EVALUATION OF MICROBIAL SOURCE TRACKING ALTERNATIVE INDICATORS IN COMPARISON WITH CONVENTIONAL INDICATORS IN HUMAN AND COW WASTES

By

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ABSTRACT

EVALUATION OF MICROBIAL SOURCE TRACKING ALTERNATIVE INDICATORS IN COMPARISON WITH CONVENTIONAL INDICATORS IN HUMAN AND COW WASTES

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Sewage or fecal input into waterbodies results is a serious concern. Direct monitoring of waters for the presence of these waterborne pathogens is expensive, laborious, and time consuming. Therefore, fecal indicators such as *E. coli* and enterococci are used as regulatory tools to monitor water as presence of these indicators indicate potential presence of enteric pathogen. But critical uncertainties such as proliferation of bacteria in places where there is no fecal contamination and strong evidence for their extended survival and re-growth in sediments have furthered water pollution science towards new approaches. Recently, members of *Bacteroides* genus are found to have the features that will enable them to be used as alternative Microbial Source Tracking (MST) fecal indicators. But before they are used for the routine monitoring for fecal contamination, they need to be evaluated and validated. The overall aim of this research was to investigate the prevalence and concentrations of recently identified human and cow specific fecal markers in comparison with *E. coli* and enterococci in both the human and cow waste environments and to understand the variation expected when cultivation methods are replaced with qPCR methods in these environmental samples. Monitoring of raw sewage, septage samples and their treated effluents for human specific MST marker, *Bacteroides thetatiataomicron* during human waste treatment processes in comparison with conventional indicators such as *E. coli* and enterococci showed that *B.*

thetaiotaomicron qPCR equivalent cells were present in significantly higher concentrations than that of *E. coli* or enterococci in raw sewage and septage and fate of these target qPCR signals were similar to *E. coli* and enterococci DNA during the treatment of these wastes. It was observed that around 10^2 to 10^3 qPCR equivalent cells/100mL of these targets were being discharged through the final effluents into surface waters. Another specific aim of this research was to study the distribution of different cow specific markers in cow manure environment and use these markers to characterize contamination of water bodies due to runoff from manure farms. Three different specific MST targets, a *Bacteroides* bovine cluster, an M2 cow specific marker, and an M3 cow specific marker were evaluated in comparison with *E. coli* and enterococci qPCR target signals using samples from manure pits just prior to the pumping of this waste for land application. It was found that these manure samples had *E. coli* and enterococci at concentrations of 10^5 -10^7 CFUs or cells/100 mL and cow specific markers in the concentrations of 10^7-10^9 qPCR target copies per 100 mL. This study suggested that M3 cow specific marker could be used as MST tools to monitor water bodies for contamination with agricultural wastes than M2 marker. This study explored the application of qPCR tools that targeted *E. coli* O157 specific *eae* and *rfbE* genes and detected these genes in human and cow waste environments. However, these assays were further inspected to evaluate their cross-specificity with other serotypes of *E. coli* that arose after some speculation about the concentration detected in environmental samples. In conclusion, qPCR is a viable alternative and it is recommended that *E. coli*, enterococci, *B. thetaiotaomicron*, and M3 marker be used for studies in future.

Dedicated to the Lotus Feet of Lord Krishna

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

4-methylumbelliferyl-β-D-glucuronide -MUG

Beaches Environmental Assessment and Coastal Health -BEACH

Biological Oxygen Demand –BOD

Clean Water Act -CWA

Coefficient of variation -CV

Colony forming unit -CFU

Combined Sewer Overflow -CSO

Crossing point –Cp

Cycle threshold -Ct

Deoxynucleotide triphosphates -dNTPs

Deoxyribonucleic acid -DNA

Enteroaggregative -EAEC

Enterohemorrhagic *Escherichia coli* –EHEC

Enteroinvasive *Escherichia coli* –EIEC

Enteropathogenic –EPEC

Enterotoxigenic *Escherichia coli* –ETEC

Ethidium Mono-Azide -EMA

Fecal Coliform to Fecal Streptococcus -FC:FS

Federal Water Pollution Control Act -FWPCA

Heat- killed -HK

Internal Transcribed Spacer -ITS

Light cycler -LC

Locus of Enterocyte Effacement -LEE

Membrane Filtration -MF

Membrane-Thermotolerant *Escherichia coli* -mTEC

National Center for Biotechnology Institute -NCBI

National Pollutant Discharge Elimination System -NPDES

Ortho-Nitrophenyl-β-galactoside -ONPG

Phosphate Buffered Saline -PBS

Phosphate Buffered Water -PBW

Polyvinylpolypyrolidone -PVPP

Primary effluent -PE

Propidium Mono-Azide –PMA

Publicly Owned Treatment Works -POTW

qPCR -quantitative Polymerase Chain Reaction

Raw sewage -RS

Reverse Transcription real-time -RT rt

Sanitary Sewer Overflow -SSO

Secondary effluent -SE

Tertiary effluent -TE

U.S Environmental Protection Agency -USEPA

United States Department of Agriculture -USDA

Viable But Nonculturable - VBNC

Waste water treatment plant -WWTP

World Health Organization –WHO

CHAPTER 1

INTRODUCTION

Among all the problems faced by the world today, access to safe water remains a major global challenge. Fecal contamination from various sources further limits the use of available water due to the threat of water-borne pathogens in such polluted waters. Apart from limiting water sources available for drinking, domestic and recreational purposes, fecal contamination of fresh or marine water bodies often leads to a heavy economic loss due to closure of beaches and implementation of other measures needed to bring the contamination under control (Henrickson *et al*. 2001; Rabinovici *et al*. 2004). In addition, in spite of implementing stringent regulations for providing a safe water supply, the total number of waterborne illnesses per year in the U.S alone is estimated to be 19.5 million, which includes 5.4 million from municipal groundwater, 1.1 million from non-community groundwater sources, and 13 million from surface water supplies (Reynolds *et al*. 2008). From a global perspective, the estimated numbers of waterborne illnesses are even more dramatic, due to the fact that many countries lack adequate infrastructure to treat their wastewater and water supplies. The World Health Organization (WHO) estimates 1.8 million deaths around the world associated with diarrheal diseases each year with most of these due to unsafe water and poor sanitation. There is uncertainty in the number of illnesses as many waterborne diseases are not reported properly to the officials in many countries**.**

1.1 An Overview of Waterborne Pathogens and Water Quality Regulations

Millions die annually worldwide due to diseases like cholera, dysentery and shigellosis mainly targeting children, elderly and poor communities (Ivnitski *et al.* 1999; Venter 2000; Leonard *et al*. 2003). Lack of efficient and inexpensive monitoring tools for water exacerbate those illnesses that are waterborne especially in heavily populated developing countries as there is often no way to measure the level of risk. The prominent waterborne pathogens of concern to human health and their diseases are given in Table 1.1.

Table 1.1: **Waterborne pathogens of concern to human health and their diseases (Arnone and Walling 2007).**

Organism	Source relevant to water exposure	Disease
Bacteria		
Aeromonas hydrophila	Aquatic environments	Gastroenteritis
Campylobacter	Bird feces	Diarrhea
E. coli O157:H7	Cattle feces	Gastroenteritis
Helicobacter pylori	Human feces	Peptic ulcer or gastric cancer
Legionella spp.	Aquatic environments	Legionnaires disease
Leptospira spp.	Urine of dogs, wild animals	Leptospirosis
Mycobacterium avium complex	Bird feces	Pulmonary disease
Salmonella spp.	Domestic and wild animal feces	Salmonellosis
Salmonella typhi	Domestic and wild animal feces	Typhoid fever
Shigella	Human feces	Shigellosis
Vibrio cholerae	Asymptomatic human feces	Cholera
Yersinia entercolitica	Animal feces	Yersinosis
Virus		
Adenovirus	Human feces	Gastroenteritis, Respiratory disease
Astrovirus	Human feces	Gastroenteritis
Calcivirus	Human feces	Gastroenteritis
Enterovirus	Human feces	Gastroenteritis, Meningitis
Hepatitis A	Human feces	Infectious hepatitis
Hepatitis E	Human and pig feces	Infectious hepatitis
Reo viris	Human feces	Gastroenteritis
Rota virus	Human feces	Gastroenteritis
Protozoa		
Cryptosporidium	Human, animal & bird feces	Cryptosporidiosis
Cyclospora	Human feces	Cyclosporiasis
Entamoeba histolytica	Human feces	Amebiasis
Giardia lamblia	Human, animal & bird feces	Giardiasis
Naegleria fowleri	Bird & aquatic mammal feces	Meningoencephalitis

In US, there are strict regulations that address waterborne disease by regulating the quality of water. The Federal Water Pollution Control Act (FWPCA) of 1948 laid the foundation for all the ambient water quality standards. There were amendments made to this Act in 1972 and U.S Environmental Protection Agency (USEPA) enforced the "Clean Water Act" (CWA) (USEPA 1972). The main objective of this Act was to restore and maintain the chemical, physical and biological integrity of nation's water and various programs have been implemented to achieve this goal. National Pollutant Discharge Elimination System (NPDES) program regulates any pollutant discharge from point sources into navigable waters. Under this permit program, secondary treatment standards for sewage are established for publicly owned treatment works (POTWs) to monitor the performance efficiency of treatment process. Based on this, the geometric mean of fecal coliforms in these secondary effluent samples collected during any 30-day period shall not exceed 200 CFU per 100 mL for fecal coliforms and the geometric mean during any seven-day period shall not exceed 400 CFU per 100 mL (US Environmental Protection Agency 1988).

Another addition to the CWA is the Beaches Environmental Assessment and Coastal Health Act (BEACH Act) that was signed into law on October 10, 2000, with primary goals to improve water quality and protect public health at coastal recreational waters. The EPA developed criteria for fecal bacterial indicators, under this Act, and coastal and Great Lakes states have to either adopt these water quality criteria into their state standards and/or design and implement water quality monitoring programs with the approval from USEPA.

Impaired water bodies are identified based on exceeding Water Quality Standards (WQS) and corrective actions are taken by developing a Total Maximum Daily Load (TMDL) that determines the level of pollutants that can be discharged into waters which would still be able to meet WQS. The water quality standards based on correlation between levels of *E. coli* and enterococci and acceptable risk of swimming related illnesses were set in 1986 by USEPA and Table 1.2 shows these threshold levels for fresh and marine water. In order for a water body to be safe for recreational purposes; for example; the enterococci level should be below 35 enterococci CFU per 100 mL, thus setting a goal for swimmers to have a 1.9 percent or less chance of becoming ill.

Table 1.2: **Threshold levels of fecal indicator bacteria based on USEPA criteria for recreational waters (USEPA 1986).**

Fecal indicator bacteria	Concentration based on geometric mean (CFU/100 mL)	Single-sample concentration (CFU/100 mL)			
		Designated beach areas	Moderate full-body contact	Lightly used full-body contact	Rare full-body contact
Fresh water*					
E. coli	33	61	78	107	151
Enterococci	126	235	298	409	575
Sea water*					
Enterococci	35	104	158	276	501

*****Acceptable swimming-associated gastroenteritis rate is 8 per 1000 swimmers for *E. coli* and enterococci in fresh water and 19 enterococci in sea water.

Pollution from non-point sources are regulated under CWA by implementing grants for the states that propose best management plans for controlling pollution discharged from various sources into surface waters. The EPA has also set standards for the use of biosolids during land application with Class B containing pathogens and Class A which are treated (USEPA 2003).

1.2. Sources of Fecal Contamination in a Watershed

Sewage contamination of water is an important source of many of the waterborne pathogens associated with fecal pollution. The most serious fecal pollution occurs in developing and undeveloped parts of the world where there is inadequate sewage treatment (Tibbetts 2000). For example, in India, a study conducted by the Central Pollution Control Board indicated that in the year 2005, 921 cities had treatment capacity developed for only 27% of the total wastewater generated (Kumar 2009). However in the US, pathogen sources include Combined Sewer Overflows (CSO), Sanitary Sewer Overflows (SSO), septage, biosolids and poorly treated sewage and animal wastes.

Combined Sewer Overflows are sewers that collect and transport rainwater runoff, domestic sewage and pre-treated industrial effluent in the same pipe to wastewater treatment plant where it it may undergo treatment before discharging into a water body. In the past all CSOs discharged directly to rivers, currently these are being fixed. Sanitary Sewer Overflows are the occasional raw sewage discharge into a water body due to severe weather, system or power failure, etc resulting in discharge of untreated waste into rivers and streams. These are serious concerns in many parts of the developed world including the US (USEPA 2004). Sludge or biosolids are the solid residues obtained after the treatment of wastewater and if not adequately treated may contain enteric pathogens.

Many of these biosolids are applied to the land with little treatment (Class B biosolids) such as in Michigan (http://www.michigan.gov/deq). Even with Class A biosolids, there is still a concern that bacterial pathogens may regrow (Zaleski *et al*. 2005a and 2005b). Leaking septic tanks are also a cause of human fecal contamination of water sources and waterborne pathogens (Fong *et al*. 2007). Many households or small communities use individual septic tanks. According to U.S Census Data on Small Community Housing and Wastewater Disposal and Plumbing Practices, eight USEPA regions in US have more than 50 percent of the small community housing on septic tanks (http://water.epa.gov/infrastructure/wastewater/septic/census_index.cfm). Septic tanks collect waste water from the house including toilets, sinks and laundry. The liquid is treated by soil infiltration and solids, known as septage are pumped out and often land applied with or without treatment.

Agricultural animal wastes (manure) are also associated with microbial pathogens (Sobsey et al. 2001). Manure is used to supplement the soils with nutrients for crops, though if not treated properly can be a major source of fecal contamination of soil and water (McFarland et al. 1998). Waterborne outbreaks have been reported due to cow manure application and runoff from farms to nearby water bodies after heavy rainfall (Kistemann *et al***.** 2002; Hrudey *et al*. 2003; McQuigge 2000). An outbreak caused by *Campylobacter* and *E. coli* O157 occurred in the Walkerton, Ontario, Canada in 2000 resulted in 2,300 illnesses and 7 deaths from contamination of the community well with cattle manure after a heavy rainfall (Hrudey *et al.* 2003). Pathogens such as *E. coli*, *Salmonella*, *Campylobacter*, *Giardia* and *Cryptosporidia* are present in manure (Pell 1997).

1.3 Fecal Indicator Organisms

Routine monitoring of water bodies in the past for the presence of waterborne pathogens has not been practical as detection tools used for these pathogens were not available. This was because cultivation was the primary detection method and many of these pathogens escaped routine cultivation as they changed into a viable but non-culturable state (VBNC) making their detection more expensive and laborious or, in the case of viruses and parasites, cultivation procedures were not available for water samples (Oliver *et al*. 1991, Oliver 2002; Colwell & Huq 1994; Stevens *et al*. 2001; Venter 2000; George *et al.* 2001). Additionally, use of cultivation methods takes longer periods of time (e.g. days) to obtain confirmed positive results for some pathogens (Sartory and Watkins 1999; Venter 2000; Leonard *et al*. 2003; Taguchi *et al*. 2005).

To assess microbial water quality, water bodies are still monitored for fecal indicator bacteria whose presence may indicate possible fecal contamination. Microbial fecal indicators are meant to represent the potential risk associated with the possible presence of other pathogens that also originate from the intestines of animals or humans. The idea of using bacterial indicator organisms for monitoring water was introduced by Frankland in 1891, and then in 1892, Schardinger suggested using *E. coli* for this purpose (Leclerc *et al*. 2001).

The desired criteria for an ideal indicator organism are as follows (Bonde 1966; Manafi 1998; Stevens et al. 2001; Pletschke et al. 2006):

- Be a member of intestinal microflora of warm blooded animals
- Be present whenever enteric pathogens are present and not be found when they are absent

- Relate its concentration directly to the degree of fecal contamination
- Have a longer survival time than enteric pathogens
- Not proliferate in water
- Be resistant to treatment processes and adverse environmental factors similar to pathogens
- Have an easy and cost-effective detection method

The sections below reviews briefly two of the bacteria that are classified and used as indicator organisms, and are used for regulatory purposes.

1.3.1 *Escherichia coli*

Bacterial fecal indicators belonging to the family *Enterobacteriaceae* which include a group known as coliforms (defined as rod-shaped, Gram-negative non-spore forming bacteria and include a sub-group known as fecal coliform bacteria which can grow at 44.5^{o}C. Coliforms are abundant in the intestines and feces of all warm blooded animals (Leclerc et al. 2001). *Escherichia coli (E. coli)*, a specific member of the coliform group, has been used to assess microbial quality of recreational water especially freshwater bodies as well as drinking water (Leclerc et al. 2001). *E. coli* is a facultative anaerobic, motile bacterium and can utilize wide variety of substrates to support their growth. This specific genus and species are present in high concentrations in digestive tract of warm blooded animals and are distinguished from other coliform group by their biochemical characteristics such as lack of urease enzyme that hydrolyses urea to carbondioxide and ammonia. Based on previous epidemiological studies, *E. coli* was found to correlate well with swimming related illnesses in recreational fresh water bodies (Cabelli *et al*. 1982; Wade *et al*. 2003; Wiedenmann *et al*. 2006) even better than that of fecal coliforms. This

has led the USEPA to recommend that *E. coli* be used to judge safe recreational waters (USEPA 1986). However, *E. coli*, like other coliforms, have been found to proliferate in soil and plants (Hardina & Fujioka 1991) in pristine areas where there is no input from fecal contamination (Niemi *et al.* 1997) capable of extended survival and re-growth in soils, especially in tropical environments (Byappanahalli & Fujioka 1998).

1.3.2 Enterococci

Enterococci, belonging to the family Enterococcaceae, are facultatively anaerobic, catalase negative, non-sporing bacteria which are able to tolerate 40% bile, a pH of 9.6 and 6.5% sodium chloride (Cai 1999). They are able to survive at a temperature range of 10-45^{o}C, with an optimum growth temperature at 37^{o}C. This group of bacteria was originally classified as *Streptococcus* species until Schleifer and Kilpper grouped them separately as *Enterococcus* in 1984, (Law-Brown and Meyers 2003; Müller *et al*. 2001) and were suggested to be indicators of fecal contamination earlier in the 1900s due to their abundance in the intestines and feces of all warm blooded animals

Various species are found to be selectively predominant in different warm blooded animal intestines. For example, *E. columbae* is found only in pigeons (Baele *et al.* 2002) whereas *E. canintestini* and *E. asini* have been isolated from dogs and donkeys respectively (DeVaux *et al.* 1998; Naser *et al.* 2005). *E. phoeniculicola* was isolated from uropygial (preen) gland secretion of Redbilled Woodhoopoes (Law-Brown and Meyers 2003). *Enterococcus faecalis* occurs in wide range of hosts but some studies suggest that occurrence of this species in a fecal polluted area is more indicative of human fecal contamination (Bonilla *et al.* 2006; Kuntz *et al.* 2004; Wheeler *et al.* 2002).

Epidemiological studies conducted by USEPA found that high densities of enterococci correlated well with swimming related gastrointestinal illness in recreational waters, and USEPA set water quality criteria using these organisms as one of the indicators (U.S. Environmental Protection Agency 1986). It has been suggested that the enterococci are better well-correlated with fecal pathogens than *E. coli* and are more suitable as an indicator in monitoring marine recreational water quality (Kinzelman *et al.* 2003). However, enterococci are also isolated from other sources and environments such as food, plants, oysters (Fortina *et al.* 2004; Müller *et al.* 2001; Ott *et al*. 2001; Silva *et al.* 2004; Whitman *et al.* 2005); and in water bodies with no fecal pollution (Svec *et al.* 2001 and 2005). They are also found to survive and replicate in sediments, fresh water, forage plants and algae (Desmarais *et al.* 2002; Hartel *et al.* 2005; Müller *et al.* 2001; Ott *et al.* 2001;Whitman *et al*. 2003, 2005). This limits their capability as an indicator of fecal contamination of water.

1.4 Detection of *Escherichia coli* and Enterococci from Water

1.4.1 Cultivation Methods: Membrane Filtration

Methods conventionally used to detect indicators from water sources include Membrane Filtration (MF) which is a cultivation based technique that requires the bacteria to be grown on a filter which is placed on specific growth media containing agar. A water sample is passed through a 47mm diameter membrane filter by applying negative pressure, with a porosity of 0.2 µm or 0.45 µm that is effective in retaining bacteria from the sample. The membrane is then placed on growth media specific for

each bacterium in petri plates and incubated under optimum conditions. After the incubation period, colonies are enumerated on the filter.

The membrane filtration methods currently used to detect *E. coli* and enterococci from water were tested and implemented by USEPA as Method 1603 and Method 1600 respectively. The initial idea of the MF technique for detection of *E. coli* was proposed by Dufour *et al.* in 1981 as a two-step membrane filtration that combined cultivation technique using membrane-Thermotolerant *Escherichia coli* agar (mTEC agar) and subsequent confirmation by urease test which was later modified by EPA in 1998 as a single step cultivation based method (EPA Method 1603) using Modified mTEC Agar. The Modified m-TEC agar medium has a chromogen (5-bromo-6-chloro-3-indolyl-β-D-glucuronide) which is broken down to glucuronic acid by *E. coli* possessing β-D-glucuronidase enzyme and forms red or magenta-colored colonies, thus providing results within 24 hours (U.S. Environmental Protection Agency 2000). The Modified m-TEC agar has Sodium lauryl sulfate and Sodium desoxycholate to inhibit the growth of Gram-positive bacteria. The false positive rate with this is medium is less than 1.0% and false negative rate is 4.0% for environmental water samples (USEPA, 2005a).

A membrane filtration method for isolation of enterococci was introduced by Slanetz et al in 1957 using selective media that could differentiate the fecal enterococci as a subpopulation of the generic fecal strepotococci. In 1975, Levin *et al*. proposed a two step method that combined incubation on mE agar for 48 hours and further incubation on Esculin Iron agar that differentiated enterococci from other streptococci. USEPA, in 1997, modified this into a one step method (USEPA, 2002) by reducing the incubation time to 24 hours and adding a chromogen, indoxyl-β-D-glucoside that is utilized by

enterococci possessing β-D-glucosidase enzyme to an insoluble indigo blue complex to form a blue halo around the colony by diffusion. This mEI agar media, in which enterococci grows by hydrolyzing esculin, has ingredients that have inhibitory effect on fungi and Gram-negative bacteria. There is a false positive rate of 6.0% and false negative rate of 6.0% for environmental water samples with this medium (Messer and Dufour 1998).

Enzymatic detection of *E. coli* based on defined substrate technology was proposed by Edberg and Edberg in 1988. This technology is based on vital nutrient growth substrates that are supplied specifically for target organisms and does not contain any substrates that support growth of other bacteria. Then during the process of substrate utilization, a chromogenic product is released from the substrate, thus indicating the presence of the target microorganism. Several commercial products are available based on this technology. Colilert (IDEXX Laboratories, Portland, ME, USA) is an example that makes use of this technology for simultaneous detection of coliforms and *E. coli*. Basically, ortho-Nitrophenyl-β-galactoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG) are carbon sources that are metabolized by *β*-galactosidase in coliforms that change the color of the medium to yellow and *β*-glucuronidase in *E. coli* to produce fluorescence. A parallel approach for detection of enterococci uses Enterolert (IDEXX Laboratories, Portland, ME, USA) where enterococci produce fluorescence when the nutrient source is metabolized due to the enzymes present in enterococci (Fricker and Fricker, 1994). The false positive rate and false negative rate associated with Enterolert was reported to be 5.1 and 0.4%, respectively, for recreational waters (Budnick et al. 1996). However, this method also lacks specificity. There is a lack of efficient tools

to identify the wide range of enterococci species in water; to date there are 47 known enterococcal species of clinical, animal and environmental origin (Brtkova 2010).

1.4.2 Molecular Methods: Polymerase Chain Reaction and Quantitative Polymerase Chain Reaction (qPCR)

Invention of polymerase chain reaction (PCR), a technique that amplifies and makes millions of copies of specific target regions of deoxyribonucleic acid (DNA), in 1983 paved the way for gene specific amplification and identification (Mullis and Faloona 1987). This tool helped to overcome many problems associated with culture based methods. PCR was quickly adapted for medical diagnostics and finally for detection of specific microbes in environmental samples including food and water (Brunk et al. 2002). Polymerase Chain Reaction is initiated with the denaturation of double stranded DNA by holding the temperature at around 95^{o}C for 10-15 minutes. Repeated cycles, usually up to 40 cycles of these three processes as described below results in amplification of target DNA.

i) Denaturation at 95^{o}C- The bond that holds two strands of DNA is broken, reducing double helix to a single strand.

ii) Annealing at 60^{o}C or a primer-dependent temperature - Primers, which are short synthetic oligonucleotides and serve as initiation point for DNA synthesis, bind to "complementary sequence spots" due to the formation of hydrogen bonds.

iii) Extension at 72^{o}C - Deoxynucleotide triphosphates (dNTPs) are added to the 3' end of the primer by polymerase enzyme, thus completing strand synthesis.

Amplified product can be visualized by a method called Agarose Gel Electrophoresis which separates DNA fragments by their length. An electric field moves the negatively charged nucleic acid molecules through an agarose matrix. Shorter molecules move farther through gel pores by a phenomenon called “sieving”. The amplified product can be visualized in the the gel as bands by addition of stains such as ethidium bromide which intercalates between DNA bases and fluoresces when viewed using a UV transilluminator **(**Voytas 2000) Quantitative PCR (qPCR) also called as real-time PCR is a modification of conventional PCR that allows the quantification of target DNA in real-time without further analysis. Quantitative PCR, using Taqman probes, was first described by Heid et al (1996). The basic principle is the same as that of conventional PCR but in this case a fluorescent DNA specific probe made up of oligonucleotides, such as Taqman® Probe, or dye, such as SYBR Green® is added to the reaction mix. Primers and probes are designed using programs such as Beacon Designer (Premier Biosoft) Primer Express Applied Biosystems or Roche Primer Design (Roche Diagnostic Corportation), in such a way that the nucleotide sequences in these short oligonucleotide fragments can bind specifically to a target sequence of DNA present in the sample during the annealing step in PCR. In the absence of target sequence, there is no binding of these primers or probes and hence there is no amplification. While designing the primers and probe sequences, care should be given to avoid any significant cross homologies by performing a BLAST search of primers and probe sequences using GenBank nucleotide database at National Center for Biotechnology Institute (NCBI) (Dieffenbach et al. 1995). It is an important step in determining the specificity of the assay as any sequence similarity of target gene

with other genes could result in cross amplification and false positives (Gunson et al. 2006)

The mechanism behind qPCR is in the design of the probe tagged with a dye termed a "reporter" at the 5' end of the DNA and at the 3' end another dye termed "quencher" is used. Both these dyes are at close proximity and when there is no amplification in progress, release of fluorescent signals by reporter dyes are suppressed by the quencher dyes. As the addition of nucleotides to the 3' end of the primers by Taq Polymerase enzyme are in progress during amplification, it reaches the annealed probe and cleaves the probe due to 5' exonuclease activity of polymerase enzyme (any base or obstacle downstream of the growing nucleotide chain is removed due to this activity). Cleavage of the probe at 5'end increases the distance between both the dyes and that increases fluorescent emissions of the reporter dye. All qPCR machines will have a detector system to capture the increase in reporter signals. The Crossing point (Cp) is the cycle at which PCR amplification curves begins and is the point that is most reliably proportional to the initial concentration of the DNA target. As the quantity of the amplified product increases, the fluorescent signals of reporter dye also increase, which is measured by the software to calculate the concentration of target DNA in the reaction mixture, thus providing rapid sensitive and quantitative results (Valasek and Repa 2005).

Efficient qPCR protocols include removal of PCR inhibitors in the sample, good primers and probe design, optimization of reagent concentration and cycling conditions, and avoidance of any external contamination to ensure improved assay sensitivity and specificity (Hoorfar et al. 2004). Sensitivity refers to the assay's capability to detect very low concentrations of a target gene. An efficient qPCR assay can detect, sometimes as

low as 2 gene copies per reaction (Nayak and Rose 2007). The success of qPCR is also in the ability of the assay to detect actual target without amplifying any other DNA present in the samples whenever the assay is run under the same reaction conditions and this is referred to as "specificity". Fluorescent dyes, such as SYBR Green, intercalates with any double-stranded DNA (including non-specific PCR products or primer-dimers) and fluoresces during qPCR; thus reducing specificity of the assay. However, when a fluorescent probe that corresponds to part of sequence of target DNA is used in qPCR, it will specifically bind to the target thus providing more accurate quantification results (Hoorfar et al. 2004).

