheep reference faecal samples. [c] Tier 3, tier 2 strains that show potential specificity to human, cattle, pig, sheep or animal reference faecal samples. [d] Tier 4, tier 3 strains detecting bacteriophage in reference faecal samples >100PFU/ml.

Thirty-eight percent of tier 1 host strains detected phage in reference samples from cattle, pig, sheep and raw municipal wastewater (tier 2 strains). A high percentage of host strains (67% and 68%) isolated from cattle and pig run-off, respectively detected phages in

reference samples, but far fewer host strains were isolated from both municipal wastewater and surface waters (34% and 35%, respectively) and only one of 76 hosts obtained from pooled cattle pats detected phages in any of the four reference samples.

One hundred and seventeen tier 2 strains (30%) were restricted to one faecal source or source group (tier 3 strains). A large proportion (44%) of the tier 3 host strains, were isolated from liquid cattle run-off. Twenty-five tier 3 strains detected phages in numbers greater than 1.0×10^4 per 100 ml of sample (tier 4 strains). Sixty-eight percent of tier 4 strains originated from cattle run-off, 24% from surface waters, 4% from pig run-off and 4% from raw municipal wastewater. Table 6.2 shows the screening results for the 25 tier 4 host strains and four additional tier 3 strains (selected for further investigation because of their 100% specificity to a particular source and their excellent plaque clarity), which were chosen for further specificity testing using a broader range of inputs representative of those present across the study catchment.

Twenty host strains appeared to be restricted to a single faecal source at this stage. Seven host strains isolated from cattle and pig-runoff detected phage in both pig and cattle faecal run-off reference samples. This could be the result of the close proximity and potential mixing of these faecal sources at the site of isolation (Wales Farm, Plumpton). Interestingly, two host strains isolated from cattle run-off demonstrated broader host ranges, also detecting phages in sheep faeces.

Table 6.2 Mean numbers of plaque forming units (PFU/100ml) detected by tier 4 (and certain tier 3) *Enterococcus* strains in pooled faecal samples from different origins

Host strain origin	Host ID.	Sample Origin (No. of samples)			
		Municipal wastewater (n=7)	Cattle run-off (n=5)	Pig run-off (n=5)	Sheep faeces (n=5)
Cattle run-off	CR1	<1[b]	1.7×10^4	<1[b]	<1[b]
Cattle run-off	CR4	<1[b]	1.4×10^4	<1[b]	<1[b]
Cattle run-off	CR6	<1[b]	2.1×10^4	<1[b]	<1[b]
Cattle run-off	CR7	<1[b]	1.3×10^4	<1[b]	<1[b]
Cattle run-off	CR15	<1[b]	8.3×10^4	<1[b]	<1[b]
Cattle run-off	CR16	<1[b]	4.4×10^4	<1[b]	<1[b]
Cattle run-off	CR17	<1[b]	7.1×10^4	<1[b]	<1[b]
Cattle run-off	CR37	<1[b]	1.2×10^5	<1[b]	<1[b]
Cattle run-off	CR45	<1[b]	1.8×10^4	6.0×10^2	<1[b]
Cattle run-off	CR47	<1[b]	4.8×10^4	1.2×10^3	<1[b]
Cattle run-off	CR51	<1[b]	5.2×10^4	2.0×10^3	<1[b]
Cattle run-off	CR58	<1[b]	1.9×10^4	<1[b]	2.0×10^2
Cattle run-off	CR61	<1[b]	2.2×10^4	1.7×10^3	<1[b]
Cattle run-off	CR63	<1[b]	3.4×10^4	<1[b]	<1[b]
Cattle run-off	CR70	<1[b]	7.3×10^4	1.0×10^3	<1[b]
Cattle run-off	CR73	<1[b]	2.3×10^4	2.1×10^3	<1[b]
Cattle run-off	CR75	<1[b]	4.8×10^4	1.0×10^2	3.0×10^2
Municipal wastewater	MW42[a]	2.7×10^3	<1[b]	<1[b]	<1[b]
Municipal wastewater	MW47[a]	3.4×10^3	<1[b]	<1[b]	<1[b]
Municipal wastewater	MW96	<1[b]	<1[b]	$>2.0 \times 10^5$	<1[b]
Newhaven-River Ouse	NRO5[a]	<1[b]	<1[b]	9.8×10^3	<1[b]
Newhaven-River Ouse	NRO8[a]	3.4×10^3	<1[b]	<1[b]	<1[b]
Newhaven-River Ouse	NRO20	<1[b]	1.0×10^5	<1[b]	<1[b]
Newhaven-River Ouse	NRO24	<1[b]	<1[b]	1.1×10^5	<1[b]
Newhaven-River Ouse	NRO30	<1[b]	1.2×10^4	<1[b]	<1[b]
Newhaven-River Ouse	NRO39	<1[b]	2.5×10^4	<1[b]	<1[b]
Pig run-off	PR3	<1[b]	2.2×10^4	1.0×10^2	<1[b]
Pellingbridge-River Ouse	PRO4	<1[b]	2.0×10^4	<1[b]	<1[b]
Wales Farm Stream	WFS7	<1[b]	6.2×10^4	<1[b]	<1[b]

[a] Tier 3 strains with 100% specificity and excellent plaque clarity, but detected lower plaque counts ($<1.0 \times 10^4$) than Tier 4 strains

[b] Phages were absent in these samples, although no detection limits were determined

6.2 Specificity vs. sensitivity

The suitability of the tier 4 enterococcal host strains (along with the subset of certain tier 3 strains) was further tested by exposing them to additional samples from ten source groups. Host performance was determined using calculated specificity and sensitivity percentages (Table 6.3).

Table 6.3 Specificity and sensitivity percentages of tier 4 (and certain tier 3) strains *Enterococcus* hosts

Host strain origin	Host ID.	Specificity (%)	Sensitivity (%)
Cattle run-off	CR1	93	33
Cattle run-off	CR4	80	33
Cattle run-off	CR6	100	33
Cattle run-off	CR7	93	22
Cattle run-off	CR15	83	33
Cattle run-off	CR16	94	33
Cattle run-off	CR17	100	33
Cattle run-off	CR37	78	33
Cattle run-off	CR45	89	50
Cattle run-off	CR47	78	83
Cattle run-off	CR51	78	83
Cattle run-off	CR58	73	40
Cattle run-off	CR61	81	83
Cattle run-off	CR63	92	17
Cattle run-off	CR70	89	83
Cattle run-off	CR73	67	78
Cattle run-off	CR75	73	60
Municipal wastewater	MW42[a]	100	33
Municipal wastewater	MW47[a]	100	67
Municipal wastewater	MW96	77	60
Newhaven-River Ouse	NRO5[a]	71	40
Newhaven-River Ouse	NRO8[a]	100	7
Newhaven-River Ouse	NRO20	74	33
Newhaven-River Ouse	NRO24	100	20
Newhaven-River Ouse	NRO30	87	33
Newhaven-River Ouse	NRO39	80	33
Pig run-off	PR3	39	71
Pellingbridge-River Ouse	PRO4	79	33
Wales Farm Stream	WFS7	100	33

[a] Tier 3 strains with 100% specificity (detection limits not determined) and excellent plaque clarity, but detected lower plaque counts ($<1.0 \times 10^4$) than Tier 4 strains

For MST purposes it is particularly important that the host strains do not detect phages from both human and non-human sources. Further testing revealed that fifteen host strains were present in animal faeces and municipal wastewater samples, even though they had previously been restricted to either human or non-human sources. This ruled them out from any further analysis. At this stage, fourteen potential host strains (just over 48%) were found to be highly specific to a particular source, or source group. The results for all fourteen host strains specific to either cattle, pig, human or mixed non-human animal faecal sources are presented in Table 6.4. Notably, seven strains appeared to be 100% specific to a single source category.

The mean specificity and sensitivity of the host strains isolated during the study was 84% and 45%, respectively (Table 6.3). Higher specificity (>70%), associated with lower sensitivity (<33%), was evident in over 50% of the tier 4 strains. Strain WFS7 isolated from surface waters downstream of a livestock farm (Wales Farm, Plumpton, UK) at which numerous animals are reared, including a large dairy herd (200+ head of animals), was 100% specific to cattle faecal samples. However, the sensitivity of strain WFS7 was only 33%, and, interestingly, appeared to be restricted to samples originating from the herd present on Wales Farm. A similar result was observed for phages infecting strain NRO24, which was found in all pig faeces (100%) from Wales Farm, but again was not detected in pig faeces from neighbouring farms, resulting in a low overall sensitivity of 20%. Phages infecting these host strains therefore appear not only to be restricted to cattle or pig sources, but to specific cattle or pig herds. This observation suggests the potential importance of diet and of geographical variations in the distribution of certain *Enterococcus* host strains and phages associated with them.

