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**COMPARATIVE DETECTION AND ENUMERATION STRENGTHS OF
QUANTITATIVE REAL-TIME PCR & FISH FOR WATERBORNE BACTERIAL
PATHOGENS IN MUNICIPAL WASTEWATER**

By

Merriam Haffar

Bachelor of Science in Biology, American University of Beirut;
Beirut, Lebanon, 2005

A thesis presented to Ryerson University
In partial fulfillment of the requirement for the degree of
Master of Applied Science and Management
In the Program of
Environmental Applied Science and Management

Toronto, Ontario, Canada, 2007

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THESIS ABSTRACT

Comparative detection and enumeration strengths of Quantitative Real-Time PCR & FISH for Waterborne Bacterial Pathogens in Municipal Wastewater

By

Merriam Haffar

Master of Applied Science

Environmental Applied Science and Management

Ryerson University, 2007

This study comparatively evaluates the detection and enumeration strengths of Real-Time PCR (RT PCR) and FISH, for selected bacterial pathogens in municipal wastewater. Both assays were performed using three primer and probe sets complementary to the same chromosomal virulence gene sequences. Primer & probe specificity was confirmed with DNA & fixed cells from each of the strains, while assay sensitivities were calculated using DNA & fixed cells from pure bacterial cultures as well as seeded wastewater samples. Detection limits calculated for the RT PCR assay were 25 to 303 *tir* gene copies for *Escherichia coli* O157:H7 and 3×10^4 to 293×10^7 *invA* gene copies for *Salmonella enterica*, using pure cultures and seeded wastewater samples, respectively. In spite of the confirmed specificity of the DNA hybridization probes with target nucleic acids, fluorescent signals from hybridized whole target cells were below the detection limit of the FISH assay, and consequently were not quantified. This research demonstrates both the utility of RT PCR in detecting bacterial pathogens and the need for further optimization with DNA-targeted FISH, using environmental samples.

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1.0 INTRODUCTION:

1.1 Background

It is estimated that every year, 1.6 million people worldwide—the equivalent of half of the entire population of the Greater Toronto Area (Statistics Canada 2007)—die from diarrhoeal disease attributed to microbially-contaminated water supplies (WHO 2004). Over 90% of the fatalities are children under the age of 5 (WHO 2004). Without a doubt, poor water quality continues to be a major threat to human health. For this reason, water quality monitoring remains an essential step in decreasing or preventing exposure to waterborne microbial pathogens, within the overall scheme of water resource management.

The assessment of water quality currently relies on the specific and rapid detection of pathogens in collected samples. These contaminants, which consist primarily of pathogenic bacteria, viruses and protozoa, are loaded into surface and groundwater upon—among other sources—the release of wastewater effluent. The classical approach to pathogenic bacterial detection involves the use of culture-based methods relying on the presence of indicator organisms. These are both time-consuming and imprecise, as the levels of these indicator organisms do not always correlate with the levels of waterborne pathogens found in the original environment.

Recent advances in the field of molecular biology have given way to more direct detection methods that are conducted at the level of the bacterial nucleic acid. These relatively rapid and precise techniques have been the focus of current research within the field of microbial water quality monitoring.

1.2 Experimental hypothesis

Two such emerging techniques, namely quantitative Real-Time Polymerase Chain Reaction (RT PCR) and Fluorescent *in situ* Hybridization (FISH) are capable of rapid and specific bacterial detection. The purpose of this study will therefore be to comparatively evaluate the strengths and limitations of either technique for the detection and enumeration of bacterial pathogens encountered in Canadian municipal wastewater effluents, using single-copy gene targets. Conclusions drawn from this work may be applied to the development of more sensitive on-site detection systems, monitoring effluent or surface water quality in real-time.

2.0 LITERATURE REVIEW:

2.1 Waterborne Bacterial Pathogens in the Environment

Each year, an astounding 200 billion liters (MacQueen 2005) worth of a “witch’s brew” (Nantel 1996) of untreated human excrement and household wastes carrying a toxic load of pathogenic microorganisms are dumped into Canadian waters. Expelled primarily from municipal wastewater treatment and collection systems (Environment Canada 2004), this waste has been identified as one of the largest sources of pollution—by volume—to Canadian water bodies (Environment Canada 2001). The pathogen load it carries poses a severe public and environmental health threat by contaminating surface and groundwater systems, subsequently endangering human, plant and animal life. Its effects also extend to the economic and social spheres, through the loss of source water for agriculture and aquaculture, in addition to recreational water areas (Edge *et al.* 2001). This degree of pathogen pollution is responsible for the staggering 90,000 cases of acute waterborne illness that are estimated to occur annually in Canada (Edge *et al.* 2001).

Pathogens commonly found in municipal wastewater span all three major microbiological classes from protozoan parasites to bacteria and viruses (Ferguson *et al.* 2003). These microorganisms either exist naturally in water sources or are released through fecal contamination (Health Canada 2004).

Measuring approximately 4 to 18 μm in size, protozoan parasites are the largest and most resistant of all waterborne pathogens (Ferguson *et al.* 2003). Unlike bacteria or viruses, these flagellated microorganisms are expelled into receiving waters in the form of cysts and oocysts that can withstand environmental stress and disinfection, yet are unable to multiply outside the gastrointestinal tract (Bitton 2005). Upon ingestion by a vertebrate host, the infective form of the organism—or trophozoite—is released from the cyst through a process known as excystment (Bitton 2005). Cysts are then re-produced through asexual division in the intestinal wall and released in feces, to be transmitted once again via water or food through the environment (Thompson 2000). It is estimated that an infected host may shed up to 5×10^5 cysts/g feces, which is enough to infect a minimum of 5,000 individuals (Bitton 2005)¹.

¹ Assuming a minimum infective dose of 100 cysts (Bitton 2005).

Viruses are similarly unable to multiply outside of a host and thus eventually die off or remain static in number once released into receiving waters (Leclerc *et al.* 2002). Over 140 different types of enteric viruses have been identified in water and wastewater samples, most of which are of fecal origin (Bitton 2005).

Bacterial pathogens account for the largest number of drinking water outbreaks in Canada, and may include both enteric and endemic aquatic bacteria (Leclerc *et al.* 2002). Enteric strains such as *Escherichia* and *Streptococcus* have been widely used as indicators of fecal contamination in water sources. This study will examine four of the most commonly isolated strains, namely *Escherichia coli*, *Escherichia coli* O157:H7, *Salmonella enterica* and *Staphylococcus aureus*.

2.1.1 *Escherichia coli*

Escherichia coli is the predominant facultative anaerobe of the human colonic flora (Nataro & Kaper 1998). As a member of the *Enterobacteriaceae* family, the species consist mostly of motile, gram negative bacilli that are serotyped on the basis of their surface antigens, O, H and K (Nataro & Kaper 1998). Although the majority of the *Escherichia coli* strains inhabiting the gastrointestinal tract of humans and warm-blooded animals are harmless, five strains bear virulence factors and are capable of producing enterotoxins. These are classified on the basis of the toxin produced, and are categorized as enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC) and enteroaggregative (EAaggEC) (Schroeder & Wuertz 2003). All the pathogenic strains cause the gastrointestinal illness known as Hemorrhagic Colitis (HC), which is characterized by severe abdominal pain and bloody diarrhoea (Schroeder & Wuertz 2003). Of these strains, *E. coli* O157:H7, or EHEC², has emerged as one of the most prominent and widespread waterborne pathogens worldwide.

EHEC was first identified as a distinct class of pathogenic *E. coli* in 1982 following a severe outbreak of enteric illness associated with the consumption of undercooked hamburger meat (Leclerc *et al.* 2002; Nataro & Kaper 1998). Currently, it is the second most frequently isolated pathogen, after both *Campylobacter* spp. and *Salmonella* spp. (Nataro &

² As the most important EHEC serotype with relation to public health, *E. coli* O157:H7 is often referred to as EHEC (WHO 2005).

Kaper 1998), causing over 20,000 infections and 250 deaths annually in the United States alone (Bitton 2005).

In addition to causing hemorrhagic colitis, infections with EHEC strains are also known to cause a case of renal failure known as Hemolytic Uremic Syndrome (HUS) (Schroeder & Wuertz 2003). Its virulence is attributed to the production of potent shiga-like toxins (also known as Vero cytotoxins) that disrupt protein biosynthesis in renal endothelial and intestinal epithelial cells, eventually leading to hemorrhaging and edema (Nataro & Kaper 1998; PHAC 2001a). One crucial step in the EHEC colonization of the gut includes the close adherence of the bacterial cells to the epithelial membranes through the formation of Attaching Effacing (A/E) lesions (Verweyen *et al.* 2000; Dean-Nystrom *et al.* 1998). This is achieved through the production of the intestinal adherence factor, intimin, as encoded for by the chromosomal *tir* translocated intimin receptor gene (Verweyen *et al.* 2000).

The spectrum of illness following EHEC infection ranges from mild diarrhoea to HC and HUS. The latter develops in approximately 2% to 7% of all cases—predominantly in young children—with an estimated mortality rate of 30% (Verweyen *et al.* 2000). It is estimated only 100 microorganisms are needed to cause an infection (Hunter 2003).

As EHEC is strongly associated with pigs and cattle, farmed animals grazing near receiving waters provide a potential source of fecal contamination and consequently EHEC bacteria (Leclerc *et al.* 2002). A surveillance study carried out by Public Health Agency of Canada from June 2005 to May 2006 in Waterloo, Ontario identified untreated surface water as a potential source of human infection primarily through recreational water activities (PHAC 2007). Major waterborne outbreaks in Bourdine Township, Missouri (December 1989) and more recently in Walkerton, Ontario (May 2000) have also shown it to be transmitted through drinking water and municipal wastewater systems (Hunter 2003).

2.1.2 *Salmonella enterica*

Another enteric bacterial genus associated with food and waterborne illness is *Salmonella*. As another member of the *Enterobacteriaceae* family, it is classified as a gram negative aerobe and facultative anaerobe with over 2300 disease-causing³ serotypes (Baudart *et al.* 2000). The three most prevalent strains implicated in food and waterborne outbreaks are the

³ The World Health Organization recognizes all serotypes of this bacterium as human pathogens (Schroeder & Wuertz 2003; WHO 2005).

three main serovars of *Salmonella enterica*: *Salmonella typhi*, *Salmonella paratyphi* and *Salmonella enteritidis* (Shcroeder & Wuertz 2003). With an estimated wastewater concentration of 10^2 to 10^9 organisms/100ml, it is considered the most predominant pathogenic bacteria in wastewater (Bitton 2005).

Symptomatic *Salmonella* infections are usually associated with two similar syndromes: Salmonellosis (an acute form of gastroenteritis) and typhoid fever (Baird-Parker 1990). Symptoms common to both illnesses include abdominal pain, diarrhoea or constipation, nausea and vomiting. Epidemiological studies have found that only 10 to 100 cells are needed to cause illness (Baird-Parker 1990).

The mechanism of infection for either illness is the same; upon ingestion, the microorganism travels past the acidic gastric environment—owing to a unique acid-tolerance response exhibited upon exposure to low pH environments—to invade epithelial cells of the gastrointestinal tract as well as surrounding macrophages (Ohl & Miller 2001). The bacterium invades the host intestinal cells by hijacking the cytoskeletal system and inducing the formation of membrane ruffles that extrude outwards to envelope the bacterium (Ohl & Miller 2001). The invasion process—known as bacterial-mediated endocytosis—happens quite rapidly, with the bacterium appearing inside membrane-bound vacuoles within minutes of cell contact (Finlay & Falkow 1997). Salmonellae are even capable of invading macrophages by the same process, and subsequently surviving and replicating intracellularly (Ohl & Miller 2001). The genetic determinant of the invasion mechanism is the invasion protein-producing, single-copy *invA* gene (Ohl & Miller 2001; Fey *et al.* 2004).

2.1.3. *Staphylococcus aureus*

Staphylococcus aureus has been identified as a powerful pathogen that is capable of infecting any organ system and is responsible for more illnesses than any other known human pathogen (Archer 1998). Some of these syndromes are listed in **Table 2.1**, with the two most common being Staphylococcal food poisoning (a form of foodborne gastroenteritis) and Toxic Shock Syndrome. As a member of *Micrococcaceae* family, this microorganism forms gram-positive cocci clusters (Archer 1998).

S. aureus is a commensal organism that is carried in certain regions of the vagina, pharynx and most significantly the nose (Lowly 1998). An estimated 30 to 50% of healthy adults are asymptomatic carriers of the bacterium and are at a risk for acquiring subsequent infections

(Lowly 1998). This occurs as a result of a breach in either the skin or mucosal barrier at the site of colonization, allowing the bacterium access to the surrounding epithelial tissue and bloodstream (Archer 1998). While there is no consensus of yet on the exact mechanism of colonization, it has been recognized that *S. aureus* adheres most strongly to mucous coated epithelial cells (Kluytmans *et al.* 1997). Once attached, it is then phagocytosed by the host cell, as with *Salmonella* (Lowly 1998).

Table 2.1: Syndromes for which *S. aureus* is the sole etiological agent. *As listed by Archer (1998).

Syndrome*	Major Characteristic	Reference
Impetigo bullosa	Localized skin boils	Amagai <i>et al.</i> 2000
Pyomyositis	Infection of striated muscle	Hall <i>et al.</i> 1990
Botryomycosis	Skin, muscle or tissue lesions containing fungus-like granules	Winslow 1959
Epidural abscess	Infection of epidural space surrounding the brain or spinal chord	Dariouche 2006
Acute or right-sided endocarditis	Inflammation of the inner lining of the heart	Dajani <i>et al.</i> 1997
Toxic shock syndrome	High, toxin-induced fever and diffuse rash	Dinges <i>et al.</i> 2000
Scalded skin syndrome (Ritter's Disease)	Widespread skin boils	Amagai <i>et al.</i> 2000
Foodborne gastroenteritis	Lesions in stomach and small intestine	Dinges <i>et al.</i> 2000

The key to the microorganism's virulence lies in its diverse arsenal of virulence proteins, which serve either to breakdown host tissue for nutrients (such as collagenases and proteases) or to suppress the host's immune response (Dinges *et al.* 2000). To fulfill the latter function, *S. aureus* produce a family of enterotoxins known as Pyrogenic Toxin Super-Antigens (PTSAs) which include Toxic shock syndrome toxin-1 (TSST-1) and the various serotypes of Staphylococcal enterotoxin (from A to I) (Dinges *et al.* 2000). These bind to Major Histocompatibility (MHC) Class II proteins which in turn attach to T-cells, thereby initiating

the overwhelming release of cytokines (Dinges *et al.* 2000). This is known to induce an autoimmune response in susceptible animal hosts leading to multi-organ failure and eventually Toxic shock syndrome (Dinges *et al.* 2000). Interestingly, PSTAgS are also capable of hindering the liver's ability to clear the endotoxins from the bloodstream thereby enhancing their effects and lowering the lethal dose by as much as 50,000 fold (Schlievert 1982). A lethal dose of 1-2 µg/kg in humans would therefore be reduced to mere picograms (Dinges *et al.* 2000).

The genes for the PSTAgS toxin family are carried either by plasmids, bacteriophages or on chromosomal pathogenicity islands. One enterotoxin in particular, Staphylococcal enterotoxin C, is encoded for on three separate low-copy chromosomal genes—*entC₁* (previously known as *sec*), *entC₂* and *entC₃*—each of which encodes a related enterotoxin C serotype (Bohach & Schlievert 1989).

S. aureus infections are most commonly acquired in clinical settings worldwide where transmission occurs through direct contact with a colonized carrier of the bacterium, usually health care workers or infected patients (Lowly 1998). Outbreaks may also result in a community setting from exposure to the pathogen or a carrier in the environment. A number of studies have identified cases of Staphylococcal transmission between individuals and their canine pets (van Duijkeren *et al.* 2004; Tanner *et al.* 2000). Cows have also been identified as carriers of the bacterium, which attaches to mammary epithelium biofilms and causes a severe form of udder inflammation termed bovine mastitis (Oliveira *et al.* 2007). Recycling filtered manure wastewater—a common practice for flushing barns on many farms—exposes other animals within the farm and ultimately the surrounding environment to the pathogen (Davis *et al.* 2006). It is therefore unsurprising to note that *S. aureus* has also been detected in dairy products, fish and sewage (Morandi *et al.* 2007; Ohlsen *et al.* 2003; Simon & Sanjeev 2007).

Treatment of Staphylococcal infections is becoming increasingly difficult owing to the emergence of antibiotic resistant strains such as MRSA, or Methicillin-Resistant *Staphylococcus aureus*. These strains are often multi-drug resistant as resistance to methicillin confers resistance to other penicillins (Lowly 1998) as well as due to horizontal gene transfer (Ohlsen *et al.* 2003).

2.2 Fate and transport in the environment:

2.2.1 Pathogen transmission routes

Infection with any of the aforementioned pathogens results from the transmission of the bacterium either from an infected individual or environmental source to a susceptible host. The latter mechanism occurs by means of complex and interrelated transmission pathways through the environment, involving animal reservoirs, food, and water sources (Bitton 2005).

E. coli O157:H7 is spread by both water- and food-borne transmission, with the latter usually occurring through meat and dairy products—primarily undercooked beef and unpasteurized milk—although the spectrum of food products associated with this pathogen is broadening rapidly (Verweyen *et al.* 2000). Domestic farm animals including cattle, poultry and pigs have been known to harbor the bacterium asymptotically, shedding an estimated 10^8 CFU/g feces without exhibiting any symptoms of infection (WHO 1989). Long-term contamination of farms is widespread despite disinfection efforts, and studies have suggested that as many as 50-80% of cattle in central Europe have been colonized by the bacterium (Verweyen *et al.* 2000). Upon excretion, the pathogen is also transmitted to susceptible hosts through water networks and reservoirs.

Salmonella is similarly transmitted by ingestion of directly or indirectly contaminated food, either from infected domestic animals or through cross-contamination from animal feed and fertilizer (PHAC 2003c). Vegetables contaminated with wastewater effluents have also been responsible for disease outbreaks (Bitton 2005; PHAC 2003b).

Despite being predominantly spread through direct person-to-person transmission with colonized individuals, *S. aureus* has also been detected in hospital and dairy manure wastewater (Scwartz *et al.* 2003; Ohlsen *et al.* 2003; Davis *et al.* 2006), which carry the potential of transmitting the pathogen to water reservoirs.