Quantitative PCR is a promising tool especially for the detection of many important pathogens in water. Some strains of pathogens such as *Vibrio cholera, Helicobacter pylori* etc. enter into a physiological state called VBNC (Viable But Nonculturable) in which the cells are metabolically active but cannot be grown in culture media (Oliver 2005). Quantitative PCR is able to detect the cells in this state, thus reducing the false negatives which would otherwise arise by using cultivation based methods. The ability to provide data in a shorter period of time is another advantage of qPCR. While cultivation based methods require at least 24-48 hours before results can be obtained, qPCR can provide results in less than an hour (Klein 2002). This is critical for prompt decision making to prevent major adverse effects on human health or economic losses associated with pollution of drinking water and recreational water. However, there are some disadvantages that need to be considered before traditional cultivation methods are replaced by qPCR methods. Some of the concerns that need to be addressed while using qPCR for detection and quantification of target organisms include the inability to

distinguish between live and dead cells, variations in the DNA recovery, misinterpretation of cell numbers based on target gene copies that vary with different bacteria, and false interpretation of target concentrations due to qPCR inhibition and inefficiency (Wilson 1997). Finally qPCR tools are often more expensive as compared to cultivation methods.

Application of qPCR for monitoring *E. coli* and enterococci in water was first published by Frahm and Obst (2003) using Taqman probe assays that targeted the uid A and 23S rRNA genes respectively. They used an overnight enrichment step to quantify the *E. coli* targets in drinking water samples as the quantification was negative with either no or short enrichment steps. This study used plasmid copies during standardization, and the detection limit of the assay was around 10-100 copies per reaction and final results were obtained within five hours following overnight incubation. Foulds et al (2002) applied a Taqman qPCR assay targeting lacZ gene to quantify *E. coli* in distilled water and river water samples seeded with *E. coli* and were able to detect three copies per reaction of target gene in each sample without the overnight enrichment steps reducing the detection time to two hours following DNA extraction. Another qPCR assay targeting Internal Transcribed Spacer (ITS) region of *E. coli* between 16S-23S rRNA subunit genes (Khan et al, 2007) had a detection limit of 70 copies /reaction in spiked agricultural water as determined from the standard curve.

Another Taqman based qPCR targeting *uidA* gene for *E. coli* was developed by Silkie *et al.* (2008) to test protocols for reagent decontamination during qPCR assays. Recently, the protocol was also applied to measure concentrations of *E. coli* in raw sewage and fresh animal feces and quantified levels of *E. coli* to be 10^7 cells per 100 mL of raw

sewage and 10^6-10^8 cells in per gram of dry weight of animal feces. Host specificity and detection limit for this assay were not evaluated. A qPCR assay (based on Scorpion qPCR chemistry in which primers are covalently linked to the probe) was utilized to target the *uidA* gene and compare to cultivation based defined substrate (IDEXX Colilert-18) and colilert in a study that monitored wastewater and surface water for *E. coli* to evaluate the capacity of the assay for future applications (Lavender and Kinzelman 2009). They found a positive correlation between the colilet and qPCR in both these samples.

The qPCR assay for enterococci developed by Frahm and Obst (2003) targeted the 23S rRNA gene from a wide range of *Enterococcus* spp. Out of 47 known enterococcal species, the assay tested positive for the following enterococci species; *E. faecalis, E. faecium, E. durans, E. hirae, E. gallinarum,* and *E. casseliflavus*. These primer sets have been applied in epidemiological studies by USEPA (Wade et al. 2006) and the qPCR for the 23S rRNA is being considered by USEPA as a potential method to be included in new regulations for monitoring recreational beach water quality (Boehm et al. 2009).

1.5 Microbial Source Tracking

Microbial source tracking (MST) is a field that has enabled differentiation of various sources of fecal contamination which in turn has assisted in addressing effective control of the pollution inputs (Stewart *et al*. 2003). MST generally involves examination of fecal indicator bacterial genetic markers which can then be associated with a specific host and it is associated with feces such as human (e.g. septage) or animal (e.g. cattle manure). Host specificity is assumed to be due to the confined occurrence of key types of fecal

indicators in animals due to nutrient selection as well as physiology of different intestines (Shanks *et al.* 2006).

1.5.1 Selected MST Approaches

There are two fundamental approaches to MST; one is based on phenotypes and other based on genotypes. Phenotypic approaches are based on some morphological or biochemical traits whereas genotypic approaches are related to the organism's nucleic acid sequences (Simpson et al. 2002). In MST using cultivation methods, target organisms from samples are grown in specific culture media and once the targets are verified; genotypic or phenotypic approach is applied. An alternative approach is to use cultivation-independent method where the nucleic acids, DNA or RNA are extracted directly from the samples and genotypic methods are applied for MST. This approach saves time and is also used to examine targets that are either present in low numbers in the sample or cannot be grown in culture media.

Another classification of MST methods is library dependent and uses reference libraries (Simpson 2002), of a large number of bacterial isolates which are collected and characterized from humans, cows, swine etc for a particular genetic or phenotypic characteristic. Isolates from one source are analyzed, compared and contrasted against isolates in the libraries using mathematical approaches.

Library-independent methods require no reference library but are based on detection of host specific target organisms in which specificity and sensitivity has been defined (USEPA 2005b). This will be described in more detail in the following section

Table 1.3: **Microbial source tracking approaches (Scott *et al*. 2002; Simpson *et al.* 2002)**

Methods	**Principle**	**Limitations**
Phenotypic Library-dependent Methods		
Antibiotic Resistance Analysis (ARA)	Different species of bacteria vary in their response to antibiotic treatment.	-Adequate database of profiles is needed. -Time consuming.
Carbon utilization Profile (Nutritional Analysis)	Different bacteria use wide range of carbon and nitrogen sources for energy and growth	-Nutrient requirements of bacteria may be influenced by environmental factors, so may not be right approach for field application. -Not many case studies done.
Phenotypic Library-independent Methods		
Fecal Coliform/Fecal Streptococci (FC/FS) Ratio	Human feces normally has a ratio of fecal coliform to fecal streptococci greater than or equal to 4.0, whereas ratios below 0.7 are associated with animal feces.	-Fecal enterococci densities vary with individuals based on diet. -Coliform survival varies with environmental factors.
Serogrouping	Bacterial strains of the same species have different somatic antigenic determinants.	- Adequate databank of anti-sera profiles is needed.
Genotypic Library-dependent Methods		
Ribotyping	Genetic differences in the genomic sequences within or flanking the 16S and 23S rRNA genes are detected.	-Slow and inconclusive results

Table 1.3 (cont'd)

Randomly Amplified Polymorphic DNA (RAPD) analysis	PCR conditions done using non-selective primers at high stringency produce a series of strain specific primer and template dependent PCR products.	-Lack of reproducibility. -Lab to lab variation.
Repetitive element sequence-based PCR (rep-PCR)	Specific fingerprints are generated using interspersed repetitive DNA sequences located in different parts of the target indicator genome.	-cell culture required. -may require large database of isolates. -variability increases as database increases.
PFGE (Pulsed-Field Gel Electrophoresis)	Variability in sequences in the genome result in DNA-fragment banding pattern detecting genetic differences between strains	-Time consuming and tedious. -Not suitable for rapid identification of large number of strains.
Genotypic Library-independent Methods		
Molecular Species-Specific (Host-Specific) Indicators	Primers are designed to match specific genes of target organisms.	-Little is known about survival and distribution in water systems -Host specificity need to be tested extensively

1.5.2 Genotypic Library-Independent Source Tracking Methods

In this section, library-independent cultivation-independent methods are reviewed as rest of the source tracking methods are beyond the scope of this research. This source tracking approach offers several advantages; saves time and cost of collecting and fingerprinting bacterial isolates from all possible fecal sources, utilizes molecular methods such as PCR or qPCR, therefore, results are obtained faster than culture-dependent methods, target organisms that are difficult to grow in culture media can also be explored for source tracking, as the DNA extract can be used for multiple assays targeting multiple markers. Although generally culture independent, some protocols in this approach required that the target organism be grown in specific culture media (due to sensitivity) prior the DNA analysis. For example, a gene for an enterococcal surface protein (esp) found in *Enterococcus faecium* was found to be specific for human fecal contamination (Scott *et al*. 2005) and has been applied for MST in water using a cultivation-PCR protocol (Ahmed *et al.* 2008; Wong *et al*. 2008).

MST assays for the detection of host-specific *Bacteroides* are currently one of the most frequently used methods (Santo Domingo and Sadowsky 2007). *Bacteroides* are obligately anerobic, Gram negative, rod shaped, and non-endospore forming bacteria and are normally commensals that constitute the most numerous members of the intestinal flora of all warm blooded animals (Wexler 2007) This group of bacteria has been suggested as alternative fecal indicators as these are found in much higher concentrations than *E. coli* in the warm-blooded humans and animals. One gram of feces may have nearly 10^{11} *Bacteroides* cells (Finegold *et al*. 1983*)* and *E. coli* is found using culture

methods at about 10^7 CFU per gram of human feces (Fiksdal 1985; Wang *et al*. 1994, 1996). They do not survive well outside the host organism (Avelar *et al*. 1998; Kreader 1998) and are less likely to re-grow in the environment (Fiksdal *et al*. 1985). Bernhard and Field (2000) utilized molecular tools such as PCR, length heterogeneity PCR (LH-PCR) and terminal restriction fragment length polymorphism (T-RFLP) to identify *Bacteroides* 16S rDNA fragments that had multiple sequences resulting in host specific gene clusters for which primers were designed for a PCR assay. It has been estimated that of the total fecal bacteria in the gut, 30% are *Bacteroides*, (Holdeman *et al*. 1976), and 10% represent of these have host-specific markers (Bernhard and Field 2000). Conventional PCR assay was designed targeting a human (HF183 marker) specific clone in the 16S rRNA of the *Bacteroides –Prevotella* group. The host specificity tested for this assay showed that out of 13 human samples tested, 11 tested positive and out of three sewage samples tested, all tested positive for the human HF183 16S rRNA marker (Bernhard and Field 2000). The host specificity for this marker was further evaluated and confirmed by Ahmed *et al.* (2008). Out of the 155 feces samples tested from various animals, none showed amplifications using the primers for HF183 marker. Quantification of the HF183 marker using a SYBR Green qPCR done by Seurinck *et al*. (2005). Even though this assay showed high specificity to human fecal contamination, it also amplified 25% and 14% of the dog and cat fecal samples tested, respectively.

Human and cow-specific assays targeting *Bacteroides* 16S rRNA were developed by Layton *et al*. (2006). Even though the human specific assay amplified the human target in 90% of the human fecal samples, it also amplified the human target in 38% of cow, 13% of horse, 88% of dog, and 71% of cat fecal samples. Similarly, host specificity tested

using primer sets developed by Okabe *et al*. (2007) targeting another region of 16S rRNA fragment from humans of *Bacteroides-Prevotella* showed cross reactivity with clones from human, cow, and pig feces. A *Bacteroides* assay by Kildare *et al*. (2007) showed cross specificity with dog feces (one fecal sample out of eight fecal samples tested) though it was positive in 32 human fecal samples tested and negative in 33 animal feces that included feces from 8 cows, 8 horses, 7 cats and 10 seagulls .

Several of these assays targeting human *Bacteroides* 16S rRNA genes are reported to show cross reactivity with fish feces (McLain *et al*. 2009). Assays that were believed to be host specific are being reported to react with other fecal sources (Layton et al., 2006; Kildare et al., 2007). This cross amplification is thought be due to the use of 16S rRNA genes (Shanks *et al* 2007). Continued research into defining the identification of host-specificity and evaluating geographic distribution of 16S rRNA *Bacteroides* markers is required before a false positive or false negative rate can be determined.

Other specific genes belonging to certain species of *Bacteroides* are being recommended as MST targets for human fecal pollution and are being evaluated. In this regard, *Bacteroides thetaiotaomicron* was suggested as a good candidate as MST marker not only for its occurrence in high concentrations in human feces but also due to its high specificity when tested with other animal feces (Carson *et al.* 2005). Testing was undertaken on 35 beef cattle, 26 dairy cattle, 24 chicken, 29 turkey, 35 horse, 44 swine, and 17 goose fecal samples and only five of 31 dog fecal samples showed a cross reaction (Carson *et al.* 2005).

Markers that have the potential to track bovine fecal pollution have also been developed. A conventional PCR assay that targeted a CF123 gene cluster of what may be

a cow specific fecal clone for *Bacteroides* 16S rRNA gene was developed by Bernhard and Field (2000). This used a forward primer (CF128) combined with a reverse primer that targeted *Bacteroides-Prevotella* group. Detection limit of this assay was found to be 1×10^{-12} grams of cow fecal DNA using 10 fecal samples in a composite (Lamendella *et al.* 2006). One study that tested specificity of the assay found cross amplification with chicken, deer, goat and sheep feces.

Layton *et al*. (2006) later developed qPCR assays targeting another specific *Bacteroides* 16S rRNA set of genes. Clone libraries from human and cow feces were produced by using a conventional PCR that targeted general *Bacteroides* and sequences of these clones were aligned to identify regions that were specific for each host fecal clone library. Primers and Taqman based probes were designed targeting these specific regions and were further evaluated for host specificity. All 11 bovine fecal samples were amplified using this assay. Bovine associated assay (Bobac assay) did not show cross amplification with any other host fecal DNA tested except with one dog sample (of four), out of 6 human, 6 swine, and 7 equine fecal samples tested. Okabe *et al.* (2007) used the same approach to design cow-specific *Bacteroides* primers for qPCR assay. However, the specificity was reported only on the basis of sequence alignment and was not tested in fecal samples from multiple hosts.

More recently, a metagenomic approach called Genome Fragment Enrichment (GFE) method using DNA hybridization was used to identify host-specific fragments that were not 16S rRNA targets (Shanks et al. 2006, 2008). In this approach, bovine-specific genome fragments were generated by hybridizing biotin labeled bovine DNA with DNA from another host fecal sample and screened further using bioinformatic tools for

confirmation. BLASTx analysis revealed potential relationship of these fragment sequences to *Bacteriodes.* Further assignment of these fragments into functional groups based on a previous *B. thetaiotaomicron* VPI-5482 genome annotation indicated that these fragments belonged to genes that encode bacterial surface and secreted proteins. Out of the three such bovine specific fragments selected after screening, qPCR assays were designed for two of these markers, termed as M2 and M3 (Shanks et al. 2006; 2008). Host specifcity of these assays were tested using 175 fecal DNA extracts from 24 different animal species and both the assays showed 100% specificity, thus no false positives were identified at a rate of 99%. M2 assay revealed 100% sensitivity whereas for M3 assay the sensitivity was 98% when tested using DNA from 247 individual bovine fecal samples representing 11 different populations, with a quantification limit of 25 copies per reaction. However, there were concerns that sensitivity and prevalence could vary for these assays with geographical distributions and cattle herd populations which should be evaluated before applying these assays in a particular location for MST purposes (Shanks et al. 2008).

Other MST methods using genotypic library-independent approaches included STIb, STII and LTIIa heat stable enterotoxin gene targets in *E. coli* to track cattle (Khatib *et al*. 2002) and swine (Khatib *et al*. 2003) feces respectively, host specific virus assays such as human adenoviruses and bovine polyomaviruses (Bofill-Mas et al. 2006), porcine adenoviruses (Hundesa *et al*. 2009) etc. These approaches have greater false negative rates or are much more expensive than examining bacterial targets.

There are limitations of any MST approach. Prevalence and concentration of these markers may vary with individuals of a species, or across different geographical

locations. There is a possibility for these markers to show up only in “pooled” samples such as raw sewage or manure lagoon samples and not be detected in individual fecal samples. Some markers could be present in such low concentrations that large volumes of sample are required to detect them. Method based limitations such as inhibition of the specific qPCR assay may also bias sensitivity of any qPCR result. Thus it is clear that while extremely promising, a better understanding of DNA targets and their concentrations associated with many of these new markers as well as agreater experience and comparison of qPCR methods to conventional standards are needed.

REFERENCES

1.6 References:

Ahmed, W., J. Stewart, D. Powell, and T. Gardner. 2008. Evaluation of Bacteroides markers for the detection of human faecal pollution. Lett Appl Microbiol **46:**237-42.

Arnone, R. D., and J. P. Walling. 2007. Waterborne pathogens in urban watersheds. J. Water Health **5:**149-162

Avelar, K. E. S., S. R. Moraes, L. J. F. Pinto, W. Souza, R. Domingues, and M. C. D. Ferreira. 1998. Influence of stress conditions on Bacteroides fragilis survival and protein profiles. Zentralbl. Bakteriol **287:**399-409.

Baele, M., L. A. Devriese, P. Butaye, and F. Haesebrouck. 2002. Composition of enterococcal and streptococcal flora from pigeon intestines. Journal of Applied Microbiology **92:**348-351.

Bernhard, A. E., and K. G. Field. 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. Appl Environ Microbiol **66:**4571-4574.

Boehm A.B, Ashbolt N.J, Colford J.M Jr, Dunbar L.E, Fleming L.E, Gold M.A, et al. 2009. A sea change ahead for recreational water quality criteria. J Water Health 7(1): 9–20.

Bofill-Mas, S., N. Albinana-Gimenez, P. Clemente-Casares, A. Hundesa, J. Rodriguez-Manzano, A. Allard, M. Calvo, and R. Girones. 2006. Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. Appl. Environ. Microbiol. **72:**7894-7896

Bonde, G. J. 1966. Bacteriological methods for estimation of water pollution. Health Lab. Sci. **3:**124−128.

Bonilla, T. D., K. Nowosielski, N. Esiobu, D. S. McCorquodale, and A. Rogerson. 2006. Species assemblages of Enterococcus indicate potential sources of fecal bacteria at a south Florida recreational beach. Marine Pollution Bulletin **52:**807-810.

Brtkova, A., M. Filipova, H. Drahovska, and H. Bujdakova. 2010. Characterization of enterococci of animal and environmental origin using phenotypic methods and comparison with PCR based methods. Veterinarni Medicina **55:**97-105.

Brunk, C., J. Li, and E. Avaniss-Aghajani. 2002. Analysis of specific bacteria from environmental samples using a quantitative polymerase chain reaction. Curr. Issues Mol. Biol. **4:**13-18.

Budnick, G. E., R. T. Howard, and D. R. Mayo. 1996. Evaluation of Enterolert for enumeration of enterococci in recreational waters. Appl Environ Microbiol. **62**:3881-3884.

Byappanahalli, M. N., and R. S. Fujioka. 1998. Evidence that tropical soil environment can support the growth of *Escherichia coli*. Water Sci. Technol. **38**:171-174.

Cabelli, V. J., A. P. Dufour, L. J. McCabe, and M. A. Levin. 1982. Swimming-associated gastroenteritis and water quality. Am J Epidemiol **115:**606-16.

Cai, Y. M. 1999. Identification and characterization of Enterococcus species isolated from forage crops and their influence on silage fermentation. Journal of Dairy Science. **82:**2466-2471.

Carson, C. A., J. M. Christiansen, H. Yampara-Iquise, V. W. Benson, C. Baffaut, J. V. Davis, R. R. Broz, W. B. Kurtz, W. M. Rogers, and W. H. Fales. 2005. Specificity of a Bacteroides thetaiotaomicron marker for human Feces. Appl Environ Microbiol **71:**4945-4949.

Colwell R. R; and Huq A. 1994. Vibrios in the environment: viable but not culturable *Vibrio cholerae.* Wachsmuth K, Blake PA, Olsvik O, eds. *Vibrio cholerae and Cholera: Molecular to Global Perspectives.* Washington, DC: American Society for Microbiology Press, 117–131.

de Vaux, A., G. Laguerre, C. Divies, and H. Prevost. 1998. Enterococcus asini sp. nov. isolated from the caecum of donkeys (Equus asinus). International Journal of Systematic Bacteriology. **48:**383-387.

Desmarais, T. R., H. M. Solo-Gabriele, and C. J. Palmer. 2002. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. Appl Environ Microbiol **68:**1165-1172.

Dieffenbach CW, Lowe TMJ, Dveksler GS .1995. General concepts for PCR primer design. In: PCR Primer, A Laboratory Manual (Dieffenbach CW, Deveksler GS eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 133-155.

Dufour, A. P., E. R. Strickland, and V. J. Cabelli. 1981. Membrane-Filter Method For Enumerating *Escherichia coli*. Appl Environ Microbiol **41:**1152-1158.

Edberg, S. C., and M. M. Edberg. 1988. A Defined Substrate Technology For The Enumeration Of Microbial Indicators Of Environmental Pollution. Yale J Biol Med. **61:**389-399.

Fiksdal, L., J. S. Maki, S. J. Lacroix, and J. T. Staley. 1985. Survival And Detection Of Bacteroides Spp, Prospective Indicator Bacteria. Appl Environ Microbiol **49:**148-150.

Finegold, S. M., Sutter, V. L., and Mathisen, G. E. 1983. Normal indigenous intestinal flora, p. 3-31. In D. J. Hentges (ed.). Human intestinal microflora in health and disease. Academic Press, New York.

Fong, T. T., L. S. Mansfield, D. L. Wilson, D. J. Schwab, S. L. Molloy, and J. B. Rose. 2007. Massive microbiological groundwater contamination associated with a waterborne outbreak in Lake Erie, South Bass Island, Ohio. Environ Health Perspect **115:**856-864.

Fortina, M. G., G. Ricci, D. Mora, and P. L. Manachini. 2004. Molecular analysis of artisanal Italian cheeses reveals Enterococcus italicus sp nov. Int J Syst Evol Microbiol. **54:**1717-1721.

Foulds, I. V., A. Granacki, C. Xiao, U. J. Krull, A. Castle, and P. A. Horgen. 2002. Quantification of microcystin-producing cyanobacteria and *E. coli* in water by 5'-nuclease PCR. J Appl Microbiol **93**, 825–834.

Frahm, E., and U. Obst. 2003. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus spp.* and *Escherichia coli* in water samples. J. Microbiol. Methods **52**, 123–131.

Fricker, E. J., and C. R. Fricker. 1994. Application Of The Polymerase Chain-Reaction To The Identification Of Escherichia-Coli And Coliforms In Water. Lett Appl Microbiol. **19:**44-46.

George, I., M. Petit, C. Theate, and P. Servais. 2001. Use of rapid enzymatic assays to study the distribution of faecal coliforms in the Seine river (France). Water Sci Technol **43:**77-80.

Gunson, R. N., T. C. Collins, and W. F. Carman. 2006. Practical experience of high throughput real-time PCR in routine diagnostic virology settings. J. Clin. Virol. **35:**355-367

Hardina, C. M., and R. S. Fujioka. 1991. Soil: the environmental source of *Escherichia coli* and enterococci in Hawaii's streams. Environ. Toxicol. Water Qual. **6:**185-195.

Hartel P. G., Rodgers K., Fisher J. A. *et al*. 2005 Survival and regrowth of fecal enterococci in desiccated and rewetted sediments. In: *Proceedings of the 2005 Georgia Water Resources Conference* (ed. K. J. Hatcher). Athens, Georgia.

Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. Genome Res. **6:**986-994.

Henrickson, S. E., T. Wong, P. Allen, T. Ford, and P. R. Epstein. 2001. Marine swimming-related illness: implications for monitoring and environmental policy. Environ Health Perspect **109:**645-50.

Holdeman, L. V., I. J. Good, and W. E. C. Moore. 1976. Human Fecal Flora - Variation In Bacterial Composition Within Individuals And A Possible Effect Of Emotional Stress. Appl Environ Microbiol **31:**359-375.

Hoorfar, J., B. Malorny, A. Abdulmawjood, N. Cook, M. Wagner, and P. Fach. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J. Clin. Microbiol. **42:**1863-1868.

Hrudey, S. E., P. Payment, P. M. Huck, R. W. Gillham, and E. J. Hrudey. 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. Water Sci Technol **47:**7-14.

Hundesa, A., C. Maluquer de Motes, N. Albinana-Gimenez, J. Rodriguez-Manzano, S. Bofill-Mas, E. Sunen, and R. Rosina Girones. 2009. Development of a qPCR assay for the quantification of porcine adenoviruses as an MST tool for swine fecal contamination in the environment. J. Virol. Methods **158:**130-135

Ivnitski, D., Abdel-Hamid, I., Atanasov, P. and E. Wilkins. 1999. Biosensors for detection of pathogenic bacteria *Biosens Bioelectron* **14**: 599-624

Khan, I. U. H.; Gannon, V.; Kent, R.; Koning, W.; Lapen, D. R.; Miller, J.; Neumann, N.; Phillips, R.; Robertson, W.; Topp, E.; Van Bochove, E.; Edge, T. A. 2007. Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agricultural watersheds J. Appl. Microbiol. Meth. **69** : 480 488

Khatib, L.A., Y.L. Tsai and B.H. Olson. 2002. A biomarker for the identification of cattle fecal pollution in water using the LTIIa toxin gene from enterotoxigenic *Escherichia coli*. Appl. Microbiol. Biotech. **59:** 97-104.

Khatib, L.A., Y.L. Tsai and B.H. Olson. 2003. A biomarker for the identification of swine fecal pollution in water, using the STII toxin gene from enterotoxigenic *Escherichia coli*. Appl. Microbiol. Biotech. **63:** 231-238.

Kildare, B. J., C. M. Leutenegger, B. S. McSwain, D. G. Bambic, V. B. Rajal, and S. Wuertz. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. Water Research. **41:**3701-3715.

Kinzelman, J., C. Ng, E. Jackson, S. Gradus, and R. Bagley. 2003. Enterococci as indicators of Lake Michigan recreational water quality: Comparison of two methodologies and their impacts on public health regulatory events. Appl Environ Microbiol **69:**92-96.

Kistemann, T., T. Classen, C. Koch, F. Dangendorf, R. Fischeder, J. Gebel, V. Vacata, and M. Exner. 2002. Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. Appl Environ Microbiol **68:**2188-2197.

Klein, D. 2002. Quantification using real-time PCR technology: applications and limitations, *Trends in Molecular Medicine* **8** :257–260.

Kreader, C. A. 1998. Persistence of PCR-detectable Bacteroides distasonis from human feces in river water. Appl Environ Microbiol **64:**4103-4105.

Kumar, M. 2009. Reclamation and reuse of treated municipal wastewater: an option to mitigate water stress. Current Science. **96**:886-889.

Kuntz, R. L., P. G. Hartel, K. Rodgers, and W. I. Segars. 2004. Presence of Enterococcus faecalis in broiler litter and wild bird feces for bacterial source tracking. Water Research. **38:**3551-3557.

Law-Brown, J., and P. R. Meyers. 2003. Enterococcus phoeniculicola sp nov., a novel member of the enterococci isolated from the uropygial gland of the Red-billed Woodhoopoe, Phoeniculus purpureus. Int J Syst Evol Microbiol. **53:**683-685.

Lamendella, R., Domingo, J.W., Oerther, D.B., Vogel, J.R., and Stoeckel, D.M. 2007. Assessment of fecal pollution sources in a small northern-plains watershed using PCR and phylogenetic analyses of Bacteroidetes 16S rRNA gene. FEMS Microbiol Ecol 59: 651–660.

Lavender, J. S.; Kinzelman, J. L. A cross comparison of QPCR to agar-based or defined substrate test methods for the determination of *Escherichia coli* and enterococci in municipal water quality monitoring programs *Water Res.* **2009**, 43 (19) 4967– 4979

Layton, A., L. McKay, D. Williams, V. Garrett, R. Gentry, and G. Sayler. 2006. Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl Environ Microbiol **72:**4214-4224.

Leclerc, H., D. A. A. Mossel, S. C. Edberg, and C. B. Struijk. 2001. Advances in the bacteriology of the Coliform Group: Their suitability as markers of microbial water safety. Annu. Rev. Microbiol. **55:**201-234.

Leonard, P., S. Hearty, J. Brennan, L. Dunne, J. Quinn, T. Chakraborty, and R. O'Kennedy. 2003. Advances in biosensors of pathogens in food and water. Enzyme and Microbial Technology**:**3-13.

Levin, M.A., J.R. Fischer and V.J. Cabelli. 1975. Membrane Filter Technique for Enumeration of Enterococci in Marine Waters. *Appl. Microbiol.* 30:66-71.

Manafi, M., and R. Sommer. 1993. Rapid Identification Of Enterococci With A New Fluorogenic-Chromogenic Assay. Wat. Sci. Tech. **27:**271-274.

McFarland, M.L., T.L. Provin and S.E. Feagley. 1998. Managing crop nutrients through soil, manure and effluent testing. Texas Agricultural Experiment Service, College Station, Texas, L-5175 5176.

McLain, J. E. T., H. Ryu, L. Kabiri-Badr, C. M. Rock, and M. Abbaszadegan. 2009. Lack of specificity for PCR assays targeting human Bacteroides 16S rRNA gene: cross-amplification with fish feces. FEMS Microbiol Lett. **299:**38-43.

McQuigge, M. 2000 The investigative report on the Walkerton outbreak of waterborne gastroenteritis. Bruce-Grey-Owen Sound Health Unit, Owen Sound, Ontario.

Messer, J. W., and A. P. Dufour. 1998. A rapid, specific membrane filtration procedure for enumeration of enterococci in recreational water. Appl Environ Microbiol **64:**678-680.