Table 6.4 Samples positive for phages from different origins detected using fourteen potential *Enterococcus* host strains[a]

Host strain ID.	No. of positive samples/ No. of samples tested										
	Cattle	**Chicken**	**Ducks and geese**	**Goat**	**Horse**	**Pig**	**Rabbit**	**Seagull**	**Sheep**	**Raw MW[b]**	**Final MW[b]**
CR1	6/18	0/6	3/6	0/3	0/3	0/6	0/3	0/3	0/6	0/6	0/3
CR6	6/18	0/6	0/3	0/3	0/3	0/6	0/3	0/3	0/6	0/6	0/3
CR7	4/18	0/6	3/6	0/3	0/3	0/6	0/3	0/3	0/6	0/6	0/3
CR15	6/18	0/6	3/3	0/3	0/3	0/6	0/3	0/3	0/6	0/6	0/3
CR16	6/18	0/6	0/3	0/3	0/3	2/6	0/3	0/3	0/6	0/6	0/3
CR17	6/18	0/6	0/3	0/3	0/3	0/6	0/3	0/3	0/6	0/6	0/3
CR70	6/9	0/6	3/3	0/3	0/3	9/9	0/3	0/3	0/6	0/6	0/3
MW42	0/15	0/6	0/3	0/3	0/3	0/12	0/3	0/3	0/6	7/18	3/12
MW47	0/15	0/6	0/3	0/3	0/3	0/12	0/3	0/3	0/6	18/18	3/12
NRO8	0/15	0/6	0/3	0/3	0/3	0/12	0/3	0/3	0/6	3/18	0/3
NRO24	0/15	0/6	0/3	0/3	0/3	3/15	0/3	0/3	0/6	0/6	0/3
NRO30	6/18	0/6	3/6	0/3	0/3	3/6	0/3	0/3	0/6	0/6	0/3
NRO39	6/18	4/6	3/6	0/3	0/3	3/6	0/3	0/3	0/6	0/6	0/3
WFS7	6/18	0/6	0/3	0/3	0/3	0/6	0/3	0/3	0/6	0/6	0/3

[a] Host strains were tested with faecal samples comprised of pooled faecal material from at least twenty individuals (collected from eight farms) and wastewater samples from seven wastewater treatment works all located within South East England
[b]MW= Municipal wastewater

Human-specific host strain MW47 demonstrated a much higher sensitivity, detecting phages in all raw municipal wastewater samples (100%) tested from six WWTW, one of which has a population equivalent of only 258. However, MW47 was only detected in 1/4 (25%) of the treated wastewaters tested, suggesting possible removal or die-off of phages during treatment. Certain host strains such as strain CR70, whilst demonstrating lower levels of phage specificity (Table 6.3), demonstrated sensitivity levels much higher than those of more specific hosts. Strain CR70 was found almost exclusively in cattle and pig samples (90% specificity), had a sensitivity of 83%, and as such could be a useful indicator of non-human faecal contamination.

Figure 6.1 shows specificity plotted against sensitivity for all twenty-nine tier 4 (and selected tier 3) enterococcal host strains. Spearman's correlation coefficient, a non-parametric statistical test was used to assess the relationship between specificity and sensitivity of the host strains, as the data were not normally distributed, even after log transformation. Results show a strong negative relationship between sensitivity and specificity (R_s= -0.531, p <0.01). As specificity increased, the sensitivities of the hosts also tended to decrease. Ideally, the specificity and sensitivity of a host for phage lysis would be 100% in each case, but in reality a compromise between the two needs to be achieved. For instance, there may be situations where specificity can be sacrificed in favour of increased sensitivity, and *vice versa.*

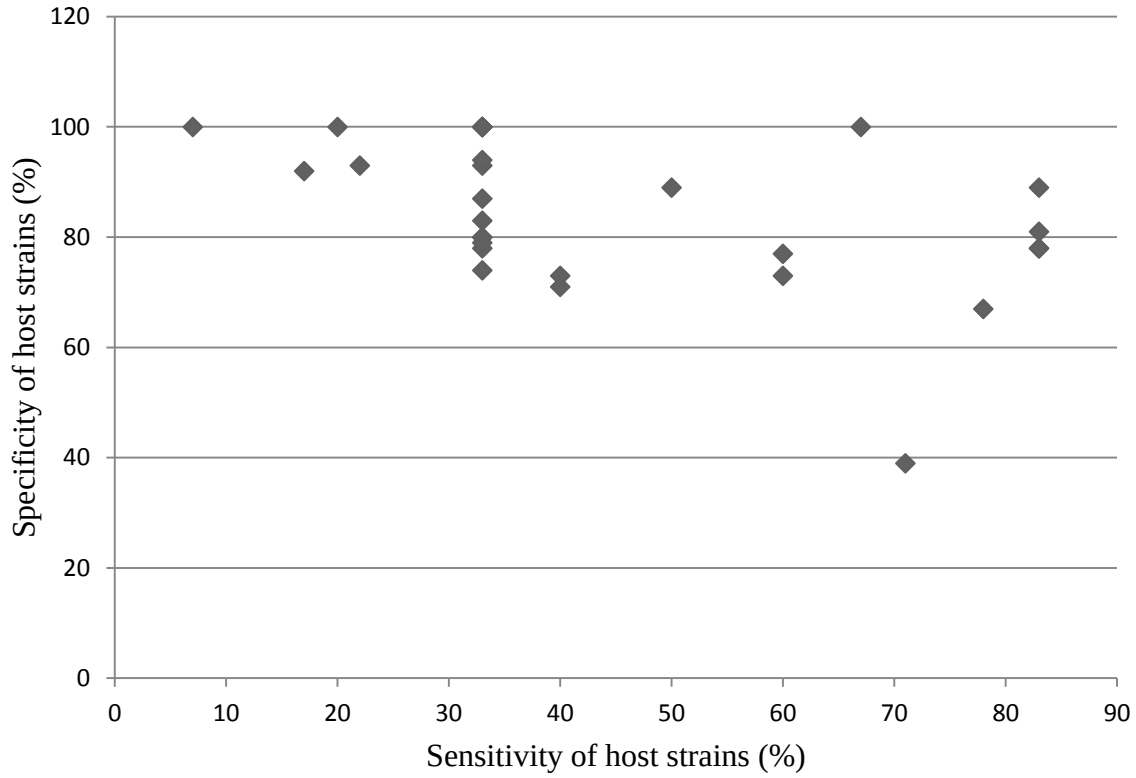

Figure 6.1 Scatter plot showing the relationship between specificity and sensitivity for twenty-nine candidate enterococcal host strains

6.3 Origin of host strains

As shown in Table 6.1, potentially useful enterococcal hosts were isolated from five samples originating from a range of sources. In order to analyse variations in host specificity and sensitivity, all enterococcal hosts were classified as being members of one of four source groups (1. pooled cattle faeces, 2. municipal wastewater, 3. cattle and pig run-off or 4. faecally impacted surface waters). Figures 6.2 and 6.3 show the initial specificity and phage numbers respectively, detected by the host strains in each source group.

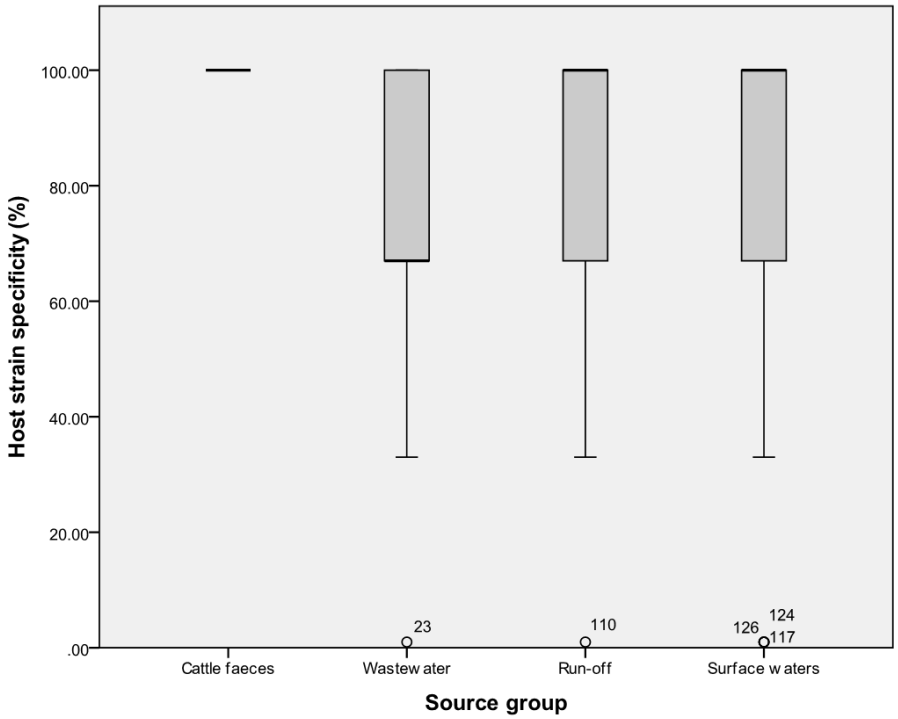

Figure 6.2 Host strain specificity (%) in four source groups (1. pooled cattle faeces, 2. municipal wastewater, 3. cattle and pig run-off, or 4. surface waters). Data points deemed to be outliers are numbered and indicated by a circle

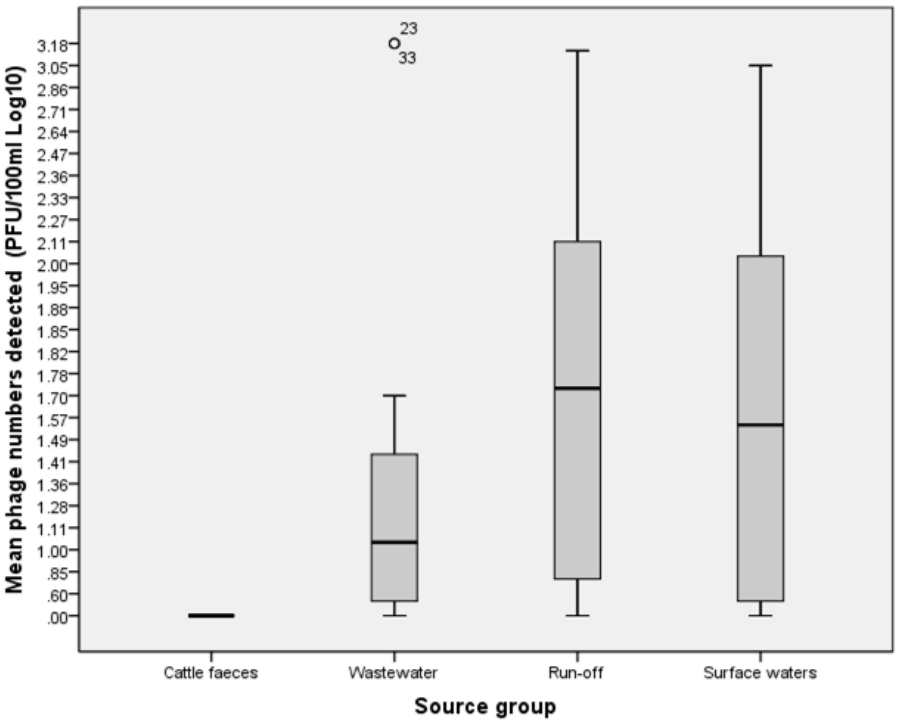

Figure 6.3 Mean phage numbers (log_{10}) detected by host strains in four source groups (1. pooled cattle faeces, 2. municipal wastewater, 3. cattle and pig faecal run-off or 4. surface waters). Data points deemed to be outliers are numbered and indicated by a circle

The Kruskal-Wallis test suggested that specificity did not vary significantly between host strains isolated from the different source groups ($p>0.05$), though statistically significant variations in the number of phages detected in each source group were apparent ($p<0.01$). *Post hoc* Mann Whitney tests revealed that numbers of phages capable of infecting host strains isolated from cattle faeces were significantly lower compared with numbers of phage infecting host strains from the other source groups ($p<0.008$). Phage numbers detected by enterococcal hosts isolated from wastewater and surface waters did not vary greatly from one another, but significantly higher numbers of phage were detected by host strains isolated from cattle and pig faecal run-off ($p<0.008$).