2.2.2 Entry into surface water

Waterborne enteric pathogens secreted in the feces of infected animal and human hosts are commonly detected in municipal wastewater, particularly in regions where diarrhoeal diseases and intestinal parasites are endemic (WHO 1989). The direct release of these pathogens into water bodies through the discharge of little or untreated effluent is a permanent point source of surface water pollution (Baudart *et al.* 2000a). Indeed the detection of various enteric bacteria such as *Salmonella* has in fact been correlated to

proximity to sewage discharge areas (Winfield & Groisman 2003; Baudart *et al.* 2000b). Runoff from agricultural land—which occurs mainly through storm events and rainfall—serves as a more diffuse, or nonpoint source of pollution, releasing the pathogenic bacteria carried in animal wastes and soil particles into nearby surface water (Baudart *et al.* 2000b). A series of studies have demonstrated that at low flow conditions in general, point sources of pollution are the most prevalent sources of pathogen contamination of surface water, while during rainfall or flood events, nonpoint sources dominate (Tyrrel & Quinton 2003).

In Canada for the period 1974 to 2001, there were a total of 288 waterborne disease outbreaks related solely to drinking water nationwide (Schuster *et al.* 2005). Of the estimated 99 (34%) outbreaks reported in municipal water sources, the most prevalent sources of contamination were identified as wild and farm animal waste (14%), inadequate water treatment (12%), and discharged wastewater effluent (3%) (Schuster *et al.* 2005).

Once released into a surface water body, the hydrodynamic distribution of pathogens is dependant on the processes of dispersion, dilution and horizontal and vertical transport (Brookes *et al.* 2004). Dispersion and horizontal transport are influenced by wind-driven currents within the surface layer and at any depth by inflows and internal waves. The speed and density of pathogen-loaded water inflows (from smaller water networks or point sources of pollution) determines the depth at which the pathogen cloud will occupy within the water body: warmer inflows will float, while cooler ones will sink beneath the surface layer of the lake to colder depths (Brooks *et al.* 2004). Here, the fate of introduced bacteria is governed mainly by the attachment and release from sediment and by inactivation (Ferguson *et al.* 2003).

The various physical and chemical factors affecting pathogen viability are summarized in **Fig. 2.1**. These principally include pressure, temperature and predation by grazing aquatic invertebrates, the latter of which allows for the bioaccumulation of the pathogen in animal tissue along the food chain (Brooks *et al.* 2004).

The vertical distribution (or sedimentation) and settling rate of pathogens is dependant on pathogen size and density (Brooks *et al.* 2004). Once attached to sediment particles, bacterial pathogens may remain viable for variable periods of time, and may subsequently be resuspended and re-distributed within the water body by sediment turbulence, such as during storm events or due to motorboat activity (Brooks *et al.* 2004).

Fig. 2.1: The fate and transport of bacterial pathogens within a surface water body. The bacteria are introduced into the water body through point and nonpoint sources of fecal contamination, and are then distributed by various transport processes, and are subsequently transmitted to human and animal hosts through a number of different water uses.

The degree of pathogen survival in response to environmental stress within water bodies varies widely, and ultimately determines the disease risk posed upon exposure. **Table 2.2** lists the key epidemiological factors of *E. coli* O157:H7, *S. enterica* and *S. aureus* that contribute to the potential risk and subsequent public health importance of their transmission within the environment. Unlike both *S. aureus* and *S. enterica*, *E. coli* O157:H7 does not survive well in non-host environments. This is offset however by the magnitude of the load continuously shed by the host organism, which maintains a stable population within the host environment (Winfield & Groisman 2003). *Salmonella* on the other hand has been known to

survive for more than 3 months in private septic systems (PHAC 2003b), outliving *S. aureus* in ground water and river water contaminated with sewage effluent (Winfield & Groisman 2003).

Table 2.2: The basic epidemiology features of the three main pathogens examined in this study, based upon WHO (1989). ^a Average number of organisms shed per gram feces (CFU/g). ^b Estimated maximum life of infective stage outside host.

Pathogen	Excretory Load (CFU/g) ^a	Persistence Outside Host ^b	Major Animal Reservoir	Minimum Infective Dose	Reference
<i>Escherichia coli</i> O157:H7	10 ⁸	< 1 month	Cattle	10-100	Winfield & Groisman 2003; PHAC 2001a
<i>Salmonella</i> spp.	10 ¹⁰	> 3 months	Poultry	100-1000	Nygaard Jensen <i>et al.</i> 2006; PHAC 2001b
<i>Staphylococcus</i> <i>aureus</i>	10 ³ -10 ⁷	< 60 days	Cattle	>10 ⁵	Lindberg <i>et al.</i> 2004; PHAC 2001c

The epidemiological characteristics of waterborne pathogens—including the load shed into the environment as well as their persistence within it—combined with their complex environmental fate and transport processes both contribute to the potential of water sources to transmit microbial pathogens to great numbers of susceptible human hosts causing subsequent illness. The monitoring of water quality as a result serves as an essential preventative measure in averting outbreaks of waterborne illness.

2.3 Pathogen detection

Water quality assessment currently relies on the rapid and specific detection of introduced pathogens, to either decrease the potential health risk associated with exposure or to evaluate

the effectiveness of pathogen control measures. However, the inherent constraints⁴ associated with direct pathogen monitoring have necessitated the use of indicator organisms as surrogates for the indirect detection of viral, protozoan and bacterial pathogens. Such an assessment of water quality involves the use of culture-based techniques that rely upon providing a combination of nutritional, physical and chemical conditions that will selectively support the growth of the target indicator (Leclerc *et al.* 2002).

2.3.1 Culture-based detection methods:

2.3.1.1 Review of indicator organisms

Coliform indicators are used to provide information on the presence of fecal material, thus indirectly indicating the potential presence of enteric pathogens. It then follows that an increase in the detected coliform density indicates a deterioration of water quality and the presence of a potential health hazard (Dutka 1979). While this assumption stands true with certain bacterial pathogens, recent studies are increasingly questioning its applicability to viruses and protozoan parasites which have often been detected in the absence of culturable indicators (Harwood *et al.* 2005; Jiang *et al.* 2001; Lipp *et al.* 2001).

In spite of these and other limitations, numerous culture-based detection approaches are in use and under continual development. The indicator group currently in use spans a broad diversity of genera and species including bacteria, bacteriophages and spores.

Although the exact definition of coliform bacteria varies among the various regulatory and monitoring agencies, they are universally described as gram-negative, non-spore forming, aerobic and facultatively anaerobic bacilli (Bitton 2005). Although members of this total coliform (TC) group are discharged in high numbers in human and animal waste, not all are of fecal origin (Bitton 2005).

As a second group of bacterial indicators, thermotolerant or fecal coliforms (FC) such as *Escherichia coli* and *Klebsiella pneumoniae*, are defined as being capable of fermenting lactose at 44.5°C. True to their name, these bacteria indicate the presence of fecal contamination, although the exact source—be it human or animal—cannot be differentiated (Bitton 2005).

⁴ Methodological limitations include low concentrations of the organisms in water samples tested and the high cost and effort of current detection technologies (Lemarchand & Lebaron 2003).

Fecal streptococci (FS) have also served as indicators of microbial pollution; these include *Streptococcus bovis*, *S. equines*, *S. avium*, *S. faecalis* and *S. faecium*—with the latter two strains comprising the Enterococci sub-group—are present in the intestinal flora and fecal waste of humans and warm-blooded animals (Bitton 2005). Members of this coliform group are capable of persisting longer in the environment than other indicator species, yet are not capable of multiplying outside of a host (Bitton 2005). The ratio of the detected densities of fecal coliforms to fecal streptococci has recently been used in bacterial source tracking to identify the origin of surface water pollution⁵ (Bitton 2005).

Other indicator organisms currently in use include anaerobic bacteria commonly found in wastewater contaminated aquatic environments, such as *Bifidobacteria* and *Clostridium* spp. (Bitton 2005). The latter—a sulfite-reducing species—has traditionally served as the indicator of choice in the European Union (Bitton 2005). However, the hardy bacterial spores formed allow it to persist for long periods of time in the environment, effectively making it a poor indicator choice for routine water quality monitoring (Payment *et al.* 2003).

Bacteriophages targeting Enterobacteriaceae such as somatic and male-specific RNA coliphages have also served as indicators of fecal contamination. Of all the indicators proposed as of yet, these organisms have exhibited the best correlation with enteric waterborne viruses (Bitton 2005).

Various governmental agencies have approved several methods of coliform detection, as illustrated in **Table 2.3**. The most commonly standardized techniques include Multiple-Tube Fermentation (or Most Probable Number), Membrane Filtration and the Presence-Absence test (Rompré *et al.* 2002).

The Multiple-Tube Fermentation or Most Probable Number technique detects the presence of total coliforms. This is expressed in terms of Most Probable Number (MPN) of organisms present, which is an estimate of the mean density of coliforms in the sample (APHA *et al.* 1998). The test is based on diluting the target coliform present in the sample to extinction, or the level at which no culturable units remain (Leclerc *et al.* 2002). The procedure involves inoculating culture media with serial dilutions of the water sample to test for the production of gas or acid or abundant growth (Rompré *et al.* 2002). To improve the

⁵ A FC/FS ratio of > 4.0 indicates a contamination of human origin, while a ratio of < 0.7 is of animal origin (Bitton 2005)

precision of the MPN value obtained, a larger number of samples (usually 3 to 12) are assayed for each dilution (APHA *et al.* 1998). Fecal coliforms are identified in samples that produce gas when grown in EC broth at 44.5°C or blue colonies when grown in m-FC agar at 44.5°C (Leclerc *et al.* 2002). This result is confirmed through a second reaction using lactose bile broth (Leclerc *et al.* 2002). This method however is limited by interference effects induced by the presence of high numbers of non-coliform bacteria as well as any inhibition from components of the culture media itself, both of which contribute to an underestimation of coliform abundance and false-negative results (Rompré *et al.* 2002; Olson 1978). The procedure is also time-consuming, taking up to 48 hours for each of the preliminary (or presumptive) and confirmation tests to be completed (Rompré *et al.* 2002).

Table 2.3: Existing water monitoring guidelines and regulations in the US, Canada, European Commission and WHO. ^a EPA 2003 ; ^b Environment Canada 1998; ^c European Commission 1998; ^d WHO 2006; ^e Rompré *et al.* 2002; ^f Köster *et al.* 2003.

	Total Coliform ^e	<i>E. coli</i> ^e	Approved Coliform Detection Methods
USA ^a	0/100 ml	0/100 ml	MTF/MPN technique; MF; P/A test
Canada ^b	0/100 ml; none above 10 CFU/ml	0/100 ml	MTF/MPN technique; MF; P/A test
European Commission ^c	0/100 ml	0/100 ml	MF; Spread/Pour Plate Count (conforms to ISO standards) ^f
WHO ^d	0/100 ml	0/100 ml	MF; Spread/Pour Plate Count (conforms to ISO standards) ^f

As one of the most commonly approved methods of detection, the Membrane Filtration (MF) technique involves filtering out coliforms from a water sample on sterile filters of variable pore size (0.45 µm on average) and incubating it on a selective medium to enumerate coliform colonies (Rompré *et al.* 2002). In North America the most widely used media for drinking water analysis is m-Endo-type, as opposed to the Tergitol-TTC more commonly used in Europe (Leclerc *et al.* 2002). This technique provides an alternative to the MPN method in assaying samples with bacterial concentrations that would require long dilution series to enumerate (Leclerc *et al.* 2002). Nevertheless, it is not applicable to turbid samples due to the interference effects of suspended solids (Köster *et al.* 2003).

Colony counts on solid media, obtained through the use of the spread or pour plate technique, also offer better precision than the MTF/MPN method (Köster *et al.* 2003). Cells are enumerated through the inoculation of solid culture media, either before (Pour plate) or after (Spread plate) plating (Leclerc *et al.* 2002). The results obtained are greatly influenced by the condition of the cultured target organisms, temperature and length of incubation, the growth medium, as well as the plating method used (Leclerc *et al.* 2002). However, for larger volume processing and faster throughput, the MF technique is preferred (Köster *et al.* 2003).

The detection of fecal indicators may also be assayed on the basis of presence or absence of enzymes *in situ*, through the addition of fluorogenic or chromogenic substrates to culture media (Leclerc *et al.* 2002). Coloured or fluorescent products indicate a positive sample containing the target indicator.

Enzymatic assays for the specific detection of *E. coli* (also known as Presence/Absence tests) are based on the hydrolysis of the fluorogenic 4-methylumbelliferone glucuronide (MUG) substrate in lauryl-tryptose broth by the intracellular *E. coli* enzyme, β -glucuronidase (Leclerc *et al.* 2002). The fluorescent end-product of the reaction can then easily be detected with a long-wave UV lamp. A similar test used to detect total coliforms involves the addition of 4-methylumbelliferone- β -D-galactoside (MUGA) or *o*-nitrophenyl- β -D-galactopyranoside (ONPG) to the growth medium (Bitton 2005). The presence of fluorescent (MUGA) or coloured (ONPG) compounds indicates a positive sample (Bitton 2005). A number of commercial tests such as Colilert[®] and Colisure[®] (IDEXX Laboratories, Inc., Westbrook, ME) have been developed to simultaneously detect both *E. coli* and total coliforms. Both tests have been approved by the US Environmental Protection Agency, and are included in the *Standard Methods for the Examination of Water and Wastewater* (APHA *et al.* 1998).

2.3.1.2 Weaknesses of culture-based pathogen detection

While the use of such culture-based pathogen detection techniques has become standardized and widespread, recent studies have increasingly questioned the validity of their application in water quality monitoring. One fundamental weakness of this approach lies in the use of indicator organisms themselves; it has been widely demonstrated that coliform bacteria are not predictive of bacterial, viral or protozoan pathogen levels, resulting in an underestimation of pathogen abundance and subsequently of the health hazard present

(Harwood *et al.* 2005). In a study conducted in a French coastal watershed, Lemarchand & Lebaron (2003) found no clear correlation between *Salmonella* (enumerated using MPN) and *Cryptosporidium* levels and those of fecal coliform and streptococci (enumerated using the Spread Plate and MTF techniques). Similarly, Polo *et al.* (1998) were able to detect *Salmonella* in 30% of sampled beach sites with low concentrations of total coliforms, fecal coliforms and fecal streptococci.

Culture-based methods are also restricted by the inability to culture organisms from complex environmental samples. These strains fall into either of two categories: cells that are partially or wholly unculturable under usual laboratory conditions, and cells that enter an unculturable state under stressful conditions (Baudart *et al.* 1999). Bacterial strains belonging to the former group include strains that are inefficiently cultured on laboratory media—such as certain marine *Pseudomonas* species identified by Button *et al.* (1993) that required a doubling time of up to 2 months—as well as those that can only be identified by cloning techniques due to unknown growth requirements (Baudart *et al.* 1999).

In contrast, the second unculturable group consists of bacterial species that are fully culturable under usual laboratory conditions yet enter a nonculturable state (known as a viable-but-nonculturable or VBNC state) when subjected to biotic stress, such as nutrient deprivation, extreme temperature shifts and exposure to toxic metals (Keer & Birch 2003). These cells retain some metabolic activity and are still capable of resuscitation (Kurokawa *et al.* 1999)—and subsequently infection—under appropriate circumstances. This retention of pathogenicity⁶ has been reported in enterotoxigenic strains of *E. coli* (Pommepuy *et al.* 1996), and *Campylobacter* spp., whereby resuscitated cells were observed to produce enterotoxins and still attach to host cells, respectively. Studies have also demonstrated that VBNC cells are capable of retaining genetic determinants of virulence for long periods of time (Chaiyanan *et al.* 2003).

It is estimated that unculturable strains belonging to both these categories make up a staggering 99% of all bacterial cells present in environmental samples (Josephson *et al.* 1993). As a result, estimates of viable bacterial pathogen abundance through culture-based techniques may be grossly underrepresented.

⁶ It is important to note that while studies have demonstrated that cells emerging from the VBNC state retain their virulence, there is no consensus as of yet on the pathogenicity of cells still within this state (Keer & Birch 2003).

2.3.2 Molecular detection methods

In an effort to overcome the known deficiencies in the current detection methods, a variety of culture-independent, molecular techniques have been developed for water-borne pathogens. These include immuno-fluorescent antibody techniques (Buswell *et al.* 1998), electro-chemiluminescence (Shelton & Karns 2002), DNA microarray technology (Lee *et al.* 2006), fluorescent *in situ* hybridization (FISH) and a wide spectrum of polymerase chain reaction (PCR) methods. It has been demonstrated that all these techniques have been able to reach a high degree of detection sensitivity and specificity, while eliminating the need for complex and time-consuming cultivation and confirmation steps (Rompré *et al.* 2002). This study will examine both FISH and PCR.

2.3.2.1 Polymerase Chain Reaction:

2.3.2.1.1 Overview of amplification process

PCR is the process of selective DNA amplification using pre-designed oligonucleotide segments known as primer pairs. Initially employed solely for amplification purposes, this method has increasingly been applied as a molecular DNA detection technique through the use of specific primer pairs targeting enteric pathogens. To date, it has been estimated that over 40,000 references to various diagnostic applications of this technology have been made, including research protocols for detecting 150 different human, veterinary and agricultural pathogens (White 1996).

Essentially, this method uses a thermo-stable polymerase enzyme (or *Taq* polymerase, originally isolated from *Thermus aquaticus* and currently from *E. coli*; Pluthero 1993) to create multiple copies of target DNA. A pair of primers flanks the region of interest within the template DNA strand to direct the amplification process over a series of 20 to 40 cycles of different temperatures (Toze 1999). The most commonly used cycling temperature profiles begin at 90-96°C to denature the double-stranded DNA template, followed by a lower temperature⁷ to allow for the annealing of the primer pair to each of the denatured target sections (on both the forward and reverse strands), followed by an intermediate temperature

⁷ The optimal Annealing Temperature is commonly between 45-65 °C. This is based on the Melting Temperatures of the primer pair used, which in turn is influenced by the length of the product, the salt concentration in the PCR reaction and the primer base composition (or GC content) (Rychlik, Spencer & Rhoads 1990).

between the two for the attachment of the polymerase enzyme and the replication of the target sequence (Toze 1999).