Muller, T., A. Ulrich, E. M. Ott, and M. Muller. 2001. Identification of plant-associated enterococci. J Appl Microbiol. **91:**268-278.

Mullis, K. and F, Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* **155**: 335-350

Naser, S. M., M. Vancanneyt, E. De Graef, L. A. Devriese, C. Snauwaert, K. Lefebvre, B. Hoste, P. Svec, A. Decostere, F. Haesebrouck, and J. Swings. 2005. Enterococcus canintestini sp nov., from faecal samples of healthy dogs. Int J Syst Evol Microbiol. **55:**2177-2182.

Nayak, A.K., and J.B Rose. 2007. Detection of *Helicobacter pylori* in sewage and water using a new quantitative PCR method with SYBRgreen. *J. Appl. Microbiol.*, **103**(5): 1931-1941.

Niemi RM, Niemela SI, Lahti K, Niemi JS. 1997. Coliforms and *E. coli* in Finnish surface waters. In *Coliforms and E. coli.Problem or Solution*? ed. D Kay, C Fricker, 13:112–19. Cambridge: R. Soc.Chem. 267 pp.

Okabe, S., N. Okayama, O. Savichtcheva, and T. Ito. 2007. Quantification of host-specific *Bacteroides-Prevotella* 16S rRNA genetic markers for assessment of fecal pollution in freshwater. Appl. Microbiol. Biotechnol. **74:**890-901.

Oliver, J. D. 2002. Public health significance of viable but nonculturable bacteria, p. 277-300. *In* R. R. Colwell and D. J. Grimes (ed.), Nonculturable microorganisms in the environment. American Society for microbiology, Washington, D.C.

Oliver, J. D. 2005. The viable but nonculturable state in bacteria. J. Microbiol. **43:**93-100

Oliver, J. D., L. Nilsson, and S. Kjelleberg. 1991. Formation of nonculturable Vibrio vulnificus cells and its relationship to the starvation state. Appl Environ Microbiol **57:**2640-2644.

Ott, E. M., T. Muller, M. Muller, C. Franz, A. Ulrich, M. Gabel, and W. Seyfarth. 2001. Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. J Appl Microbiol. **91:**54-66.

Pell, A. N. 1997. Manure and microbes: public and animal health problem? J. Dairy Sci. **80:**2673-2681

Pletschke, B, Togo, C. and Wutor V. 2006. On-line real-time enzyme diagnostic system for the detection and monitoring of faecal contamination of water intended for drinking purposes. WRC Report No. 1446/1/06.

Rabinovici, S. J., R. L. Bernknopf, A. M. Wein, D. L. Coursey, and R. L. Whitman. 2004. Economic and health risk trade-offs of swim closures at a Lake Michigan beach. Environ Sci Technol **38:**2737-2745.

Reynolds, K. A., K. D. Mena, and C. P. Gerba. 2008. Risk of waterborne illness via drinking water in the United States. Rev Environ Contam Toxicol **192:**117-58.

Santo Domingo, J. W., and M. J. Sadowsky. 2007. Microbial source tracking. ASM Press, Washington, DC.

Sartory, D. P., and J. Watkins. 1999. Conventional culture for water quality assessment: is there a future? J. Appl. Bact. Symp. Supp. **85:**225S-233S.

Scott, T. M., J. B. Rose, T. M. Jenkins, S. R. Farrah, and J. Lukasik. 2002. Microbial source tracking: current methodology and future directions. Appl. Environ. Microbiol. **68:**5796–5803.

Scott, T. M.; Jenkins, T. M.; Lukasik, J.; Rose, J. B. 2005. Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution Environ. Sci. Technol.39, 283– 287

Seurinck, S., T. Defoirdt, W. Verstraete, and S. D. Siciliano. 2005. Detection and quantification of the human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Environ Microbiol. **7:**249-259.

Shanks, O. C., J. W. Santo Domingo, R. Lamendella, C. A. Kelty, and J. E. Graham. 2006. Competitive metagenomic DNA hybridization identifies host-specific microbial genetic markers in cow fecal samples. Appl. Environ. Microbiol. **72:**4054–4060.

Shanks, O. C., J. W. Santo Domingo, J. R. Lu, C. A. Kelty, and J. E. Graham. 2007. Identification of bacterial DNA markers for the detection of human fecal pollution in water. Appl Environ Microbiol **73:**2416-2422.

Shanks, O. C., E. Atikovic, A. D. Blackwood, J. Lu, R. T. Noble, J. Santo Domingo, S. Siefring, M. Sivaganesan, and R. P. Haugland. 2008. Quantitative PCR for detection and enumeration of genetic markers of bovine fecal pollution. Appl. Environ. Microbiol. **74:**745-752.

Silkie, S.S., Tolcher, M.P., and K.L Nelson. 2008. Reagent decontamination to eliminate false-positives in Escherichia coli qPCR. Journal of Microbiological Methods 72, 275–282.

Silva, A. I. M., R. Vieira, F. G. R. Menezes, A. A. Fonteles, R. C. O. Torres, and E. S. Sant'Anna. 2004. Bacteria of fecal origin in mangrove oysters (Crassostrea rhizophorae) in the Coco River estuary, Ceara State, Brazil. Brazilian Journal of Microbiology. **35:**126-130.

Simpson, J. M., Santo Domingo, J. W., and Reasoner, D. J. 2002. Microbial source tracking: State of the science. Environ. Sci. Technol. 36:5279-5288.

Stevens, M., Ashbolt, N. and Cunliffe, D. 2001. Microbial Indicators of Water Quality: An NHMRC Discussion Paper. National Health and Medical Council Act, Canberra.

Stewart, J.R., R.D. Ellender, J.A. Gooch, S. Jiang, S.P. Myoda, and S.B. Weisberg. 2003. Recommendations for microbial source tracking: Lessons learned from a methods comparison study. J. Water Health **1**:225–231.

Sobsey, M.D., Khatib, L.A., Hill, V.R., Alocilja, E. *and* Pillai, S. 2001 Pathogens in animal wastes and the impact of waste management practices on their survival, transport and fate. *In* Proceedings of the International Symposium Addressing Animal Production and Environmental Issues, *Research Triangle, NC.*

Ŝvec, P., L.A. Devriese, I. Sedláèek, M. Baele, M. Vancanneyt, F. Haesebrouck, J. Swings, and J. Doŝkaø. 2001. *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis* sp. nov., Isolated from Water. Int J Syst Evol Microbiol. **51**:1567-1574

Ŝvec, P., M. Vancanneyt, L.A. Devriese, S.M. Naser, C. Snauwaert, K. Lefebvre, B. Hoste, and J. Swings. 2005. *Enterococcus aquimarinus* sp. nov., Isolated from Seawater. Int J Syst Evol Microbiol. **55**:2183-2187

Taguchi, T., H. Takeyama, and T. Matsunaga. 2005. Immuno-capture of Cryptosporidium parvum using micro-well array. Biosensors and Bioelectronics. **20:**2276-2282.

Tibbetts J. Waterworld 2000. Environmental Health Perspectives, Vol. 108, No. 2 Feb., pp. A69-A73.

USEPA. 1972. Clean Water Act. 33USC 1251-1387

USEPA. 2002**.** *Enterococcus spp.* in Water by Membrane Filter using membrane Enterococcus Indoxyl-B-D-Glucoside Agar (mEI). EPA-821-R-02-022. Office of Water, Washington D.C.

USEPA 2003. . Standards for the Use or Disposal of Sewage Sludge; Agency Response to the National Research Council Report on Biosolids Applied to Land and the Results of EPA's Review of Existing Sewage Sludge Regulations. Fed Reg 68: 17379–17395.

USEPA. 2004. "Report to Congress: Impacts and Control of CSOs and SSOs," April 26, 2004, EPA 833-R-04-001, 4-29 http://cfpub.epa.gov/npdes/cso/cpolicy_report2004.cfm.

USEPA. 2005. *Escherichia coli* (*E. coli*) in Water by Membrane Filtration using Modified membrane-Thermotolerant *Escherichia coli* Agar (modified mTEC). EPA 821-R-04-025. Office of Water, Washington D.C.

USEPA. 2005b. Microbial Source Tracking Guide Document. EPA-600/R-05/064, Office of Research and Development, Washington D. C.

U.S. Environmental Protection Agency. 1986 Bacteriological ambient water quality criteria for marine and fresh recreational waters. Washington, D.C.: U.S. Environmental Protection Agency.

US Environmental Protection Agency (1988) Water quality standards criteria summaries: A compilation of state/federal criteria. EPA 440/5-88-007. US Environmental Protection Agency, Office of Water, Washington DC.

U.S. Environmental Protection Agency. 2000. Improved enumeration methods for the recreational water quality indicator: enterococci and *Escherichia coli*. Publication EPA/821/R-97/004. Office of Science and Technology, USEPA, Washington, D.C.

U.S. Environmental Protection Agency. 2002. *National Beach Guidance and Required Performance Criteria for Grants*. EPA Document Number EPA-823-B-02-004, Office of Water, Washington, D.C.

Valasek, M. A., and J. J. Repa. 2005. The power of real-time PCR. Adv. Physiol. Educ. **29:**151-159.

Venter, S.N. 2000. Rapid microbiological methods: the status quo. Blue Pages, International Water Association, London, UK.

Voytas D. Agarose gel electrophoresis. Curr Protoc Mol Biol. 2000;**51**: 2.5A.1–2.5A.9.

Wade, T. J., N. Pai, J. N. Eisenberg, and J. M. Colford, Jr. 2003. Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environ. Health Perspect. **111:**1102-1109.

Wade TJ, Calderon RL, Sams E, Beach M, Brenner KP, Williams AH, et al. 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ Health Perspect 114: 24–28.

Wang, R. F., W. W. Cao, W. L. Campbell, L. Hairston, W. Franklin, and C. E. Cerniglia. 1994. The Use Of Pcr To Monitor The Population Abundance Of 6 Human Intestinal Bacterial Species In An In-Vitro Semicontinuous Culture System. FEMS Microbiol Lett. **124:**229-237.

Wang, R. F., W. W. Cao, and C. E. Cerniglia. 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl Environ Microbiol **62:**1242-1247.

Wexler H. M. Bacteroides: the good, the bad, and the nitty-gritty.*Clin Microbiol Rev* 2007, **20:**593-621.

Wheeler, A. L., P. G. Hartel, D. G. Godfrey, J. L. Hill, and W. I. Segars. 2002. Potential of Enterococcus faecalis as a human fecal indicator for microbial source tracking. Journal of Environmental quality. **31:**1286-1293.

Whitman, R. L., D. A. Shively, H. Pawlik, M. B. Nevers, and M. N. Byappanahalli. 2003. Occurrence of Escherichia coli and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan. Appl Environ Microbiol **69:**4714-4719.

Whitman, R. L., S. E. Byers, D. A. Shively, D. M. Ferguson, and M. Byappanahalli. 2005. Occurrence and growth characteristics of Escherichia coli and enterococci within the accumulated fluid of the northern pitcher plant (Sarracenia purpurea L.). Canadian Journal of Microbiology. **51:**1027-1037.

Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. **63:**3741-3751

Wiedenmann A, Krüger P, Dietz K, Lopez-Pila JM, Szewzyk R, Botzenhart K. A. 2006. randomized controlled trial assessing infectious diseases risks from bathing in fresh recreational waters in relation to the concentration of *Escherichia coli*, intestinal enterococci, *Clostridium perfringens*, and somatic coliphages. Environ Health Perspect. 228–236.

Wong, M., Kumar, L., Jenkins, T. M., Xagoraraki, I., Phanikumar, M. S., Rose, J. 2008. Evaluation of public health risks at recreational beaches in Lake Michigan via detection of enteric viruses and a human-specific bacteriological marker *Water Res.* **43**: 1137– 1149.

Zaleski, K. J., K. L. Josephson, C. P. Gerba, and I. L. Pepper. 2005a. Survival, growth, and regrowth of enteric indicator and pathogenic bacteria in biosolids, compost, soil, and land applied biosolids. Appl Environ Microbiol **2:**49-63.

Zaleski, K.J., K.L. Josephson, C.P. Gerba, and I.L. Pepper. 2005b. Potential regrowth and recolonization of *Salmonella* and indicators in biosolids and biosolid amended soil. Appl. Environ. Microbiol **71**:3701–3708.

CHAPTER 2

RESEARCH OBJECTIVES

2.1 Problem Statement

Risks to human health are imposed by both human and non-human sources of fecal contamination of our waterways, although these risks may differ with different fecal sources. Indictor organisms play an important role in minimizing or avoiding these risks by implying the occurrence of fecal contamination in a water body. But very often, the sources of fecal inputs remain unknown and the pollution problems persist for longer periods of time or keep occurring intermittently. Alternative indicators, such as those belonging to genus *Bacteroides*, due to their source tracking capability are being considered for water quality evaluation and may be included in the new or revised water quality criteria for recreational waters by U.S. EPA (USEPA 2007). Even though some studies have been conducted to evaluate the host specificity, sensitivity, and geographical distribution of some of these host-specific markers (USEPA 2005), there are still significant data gaps that need to be addressed to obtain a better understanding of these markers and their applications. Among several research gaps that need to be addressed, one of the priorities is to determine relationships between these markers and current regulatory indicators such as *E. coli* and enterococci in various fecal sources of water pollution using standard and new detection methods. These gaps, if addressed, will help regulatory agencies provide recommendations for use of these markers in NPDES permits for wastewater treatment plants or Best Management Practices (BMP) for non-point sources of fecal contamination.

2.2 Research Goals, Hypothesis and Objectives

The overall aims of this research were to investigate the prevalence and concentrations of recently identified human and cow specific *Bacteroides* markers in comparison with *E. coli* and enterococci in both the human and cow waste environments and to monitor these environments for occurrence of *E. coli* O157:H7. In this study, there was also an attempt made to understand the variation expected when cultivation methods are replaced with qPCR methods in different samples.

The main hypotheses were: 1) Human specific *Bacteroides thetaiotaomicron* DNA target is present in similar levels as that of *E. coli* and enterococci and is a good substitute for conventional indicators in predicting human fecal contamination. 2) High concentrations of human specific *Bacteroides thetaiotaomicron* DNA markers are present throughout the wastewater treatment processes; 4) Prevalence and concentration of cow markers such as cow specific *Bacteroides* 16S rRNA markers, cow M2 and M3 markers differ in different cow manure samples.

The specific objectives were: 1) to monitor concentrations of *Bacteroides thetaiotaomicron*, *E. coli* and enterococci in raw sewage and septage samples and evaluate fate of these organisms and/or their DNA during wastewater treatment processes; 2) to evaluate the prevalence and concentrations of different *Bacteroides* cow markers in cow manure samples; 3) to monitor cow manure, sewage and septage samples for occurrence of *E. coli O157*; and 4) to evaluate and compare cultivation methods with qPCR methods for monitoring different environments.

2.3 Thesis Outline

After chapter 1 (included as a brief literature review of water quality) and chapter 2 (on objectives), chapter 3 addresses methods development. This was an evaluation of a Taqman qPCR assay for generic *E. coli* that was used in all monitoring studies, comparing and evaluating an automated DNA extraction method with manual extraction kits for environmental samples. An ethidium monoazide-qPCR method to distinguish live from dead cells in treated and untreated waste samples was explored. In addition, a preliminary evaluation of the 16S rRNA *Bacteroides* human marker by conventional PCR was undertaken.

Chapter 4 details the goals, methods and results from samples collected from waste water and septage treatment facilities. This was focused on understanding the fate of human specific DNA markers and conventional indicators throughout treatment process and characterizing effluent quality using these DNA targets, as well as comparing qPCR to cultivation methods. Chapter 5 describes the methods and findings of the monitoring of cow manure lagoons using three different cow specific *Bacteroides* markers, and conventional indicators. Comparison between cultivation methods and qPCR methods were performed using two targets, *E. coli* and enterococci using individual cow feces and manure lagoon samples. Chapter 6 focuses on the monitoring of cow manure lagoons, sewage, and septage samples for *E. coli* O157. However, this chapter also discusses the issues with specificity of qPCR assays used for the detection of this pathogen.

Chapter 7 summarizes the conclusions of this research and addresses future directions.

REFERENCES

2.4 References

USEPA. 2005. Microbial Source Tracking Guide Document. EPA-600/R-05/064, Office of Research and Development, Washington D. C.

USEPA. 2007. Report of the experts scientific workshop on critical research needs for the development of new or revised recreational water quality criteria, EPA-823-R-07-007. United States Environmental Protection Agency, Washington, DC.

CHAPTER 3

METHODS DEVELOPMENT, EVALUATION AND APPLICATION

3.1 Introduction

Recent advances in molecular biology such as polymerase chain reaction (PCR) have revolutionized microbiology and are beginning to be used to develop a tool box to address the microbial quality of recreational and drinking water environments (Noble et al. 2006). Quantitative PCR known as qPCR has many advantages such as producing results more rapidly (30 minutes to 2 hours), detection of non-cultivatable microbes compared to standard cultivation methods, and providing quantitative results with a wider detection range (10^0 - 10^8 copies/reaction) (Mackay 2004).

Escherichia coli (E. coli) and *Enterococcus* spp. have long been used as indicators of water pollution. Cultivation methods are used to enumerate these indicator organisms such as EPA Method 1600 for *Enterococcus* spp. and 1603 for *E. coli* (Messer and Dufour 1998; USEPA 2002; 2005)**.** However, it takes at least 24 hours to obtain the results using these methods. Multiplex PCR targeting *lacZ* and *lamB* followed by detection of amplified genes using specific probes were the initial molecular approaches for detection of *E. coli* in water (Bej *et al*. 1991a). Other genes such as *phoE* genes and 16S rRNA genes have also been targeted for molecular detection of *E. coli* (Spierings *et al*. 1993; Tsai and Olson 1992; Tsen *et al.* 1998**)** by conventional PCR**.** One of the most widely studied targets is the *beta*-glucuronidase in *E. coli* which is coded for by the *uidA* gene (*Bej et al.* 1991a, b; Buckalew *et al*. 2006; Gracias and McKillip 2004; Tryland and Fiksdal 1998) and PCR methods targeting that gene have been developed and evaluated

to monitor water quality (Foulds *et al*. 2002, Frahm and Obst 2003). A new qPCR method targeting the *uidA* gene was developed and used in the research presented in this dissertation for quantification of *E. coli* in different samples. This assay was initially designed with an idea to develop a multiplex qPCR assay to detect generic *E. coli* and *E. coli* O157 simultaneously in one single run from different samples. But further evaluation during assay development did not give satisfactory results and the idea of multiplexing assays was abandoned. However, the qPCR assay for detection of generic *E. coli* was found to be satisfactory based on the specificity testing and therefore was further evaluated and applied throughout this research.

The qPCR assay used in this study for *Enterococcus* spp., targeting 23S rRNA gene, was originally developed by Ludwig & Schleifer (2000) and had been further evaluated by Frahm & Obst (2003). They tested specificity of the assay and most strains of *Enterococcus* spp. were detected that are commonly found in the environment. Many studies have used this qPCR assay to detect *Enterococcus* spp. (He and Jiang 2005; Noble *et al.* 2006; Shivaganesan *et al*. 2008; Viau and Peccia 2009; Wade *et al.* 2006). Noble *et al.* (2006) included this assay in a multi-tiered approach to assess the level of contamination in Santa Monica Bay, California. Despite the fact that qPCR equivalent cells of *Enterococcus* spp. remained steady even when their cultivable levels were reduced due to inactivation by sunlight, the advantage of using *Enterococcus* spp. qPCR assay in the tool-box approach to provide quantitative and rapid water quality test results to beach quality managers was illustrated in their study. Wade *et al.* (2006) used this *Enterococcus* spp. qPCR assay and found a strong correlation between *Enterococcus* spp. qPCR daily average concentrations (cells/100mL) and swimming related gastrointestinal

illnesses in two of the Great Lakes beaches. This assay has also been applied for evaluating levels of *Enterococcus* spp. in biosolid samples (Viau and Peccia 2009), sewage and river water samples (He and Jiang 2005) and currently, U. S. EPA is validating this assay for monitoring marine and fresh ambient waters. The *Enterococcus spp.* qPCR assay protocol used by U. S. EPA is in the following link:

http://www.epa.gov/waterscience/methods/method/biological/rapid1.pdf

"Method A: *Enterococcus* spp. in Water by TaqMan Quantitative Polymerase Chain Reaction (qPCR) Assay". This *Enterococcus* spp. qPCR targeting 23S rRNA gene was standardized and applied for all monitoring studies in this research.

One of the most important steps in obtaining reliable data from qPCR methods is cell lysis and DNA extraction. It was noted that the lysis method used should ensure the disruption of thick Gram-positive cell walls (i.e., enterococci) but should not be so harsh that it causes shearing of released DNA from Gram-negative cells (i.e., *E. coli*) (Schneegurt *et al.* 2003). Early in methods development for extraction of DNA, Holben (1997) included separation of cells from soils by centrifugation and then extracted DNA from each cell fraction. This protocol included lysozyme for lysis of the cells, followed by caesium chloride-ethidium bromide density centrifugation and ethanol precipitation for DNA recovery. This method was tedious and included an overnight incubation to recover the DNA. Another popular approach was a direct lysis method (Ogram 2000) which included physically breaking the cells (bead beating) followed by a chemical lysis step (sodium dodecyl sulphate) and precipitation of DNA using polyethylene glycol and finally elution of DNA by phenol-chloroform extraction. This method was observed later to shear DNA with poor yields (Leff 1995).

Today most laboratories use commercial kits such as Qiagen Stool kit, MoBio Soil or Fecal extraction kits (Lebuhn *et al*. 2005; Rose *et al.* 2003) but these methods can still be laborious, time-consuming, with an increased risk of cross contamination, variability and errors. Automated DNA extraction methods have been evaluated for clinical specimens (Beuselinck *et al*. 2005; Wilson *et al*. 2004), to screen for viral pathogens in routine blood donations but have not been used often for environmental samples (Fafi-Kremer *et al.* 2004; Hourfar *et al.* 2005; Mengelle *et al*. 2003; Pichl *et al*. 2005). Rasmussen *et al.* (2009) compared different automated extraction instruments for viral nucleic acids in an interlaboratory study and found consistency between different instruments. They reported that automated DNA extraction was rapid, sensitive, reproducible and highly efficient from clinical samples. In this study, an automated DNA extractor was evaluated and compared to commercial extraction kits, QIAmp DNA mini kit (Qiagen, Valencia, CA, USA) and QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) using treated and untreated sewage and cow feces for the quantification of *E. coli* and *Enterococcus spp.* by qPCR.

Bacteroides-Prevotella 16S rRNA HF 183 human marker has been reported to be an effective marker to track human fecal contamination (Bernhard and Field 2000b) and its geographical stability has been evaluated in different locations such as in the USA (Bernhard *et al*. 2003), France (Gourmelon *et al.* 2007), United Kingdom, Portugal, Ireland (Gawler *et al*. 2007), and Australia (Ahmed *et al*. 2008) where it was found to be suitable based on specificiy for tracking human fecal contamination in surface water bodies thus far in the US, Europe and Australia.

Although molecular methods such as qPCR are a promising tool for environmental monitoring, one disadvantage of this method is its inability to differentiate between live and dead cells. When the cells die in the environment, their DNA persists and can be detected by molecular methods (Young et al. 2007). Thereby suggesting the levels of fecal contamination is commensurate with live pathogens, and could be considered false-positive results. One of the strategies used to overcome this issue has been to detect less stable RNA using Reverse-Transcription real-time PCR (RT-rtPCR) (Bustin 2000), which has been applied to detect pathogens from environmental samples but viability has not been verified (Gonzalez-Escalona *et al.* 2009; Sharma 2006). Lower recovery of RNA and reduced efficiency in amplification were the disadvantages with this approach (McKillip *et al*. 1999). Another strategy proposed to differentiate live and dead cells in qPCR was the use of Ethidium Mono-Azide (EMA) or Propidium Mono-Azide (PMA) dyes (Rudi et al. 2005). These dyes penetrate the damaged membranes of dead cells but cannot do so in the live cells whose membranes are intact. Once inside the cells, the dyes intercalate between the double strands of DNA upon photoactivation thus preventing amplification during PCR, in theory producing amplification of DNA from only live cells. EMA-qPCR has been applied in pure cultures and in other samples such as food (Wang and Levin 2005), soil and biofilms (Pisz *et al.* 2007) and water samples (Delgado et al. 2009; Gedalanga and Olson 2009). In this study, EMA-qPCR was evaluated using pure cultures of *E. coli* and *Enterococcus* spp., and raw sewage and final chlorine disinfected effluent samples from a wastewater treatment plant.

The specific objectives of this chapter are summarized below;

i) Standardisation of *E. coli* and *Enterococcus* spp. qPCR assays;

ii) Comparison of an automated DNA extraction method with manual methods using environmental samples;

iii) Application and evaluation of *Bacteroides-Prevotella* 16S rRNA HF 183 human marker conventional PCR assay; and

iv) Evaluation of EMA-qPCR using pure cultures and wastewater treatment samples.

3.2 Materials and Methods

3.2.1 Development of a qPCR Assay for *E. coli uid*A gene and *Enterococcus spp.* 23S rRNA gene

3.2.1.1 Bacterial Strains and DNA extraction

E. coli ATCC strain 15597, *Enterococcus faecalis* ATCC strain 19433, *Klebsiella pneumoniae* ATCC strain 13883, *Shigella flexneri* ATCC 12022, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Citrobacter freundii* ATCC 8090 were used as part of the initial development and testing of the qPCR method. *E. coli* strains used for specificity testing were provided by Microbial Evolution Laboratory, National Food Safety and Toxicology Center, at MSU. All the bacterial strains were grown in Trypticase Soy Broth at 37^{o}C for 18-24 hours. DNA was extracted from these cultures using QIAmp DNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

3.2.1.2. Preparation of qPCR Standards

Inorder to prepare the standards, the *uidA* gene of *E. coli* was amplified separately using a primer set that flanked the qPCR target amplicon sequences; the sequence for forward primer was 5'-GCAGTCTTACTTCCATGATTTCTTTA-3' and for the reverse

primer, it was 5'-TAATGCGAGGTACGGTAGG-3'. These primers were developed using the Roche Light Cycler Primer Design Software. Polymerase chain reaction (PCR) was performed in a 25 μL total reaction mix which contained 14 μL Hotstart DNA Polymerase Mastermix, 0.4 μM of each primer, 3 μL of the template DNA and molecular graded water (QIAgen, Valencia, CA, USA) to make up a final volume of 25 μL. The PCR was initiated with a denaturation step for 10min at 95^{O}C followed by 35 cycles of 30 sec at 95^{O}C; 30 sec at 57^{O}C and 60 sec at 72^{O}C and a final extension cycle at 72^{O}C for 8 min. Negative controls (reaction mix with molecular graded water instead of DNA) were included in the PCR runs. The PCR product of 522 bp size was visualized in 1.2% agarose gel stained with Gel-red by means of UV-trans illumination and a 100-bp DNA ladder (Promega, Madison, USA) was used as a marker.

Similarly, the 23SrDNA gene of *Enterococcus* spp. was amplified using the forward primer 5'ATCTACCCATGTCCAGGTTGAAG3' and reverse primer 5'CCATCTCGGGTTACCGAATTCAG3'. These primers were developed using the Roche Light Cycler Primer Design Software. Polymerase chain reaction (PCR) was performed in a 25μL total reaction mix which contained 15 μL Hotstart DNA Polymerase Mastermix, 0.4 μM of each primer, 2 μL of the template DNA and molecular graded water (QIAgen, Valencia, CA, USA) for a final volume of 25 μL. The PCR was initiated with a denaturation step for 10min at 95^{O}C followed by 35 cycles of 30 sec at 94^{O}C; 30 sec at 60^{O}C and 30 sec at 72^{O}C and a cycle of final extension at 72^{O}C for 10 min. Negative controls (reaction mix with molecular graded water instead of DNA) were

included in the PCR runs. The product of size 223 bp was visualized in 1.2% agarose gel stained with Gel-red as described previously.

The amplified PCR products for both *E. coli* and *Enterococcus* spp. denaturation step were cloned into TOPO PCR 2.1 and transformed with the TOPO10 F' competent cells (Invitrogen Inc., Carlsbad, CA, USA), according to the protocol provided by the manufacturer and the transformants were carefully picked, and inoculated in Luria–Bertani broth plus ampicillin (50mg/ml). The transformed bacteria were grown for 12-16 h in a shaker incubator set at 200rpm at 37^{o}C. Plasmids were extracted using the QIAGEN spin kit (Valencia, CA, USA) and were sequenced at the Research Technology Support Facility (RTSF) at Michigan State University that confirmed the insertion of the target inside the vector. The concentration of the plasmids was measured on a nano-spectrophotometer several times and the mean value was calculated. These were then serially diluted ten-fold to construct qPCR standard curves.