6.4 Identification of enterococcal host strains

The fourteen potential host strains demonstrating specificity to a single faecal source or source group, were identified to species level using both API 20 Strep identification strips and the biochemical key (Table 6.5) described by Manero and Blanch (1999).

Human-specific host strains NRO8 and MW42 were both identified as being members of the species *E. faecalis,* and MW47 as being *E. faecium*. This was in accordance with previous studies that have found *E. faecalis* and *E. faecium* to be the most abundant *Enterococcus* species in human faeces and wastewaters (Ruoff *et al*., 1990; Manero *et al*., 2002; Gelsomino *et al*., 2003). Pig-specific host strain (NRO24) was also identified as *E. faecium.* Very few studies have focused on the flora of swine. Results from Devriese *et al*. (1987) found that the *Enterococcus* species most commonly isolated from pig faeces were *E. faecalis* and *E. faecium*. *E. faecium* was however detected in lower numbers. All cattle-specific host strains were identified as *E. mundtii, E. casseliflavus* or *E. gallinarum*. *E.*

casseliflavus and *E. gallinarum* are commonly isolated from environmental samples, but it has been suggested that these species could be incorporated into the microbiota of the digestive tract following ingestion by grazing ruminants and that their presence could therefore be related to diet (Layton *et al*., 2010). The species classification of the host strains CR1 and NRO39 differed depending on which of the two identification methods were used. Host strains CR1 and NRO39 were identified as being members of the species *E. casseliflavus* and *E. gallinarum* respectively, by API 20 Strep, whereas the simplified biochemical key of Manero and Blanch (1999) identified the hosts as being members of the species *E. mundtii* and *E. faecium*, respectively. All other classifications by both methods were in agreement, however API 20 Strep failed to provide identification for three isolates, all of which were identified as *E. gallinarum* by the biochemical key. As there were discrepancies between the two methods, DNA based characterisation should also be completed on the host strains in the future to ensure identifications are accurate.

Table 6.5 Identification of *Enterococcus* hosts strains with API 20 Strep and a biochemical key

Host origin	**ID.**	**API 20 Strep Identification**	**Biochemical Key Identification**
Cattle run-off	CR1	*E. casseliflavus*	*E. mundtii*
Cattle run-off	CR6	CNI[a]	*E. gallinarum*
Cattle run-off	CR7	*E. casseliflavus*	*E. casseliflavus*
Cattle run-off	CR15	*E. casseliflavus*	*E. casseliflavus*
Cattle run-off	CR16	CNI[a]	*E. gallinarum*
Cattle run-off	CR17	CNI[a]	*E. gallinarum*
Cattle run-off	CR70	*E. gallinarum*	*E. gallinarum*
Municipal wastewater	MW42	*E. faecalis*	*E. faecalis*
Municipal wastewater	MW47	*E. faecium*	*E. faecium*
Newhaven (River Ouse)	NRO8	*E. faecalis*	*E. faecalis*
Newhaven (River Ouse)	NRO24	*E. faecium*	*E. faecium*
Newhaven (River Ouse)	NRO30	*E. casseliflavus*	*E. casseliflavus*
Newhaven (River Ouse)	NRO39	*E. gallinarum*	*E. faecium*
Wales Farm Stream	WFS7	*E. gallinarum*	*E. gallinarum*

[a] CNI = API 20 Strep could not identify

Chapter Seven Results: Phage isolation, characterisation and survival

A potential limitation of phage-based MST techniques, particularly those using somatic or F-specific coliphage, has been the differential survival of the various phages capable of infecting the host strain (discussed in detail in Chapter Two). Phages with different morphologies have been shown to differ with respect to their abundance and survival in the environment, which may hinder the interpretation of results in MST studies (Muniesa *et al*., 1999; Muniesa *et al*., 2009). An effective MST marker should demonstrate consistent responses to environmental stressors (Diston *et al*., 2012). Homogeneous groups of phages are more likely to demonstrate consistent ecological behaviour and survival in the environment (Queralt *et al*., 2003). It was beyond the scope of this research to investigate the diversity and inactivation of phages infecting all fourteen potential host strains isolated during this study. However, the diversity of phages infecting one of the most promising *Enterococcus* host strains, namely MW47 (specific to human faeces), was further investigated.

7.1 Phage isolation

In total, twenty-two single distinct plaques were picked and purified from double agar-layers of host strain MW47 infected with phages from raw municipal wastewater obtained from a wastewater treatment works with a population equivalent of 37,327 (Scaynes Hill, UK). All plaques were successfully propagated to a high titre (at least 10^8 PFU/ml) in accordance with the plate propagation method described in Chapter Three. To determine the titre, phage lysates were tested in duplicate using spot test assays. Twelve of the picked

phage required only one round of plate propagation to achieve the necessary titre, whilst seven required two rounds, and phages MW47-4, -10 and -19 required three rounds of propagation. The final 22 phage cultures had titres of between 5.0 x 10^8 and 1.1 x 10^{10} PFU/ml (Figure 7.1).

Table 7.1 Titres and plaque size of MW47 phages

Phage ID (MW47-)	**MW47 (*E. faecium*) PFU/ml**	**Plaque size range (mm) (n=10)**	**Mean plaque size (mm) (n=10)**
1	1.1 x 10^{10}	0.7-1.8	1.13
2	4.5 x 10^9	0.5-1.8	1.06
3	7.3 x 10^9	0.9-1.8	1.37
4	5.0 x 10^8	0.3-0.8	0.55
5	1.1 x 10^{10}	0.5-1.6	1.00
6	4.6 x 10^9	0.9-1.3	1.04
7	9.0 x 10^8	1.0-2.3	1.74
8	8.6 x 10^9	0.9-2.0	1.39
9	5.7 x 10^9	0.2-0.6	0.37
10	2.1 x 10^9	0.6-0.8	0.70
11	2.5 x 10^9	0.9-1.3	1.13
12	5.6 x 10^8	0.8-1.4	1.12
13	3.4 x 10^9	0.9-1.9	1.18
14	7.1 x 10^9	0.9-2.0	1.43
15	8.6 x 10^8	0.1-0.3	0.18
16	1.3 x 10^9	1.1-1.6	1.28
17	4.1 x 10^9	0.7-0.9	0.81
18	4.7 x 10^9	1.0-1.6	1.35
19	7.3 x 10^8	0.2-0.5	0.36
20	6.9 x 10^8	0.8-1.8	1.41
21	3.7 x 10^9	0.7-1.9	1.38
22	8.0 x 10^8	0.9-1.2	1.01

During phage isolation and propagation it was evident that different phage produced plaques of varying size. Plaque sizes appeared to vary not only between the different plates, but also within certain plates infected with a single phage isolate. Figure 7.1 shows examples of the diversity of plaque sizes observed. Phages MW47-4, -9, -10, -15 and -19,

produced particularly small ‘pin-hole’ sized plaques. Although these plaques were small, they were still clear and readable. MW47-15 produced the smallest plaques with a mean size of 0.18 mm (*n*=10) and MW47-7 produced the largest plaques with a mean size of 1.74 mm (*n*=10). A number of the phage isolates producing larger plaques (>1 mm diameter), also exhibited a halo formation around the plaque, where secondary lysis had occurred (displayed by phage isolate MW47-22 in Figure 7.1).