2.3.2.1.2 Primer design and selection

The specificity of any type of PCR reaction depends primarily on the choice of the primer pair. Depending on the application, these may be species- (Sharma & Dean-Nystrom 2003), genus- (Linton *et al.* 1996) or group-specific (Matsuki *et al.* 2002). Pre-designed and evaluated sets targeting eukaryotic and prokaryotic genes for various applications are available on databases online—namely Primer Bank (Wang & Seed 2003) and RTPrimerDB (Pattyn *et al.* 2006)—or in previous studies. Alternatively, primers may be designed using gene sequences deposited in online databanks such as GenBank (National Center for Biotechnology Information; MD, USA) and Comprehensive Microbial Resources (The Institute for Genomic Research; MD, USA). Key design criteria to be considered include: the melting temperature and base composition (including GC content) of each primer, the size of the product generated, formation of secondary structures such as hairpin loops, and the uniqueness of each primer (Dieffenbach *et al.* 1993). Primer design software is commercially available from a number of manufacturers, most notably Roche Diagnostics (Primer Design[®] 2.0; Mannheim, Germany), Applied Biosystems (Primer Express[®] 2.0; CA, USA) and Premier Biosoft International (Primer Premier[®]; CA, USA). Although the degree of specificity of the primer pair is tailored to the specific application, primers with poor overall specificity generate unintended PCR products by either mispriming (or attaching to sequences other than those targeted) or dimerizing (Dieffenbach *et al.* 1993).

2.3.2.1.3 PCR detection systems

Standard amplification reactions are known as end-point PCR. In this procedure, the analysis of the products generated is carried out by Ethidium Bromide staining and agarose gel electrophoresis, or non-radioactive labeling and membrane hybridization such as Southern Blotting (Hallier-Soulier & Guillot 1999; Sharma *et al.* 2000). This conventional form of PCR requires relatively low-cost equipment, but is only capable of providing a semi-quantitative estimate of the gene products generated.

The progression of the reaction may also be monitored in real-time through a Real-Time (RT) PCR reaction. As a faster and more sensitive alternative to end-point reactions, this approach integrates the amplification and analysis steps by monitoring the products

generated during each PCR cycle. Unlike end-point PCR, this system measures product accumulation throughout the logarithmic phase of the reaction, rather than at its completion, which provides for a more accurate estimate (Heid *et al.* 1996). In this method, products are detected in either of two ways: molecular beacons or TaqMan[®] Probes, or double-stranded DNA-binding dyes such as SYBR Green I.

The TaqMan[®] system involves the use of an internal oligonucleotide, or TaqMan[®] probe that is complementary to a sequence on the template DNA between the sites targeted by the two primers. This probe is dual-labeled with a reporter dye (such as FAM, or 6-carboxyfluorescein) at one end and a quencher dye (such as TAMRA, or 6-carboxytetramethylrhodamine) at the other (Giulietti *et al.* 2001). In an intact probe, the fluorescent signal emitted by the reporter dye is suppressed by the quencher dye (Sharma & Dean-Nystrom 2003). During the reaction, as the *Taq* DNA polymerase moves along the denatured template strands extending the newly-formed product segment, it cleaves the TaqMan probe, effectively separating the reporter dye from the quencher. This allows for the detection of the fluorescent signal emitted from the reporter dye, and ultimately the quantification of the products at the end of the reaction. This system has also been known as a 5' Nuclease Assay as a result of the exonuclease activity of the *Taq* DNA polymerase (Malinen *et al.* 2003).

Alternatively, the SYBR Green I system involves the use of a fluorochrome, namely SYBR Green I, that binds exclusively to double-stranded DNA. When the dye molecules are in free-form, they remain undetectable, but upon attachment, they fluoresce (Giulietti *et al.* 2002). However, herein lies the fundamental weakness of this system; it cannot differentiate between intended and unintended PCR products, both of which are double-stranded (Vandesompele *et al.* 2002). Thus, while SYBR Green I allows for higher detection sensitivity than end-point PCR systems, it does not alone allow for the verification of product specificity and may therefore result in an overestimation of the amount of intended product generated (Ririe *et al.* 1996).

In an effort to overcome this limitation, Real-Time systems using SYBR Green I have been programmed to generate melting curves for each of the products. This is performed at the end of the PCR reaction, whereby the temperature is raised gradually while monitoring fluorescence (Ririe *et al.* 1996; Schnerr *et al.* 2001). Once the melting temperature of each of

the different products generated is reached, the strands denature and a marked drop in the fluorescent signal occurs (Ririe *et al.* 1996). This appears as a peak in a plot of the derivative of the fluorescence versus temperature, for each product present (Ririe *et al.* 1996). Ideally, a specific PCR reaction ought to generate melting curves with a single peak, corresponding to the sole intended product.

Over the past three decades, RT PCR has emerged as a powerful tool for the sensitive quantification of gene targets at the end and at the start of the reaction. Minimum starting quantities detected using either of these techniques have been as low as one bacterial cell or viral particle equivalent per milliliter of water or wastewater sample (Campbell *et al.* 2001; Chen *et al.* 2001; Tsai *et al.* 1994).

2.3.2.1.4 PCR amplification systems

As illustrated in Fig. 2.2, various modifications of the standard PCR amplification procedure exist. PCR reactions may be designed with various combinations of the listed amplification techniques coupled with either RT or end-point detection system.

In Nested or Semi-Nested PCR reactions, a second amplification reaction is performed with either the original primer pair (Semi-Nested) or a completely new set (Nested) (Toze 1999). This improves the efficiency of the reaction by selectively amplifying the template sequences targeted by the first reaction, while avoiding any unexpected products and overcoming any inhibitory components present in the sample tested (Mayer & Palmer 1996; Tuinen *et al.* 1998).

Grote *et al.* (2002) demonstrated that this method is up to 1000 times more sensitive than conventional PCR for the detection of bacterial pathogens. This has also been demonstrated using viral pathogen strains. A study conducted by Puig *et al.* (1994) on adenovirus and poliovirus strains in sewage was able to detect 1 to 10 virus particles per reaction. In comparing the results of the Nested PCR assay with conventional PCR and indicator-based Plaque Forming Unit (PFU) counts, it was also found that over half of the samples analyzed gave false-positive results when assayed with the latter two methods. Other Nested PCR assays have also targeted protozoan parasites. For instance, the study conducted by Moris & Saint (2000) employed Nested PCR to monitor *Cryptosporidium parvum* levels in wastewater effluent. This assay was able to detect a minimum of five oocysts per reaction.

Fig. 2.2: The various amplification and detection systems available for both end-point and RT PCR reactions.

Gene targets may also be quantified through the use of internal controls in a Competitive PCR (cPCR) assay. In this reaction, a single primer pair co-amplifies two DNA sequences: the target sequence to be quantified, as well as an added competitor sequence known as an Internal Positive Control (IPC), of known concentration (Li & Drake 2001). As no means of determining the amplification rate are yet available, the amount of gene target present in the initial sample can only be estimated by comparing the amount of target (unknown) and competitive (known) products at the end of the reaction, through the use of standard curves and the visual analysis of DNA product bands on an agarose gel using image analysis software (Chung *et al.* 1999). Despite the subjectivity of cPCR assays, they have often been described as Quantitative or qPCR.

A critical factor in ensuring the accuracy of this technique is the co-amplification of both target and competitor sequences with the same individual efficiencies (Heid *et al.* 2007), such that fluctuations of the amplification efficiency of the entire PCR reaction should ideally be reflected in both sequences to the same degree (Ferre 1992). However, this is often difficult to achieve and requires extensive validation tests (McCulloch *et al.* 1995).

Such setbacks have limited the application of this technique as a diagnostic procedure within the realm of pathogen detection. Nevertheless, it has been applied in the detection of protozoan cysts in drinking water. In 1999, Chung *et al.* developed a cPCR assay to detect a minimum of 1000 *C. parvum* oocysts in a single liter of treated drinking water samples. A similar cPCR assay developed by Udeh *et al.* (2000) in the following year improved the sensitivity of the assay by almost 10 fold, by detecting a minimum of 130 oocysts.

The quantification of RNA targets can be performed through the use of a Reverse Transcriptase PCR (RVT PCR) assay. This involves a two-step process: initially, a complementary DNA (cDNA) strand is generated through the RNA-dependant DNA polymerase activity of a Reverse Transcriptase (RVT) enzyme, and is then used as a template in a PCR reaction. As a diagnostic procedure, this technique has found a comfortable niche within the field of viral detection. RVT PCR assays have commonly been evaluated for a number of RNA enteric viruses such as Hepatitis A, adenovirus and Norwalk virus. Studies have demonstrated that detection limits as low as 1 PFU per milliliter in water samples have been achieved using this technique (Schwab *et al.* 1994).

Unfortunately, many RVT PCR applications have suffered from variability and a lack of reproducibility, stemming primarily from the initial RNA conversion step. Fluctuations in the efficiency of this step have been reported to range from 5% to 90% between different methods, and an astounding 200% to 300% within the same study (Ferre 1992). In general, the synthesis efficiency of any RVT enzyme is a function of the amount of template RNA as well as the concentrations of salt, alcohol and phenol present in the extraction solution (Freeman *et al.* 1999). It is also dependant on the conversion approach adopted. The three methods currently available involve the use of gene-specific (specific priming) or non-specific (hexa- or oligodT-priming) primers targeting specific or all extracted mRNA strands, respectively (Freeman *et al.* 1999).

In an attempt to overcome the inherent variability present in the RVT step, RVT PCR assays have often been coupled with cPCR through the use of RNA controls. As for DNA controls, these are usually homologous RNA sequences that share the same binding sites and nucleotide sequences as the target templates, except for small base insertions, deletions or mutations (Freeman *et al.* 1999). Unfortunately, problems in efficiency and uncertainty

persist and have, as a consequence, caused many reviewers and researchers to view this technique with skepticism (Bustin 2002; Freeman *et al.* 1999; Ferre 1992).

Nevertheless, one key advantage held by RVT PCR assays lies in the ability to differentiate between viable and nonviable cells. Theoretically, DNA-based PCR amplification systems detect intact target sequences from both viable and nonviable cells (Klein & Juneva 1997). This drawback has often been circumvented through the use of an enrichment step prior to the amplification reaction (Campbell *et al.* 2001). However, such techniques have increased the cost and length of the analysis procedure. Alternatively, by targeting mRNA, RVT PCR offers a direct method of determining cell viability, and has been assessed for the direct detection a number of waterborne pathogens, including *Listeria* spp. (Klein & Juneja 1997). Stinear *et al.* (1996) also developed a RVT PCR assay that was able to detect a single viable *C. parvum* oocyst in concentrated environmental water samples. When evaluating the performance of this assay in comparison to another common viability test, the immunofluorescence or IF technique, Kaucner & Stinear (1998) found that the number of viable oocysts detected increased from 24% using the IF method to 69% using RVT PCR.

To simultaneously detect different DNA segments (or loci) with several primer pairs in a single reaction, a Multiplex (MP) PCR system is used. To overcome poor specificity and the preferential amplification of different loci, rigorous optimization of both cycling conditions, including annealing temperatures, as well as the concentration of reaction components is necessary. For cycling conditions, studies have demonstrated that a 4°C to 6°C decrease in the annealing temperature used in the single PCR reactions for each primer set is required to allow for the same loci to be amplified in the presence of other primer pairs within a multiplex reaction (Henegariu *et al.* 1997). Individual primer pairs are usually added in varying amounts relative to the other sets, along with a number of PCR efficiency components such as Bovine Serum Albumin (BSA; Al-Saoud & Rådström 2000; Hoshino *et al.* 1998), Dimethyl sulphoxide (DMSO; Kanakaraj *et al.* 1998), or glycerol (Cerna *et al.* 2003) in concentrations varying from 5-10% (v/v).

Of all the amplification systems available, this method is currently the most commonly employed for pathogen detection. The cost effectiveness and high throughput associated with detecting a range of gene targets within the same reaction have made this system a

convenient screening assay for water quality monitoring (Ibekwe *et al.* 2002). RT MP PCR systems, in particular, have been increasingly used for the automated detection of a range of enteric pathogens in water, food, clinical and environmental samples as a means of detecting contamination or of monitoring the effectiveness of disinfection and other pathogen control methods.

For the detection of single pathogen strains, common multiplexed gene targets include genetic determinants for a group of bacterial toxins released by a single species, such as the *E. coli* O157:H7 Shiga-like toxins I and II (Call *et al.* 2001; Campbell *et al.* 2001; Sharma & Dean-Nystrom 2003) and the Staphylococcal enterotoxins A to D (Chen *et al.* 2001; Mehrota *et al.* 2001; Sharma *et al.* 2000). Multiplex assays have also been developed to detect multiple waterborne pathogens in a single reaction. Kong *et al.* (2002) developed a rapid 12 hour MP PCR assay to detect six bacterial pathogens in marine water samples. *Aeromonas*, *Shigella*, *Salmonella* and *Yersinia* species, along with two strains of *Vibrio* were detected in levels as low as one to 100 cells per reaction. In the following year, Fukushima *et al.* (2003) developed a duplex Real-Time PCR assay targeting 17 bacterial pathogens, including five virulent *E. coli* strains, using two sets of 20 different primer pairs per reaction. Conducted in under two hours, this assay was able to detect 10^4 cells per reaction in stool samples.

Problems with amplification efficiency and specificity have been countered by combining MP PCR assays with any number of the other forms of amplification systems available (as listed in Fig. 2.2). When coupled to Reverse Transcriptase assays, this system has been used for the detection of waterborne viruses, such as rotaviruses, Hepatitis A and poliovirus. A study conducted on sewage and ocean samples found that a Triplex RVT PCR assay screening for these three strains was as sensitive as either of the individual (Monoplex) Hepatitis A and rotavirus assays, by detecting less than one Plaque Forming Unit (PFU) per reaction (Tsai *et al.* 1994).

2.3.2.1.5 Application in pathogen detection

Various combinations of PCR amplification and detection systems have been tested for the rapid detection of *E. coli* O157:H7 in a number of studies, as listed in Table 2.4. As an increasingly prevalent food- and water-borne pathogen, *E. coli* O157:H7 has become the focus of epidemiological investigations, clinical diagnosis and the routine monitoring of food and water quality.

Table 2.4: Applications of PCR in the detection of *E. coli* O157:H7 in fecal and environmental samples.

Reference	Test Samples	Gene Target	Detection Limit	PCR
Ramotar <i>et al.</i> 1995	Culture strains; fecal samples	<i>stx1, stx2</i>	10 ³ – 10 ⁸ CFU/g feces	Multiplex Real- Time PCR
Fratamico <i>et al.</i> 1995	Culture strains	<i>eae, stx1, stx2</i>	-	Multiplex Real- Time PCR
Witham <i>et al.</i> 1996	Culture strains	<i>stx1</i>	0.5 CFU/ml	Real-Time PCR
Tsen & Jian 1997	Culture strains	LTI, <i>stx1, stx2</i> , STII	1 CFU/ml; 1 CFU/100ml with enrichment	Multiplex Real- Time PCR (with enrichment)
Paton & Paton 1998	Culture strains; fecal samples	<i>eae, stx1, stx2</i> , <i>hlyA</i>	-	Multiplex Real- Time PCR
Call <i>et al.</i> 2001	Culture strains	<i>eae, stx1, stx2</i> , <i>hlyA</i>	10 fg of genomic DNA per 50 µl reaction	Multiplex PCR
Sharma & Dean- Nystrom 2003	Culture strains; fecal samples	<i>eae, stx1, stx2</i>	10 ⁴ CFU/g feces; 1 CFU/g feces with enrichment	Multiplex Real- Time PCR (with enrichment)
Ibekwe <i>et al.</i> 2002	Culture strains; dairy wetland samples	<i>eae, stx1, stx2</i>	6.4 x 10 ³ CFU/ml	Multiplex Real- Time PCR (with enrichment)
Campbell <i>et al.</i> 2001	Culture strains; drinking water and soil samples	<i>eae, stx1, stx2</i>	1 CFU/ml	Multiplex Real- Time PCR (with enrichment)

A significant number of these studies have relied upon the use of MP PCR targeting toxin—specifically Shiga-like or *stx*, and intimin or *eae*—genes (Ibekwe *et al.* 2002; Ramotar *et al.* 1995; Sharma & Dean-Nystrom 2003; Whitham *et al.* 1996), other virulence genes such as the intimin or *tir* gene (Lee *et al.* 2006) or a combination of both (Call *et al.*

2001; Fratamico *et al.* 1995; Paton & Paton 1998; Tsen & Jian 1997) from food, water and fecal samples.

Detection limits have ranged from a single CFU/ml (Campbell *et al.* 2001; Tsen & Jian 1997) to 6.4×10^3 CFU/ml (Ibekwe *et al.* 2002). Enrichment of environmental samples in broth solutions for 4 to 16 hours lowered the detection range, on average, by 10^2 (Tsen & Jian 1997) to 10^4 (Ibekwe *et al.* 2002) fold.

As compared to *E. coli* O157:H7, the application of PCR-based assays to detect *Salmonella* spp. has been more diverse in terms of both the amplification system used as well as the virulence genes targeted (Table 2.5). Predominantly employed within the food industry, countless PCR studies have identified *Salmonella* spp. in a range of contaminated food products, including fish (Lin & Tsen 1996), chicken (Tuchili *et al.* 1994), eggs and dairy products (Cohen *et al.* 1996) and oysters (Brasher *et al.* 1998). The specificity of such assays has led to the development of a number of commercial USDA-approved RT PCR *Salmonella* detection kits including BAX[®] (Bailey 1998; DuPont Qualicon; DE, USA), TaqMan[®] *Salmonella enterica* Detection Kit (Applied Biosystems; CA, USA), LightCycler[®] Foodproof *Salmonella* Detection Kit (Roche Diagnostics; Mannheim, Germany).

Currently, the majority of recent research has focused on refining the specificity and length of PCR assays. As a consequence, a number of studies have comparatively evaluated the specificity of previously described sets of *invA* primer pairs (Csordas *et al.* 2005; Fey *et al.* 2004; Hoorfar *et al.* 2000). Assays conducted in shorter amounts of time have also been described, such as the Real-Time PCR assay developed by Eyigor *et al.* (2002) that was conducted in 25 minutes to screen infected poultry flocks.

As for *E. coli* O157:H7, PCR assays developed for the detection of *S. aureus* have centered on the amplification of multiplexed enterotoxin gene targets in food products or clinical samples for diagnostic and epidemiological purposes (Table 2.6). Commonly amplified targets include staphylococcal enterotoxin genes, *entA* to *entE*, exfoliative toxin genes, *etaA* and *etaB*, toxic shock syndrome toxin I gene, *tst*, as well as the methicillin resistance *mecA* gene (Mehrota *et al.* 2000). Although assays have been developed to identify subtypes and MRSA strains isolated from food and waterborne outbreaks, few have yet been described to differentiate between staphylococcal enterotoxin C subtypes (Chen *et al.* 2001).

Table 2.5: Applications of PCR in the detection of *Salmonella* spp. in fecal and environmental samples.