3.2.1.3 Assay Performance

The quantification of *uidA* gene for *E. coli* was optimized by using the primers and probes described in Table 3.1 and were designed using the Roche Light Cycler Primer Design Software and these primer designs were further checked for presence of any secondary structures that could lead to qPCR inefficiency by an Oligonucleotide company (TIB Molbiol, Freehold, NJ, USA). Ten-fold dilutions of plasmids were prepared ranging from $1.2x10^{6}$ to $1.2x10^{1}$ copies per reaction and were used as templates to prepare the standard curves. The reaction mix for amplification of *uidA* gene consisted of 2µL of the Fast Start Light Cycler Mastermix (Roche, Indianapolis, IN), 0.5 µM of each of forward and reverse primers, 0.2 µM of the probe A (Table 3.1), 3.2 µM $MgCl_2$

and nuclease free water to a final volume of 20 µL. The assay was carried out in LightCycler 2.0® (Roche, Indianapolis, IN) through the following temperature profiles; initial denaturation at 10 minutes at 95^{o}C followed by 45 cycles of denaturation for 6 seconds at 94^{o}C; annealing for 8 seconds at 58^{o}C and extension at 72 ^{o}C for 8 seconds. Triplicate analysis was done for each dilution and negative controls.

The primers and the probes used for the *Enterococcus* spp. targeting 23S rRNA gene were originally described by Ludwig & Schleifer (2000). The sequences for primers and probes used in this study are described in Table 3.1. A series of ten-fold dilutions of corresponding plasmids were prepared ranging from $3x10^{6}$ to $3.2x10^{0}$ and was used as template to prepare the standard curve. The reaction mix for qPCR consisted of 4µL of Taqman Light Cycler Mastermix (Roche, Indianapolis, IN), 0.5 µM of each of forward and reverse primers, 0.4 µM of the probe A (Table 3.1), 5 µL of plasmid DNA and nuclease free water to a final volume of 20 µL. The assay was carried out in LightCycler 2.0® (Roche, Indianapolis, IN) through the following temperature profiles; initial denaturation at 10min at 95^{o}C followed by 35 cycles of denaturation for 30 sec at 94^{o}C; annealing for 30 sec at 60^{o}C and extension at 72 ^{o}C for 8 sec. Triplicate analysis was done for each dilution and all negative controls.

For both assays, Cycle threshold (Ct) was measured during each amplification and was analyzed by absolute quantification method (Ginzinger 2002) Efficiency of amplification (E) was also estimated from standard curve by applying the formula, $E= (10^{-1/slope}) -1$.

Table 3.1: Primers and probes used for *E. coli* and Enterococci qPCR assay

Bacteria	Target gene	Primer/probe sequence	Amplicon size	Reference
E. coli	*uidA*	FP 5'-CAATGGTGATGTCAGCGTT-3' RP 5'-ACACTCTGTCCGGCTTTTG-3' Probe 6FAM-TTGCAACTGGACA AGGCACCAGC-BBQ	163bp	This study
Enterococci	23S rRNA	5'-AGAAATTCCAAACGAACTTG-3' 5'-CAGTGCTCTACCTCCATCATT-3' 6FAM-TGGTTCTCTCCGAAA TAGCTTTAGGGCTA-TAMRA	91bp	Ludwig & Schleifer (2000)

3.2.2 Comparison between Auto and Manual DNA Extraction Methods using Environmental Samples

3.2.2.1 Sample Processing and DNA extraction

a) Treated and untreated sewage samples were collected from a wastewater treatment plant (WWTP) in Michigan. The untreated sewage refers to raw sewage collected from a facility that serves around 90,000 people. Treated sewage included effluents from 1) primary treatment, after the solids have been settled, 2) secondary treatment, where primary clarified sewage has undergone activated sludge process and chlorination, and 3) tertiary treatment, effluent from secondary treatment post sodium bisulfate dechlorination and filtration through rapid sand filters. Raw sewage (n=9) and primary effluent samples (n=9) secondary and tertiary effluents (n=9 and 9, respectively) were used for the study, for a total of 36 samples from the wastewater environment.

b) Fecal samples were also collected from individual cows from a cattle dairy farm at Michigan State University (n=9).

Samples were stored and transported on ice and processed within 2 hrs after collection. Fifty milliliters of raw sewage sample were centrifuged at 8000xg for 20 minutes. Around 48 mL of the supernatant was discarded and remaining sample was mixed well by vortexing. The volume was recorded and from this, 400 µL of the pellet was used for manual DNA extraction with the QIAmp DNA mini kit (Valencia, CA, USA), another 400 µL of the aliquot was used for extraction by Roche MagNaPure LC instrument (Roche Applied Sciences, Indianapolis, Ind.) and remainder was stored at -80 ^{o}C. These volumes were included in the calculation when final concentrations of targets were calculated after qPCR.

For treated sewage, one liter of secondary and tertiary effluents was filtered using 90 mm, 0.45 µm pore size nitrocellulose membrane filters (Millipore, Billerica, Mass.) after which the filters were folded and immersed to the 45 mL mark with sterile phosphate buffered saline (PBS) in 50 mL centrifuge tubes. The tubes were then vortexed at high speed for 30-45 seconds to detach the cells from membrane. The filters were removed and the tubes were centrifuged at a speed of 8000xg for 15 minutes. Around 48 mL of the supernatant was discarded and remaining sample was mixed well by vortexing; from this 400 µL of the pellet aliquot was used for manual DNA extraction by using the QIAmp DNA mini kit (Valencia, CA, USA). Another 400 µL of the aliquot was used for extraction by Roche MagNaPure LC instrument (Roche Applied Sciences, Indianapolis, Ind.).

For DNA extraction using Roche MagNaPure automated instrument, an additional lysis was performed by mixing the 400 µL of concentrated samples with 180 µL of the MagNaPure lysis buffer and 20 µL of the Proteinase K (20 mg/mL) which was incubated at 65 ^{o}C for 30 minutes. The mixture was then centrifuged at 500g for 30 seconds to settle down the particles and the supernatant was used for DNA extraction by the instrument. This autoextractor employs a magnetic-bead technology for extraction. The initial steps are cell lysis and protein digestion followed by nucleic acid binding to the surface of the magnetic glass particles. The nucleic acid-bead complex formation is further separated by magnetism. After the removal of cellular debris by multiple washing steps, there is detachment of nucleic acids from the beads and DNA is eluted out. Simultaneously, a manual DNA extraction was carried out on the same samples using the QIAmp DNA mini kit (Qiagen, Valencia, CA, USA).

Both extraction methods resulted in a final volume of 200 µL of DNA suspended in TE buffer. Negative controls (molecular grade water) were used to check for cross contamination in both extraction methods. The concentrations of extracted DNA were determined by using Nanodrop ND-1000 Spectrophotometer.

Two hundred milligrams of cow feces were weighed and placed in 2 mL micro centrifuge tubes for DNA extraction using QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) and similarly for extraction by Roche MagNaPure LC (Roche Applied Sciences, Indianapolis, Ind.) instrument as described above.

3.2.2.2 Reproducibility

In order to assess the intervariability in extraction, eight replicates of concentrated raw sewage samples were subjected to DNA extraction by both instrument and manual methods following the same procedures described above. For all the replicates, the same volume of lysis buffer (180 µL) and proteinase K (20 µL) were added to the tubes and subjected to auto extraction. DNA concentrations in all extracts were analyzed by qPCR methods for both *E. coli* and *Enterococcus* spp. The extractions were also carried by two different people to assess the variability in both instrument and manual methods.

3.2.2.3 qPCR Analyses

DNA samples extracted from all wastewater samples and cow feces were analyzed by qPCR for *E. coli* and *Enterococcus* spp. One of the plasmid dilutions used for creating the standard curve was also included in the assay as a positive control. The qPCR analysis was performed using the same reaction mix components and cycling conditions as used for the plasmid standard curves. Five microlitres of the extracted sample were used as the template. The reaction was performed for duplicates for all samples and

negative controls were included. The copies of *uidA* gene of *E. coli* and 23S rRNA of *Enterococcus* spp. present in the sample were quantified from the standard curves obtained earlier. The copies of the corresponding genes were converted to cell equivalents; in the case of *E. coli*, only one copy of the *uidA* gene is present in a cell, thus one copy number corresponds to one cell. However in case of *Enterococcus* spp., there are four copies of 23S rRNA present in a cell, therefore four copies in *Enterococcus* spp. qPCR assay corresponds to one cell. All the final concentrations for qPCR analyses were reported as qPCR equivalent cells/100mL for all sewage samples and qPCR equivalent cells/gram of wet weight for fecal samples after conversion of copy numbers to cells.

3.2.2.4 Statistical Analysis

All statistical analyses were performed using SAS software 9.2 (SAS Inc, 2002). The data were log-transformed to achieve normal distribution and meet the assumptions of a parametric test. Simple t-tests were used to compare the means of concentrations of the qPCR and the cultivation method results ($p < 0.05$). The coefficient of variation (CV %) was calculated to evaluate the intervariability in extraction procedures using the formula;

CV%= (standard deviation/ mean) x 100.

Linear regression analysis was performed using scatter plots of $\log_{10}$ cells/100 mL of *E. coli* and *Enterococcus* spp. for comparison between manual and instrument DNA extraction from the samples tested.

3.2.3 Application of Ethidium Monoazide-qPCR for Differentiating between Live and Dead cells in Environmental Samples

E. coli ATCC strain 15597, *Enterococcus faecalis* ATCC strain 19433 were used to evaluate EMA-qPCR using pure cultures. Freezer stocks of these bacteria were revived overnight in Trypticase Soy Broth (TSB) by incubating the tubes at 37^{o}C in a shaker-incubator (NewBrunswickScientific, New Jersey, USA) at 150rpm. From this, cultures were further inoculated into 25 mL of TSB and incubated again in the same conditions for 6 hours. Cultures were serially diluted in Phosphate Buffered Saline (PBS) and Colony Forming Units (CFU/mL) of *E. coli* and *Enterococcus* spp. were enumerated by plating 0.1ml of dilutions of cultures on Trypticase Soy Agar (TSA) plates and incubating at 37 ^{o}C for 18-24 h.

Microcentrifuge tubes containing one mL aliquots of the culture were immersed in water bath set at 80 ^{o}C for 15 minutes for heat-killing. These conditions for heat-killing were used after testing different combinations of temperature and time. Loss of viability was tested by plating 100 µL from the heat-killed aliquote on TSA plates and incubating at 37 ^{o}C for 18-24 h.

EMA (Biotium, Hayward, CA, USA) was dissolved in sterile distilled water to a stock concentration of 1 mg/mL and aliquoted into microcentrifuge tubes covered with aluminum foil for storage in the dark at −80°C. Further processing using EMA was done under minimal light conditions as EMA is sensitive to degradation by light. Fresh and heat-killed aliquots of *E. coli* and *Enterococcus* spp. were centrifuged at 13,000 x g for 5 min, the supernatants were discarded and the pellets were resuspended in 1mL of EMA at

a concentration of 2.5 µg/mL. The tubes were then incubated in the dark for 5 min after which they were placed at a distance of 20cm away from light source using 500 W, 120 V halogen T2.5 lamp. This light exposure for 2 minutes was done to photoactivate EMA to form covalent bonds between DNA strands. Cultures exposed to EMA were centrifuged at 13,000 x g for 3 minutes and washed with one mL sterile PBW to remove any unbound EMA and the tubes were kept at 4 ^{o} C in the dark until further processing. Another set of the aliquots of fresh and heat-killed cultures were stored at 4 ^{o} C until further processing to be used as controls without addition of EMA.

Raw sewage (100 mL) and final chlorine disinfected effluent (2 L) samples were collected in triplicates from the wastewater treatment plant at East Lansing, MI. Viable counts of *E. coli* and *Enterococcus* spp. were enumerated by following U. S. EPA Method 1603 and 1600. Serial dilutions of the raw sewage (10^{-1} through 10^{-5}) were made and one mL from these dilutions was filtered through 47 mm diameter, 0.45 µm pore size, membrane filters. For treated effluents, one hundred milliliters of samples were filtered. The filters were placed on mTEC agar and mEI agar plates. The mTEC agar plates were incubated 35 ± 0.5°C for 2 h, followed by incubation in a water bath at 44.5 ± 0.2°C for 22 h and mEI plates were incubated for 24 h at 41°C and colonies were counted.

Fifty milliliters of raw sewage and effluent samples (1L) were processed as previously described in section 3.2.2.1. Both raw sewage and effluent pellet suspensions were vortexed and one mL from each sample was transferred into 5mL glass centrifuge tubes for EMA exposure for 5 minutes and another one mL was used as a control without

EMA addition. Sterile distilled water with EMA treatment and without EMA were used as negative controls.

DNA was extracted from all the EMA exposed and unexposed pure culture and sewage samples using the QIAmp DNA mini kit (Valencia, CA, USA) following manufacturer's instructions. The DNA was stored at -20 ^{O}C until analysis. Quantitative PCR was performed on the DNA extract to quantify *E. coli* uidA gene and *Enterococcus* spp. 23S rRNA gene by following the same protocol as described in section 3.2.1.3 using the primers and probes given in Table 3.1.

3.2.4 Preliminary Evaluation of 16S rRNA Bacteroides Human HF183 Markers by conventional PCR Assay

3.2.4.1 Application of HF 183 PCR Assay for Monitoring Water Quality

A total of 48 surface water grab samples were collected at four different locations in Michigan in 2007 and 2008 ; five sampling sites at Silver Lake (SL), five sites at Saginaw Bay (SB), four sites at Coldwater Creek on six sampling dates (CCA, CCB, CCC, CCD), and two sites at Buck Creek on seven sampling dates (BC11 and BC12). These sampling sites were selected based on concerns from the local governments and citizens about the quality of the water. Water samples were collected in sterile containers and were stored at 4^{O}C until further processing which was always less than 24 hours.

Different volumes of the water samples were filtered and analyzed for *E.coli* and *Enterococcus* spp. using U.S.EPA method 1603 and 1600 respectively. Water (1L-4L) was filtered through the 0.45-µmpore size, 90mm diameter membrane filter and filtered volume was included in the final calculation. New filters were used whenever the filters were clogged due to the suspended solids. These filters were then soaked in 45mL

Phosphate Buffered Saline (PBS) in 50mL disposable centrifuge tubes, vortexed for 2-3 minutes. The tubes were centrifuged for 2-3 minutes at 8,000xg for 20 minutes. The supernatant was gently removed to a volume of five mL of pellet. From this, one mL of the sample was transferred into a 2mL centrifuge tube and after centrifugation at 20,000 x g for 2 minutes, the supernatant was discarded. The process was repeated until a total volume of 3 mL was pelleted down for DNA extraction using the QIAamp DNA kit (QIAGEN) extraction kit following the manufacturer's instructions. The remaining two mL of the pellet was stored at -80 ^{O}C for any future analysis. The eluted volume containing the DNA was in 200 µL volumes and stored at -20^{O}C..

The human *Bacteroides* 16S rRNA HF 183 marker was amplified with the forward primer 5′ ATCATGAGTTCACATGTCCG3′ and Bac708 reverse primer 5′ CAATCGGAGTTCTTCGTG 3′ (Bernhard and Field 2000b). The PCR reaction was carried out in a 25 µL reaction mixture containing 14 µL HotStar Taq Master Mix, 1 µL of each primer (10µM concentration), 6 µL of molecular grade water and 3 µL of template DNA. Amplification was carried out with the following cycling conditions: 15 min at 95^{O}C for initial denaturation and 35 cycles of amplification steps consisting of denaturation at 94^{O}C for 30 s, annealing at 58^{O}C for 30 s and extension at 72^{O}C for 60 s. Following the amplification cycles, the final extension was done for 8 minutes at 72 ^{O}C. DNA from sewage samples that was confirmed to be positive for human specific *Bacteroides* markers by sequencing the amplified products and BLAST analysis were used as the positive control and molecular grade water was used as the negative control. Amplified products were detected by visualizing the PCR product by electrophoresis

through 1.2% agarose gel and then exposing it to UV transilluminator. The samples were recorded as positive when the bands corresponded to the positive control at around 585bp.

3.2.4.2 Detection limit of HF183 PCR Assay

Raw sewage sample was collected in 50mL sterile centrifuge tubes from wastewater treatment plant in East Lansing and was transported to laboratory on ice. The sample was mixed well by vortexing for two minutes and serial dilutions from 10^{-1} through 10^{-4} were made using sterile PBS. One milliliter from each dilution was suspended in one liter sterile distilled water (triplicates were made for each dilution). The mixture was stirred using a magnetic stirrer for 15- 20 minutes. One milliliter from each of the suspensions was directly used for DNA extraction. Also, different volumes (1mL and 1000mL) were filtered using 0.45 µm pore size nitrocellulose membrane filters (Millipore, Billerica, Mass.) and the filters were folded and immersed to the 45 mL mark with sterile phosphate buffered saline (PBS) in 50 mL centrifuge tubes. The tubes were then vortexed at high speed to detach the cells from membrane. The volumes were centrifuged at a speed of 8000xg for 20 minutes and the supernatant was gently removed to a one mL volume. From this pellet, 400 µL was used for DNA extraction using the QIAmp DNA mini kit (Valencia, CA, USA) following the manufacturer's instructions. The total final volume extracted was 200 µL per sample.

The extracted DNA samples were subjected to PCR for detecting the human *Bacteroides* marker HF 183 amplified as described previously. A sample consisted of three replicates and each was reported as positive or negative.

3.3 Results

3.3.1 Standardization and Specificity of *E. coli uid*A qPCR Assay

The standard curve for the *E. coli* qPCR assay is shown in Figure 3.1. A linear relationship was observed between the log concentration (copies per reaction) and the Cycle threshold (Ct). The efficiency of amplification calculated for the *E. coli* assays based on the slopes of -3.32 was 104%. In qPCR standardization, with ten-fold serial dilution of plasmids, the Ct should be separated by approximately 3.3 cycles. With this slope value, amplification efficiency of a robust, reproducible qPCR assay will be 100%. Any efficient qPCR reaction should have amplification efficiency of 90-105%; efficiency below 90% or above 105% indicates poor primer design, suboptimal cycling conditions, pipetting errors, etc (Rasmussen 2001). *E. coli* qPCR assay specificity was also tested by performing qPCR on different organisms. The results are summarized in Table 3. 2. The *E. coli* qPCR assay was found to detect all six strains of *E. coli* tested with strong amplification signals and did not detect eight of the other bacteria including enterococci, *Enterobacter, Klebsiella, Proteus, Pseudomonas* and *Salmonella*, but did test positive with *Shigella flexneri*.

Figure 3.1: Standard curve for 10-fold serial dilutions of *E. coli uidA* gene.

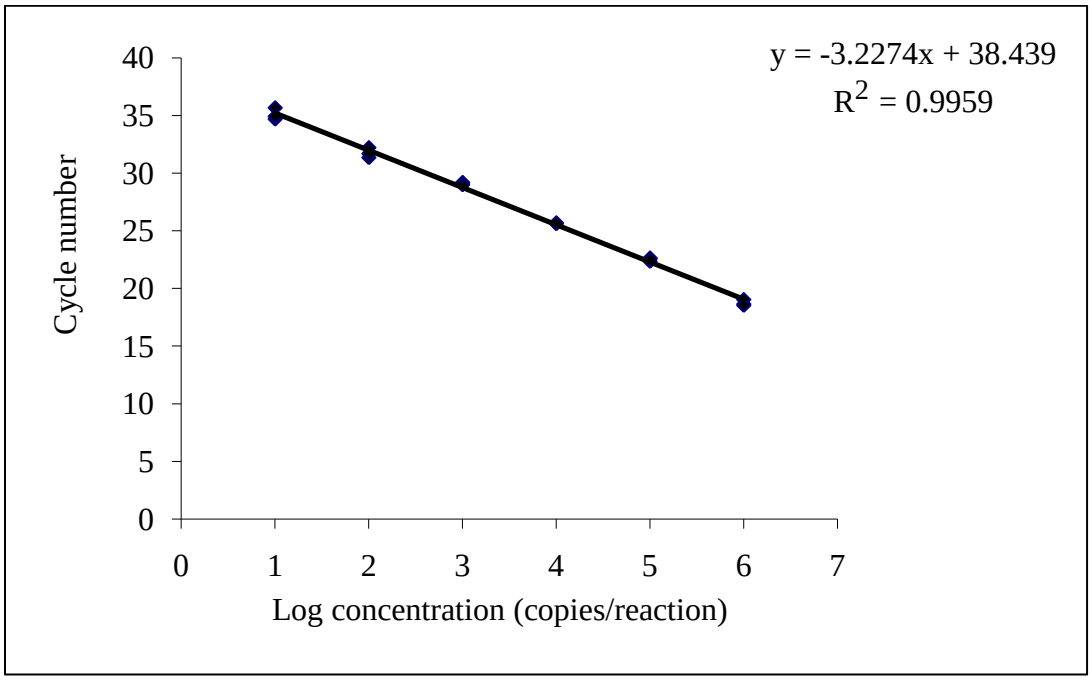


Table 3.2: Evaluation of the *E. coli uid*A gene qPCR assay against a variety of bacteria

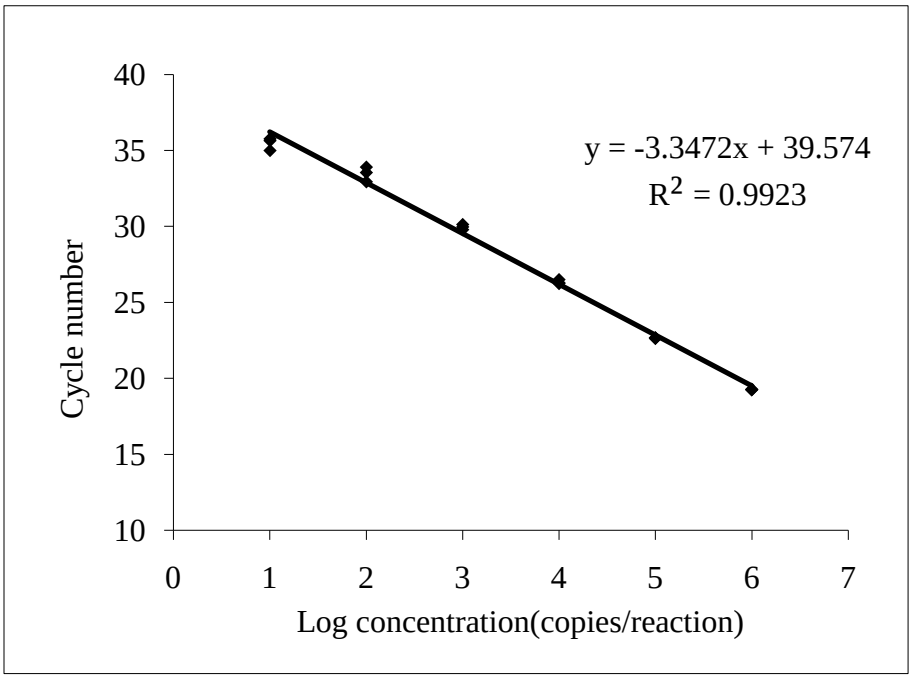
Organisms*	Source	+/- for qPCR
E. coli C3000	ATCC	+
E. coli O26:H11	Human	+
E. coli O55.H7	Meat	+
E. coli O111:H8	Human	+
E. coli O118:H16	Human	+
E. coli O76:H19	Human	+
Klebisiella pneumonia	ATCC	-
Citrobacter freundii	ATCC	-
Enterobacter aerogenes	ATCC	-
Salmonella paratyphi	ATCC	-
Proteus vulgaris	ATCC	-
Pseudomonas aeruginosa	ATCC	-
Shigella flexneri	ATCC	+
Enterococcus faecium	ATCC	-
Enterococcus faecalis	ATCC	-

*All non-ATCC strains were obtained from Microbial Evolution Laboratory, National Food Safety and Toxicology Center, at MSU

3.3.2 Standardization of *Enterococcus spp.* 23S rRNA Gene qPCR Assay

The amplification curve generated for *Enterococcus* spp. 23SrDNA gene is shown in Figure 3.2. A linear relationship was observed between the log concentration and the Cycle threshold. The amplification efficiency calculated for the 23SrDNA *Enterococcus* spp. assay based on the slopes of the -3.34 was 99%.

Figure 3.2: Standard curve for 10-fold serial dilutions of *Enterococcus* spp. 23SrDNA gene.



Linear regression analysis shows an R^2 of 0.992, a slope of -3.34 and an intercept of 39.574.

3.3.3 Comparison of Concentrations of *E. coli* and *Enterococcus* spp. DNA by Manual and Automated Extraction

The concentrations of *E. coli* and *Enterococcus spp.* were determined by qPCR and compared for both instrument and manual DNA extracts of treated and untreated sewage, and cow feces. Additional lysis step was used for all samples with the automated system. All results were $\log_{10}$ transformed before statistical analyses. In order to determine if there was a statistically significant difference between the methods, a simple t-test for comparing means was used. Statistical analyses showed that qPCR equivalent cells of *E. coli*/100 mL of raw sewage, primary effluent and secondary effluent were statistically higher for the autoextractor as compared to manual extraction ($p<0.05$) (Table 3.3). In tertiary effluent samples and cow feces both methods returned equivalent cells/100ml. For *Enterococcus* spp., there was no statistically significant difference found between extraction methods in any of the samples except for cow feces, where the manual extraction was found to show significantly higher (0.44 $\log_{10}$) concentrations of *Enterococcus* spp. ($p<0.05$) (Table 3.4). The mean difference of qPCR equivalent cell concentrations of *E. coli* and *Enterococcus* spp. between instrument and manual extracted DNA ranged from 0.31 to 0.61 and 0.09 to 0.26 $\log_{10}$ units respectively in various samples.

Table 3.3: t-test results for comparison of means between the qPCR equivalent concentrations of *E. coli* from DNA extracted by auto and manual methods.

	Average cells/100 mL				
Samples[a]	Automated method	Manual method	Mean difference	Standard error for mean difference	t-statistic (p)
Raw sewage	4.62E+06	2.18E+06	0.31	0.07	4.09 (0.001)
Primary effluent	6.79E+06	2.24E+06	0.55	0.16	3.51 (0.004)
Secondary effluent	3.20E+03	1.22E+03	0.61	0.23	2.60 (0.02)
Tertiary effluent	2.58E+03	1.50E+03	0.42	0.38	1.11 (0.28)
Cow feces	7.97E+05	3.75E+05	0.34	0.38	0.62(0.54)

[a] n = 9 for each type of sample.

Table 3.4: t-test results for comparison of means between the concentrations of *Enterococcus* spp. from DNA extracted by auto and manual methods

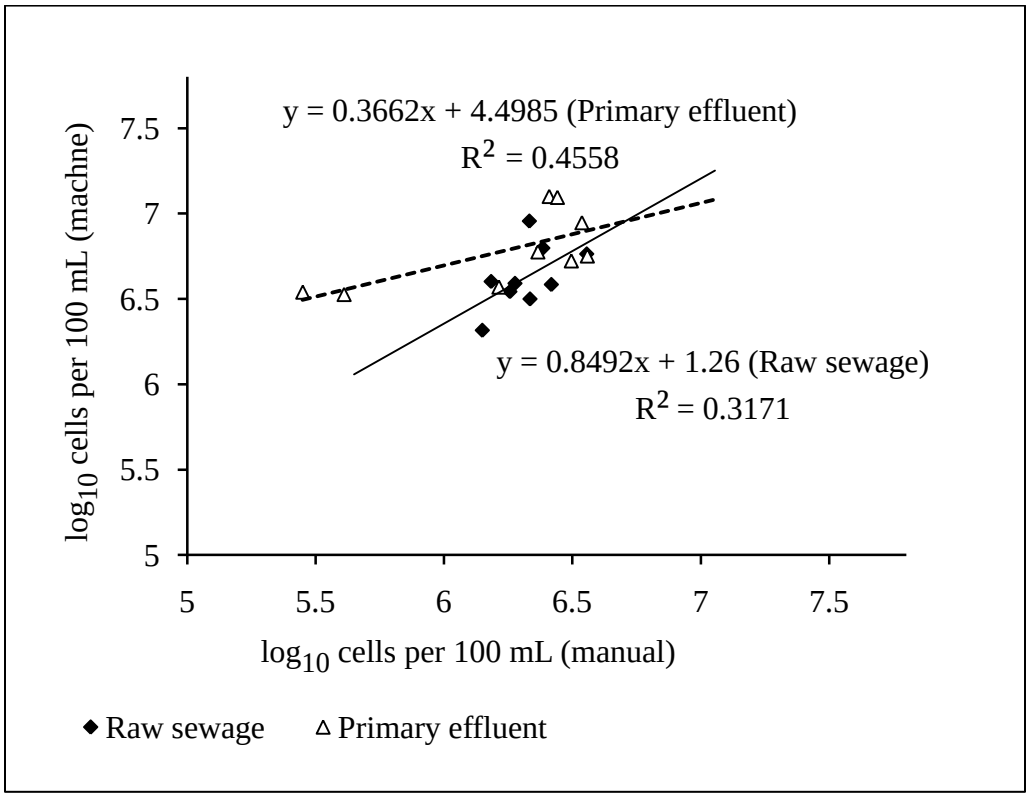
	Average cells/100 mL				
Samples[a]	Automated method	Manual method	Mean difference	Standard error for mean difference	t-statistic (p)
Raw sewage	2.23E+07	2.10E+07	0.26	0.29	0.57 (0.57)
Primary effluent	1.99E+07	1.58E+07	0.24	0.23	1.02 (0.32)
Secondary effluent	1.34E+05	7.16E+04	0.23	0.29	0.80 (0.43)
Tertiary effluent	1.49E+05	7.96E+04	0.09	0.30	0.25 (0.80)
Cow feces	1.79E+04	4.69E+04	-0.44*	0.14	-3.95 (0.001)

[a]n = 9 for each kind of sample.