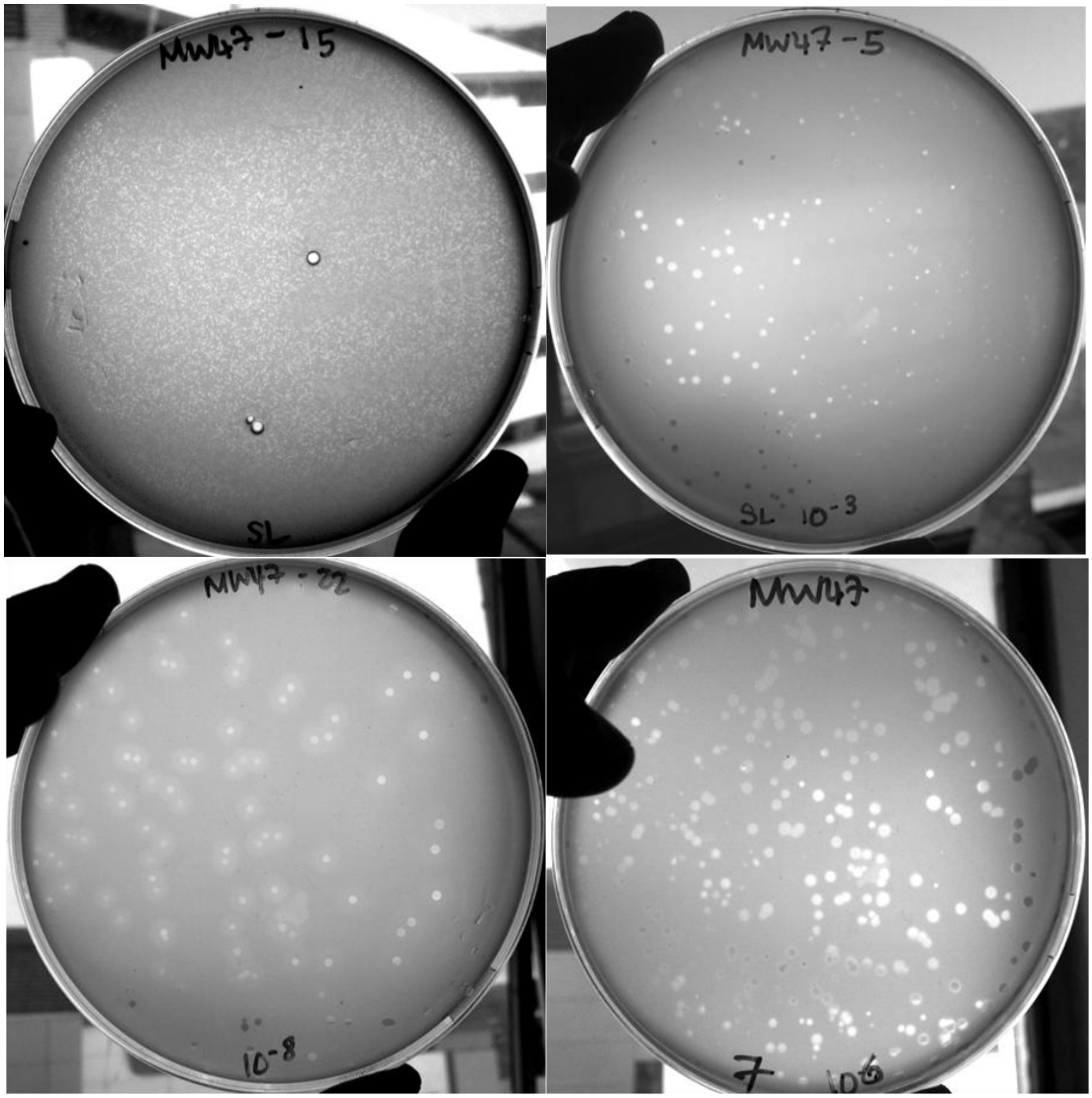

Figure 7.1 Examples of the plaque size diversity produced by MW47 phages. MW47-15 phage with almost complete lysis producing uniform ‘pin-hole’ sized plaques (top left), MW47-5 phage producing varied plaque sizes ranging from 0.5 to 1.6 mm (top right), MW47-22 phage displaying halo formation (bottom left), and MW47-7 phage producing larger plaques ranging from 1.0 to 2.3 mm (bottom right)

7.2 Phage characterisation

In order to determine the morphological diversity of phages capable of infecting host strain MW47, all 22 high titre phage cultures were viewed using electron microscopy (TEM). Staining with uranyl acetate produced both negatively (appearing white with a dark background) and positively (deep black) stained phage particles, as shown in Figure 7.2.

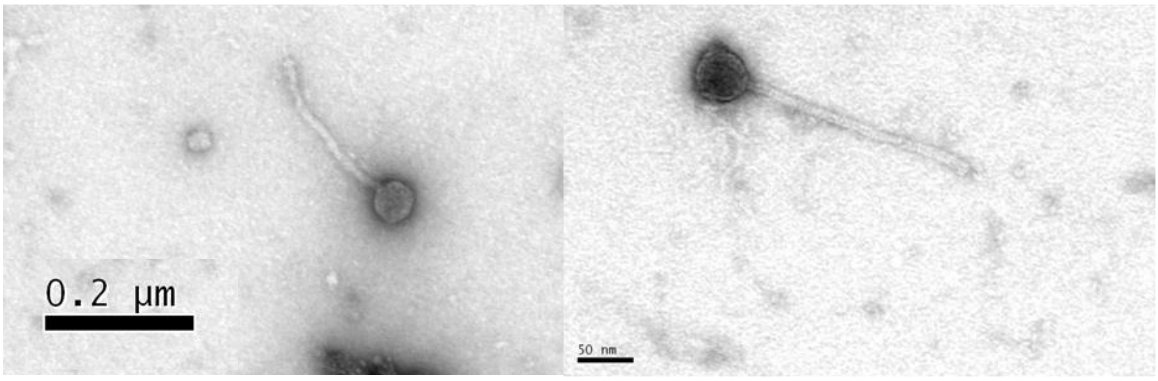

Figure 7.2 Negatively (left) and positively stained (right) siphovirus phage (MW47-1) with icosahedral capsid morphology

The large majority of the phage particles were positively stained. Positive staining is the result of the strong affinity of uranyl acetates for double-stranded DNA (dsDNA). Positively stained capsids can be up to 30% smaller than negatively stained capsids (Ackermann, 2009). Measurements may therefore be subject to inaccuracies. Negative and positive staining on the same grid was only observed for one phage (MW47-1; Figure 7.2). The negatively stained capsid diameter of phage MW47-1 was 10 nm larger than the mean diameter of all positively stained capsids. As the majority of phages were positively stained, measurements would prove unreliable and were therefore not taken. However major morphological characteristics could be observed from the TEM phage micrographs

and are shown in Table 7.2. TEM phage micrographs of the three distinct phage morphologies observed are shown in Figures 7.2-7.4.

Table 7.2 Morphology of phages isolated from host strain MW47

Phage ID (MW47-)	**Capsid morphology**	**Tail morphology**	**Family**
1	Icosahedral	Straight/slightly curved	*Siphoviridae*
2	Elongated icosahedral	Slightly curved/ curved	*Siphoviridae*
3	Icosahedral	Slightly curved	*Siphoviridae*
5	Elongated icosahedral	Curved	*Siphoviridae*
6	Icosahedral	Straight/slightly curved/ wavy	*Siphoviridae*
7	Elongated icosahedral	Slightly curved/ curved	*Siphoviridae*
8	Elongated icosahedral	Straight/ wavy	*Siphoviridae*
9	Elongated icosahedral	Straight	*Siphoviridae*
10	Icosahedral	Straight/slightly curved	*Myoviridae*
11	Icosahedral	Slightly curved	*Siphoviridae*
12	Icosahedral	Curved	*Siphoviridae*
13	Icosahedral	Curved/ wavy	*Siphoviridae*
14	Icosahedral	Straight/ curved	*Siphoviridae*
15	Icosahedral	Straight	*Myoviridae*
16	Elongated icosahedral	Straight/ curled	*Siphoviridae*
17	Icosahedral	Slightly curved	*Siphoviridae*
18	Icosahedral	Straight	*Siphoviridae*
20	Icosahedral	Slightly curved	*Siphoviridae*
21	Icosahedral	Slightly curved/ curved/ wavy	*Siphoviridae*
22	Icosahedral	Straight/slightly curved	*Siphoviridae*

The TEM data revealed all phages to have helical tails and thus to belong to the order *Caudovirales*. As described in Chapter Two, the *Caudovirales* order is divided into three families (*Myoviridae*, *Siphoviridae* and *Podoviridae*) all of which are dsDNA phages. Of the 20 phages viewed by TEM, 18 (90%) had simple non-contractile tails, placing them in the *Siphoviridae* family (Figures 7.2 to 7.3). Two phages (10%), MW47-10 and -15, exhibited tails with a contractile sheath (Figure 7.4). This tail structure identified the phage as belonging to the *Myoviridae* family.

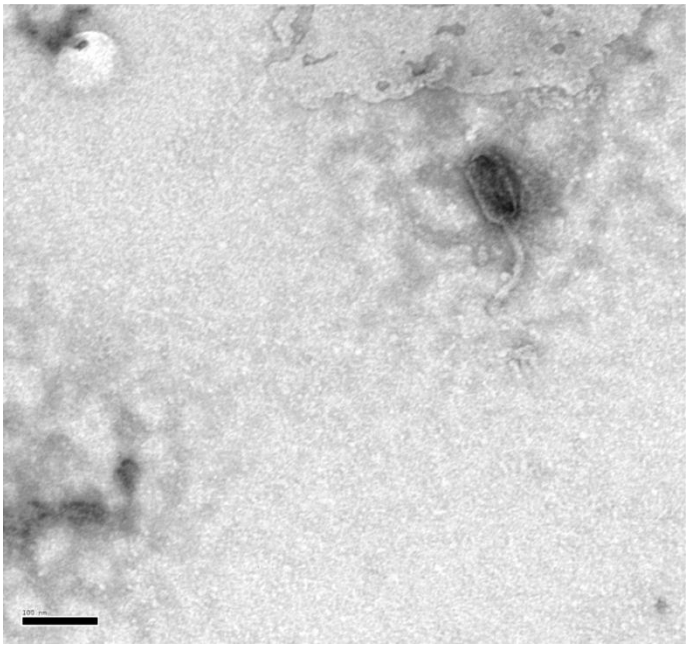

Figure 7.3 Micrograph of a positively stained siphovirus (MW47-5) with elongated icosahedral capsid morphology (bar=100 nm).

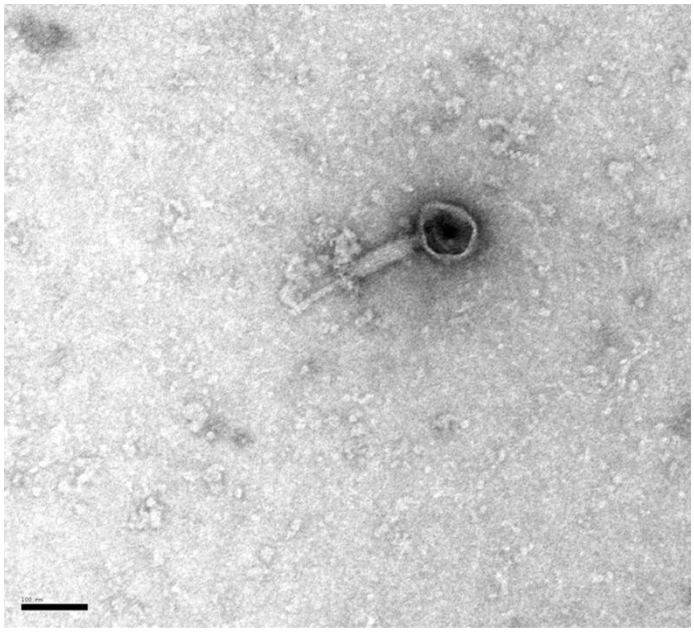

Figure 7.4 Micrograph of a positively stained myovirus (MW47-15) with an icosahedral capsid and contracted tail sheath (bar=100 nm).