Reference	Test Samples	Gene Target	Detection Limit	PCR
Way <i>et al.</i> 1993	Culture strains; pond water samples	<i>phoP</i> , <i>Hin</i> , <i>H-li</i>	100 CFU per 50 µl reaction	Multiplex PCR
Chiu & Ou 1996	Fecal samples	<i>invA</i> , <i>spvC</i>	200 CFU per 50 µl reaction	Multiplex PCR (with enrichment)
Waage <i>et al.</i> 1999	Culture strains; seeded sewage and drinking water samples	Randomly cloned chromosomal fragment (regions ST14 and ST11)	2-10 CFU/100 ml	Nested PCR (with enrichment)
Hoorfar <i>et al.</i> 2000	Culture strains	<i>invA</i>	-	Taqman End-point (5' Nuclease) PCR
Eyigor <i>et al.</i> 2002	Culture strains; fecal samples	<i>invA</i>	-	Real-Time PCR (with enrichment)
Fey <i>et al.</i> 2004	Culture strains	<i>invA</i>	-	Real-Time Reverse Transcriptase PCR
Csordas <i>et al.</i> 2004	Culture strains	<i>invA</i> , <i>invB</i> , <i>fimA</i> , <i>iroB</i> , randomly cloned chromosomal fragment (regions ST15 and ST11)	35CFU, 3000 CFU per 50 µl reaction with previously published and newly-designed primer pairs respectively	Real-Time PCR

2.3.2.2 Fluorescent *in situ* hybridization:

2.3.2.2.1 Overview of hybridization process

As a further improvement on molecular detection methods in recent years, fluorescent *in situ* hybridization (FISH) with RNA or DNA oligonucleotide probes has developed into an invaluable tool with which to evaluate the phylogenetic identity, morphology, number and spatial distributions of microorganisms in environmental samples (Hugenholtz *et al.* 2001). Unlike both culture-dependant and PCR techniques, FISH allows for the identification of

morphologically intact cells *in situ*, eliminating the need for culturing or extraction steps. Used in combination with confocal laser scanning microscopy (CLSM), this technique has been widely described for the analysis of bacterial community structure in sludge samples (Snaidr *et al.* 1997), as well as for the detection and enumeration of pathogenic bacterial species in environmental and clinical samples (Licht *et al.* 1996; Jansen *et al.* 2000; Waar *et al.* 2005).

Table 2.6: Applications of PCR in the detection of *S. aureus* in fecal and environmental samples.

Reference	Test Samples	Gene Target	Detection Limit	PCR
Mehrota <i>et al.</i> 2000	Culture strains	<i>entA, entB, entC, femA, mecA, eta, etb, tst, femA</i>	-	Multiplex PCR
Reischl <i>et al.</i> 2000	Culture strains	<i>mecA</i>	25 CFU per 20 µl reaction	Multiplex Real-Time PCR
Sharma <i>et al.</i> 2000	Culture strains	<i>entA, entB, entC, entD, entE, tst, speA</i>	-	Multiplex PCR
Chen <i>et al.</i> 2001	Culture strains isolated from fecal samples	<i>entA, entB, entC1, entC2, entC3, entD, entE, tst</i>	1 CFU per 50 µl reaction	Multiplex PCR

2.3.2.2.2 Principles of whole cell hybridization

FISH involves the application of labeled probes to treated whole cells *in situ*. Cells are simultaneously permeabilized and fixed with an aldehyde or alcohol to maintain morphological integrity throughout the hybridization process (Amann *et al.* 1992). Upon entering the cells, probes hybridize to their complementary target ribosomal (RNA) or chromosomal (DNA) sequences. These are applied in a high-salt hybridization buffer and incubated for a number of hours (Amann *et al.* 1997). Weakly or unbound probe molecules are removed by a subsequent washing step with low salt buffers containing a denaturing detergent (Hilario 2007). The specificity of the hybridization reaction depends primarily on the stringency of these two stages. The sample can subsequently be analyzed by epifluorescence microscopy or flow cytometry (Amann *et al.* 1997).

2.3.2.2.3 Oligonucleotide probes

Probes currently available for FISH analysis are grouped into two major categories: double or single-stranded DNA probes and single-stranded RNA probes. Recently, a third PNA (Peptide Nucleic Acid) probe⁸ has also been developed for use in clinical diagnosis of viruses and protozoan pathogens (Bonvicini *et al.* 2006). Probes targeting ribosomal (r)RNA have been the most commonly used in the analysis of environmental samples. This is due to the large amount of rRNA available for hybridization present in most cells, and the wide range of highly conserved as well as highly variable regions available (Amann *et al.* 1997). On the other hand, gene-specific DNA probes confer a higher degree of specificity, yet in turn result in slightly lower detection sensitivity as a result of the inherently low-copy number of target nucleic acids present. Applications of these probes have consequently been restricted to clinical samples (He *et al.* 1998).

As for PCR primers, FISH probes are available in online databases, such as probeBase (Vienna Ecology Center; Vienna, Austria) for rRNA probes, or in published literature. They may also be designed on chromosomal or ribosomal sequences deposited in online databanks, such as the Ribosomal Database Project II (Cole *et al.* 2007) and the European Ribosomal RNA Database (University of Antwerp; Antwerp, Belgium) for RNA sequences, and GenBank (NCBI; MD, USA) for DNA sequences. The specificity of the probe is tailored to the specific application and may be adjusted to different phylogenic levels (Loy *et al.* 1998).

Probes are either fluorescently labeled at the 5' end with fluorescent dyes such as the carboxycyanines Cy3 and Cy5 (Amann *et al.* 1997), or non-radioactively labeled with digoxigenin (DIG)-labeled nucleotides (Roche 2006). Subsequently, these may be hybridized to whole cells or to membrane or gel-bound nucleic acids (Khan & Nawaz 2007).

2.3.2.2.4 Application in pathogen detection

Over the past two decades, nucleic acid probes have revolutionized the field of environmental microbiology, and have been employed to study microbial diversity and bacterial community structures in a range of environmental samples including, soils and sediments (Hahn *et al.* 1992; Llobet-Brossa *et al.* 1998; Zarda *et al.* 1997), wastewater

⁸ As a synthetic molecule that structurally imitates DNA, PNA is known to bind complementary nucleic acid sequences with a much stronger affinity than DNA or RNA molecules, making it a highly specific choice for hybridization probes (Bonvicini *et al.* 2006).

biofilms (Amann *et al.* 1992), and water samples (DeLong *et al.* 1994; Harmsen *et al.* 1997; Fuhrman & Ouverney 1998). Protocols have been optimized and described for the detection of a variety of bacterial strains in environmental samples. Tang *et al.* (2005) developed a rapid and specific FISH assay using fluorescein-labeled 16S rRNA probes to detect *E. coli* cells in seawater samples. Conducted in under 2 hours, this assay was able to positively identify 90% of the seeded seawater samples. Similarly, Brehm-Stecher *et al.* (2005) were able to detect the food-borne pathogen, *Listeria monocytogenes* using specific fluorescein-labeled PNA probes. Numerous genus-specific 16S rRNA assays have also been described for *Helicobacter pylori* and *Legionella* spp. in water samples (Buchbinder *et al.* 2002; Moreno *et al.* 2003), *Enterococcus* spp. in fecal samples (Waar *et al.* 2005) and *S. aureus* in blood samples (Jansen *et al.* 2000). To date, no studies have been conducted on the detection of bacterial strains (both pathogenic and nonpathogenic) using DNA-based and -targeted FISH probes. The sole application of these probes has been within a clinical diagnostic setting for the detection of DNA or RNA viruses such as Human Immunodeficiency Virus type 1, hepatitis B and human papillomavirus (He *et al.* 1998; Player *et al.* 2001)

The application of FISH has also been hindered by its restricted sensitivity in environmental samples (Pernthaler & Amann 2004). As the intensity of the fluorescent signal emitted by the hybridized cells depends on the amount of probe target present within them, signal intensities in environmental samples are often below the microscopic detection limits or lost among high background levels (Pernthaler & Pernthaler 2007). As a consequence, rigorous optimization of hybridization conditions is often needed, as well as the application of signal amplification techniques such as Catalyzed Reporter Deposition or CARD (Bobrow *et al.* 1991). Initially applied to immunoassays (such as ELISA and immunoblotting), this technique has recently been coupled to *in situ* hybridization to improve detection sensitivities for both DNA and RNA assays by up to 100 fold (Speel *et al.* 1999) in the analysis of bacterial communities in sludge samples (Snaird *et al.* 1997) and the natural environment (Ainsworth *et al.* 2006). This method, along with other signal amplification techniques will be discussed in more detail in the coming sections.

2.3.2.3 Comparative evaluation

As two of the most prominent molecular detection techniques, both PCR and FISH have each been successfully applied to the detection of a wide range of wastewater pathogens.

Both have been shown to be capable of rapid and sensitive enumeration, independent of any cultivation steps. As a result, both have been capable of overcoming the weaknesses commonly encountered by conventional indicator-based assays. In spite of such comparable performance, few studies to date have comparatively evaluated the detection capabilities of both techniques in any diagnostic applications. Of the studies available, all have focused primarily on RNA-based assays with unculturable bacterial strains. In 2003, Moreno *et al.* (2003b) compared RNA-based end-point PCR and FISH for the detection of *Arcobacter* and *Campylobacter* in river and wastewater samples. Semi-quantitative assay sensitivity estimates were determined based on minimum PCR detection limits in CFU/ml, and the presence or absence (defined as above or below an arbitrary intensity threshold) of the hybridization signal for FISH. Out of the 30 samples assayed for *Campylobacter*, 40% of the samples testing positive with FISH, were undetectable with PCR, while for *Arcobacter*, both assays had equivalent detection strengths. Overall, it was concluded that both techniques were capable of rapid, sensitive and specific enumeration. A similar study conducted by Buchbinder *et al.* (2002) for the detection of *Legionella* spp. in water samples using the same detection and enumeration methodologies concluded that PCR was the least sensitive of the two assays.

2.3.3.2.4 Experimental objectives

In light of these results, this study will attempt to quantitatively evaluate the comparative strengths of DNA-based FISH and PCR for the enumeration of selected wastewater bacteria. It is hypothesized that both techniques will be capable of rapid and specific detection of single-copy gene targets. Therefore, the specific objectives of this work are to:

1. Test the specificity of primer and probe sets targeting single-copy, chromosomal virulence genes
2. Determine the minimum detection limits in gene copy numbers of either assay in pure cultures and seeded wastewater samples
3. Examine the inhibitory effect of wastewater components on the detection sensitivity of either assay
4. Compare the strengths and limitations of either assay.

3.0 MATERIALS & METHODS:

3.1 Collection of wastewater samples

Wastewater samples analyzed in both FISH and PCR assays were collected from the activated sludge digester tank at the Ashbridges Bay Wastewater Treatment Plant, located in Eastern Toronto. As the city's main treatment facility and one of the largest nation-wide, this plant receives up to 200,000 m³ of municipal wastewater daily (City of Toronto 2007).

3.2 Bacterial Culturing

Pure cultures of *Escherichia coli* ATCC 25922, *Escherichia coli* O157:H7 ATCC 25928, *Escherichia coli* O157:H7 85170⁹, *Salmonella enterica* subsp. *enterica* ATCC 14028, *Staphylococcus aureus* subsp. *aureus* ATCC 25923 were supplied by Dr. Dae-Young Lee of the Wastewater Technology Center (Environment Canada, Burlington, ON) and Dr. Deborah Foster of Ryerson University. Frozen stock strains were made with 40% glycerol and 60% of a 5% w/v Trisodium Citrate solution in water. These were stored at -80°C and were routinely subcultured onto Nutrient Agar (DIFCO Laboratories, MI, USA) plates. These cultures were stored at 4°C and subcultured every three weeks to maintain viability and avoid contamination.

Broth cultures of all strains were prepared by inoculating 5 ml of Nutrient Broth (Scott Laboratories Inc., CA, USA) with a single colony from the respective Nutrient Agar plate culture and incubated overnight in a shaker-incubator at 37°C at 200 rpm.

3.3 PCR Assay:

3.3.1 DNA extraction

For use in the PCR assay, actively growing cells in 1 ml of broth culture were harvested by centrifugation at 10,000 g for 2 minutes prior to extraction. Wastewater samples were stored in sealed containers at 4°C upon collection and used for extraction without pre-enrichment. The deposited sludge was thickened by centrifugation at 10,000 g for 2 minutes, after which most of the supernatant was removed.

Genomic DNA was extracted from both wastewater samples and bacterial cultures using three commercial DNA isolation kits: PureLink[®] Genomic DNA Purification kit (Invitrogen, CA, USA), UltraClean[®] Soil DNA Isolation kit (MO BIO Laboratories Inc., CA, USA) and

⁹ As a derivative of the parent strain *E. coli* O157:H7 84-289, this mutant strain lacks the Vero toxin producing genes, VT1 and VT2 (Tzipori *et al.* 1987)

Wizard® Genomic DNA Purification kit (Promega Corp., WI, USA) according to the manufacturers' protocol. For wastewater samples (including seeded wastewater samples) a modified protocol was followed that combined the extraction-purification procedure of the Purelink® Genomic DNA Purification kit with the bead-beating technology of the UltraClean® Soil DNA Isolation kit. The Wizard® Genomic DNA Purification kit was used with bacterial cultures according to the manufacturer's protocol.

The purity of the isolated DNA was then assessed by comparing the optical density (spectrophotometric absorbance) of the sample at 260 nm and at 280 nm. Relatively pure nucleic acid samples are expected to have $A_{260/280}$ ratio values from 1.5 - 2.0 (Declerck *et al.* 2006).

3.3.2 Primer design

Three sets of primer pairs were analyzed in this study (Table 3.1), all of which target low copy number, species-specific genes. As such the PCR reaction has been made highly specific yet less sensitive. The first set (set 1) targeting *E. coli* (Appendix A), *E. coli* O157:H7 (Appendix B), *S. enterica* (Appendix C) and *S. aureus* (Appendix D) genes was adopted from Lee *et al.* (2005). The second set (set 2) adopted from Csordas *et al.* (2004) targeted the same *S. enterica* and *S. aureus* genes used in the previous study. The third set (set 3) were designed specifically for use in this study using Primer Design II (Roche Diagnostics, Mannheim, Germany) against the same *E. coli* (Appendix A), *E. coli* O157:H7 (Appendix B) and *S. enterica* (Appendix C) genes, using sequences deposited in the NCBI GenBank database.

For use with the SYBR Green I qPCR detection system, individual primers were designed following a standardized list of key selection criteria that included: primer length (18-24 bp), GC content (over 50%, with a terminal 'GC clamp'), PCR amplicon size (200-400 bp to be effectively resolved on a gel and clearly differentiated from any primer-dimers) and melting temperature, T_m (58-60°C). Within the individual pairs, it was necessary to have comparable T_m and GC content values to avoid a loss of specificity, as well as minimal homology, including dimerization or formation of hairpin loops and secondary structures, particularly at the 3' end.

Primer pairs EUID, ETIR and SINV were finally selected to amplify target *E. coli*, *E. coli* O157:H7 and *S. enterica* gene sequences, respectively. A list of key primer design

parameters, as well as the target genes and species, are shown in **Table 3.2**. All primers were subjected to a BLAST search as a preliminary indicator of specificity.

All primers were synthesized at the DNA Synthesis Facility at the Hospital for Sick Children (MaRS Center, Toronto, ON) in de-salted form. These were individually reconstituted in TE buffer to a stock solution of 100 µM, and then diluted in PCR-grade water to working solution of 5 µM. Both solutions were stored at -20°C.

Table 3.1: Primer sets used in this study, along with the respective target organism and gene of interest.

Organism	Gene	Function	Primer set	Sequence (5'→ 3')	Reference
<i>Escherichia coli</i>	<i>uidA</i>	Glucuronidase	Eco-F	GTCCAAAGCGGCGATTTG	Lee <i>et al.</i> 2005
			Eco-R	CAGGCCAGAAGTTCTTTTCCA	
			EUID-F	GCGTCAATG TAATGTGCG	This study
			EUID-R	GGTGATACATATCCAGCCATGC	
<i>Escherichia coli</i> O157:H7	<i>Tir</i>	Translocated intimin receptor	EcoOH-F	TCGAGCGGACCATGATCA	Lee <i>et al.</i> 2005
			EcoOH-R	GGCGGCGTCTGAGATAACA	
			ETIR-F	GTCAGCTCATTA ACTCTACGGG	This study
			ETIR-R	GCCTGTTAAGAGTATCGAGCG	
<i>Salmonella enterica</i>	<i>invA</i>	Invasion protein	Sal-F	CGTTTCCTGCGGTACTGTTAATT	Lee <i>et al.</i> 2005
			Sal-R	AGACGGCTGGTACTGATCGATAA	
			SEN-F	TTTCAATGGGA ACTCTGC	Csordas <i>et al.</i> 2004
			SEN-R	AACGACGACCCTTCTTTT	
			SINV-F	TATGCCCCGTAAACAGATGAG	This study
			SINV-R	GTATAAGTAGACAGAGCGGAGG	
<i>Staphylococcus aureus</i>	<i>entC1</i>	Enterotoxin C gene	Saur-F	CGTATTAGCAGAGAGCCAACCA	Lee <i>et al.</i> 2005
			Saur-R	AGACGGCTGGTACTGATCGATAA	

3.3.2 Determination of Assay specificity:

3.3.2.1 End-point PCR amplification

In determining the specificity of the PCR assay, primer pairs of each strain were individually tested against both target and non-target strains in an end-point PCR reaction.

Template genomic DNA, extracted as described earlier, and PCR primers were added to TITANIUM® *Taq* PCR kit reagents (Clontech Laboratories, CA, USA) to a final volume of 25 µl. The reaction contained a final concentration of 1 µM of each primer, 0.2 mM dNTP mix, 0.5 X TITANIUM® *Taq* DNA Polymerase, 0.4 X TITANIUM® *Taq* PCR Buffer, PCR-grade water, 2 mg/ml Bovine Serum Albumin (BSA) (New England BioLabs Inc., MA, USA) and 100-200 ng of template DNA. Negative controls run with each reaction contained PCR-grade water in place of DNA. The end-point PCR reaction was conducted in a MyCycler® Thermal Cycler (BIO RAD Laboratories Inc., CA, USA) with the following temperature profiles: denaturation at 95°C for 2 min followed by 36 cycles of 95°C for 1 min, 59°C for 30 sec and 72°C for 2 min, followed by a 2 min extension at 72°C and a final hold at 4°C.

Table 3.2: Target genes and species, along with GC content, T_m , amplicon size values for each of the selected EUID, ETIR and SIN V primer pairs.