* - sign indicates that the concentration of *Enterococcus spp.* was more in manual extracted DNA than the machine.

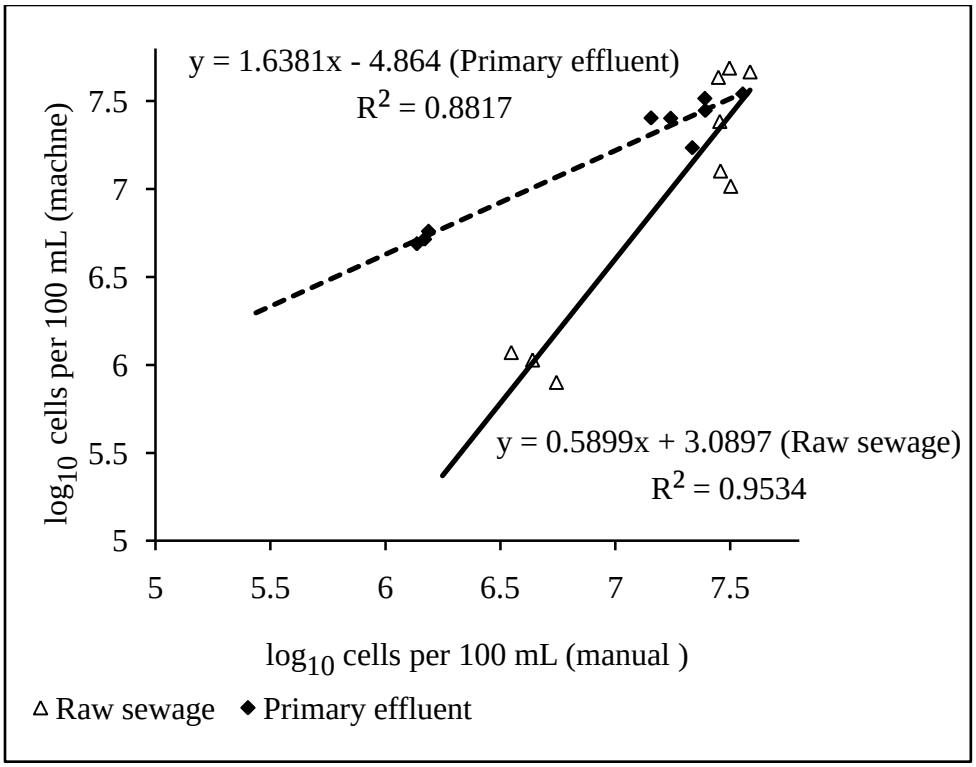
Correlation and regression analysis were also performed on the qPCR equivalent concentrations of *E. coli* and *Enterococcus* spp. from DNA by manual and automated extraction for *E. coli* in raw sewage and primary effluent (Fig 3.3), in secondary and tertiary effluent (Fig 3.5), and in cow feces (Fig 3.7), and for enterococci in raw sewage and primary effluent (Fig 3.4), in secondary and tertiary effluent (Fig 3.6), and in cow feces (Fig 3.8). A positive correlation was found between qPCR equivalent cells of *E. coli* and *Enterococcus* spp. in DNA from instrument and manual extraction from all samples except for *Enterococcus* spp. from cow feces. Strong correlations were found between both the extraction methods for *E. coli* cell equivalent concentrations in tertiary effluents ($r=0.98$, $p<0.05$), and cow feces ($r= 0.97$, $p<0.05$), and *Enterococcus* spp. cell equivalent concentrations in raw sewage ($r=0.93$, $p<0.05$) and primary effluent ($r=0.97$, $p<0.05$).

Figure 3.3: Regression analysis for concentrations of *E. coli* from both types of DNA extraction in raw sewage and primary effluent*



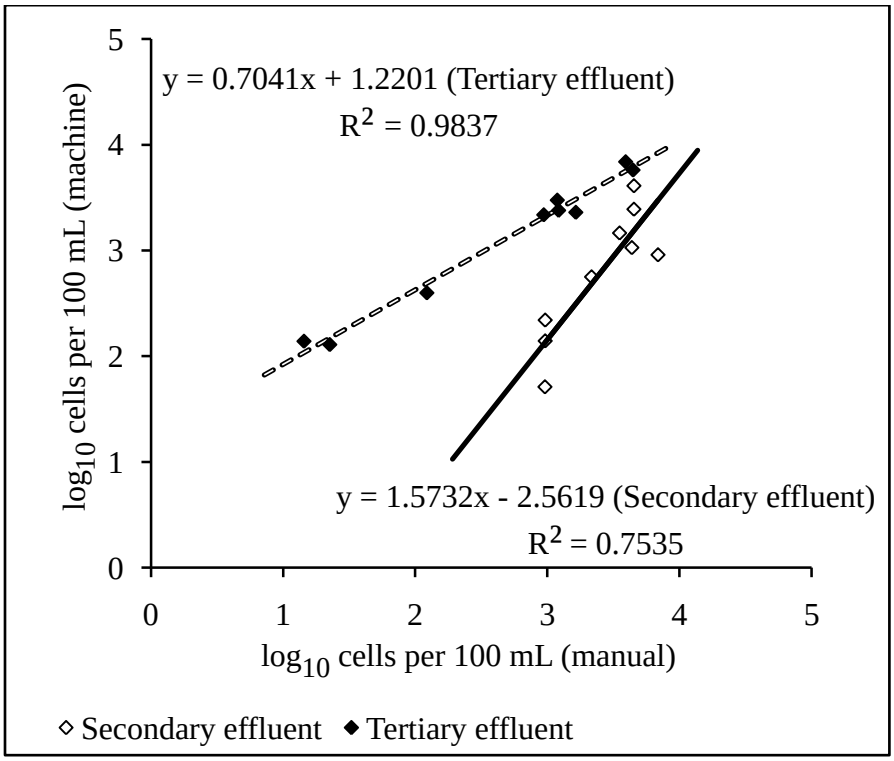
*****number of raw sewage samples, n=9, number of primary effluent samples, n=9

Figure 3.4: Regression analysis for concentrations of *Enterococcus* spp. from both types of DNA extraction in raw sewage and primary effluent*****



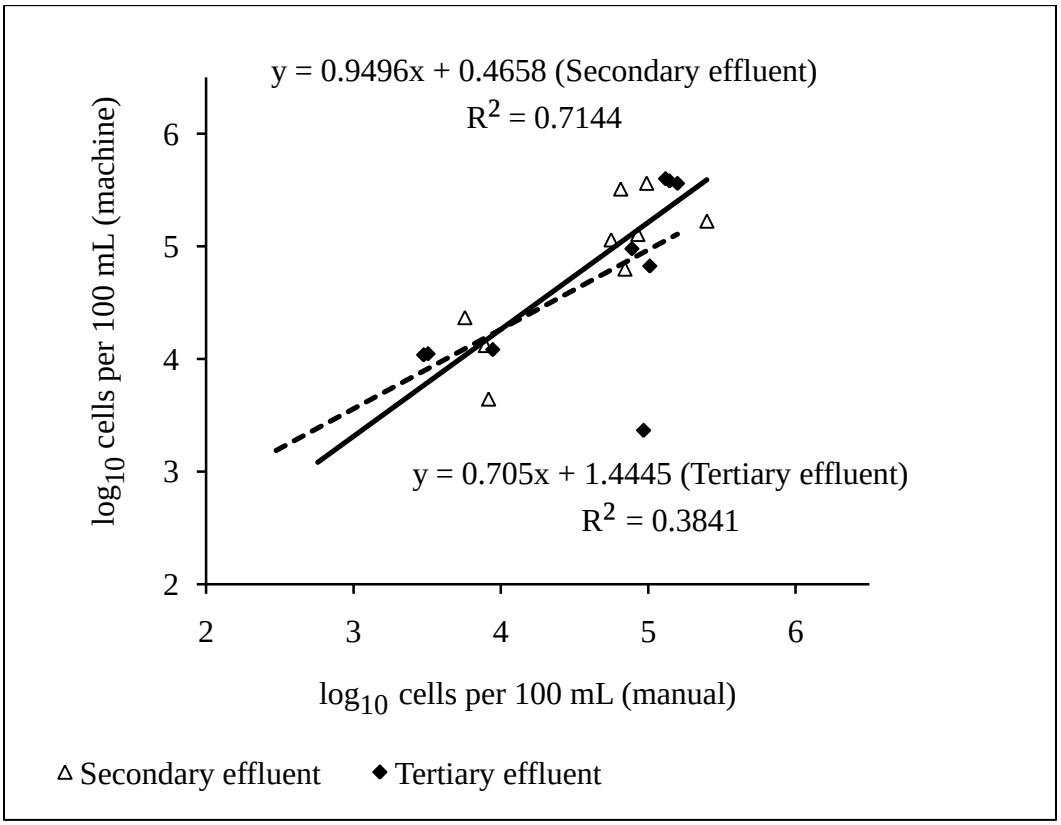
*****number of raw sewage samples, n=9, number of primary effluent samples, n=9

Figure 3.5: Regression analysis for concentrations of *E. coli* from both types of DNA extraction in secondary and tertiary effluents*****



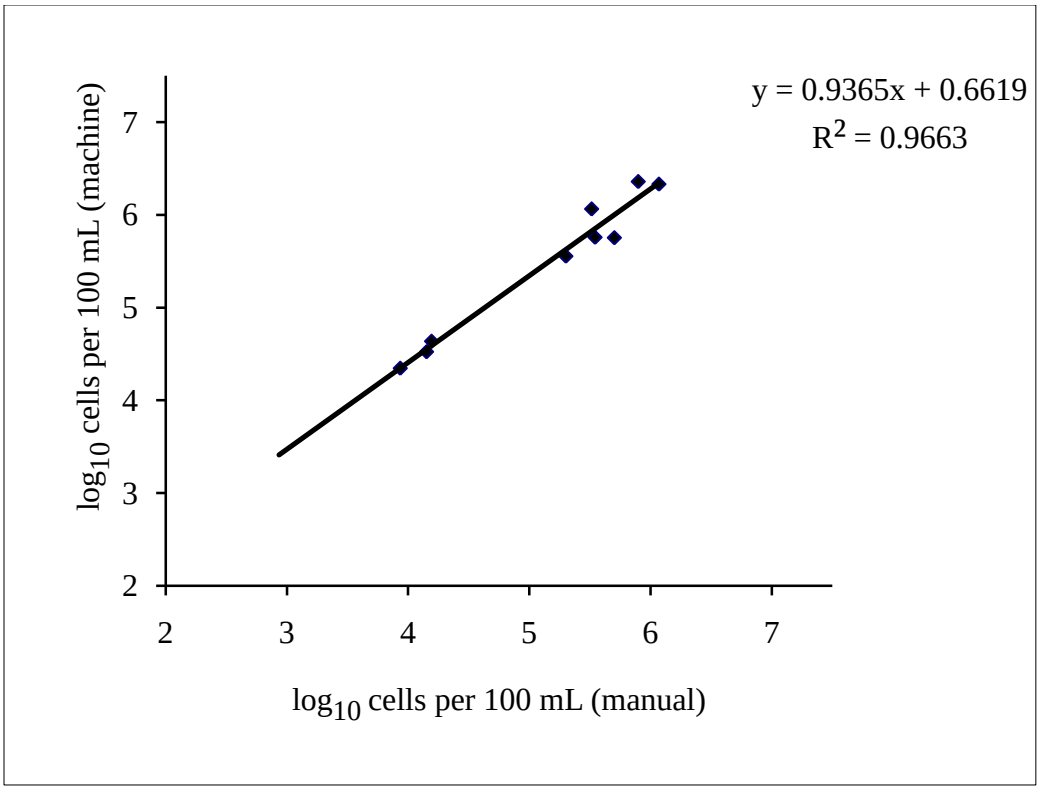
*number of secondary effluents, n=9; number of tertiary effluents, n=9

Figure 3.6: Regression analysis for concentrations of enterococci from both types of DNA extraction in secondary and tertiary effluents*****



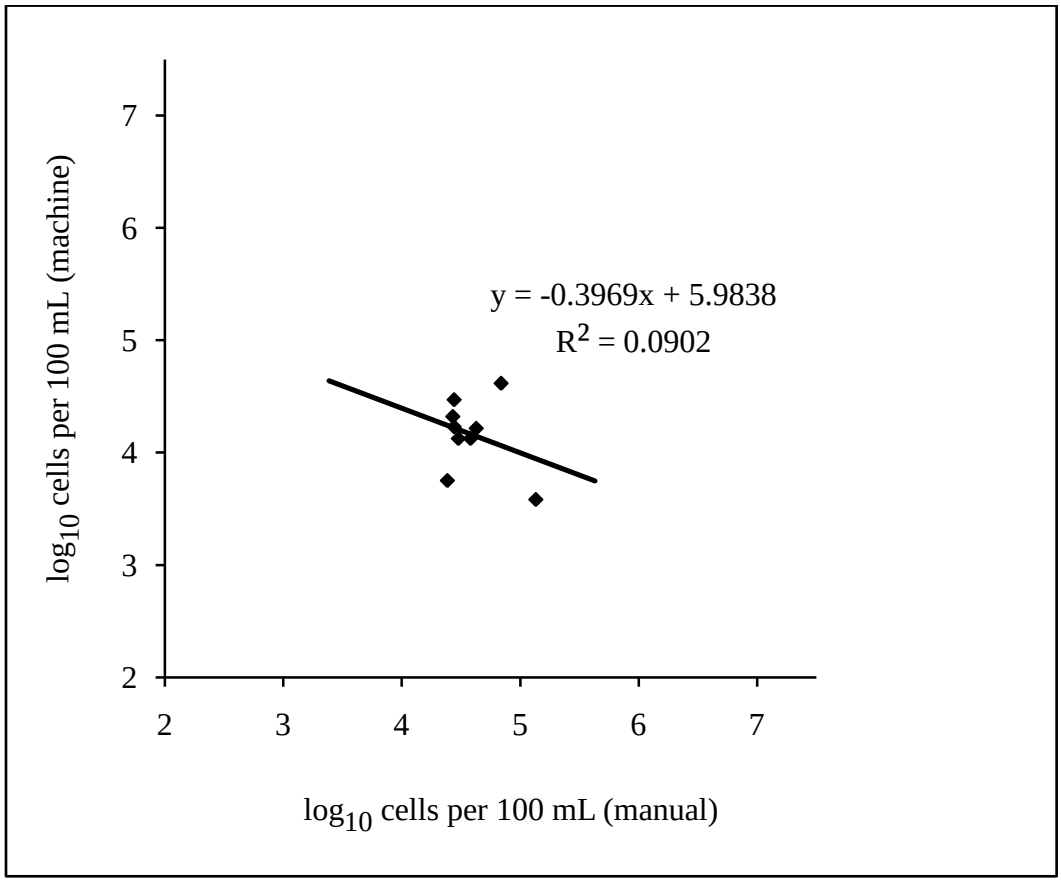
*number of secondary effluents, n=9; number of tertiary effluents, n=9

Figure 3.7: Regression analysis for concentrations of *E. coli* from both types of DNA extraction in cow feces*****



*Number of cow fecal samples, n=9

Figure 3.8: Regression analysis for concentrations of *Enterococcus* spp. from both types of DNA extraction in cow feces



*Number of cow fecal samples, n=9

3.3.3.1 Performance of Extraction Procedure

Variation between the auto extraction and the manual method was evaluated with replicates of raw sewage for qPCR equivalent cell concentrations of *E. coli* and *Enterococcus* spp. and for DNA concentration as measured by Nanodrop Spectrophotometer. It was found that nanograms of DNA per microliter extracted by the instrument had less variation with coefficient of variation (CV) of 4.50% as compared to manual DNA extract concentration with a CV of 13.29%. The qPCR equivalent cell concentration of *E. coli* per reaction for raw sewage showed a CV (%) of 1.31 for instrument and 1.67 for manual DNA extraction whereas cell concentration of *Enterococcus* spp. per reaction for raw sewage showed a CV(%) of 1.48 for instrument and 1.71 for manual DNA extraction.

Intervariability in extraction by both auto and manual methods performed by two different individuals was also tested. DNA concentrations (nanograms of DNA per microliter) of samples extracted by instrument showed a CV of 5.07% and that of manual DNA extraction showed a CV (%) of 20.65. The qPCR equivalent cell concentration of *E. coli* per reaction for raw sewage showed a CV (%) of 0.86 for instrument and 1.88 for manual DNA extraction whereas cell concentration of *Enterococcus* spp. per reaction showed a CV (%) of 1.36 for instrument and 1.83 for manual DNA extraction by the second individual.

Table 3.5: Coefficient of variation (CV %) for DNA concentrations and cell equivalent concentrations of *E. coli* and *Enterococcus* spp. performed by two individuals for auto and manual extraction methods

Sample (raw sewage)	Individual 1		Individual 2	
	Instrument CV%	Manual CV%	Instrument CV%	Manual CV%
DNA concentration (ng/µL)	4.50	13.29	5.07	20.65
Enterococcus spp. concentration (cells/rxn)	1.48	1.71	1.36	1.83
E. coli concentration (cells/rxn)	1.31	1.67	0.86	1.88

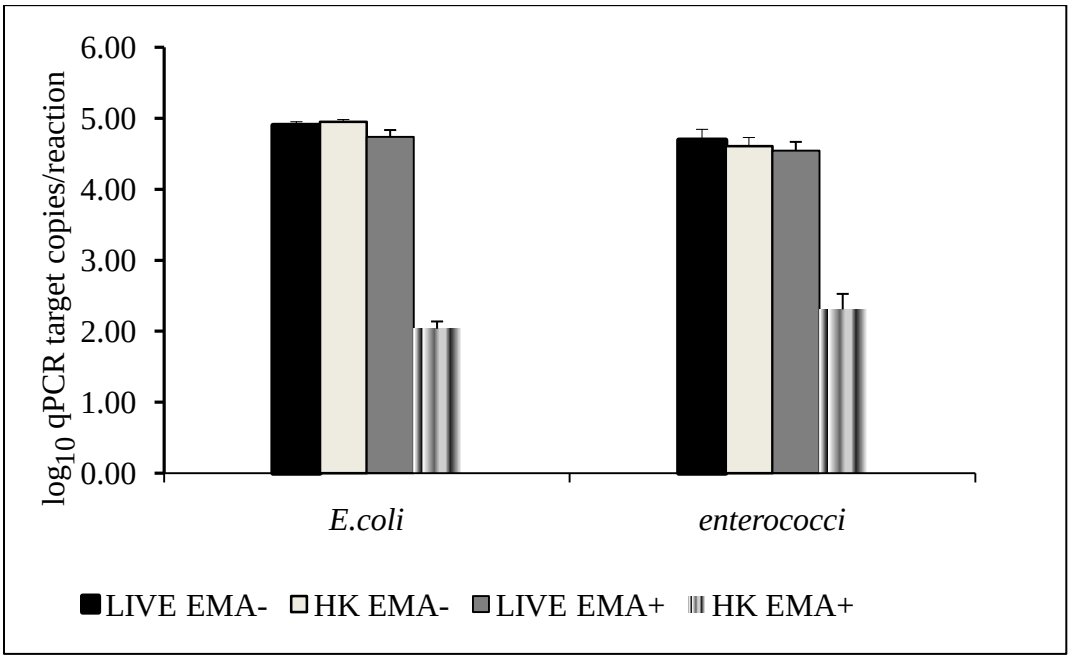
* Raw sewage (n=9) from the same sample concentrate was used for the experiment.

3.3.4 Differentiation between Live and Dead cells using Ethidium Monoazide-qPCR in Environmental Samples

Heat-killed and live cells of *E. coli* and *Enterococcus* spp. were treated with 2.5µg/mL of EMA, followed by exposure to light for 2 minutes for photolysis. As shown in Fig 3.9, this resulted in a 2-3 log reduction in qPCR target copies as compared to the controls that were not treated with EMA. There was no reduction in the concentrations of both the targets in cells that were not heat-killed but were exposed to EMA. Concentrations of viable *E. coli* and *Enterococcus* spp. (CFU/mL) enumerated in TSA plates were around 10^7 CFU/mL and were comparable to the qPCR equivalent cells/mL in live and heat-killed cells that were not pre-treated with EMA.

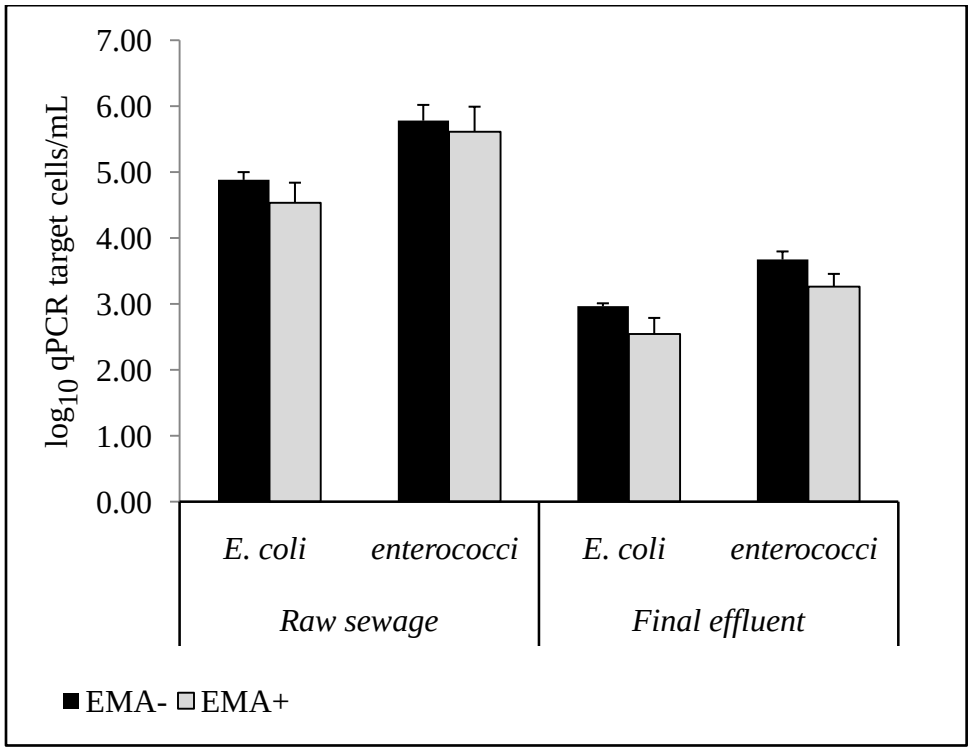
Raw sewage and final effluent samples were treated with EMA and as shown in Fig 3.10, there was not much difference between qPCR target copies of *E. coli* and *Enterococcus spp.* in EMA treated samples and samples without EMA added. Viable counts of *E. coli* and *Enterococcus spp.* as enumerated by cultivation on mTEC and mEI media were 1.09 x 10^5 CFU/mL and 2.47x10^5 CFU/mL respectively in raw sewage and qPCR equivalent concentrations for these targets were 2.26 x 10^5 cells/mL and 3.27 x 10^5 cells/mL respectively. Cultivable counts of *E. coli* and *Enterococcus spp.* in final effluent were only 0.13 CFU/mL and 0.66 CFU/mL respectively whereas the qPCR equivalent cells of these targets were 1.80 x 10^2 cells/mL and 2.43 x10^2 cells/mL respectively. Negative controls with and without EMA treatment did not show any amplification signals.

Fig 3.9: Average $\log_{10}$ qPCR target copies/ reaction for pure cultures of *E. coli* and *Enterococcus spp.* in EMA treated and untreated live and heat-killed (HK) cells*****



*Number of replicates for each treatment=3

Figure 3.10: Average $\log_{10}$ concentrations of *E. coli* and *Enterococcus* spp. in EMA treated and untreated raw sewage and treated effluent samples.



*Number of replicates for raw sewage=3

*Number of replicates for treated effluent=3

3.3.5 Occurrence of 16S rRNA *Bacteroides* (HF183) Human Marker in Surface Water Samples

Forty eight surface water samples from different locations in Michigan were collected evaluated for *E. coli* and *Enterococcus* spp. concentration by cultivation methods and for the presence of 16S rRNA *Bacteroides* (HF183) Human Marker by conventional PCR (Table 3.6). *E. coli* concentrations ranged from $5.7x10^{0}$ to $1.51x10^{4}$ CFU/100mL and *Enterococcus* spp. ranged from $9.70x10^{1}$ to $2.20x10^{3}$ CFU/100mL. Seven samples showed the presence of human specific *Bacteroides* marker; three of them in samples collected from Silver Lake, two from Saginaw Bay and one each from Cold Water Creek and Buck Creek.

Table 3.6: Occurrence of 16S rRNA *Bacteroides* (HF183) human marker as detected by conventional PCR and their comparison with *E. coli* and *Enterococcus* spp. (CFU/100mL) in surface water samples.

Surface water Sample ID*	*Bacteroides* 16SrRNA human marker +/-	*E. coli* cfu/100mL	Enterococci cfu/100mL
SL1	+	4.03E+01	7.47E+01
SL2	-	9.20E+01	9.10E+01
SL3	+	1.35E+01	2.10E+01
SL4	-	8.10E+00	4.40E+01
SL5	+	5.70E+00	9.70E+01
SB1	+	5.33E+02	3.60E+02
SB2	-	2.42E+03	7.33E+02
SB3	-	2.08E+03	6.67E+02
SB4	+	1.56E+02	5.00E+01
SB5	-	1.47E+02	8.50E+01
CCA1	-	7.40E+02	7.90E+03
CCA2	-	4.00E+02	7.90E+02
CCA3	-	4.70E+02	5.50E+02
CCA4	-	7.07E+02	9.93E+02
CCA5	-	5.90E+02	8.30E+02
CCA6	-	1.93E+02	5.73E+02
CCB1	-	1.80E+03	2.32E+03
CCB2	-	2.00E+02	4.70E+02
CCB3	-	2.45E+02	2.60E+02
CCB4	-	8.57E+02	1.74E+03
CCB5	-	7.80E+01	2.67E+02
CCB6	-	1.32E+02	1.63E+02
CCC1	-	4.40E+02	9.30E+02
CCC2	-	1.51E+04	2.20E+03
CCC3	-	2.80E+02	5.50E+02
CCC4	-	6.27E+02	1.36E+03
CCC5	-	1.12E+02	3.97E+02
CCC6	-	8.30E+01	1.80E+02
CCD1	-	1.45E+02	6.80E+02
CCD2	-	6.70E+01	9.80E+02
CCD3	-	1.30E+02	2.00E+03
CCD4	-	1.50E+01	4.10E+01
CCD5	-	8.00E+00	6.40E+01

Table 3.6 (cont'd)

CCD6	+	5.10E+01	4.70E+01
BC11-1	-	3.50E+02	3.30E+02
BC11-2	-	2.80E+02	6.90E+03
BC11-3	-	3.90E+02	4.60E+02
BC11-4	-	2.50E+02	4.30E+02
BC11-5	-	2.40E+02	5.40E+01
BC11-6	-	3.00E+02	8.50E+02
BC11-7	-	1.20E+02	1.10E+02
BC12-1	+	1.40E+03	4.10E+02
BC12-2	-	7.60E+02	1.50E+04
BC12-3	-	2.10E+02	6.80E+02
BC12-4	-	4.60E+02	1.00E+03
BC12-5	-	4.20E+02	7.70E+02
BC12-6	-	8.50E+02	3.90E+03
BC12-7	-	1.40E+02	2.20E+02

Highlighted rows are the samples that showed positive for 16S rRNA Bacteroides (HF183) human marker
*SL-Silver lake
*SB=Saginaw Bay
*CCA=Coldwater Creek at location A
*CCB= Coldwater Creek at location B
*CCC= Coldwater Creek at location C
*CCD= Coldwater Creek at location D
*BC-Buck Creek

3.3.6 Detection Limit of 16S rRNA *Bacteroides* (HF183) PCR Assay Using Raw Sewage

The limit of detection was tested for the 16S rRNA *Bacteroides* HF183 assay using tenfold dilutions of raw sewage in sterile distilled water which also gave the efficiency of the method for detecting low levels of fecal contamination of water. Results are summarized in Table 3.7. When 1mL of sewage was suspended in 1L of water, the signal was present in all the volumes that were analyzed, whether 1 mL or 1000 mL was membrane filtered, or whether there was direct DNA extracted from 1 mL. Same results were observed with 100 µL of sewage seeded in 1L of water. With 10 µL of sewage seeded into 1 L water (10^{-5} dilution), positive signals were observed with 1mL DNA extracted directly as well as 1000mL filtered but not with 1mL membrane filtered which suggests the possible loss of targets while processing the samples by membrane filtration. The signal was present in 1 µL sewage seeded into water but only with 1000 mL filtered and there were no markers detected in the 1 mL samples that were processed directly. There was no signal obtained with any of the volumes processed for 0.1 µL into 1 L of water (10^{-7} dilution).