Within the 18 recognised *Siphoviridae* phages, two distinctive capsid morphologies were apparent. Twelve of the phage had icosahedral capsids (Figures 7.2), and six possessed elongated icosahedral capsids (Figure 7.3). Both recognised *Myoviridae* phages had icosahedral capsids which appeared to be much larger than the capsids observed for *Siphoviridae* phages. Tail shapes observed included straight, slightly curved, curved, wavy, and curled. Tail fibres were only observed in one micrograph of phage MW47-6 (Figure 7.5).

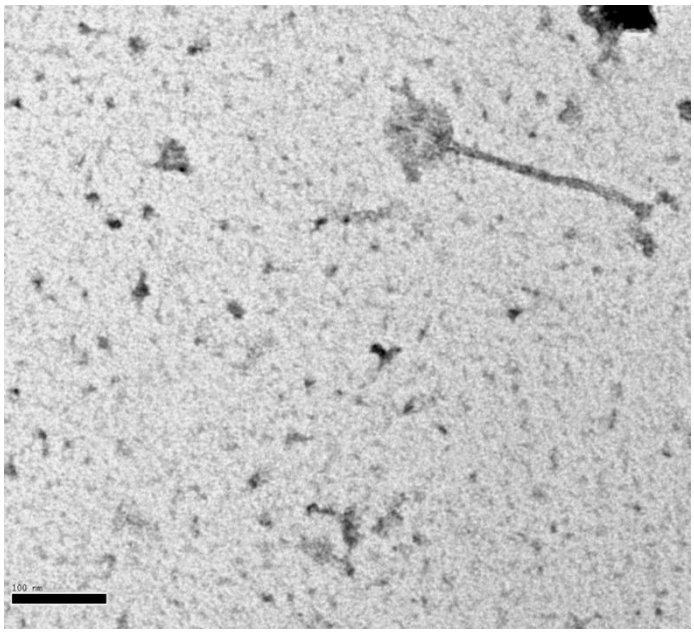

Figure 7.5 Micrograph of a positively stained siphovirus (MW47-6) with straight tail and visible tail fibres (bar=100 nm)

7.3 Host range

The host ranges of MW47 phages, determined using 11 *Enterococcus* type strains, an *E.coli* host strain (WG5) and a *Bacteroides* host strain (GB124), are shown in Table 7.3. No phages were capable of infecting the *E.coli* host strain (WG5) or the *Bacteroides* host strain (GB124). Fifteen of the phage isolates were unable to infect any other *Enterococcus* host type strains. Interestingly, no phages of MW47 (identified as *E. faecium*) were able to

infect the *E. faecium* type strain (DSM 20477). These results are interesting in that they provide evidence to suggest that phages capable of infecting and lysing certain *Enterococcus* host strains have very narrow host ranges.

Phages MW47-9, -10 and -20 displayed broader host ranges. Phages MW47-9 and -10 were also capable of infecting the *E. faecalis* type strain (DSM 20478), and MW47-20 was able to infect *E. asini* (DSM 11492), but the phage numbers detected were low (1.0×10^3 PFU/ml). Phages MW47-8 and -15 displayed the broadest host ranges and were able to infect type strains *E. faecalis* and *E. asini*. Although these five phages exhibited broader host ranges, the number of phages detected on the alternative *Enterococcus* host strains were significantly lower than were detected on host strain MW47 (Wilcoxon signed ranks test, $P<0.05$).

Phages MW47-8, -9, -10, -15, and -20, represent a morphologically diverse group, including phage from the families *Siphoviridae* and *Myoviridae* with both icosahedral and elongated icosahedral capsids (Section 7.2). These results indicate variation in host ranges, which may be linked to the diversity of phage morphology. *E. faecalis* is commonly found in human faeces and in raw wastewater. *E. asini* however is reported to be found exclusively in donkey faeces (de Vaux *et al.*, 1998). In southern England, *E. asini* is less likely to be encountered in inland surface waters and it is therefore probable that the primary host of the phage is *E. faecium*.

Table 7.3 Host ranges of phages capable of infecting host strain MW47

Phage ID. (MW47-)	MW47 (*E. faecium*) PFU/ml	*Enterococcus* type strains (DSM No.)					
		***asini* (20681)**	***casseliflavus* (20680)**	***durans* (20633)**	***faecalis* (20478)**	***faecium* (20477)**	***gallinarum* (24841)**
1	1.1×10^{10}	N[a]	N	N	N	N	N
2	4.5×10^{9}	N	N	N	N	N	N
3	7.3×10^{9}	N	N	N	N	N	N
5	1.1×10^{10}	N	N	N	N	N	N
6	4.6×10^{9}	N	N	N	N	N	N
7	9.0×10^{8}	N	N	N	N	N	N
8	8.6×10^{9}	6.1×10^{7}	N	N	5.4×10^{6}	N	N
9	5.7×10^{9}	N	N	N	1.1×10^{7}	N	N
10	2.1×10^{9}	N	N	N	1.7×10^{6}	N	N
11	2.5×10^{9}	N	N	N	N	N	N
12	5.6×10^{8}	N	N	N	N	N	N
13	3.4×10^{9}	N	N	N	N	N	N
14	7.1×10^{9}	N	N	N	N	N	N
15	8.6×10^{8}	4.7×10^{8}	N	N	3.7×10^{8}	N	N
16	1.3×10^{9}	N	N	N	N	N	N
17	4.1×10^{9}	N	N	N	N	N	N
18	4.7×10^{9}	N	N	N	N	N	N
20	6.9×10^{8}	1.0×10^{3}	N	N	N	N	N
21	3.7×10^{9}	N	N	N	N	N	N
22	8.0×10^{8}	N	N	N	N	N	N

[a]N= Phage lysis not detected; DSM= *Deutsche Sammlung* von Mikroorganismen

Table 7.3 (cont.) Host ranges of phages capable of infecting host strain MW47

Phage ID. (MW47-)	*Enterococcus* type strains (DSM No.)					WG5 (somatic coliphage)	GB124
	***hirae* (20160)**	***mundti* (4838)**	***pseudoavium* (5632)**	***saccharolyticus* (20726)**	***sulfureus* (6905)**		
1	N[a]	N	N	N	N	N	N
2	N	N	N	N	N	N	N
3	N	N	N	N	N	N	N
5	N	N	N	N	N	N	N
6	N	N	N	N	N	N	N
7	N	N	N	N	N	N	N
8	N	N	N	N	N	N	N
9	N	N	N	N	N	N	N
10	N	N	N	N	N	N	N
11	N	N	N	N	N	N	N
12	N	N	N	N	N	N	N
13	N	N	N	N	N	N	N
14	N	N	N	N	N	N	N
15	N	N	N	N	N	N	N
16	N	N	N	N	N	N	N
17	N	N	N	N	N	N	N
18	N	N	N	N	N	N	N
20	N	N	N	N	N	N	N
21	N	N	N	N	N	N	N
22	N	N	N	N	N	N	N

[a]N= Phage lysis not detected; DSM= *Deutsche Sammlung* von Mikroorganismen

7.4 Phage survival

The results demonstrated that phages isolated from MW47 have diverse plaque size, morphology and host ranges. Previous investigations have shown that the inactivation characteristics of phages appear to vary with respect to phage morphology (Muniesa *et al*., 1999; Muniesa *et al*., 2009). It was considered beyond the scope of this research to investigate the inactivation of all 22 MW47 phage. However, as an initial investigation, *in-vitro* inactivation experiments were conducted on a subset of three phages that showed the greatest morphological diversity. The aim of this investigation was to determine whether these phages exhibited varying inactivation rates in fresh water and sea water. Two phages of the *Siphoviridae* family, one with an icosahedral capsid (MW47-1; Figure 7.2) and one with an elongated icosahedral capsid (MW47-5; Figure 7.3), were selected for investigation alongside one phage of the *Myoviridae* family (MW47-15; Figure 7.4). The chemo-physical composition of the fresh and sea waters used in these experiments are shown in Table 7.4. Inactivation experiments were conducted at 4 ^{o}C in the dark in order to factor out the impact of elevated temperatures and solar radiation on phage survival.

Table 7.4 Chemo-physical properties of surface waters at the time of sampling

Water type	Temperature (^{o}C)	Turbidity (NTU)	pH	DO (%)	Salinity (ppt)
Fresh	11.0	47.9	8.4	88.9	0.51
Sea	8.3	14.2	8.2	71.6	14.80

The results of the inactivation experiments for phages MW47-1, -5, and -15 in fresh and sea waters are shown in Figures 7.6 and 7.7. Inactivation rate coefficients (k_D) in log units per day, derived by linear regression, and the time taken to achieve a 90% reduction in PFU concentrations (T_{90}), calculated by dividing In (0.1)/ k_D (Noble *et al*., 2004; Santiago-Rodríguez *et al*., 2010) are shown in Table 7.5.