Primer	Target species	Target gene	Length (bp)	GC content (%)	T_m (°C)	Primer-dimer size (bp)	PCR amplicon size (bp)
EUID-F	<i>E. coli</i>	<i>uidA</i>	18	50.0	58.1	40	275
EUID-R			22	50.0	63.2		
ETIR-F	<i>E. coli</i> O157:H7	<i>Tir</i>	22	50.0	60.1	43	207
ETIR-R			22	50.0	61.5		
SINV-F	<i>S. enterica</i>	<i>invA</i>	21	47.6	62.1	43	252
SINV-R			22	50.0	57.3		

Specificity was determined by the presence of the intended PCR product in end-point PCR reactions containing target and non-target DNA extracted from *E. coli* ATCC 25922, *E. coli* O157:H7 ATCC 25928, *S. enterica* and *S. aureus*.

3.3.2.2 Analysis of PCR products using Gel Electrophoresis

PCR products were analyzed on a 2% agarose gel, under 100V for 20 min, alongside a 100 bp molecular marker (MBI Fermentas International Inc., Burlington, ON). Gel images

were captured using a UV Transilluminator and BioDoc-It Imaging System (UVP, CA, USA).

3.3.3 Determination of RT PCR Assay sensitivity

The sensitivity of the RT PCR assay was evaluated on the basis of the minimum amount of genomic template DNA detected in the PCR reaction, using pure cultures and seeded wastewater samples.

To determine the sensitivity of the assay using bacterial cultures, DNA extracted from *E. coli* ATCC 25922, *E. coli* O157:H7 ATCC 25928, *S. enterica* and *S. aureus* was quantified by spectrophotometry, then serially diluted in water (from over 100 ng to 1 fg of genomic DNA per reaction) and used as template in a RT PCR reaction.

The reactions were performed using a 2.0 LightCycler[®] (Roche Diagnostics, Mannheim, Germany) in a 20 µl reaction volume containing 1 X LightCycler[®] FastStart DNA Master^{PLUS} SYBR Green I Master Mix (Roche Diagnostics, Mannheim, Germany), 0.5 µM of each primer and 10-100 ng of template DNA. As for the end-point PCR reaction, negative controls run with each reaction contained PCR-grade water in place of DNA. Cycling conditions included preincubation for 10 min at 95°C and amplification for 40 cycles of 10 sec at 95°C, 5 sec at 59°C and 15 sec at 72°C.

The presence of PCR products was determined through melting curve analysis using LightCycler[®] Software 4.1 (Roche Diagnostics, Mannheim, Germany). Melting curves of the PCR products were generated by measuring the fluorescent signal emitted by the products of the reaction while automatically raising the temperature. This was performed after the amplification cycles as follows: 0 sec at 95°C, 65°C for 30 sec, and an increase from 65°C to 95°C at increments of 0.1°C per sec.

To determine assay sensitivity using seeded wastewater, DNA extracted from a series of 10-fold dilutions of wastewater samples seeded with a known number of bacterial cells was used as template in a RT PCR reaction.

Concentration curves were first constructed to calculate the number of cells seeded into the wastewater dilution series (see **Appendix E**). A series of 10-fold dilutions of 10 ml overnight broth cultures of each of the four bacterial strains were quantified with spectrophotometry and cultured on nutrient agar plates using the spread plate technique. The spectrophotometric absorbance and cell concentration (CFU/ml) of each dilution were then

used to construct concentration curves for each strain. The concentration (CFU/ml) of the bacterial cells present in the broth culture was then interpolated from the generated curves and used to calculate the approximate number of cells seeded into the wastewater dilutions.

A series of 10-fold seeded wastewater dilutions was prepared by the addition of 0.1 ml of overnight broth culture of each of the four bacterial strains—quantified through the use of spectrophotometry and concentration curves—to 0.9 ml of wastewater with further dilutions in wastewater. DNA was extracted from each seeded dilution and used as template in a RT PCR reaction, using the same reaction and cycling conditions described earlier. The level of specificity of the RT PCR assay was determined as the minimum cell concentration (CFU/ml) detected in the RT PCR reaction.

One ml samples of broth cultures of all four bacterial strains were quantified by spectrophotometry before and after extraction to determine the mass of genomic DNA per cell, in order to express the RT PCR detection limits in seeded wastewater in terms of the concentration of DNA template present per reaction. These values are listed in **Appendix F**.

3.4 FISH/CSLM Assay:

3.4.1 Oligonucleotide probe design

Two sets of fluorescently-labeled oligonucleotide probes were used in the FISH assay. These were designed to target the same chromosomally-encoded *E. coli*, *E. coli* O157:H7 and *S. enterica* genes used for primer design in the PCR assay. Probe set A included the *E. coli* O157:H7 probe, EcoOH-P, complementary to the corresponding EcoOH forward primer, while probe set B included the *E. coli* O157:H7 and *S. enterica* probes, Ecotir and Salinv, complementary to the respective ETIR and SINV primer sequences from primer set 3. To aid in the comparative evaluation of the two assays, the probes were designed on the sequences of the ETIR-F, SINV-F and EcoOH-F primers. The universal EUB338 DNA probe, complementary to a region of the 16S rRNA of most of the domain *Bacteria*, was used as a positive control to detect all eubacteria in the samples tested (Amann *et al.* 1990).

All the probes were synthesized at the DNA Synthesis Facility at the Hospital for Sick Children (MaRS Center, Toronto, ON) as single-stranded, fluorescently-labeled DNA probes. These were individually reconstituted in sterile distilled and deionized water to a stock concentration of 2mM, and then diluted further to a working concentration of 50 ng/μl. Both solutions were covered and stored at -20°C. All probes were subjected to a BLAST

search as a preliminary test of specificity. A summary of the probe name, sequence, fluorescent label and target species is listed in **Table 3.3**.

Table 3.3: The sequences, labels, and target genes and species of the four oligonucleotide probes used in this study.

Probe Name	Sequence (5' → 3')	Target species	Gene	Fluorescent label	Reference
EcoOH-P	TCGAGCGGACCATGATCA	<i>Escherichia coli</i> O157:H7	<i>tir</i>	6-FAM	This study
Ecotir	GTCAGCTCATTAACCTACGGG	<i>Escherichia coli</i> O157:H7	<i>tir</i>	Cy5	This study
Salinv	TATGCCCGGTAAACAGATGAG	<i>Salmonella enterica</i>	<i>invA</i>	HEX	This study
EUB338	GCTGCCTCCCGTAGGAGT	Most eubacteria	16S rRNA	HEX	Amann <i>et al.</i> 1990

3.4.2 FISH analysis

For *in situ* analysis, wastewater and culture samples were treated with a fixative then applied onto gelatin coated slides and hybridized with the respective oligonucleotide probe overnight.

3.4.2.1 Gelatin coated slides

As directed in the Methods in Gene Biotechnology manual (Wu *et al.* 1997), glass slides were sterilized in 10% KOH in 95% ethanol for an hour, then rinsed in distilled water and air-dried. The dried slides were then immersed in a gelatin solution of 0.1% w/v gelatin and 0.01% w/v KCr(SO₄)₂ in sterile distilled water for 10 min at 70°C, then dried at a slant overnight to obtain an even gelatin coating. This coating ensures the adhesion of the cell sample to the slide despite repeated washing and hybridization steps. The gelatin coated slides were stored in a dust-free container at room temperature for up to 2 months.

3.4.2.2 Cell fixation

One ml of overnight broth culture or wastewater sample was centrifuged at 10,000 *g* for 2 min. The supernatant was decanted and the pellet was washed in 1 X PBS buffer (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl in sterile distilled water at pH 7.4)

by vortexing for 5 min. The sample was centrifuged once more and the pellet was resuspended in 4% w/v paraformaldehyde (PFA) in 1 X PBS (heated to 60°C). The samples were incubated at 4°C for 4 hours.

After fixation, the cells were pelletized by centrifugation at 10,000 g for 2 min, washed twice with 1 X PBS to get rid of any remaining fixative solution, then fully resuspended in 70% ethanol in 1 X PBS. The samples were stored at -20°C for up to two months.

3.4.2.3 Whole cell hybridization:

3.4.2.3.1 Pre-hybridization treatment

Frozen samples were centrifuged and washed with 1 X PBS and left to rehydrate in 300 µl of 1 X PBS for 20 min at room temperature. After a second round of centrifugation for 2 min at 10,000 g, the cell pellets were resuspended in 300 µl of 1 X PBS and a drop of TWEEN 20 (Promega Corp., WI, USA) detergent to permeabilize the cell membrane. The samples were pelletized once again and fully resuspended in 400 µl of 0.1% v/v TWEEN 20 in 1 X PBS. Ten µl of this solution were spotted onto each well of a gelatin coated slide and spread with the flat edge of a pipette tip to avoid scratching the gelatin coating. Slides were dried in a hybridization oven at 46°C for 10 to 15 min until dry. Another 10 µl were spotted onto the slide wells and then dried.

3.4.2.3.2 DNA Denaturation

DNA of the fixed cells spotted onto the slides was denatured by adding 100 µl of 70% v/v formamide solution in 2 X SSC (0.3M NaCl, 0.03M Sodium Citrate in distilled water) to each well for 2 min at 70°C. The denaturation solution was rinsed off with 2 X SSC and the slides were left to dry at room temperature. This step was omitted with the EUB338 probe as the rRNA sequence targeted is present within the cell in single-stranded form.

3.4.2.3.3 *In situ* hybridization

Dried slides were then dehydrated in a graded ethanol series (50%, 80% and 95% in distilled water) for 3 min each then dried for 10 min at 46°C. Ten µl of a hybridization cocktail of 2 µl of the hybridization probe (at a concentration of 50 ng/µl) and 8 µl of the appropriate hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl buffer at pH 7.2, 0.01% w/v SDS in distilled water and formamide concentration as shown in **Table 3.4**) were then added to each well. This was spread evenly with the flat side of a pipette tip and covered with a sterile glass coverslip, taking care to avoid trapping any air bubbles. Slides were placed in an

isotonically equilibrated humidity chamber consisting of a sealed Petri dish containing paper towels soaked in a 1M NaCl solution, and incubated for 16-18 hrs at 46°C.

Table 3.4: Concentrations of formamide and NaCl in the hybridization and washing buffers respectively for each of the four hybridization probes.

Probe Name	EcoOH-P	Ecotir	Salinv	EUB338
Formamide Concentration (v/v %)	30	22	25	39
NaCl Concentration (mM)	135	135	135	135

3.4.2.3.4 Probe washing

After hybridization, slides were rinsed with 5 ml of the appropriate washing buffer (NaCl in concentrations shown in Table 6, 20 mM Tris/HCl buffer at pH 7.2, 0.01% SDS and 5 mM EDTA) and incubated at 48°C for 2 min. Slides were then rinsed twice in the dark in sterile distilled water for 30 sec, and left and to dry in a dark container.

3.4.2.3.5 Optimizing probe specificity

Stringency conditions for each probe were optimized to avoid nonspecific binding. Although this is commonly achieved by adjusting each of the formamide and salt concentrations (in the hybridization and washing buffers respectively) as well as the hybridization temperature, in various combinations, this was found to be too time-consuming and inefficient once applied to previously undescribed probe sets. Accordingly, this study followed the optimization protocol adopted by Pogue (2004) that involved adjusting only the formamide concentrations for each probe at fixed NaCl and temperature conditions. As such, the EUB338 formamide concentration used in this study does not correspond to that used in other studies (Amann *et al.* 1990).

Hybridizations were performed at a fixed temperature of 46°C and 135 mM NaCl concentration with the optimal formamide concentration (listed in **Table 3.4**) as calculated through the Lathe equation below (Lathe 1985; Pogue 2004):

$$T_m = 81.5 + 16.6 \log [Na^+] + 0.41 (\%GC) - 0.63 (\%F) - [300 + 200 (Na^+)]/N$$

EQUATION 1

T_m: Melting temperature of the hybridization probe, °C

[Na⁺]: Molar concentration of Na⁺ ions in the washing buffer, M

%GC: Percentage of GC base pairs in the hybridization probe

%F: Concentration of formamide in the hybridization buffer, v/v%

N: Length of the hybridized probe, bp

These stringency conditions were confirmed by comparing the level of fluorescence in hybridized samples of both target and non-target strains.

3.4.2.4 Confocal microscopy and image analysis

Hybridized samples were analyzed by confocal microscopy using a Carl Zeiss (Oberkochen, Germany) LSM 510 confocal laser scanning microscope (CLSM) equipped with a helium neon (HeNe) laser and an argon (Ar) ion laser with emission wavelengths of 543 nm and 433 nm respectively. The laser configuration, filter and pinhole settings calibrated for use with each of the four probes are listed in **Table 3.5**. Microscopic analysis was performed using the Achroma 40 X and 100 X objective lenses, along with the image analysis features of the LSM 510 software package.

Slides were first viewed with transmitted light, followed by fluorescence. The two images were then overlaid to visualize the fluorescence within the sample. Images were exported in a JPEG format using the LSM 510 Image Browser software (Carl Zeiss, Oberkochen, Germany).

3.4.3 Determination of assay specificity:

3.4.3.1 Dot blot analysis

All probes were subjected to a BLAST search as a preliminary test of specificity. This was then confirmed through the use of a modified dot blot assay adapted from Harmsen *et al.* (1997). End-point PCR reactions were performed with template DNA extracted from *E. coli* O157:H7 ATCC 25928 and *S. enterica* broth cultures along with the corresponding PCR primer pair from the ETIR and SINV primer set. As the FISH probes were designed on the sequence of the forward primers, the double-stranded PCR products generated were complementary to the corresponding Ecotir and Salinv probes. Products were run on a 2%

agarose gel as detailed previously, and the expected PCR product bands were identified. These were then transferred onto separate nitrocellulose membranes by means of standard Southern blotting methods (Oss *et al.* 1987). Nitrocellulose membranes containing the incorporated PCR products were then separately immersed in 15 ml of the corresponding hybridization buffer containing 500 µl of either the Ecotir or Salinv probe, for 4 hours at 46°C. Hybridized membranes were then immersed in the appropriate washing solutions for 20 mins at 48°C, then mounted onto sterile glass slides and dried at 48°C. These were then analyzed by confocal microscopy. Specificity of the Ecotir and Salinv probes was determined by the presence of fluorescence on membranes spotted with DNA from PCR reactions with template DNA from the corresponding bacterial culture (*E. coli* O157:H7 and *S. enterica* respectively).

Table 3.5: CLSM configuration settings for viewing probes labeled with each of the three fluorescent labels. Fluorescence data is inputted through Channel 1, while transmitted light data is inputted through Channel D.

Probe Name	EcoOH-P	Ecotir	Salinv	EUB338
Fluorescent label	6-FAM	HEX	Cy5	HEX
Excitation wavelength (nm)	492	535	650	535
Emission wavelength (nm)	517	556	670	556
Laser	Ar (488nm)	Ar (488nm)	HeNe (543nm)	Ar (488nm)
Filter	LP530	LP530	LP650	LP530
Pinhole (AU)	1.00	1.00	1.00	1.00
Channel 1				
Detector Gain	981	981	1149	981
Amplification Offset	-0.24	-0.24	-0.27	-0.24
Amplification Gain	1.10	1.10	1.00	1.10
Channel D				
Detector Gain	235	235	416	235
Amplification Offset	-0.73	-0.73	-1.24	-0.73
Amplification Gain	1.14	1.14	1.60	1.14

3.4.3.2. Whole cell hybridization using bacterial cultures

The specificity of the FISH assay was further evaluated using whole-cell hybridization of bacterial cultures. One ml broth samples of *E. coli*, *E. coli* O157:H7, *S. aureus* and *S. enterica* strains were fixed as described previously, and then individually hybridized to the Ecotir and Salinv probes. As a control, samples of the same cultures were simultaneously pretreated and hybridized to the EUB338 to confirm the validity of the pretreatment and hybridization protocols. Specificity of the two probes was evaluated on the basis of the extent of hybridization with target and non-target bacterial strains.

As for the RT PCR assay, the sensitivity of the FISH assay was to be similarly evaluated by whole-cell hybridization with samples of bacterial cultures and wastewater samples seeded with bacterial cultures. As will be discussed, this was not conducted in this study. A description of the method to be followed is listed in **Appendix G**.

4.0 RESULTS:

4.1. PCR assay

As described in the Methods section, specificity and sensitivity tests were first conducted on primer sets 1 and 2 simultaneously, using end-point and RT PCR, respectively. Following this, primer set 3 was then designed and similarly evaluated.

Specificity tests with end-point PCR confirmed the specificity of the primer pairs, permitting the determination of RT PCR sensitivity using dilutions of pure culture DNA and seeded wastewater.

4.1.1. Primer sets 1 and 2:

4.1.1.1. Evaluation of specificity

Overall, all primer pairs from both sets exhibited poor specificity; primer-dimer formation was widely observed in all reactions, and gene targets were poorly amplified resulting in either the absence of intended products or the presence of misprimed products.

For primer set 1, the products of the Eco reaction are shown in **Fig. 4.1**. As detailed in **Appendix A**, the expected product and primer-dimer sizes for the Eco reaction with target *E. coli* DNA are 59 bp and 22 bp, respectively. As apparent in **Fig. 4.1**, primer-dimer product bands were observed in all reactions. The intended product was generated with target DNA (**Lane 1**), while multiple non-specific products were also generated with nontarget *S. aureus* (**Lane 3**) and *S. enterica* (**Lane 4**) DNA as indicated by the presence of bright smears and multiple product bands.

The EcoOH primer pair exhibited similarly poor specificity (**Fig. 4.2**). Several misprimed nonspecific products were generated with target *E. coli* O157:H7 (**Lane 3**) and nontarget *E. coli* (**Lane 2**) and *S. enterica* (**Lane 4**) DNA. Primer-dimer was generated in all reactions (40 bp in size; **Appendix B**).

For the Sal primer pair (**Fig. 4.3**), the intended product (65 bp in size; **Appendix C**) was not generated in any Sal reactions with either target (**Lane 4**) or nontarget (**Lanes 1 - 3**) DNA. As for the EcoOH primer pair, primer-dimer formation (46 bp in size; **Appendix C**) was also observed in all reactions, including the No Template Control (NTC) containing no DNA (**Lanes 1 - 5**). The positive control reaction (**Lane 4**; Sal with *S. enterica* template) generated the highest amount of dimerization.

Fig. 4.1: Products of the end-point PCR reaction using the Eco primer set with template DNA extracted from: (Lane 1) *E. coli* (+), (Lane 3) *S. aureus*, (Lane 4) *S. enterica* and (Lane 2) the No Template Control (no DNA added). Products were run on a 2% agarose gel under 100V for 20 minutes, alongside a 100 bp Molecular Marker, shown on the left.