Table 3.7: Detection limit of 16S rRNA *Bacteroides* HF183 assay using water seeded with raw sewage

Volume of sewage seeded into 1L sterile distilled water (dilutions)	Number of positives/ number of replicates		
	DNA extracted from		
	1mL directly	1mL MF	1000 mL MF
1000 µL (10^{-3})	3/3	3/3	3/3
100 µL (10^{-4})	3/3	3/3	3/3
10 µL (10^{-5})	3/3	1/3	3/3
1 µL (10^{-6})	2/8	0/8	6/8
0.1 µL (10^{-7})	0/8	0/8	2/8

3.4 Discussion

In this study, a qPCR assay that targeted the *uidA* gene of *E. coli* was developed and evaluated. This gene codes for the enzyme, β-glucuronidase (GUS) that catalyzes the hydrolysis of glucuronide compounds and phenotypic detection methods have utilized this enzyme activity (Bej *et al.* 1991a, b; Buckalew *et al*. 2006; Chao *et al*. 2004; Gracias and McKillip 2004; Servais *et al.* 2005; Tryland and Fiksdal 1998).

The assay developed in this dissertation had a detection limit of 10 copies/reaction as determined from the standard curve. The sensitivity of the *uidA E. coli* qPCR assay developed in this study was better than the *uidA* qPCR developed by Frahm et al (2003) and ITS qPCR assay described above but did not match up with sensitivity of *lacZ* assay. The *uidA* qPCR developed by Frahm et al 2003 had a detection limit 10-100 copies per reaction. The improved detection limit of the assay developed in this study could be due to better primer/probe design or reaction conditions.

The *uidA* qPCR assay used in this study has been tested for its specificity with *E. coli* and non-*E. coli* strains and was specific except with *Shigella sp* with which some amplification signals were detected. The *uidA* assay by Frahm et al (2003) was reported to be specific, however, the cross reactivity of the assay was tested only with *Aeromonas sp, Enterobacter sp, Pantoea agglomerans, Klebsiella sp, and Serratia sp*.; and *Shigella* spp. was not included in their study. Bej *et al.* (1991a) also reported the amplification of *Shigella flexneri uid*A gene with the primers developed for *E. coli* and negative for other members of coliform group. The ITS qPCR assay showed no cross reactivity with isolates of 22 different bacteria (Khan et al. 2007). Specificity was not evaluated in any other assays described here. *Shigella* is closely related to *E. coli* and shows 99%

homology for *uidA* gene sequence. Despite this cross-reation, the detection of *Shigella* in the assay developed in this dissertation for *E. coli* will not cause false positives in polluted waters due the high ratio of *E. coli* to *Shigella.*

One of the key issues for qPCR assays for water samples is reproducibility, interferences and cross-contamination. An automated method for extracting DNA from environmental samples was evaluated in this study. Other studies have compared the extraction of DNA for real-time PCR detection of *Clostridium difficile* from human fecal samples using QiAamp DNA blood mini kit (after a pretreatment with polyvinylpolypyrolidone (PVPP)) compared to automated extraction with the Roche MagnaPure LC (pre-treatment using the Stool Transport and Recovery (STAR) buffer). That study reported no difference in Ct values between the two extraction methods (van den Berg *et al.* 2006) which indicated that there was no difference between the concentrations of *Clostridium difficile* DNA extracted by manual and machine. The same instrument has also been applied for extraction of DNA from drinking water while studying the occurrence and genetic diversity of *Legionella* spp. (Wullings & van der Kooij 2006). Since method evaluation was not their goal, the autoextraction method was not evaluated or compared with any other existing methods.

The results in this study indicate that autoextractor showed significantly higher or equal efficiency compared to manual kits in extracting DNA from treated and untreated sewage, but with cow feces for enterococci, the autoextraction was not efficient. This could be due high fiber content of the cow feces and other factors. The fibrous nature of cow feces could facilitate attachment of cells, thus resulting in low recovery of DNA

during extraction specifically for Gram-positive cells perhaps due to the thick peptidoglycan biochemistry; however the *Clostridium* study cited above reported good extraction of this gram positive bacterium using the machine for human feces. Even though there are no reports that gives direct evidence for this assumption, there is evidence of that enteric bacteria attach to sediments and different soil fractions with varying organic content (Guber *et al*. 2007; Jeng *et al.* 2005).

Efficient DNA extraction (concentration and level of purity) from environmental matrices is influenced by various parameters as inhibitors are concentrated along with the DNA which influences the PCR reaction (Lakay *et al*. 2007). The importance of the lysis step in recovering DNA from organisms possessing rigid cell walls has been reported before through studies which contrasted different DNA extraction methods for fungi such as *Aspergillus* sp (Fredericks *et al*. 2005) and *Rhizopus oryzae* (Francesconi *et al*. 2008). DNA recovery from cow fecal samples using the autoextractor improved when a pretreatment step using lysis buffer and Proteinase K was included. The lysis step included in the autoextraction process may not be sufficient to lyse the maximum numbers of cells in complex samples. In this investigation, optimal concentration of lysis buffer for pre-treatment of samples was found to be 180 µL. Without the lysis step or even with lesser volume of lysis buffer (60 µL), the qPCR concentrations of *Enterococcus* spp. in autoextracted DNA from cow fecal samples were reduced by at least one log. Increasing the volume of lysis buffer in the pretreatment did not further improve DNA recovery from *E. coli* or *Enterococcus spp.* cells, thus the volume used in our protocol was adequate. It has been shown that excess buffer in the lysis step during

extraction can result in shearing of DNA and may not give favorable results (Leuko *et al*. 2007).

Advantages in using the autoextractor included improved consistency and decreased variability. With the pre-treatment step included, the time it takes for extracting DNA from eight samples was slightly less when using the automated method (1 hour) as compared to the manual kit (1.5 hours). Previous studies in clinical samples have also compared the cost of DNA extraction using commercially available manual kits and automated methods (Knepp *et al*. 2003). Roche MagNaPure LC extraction is currently more expensive than the manual QIAamp DNA extraction with QIAamp kit reported to cost $2.64 per specimen with the MagNaPure costing around $3.58 per specimen (the price excludes cost of plastics). Wastewater samples worked well with the automated protocol; however, application of any automatic extracting system for recovering DNA from other challenging matrices such as soil would need further evaluation (Liles *et al.* 2008).

Scientific developments such as qPCR will likely yield changes in future regulations and monitoring for drinking and recreational waters. Therefore efficient and reliable data becomes an important issue for these emerging techniques. These preliminary studies have demonstrated that automation can be used to improve efficiency and reproducibility of DNA extraction and that qPCR can be used to describe bacterial concentrations in wastewater. New instrument configurations for automation which can handle more samples and greater diversity of matrices for environmental testing would be beneficial in analysis of wastewater and recreational waters.

Ethidium MonoAzide (EMA) was chosen for this study because of its lower costs as compared to Propidium MonoAzide. EMA has been found to penetrate into live cells (Nocker et al. 2006).but the treatment of samples with low concentrations of EMA has also been shown that the DNA from live cells can still be amplified (Lee and Levin 2006).

EMA-qPCR was found to distinguish between live cells and heat-killed cells in this study and some other studies as well (Nocker *et al.* 2006; Varma et al. 2009; Gedalanga and Olson 2009). However, with raw sewage and chlorine treated wastewater effluents, there was no distinction found between live and dead cells. There are several possibilities for this. One reason could be due to the effect of the turbidity on the samples In this study large volume concentrated samples from sewage were used for the EMA-treatment and due to the high turbidity of these samples, light penetration may have diminished and reduced the photolysis of the dyes and binding with DNA. The effect of turbidity on the efficiency of EMA-qPCR method has been studied before (Gedalanga and Olson 2009) and at turbidities higher than 10 NTU, the method was not able to distinguish between live and dead. The high turbidity of the concentrated effluent samples could have interfered with the method in this dissertation study.

Another possibility could be the presence of viable but nonculturable cells present in the disinfected effluents whose cell membranes are intact which was preventing penetration of EMA or PMA into the cells. A previous study has recommended UV irradiation as a wastewater disinfection method due to the observation that bacterial cells move into a non-cultivable state at chlorine levels used for wastewater disinfection and are recovered later (Blatchey et al. 2007). Previously, two groups have applied the

combination of EMA or PMA and qPCR method in wastewater treatment samples. One study spiked the secondary activated effluent with heat-killed *E. coli* O157 cells found a 1.5 $\log_{10}$ difference between qPCR equivalent cells in samples treated with EMA and those not treated with EMA. *E. coli* O157 concentrations by qPCR on samples treated with EMA were in the same levels as that of plate counts from these samples (Gedalanga and Olson 2009). Another study applied the method using PMA dye in wastewater effluents spiked with heat-killed enterococci cells and by using samples directly (Varma et al. 2009). They found that the even though PMA treatment on spiked samples showed a difference of 0.5 $\log_{10}$ units as compared to spiked PBS controls, when waste water and effluents were monitored directly, there was no difference between samples treated with dye and those untreated.

A significant difference between colony counts (CFU) and qPCR equivalent cells in disinfected effluents was observed in both those studies and this study. But inspite of treatment of samples with EMA or PMA, there appeared little reduction in qPCR signals and was definitely not comparable to colony counts suggesting that there may still be some cells present in the disinfected effluent samples that are not penetrated by EMA or PMA. More work is warranted on chlorine-killed bacteria and the use of such dyes for measuring viability by qPCR.

Surface water samples collected from different locations in Michigan showed that human fecal marker signals were absent even when concentrations *E. coli* and *Enterococcus* spp. were higher than the critical levels. This could be due to several reasons. The higher levels of *E. coli* and enterococci may be due to some other source of fecal input which was not detected by HF 183 assay, or could have been due to the

regrowth of these indicators in the sediments. Four of the sites (SL1, SL3, SL5 and CCD6) that showed positive for human specific HF183 marker had *E. coli* levels below the Michigan water quality standards for recreational waters based on a single sample maximum (300CFU/mL). Detection of HF183 marker in water when the levels of fecal indicator bacteria are below the standards have been reported before and they also found no direct relationship between indicator bacterial levels and detection of HF183 marker (Santoro and Boehm 2007). This lack of correlation could be due to the variation in the rate of inactivation of cultivable cells and DNA. Human specific Bacteroides 16S rRNA markers have been reported to persist for a longer time in waters as compared to cultivable cells of fecal indicator bacteria (Okabe et al. 2007).

For the water samples tested in this study, 1000-4000 mL was filtered and the limit of detection determined in this study was around 10^{-6}. However, the study was conducted using spiked distilled water and detection limit in surface water matrix may vary. Previous studies have also observed that the HF 183 marker was detected when raw sewage was diluted up to 1.4×10^{-6} serial dilutions but seeded into a large volume of 1000 mL of sterilized bay water (Bernhard and Field 2000a). Another study has detected the HF183 marker up to dilution 1×10^{-7} in sewage samples serially diluted and 300 mL of the seeded water was tested by the PCR assay (Ahmed et al. 2008).

These preliminary studies lead the way for addressing the objectives of this research. Based on the results, it was concluded that:

1) The new protocol, primers and probes for *E. coli* was a good method for characterizing human and cow waste environments.

2) Autoextractor protocol was a good approach to extract DNA from wastewater samples; however for cow waste samples, manual DNA extraction was found to be better.

3) Distinction between viable and non-viable cells using EMA proved to be efficient for pure cultures; however with samples such as raw sewage and treated effluent samples, the method did not produce satisfactory results. Therefore, this method was not used in this study.

4) The human marker developed by Bernhard and Field (2000b) had good detection limits but was not quantitative and not found in more than 14.5% of Michigan surface water tested where sewage was detected, therefore alterative markers were identified.

REFERENCES

3.5 References:

Ahmed, W., J. Stewart, D. Powell, and T. Gardner. 2008. Evaluation of Bacteroides markers for the detection of human faecal pollution. Letters in Applied Microbiology 46:237-242.

Bernhard, A. E., and K. G. Field. 2000a. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Appl. Environ. Microbiol. 66: 1587-1594.

Bernhard, A. E., and K. G. Field. 2000b. A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. Appl. Environ. Microbiol. 66:4571-4574.

Bernhard, A.E., Goyard, T., Simonich, M.T. and K. G. Field. 2003. Application of a rapid method for identifying fecal pollution sources in a multi-use estuary. Water Res **37**: 909–913.

Bej, A. K., J. L. Di Cesare, L. Haff, and R. M. Atlas. 1991a. Detection of *Escherichia coli* and *Shigella spp.* in water by using the polymerase chain reaction and gene probes for uid. Appl. Environ. Microbiol. **57/4**, 1013– 1017.

Bej, A. K., S. C. McCarty, and R. M. Atlas. 1991b. Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: comparison with defined substrate and plating methods for water quality monitoring. Appl. Environ. Microbiol. **57**, 2429– 2432.

Beuselinck, K., M. van Ranst, and J. van Eldere 2005. Automated extraction of viral-pathogen RNA and DNA for high-throughput quantitative realtime PCR. J Clin Microbiol; **43**: 5541–6.

Blatchley III, E.R., Gong, W.-L., Allernan, J.E., Rose, J.B.,Huffman, D.E., Otaki, M., Lisle, J.T. 2007. Effects of Wastewater disinfection on waterborne bacteria and viruses. Water Environ. Res. 79, 81–92.

Buckalew, **D. W.**, **L. J.** Hartman, **G. A.** Grimsley, **A. E**. Martin, and **K. M.** Register. 2006. A long-term study comparing membrane filtration with Colilert defined substrates in detecting faecal coliforms and Escherichia coli in natural waters. J Environ Manage **80**, 191–197.

Bustin, S.A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **25**: 169–193.

Chao, K. -K., C. –C. Chao, and W. –L. Chao. 2004. Evaluation of Colilert-18 for Detection of Coliforms and *Eschericha coli* in Subtropical Freshwater. Appl. Environ. Microbiol. **70**: 1242-1244.

Delgado-Viscogliosi, P., L. Solignac, and J. M. Delattre. 2009. Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Appl. Environ. Microbiol. **75:**3502-3512

Fafi-Kremer, S., K. Brengel-Pesce, G. Bargues, M. J. Bourgeat, O. Genoulaz and J. M. Seigneurin. 2004. Assessment of automated DNA extraction coupled with real-time PCR for measuring Epstein–Barr virus load in whole blood, peripheral mononuclear cells and plasma. J Clin Virol; **30**:157–64.

Foulds, I. V., A. Granacki, C. Xiao, U. J. Krull, A. Castle, and P. A. Horgen. 2002. Quantification of microcystin-producing cyanobacteria and *E. coli* in water by 5'-nuclease PCR. J Appl Microbiol **93**, 825–834.

Frahm, E., and U. Obst. 2003. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus spp.* and *Escherichia coli* in water samples. J. Microbiol. Methods **52**, 123–131.

Francesconi, A., M. Kasai, S. M. Harrington, M. G. Beveridge, R. Petraitiene, V. Petraitis, R. L. Schaufele, and Walsh T. J. 2008. Automated and manual methods of DNA extraction for *Aspergillus fumigatu*s and *Rhizopus oryzae* analyzed by quantitative real-time PCR. J. Clin. Microbiol. **46**:1978-1984.

Fredricks, D. N., C. Smith, and A, Meier. 2005. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J Clin Microbiol **43**, 5122–5128.

Gawler, A.H., Beecher, J.E., Brandaˆo, J., Carroll, N.M., Falcaˆo, L., Gourmelon, M., Masterson, B., Nunes, B. et al. 2007.Validation of host-specific Bacteriodales 16S rRNA genes as markers to determine the origin of fecal pollution in Atlantic Rim countries of the European Union. Water Res 41: 3780–3784.

Gedalanga P. B, Olson B. H.2009. Development of a quantitative PCR method to differentiate between viable and nonviable bacteria in environmental water samples. Appl Microbial Biotechnol **82**:587–596

Ginzinger,D.G. 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp. Haematol.*, **30**, 503–512

Gonzalez-Escalona, N., Hammack, T. S., Russell, M., Jacobson, A. P., De Jesus, A. J., Brown, E. W., and Lampel, K. A. 2009. Detection of Live Salmonella sp. Cells in Produce by a TaqMan-Based Quantitative Reverse Transcriptase Real-Time PCR Targeting invA mRNA. *Appl. Environ. Microbiol.* **75**: 3714-3720.

Gourmelon, M., Caprais, M.P., Se´gura, R., Mennec, C.L., Lozach, S., Piriou, J.Y. and R.A Rince´. 2007. Evaluation of two library-independent microbial source tracking methods to identify sources of fecal contamination in French estuaries. Appl Environ Microbiol 73 : 4857–4866.

Gracias K. S., and **J. L**. McKillip**.** 2004. A review of conventional detection and enumeration methods for pathogenic bacteria in foods. Canadian Journal of Microbiology **50** 883–890.

Guber, A.K., Y.A. Pachepsky, D.R. Shelton, and O. Yu. 2007. Effect of bovine manure on fecal coliform attachment to soil and soil particles of different sizes. Appl. Environ. Microbiol. **73**:3363–3370.

He, J.-W., and S. Jiang. 2005. Quantification of *Enterococcus spp.* and human adenoviruses in environmental samples by real-time PCR. Appl. Environ. Microbiol. **71:**2250-2255.

Holben, W. E. 1997. Isolation and purification of bacterial community DNA from environmental samples, p. 431-436. *In* C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter (ed.), Manual of environmental microbiology. American Society for Microbiology, Washington, D.C.

Hourfar M, K., M. Schmidt, E. Seifried, and W. Roth. 2005. Evaluation of an automated high-volume extraction method for viral nucleic acids in comparison to a manual procedure with preceding enrichment. Vox Sang; **89**:71–6. 4.

Jeng, H. C., A. J. H. England, and H. B. Bradford. 2005. Indicator organisms associated with stormwater suspended particles and estuarine sediment. J. Environ. Sci. Health **40**:779-791

Khan, I., V. Gannon, R. Kent, W. Koning, D. Lapen, J. Miller, N. Neumann, R. Phillips, W. Robertson, E. Topp, E. van Bochove, and T. Edge. 2007. Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds. Journal of Microbiological Methods **69 (3),** 480–488.

Knepp, J. H., M. A. Geahr, M. S. Forman, and A. Valsamakis. 2003. Comparison of automated and manual nucleic acid extraction methods for detection of enterovirus RNA.J. Clin. Microbiol. **41**:3532-3536.

Lakay, F. M., A. Botha, and B. Prior. 2007. Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils. J Appl Microbiol **102**:265–273.

Lebuhn, M., M. Effenberger, G. Garce´s, A. Gronauer, and P.A. Wilderer. 2005. Hygienization by anaerobic digestion: comparison between evaluation by cultivation and quantitative real-time PCR. Water Science and Technology **52 (1–2)**, 93–99.

Lee, J.L; R.E. Levin. 2006. Use of ethidium bromide monoazide for quantification of viable and dead mixed bacterial flora from fish fillets by polymerase chain reaction. J Microbiol Methods 67:456–462

Leff, L.G. 1995. Comparison of methods of DNA extraction from stream sediments. Appl. Environ. Microbiol. **61**, 1141–1143.

Leuko, S., F. Goh, R. Ibáñez-Peral, B. P. Burns, M. R Walter, and B. A Neilan. 2007. Lysis efficiency of standard DNA extraction methods for *Halococcus* spp. in an organic rich environment. Extremophiles **12**:301–308.

Liles, M. R., Williamson, J. Rodbumrer, V. Torsvik, R. M Goodman, and J. Handelsman. 2008. Recovery, purification and cloning of high molecular weight DNA from soil microorganisms. Appl Environ Microbiol **74**: 3302–3305.

Ludwig, W., and K.-H. Schleifer. 2000. How quantitative is quantitative PCR with respect to cell counts? Syst. Appl. Microbiol. **23**, 556–562.

Mackay, I. M. 2004. Real-time PCR in the microbiology laboratory. Clinical Microbiology and Infection, 10: 190–212.

McKillip, J., Jaykus, L., Drake, M. 1999. Nucleic acid persistence in heat-killed Escherichia coli O15:H7 from contaminated skim milk. J. Food Prot. **62**: 839–844.

Mengelle, C., K. Sandres-Saune, C. Pasquier, L. Rostaing, J. M. Mansuy, M. Marty, I. Da Silva, M. Attal, P. Massip, and J. Izopet. 2003. Automated extraction and quantification of human cytomegalovirus DNA in whole blood by real-time PCR assay.J. Clin. Microbiol. **41:**3840-3845.

Messer, J.W., and A.P. Dufour. 1998. A rapid, specific membrane filtration procedure for enumeration of *Enterococcus spp.* in recreational water. Appl. Environ. Microbiol. **64**: 678–680.

Method A: *Enterococcus* spp. in Water by TaqMan Quantitative Polymerase Chain Reaction (qPCR) Assay.
http://www.epa.gov/waterscience/methods/method/biological/rapid1.pdf

Noble, R. T., J. F. Griffith, A. D. Blackwood, J. A. Fuhrman, J. B. Gregory, X. Hernandez, X. Liang, A. A. Bera, and K. Schiff. 2006. Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California. Appl. Environ. Microbiol. **72:**1604-1612.

Nocker, A., Cheung, C.-Y., Camper, A.K. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods 67, 310–320

Ogram, A. 2000. Soil molecular microbial ecology at age 20: methodological challenges for the future, *Soil Biology & Biochemistry* **32**: 1499–1504.

Okabe, S., Okayama, N., Savichtcheva, O., and Ito, T. (2007) Quantification of host-specific Bacteroides-Prevotella 16S rRNA genetic markers for assessment of fecal pollution in freshwater. Appl Microbiol Biotechnol 74: 890–901.

Pichl L, A. Heitmann, P. Herzog, J. Oster, H. Smets, V. Schottstedt. 2005. Magnetic bead technology in viral RNA and DNA extraction from plasma minipools. Transfusion; **45**:1106–10.

Pisz, J. M., J. R. Lawrence, A. N. Schafer, and S. D. Siciliano. 2007. Differentiation of genes extracted from non-viable versus viable micro-organisms in environmental samples using ethidium monoazide bromide. J. Microbiol. Methods **71:**312-318.

Rasmussen,R. 2001. Quantification on the LightCycler. In Meuer,S., Wittwer,C. and Nakagawara,K. (eds), *Rapid Cycle Real-time PCR, Methods and Applications.* Springer Press, Heidelberg, pp. 21–34.

Rasmussen, T. B., A. Uttenthal, M. Hakhverdyan, S. Belak, P.R. Wakeley, S.M. Reid, K. Ebert and D.P. King. 2009. Evaluation of automated nucleic acid extraction methods for virus detection in a multicenter comparative trial. J. Virol. Methods. **155:** 87–90.

Rose, P., J. M. Harkin, W. J. Hickey. 2003. Competitive touchdown PCR for estimation of *E. coli* DNA recovery in soil DNA extraction. Journal of Microbiological Methods. **52**: 29–38.

Rudi, K., B. Moen, S. M. Dromtorp, and A. L. Holck. 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl. Environ. Microbiol. 71**:**1018-1024.

Santoro, A.E., and A.B.Boehm. 2007. Frequent occurrence of the human-specific Bacteroides fecal marker at an open coast marine beach: relationship to waves, tides and traditional indicators. Environ Microbiol **9**: 2038–2049.

Servais, P., T. Garcia-Armisen, A.S. Lepeuple and P. Lebaron. 2005. An early warning method to detect fecal contamination of river waters. Ann Microbiol. **55:** 67–72.

Schneegurt, M.A., S.Y. Dore and C.F. Kulpa Jr. 2003. Direct extraction of DNA from soils for studies in microbial ecology. Curr. Issues Mol. Biol. **5**: 1-8.

Sharma, V. K. 2006. Real-time reverse transcription-multiplex PCR for simultaneous and specific detection of *rfbE* and *eae* genes of *Escherichia coli* O157: H7. Mol. Cell. Probes **20:**298-306

Sivaganesan, M., S. Seifring, M. Varma, R. A. Haugland, and O. C. Shanks. 2008. A Bayesian method for calculating real-time quantitative PCR calibration curves using absolute plasmid DNA standards. BMC Bioinformatics. **9**:120.

Spierings, G., C. Ockhuijsen, H. Hofstra, J. Tommassen. 1993. Polymerase chain reaction for the specific detection of *Escherichia coli*/Shigella. Res. Microbiol. **144**: 557–564.

Tryland, I., and L. Fiksdal. Enzyme characteristics of beta-d-galactosidase- and beta-d-glucuronidase-positive bacteria and their interference in rapid methods for detection of waterborne coliforms and *Escherichia coli.* 1998. Appl. Environ. Microbiol. **64:**1018–1023.

Tsai Y-L, and B. H. Olson. 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58**:2292–2295.

Tsen, H. Y., C. K. Lin, and W. R. Chi. 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of Escherichia coli cells in water.J. Appl. Microbiol. **85**: 554–560.

USEPA. 2002. Method 1600: *Enterococcus spp.* in Water by Membrane Filter using membrane Enterococcus Indoxyl-B-D-Glucoside Agar (mEI). EPA-821-R-02-022. Office of Water, Washington D.C.

USEPA. 2005. **Method 1603:** *Escherichia coli* (*E. coli*) in Water by Membrane Filtration using Modified membrane-Thermotolerant *Escherichia coli* Agar (modified mTEC). EPA 821-R-04-025. Office of Water, Washington D.C.

van den Berg, R.J., Kuijper, E.J., van Coppenraet, L. and Claas, E.C.J. 2006. Rapid diagnosis of toxinogenic Clostridium difficile in faecal samples with internally controlled real-time PCR. Clinical Microbiology and Infection 12(2), 184-186.

Varma M, Field R, Stinson M, Rukovets B, Wymer L, Haugland R. 2009. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. Water Res **43**:4790–4801.

Viau, E., and J, Peccia. 2009. Survey of Wastewater Indicators and Human Pathogen Genomes in Biosolids Produced by Class A and Class B Stabilization Treatments. Appl. Environ. Microbiol.**75**: 164-174.

Wade, T. J., R. L. Calderon, E. Sams, M. Beach, K. P. Brenner, A. H. Williams, and A. P. Dufour. 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspect. **114**:24-28.

Wang, S., and R. E Levin .2005. Discrimination of viable Vibrio vulnificus cells from dead cells in real-time PCR. J Microbiol Methods **64**:1–8

Wilson, D., B. Yen-Lieberman, U. Reischl, I. Warshawsky, and G. W. Procop. 2004. Comparison of five methods for extraction of *Legionella pneumophila* from respiratory specimens. J. Clin. Microbiol. **42:**5913-5916.

Wullings, B. A.; D. van der Kooij. 2006. Occurrence and genetic diversity of uncultured *Legionella* spp. in drinking water treated at temperatures below 15 °C. Appl. Environ. Microbiol. **72(1):** 157-166.

Young G., Turner S., Davies J.K., Sundqvist G., Figdor D. 2007. Bacterial DNA Persists for Extended Periods after Cell Death. *Journal of Endodontics*, 33 (12), pp. 1417-1420.

CHAPTER 4

EVALUATION OF FATE OF *BACTEROIDES THETAIOTAOMICRON* IN COMPARISON WITH CONVENTIONAL INDICATORS DURING SEWAGE AND SEPTAGE TREATMENT PROCESSES

4.1 Introduction

Adequate wastewater treatment and its disposal play a critical role in minimizing public health risks. Wastewater is reported to have many pathogens belonging to different groups such as bacteria, viruses and protozoa, that are discharged to waste stream by infected humans (Nayak and Rose 2007; Lee et al. 2006; Kamel et al. 2010; Robertson et al. 2006). These pathogens can then enter into water bodies when inadequately treated waste is discharged, facilitating widespread transmission of waterborne diseases. Changing climatic patterns and urbanization are forcing communities to resort to alternative water supplies dominated by wastewater or directly to reclaimed wastewater which highlights the importance of proper treatment as a key barrier to transmission. Though strong regulations exist in many places such as the US and the European Union to regulate wastewater discharges; many countries lack such regulations and proper waste disposal systems. According to the 2006 United Nations Human Development Report, 2.6 billion people lack access to basic sanitation, and lack adequately treated wastewater.