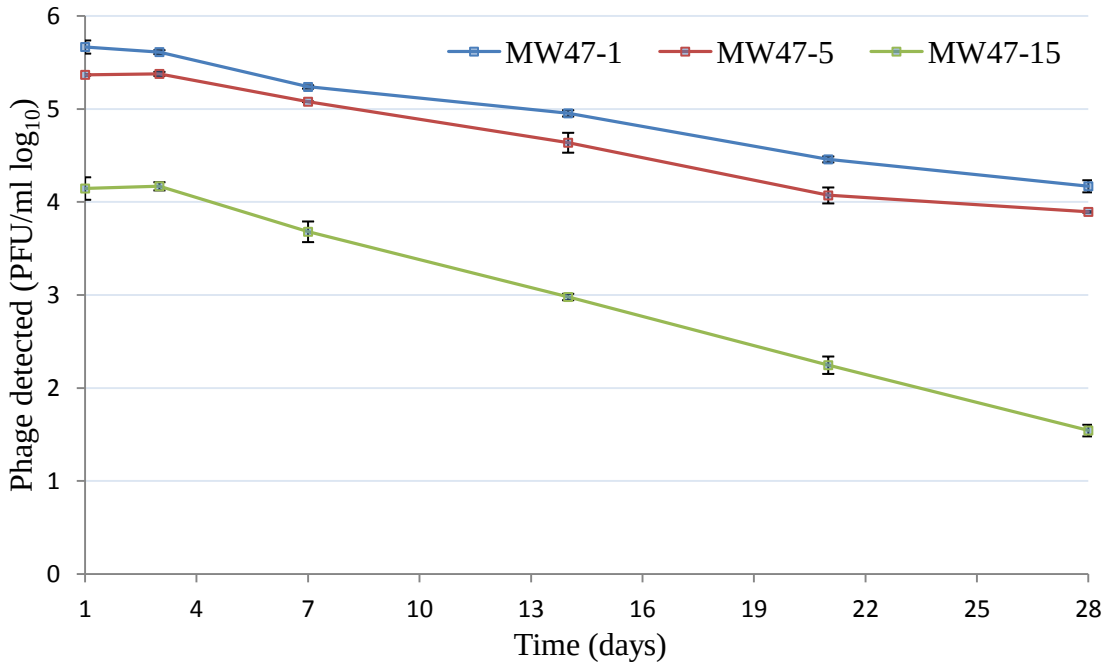

Figure 7.6 Inactivation of phages MW47-1, -5, and -15 in fresh water at 4 ^{o}C in the dark. Error bars represent the standard error of duplicate samples

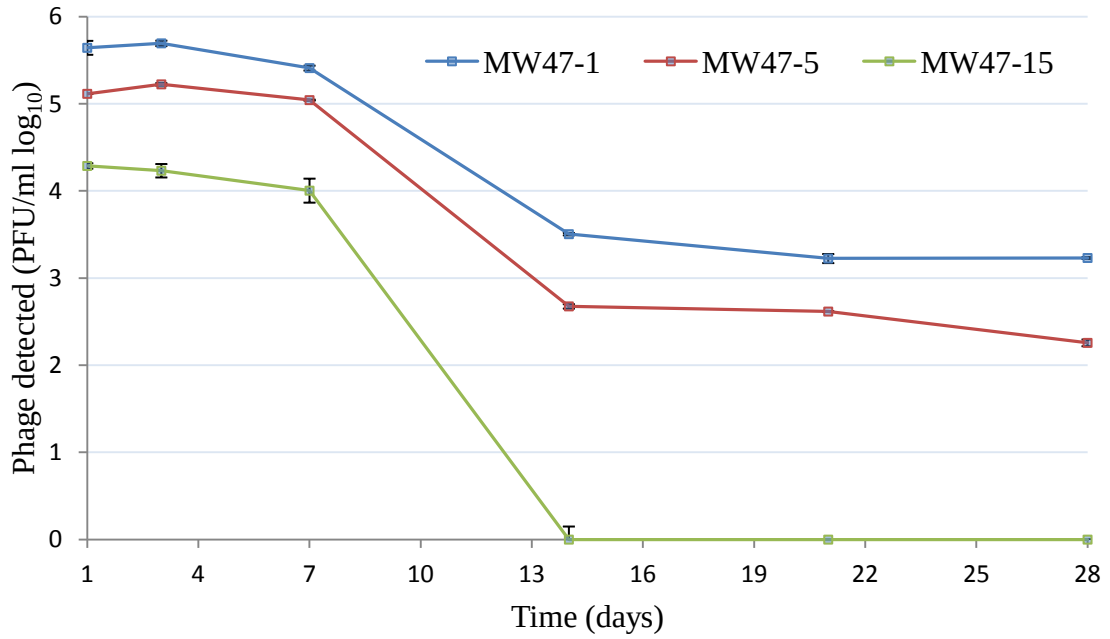

Figure 7.7 Inactivation of phages MW47-1, -5, and -15 in sea water at 4 ^{o}C in the dark. Error bars represent the standard error of duplicate samples

Original in Colour

Table 7.5 Inactivation rate coefficients (k_D) in log units per day and predictions of time taken to reach a 90% reduction in phage concentration (T_{90} values)

Phage ID	Marine Waters (Newhaven)		Fresh Waters (Spatham Lane)	
	Inactivation rate coefficient at 4°C (S.E./ n)	**T_{90} (days)**	**Inactivation rate coefficient at 4°C (S.E. / n)**	**T_{90} (days)**
MW47-1	0.107 (0.021/ 6)	21.5	0.056 (0.002/ 6)	41.1
MW47-5	0.124 (0.024/ 6)	18.6	0.059 (0.004/ 6)	39.0
MW47-15	0.324 (0.097/ 4)	7.1	0.099 (0.002/ 6)	23.3

The inactivation rate coefficients of both siphovirus (MW47-1 and -5) were similar for fresh water (Table 7.5). After 28 days in fresh water, MW47-1 and MW47-5 concentrations had both reduced by 1.5 $\log_{10}$. In comparison, the myovirus MW47-15 demonstrated more rapid inactivation and after 28 days in fresh water, experienced a decrease of 2.6 $\log_{10}$. The paired t-test revealed that survival of all three phages was significantly greater in fresh waters compared with sea waters ($P>0.05$). Most notably, all three phage experienced a considerable reduction in sea water after one week. T_{90} predictions for MW47-1 and -5 suggested a 90% reduction of PFU densities would take approximately 20 additional days in fresh water. Whilst MW47-5 was predicted to have 90% reduction in PFU concentrations after a further 16 days in fresh water. After 28 days in sea waters MW47-1 and -5 concentrations had decreased by 2.4 $\log_{10}$ and 2.9 $\log_{10}$. Again, MW47-15 appeared less persistent, with an inactivation rate coefficient of 0.324 $\log_{10}$ per day. After day 14, MW47-15 fell below detection limits.

Although there were apparent differences between the inactivation of *Siphoviridae* and *Myoviridae* phages (MW47-1, -5 and -15), statistical analysis revealed that variations between the inactivation of the three phage, were not significant (ANOVA, sea water *p*=

0.989; fresh water p= 0.650). Differences in inactivation of the two phage families were also insignificant (independent t-test, sea water p=0.922; fresh water p=0.346). The limited data available for myovirus in this pilot experiment, particularly in sea water where levels dropped below detection limits, undoubtedly had an effect on the significance of variance. This pilot study therefore highlights the need for further in-depth investigations into the survival of the different phage morphologies capable of infecting host strain MW47.

Chapter Eight: Discussion, conclusions and future work

8.1 Discussion

This research programme was designed with the aim of evaluating the suitability and effectiveness of bacteriophages (phages) infecting host strains of *Enterococcus* species as a low-cost tool for MST. In this chapter, the methods and protocols presented in earlier chapters are evaluated critically and recommendations are presented both for future research and for practical application of the methods as they currently stand. This chapter also discusses how the results reported in the three preceding chapters influence the suitability of phages infecting host strains of *Enterococcus* species as a tool for MST. This research programme has provided a protocol for the isolation of *Enterococcus* spp. host strains suitable for MST and is the first study to assess the ability of phages infecting enterococcal host strains to detect both human and non-human sources of faecal pollution. Additionally this study has provided important information on the diversity and survival of these phages.

8.1.1 Protocol evaluation

The double agar-layer assay for phage detection on *Enterococcus* host strains used in this study provided clearly visible plaques. The assay proved to be rapid, with all *Enterococcus* host strains reaching the desired optical density (from frozen culture) within 1.5-3 h, and plaques becoming visible within 4h of incubation. These incubation times are comparable with those currently required for facultatively anaerobic host strains (e.g. the *E. coli* host of somatic coliphage) and are considerably shorter than those required by strict anaerobes, such as *Bacteroides* host strains (Ebdon *et al*., 2007; Santiago-Rodríguez *et al*., 2010). It

was previously reported by Bonilla *et al*. (2010) that, for a human-specific *E. faecalis* strain, plaques were not visible to the human eye without the addition of sodium azide (NaN_3) and that optimal plaque formation was evident with a calcium chloride ($CaCl_2$) concentration of 2.6 mg/ml. However, in this study good plaque clarity was observed with the large majority of host strains isolated without the addition of either NaN_3 or $CaCl_2$. It is therefore advantageous to isolate host strains that do not require the addition of NaN_3 in particular, as this compound is hazardous to human health and as such, requires the use of specialist safety equipment.

The application of the simplified tiered screening approach (Purnell *et al*., 2011) improved the isolation efficiency of new enterococcal host strains and provided a useful protocol to support the isolation of future phage hosts. The approach effectively identified host strains that exhibited rapid growth and high specificity to a single faecal source (or source group). Initial screening with four key reference samples, representing the dominant faecal sources in the study area (e.g., human, cattle, pig and sheep faeces), enabled large numbers of enterococcal isolates to be screened. Using this approach, at least one potential host strain was isolated for every sample analysed (approximately one host strain for every 40 isolates analysed), with the exception of faecal samples from cattle. This is higher than the rates of successful isolation reported in previous studies involving the isolation of *Bacteroides* hosts for MST (Payan *et al*., 2005; Gómez-Doñate *et al*., 2011). Although the geographical stability of the enterococcal host strains isolated in this study was not fully determined, other bacterial host strains used in phage-based MST techniques have failed to demonstrate geographical stability (Tartera *et al*., 1989; Puig *et al*., 1999; Payan *et al*., 2005). However, the rapid and successful approach to host strain isolation presented here lends itself well to

implementation in different geographical settings. This would therefore help to ensure that appropriate hosts are used in specific regions.

8.1.2 Cost evaluation of the approach

The primary focus of the emerging field of MST has been the development of methods that demonstrate high specificity and sensitivity. This had led to the development of sophisticated molecular MST techniques. These techniques often require specialist equipment and skills, and their routine application may be limited in many less-economically developed countries (LEDC) because of the relatively high costs associated with their development and implementation. Cost analysis of the isolation and screening stages used throughout this study (Appendix 1) suggested that four or more host strains could be isolated for between £600 and £910 (US $949-1440). This is considerably lower than the cost of developing molecular MST techniques, especially where the development of a library of isolates from known sources is required (Malakoff, 2002). This cost estimate also compares favourably with phage-based techniques using *Bacteroides* host strains (Payan *et al*., 2005).