For the Saur primer set, the intended product (63 bp in size; **Appendix D**) was not generated with target *S. aureus* template (**Fig. 4.4; Lane 3**). Instead, primer-dimer formed with both the target and nontarget (Lane 1) template. Additionally, a misprimed product band of approximately 1,100 bp in size was observed with both reactions.

Fig. 4.2: Products of the end-point PCR reaction using the EcoOH primer set with template DNA extracted from: (Lane 2) *E. coli*, (Lane 3) *E. coli* O157:H7 (+), (Lane 4) *S. enterica*, (Lane 5) *S. aureus* and (Lane 1) the No Template Control (no DNA added). Products were run on a 2% agarose gel under 100V for 30 minutes, alongside a 100 bp Molecular Marker, shown on the left.

Fig. 4.3: Products of the end-point PCR reaction using the Sal primer set with template DNA extracted from: (Lane 1) *Klebsiella pneumoniae*, (Lane 2) *E. coli*, (Lane 3) *S. aureus*, (Lane 4) *S. enterica* (+) and (Lane 5) the No Template Control (no DNA added). Products were run on a 2% agarose gel under 100V for 20 minutes, alongside a 100 bp Molecular Marker, shown on the left.

Fig. 4.4: Products of the end-point PCR reaction using the Saur primer set with template DNA extracted from: (Lane 1) *K. pneumoniae*, (Lane 2) *E. coli*, (Lane 3) *S. aureus* (+) and (Lane 4) *S. enterica*. Products were run on a 2% agarose gel under 100V for 30 minutes, alongside a 100 bp Molecular Marker, shown on the left.

Although PCR conditions were corrected for cycling parameters, $MgCl_2$ concentration, and annealing temperatures, primer-dimer formation persisted in reactions with all four primer pairs.

As described earlier for primer set 2, the specificity of the SEN primer pair was evaluated using RT PCR reactions with target and nontarget DNA.

Typically, the start of amplification of the gene target is marked by an increase in fluorescence after 10-20 cycles. However, the RT PCR reaction with the SEN primer set actually produced a drop in fluorescence at this point, as shown in **Fig. 4.5**.

Fig. 4.5: The Amplification curves generated from the RT PCR reaction of the SEN primer pair with target and nontarget DNA from *E. coli*, *E. coli* O157:H7, *S. enterica* and *S. aureus*. The amplification curve of the RT PCR reaction with the EcoOH primer pair and *E. coli* O157:H7 DNA has been plotted alongside for comparison.

To confirm this, the amplification curves of the SEN reactions have been plotted here alongside a positive control RT PCR reaction using the EcoOH primer pair with target *E. coli* O157:H7 DNA for comparison. As both the EcoOH and SEN reactions were conducted using the same RT PCR reagents and target DNA, it was determined that the observed drop in fluorescence was not a result of any interfering components present along with the RT PCR reagents, or of defective reagents themselves (including SYBR Green I dye). Due to the amplification inconsistencies exhibited, this primer pair was not evaluated further.

4.1.1.2. Evaluation of sensitivity

Primer sets 1 and 2 were simultaneously evaluated for the ability to amplify decreasing concentrations of the intended gene targets in serial dilutions of template DNA, from pure culture and seeded wastewater samples.

For the EcoOH primer pair from set 1, RT PCR reactions were conducted using dilutions of target *E. coli* DNA ranging from 0 ng to 80 ng per reaction. As shown from the amplification curves in **Fig. 4.6**, the amplification of the intended gene target began at regular cycle intervals for each reaction along the dilution series, as expected.

Fig. 4.6: The amplification curves generated from the RT PCR reaction of the EcoOH primer pair with dilutions of *E. coli* O157:H7 template DNA from 0 ng to 80 ng per reaction.

This was also observed with the Sal (**Fig. 4.7**) and Saur (**Fig. 4.8**) primer pairs with similar dilutions of target *S. enterica* and *S. aureus* template, respectively. From these results, template concentrations that generated overlapping amplification curves (0.0012 ng to 1.2 ng of *S. enterica* template in **Fig. 4.7**; 0.0024 ng to 0.24 ng of *S. aureus* template in **Fig. 4.8**) were determined to be below the detection range of the RT PCR assay. The SEN primer pair from primer set 2 was not tested due to the amplification problems exhibited during the specificity testing described earlier.

However, the further analysis of the melting curves of the individual products generated revealed that in actual fact, the amplified products were not the intended gene targets but rather, primer-dimers of various sizes.

Fig. 4.7: The amplification curves generated from the RT PCR reaction of the Sal primer pair with dilutions of *S. enterica* template DNA from 0 ng to 120 ng per reaction.

Fig. 4.8: The amplification curves generated from the RT PCR reaction of the Saur primer pair with dilutions of *S. aureus* template DNA from 0 ng to 24 ng per reaction.

For the EcoOH primer pair, the analysis of the melting peaks (represented by the maxima of the first derivative of the melting curves) generated at the end of the amplification shown

in **Fig. 4.6** revealed that three separate, yet similar¹⁰ products of consecutive melting temperatures of 78°C, 80°C and 83°C, were produced (**Fig. 4.9**). The 80°C product was generated in the presence of the undiluted 80 ng and template, as well as the 0.8 ng dilution. The remaining 78°C and 83°C products were produced in all the remaining template dilutions as well as in the NTC. As reflected by the height of the individual melting peaks, all three products were generated inconsistently in the reactions along the dilution series. Thus, at 0.008 ng, the relative amount of the 78°C product generated was higher than that of the 83°C product, while at 0.0008 ng, the relative amount of the 83°C product was higher.

Fig. 4.9: The melting peaks generated from the RT PCR reaction of the EcoOH primer pair with dilutions of *E. coli* O157:H7 template DNA from 0 ng to 80 ng per reaction.

In the absence of contamination, the presence of the 80°C and 83°C product peaks in the NTC indicate the formation of a primer-dimer, and the further dimerization of this dimer. The location of the third 78°C peak at an equal distance from the other two, in addition to its presence in both the undiluted and diluted template indicates that it too corresponds to the formation of yet another dimer. It then follows that the inconsistent peak heights may have been a consequence of the preferential binding of the SYBR Green I dye to different dimers. This phenomenon has been previously reported by Gudnason *et al.* (2007), Branfuss & Karlovsky (2006) and Giglio *et al.* (2003).

¹⁰ The melting temperature of a PCR product depends primarily on its length and GC content (Brandfuss & Karlovsky 2006). As such, similar product sequences tend to have similar melting temperatures.

This extensive dimerization was verified by analyzing the products of the RT PCR reaction by agarose gel electrophoresis (**Fig. 4.10**). As suspected, multiple products between 50 bp and 500 bp in size—within one to ten multiples of the 40 bp primer-dimer (**Appendix B**)—were generated. Thus, rather than precisely amplifying and detecting the intended products, the RT PCR reactions simply reflected the results obtained with the end-point PCR tests, both of which amplified nonspecific products.

Fig. 4.10: Products of the RT PCR reaction using the EcoOH primer set with (**Lane 1**) NTC and template *E. coli* O157:H7 DNA dilutions containing: (**Lane 2**) 800 ng, (**Lane 3**) 80 ng, (**Lane 4**) 8 ng, (**Lane 5**) 0.8 ng, (**Lane 6**) 0.08 ng and (**Lane 7**) 0.008 ng. Products were run on a 2% Agarose gel under 100V for 20 minutes, alongside a 100 bp Molecular Marker, shown on the left.

Primer-dimer formation was also revealed in the Sal and Saur primer pair reactions. For the Sal primer pair, amplification products were observed both in the presence and absence of template DNA (**Fig. 4.11**). The melting peaks generated for all dilutions as well as the NTC were all within the same 3°C temperature range, once again indicating the formation of multiple closely-related—if not a single—products in all reactions. As for the previous EcoOH set, in the absence of contamination and without the use of PCR product sequencing, this was interpreted as the formation of several primer-dimer products in all reactions. This was verified once again through agarose gel electrophoresis, by the appearance of multiple product bands and smears (**Fig. 4.12**).

Fig. 4.11: The melting peaks generated from the RT PCR reaction of the Sal primer pair with dilutions of *S. enterica* template DNA from 0 ng to 120 ng per reaction.

Fig. 4.12: Products of the RT PCR reaction using the Sal primer set with (**Lane 1**) NTC and template *S. enterica* DNA dilutions containing: (**Lane 2**) 120 ng, (**Lane 3**) 12 ng, (**Lane 4**) 1.2 ng, (**Lane 5**) 0.12 ng, (**Lane 6**) 0.012 ng and (**Lane 7**) 0.0012 ng. Products were run on a 2% agarose gel under 100V for 20 minutes, alongside a 100 bp Molecular Marker, shown on the left.

Similar dimerization was also observed for the Saur primer set. As for the previous two primer sets, amplification was observed in all reactions including the NTC (**Fig. 4.13**). Three melting peaks—at 85°C, 88°C and 90°C—corresponding to three or more products, were

generated. The product peaks of 85°C and 90°C present in the NTC were identified as primer-dimers. The location of the 88°C peak equidistant from the other two peaks, in addition to its presence in the undiluted 240 ng reaction and the 2.4 ng dilution indicate that it too may correspond to yet another primer-dimer.

Fig. 4.13: The melting peaks generated from the RT PCR reaction of the Saur primer pair with dilutions of *S. aureus* template DNA from 0 ng to 24 ng per reaction.

Overall, the results of the RT PCR reactions revealed the poor amplification efficiency of the individual primer pairs from sets 1 and 2, thereby confirming the results of the end-point PCR reactions conducted simultaneously. Both primer sets were designed for use in the specific detection of the selected bacterial pathogens under optimized conditions that could not be replicated in this study; Lee *et al.* (2006) used primer set 1 with a TaqMan[®] RT PCR assay, while Csordas *et al.* (2004) used primer set 2 with different reaction and cycling conditions. In such sensitive assays, the precise amplification of the intended target demands the use of specific optimal priming conditions. This need is only magnified by the additional constraint of a low-copy target.

As a result, primer pairs from both primer sets were not considered for further sensitivity testing, and were not deemed suitable for detecting low-copy gene targets, under the reaction and cycling conditions specified in this study. Instead, a new set of primer pairs were designed, optimized and evaluated.

4.1.2. Primer set 3:

4.1.2.1. Primer design and evaluation

The three primer pairs from set 3 were evaluated for specificity using end-point PCR reactions with target and nontarget template DNA. With the exception of the EUID primer pair, this final set was capable of exclusively amplifying target gene sequences without the possibility of mispriming or dimerization.

As shown in **Fig. 4.14**, the EUID primer pair generated unintended products with both target and nontarget template. The expected product band of 275 bp in size (**Appendix A**) was not present in any reactions, including the positive controls containing target *E. coli* (**Lane 2**) and *E. coli* O157:H7 (**Lane 3**), both of which carry the *uidA* gene. A misprimed product band of approximately 700 bp in size was generated instead in reactions with *E. coli* O157:H7 (**Lane 3**) and *S. enterica* (**Lane 4**) template. Additionally, primer-dimer formation was apparent in the NTC and in the reactions with *E. coli* O157:H7 (**Lane 3**) and *S. aureus* (**Lane 5**) template.

Fig. 4.14: Products of the end-point PCR reaction using the EUID primer set with (**Lane 1**) NTC and template DNA extracted from: (**Lane 2**) *E. coli* (+), (**Lane 3**) *E. coli* O157:H7 (+), (**Lane 4**) *S. enterica*, and (**Lane 5**) *S. aureus*. Products were run on a 2% agarose gel under 100V for 20 minutes, alongside a 100 bp Molecular Marker, shown on the left.

On the other hand, both the ETIR and SINV primer sets were shown to be highly specific, as illustrated in **Figs. 4.15** and **4.16**. For the former set (**Fig. 4.15**), an expected product band of 207 bp in size (**Appendix B**) was clearly produced in a reaction with the target *E. coli*

O157:H7 DNA, and is clearly absent in the remaining four reactions with nontarget *E. coli*, *S. enterica* and *S. aureus* DNA (Lanes 2, 4 and 5 respectively). Misprimed and primer-dimer products are also clearly absent from all samples, including the NTC (Lane 1).

Fig. 4.15: Products of the end-point PCR reaction using the ETIR primer set with (Lane 1) NTC and template DNA extracted from: (Lane 2) *E. coli*, (Lane 3) *E. coli* O157:H7 (+), (Lane 4) *S. enterica*, and (Lane 5) *S. aureus*. Products were run on a 2% agarose gel under 100V for 20 minutes, alongside a 100 bp Molecular Marker, shown on the left.

Fig. 4.16: Products of the end-point PCR reaction using the SINV primer set with (Lane 1) NTC and template DNA extracted from: (Lane 2) *E. coli*, (Lane 3) *E. coli* O157:H7, (Lane 4) *S. enterica* (+), (Lane 5) and *S. aureus*. Products were run on a 2% agarose gel under 100V for 20 minutes, alongside a 100 bp Molecular Marker, shown on the left.

Similarly, for SINV (Fig. 4.16), the expected product band of 252 bp (Appendix C) in size is also produced exclusively with target *S. enterica* DNA (Lane 4). The remaining SINV reactions with no (Lane 1) or nontarget (Lanes 2, 3 and 5) DNA are also clear of any primer-dimer formation.

Once the specificity of the ETIR and SINV primer pairs was confirmed, they were considered suitable for sensitivity testing using dilutions of pure culture and wastewater samples. Due to the poor amplification specificity exhibited by the EUID primer pair, it was not evaluated further.

4.1.2.2. Evaluation of sensitivity:

4.1.2.2.1. Pure culture dilutions

For the ETIR primer pair, RT PCR reactions were conducted with dilutions of template *E. coli* DNA in concentrations ranging from 0 ng to 75 ng per reaction, to determine the minimum level of detection of the RT PCR assay. As expected, the amplification curves (Fig. 4.17) were generated at regular cycle intervals, and the melting peaks (Fig. 4.18 A & B) indicated the production of a single PCR product (of melting temperature of 83°C).

From the melting peaks, the intended gene target was amplified and detected in all template dilutions down to 0.00075 ng of genomic DNA per reaction. From the DNA concentration calculated per *E. coli* O157:H7 cell listed in Appendix E, this detection limit translates to approximately 25 CFU, or 25 copies of the single-copy *tir* gene.

For the SINV primer pair, RT PCR reactions were conducted with dilutions of *S. enterica* DNA in water ranging in concentration from 0 ng to 100 ng per reaction. As for the previous ETIR pair, the amplification phase began at regular cycle intervals along the dilution series (Fig. 4.19), and a plot of the melting peaks (Fig. 4.20) indicated the production of a single PCR product of melting temperature of 85°C.

From the melting peaks shown in Fig. 4.20, the intended gene target was amplified and detected in all template dilutions down to 0.01 ng of genomic DNA per reaction. From the DNA concentration calculated per *S. enterica* cell listed in Appendix E, this detection limit translates to approximately 3×10^4 CFU, or 3×10^4 copies of the single-copy *invA* gene. The RT PCR assay using this primer pair was almost 1000 fold less sensitive than that using the ETIR pair.

Fig. 4.17: The amplification curves generated from the RT PCR reaction of the ETIR primer pair with dilutions of *E. coli* O157:H7 template DNA from 0 ng to 75 ng per reaction.

4.1.2.2.2. Seeded wastewater dilutions

As detailed in the Methods section, the sensitivity of the RT PCR assay was also assessed using both ETIR and SINV primer pairs with dilutions of wastewater samples seeded with *E. coli* O157:H7 and *S. enterica* broth cultures. For the ETIR primer pair, RT PCR reactions were conducted on template DNA extracted from wastewater samples seeded with a series of increasing concentrations of target *E. coli* O157:H7 cells, ranging from 0 CFU/ml to 11.5×10^7 CFU/ml.

From the melting peaks shown in **Fig. 4.21**, a single PCR product of a melting temperature of 84°C , which corresponds to that generated in pure culture dilutions (**Fig. 4.18 A & B**) was produced. This was amplified and detected down to 11.5×10^3 CFU/ml. Assuming pre-extraction and post-extraction volumes of 1 ml and 190 μl respectively, this limit effectively translates to approximately 303 CFU or 303 *tir* gene copies per PCR reaction. Accordingly, the RT PCR assay of the ETIR primer pair was approximately 10 fold less sensitive using seeded wastewater samples.

Fig. 4.18: The melting peaks (A & B) generated from the RT PCR reaction of the ETIR primer pair with dilutions of *E. coli* O157:H7 template DNA from 0 ng to 75 ng per reaction.

For the SINV primer pair, RT PCR reactions were conducted on template DNA extracted from wastewater samples seeded with a series of increasing concentrations of target *S. enterica* cells, ranging from 0 CFU/ml to 293×10^7 CFU/ml. The analysis of the melting peaks revealed that the intended product (of melting temperature of 85 °C, as observed in **Fig. 4.22**) was only detected in the unseeded reaction (293×10^7 CFU/ml). This limit corresponds to approximately 7.1×10^7 CFU or 7.1×10^7 *invA* gene copies per PCR reaction, which is almost 10^6 fold lower than that achieved with template dilutions (**Fig. 4.20**).

Fig 4.19: The amplification curves generated from the RT PCR reaction of the SINV primer pair with dilutions of *S. enterica* template DNA from 0 ng to 100 ng per reaction.

Fig 4.20: The melting peaks generated from the RT PCR reaction of the SINV primer pair with dilutions of *S. enterica* template DNA from 0 ng to 100 ng per reaction.

Fig 4.21: The melting peaks generated from the RT PCR reaction of the ETIR primer pair with template DNA extracted from wastewater samples seeded with dilutions of *E. coli* O157:H7 cells of concentrations ranging from 0 CFU/ml to 11.5 x 10⁷ CFU/ml.

Fig 4.22: The melting peaks generated from the RT PCR reaction of the SINV primer pair with template DNA extracted from wastewater samples seeded with dilutions of *S. enterica* cells of concentrations ranging from 0 CFU/ml to 293 x 10⁷ CFU/ml.

4.1.2.2.3. Wastewater inhibition test

The drop in detection sensitivity exhibited upon the use of wastewater samples is a consequence of either the decreased efficiency of the extraction process—and thus the extraction of less genomic DNA per culture volume—or of the presence of inhibitory wastewater components co-extracted along with the genomic DNA.

To study this phenomenon further, a Wastewater Inhibition test was conducted. As previously described, this involved a RT PCR reaction of the *E. coli* O157:H7 primer pair, ETIR, with a constant amount of target *E. coli* O157:H7 genomic DNA along with serial dilutions of DNA extracted from wastewater samples, in water. Two products were generated, as revealed by the presence of two peaks at 75°C and 84°C in the Melting peaks produced (**Fig. 4.23**).