On-site wastewater disposal such as septic tanks has also been an issue regarding pathogen entry into and transmission through water. Leaking septic tanks is another cause of human fecal contamination of water sources (Fong et al. 2007), especially groundwater

sources. Many households or small communities use individual septic tanks. Septic tanks collect waste water from the toilets, showers, sinks, and laundry. The liquid is treated by soil infiltration and solids are pumped out and often land applied, with or without treatment.

Monitoring the sewage environment and validating waste treatment by testing for all pathogens is not practical. Many pathogen methods are laborious, time consuming and expensive. Therefore microbial indicators of fecal contamination have been used instead to examine the performance of these waste treatment systems and the quality of effluents discharged or reused. Fecal coliform bacteria are generally the standard for addressing wastewater effluents and prevention of deterioration of water quality (Elmund et al. 1999). The standard for Michigan as stated in Rule 62 is "Discharges containing treated or untreated human sewage shall not contain more than 200 fecal coliform bacteria per 100 milliliters, based on the geometric mean of all of 5 or more samples taken over a 30-day period, nor more than 400 fecal coliform bacteria per 100 milliliters, based on the geometric mean of all of 3 or more samples taken during any period of discharge not to exceed 7 days" (Michigan Department of Environmental Quality).

Traditionally, *Escherichia coli* (*E. coli*) and enterococci have been used to help regulate microbial water quality, and research related to these indicator bacteria has developed new criteria or standards around the globe on the acceptable levels of indicator bacteria in recreational or drinking water limiting possible health risks (Michigan Natural Resources and Environmental Protection Act 1994; USEPA 1986; WHO 2008). However, when these indicator bacteria are found, it is not possible with routine methods

to discriminate between various fecal sources as they are present in intestines of all warm-blooded animals (Field et al, 2003).

Microbial source tracking (MST) is a field that has enabled the identification of sources of fecal contamination (Scott et al. 2002; Simpson et al. 2002; US EPA 2005) via generally genetic methods for *E. coli* and enterococci. Currently, enteric bacteria in the order of *Bacteriodales* have been proposed as an alternate indicator of fecal contamination along with identifiable genetic host specificity (Bernhard & Field 2000). Some of the members are bacteria in the genus *Bacteroides* which are obligately anaerobic, Gram negative, rod shaped, and non-endospore forming including *Bacteroides fragilis* and closely related species namely, *B. caccae, B. distasonis, B. eggerthii, B. merdae, B. ovatus, B. stercoris, B. thetaiotaomicron, B. uniformis,*and *B. vulgates* are included (Shah and Collins 1990). *Bacteroides sp* are normally commensals and make up more than one-quarter of the total gut bacteria in humans and many warm blooded animals (Daley and Shirazi-Beechey 2003; Franks et al. 1998; Harmsen et al. 2002, Holdman 1976, Wood et al. 1998). As *Bacteroides* are strict anaerobes, they do not survive long or reproduce outside the host organism in aerobic environments. This characteristic prevents these bacteria from proliferating in waters and sediments, unlike *E. coli* and enterococci (Avelar et al. 1998, Kreader 1998). However, it is more difficult to cultivate *Bacteroides*, thus molecular methods such as Polymerase Chain Reaction (PCR) and quantitative PCR (qPCR) targeting specific DNA from both live and dead cells have been relied upon for their detection. There are many specific target assays within *Bacteroides* available based on multiple sequences forming host specific 16SrRNA gene clusters. Recent studies have shown that there is cross reactivity

associated with these highly conserved regions of 16S rRNA gene clusters when it comes to distinguishing host specific targets (Sadowsky 2007). Thus, targeting other more specific genes as microbial source tracking markers involved directly in host microbial interaction has been suggested as an alternative approach (Shanks et al. 2007).

Bacteroides thetaiotaomicron is a species which has been proposed to be examined for genes that may be more specific to a human host (Yampara-Iquise et al. 2008). This organism is ranked third highest in relative frequency (8.9%) of isolation from human feces in a study that looked at fecal flora of three men over 5 months with a total of 1442 isolates and their concentration was around 2.29×10^{10} per gram of dry fecal matter (Holdeman et al. 1986). This organism is a symbiotic organism and its role in the intestine is to break down complex polysaccharides (Xu et al. 2003). Host specificity of these markers has been tested by Yampara-Iquise *et al.* (2008) by targeting putative mannanase 1-6 genes of these bacteria. Sequence analysis has suggested they are reliable human fecal specific markers that could be used to track human fecal contamination in water (Yampara-Iquise *et al.* 2008).

In its report of the "Experts Scientific Workshop on Critical Research Needs for the Development of New or Revised Recreational Water Quality Criteria", the US EPA (2007) stated as high/medium priorities to "conduct microbial fate and transport studies to determine relationships between traditional and new fecal indicators, index pathogens, and priority pathogens in treated effluents and in downstream recreational waters to compare and validate their applicability for specific criteria uses".

The specific objectives of this portion of the research were to:

1) Determine the concentrations of *Bacteroides thetaiotaomicron* α-mannanase 1-6 gene in raw sewage and septage and to study the fate of the molecular signal during treatment, and

2) Compare this target with conventional indicators such as *E. coli* and enterococci. This research hypothesized that a correlation exists between the *Bacteroides thetaiotaomicron* human marker and *E. coli* or enterococci as measured by cultivation or qPCR.

4.2 Materials and Methods

4.2.1 Sample Collection and Processing

a) Wastewater treatment facility:

Samples were collected from a municipal wastewater treatment plant, located in East Lansing, Michigan that serves around 90,000 people. The plant receives, on an average basis, a little less than 13.40 MGD wastewater inflow. Samples were collected during 18 sampling events that lasted from January 2009 to January 2010. Samples collected from this facility included:

i) Raw sewage (RS)

ii) Primary effluent (PE), after the solids have settled

iii) Secondary effluent (SE), after activated sludge process and chlorination, and

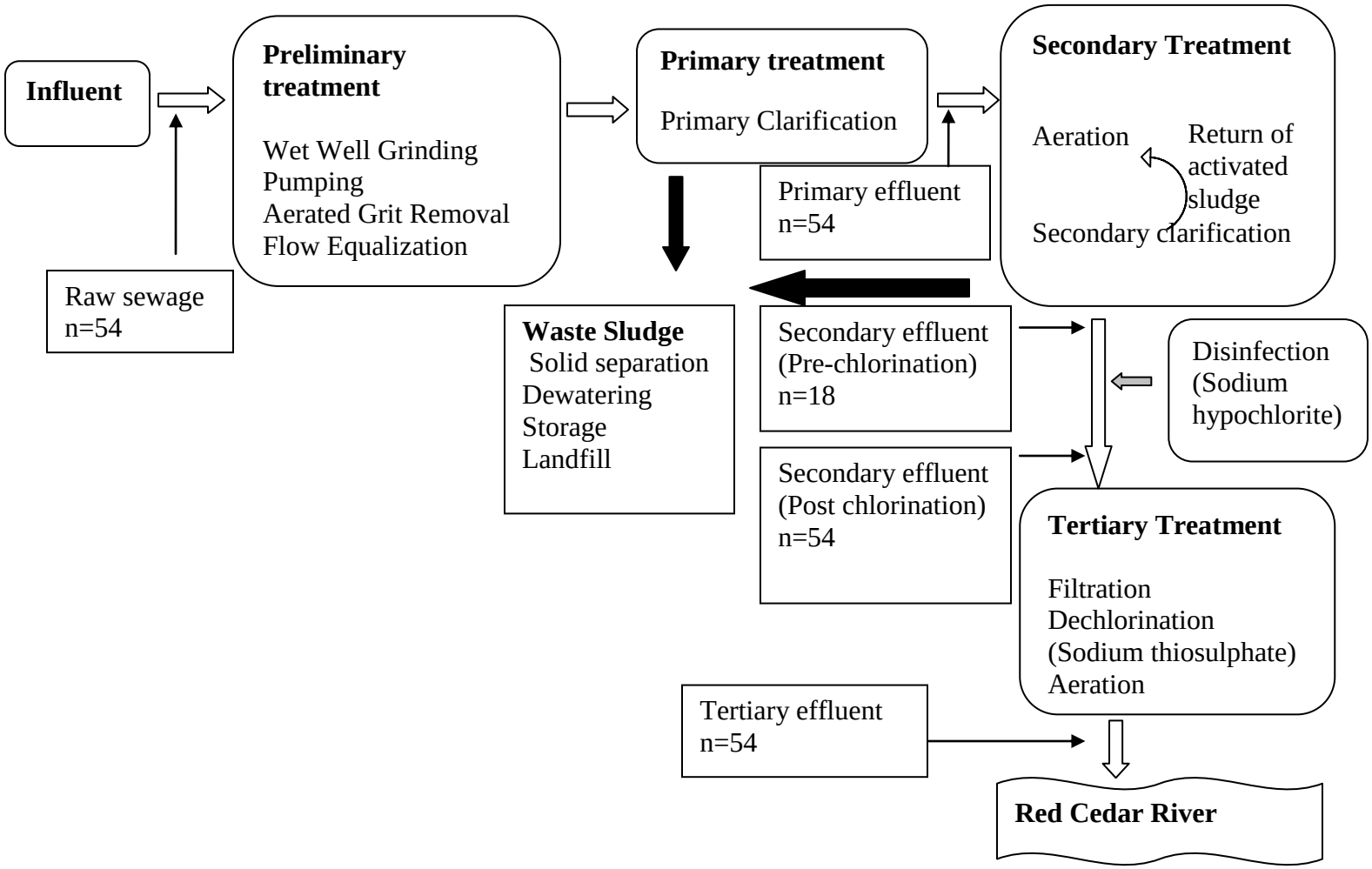
iv) Tertiary effluent (TE), effluent from secondary treatment post sodium bi-sulfite dechlorination and filtration through rapid sand filters.

Apart from four types of samples described above, secondary treated samples in triplicates prior to the chlorination step were also collected during six sampling events in January 2010 (included in the 18 sampling events). The sampling locations are

schematically presented in Figure 4.1. During each sampling event, one hundred milliliters of raw sewage and primary effluent, 500 milliters of pre-chlorinated secondary effluents and two liters of secondary and tertiary effluents were collected in triplicates, transported to the laboratory on ice and processed immediately. Chlorinated effluents were collected in bottles added with Sodium Thiosulphate (1mL of 10% solution) to neutralize any residual chlorine present in the effluents.

The residual chlorine on all sampling dates was found to be 0.01 mg/L in the final effluent. Data for physicochemical characteristics of raw sewage and final effluent samples were collected on all sampling dates.

Figure 4.1: Schematic representation of sampling locations in the waste water treatment plant at East Lansing.



b) Septage treatment plant:

Samples were collected from a septage treatment plant located in Charveloix, Michigan that utilizes an aerobic biological treatment system to treat septage wastes and discharges the treated effluent to the municipal sewer system. Briefly, septage is pumped from the trucks into screens and a de-grit chamber, which then flows into an equalization tank. The waste then goes through lime treatment process, after which it is pumped through a flocculation tank and a rotary screen thickener for biosolid production. Solids produced are processed in a screw press that heats up to a minimum of 100^{O}C for a minimum of 20 minutes; where the combination of pH during lime stabilization and high temperature treatment reduces microorganisms in the solids. Water extracted during solid production is then discharged into series of aerobic treatment tanks. These large tanks have microbial generators that provide a source of microorganisms. The organic wastes are reduced from the wastewater by these organisms in combination with naturally occurring microorganisms. Water then enters into settling tanks, the solid collected goes through lime treatment and a screw press processes. The clarified water is aerated further after which it is discharged as effluent into the municipal sewer system. The treatment also produces Class A biosolids after the dewatering stage.

Samples were collected during eight sampling events that lasted from January 2009 until November 2009. During each event, triplicates of 50 mL raw septage and 500 mL of effluent were collected from the plant, placed on ice and shipped to Water Quality and Health Laboratory at Michigan State University, East Lansing, MI.

4.2.2 Analyses

All samples were analyzed by cultivation methods for *E. coli* and enterococci and by qPCR methods for *E. coli*, enterococci and *Bacteroides thetaiotaomicron.*

4.2.2.1 Cultivation Methods

U.S.EPA Membrane Filtration methods 1603 and 1600 were used for enumerating *E. coli* and *Enterococci* respectively (USEPA 2002; 2005). Serial dilutions of the raw sewage and primary effluents (10^{-1} through 10^{-5}) were made and one mL from these dilutions was filtered through 47 mm diameter, 0.45 µm pore size, membrane filters. For pre-chlorinated secondary effluents, one mL of the sample was filtered directly and for secondary and tertiary treated effluents, one hundred milliliters of samples were filtered. For samples from the septage plant, one mL of raw septage samples was serially diluted and these dilutions were used for further bacterial indicator analysis. For effluent samples, volumes of 0.1 mL, 1 mL and 10 mL were used for filtration.

The filters were placed on mTEC agar and mEI agar plates. The mTEC agar plates were incubated 35 ± 0.5°C for 2 h, followed by incubation in a water bath at 44.5 ± 0.2°C for 22 h and mEI plates were incubated for 24 h at 41°C and colonies were counted. The concentrations of *E. coli* and enterococci from all tested samples were reported as colony forming units (CFU) per 100 mL.

4.2.2.2 DNA Extraction and qPCR Analyses

Waste water treatment plant samples: Fifty milliliters of raw sewage sample were centrifuged at 8000xg for 20 minutes. Around 48 mL of the supernatant was discarded and remaining sample was mixed well by vortexing. The volume was recorded and from this, 400 µL of the pellet was used for extraction using Roche MagNaPure LC instrument

(Roche Applied Sciences, Indianapolis, Ind.) and remainder was stored at -80 ^{o}C. These volumes were included in the calculation when final concentrations of targets were calculated after qPCR. For treated sewage, 200 mL of the pre-chlorinated secondary effluent sample and one liter of secondary and tertiary effluents was filtered using 90 mm, 0.45 µm pore size nitrocellulose membrane filters (Millipore, Billerica, Mass.) and the filters were folded and immersed to the 45 mL mark with sterile phosphate buffered saline (PBS) in 50 mL centrifuge tubes. The tubes were then vortexed at high speed to detach the cells from membrane for two minutes. The filters were removed and the tubes were centrifuged at a speed of 8000xg for 15 minutes and around 48 mL of the supernatant was discarded. The remaining sample was mixed well by vortexing; 400 µL of the pellet was used for extraction.

DNA extraction using Roche MagNaPure automated machine included an external lysis and was performed by mixing 400 µL of the concentrated samples with 180 µL of the MagNaPure lysis buffer and 20 µL of the Proteinase K (20 mg/mL) incubated at 65^{o}C for 30 minutes. The mixture was then centrifuged at 500g for 30 seconds to settle down the particles in a microcentrifuge and the supernatant was used for DNA extraction in the machine. The eluted volume containing the DNA from all the samples was in 200 µL volumes and stored at -20^{o}C.

Septage treatment plant samples: Raw septage samples were mixed thoroughly by vortexing for one minute and 600µL was taken from this for DNA extraction. For effluent samples, 50 mL of the sample was centrifuged at 8000g for 20 minutes. The supernatant was discarded and 1 mL of the pellet was left behind. From this, 600 µL was

used for DNA extraction. DNA extraction was carried out using Roche MagNaPure automated machine following the protocol described above.

The bacteria genes, amplicons used given in Table 4.1 and the primers/probes and corresponding PCR programs are described in Table 4.2. DNA extracted from all samples was analyzed by qPCR using Roche LightCycler® 2.0 Instrument (Roche Applied Sciences, Indianapolis, IN). The reaction mixture for amplification of *E. coli uidA* gene consisted of 2µL of Roche Fast Start Light Cycler Mastermix, forward and reverse primers, probe, 3.2 mM $MgCl_2$ and nuclease free water to make up the final volume to 15 µL. Cycle threshold temperature (Ct) was measured during each amplification and target gene concentration was analyzed automatically by absolute quantification method by the LightCycler® Software 4.0. The enterococci 23S rRNA and *B. thetaiotaomicron* α-mannanase 1-6 qPCR assays were carried out using 10 µL of Light Cycler 480 Probes Mastermix (Roche, Indianapolis, IN), forward and reverse primers, probe, and nuclease free water to make up the final volume to 15 µL.

One of the plasmid dilutions used for creating the standard curve for the targets was included in the assay as a positive control. Five microliters of the extracted sample were used as the template. The reaction was performed in duplicate for samples and negative controls were included. The copies of *uidA* gene of *E. coli,* 23S rRNA of enterococci gene and α-mannanase 1-6 gene of *Bacteroides thetaiotaomicron* present in the sample were quantified from the standard curves obtained earlier. The copies of the corresponding genes were converted to cell equivalents; in the case of *E. coli* and *B. thetaiotaomicron,* only one copy of the target gene is present in a cell, thus one copy number corresponds to one cell. However in case of enterococci, there are four copies of

23S rRNA present in a cell; therefore when final copies to cell conversions are done, four copies of enterococci qPCR targets corresponds to one cell. All final concentrations for qPCR analyses were reported as qPCR equivalent cells/100 mL for all samples.

Table 4.1: The bacteria and gene targets used for qPCR assays in the monitoring of raw sewage, septage, and treated effluents.

Bacteria	**Gene**	**Amplicon size**	**Reference**
E. coli	*uidA*	163 bp	Developed in this study
Enterococci	23S RNA	91 bp	Frahm & Obst (2003)
B. thetaiotaomicron	α-1-6 mannanase	63 bp	Yampara-Iquise et al. (2008)

Table 4.2: Primer/ probe sequences, concentrations and PCR programs used for bacterial qPCR assays (References in Table 4.1).

Bacteria	Primer/probe sequence	Primer/probe concentrations	PCR programs [a] [b]
E. coli	5'-CAATGGTGATGTCAGCGTT-3'	0.5 µM	6 s at 95°C
	5'-ACACTCTGTCCGGCTTTTG-3'	0.5 µM	8 s at 58°C
	6FAM-TTGCAACTGGACAAGGCACCAGC-BBQ	0.2 µM	8 s at 72°C
Enterococci	5'-AGAAATTCCAAACGAACTTG-3'	0.5 µM	15 s at 95°C
	5'-CAGTGCTCTACCTCCATCATT-3'	0.5 µM	30 s at 60°C
	6FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA	0.4 µM	15 s at 72°C
B. thetaiotaomicron [b]	5'-CATCGTTCGTCAGCAGTAACA	0.2 µM	15 s at 94°C
	5'-CCAAGAAAAAGGGACAGTGG	0.2 µM	60 s at 60°C
	6FAM-ACCTGCTG-NFQ	0.1 µM	5 s for 72°C

[a] Repeated for 40 cycles, after an initial cycle of 10 min at 95°C (For *E. coli and* enterococci)

[b] Repeated for 45 cycles, after an initial cycle of 15 min at 95°C (For *B. thetaiotaomicron*)

4.2.3 Statistics

Statistical analyses were performed using SAS software 9.2 (SAS Inc, 2002) and significance level was set at α=0.05. In order to fulfill the normality assumptions of tests used in the analysis, data were $\log_{10}$ transformed before conducting the tests. Analysis of variance (ANOVA) was performed to determine the differences in mean concentrations of target organisms in different treatment groups. If ANOVA was significant, multiple pairwise comparisons were carried out using Fisher's Least Square Difference (LSD) test. Covariate analysis was performed to decide if correlation needed to be adjusted due to the effect of untreated (raw sewage and primary effluent) and treated (secondary effluent and tertiary effluent) groups from the waste water treatment plant. If there was an effect due to these variables, correlation was performed separately for both treated and untreated set of samples. Pearson's correlation coefficient was used to test the relationship between conventional indicators and *B. thetaiotaomicron.* Linear regression analysis was used to estimate the coefficients of the linear equation for conventional indicators that best predicted the concentrations of *B. thetaiotaomicron* after various waste water treatment processes.

4.3 Results

4.3.1 Wastewater treatment samples

The physicochemical characteristics for raw sewage and final effluent were recorded on all sampling dates and the ranges for these characteristics are summarized in Table 4.3. The pH was found consistent ranging from 6.6 to 7.2. Biological Oxygen Demand (BOD), suspended solids, phosphorus, and ammonia levels were all decreased by the activated sludge treatment as expected.

Table 4.3: Range of physical and chemical characteristics for raw sewage and final effluent samples for all sampling dates.

Samples	pH	BOD_5 mg/L	Suspended solids mg/L	Total-Phosphorus mg/L	Ammonia mg/L
Raw sewage	6.6-7.0	12-223	20-274	1.5-6.7	3.1-36.2
Tertiary effluent	6.8-7.2	1.0-6.0	1.0-7.0	0.5-2.1	0.1-2.4

Table 4.4 shows the average $\log_{10}$ transformed concentrations with the standard deviations for the 216 samples (54 after each treatment location) collected from the wastewater treatment plant. The qPCR equivalent concentrations of *E. coli* in raw sewage ranged from $1.47x10^5$ to $1.48x10^7$ cells/100 mL, enterococci ranged from $7.08x10^5$ to $5.75x10^7$ cells/100 mL and for the *B. thetaiotaomicron* ranged from $7.76x10^6$ to

$5.68x10^7$ cells/100 mL (Table 4.4). The final effluent, after the tertiary treatment had a range of concentration of $2.16x10^1$ to $4.39x10^4$ of *E. coli*, $6.35x10^0$ to $3.81x10^5$ of enterococci and $9.79x10^2$ to $1.59x10^5$ of *B. thetaiotaomicron* qPCR equivalent cells/100mL (Table 4.4).

The data for fecal coliform counts for final effluents were obtained for these sampling dates from the treatment facility and ranged from $3.0x10^0$ to $2.3x10^3$ CFU/100 mL and the mean concentration was $1.91x10^2$ CFU/100 mL. Concentrations of cultivatible *E. coli* in the same effluent samples ranged from below detection limit to $3.34x10^2$ CFU/100 mL and that of enterococci were below detection limit to $2.31x10^2$ CFU/100 mL. The mean concentrations of both were $4.64x10^1$ and $3.03x10^1$ CFU/100 mL respectively in final tertiary effluent.

There were statistically significant differences in concentrations of all targets either by cultivation or qPCR methods between samples collected prior to disinfection and after disinfection ($p<0.05$). Ficher's LSD showed that concentrations of *B. thetaiotaomicron* were significantly higher than *E. coli* or enterococci in all samples except in secondary and tertiary effluents where their concentrations were not significantly different from that of enterococci ($p<0.05$). Concentrations of *E. coli* were significantly higher than enterococci in all treated and untreated sewage samples by cultivation methods; however by qPCR method enterococci concentrations were found to be significantly higher than *E. coli* in secondary and tertiary effluents ($p<0.05$).

Table 4.4: Average $\log_{10}$ concentrations for *E. coli*, enterococci by cultivation and qPCR and *B. thetaiotaomicron* by qPCR throughout wastewater treatment.

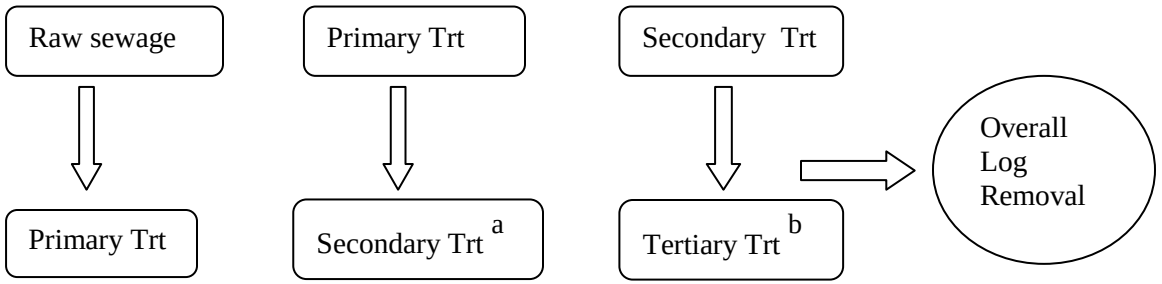
Samples[a]		*E. coli* ($\log_{10}$ CFU/100 mL)	Enterococci ($\log_{10}$ CFU/100 mL)	*E. coli* ($\log_{10}$ cells/100 mL)	Enterococci ($\log_{10}$ cells/100 mL)	*B. thetaiotaomicron* ($\log_{10}$ cells/100 mL)
RS [b]	Mean	6.21	5.72	6.46	6.63	7.26
	Std dev	0.26	0.42	0.59	0.51	0.24
PE [c]	Mean	6.17	5.58	6.48	6.75	7.31
	Std dev	0.26	0.27	0.72	0.40	0.41
SE [d]	Mean	1.01	0.64	3.05	4.13	4.19
	Std dev	0.84	0.91	0.95	0.84	0.42
TE [e]	Mean	1.04	0.75	2.82	3.59	3.67
	Std dev	0.93	1.01	1.19	1.12	0.60

[a] n=54 for each treatment location

[b] RS- Raw sewage, [c] PE- Primary effluent, [d] SE-secondary effluent, [e] TE-Tertiary effluent

The overall average $\log_{10}$ reductions through the waste water treatment process and $\log_{10}$ reduction of each target cell during each step of treatment by cultivation and qPCR methods are shown in Figure 4.2. *E. coli* concentrations as evaluated by membrane filtration were found to show the highest overall $\log_{10}$ reduction of 5.17, followed by enterococci CFU with an average $\log_{10}$ reduction of 4.97. By qPCR, the overall $\log_{10}$ reductions were 3.64 for *E. coli*, 3.59 for enterococci and 3.03 for *B. thetaiotaomicron*. Primary treatment process of raw sewage did not significantly change the concentrations of any target organismss. During secondary treatment process, there was a significant reduction in all bacteria. There was an average $\log_{10}$ removal of 5.16 and 4.94 for *E. coli* and enterococci CFU concentrations, respectively by the secondary treatment process. The $\log_{10}$ reductions in qPCR equivalent cell concentrations were 3.43, 2.62 and 3.12 for *E. coli*, enterococci and *B. thetaiotaomicron*, respectively during this treatment step. After this step, there were no significant reductions in any of the targets during tertiary treatment.

Figure 4.2: Average $\log_{10}$ reductions of *E. coli*, e*nterococci* CFUs and cell equivalents *and B. thetaiotaomicron* cell equivalents by waste water treatment.