Culture-based phage MST techniques, such as the one featured here, are relatively simple to perform, do not require specialist expertise (e.g., anaerobic facilities) and can therefore be performed in laboratories equipped with basic microbiological apparatus. A high proportion of the costs associated with the isolation and screening of potential host strains was related to labour. The average hourly rate for a laboratory technician in the UK (calculated as £9.00 an hour) is substantially higher then would be expected in LEDC. In the UK, labour expenses would amount to over 50% of the total cost. The overall cost of

the method may therefore be further reduced in LEDC. These findings support those from other previously reported phage-based MST techniques, suggesting that they may be applicable in countries with limited financial resources (Ebdon *et al*., 2007; Nnane *et al*., 2011).

8.1.3 Performance of *Enterococcus* host strains

Specificity (the probability that a sample that is not from a source is correctly identified as not originating from that source), sensitivity (the probability that a sample from a source will be correctly identified as originating from that source), and phage detection levels were the three performance parameters used in this study to assess the potential of *Enterococcus* host strains for MST application. It was considered important to assess the method against standard criteria, so that comparisons with other available MST methods could be made.

Enterococcus host strains demonstrated a broad range of specificity and sensitivity levels. The range of specificity values displayed by host strains isolated in this study (0-100%) is similar to those reported for other phage-based culture methods (Ebdon *et al*., 2007; Vijayavel *et al*., 2010; Gómez-Doñate *et al*., 2011; Wicki *et al*., 2011). A small proportion of the host strains tested were highly specific (100%) to a particular faecal source (or source group). However, results showed a strong negative relationship between host strain specificity and sensitivity. Consequently, higher specificity was often associated with lower sensitivity. Indeed, low sensitivity appears to be a major limitation of the *Enterococcus* host strains isolated within this study. Although the final fourteen host strains produced very few false positive results, false negative results were relatively common. Low sensitivity was particularly evident with cattle-specific host strains, with many of these strains

appearing to be specific to a single herd or group of animals. This observation may be the result of differences between the breeds and diets of cattle from different farms, as both factors have been shown to affect the intestinal microbial composition of cattle (Laugalis *et al*., 2007; Durso *et al*., 2010; Pitta *et al*., 2010).

These results highlight potential geographical instabilities among certain *Enterococcus* host strains, particularly those associated with livestock sources. Whilst specificity to a single herd, or group of animals may not be desirable for MST purposes, the high level of specificity of phages witnessed in this study, suggests that *Enterococcus* hosts are sufficiently specific to have a potential role as MST tools. Host strains with lower sensitivity (<70%) may have limited use as MST markers. However, some non-human, animal-specific strains displayed lower specificity, but higher sensitivity. Strains with higher sensitivity (such as CR70) may prove more useful for MST applications in surface waters than more 'specific' strains (such as WFS7). Currently, the use of one marker alone may not be sensitive enough. Ideally, a 'toolbox' approach to the detection of human and non-human sources, utilising several strains (with a range of specificities and sensitivities) in parallel, may be advisable for future MST studies, both in terms of improved sensitivity and specificity, and on the grounds of cost. This research also provides further evidence that the guts of humans and ruminants appear to harbour distinct and diverse microbial communities. However, further metagenomic analysis is required in order to understand fully the role of bacteriophages in the development and function of these communities.

8.1.4 Host strain origin

Host strains were isolated from five faecal sources (pooled cattle faeces, cattle run-off, pig run-off, municipal wastewater and surface waters impacted by non-human and human faecal contamination). Traditionally, either individual faecal material or wastewater (for human-specific strains) samples have been used to isolate new host strains suitable for MST. Using fresh faecal samples increases the chances of finding host strains with high specificity to a source. However, results from this study demonstrated that, although some host strains isolated from pooled faecal samples and wastewater had high degrees of host specificity, they generally detected lower phage numbers (e.g. 1.0×10^2 PFU/100ml to 3.4×10^3 PFU/100ml) when compared with host strains isolated from run-off and surface water sources.

Therefore the results reported in Chapter Six (section 6.3) suggest that when isolating potential enterococcal host strains for phage-based MST applications, it is better to isolate agricultural hosts directly from liquid farm run-off, rather than from fresh pooled stools from individual animals. In fact, only one host strain isolated from individually pooled cattle faeces detected any phage in reference samples. Host strains isolated from run-off displayed similar ranges of host specificity to those isolated from faecal and wastewater samples, but detected higher numbers of phage. Isolation of enterococcal hosts directly from impacted surface waters also led to the discovery of strains useful for MST application, without negatively impacting host specificity. This may be due to the selection of more ‘dominant’, ‘environmentally tolerant’ phages and enterococcal hosts.

8.1.5 Host strain identification

The identification of host strains was undertaken in order to determine whether certain *Enterococcus* species exhibiting high specificity were more frequently associated with particular faecal sources. Both API 20 Strep strips (BioMérieux, UK) and the biochemical key developed by Manero and Blanch (1999) were used to identify *Enterococcus* host strains. However, some discrepancies between the two methods were observed. Of the fourteen host strains identified, the two methods were in agreement in only nine cases. The biochemical key appeared to be more effective than API 20 Strep strips in that it was able to identify all host strains, whereas API 20 Strep failed to identify three of the host strains. Cattle-specific host strains were most commonly mis-identified. The results of this research suggest that for future identification of enterococcal host strains the biochemical key would be more effective than API 20 strips. Commercially available API 20 Strep strips were not able to identify a broad enough group of species. The biochemical key did perform well, but a combination of phenotypic and molecular identification protocols may prove more reliable for future studies.

It is notable that both methods were in agreement when identifying host strains with specificity to human faeces. Human-specific host strains were all identified as either *E. faecium* or *E. faecalis*. These findings are in accordance with those reported in previous studies focussing on human faeces and wastewater (Ruoff *et al*., 1990; Manero *et al*., 2002; Gelsomino *et al*., 2003). A pig-specific host strain was also found to be *E. faecium*, by both identification tests. The most common species encountered in the intestines of farm animals are *E. faecalis*, *E. faecium*, *E. hirae* and *E. durans* (Devriese *et al*., 1987; Devriese *et al*., 1992[a]; Devriese *et al*., 1994). Interestingly, all non-human or cattle- specific host strains

(excluding pig-specific host strain NRO24) were identified as *E. gallinarum*, *E. casseliflavus* or *E. mundtii*. Research suggests that these species are not the most abundant species found in the faeces of agricultural livestock animals, and cattle in particular (Devriese *et al*., 1992; Jackson *et al*., 2010). *Enterococcus* species, *E. casseliflavus* and *E. mundtii* (yellow pigmented enterococci) are generally associated with plant material (Ulrich and Müller, 1998; Müller *et al*., 2001). It has been suggested that species commonly associated with environmental samples, such as *E. casseliflavus* could be incorporated into the microbiota of the digestive tract following ingestion by grazing ruminants and that their presence could therefore be related to diet (Layton *et al*., 2010). The identification of plant-associated enterococci from animal faecal sources therefore suggests that the presence of these species in environmental samples should not be attributed solely to non-faecal sources.

The tiered screening approach preferentially selected host strains with higher specificity to source(s). Although not the most abundant species found in livestock animals, *E. gallinarum*, *E. casseliflavus* or *E. mundtii* may offer host strains with higher specificity to non-human ruminant faecal sources. Therefore, in future studies it may be advantageous to target particular enterococcal species, such as *E. faecalis* for human sources and *E. gallinarum* for non-human sources, when isolating future strains for MST application.

8.1.6 Implications of phage homogeneity for MST

Phages infecting host strains used for MST should ideally belong to morphologically homogeneous groups, sharing similar survival characteristics (see Chapter Two). Although it was considered beyond the scope of this study to evaluate the homogeneity of phages

infecting all the potential MST host strains isolated, twenty phages capable of infecting one strain (MW47) were further analysed for their morphology and host range homogeneity.

MW47 phages isolated in this study displayed a range of morphologies. From TEM micrographs it was visually evident that the phages belonged to two distinct families within the order *Caudovirales*. The majority of the phages belonged to the *Siphoviridae* family, having long simple non-contractile tails. Two phages were also identified as belonging to the *Myoviridae* family, having tails that are contractile and consisting of a neck, a contractile sheath, and a central tube. These distributions are consistent with previous studies of *Enterococcus* phage that demonstrated *Siphoviridae* to be the most commonly isolated family, followed by phages of the *Myoviridae* family (Ackermann, 2007).

These *Enterococcus* phages also displayed 'within-family' variation. Icosahedral and elongated icosahedral capsids were observed, as well as straight, curved and curled tail morphologies. Host range investigations also highlighted some differences between the 22 phage. Although, most phages appeared to possess very narrow host ranges, phages MW47-8, -9, -10, -15, and -20 (including both myoviruses) were also capable of infecting either an *E. asini* type strain (DSM 20681), an *E. faecalis* type strain (DSM 20478), or both strains.

The results of the morphology and host range investigations show that a heterogeneous group of phage are able to infect MW47. Although the morphology of only a limited number of phages was determined in this study, initial findings suggest that phages infecting this enterococcal host strain are morphologically more heterogeneous than phages

infecting *Bacteroides* host strains (Queralt *et al*., 2003). These results therefore prompted further investigations into the inactivation of phage of different morphologies capable of infecting host strain MW47, because differential inactivation of phage may hinder the interpretation of results (Muniesa *et al*., 2009) and limit its suitability as an MST tool.

All phages identified were tailed, containing dsDNA. Phages viewed by TEM were largely positively stained because uranyl acetate has a strong affinity for dsDNA. Positively stained phage particles often have shrunken capsids. Measurements of positively stained phage particles should therefore not be used in comparison with phage from other studies. When determining enterococcal phage morphology in future research it may be advisable to use an alternative stain such as phosphotungstate (Ackermann, 2009). Other sources of error when using uranyl acetate include swollen tails and the production of false envelopes.

8.1.7 Phage inactivation

An investigation into the inactivation of three morphologically different phages (MW47-1, MW47-5 and MW47-15) was conducted in order to determine whether the phages shared similar survival characteristics in fresh water and sea water. Two *Siphoviridae* phages (one with an icosahedral and one with an elongated icosahedral capsid), and one *Myoviridae* phage, were selected for inactivation experiments as they represented the three most distinctive morphologies observed in the TEM micrographs. A major concern relating to the use of bacteriophage infecting facultatively anaerobic host strains such as *E. coli* has been their potential for replication in the environment. Throughout the inactivation experiment there was no evidence of replication, supporting recent findings that suggest that replication in the environment has a negligible impact on the numbers of bacteriophage detected (Jofre, 2009).