Fig 4.23: The melting peaks generated from the RT PCR reaction of the ETIR primer pair with template *E. coli* O157:H7 DNA along with dilutions of DNA extracted from wastewater samples.

All reactions containing template DNA generated the intended product, with a melting peak at 84°C. This corresponds to the peak generated in ETIR reactions with target *E. coli* O157:H7 DNA in both the pure culture and seeded wastewater dilution tests (**Fig. 4.18 A & B**). In comparing the relative heights of the peaks generated in the reactions containing both template and extracted wastewater DNA, it is clear that the presence of the wastewater mix hindered the amplification of the template gene target. This inhibition was only reversed at a 100,000 fold dilution (10^{-5}) of the extracted wastewater solution.

4.2. FISH assay

As described earlier, the FISH assay was evaluated with two probe sets designed on the same DNA sequence as the selected forward primers from primer sets 1 and 3. To maintain the premise of complementary primer and probe target sequences, it was necessary to re-design and evaluate new probes for every new PCR primer set adopted. Probe set A was

evaluated first, followed by probe set B upon the observation of the poor amplification efficiency exhibited by primer pairs of this set in RT PCR sensitivity tests, as described above.

4.2.1. EUB338

Fluorescence was observed in all *E. coli* O157:H7, *S. enterica* and *S. aureus* cells upon hybridization with the universal rRNA-targeted EUB338 DNA probe, as shown in **Fig. 4.24**, indicating the validity of the protocol adopted.

4.2.2. Probe set A

Cells from all three strains were then hybridized to the *E. coli* O157:H7 DNA probe, EcoOH-P, from probe set A, to evaluate the specificity of the FISH assay. As shown in **Fig. 4.25**, the EcoOH-P probe hybridized to target *E. coli* O157:H7 as well as to nontarget *S. enterica* and *S. aureus* cells, both of which do not carry the targeted *tir* gene. A high level of background fluorescence was also observed.

This probe set was not evaluated further as the results of the RT PCR assay confirmed the need to design a new set of primers, and accordingly, a new set of probes.

Fig. 4.24: CLSM images of the EUB338 probe hybridized to: (A) *E. coli* O157:H7, (B) *S. aureus* cells *in situ*, at a magnification of x630 (A) and x400 (B). Fluorescent signals (left column) have been overlaid onto visible light microscope images (middle column) to locate and identify hybridized cells.

4.2.3. Probe set B

As shown in **Fig. 4.26 (A & B)**, both the Ecotir and Salinv probes hybridized successfully to the intended membrane-bound target gene sequences in the dot blot assay, consequently permitting the further evaluation of probe specificity using whole-cell hybridization. Weak background fluorescence generated in the absence of any DNA sequences (both target and nontarget; **Fig. 4.26 A.2 & B.2**) was attributed to the presence of unwashed probes hybridized to membrane fibers.

Fig. 4.25: CLSM images of the EcoOH-P probe hybridized to: (A) *E. coli* O157:H7 (+), (B) *S. enterica* and (C) *S. aureus* cells *in situ*, at a magnification of x400 (A) and x630 (B & C). Fluorescent signals (left column) have been overlaid onto visible light microscope images (middle column) to locate and

Fig. 4.26: CLSM image of the amplified, membrane-bound target *E. coli* (A.1) and *S. enterica* (B.1) nucleic acids hybridized to Ecotir and Salinv probes respectively, at a magnification of x1000. Negative control membranes carrying no DNA products are shown on the right (A.2 & B.2) for comparison.

In spite of the ability of the Ecotir and Salinv probes to hybridize to pure target DNA, neither was able to produce a detectable fluorescent signal when used on whole cells. As shown in **Fig. 4.27 (A & B)**, Ecotir was particularly problematic; probe hybridization was not detectable with any target *E.coli* O157:H7 (A) cells. Salinv, on the other hand, was able to hybridize sufficiently to target *S. enterica* (**Fig. 4.28 A**) cells to produce a detectable signal. Nevertheless, the hybridization was not uniform, and numerous confirmed target cells remained undetected.

In consequence, the detection sensitivities of the FISH assay using both probes of set B could not be adequately quantified, either in pure culture dilutions or subsequently in seeded wastewater samples.

Fig. 4.27: CLSM images of the Ecotir probe hybridized to: (A) *E. coli* O157:H7 (+), (B) *S. enterica* cells *in situ*, at a magnification of x400. Fluorescent signals (left column) have been overlaid onto transmitted light microscope images (middle column) to locate and identify hybridized cells (right column).

Fig. 4.28: CLSM images of the Salinv probe hybridized to: (A) *S. enterica* (+), (B) *E. coli* O157:H7 cells *in situ*, at a magnification of x630. Fluorescent signals (left column) have been overlaid onto transmitted light microscope images (middle column) to locate and identify hybridized cells (right column).

5.0 DISCUSSION:

5.1. PCR assay:

Overall, the RT PCR assay evaluated in this study suffered from a lack of sensitivity and specificity brought about by the poor amplification efficiency of the chosen primer sets. Results of the RT PCR reactions for each primer pair within primer sets 1 and 2 confirmed those obtained with the end-point reactions, in that primer-dimer was the sole amplification product. It was then concluded that with the reaction conditions used in this study, primer sets 1 and 2 were unable to amplify the intended virulence gene targets and as such, were deemed an inadequate choice for determining the strength of the PCR assay in detecting bacterial pathogens.

Of all three sets evaluated, only the final primer set—namely the ETIR and SIN V primer pairs—designed specifically for this study were able to exclusively and efficiently amplify the intended *E. coli* O157:H7 and *S. enterica* virulence gene targets, under the specific optimization conditions adopted. The detection limits established for the assay using these two primer pairs ranged from 25 to 303 copies of the *tir* gene and 30×10^3 to 29.3×10^8 copies of the *invA* gene, using both pure culture and seeded wastewater dilutions.

5.1.1. Reaction conditions and reproducibility

PCR assays are being increasingly implemented as precise and reliable alternatives to culture-based pathogen detection. Nevertheless, poor inter-laboratory reproducibility of the reaction conditions, and subsequently the results obtained, has begun to jeopardize their application as standardized monitoring techniques. Numerous factors affecting the performance of the reaction have been identified at all stages of the amplification procedure, including: the method of DNA extraction and template quality, concentration and type of DNA polymerase, primer design and concentration, as well as primer annealing temperature and cycling conditions (Tyler *et al.* 1997). Given these variables, almost any PCR assay may perform satisfactorily at the hands of the original developers, but poorly under the evaluation of other researchers.

In this study, under the reaction conditions followed, neither of the two pre-designed primer sets were able to effectively amplify the intended virulence gene targets as previously described. Primer set 1 adopted from Lee *et al.* (2006) were initially designed for use in a TaqMan® RT PCR assay for the detection of bacterial pathogens, as part of a larger

comparative evaluation of PCR and microarray detection systems. Using a single representative primer pair, the assay was capable of specifically detecting 2.2 gene copies per reaction, with no mention of the generation of nonspecific products. Primer set 2, on the other hand, was designed by Csordas *et al.* (2004) in the course of an evaluation of available RT PCR primer pairs for the specific detection of *Salmonella enterica* serovars. Using SYBR Green I, the RT PCR assay developed for use with this primer pair was capable of detecting a minimum of 35 CFU, or 35 copies of the single-copy *invA* gene per reaction. However, the concentrations of the individual PCR reagents had to be optimized to avoid the generation of nonspecific PCR products.

In adopting both primer sets in this work, reaction conditions originally described in both studies had to be re-optimized for use with either a different detection system (primer set 1) or different reagent concentrations (primer set 2). It is unsurprising to note therefore that the individual assay specificity and sensitivity limits previously described for both primer sets could not be reproduced, and that all primer pairs of set 1 displayed extensive dimerization, while primer set 2 was not capable of any amplification. Such variability in the performance of the PCR assay was only more pronounced in this study in particular, due to the sensitivity of the RT PCR system used, in addition to the low copy number of the genes targeted.

The results observed here serve to highlight the dire need for the standardization of PCR methodologies for the successful adoption of previously developed assays. While this is yet to be realized, progress has been made in the form of the European Commission-approved research project known as FOOD-PCR. Initiated in 1999, this project was aimed at validating noncommercial PCR assays for the detection of a number of bacterial pathogens—namely *Campylobacter* spp., *Yersinia* spp., *Listeria* spp., *Salmonella* spp. and *E. coli* O157:H7—in food samples, with the ultimate goal of producing standardized PCR operating guidelines and protocols (Lübeck *et al.* 2003; Joefsen *et al.* 2003). Currently in the final development phase, FOOD-PCR has established standardized real-time detection protocols, but has yet to validate these through a multi-laboratory evaluation. Thus, until then, PCR reproducibility, and ultimately reliability, remains problematic.

5.1.2. Interpretation of results

In light of this, caution must be exercised when comparing and interpreting results of similar PCR assays both within individual, and between different studies. This holds true

particularly in the application of SYBR Green I detection systems that inherently lack the specificity and reliability usually conferred by the validation of the PCR products generated.

In the presence of several products, a thorough investigation of the melting curves generated is required to differentiate between intended and unintended products. Such an evaluation is rooted in subjectivity: the amount of product generated is indicated by peak height ratios, the formation of primer-dimer is identified by the comparison of peaks generated in the undiluted and NTC reactions and the presence of intended product is identified by the consistent appearance of a melting peak in various template dilutions.

As a consequence, agarose gel electrophoresis is often used to confirm the interpretation of the melting curves generated, as described for the evaluation of primer set 1 in this study. This however, is simply not feasible in a routine diagnostic setting. For not only does such an additional confirmatory step increase the overall detection time of the assay, but it also counters the increased detection sensitivity of the RT assay by coupling it to the inherently less sensitive—and semi-quantitative—agarose gel detection system.

Ultimately, SYBR Green I PCR assays are best suited for use with primers of confirmed specificity, in the “generic detection” of amplified DNA (Gudnason *et al.* 2007). It is only here, in the absence of primer-dimer formation and additional confirmatory steps, that the cost-effectiveness of this technique and its potential for rapid, high-throughput analysis are fully realized (Vandesompele *et al.* 2002).

5.1.3. Application in detection

The minimum detection limits achieved by the RT PCR assay using primer set 3 illustrate the potential strength of PCR in sensitively and specifically detecting bacterial pathogens in environmental samples, given that the optimal reaction conditions have been met. This primarily refers to the design and specificity of the primer pair used, for a PCR assay can only be as specific and sensitive as the primer pair chosen. The optimization of the reaction conditions may only enhance the performance of a primer pair; ultimately, the strength of the assay is dictated by the efficacy of the primers themselves. Thus while the RT PCR assay developed in this study was similarly optimized for both ETIR and SINV primer pairs, the former set was 1000 fold more sensitive in detecting comparable single-copy gene targets.

PCR sensitivity limits have nevertheless been shown to decrease in the presence of environmental samples. From the results of this study alone, a 10^3 to 10^5 fold drop was

observed on average between the application of the same RT PCR assay with dilutions of pure culture template and extracted dilutions of seeded wastewater samples. This has been partly attributed to the difficulty in isolating detectable quantities of bacterial genomic DNA from environmental samples (Josephson *et al.* 1993). For the SINV primer pair, none of the seeded wastewater samples generated enough DNA to meet the detection limit of the RT PCR assay. Thus, unlike the ETIR assay, only the pure culture sample was detectable. This however, was unsurprising given the weak detection sensitivity exhibited by the assay with pure culture dilutions.

Such a drop in sensitivity may also have been due in part to the presence of inhibitory sample components co-extracted along with the genomic DNA. Predominantly isolated from wastewater samples, these include humic compounds, divalent cations and salts, all of which interfere with DNA polymerase activity during amplification (Toze 1999). As the chemical removal of these substances often requires costly and sophisticated machinery, it is unsuitable for use in routine screening (Toze 1999). However, a simple and cost-effective alternative involves the dilution of the wastewater template sample, as demonstrated in the Wastewater Inhibition test described previously.

Yet in spite of the decreased sensitivity of the PCR assays with environmental samples, many researchers have contentiously argued that it often overestimates the potential hazard present (Josephson *et al.* 1993). Thus, while it does offer a suitable alternative to culture-based techniques that under-represent densities of nonculturable cells, this technique has been reported to overshoot the detection mark by amplifying genomic DNA from nonviable (and consequently noninfectious) bacterial cells, generating false-positive results. In an effort to circumvent this, a number of studies have turned to Reverse Transcriptase PCR assays targeting mRNA as a more suitable means of reflecting the levels of viable (culturable or nonculturable) bacterial pathogens present in water or wastewater samples (Toze 1999). However, such assays are often incorrectly built upon the assumption that the targeted mRNA is always expressed (and thus available for detection) or intact. Nevertheless, for the purposes of routine monitoring, the detection of either the viable or nonviable forms of all pathogens indicate the immediate or imminent presence of a potential hazard, both of which necessitate the activation of the appropriate pathogen control measures.

5.2. FISH assay:

5.2.1. Protocol optimization

Oligonucleotide probe hybridization of whole cells *in situ* has been widely adopted for the detection of various bacterial pathogen species in mixed populations. Within the fields of bacterial pathogen detection and community analysis, the use of rRNA probe sequences is now well established, driven partly by advances in rRNA sequencing. With sequence data currently available for over 2500 bacterial species, custom FISH probes with any range of specificities, targeting various phylogenetic levels, may now be designed (Rompré *et al.* 2002). As the probes of choice in environmental screening applications, rRNA probes are capable of generating sufficiently strong hybridization signals due to the presence of multiple target copies—which may be as many as 100,000—within a cell (Rompré *et al.* 2002).

Although DNA probes are also fundamentally capable of *in situ* hybridization, the low copy number of DNA targets within cells has influenced the sensitivity of DNA FISH assays. As such, their application has been severely limited outside of any clinical setting. To date, no FISH assays have yet been developed for use with DNA probes and targets in the detection of bacterial strains in any environmental samples. As a result, no standard DNA FISH protocols for environmental samples are available. For the purposes of this study, a previously evaluated 16S rRNA FISH protocol was modified for use with DNA probes and targets through the addition of a DNA denaturation step.

For this reason, rigorous optimization of hybridization stringency conditions is essential. This is particularly problematic in the case of previously undescribed probes that require extensive stringency testing with different combinations of hybridization temperatures and formamide and salt concentrations in the hybridization and wash buffers. For, while the Lathe equation offers a calculated estimate of the optimal values, these will still need to be verified experimentally, often through trial-and-error. This process has nevertheless proven to be time-consuming, tedious and somewhat subjective in the analysis of hybridization signal intensities (against an arbitrary threshold) for enumeration, making it unsuitable in routine monitoring. In this study, an attempt was made at simplifying this task through fixing the hybridization temperature and salt concentrations at a constant value throughout all of the reactions, while adjusting the formamide concentrations accordingly.

5.2.2. Application in detection

The rigorous optimization of these stringency conditions in this study was necessary to enhance the fluorescence signal in positive control reactions with pure culture target strains. In spite of this, neither of the two probe sets was capable of generating a detectable signal. Dot blot hybridization tests did, however, confirm the specificity of the DNA probes to the intended, amplified target sequences.

As observed in the results obtained, the detection sensitivity of low copy targets weakened greatly when evaluated *in situ*, and even more so if applied to mixed culture samples. Owing to this, the detection sensitivity of the FISH assay could not be determined in seeded samples, as for the PCR assay. Here, the fluorescent signal—already at the limit of detection—may be lost either to high levels of background noise, or among the very few hybridized cells present. This has been overcome through the use of Catalyzed Reporter Deposition (CARD) FISH that employs the use of horseradish peroxidase (HRP) in catalyzing the deposition of labeled tyramide molecules that are responsible for the signal amplification. The HRP is conjugated to antibodies targeting the particular hybridization probe label so as to guide the attachment of the tyramide molecules close to the site of hybridization, effectively magnifying the signal emitted around the hybridized probe (Speel *et al.* 1999). These tyramide intermediates may be labeled either with biotin or fluorochromes such as fluorescein and rhodamine, and are used in conjunction with streptavidin or the corresponding antibody, respectively (Speel *et al.* 1999). Further conjugation with yet another labeled antibody has also been used to boost the amplification further.

Since 1995, CARD FISH has been applied in the detection of single-copy DNA targets, enabling the detection of up to 1 kbp sequences in tissue sections. It has been reported to increase assay sensitivity by up to two- to 100-fold (Speel *et al.* 1999). Nevertheless, this technique may only be effectively applied after optimization of hybridization conditions, to avoid the amplification of nonspecific or background signals. Thus it would not be applicable for use with probe set A, due to the high level of background signal in addition to the nonspecific hybridization to nontarget strains. On the other hand, further research is needed to determine whether or not it would improve the *in situ* detection sensitivities of Ecotir and Salinv of probe set B, of confirmed specificity.

Ultimately, the application of DNA-based FISH in environmental samples is on the whole too challenging for practical use; for the enhanced specificity conferred by the use of single-copy DNA sequences is ultimately countered by the low detection sensitivity of the assay, and the need for rigorous optimization. Moreover, with the current rRNA sequencing information, it may be possible to design RNA-based probes with a comparable level of specificity, yet with the added benefit of multiple hybridization targets.

5.3. Comparative evaluation

While applications of either assay have been widespread, both are in need of more thorough development before being applied for routine detection. As of yet, the application of either assay in the automated screening of large numbers of environmental samples is not yet possible, owing to the need for standardized protocols and the heavy interpretation of results. For while both technologies offer a more direct and reliable alternative to culture-based detection techniques, they currently cannot compete with the simplicity and ease-of-use that have originally popularized the application of indicator methods. Both assays for instance, require the presence of experienced personnel; to extract template DNA in PCR as well as to analyze the fluorescent signals in FISH.

Nevertheless, the results of this study suggest that despite problems with standardization and inhibition, RT PCR is potentially a stronger detection technique than FISH, when applied to low-copy DNA targets in environmental samples. As an inherently quantitative assay, it is capable of enumerating target gene or genome copies present within the sample tested directly, without the need for an additional quantitative analysis step. In contrast, FISH assays often need to be coupled to automated cell counting methods such as flow cytometry, and as such would be more suited for more routine monitoring that screens simply for the presence and absence of target pathogens.