In this study, the survival of all three phages was greater in fresh water compared with sea water. These results contradict findings from similar studies using phage capable of infecting *Enterococcus* and *E. coli* host strains. Santiago-Rodríguez *et al*. (2010) determined the inactivation of a group of siphovirus (capable of infecting an *E. faecalis* host strain) in sea water and fresh water. Their results showed that the phage were able to survive for longer periods in sea water (13 days) compared with fresh water (6-8 days). The greater survival in sea water was attributed to the presence of high concentrations of inorganic salts that may have increased phage absorption. Although the experimental design of this study was not intended to elucidate potential drivers of inactivation, it is unlikely that higher salinity was the determining factor causing faster inactivation of MW47 phages in sea water. Survival studies have previously demonstrated that inactivation of phage is largely unrelated to salinity levels (Berry and Noton, 1975; Borrego and Romero, 1985). More recent research conducted by Noble *et al*. (2004) also showed no apparent differences between the inactivation of F+ specific coliphage in fresh water and sea water. However, it was notable that all three phages experienced considerable reductions after one week in sea water. Inactivation appears to be the result of a complex blend of factors including the association of phage with bacteria and solids, presence of organic matter, temperature, pH level, the ionic environment, metabolic activity of other micro-organisms, hydrostatic pressure, and solar radiation (Grabow, 2001). More advanced phage survival experiments, in which potential variables are independently investigated, would be required to reach more in-depth conclusions. However, it was notable that the fresh water sample used in inactivation experiments had a higher level of turbidity than the sea water, which may have also influenced phage survival.

The main purpose of the investigation was to determine whether differences in survival times existed between the three morphologically different phages tested. In previous studies, phages of the *Siphoviridae* family have demonstrated greater resistance to inactivation than other phage families, including *Myoviridae* (Muniesa *et al*., 1999; Duran *et al*., 2003). The results of the study suggested that differences in inactivation between the three phages did not vary significantly. Inactivation rates of both siphoviruses were very similar. However, although no clear statistical link between morphology and inactivation times could be established, MW47- 15, a member of the *Myoviridae* family, did show more rapid inactivation in both fresh water and sea water than the two siphovirus. These results suggest that survival times of the different phage are similar and therefore that differential inactivation appeared not to be an issue in this instance. However, conclusions from this preliminary work should be interpreted with caution. The greater inactivation rate observed for the myovirus (MW47-15) indicates that further research should be conducted into the inactivation of different phage families infecting enterococcal host strains. A larger data set would provide more conclusive evidence of any potential differences in survival characteristics.

8.2 Conclusions and recommendations for future work

The findings of this study are significant in that they offer an insight into host-phage interactions, specificity, sensitivity, and suitability of phages infecting different *Enterococcus* strains for MST application. They therefore offer new knowledge on the ecology of the interactions between enteric bacteria and their phages in the natural environment. More specifically they support the development of the emerging science of

MST. The principal conclusions and outputs of this programme of research can be summarised as follows:

1. The high specificity of enterococcal hosts isolated in this study demonstrates that phages infecting *Enterococcus spp*. possess narrow host ranges, similar to those reported for anaerobes such as *Bacteroides* spp.

2. The demonstrated strong negative relationship between the specificity and sensitivity of the host strains isolated in this study suggests that a compromise between specificity and sensitivity may provide the best approach to isolating host strains that are most useful for MST applications in impacted surface waters.

3. From the fourteen tier 4 host strains showing potential for MST, the human-specific host strain MW47 and the non-human animal-specific host strain CR70 were the most promising candidates for future MST application. These two host strains could be used in conjunction as part of an MST toolbox to detect human from non-human animal faecal contamination.

4. Human specific *Enterococcus* host strains were identified as *E. faecalis* or *E. faecium*, whilst predominantly animal-specific host strains were identified as *E. casseliflavus*, *E. mundtii* or *E. gallinarum*. When isolating future host strains it may be advantageous to target these species.

5. A phage inactivation investigation demonstrated that the survival of three morphological different phages was not significantly different. However, the results did indicate that the myovirus (MW47-15) was inactivated more rapidly in fresh water and sea water compared with the two siphoviruses.

In addition to these principal outputs an effective protocol for the isolation of new enterococcal host strains suitable for MST has been presented here, which may be effectively used by others to isolate bacterial hosts for phage lysis work in other parts of the world. The non-molecular culture-based laboratory protocol (utilising existing ISO phage methods) is low-cost, simple and rapid. Rapid visualisation of plaques (4 h) suggests that this protocol may allow same day results. Whilst the focus of this research programme was to isolate host strains with high specificity to faecal sources, markers of general faecal pollution are vital for determining the overall level of faecal contamination in a water body and for quantifying source contributions. There were many host strains in this study that were not investigated further on the basis of their low specificity. Future research should evaluate the potential of host strains with low specificity (determined in the screening stage of this approach) as general faecal markers.

In order for a novel MST method to be applied with confidence in regulatory applications, it should satisfy a larger group of performance criteria than were evaluated in this research programme. These criteria should include the host distribution (within host sources), temporal stability, geographical stability, limits of detection, and the survival of the marker in the environment, relative to faecal indicator organisms and pathogens (Field and Samadpour, 2007; Santo Domingo *et al*., 2007). Future research should therefore focus on determining the performance of *Enterococcus* host strains against all these performance criteria.

The results of Chapter Seven indicate that host strain MW47 can be infected by a morphologically diverse group of phages. As the literature suggests that a broad range of

phage families have been shown to infect *Enterococcus* host strains (Chapter Two), the homogeneity of phage infecting all host strains with potential for MST application should be assessed. Furthermore, the data presented in Chapter Seven suggest that further research is required to determine whether phages with different morphologies (particularly those from different families), survive for different periods of time in the environment. This should be determined by *in situ* inactivation experiments, which are impacted by a wide variety of natural stressors, including solar radiation and temperature. Further work should also include the inactivation of these phages by wastewater treatment processes. If the morphological homogeneity of phages infecting enterococcal host strains is varied, host strains infected by homogeneous groups of phages should be preferentially selected.

If and when these questions are satisfactorily answered, phage lysis of *Enterococcus* spp. may well offer an important tool to support global efforts to reduce the burden of human waterborne disease transmission.

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Appendices

Appendix 1 - Cost analysis of the approach

Table A1 Estimated duration of presumptive *Enterococcus* isolation (based on approximately 100 isolates)

Procedure	Duration (h)
Preparation of sample (including dilution series)	0.5
Membrane filtration of samples onto M-*Enterococcus* agar plates	0.5
Incubation of M-*Enterococcus* agar plates	48
Transfer of membrane filters onto BEA plates	0.5
Incubation of BEA plates	4
Picking and streaking of colonies onto M-*Enterococcus* agar	2
Incubation of M-*Enterococcus* agar plates	48
Gram stains and observation under a light microscope	3
Catalase tests	1.5
Sub-culturing of isolates into TSB	1.5
Incubation of isolates in TSB	18
Mixing with 50% glycerol, transferring isolates into cryovials	3
Total duration (including incubation time)	130.5
Labour (total duration-incubation time)	12.5

Table A2 Duration of double agar-layer assays (based on screening of approximately 100 isolates)

Procedure	Duration (h)
Reference sample preparation (dilution, centrifugation, filtration)	1.5
Thawing of host strains, followed by sub-culturing into fresh TSB	1
Incubation of host strain in TSB	2.5-3.5
Melting of TSAss and distribution into test tubes	2
Double agar-layer assays	7
Incubation of double agar-layer assays	18
Plaque enumeration	2
Total duration (including incubation time)	34-35
Labour (total duration-incubation time)	13.5

Table A3 Estimated costs of consumables for host strain isolation (based on approximately 100 isolates)

Consumables (manufacturer)	**Cost £ (UK)**	**Cost/100 isolates**
Bile aesculin agar (Oxoid)	0.20/plate	2.00
Cryobox (Nalgene)	6.07/box	18.21
Cryovials (Nalgene)	0.20/vial	40.00
Glass slides (Fisher)	0.03/slide	3.00
Glycerol (Fisher)	4.19/litre	0.84
Gram stain kit (Pro-Lab Diagnostics)	40.95/250ml	16.38
Hydrogen peroxide (Fisher)	8.56/litre	0.02
M- *Enterococcus* agar (Difco)	0.14/plate	4.90
Nitrocellulose membrane filters (0.45µm) (Nalgene)	0.46/filter	9.20
Petri-dishes 90mm (Fisher)	0.06/plate	2.70
Pipette tips (Fisher)	0.02/tip	2.40
Ringers solution (¼ strength)(Oxoid)	0.16/litre	0.24
Sampling containers (Fisher)	0.20/container	0.40
Sterile swabs (Fisher)	0.10/swab	0.20
Tryptone soya broth (Oxoid)	1.90/litre	0.95
Total consumable cost:		101.44
Total cost including labour in the UK(12.5 x £9.00):		213.94
Total cost including labour in Malawi (12.5 x £3.00):		138.94

Table A4 Estimated costs of consumables for double agar-layer phage enumeration assays (based on the screening of approximately 100 isolates)

Consumables (Manufacturer)	**Cost £ (UK)**	**Cost/100 isolates £**
Agar bacteriological (Oxoid)	0.14/gram	1.12
Centrifuge tubes (50ml) (Fisher)	0.08/tube	0.64
Petri-dishes 90mm (Fisher)	0.06/plate	24.00
Pipette tips (Fisher)	0.02/tip	4.80
Ringers solution (¼ strength)(Oxoid)	0.16/litre	0.08
Sterile disposable syringes (Becton Dickinson)	0.06/syringe	0.24
Syringe filter units (0.22µm) (Millipore)	0.31/filter	24.80
Sterile disposable test tubes (Sterilin)	0.10/tube	40.00
Tryptone soya agar (Oxoid)	0.05/plate	20.00
Tryptone soya broth (Oxoid)	1.90/litre	3.80
Total consumable cost:		119.48
Total cost including labour (13.5 x £9.00):		240.98
Total cost including labour in Malawi (13.5 x £3.00):		159.98