Both assays have been combined into a single *in situ* PCR assay to visualize amplified target DNA sequences within cell and tissue samples (Speel *et al.* 1999). As the name implies, this emerging technique involves the amplification of gene targets within cells prior to hybridization. The reaction mix is applied directly onto fixed tissues or cells, which are then incubated in a thermal cycler or oven for the length of the amplification process. Nevertheless, the increase in detection sensitivity compared with conventional FISH is rather

limited, and does not necessarily warrant the extra time and effort of the pretreatment and thermal cycling steps required (Speel *et al.* 1999).

Ultimately, further study is required to fully evaluate the precise detection strengths of either assay, when applied to bacterial pathogen enumeration in wastewater samples

6.0 SUMMARY:

The purpose of this study was to evaluate the performance of RT PCR and FISH assays in the detection of selected waterborne bacterial pathogens, using single copy DNA targets as a means of enhancing the specificity of the individual assays. The specific objectives of the study were to evaluate the specificity of the primer and probe sets chosen, determine the minimum detection limits of either assay in pure cultures and seeded wastewater samples, and from that infer the effect of wastewater components—both within the sample itself or co-extracted along with it—interfering with either reaction.

For the RT PCR assay, three primer sets were evaluated. Previously established detection sensitivities using both sets of adopted primer pairs proved difficult to reproduce under the reaction conditions followed in this work. This reflected the reproducibility and reliability problems undermining the current application of PCR assays, and highlighted the need for more standardized amplification protocols. Future work is needed on the development and validation of such techniques in view of their potential future use in on-site monitoring systems.

After careful selection, the third primer set was subsequently designed and evaluated. This set was capable of exclusively amplifying the intended gene targets, with a minimum detection limit of 25 to 303 *tir* gene copies with *E. coli* O157:H7 and 3×10^4 to 293×10^7 *invA* gene copies for Salmonella, using pure culture and seeded wastewater samples respectively. The 10 to 100,000-fold decrease in detection sensitivity of the assay with both primer pairs was partly a result of the presence of amplification inhibitors in the extracted solution. Their effect was relieved by dilution after 100,000 fold.

For the FISH assay, probe specificity was confirmed with hybridization with membrane-bound target nucleic acids. However, due to the low copy number of the DNA target, this specificity did not translate to whole cells *in situ*. Poor or no hybridization was observed with both *E. coli* O157:H7 and *S. enterica* probes.

The results of this study provide valuable insight on the methodological application of DNA-based molecular detection techniques to environmental samples. Although a true comparative evaluation was not possible, the results obtained suggest that RT PCR has a stronger detection potential than DNA-based FISH when targeting chromosomal virulence genes. Despite the presence of target inhibitors, it was possible to enumerate a target copy

number. In contrast, enumeration was not possible with FISH due to the lack of *in situ* hybridization specificity exhibited.

Further studies should build on the findings documented for DNA-based FISH, in the specific application of CARD-FISH to DNA probes of confirmed specificity.

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APPENDIX A

LOCATION OF *ESCHERCHIA COLI* PRIMER SETS WITHIN *uidA* GENE

	Eco (adopted from Lee <i>et al.</i> 2006)	EUID (designed for this study)
Forward (5'-3')
Reverse (3'-5')	CAGGCCAGAAAGTTCTTTTCCA	GCATGGCTGGATATGTATCACC
Reverse Complement
Intended product size	59 bp	275 bp
Primer-dimer product size	22 bp	40 bp

Table A. 1: List of strand sequence and product sizes of *E. coli* Eco and EUID primer sets used in this study.

Location of the primer sets within the *uidA* gene (GenBank Accession # S69414):

5' –GGAAAAGTGTACGTATCACC GTTTGTGTGAACAACGAACTGAACTGGCAGACTATCCCG
 CCGGGAATGGTGATTACCGACGAAAACGGCAAGAAAAAGCAGTCTTACTTCCATGATTTCTTTAA
 CTATGCCGGAATCCATCGCAGCGTAATGCTCTACACCACGCCGAACACCTGGGTGGACGATATCA
 CCGTGGTGACGCATGTCGCGCAAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAA
 GGTGATGTCAGCGTTGAACTGCGTGATGCGGATCAACAGGTGGTTGCAACTGGACAAGGCACTAG
 CGGGACTTTGCAAGTGGTGAATCCGCACCTCTGGCAACCGGGTGAAGGTTATCTCTATGAACTGTG
 CGTCACAGCCAAAAGCCAGACAGAGTGTGATATCTACCCGCTTCGCGTCGGCATCCGGTCAGTGG
 CAGTGAAGGGCGAACAGTTCCTGATTAACCACAAACCGTTCTACTTTACTGGCTTTGGTCGTCATG
 AAGATGCGGACTTGCGTGGCAAAGGATTCGATAACGTGCTGATGGTGCACGACCACGCATTAATG
 GACTGGATTGGGGCCAACTCCTACCGTACCTCGCATTACCCTTACGCTGAAGAGATGCTCGACTGG
 GCAGATGAACATGGCATCGTGTTGATTGATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTTAGGC
 ATTGGTTTTCGAAGCGGGCAACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTCAACGGGGGAAA
 CTCAGCAAGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAAAACCACCAAGCGTG
 GTGATGTGGAGTATTGCCAACGAACCGGATACCCGTCCGCAAGGTGCACGGGAATATTTCCGCC
 ACTGGCGGAAGCAACGCGTAACTCGACCCGACGCGTCCGATCACCT

GAAACGGCAGAGAAGGTAC G
 CAGGAGAACTGCATCAGCCGATTATCATCCGAATACGGCGTGGATACGTTAGCCGGGCTGCA
 CTCATGTACACCGACATGTGGAGTGAAGAGTATCAGTGT CGCG
 TCTTTGATCGCGTCAGCGCCGTCGTCGGTGAACAGGTATGGAATTTCCCGGATTTTTCGACCTCGC
 AAGGCATATTGCGCGTTGGCGGTAACAAGAAAGGGATCTTCACTCGCGACCGCAAACCGAAGTCG
 GCGGCTTTTCTGCTGCAAAAACGCTGGACTGGCATGAACTTCGGTGAAAAACCGCAGCAGGGAGG
 CAAACAATGA –3'

APPENDIX B

LOCATION OF *ESCHERCHIA COLI* O157:H7 PRIMER SETS WITHIN *tir* GENE

	EcoOH (adopted from Lee <i>et al.</i> 2006)*	ETIR (designed for this study)
Forward (5'-3')	GTCCAAAGCGGCGATTTG	GTGAGGTCATTAACTGTACGGG
Reverse (3'-5')	TGGAAAAAGAACTTCTGGCCTG	CGCTCGATACTCTTAACAGGC
Reverse Complement	CAGGCCAGAAGTTCTTTTCCA	CGCTCGATACTCTTAACAGGC
Intended product size	-	207 bp
Primer-dimer product size	40 bp	43 bp

Table B. 1: List of strand sequence and product sizes of *E. coli* O157:H7 EcoOH and ETIR primer sets used in this study. * Primer sequences not present within *tir* gene sequence.

Location of the primer sets within the *tir* gene (GenBank Accession # AF125993):

5' -ATGCCTATTGGTAACCTTGGTCATAATCCCAATGTGAATAATTCAATTCCTCCTGCACCTC
CATTACCTTCACAAACCGACGGTGCAGGGGGGCGTGG**GTGAGGTCATTAACTGTACGGG**GCCGTTG
GGATCTCGTGCGCTATTTACGCCTGTAAGGAATTCTATGGCTGATTCTGGCGACAATCGTGCCAGT
GATGTTCTTGACTTCCTGTAAATCCGATGCGCCTGGCGGCGTCTGAGATAACACTGAATGATGGA
TTGAAGTTCCTCATGATCATGGTC**CGCTCGATACTCTTAACAGGC**AGATTGGCTCTTCGGTATTTT
GAGTTGAAACTCAGGAAGATGGTAAACATATTGCTGTCCGTCAGAGGAATGGTGTGAGACCTCT
GTTGTTTTAAGTGATCAAGAGTACGCTCGCTTGCAAGTCCATTGATCCTGAAGGTAAAGACAAATTT
GTATTTACTGGAGGCCGTGGTGGTGCTGGGCATGCTATGGTCACCGTTGCTTCAGATATCACGGAA
GCCCCGCAAAGGATACTGGAGCTGTTAGAGCCCAAAGGGACCGGGGAGTCCAAAGGTGCTGGGG
AGTCAAAAGGCGTTGGGGAGTTGAGGGAGTCAAAATAGCGGTGCGGAAAAACACCACAGAACTCA
GACCTCAACCTCAACTTCCAGCCTTCGTTTCAGATCCTAAACTTTGGTTGGCGTTGGGGACTGTTGCT
ACAGGTCTGATAGGGTTGGCGGCGACGGGTATTGTACAGGCGCTTGCAATTGACGCCGAGCCGGA
TAGCCCAACCACGACCGACCCTGATGCAGCTGCAAGTGCAACTGAACTGCGACAAGAGATCAGT
TAACGAAAGAAGCGTTCCAGAACCCAGATAATCAAAAAGTTAATATCGATGAGCTCGGAAATGCG
ATTCCGTCAGGGGTATTGAAAGATGATGTTGTTGCGAATATAGAAGAGCAGGCTAAAGCAGCAGG
CGAAGAGGCCAAACAGCAAGCCATTGAAAATAATGCTCAGGCGCAAAAAAATATGATGAACAA
CAAGCTAAACGCCAGGAGGAGCTGAAAGTTTCATCGGGGGCTGGCTACGGTCTTAGTGCGCATT
GATTCTTGGTGGGGGAATTGGTGTGTCGCTCACCGCTGCGCTTCATCGAAAAAATCAGCCGGTAGA
ACAAACAACAACACTACTACTACAACACTACAAGCGCACGTACGGTAGAGAATAAGCCTG
CAAATAATACACCTGCACAGGGCAATGTAGATACCCCTGGGTGAGAAGATACCATGGAGAGCAGA
CGTAGCTCGATGGCTAGCACCTCGTCGACTTTCTTTGACACTTCCAGCATAGGGACCGTGCAGAAT
CCGTATGCTGATGTTAAACATCGCTGCATGATTCGCAGGTGCCGACTTCTAATTCTAATACGTCT
GTTGAGAAATATGGGAATACAGATTCTGTTGTATATAGCACCATTCAACATCCTCCCCGGGATACT
ACTGATAACGGCGCACGGTTATTAGGAAATCCAAGTGCGGGGATTCAAAGCACTTATGCGCGTCT
GGCGCTAAGTGGTGGATTACGCCATGACATGGGAGGATTAACGGGGGGAGTAATAGCGCTGTGA
ATACTTCGAATAAACCACAGCGCCGGGATCCCATCGTTTCGTCTAA-3'

LOCATION OF *SALMONELLA ENTERICA* PRIMER SETS WITHIN *invA* GENE

Table C. 1: List of strand sequence and product sizes of *S. enterica* Sal, SEN and SINV primer sets used in this study.

5' -AACAGTGCTCGTTTACGACCTGAATTACTGATTCTGGTACTAATGGTGATGATCATTCTA
TGTTTCGTCATTCCATTACCTACCTATCTGGTTGATTTCTGATCGCACTGAATATCGTACTGGCGAT
ATTGGTGTTTATGGGGTCGTTCTACATTGACAGAATCCTCAGTTTTTCAA
ACCACGCTCTTTTCGCTGGCA
TATCTTGATTGAAG
CCGATGCCGGTGAAATTATCGCCACGTTCCGGCAATTTCGTTATTGGCGATAGCCTGGCGGTGGGTT
TTGTTGTCTTCTCTATTGTCACCGTGGTCCAGTTTATCGTTATTACCAAAGGTTTACAGAACGCGTCGC
GGAAGTCGCGGCCCGATTCTCTGGATGG
TATTGATGCCGATT
TGAAGGCCGGTATTATTGATGCGGATGCTGCGCGCAACGGCGAAGCGTACTGGAAAGGGAAG
CCAGCTTTACGGTTCCTTTGACGGTGCGATGAAGTTTATCAAAGGTGACGCTATTGCCGGCATCAT
TATTATCTTTGTGAACTTTATTGGCGGTATTTCCGGTGGGGATGGCCCGCCATGGTATGGATTTGT
CATGCTGACCATTGGTGATGGTCTTGTGCGCCAGATCCCCGCATT
GTTGATTGCGATTAGTGCCGGTTTTATCGTGACTCGCGTAAATGGCGATAGCGATAATATGGGGCG
GAATATCATGACGCAGCTGTTGAACAACCCATTTGTATTGGTTGTTACGGCTATTTTGACCA
AATGGGAACCTCTCCGCGGATTCCCGCTGCCGGTATTTGTTATTTTATCGGTGGTTTTAAGCGTACT
CTTCTATTTTAAATTCCGTGAAGCAAAACGTAGCGCCGCCAAACCTAAAACCAGCAAAGGCGAGC
AGCCGCTTAGTATTGAGGA
GGGACTGATTGGCGATCTCGATAAAGT
CTCTAC-3'

APPENDIX D

LOCATION OF *STAPHYLOCOCCUS AUREUS* PRIMER SET WITHIN *entC1* GENE

Saur (adopted from Lee <i>et al.</i> 2006)	
Forward (5'-3')	ATCATTAAATATAATTAATTTCTTTTAATATTTTTTTAATTGAATATTTAAGATTATAAGA
Reverse (3'-5')	GTGAATTTACTCGCTTTGTGCAA
Reverse Complement	ATCATTAAATATAATTAATTTCTTTTAATATTTTTTTAATTGAATATTTAAGATTATAAGA
Intended product size	63 bp
Primer-dimer product size	45 bp

Table D. 1: List of strand sequences and product sizes of *S. aureus* Saur primer set used in this study.

Location of the primer sets within the *entC1* gene (GenBank Accession # X05815):

5' – ATCATTAAATATAATTAATTTCTTTTAATATTTTTTTAATTGAATATTTAAGATTATAAGA
TATATTTAAAGTGTATCTAGATACTTTTTGGGAATGTTGGATGAAGGAGATAAAAATGAATAAGA
GTCGATTTATTTTCATGCGTAATTTTGATATTCGCACTTATACTAGTTCTTTTACACCCAA
GACCCTACGCCAGATGAG TGGTTTGA
TGGAAAATATGAAAGTTTTATATGATGATCATTATGTATCAGCAACTAAAGTTAAGTCTGTAGATA
AATTTTTGGCACATGATTTAATTTATAACATTAGTGATAAAAAAACTGAAAAATTATGACAAAGTGA
AAACAGAGTTATTAATGAAGGTTTAGCAAAGAAGTACAAAGATGAAGTAGTTGATGTGTATGGA
TCAAATTACTATGTAAACTGCTATTTTTCATCCAAAGATAATGTAGGTAAAGTTACAGGTGGCAAA
ACTTGTATGTATGGAGGAATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACCTTACAAAA
TGTACTTATAAGAGTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGA
AAAGTGTAACAGCTCAAGAACTAGACATAAAAGCTAGGAATTTTTTAATTAATAAAAAAAATTTG
TATGAGTTTAACAGTTCACCATATGAAACAGGATATATAAAATTTATTGAAAATAACGGCAATACT
TTTTGGTATGATATGATGCCTGCACCAGGCGATAAGTTTGACCAATCTAAATATTTAATGATGTAC
AACGACAATAAAACGGTTGATTCTAAAAGTGTGAAGATAGAAGTCCACCTTACAACAAAGAATGG
ATAATGTTAATCCGATTTTGATATAAAAAGTGAAAGTATTAGATATATTTGAAAGGTAAGTACTTC
GGTGCTTGCTTTTTAGGATGCATATATATAGATTAAACCGCACTTCTATATTAATAGAAAGTGCG
GTTATTTATACACTCAATCTAAACTATAATAATTGGAATCATCTTCAA–3'

APPENDIX E
BACTERIAL CONCENTRATION CURVES

E. 1 *Escherichia coli* ATCC 25922 Cell Concentration vs. Spectrophotometric Absorbance

E. 2 *Escherichia coli* O157:H7 ATCC 25928 Cell Concentration vs. Spectrophotometric Absorbance

Figs. E. 1 - E. 4: Growth curves of cell concentrations of individual broth cultures of *E. coli* (E. 1), *E. coli* O157:H7 (E. 2), *S. enterica* (E. 3) and *S. aureus* (E. 4) versus the spectrophotometric absorbance readings. Trend lines and linear equations are shown in bold.

APPENDIX F

CALCULATION OF DNA CONCENTRATIONS PER CELL

Table F. 1: Concentrations of genomic DNA per cell for *E. coli*, *E. coli* O157:H7, *S. enterica* and *S. aureus*, calculated using an initial broth culture volume of 1 ml and a final extraction volume of 190 µl.

Strain	Cell Concentration (CFU/ml)	Concentration of Extracted DNA (ng/µl)	Mass of genomic DNA per cell (fg/cell)
<i>E. coli</i> ATCC 25922	26.9 x 10 ⁷	56.9	40.2
<i>E. coli</i> O157:H7 ATCC 25928	16.25 x 10 ⁷	25.7	30.1
<i>S. enterica</i> ATCC 14028	32.35 x 10 ⁷	56.6	33.2
<i>S. aureus</i> ATCC 25923	31.4 x 10 ⁷	52.4	31.7

APPENDIX G

DETERMINATION OF FISH ASSAY SENSITIVITY

For *in situ* analysis using pure cultures, broth cultures of each of the four bacterial strains were to be quantified by spectrophotometry and the cell concentration (CFU/ml) was then interpolated from the respective concentration curve. One ml samples of each of the four cultures were to be fixed as described earlier and hybridized to the Ecotir and Salinv probes separately. A third control sample of each culture was to be similarly fixed and hybridized to EUB338.

Hybridized samples would then have been visualized with CLSM as described earlier and quantified through the use of the z-stacking feature of the LSM 510 software package. This function allows for the production of series of scanned images in different focus positions, termed Z-slices (Carl Zeiss 1999). The number and thickness of the individual slices, along with the diameter of the pinhole used determines the volume of the overall optical slice analyzed. To quantify the sensitivity of the FISH assay, the total number of cells detected (fluorescing) in several optical slices were to have been visually enumerated and then averaged. This cell count would then be divided by the volume of the slice used to ultimately obtain a minimum detection limit in CFU/ml.

For *in situ* analysis using seeded wastewater, broth cultures of all four bacterial strains were to be quantified and seeded into 1 ml wastewater samples, as previously detailed for the RT PCR assay. One ml samples of each seeded wastewater dilution would then be fixed and hybridized to each of the Ecotir, Salinv and EUB338 probes separately. The minimum detection limit of the FISH assay using seeded wastewater samples would then be determined through confocal microscopy and z-stacking, as described earlier.

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