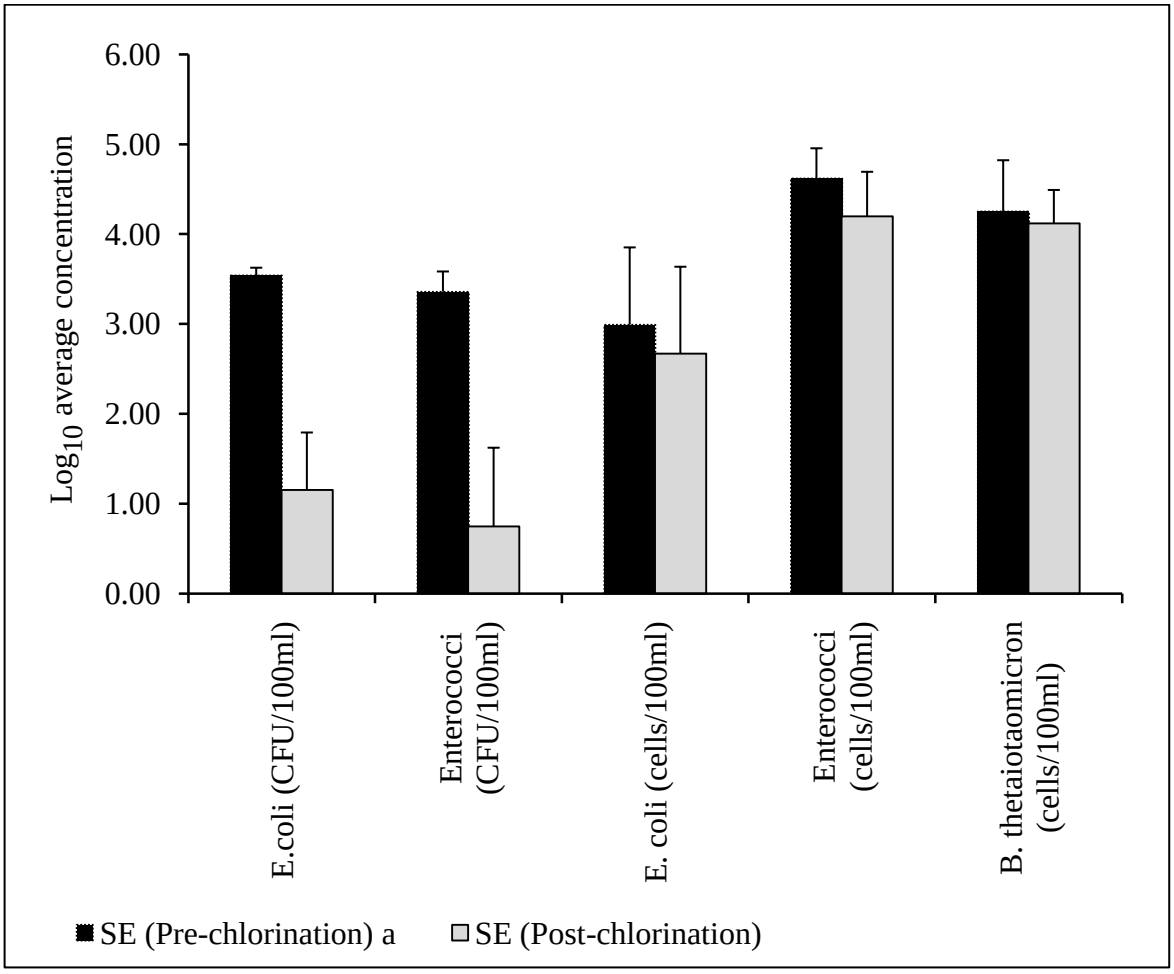
E. coli (CFU/100 mL)	0.04	5.16	-0.03	5.17
Enterococci (CFU / 100 mL)	0.14	4.94	0.11	4.97
E. coli (cells/100 mL)	-0.01	3.43	0.23	3.64
Enterococci (cells/100 mL)	-0.13	2.62	0.54	3.59
B. thetaiotaomicron (cells/100 mL)	-0.05	3.12	0.52	3.03

[a] includes sedimentation and disnfection

[b] includes filtration

Of the 18 sampling events, 6 events included collection of samples after the secondary clarification prior to chlorination step, along with the other locations throughout the treatment plant by cultivation and qPCR methods in order to examine specifically the disinfection step (Figure 4.3). During secondary activated sludge treatment prior to the chlorination step, there was a significant reduction in all target organisms. There was an average log removal of 2.75 and 3.11 for *E. coli* concentrations by cultivation method and qPCR respectively. For enterococci, the log reduction was 2.42 and 1.88 by cultivation method and qPCR respectively. The highest log removal in this stage was found to be for *B. thetaiotaomicron*, at a value of 3.31, as measured by qPCR. When chlorine was added to the effluent, there was a further significant reduction in the average concentrations; 2.42 for *E. coli* and 2.63 for enterococci as measured by the cultivation method. But as expected by qPCR methods, this reduction was only 0.31 and 0.40 for *E. coli* and enterococci respectively, and only 0.12 for *B. thetaiotaomicron* cells, none of which were found to be significant.

Figure 4.3: Average $\log_{10}$ concentrations of bacteria in secondary effluents before and after chlorination using cultivation and qPCR.



*number of samples, n=18 for each treatment location

[a] secondary treatment pre-chlorination involves activated sludge process and secondary clarification.

Covariate analysis indicated that to determine the correlation between *B. thetaiotaomicron* and each conventional indicator, analysis had to be done on the untreated (raw sewage and primary effluent) group and treated (secondary effluent and tertiary effluent) group of samples separately. However, correlation between *B. thetaiotaomicron* and conventional indicators was also determined using pooled data from 216 samples from waste water treatment plant at all four locations to see the trend in the overall process. Significant correlations between each of the conventional indicators and *B. thetaiotaomicron* were observed using the pooled data. Table 4.5 presents Pearson's correlation coefficients for both the conventional indicators and *B. thetaiotaomicron* in treated and untreated groups as well as pooled data.

Correlation coefficient (r) between *E. coli* and enterococci was 0.9, correlation coefficient between *E. coli* and *B. thetaiotaomicron* was found to be 0.94 and between enterococci and *B. thetaiotaomicron* was 0.88 and these correlations were found to be statistically significant ($p<0.05$).

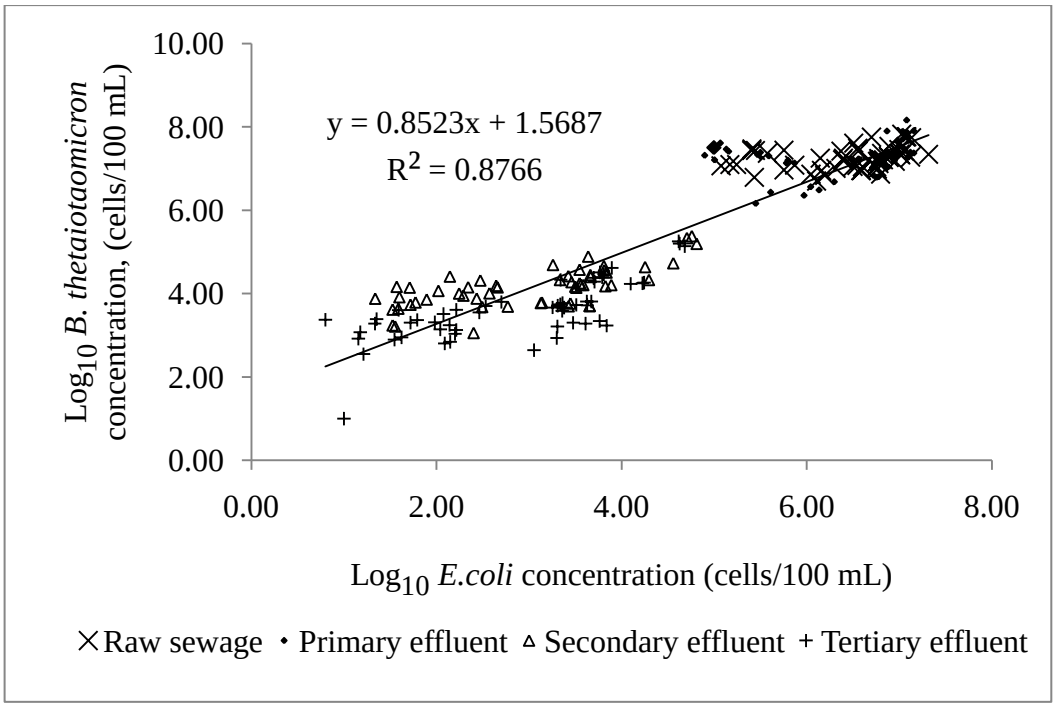
Table 4.5: Pearman's correlation between conventional indicators and *B. thetaiotaomicron* in treated, untreated groups of data and pooled data by qPCR

Pearson's correlation coefficient (R)			
Indicator bacteria	Untreated [a]	Treated [b]	Pooled [c]
E. coli	0.33	0.66	0.93
Enterococci	0.37	0.53	0.88

[a] raw sewage and primary effluent data combined (n=108 samples)
[b] secondary and tertiary effluents data combined (n=108 samples)
[c] treated and untreated data combined (n=216 samples)

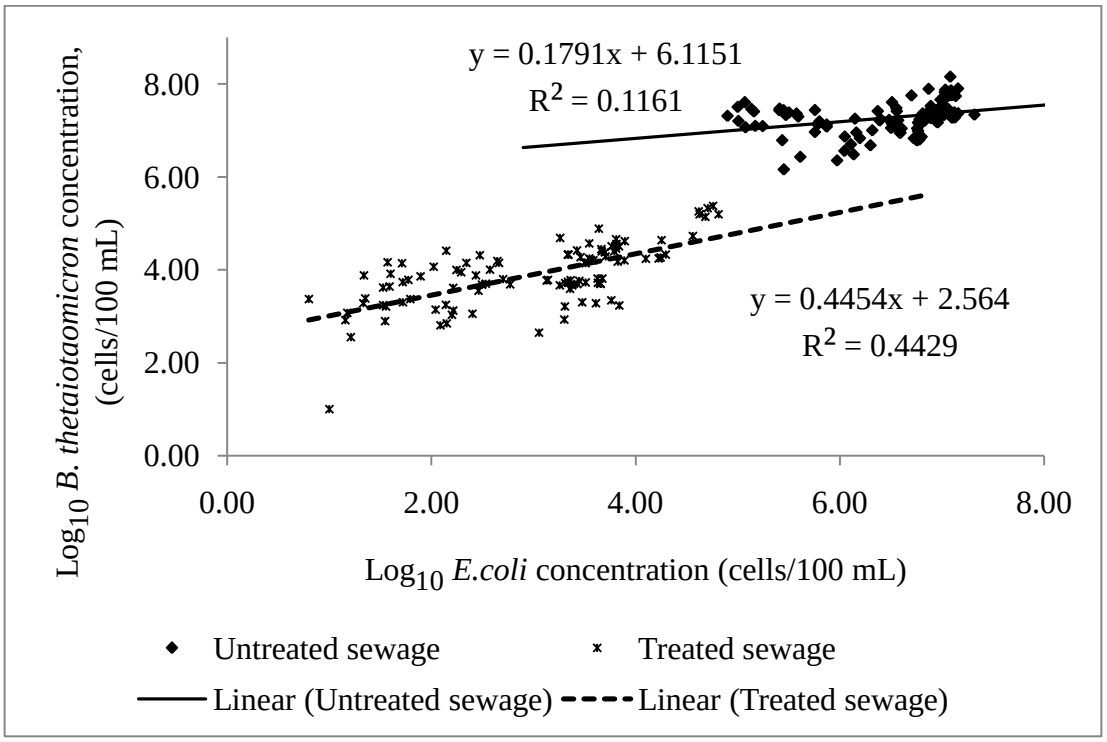
Regression analysis was used to test the strength of association between conventional indicators and *B. thetaiotaomicron.* The equation for each regression is displayed on each chart. Figure 4.4 shows scatter-plot for *E. coli* and *B. thetaiotaomicron* concentrations from pooled data from four treatments which displayed strong correlations with R^2=0.87 and figure 4.5 shows scatter-plots for *E. coli* and *B. thetaiotaomicron* concentrations for data from untreated and treated sets. For untreated data set, R^2 was only 0.11 and for treated data set, it was 0.44. R^2 ranges from 0 to 1 and values close to 1 denote strong association between variables.

Figure 4.4: Correlation between *E. coli* and *Bacteroides thetaiotaomicron* by qPCR in pooled data from wastewater treatment samples.



Treated and untreated data pooled, n=216

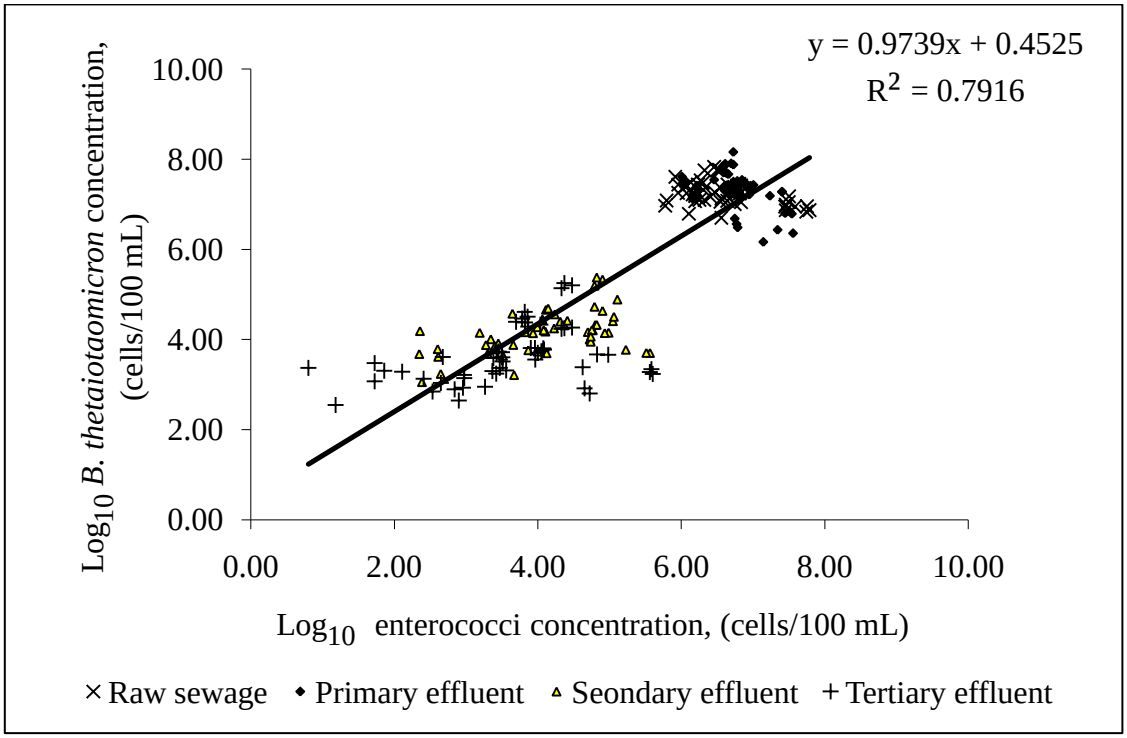
Figure 4.5: Correlation between *E. coli* and *B. thetaiotaomicron* by qPCR in untreated and treated groups of wastewater samples



n=108 samples (raw sewage and primary effluent data combined)
n=108 samples (secondary and tertiary effluents data combined)

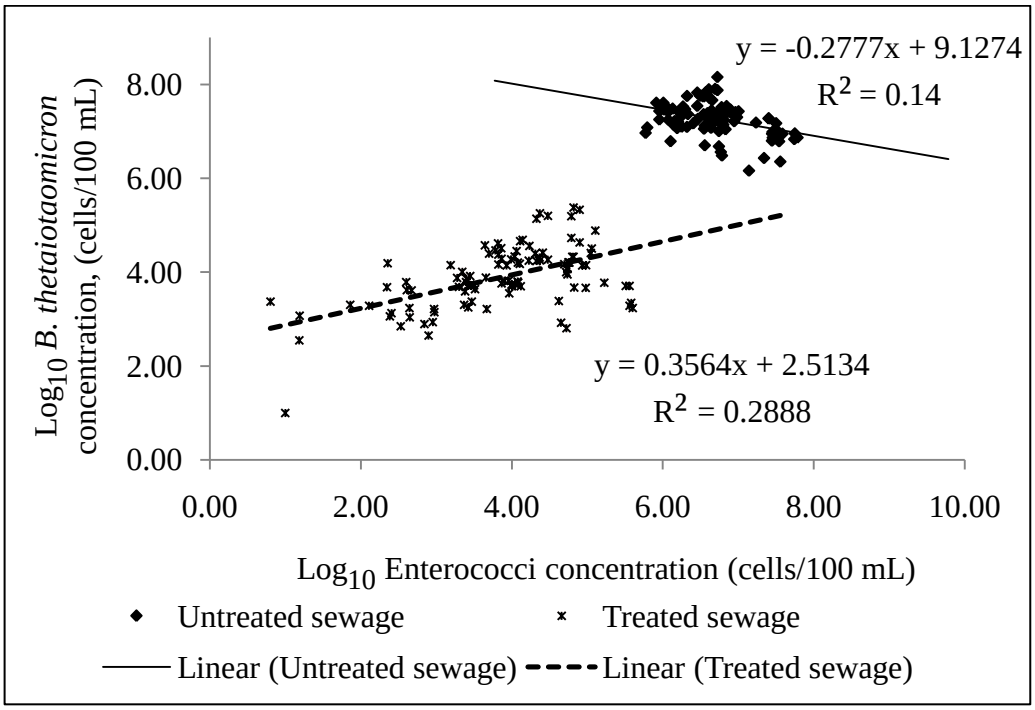
The scatter-plot for enterococci and *B. thetaiotaomicron* from pooled data from four treatments which displayed strong correlation with R^2=0.79 is displayed in figure 4.6 and figure 4.7 shows scatter-plots for enterococci and *Bacteroides* concentrations for data from untreated and treated groups of samples. For untreated data set, R^2 was only 0.14 and R^2 for treated group was 0.28.

Figure 4.6: Correlation between enterococci and *Bacteroides thetaiotaomicron* by qPCR in pooled data from wastewater treatement samples.



Treated and untreated data pooled, n=216

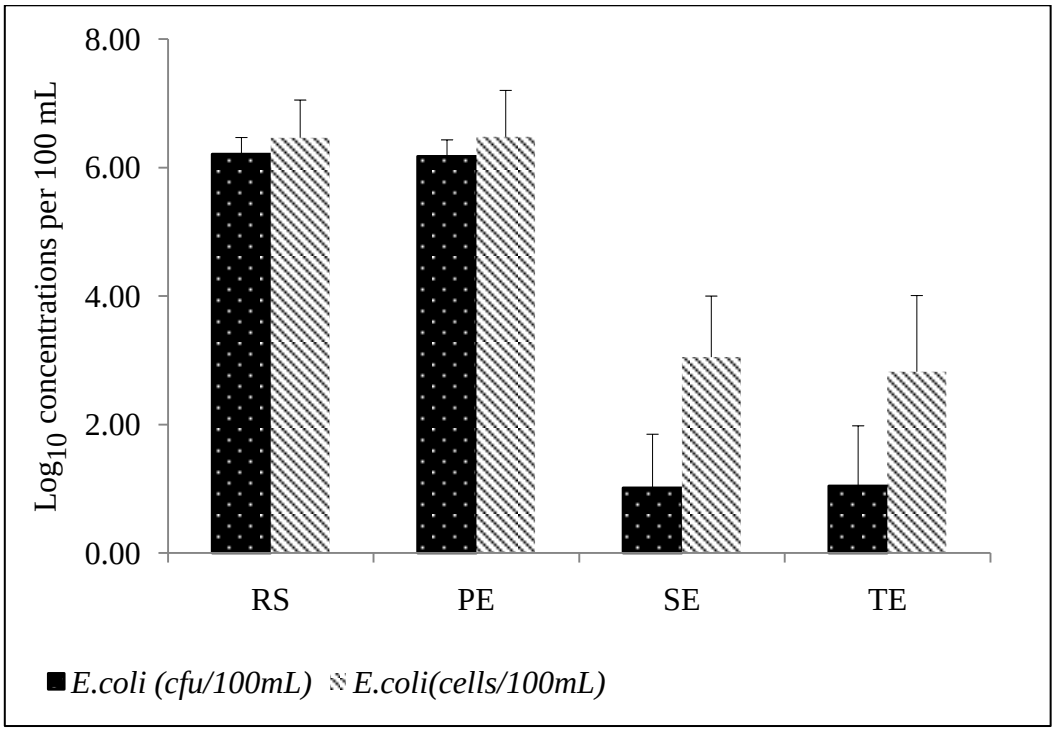
Figure 4.7: Correlation between enterococci and *B. thetaiotaomicron* by qPCR in untreated and treated data sets of wastewater samples



n=108 samples (raw sewage and primary effluent data combined)
n=108 samples (secondary and tertiary effluents data combined)

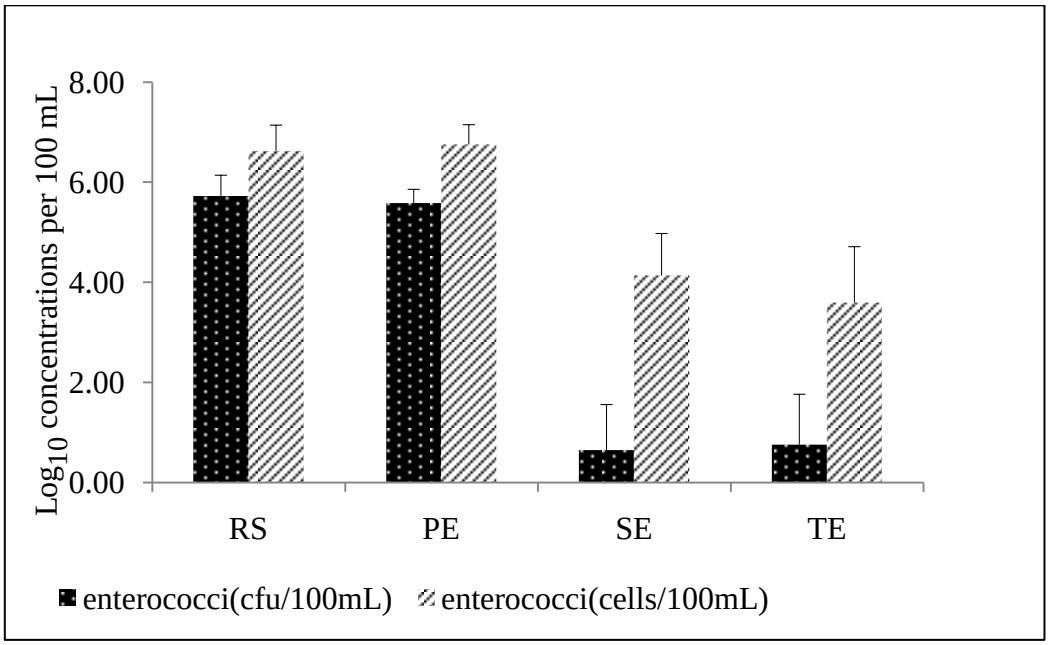
Figures, 4.8 and 4.9 contrast the average concentrations of *E. coli* and enterococci respectively, by CFU and qPCR throughout the treatment process. The mean $\log_{10}$ difference between concentrations of *E. coli* by cultivation methods and by qPCR prior to disinfection ranged between 0.25 and 0.31 whereas this difference in samples from post-disinfection step was around 1.78 to 2.04. The mean $\log_{10}$ difference between concentrations of enterococci by cultivation methods and that by qPCR prior to disinfection ranged between 0.81 and 1.17 whereas this difference in samples from post-disinfection step ranged from 2.84 to 3.49. There was a statistically significant difference in concentrations of *E. coli* between both cultivation and qPCR methods in all samples from wastewater treatment except raw sewage ($p<0.05$) and concentrations of enterococci differed significantly by both methods from all wastewater treatment samples ($p<0.05$).

Figure 4.8: Comparison between log_{10} transformed average concentrations of *E. coli* by cultivation based (CFU/100 mL) and qPCR based methods (cells/100 mL) in waste water treatment plant samples.



RS- Raw sewage
PE- Primary effluent
SE- Secondary effluent
TE- Tertiary effluent
Total number of samples, N=216 (n=54 for each treatment)

Figure 4.9: Comparison between log_{10} transformed average concentrations of enterococci by cultivation based (CFU/100 mL) and qPCR based methods (cells/100 mL) in waste water treatment plant samples.

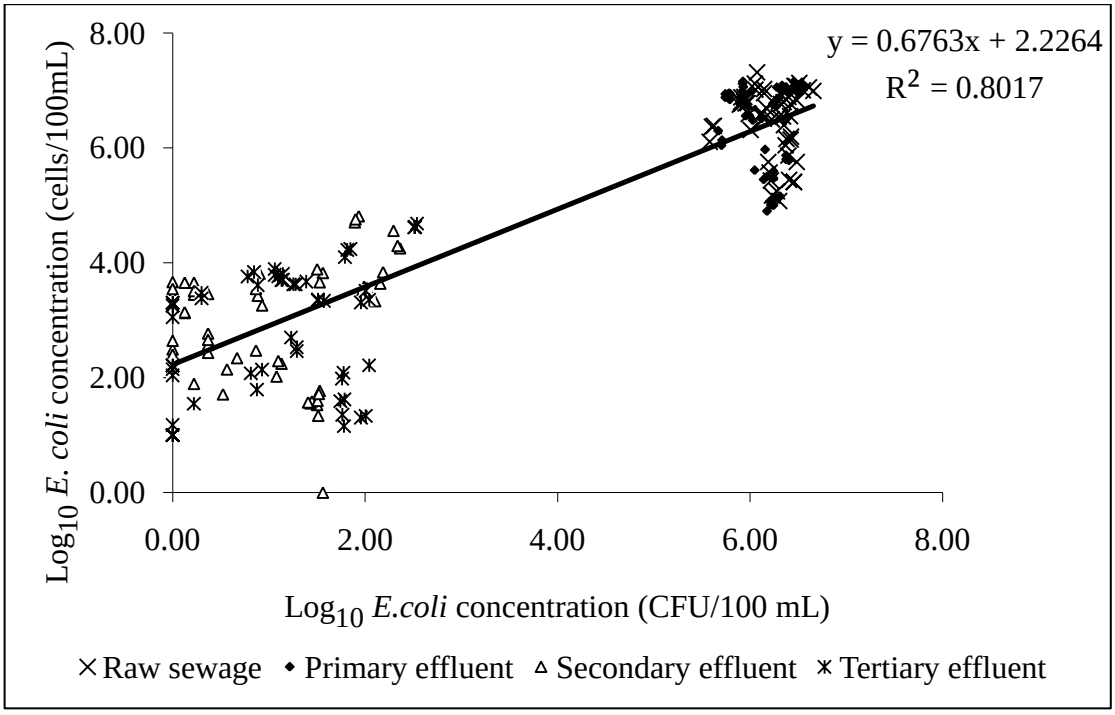


RS- Raw sewage
PE- Primary effluent
SE- Secondary effluent
TE- Tertiary effluent
Total number of samples, N=216 (n=54 for each treatment)

Correlation between concentrations by cultivation and qPCR methods for *E. coli* and enterococci in wastewater treatment plant samples were determined. There was a strong correlation between two methods when pooled data from all treatments was used for analysis for *E. coli* and enterococci, with R=0.89 and 0.87, respectively. Regression analysis was used to test the strength of this association. Figure 4.10 shows the scatter-plot for *E. coli* CFU/100mL by cultivation and *E. coli* cells/100mL by qPCR from pooled data from four treatments which displayed significant coefficient of determination, R^2=0.80 ($p<0.05$) and

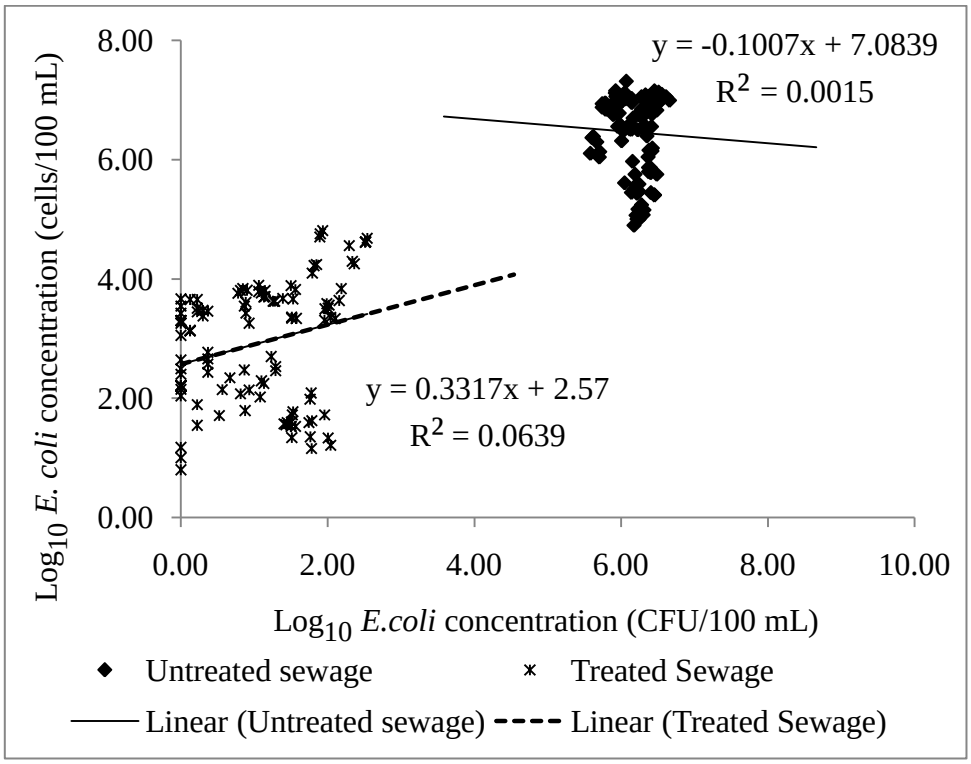
figure 4.12 shows the scatter-plot for enterococci concentrations by both methods with a lower but yet significant R^2 of 0.75 ($p<0.05$). This significance was due to the similar trends associated with reduction through the treatment process. However, there was no significant correlation found between both methods for concentrations of *E. coli* with data from untreated and treated sets (Figure 4.11). For untreated data set, correlation coefficient, R was only 0.01 and for treated data set, it was 0.24. Value of R close to +1 denotes positive correlation, close to -1 denotes negative correlation and that of zero denotes no correlation. A similar trend was observed for enterococci also with no significant correlation found between both methods with data from untreated, where R was only 0.01 and treated sets, where R was equal to 0.21 (Figure 4.13).

Figure 4.10: Correlation between concentrations of *E. coli* by cultivation methods (CFU/100 mL) and qPCR (cells/100 mL) in pooled data from wastewater treatment samples.



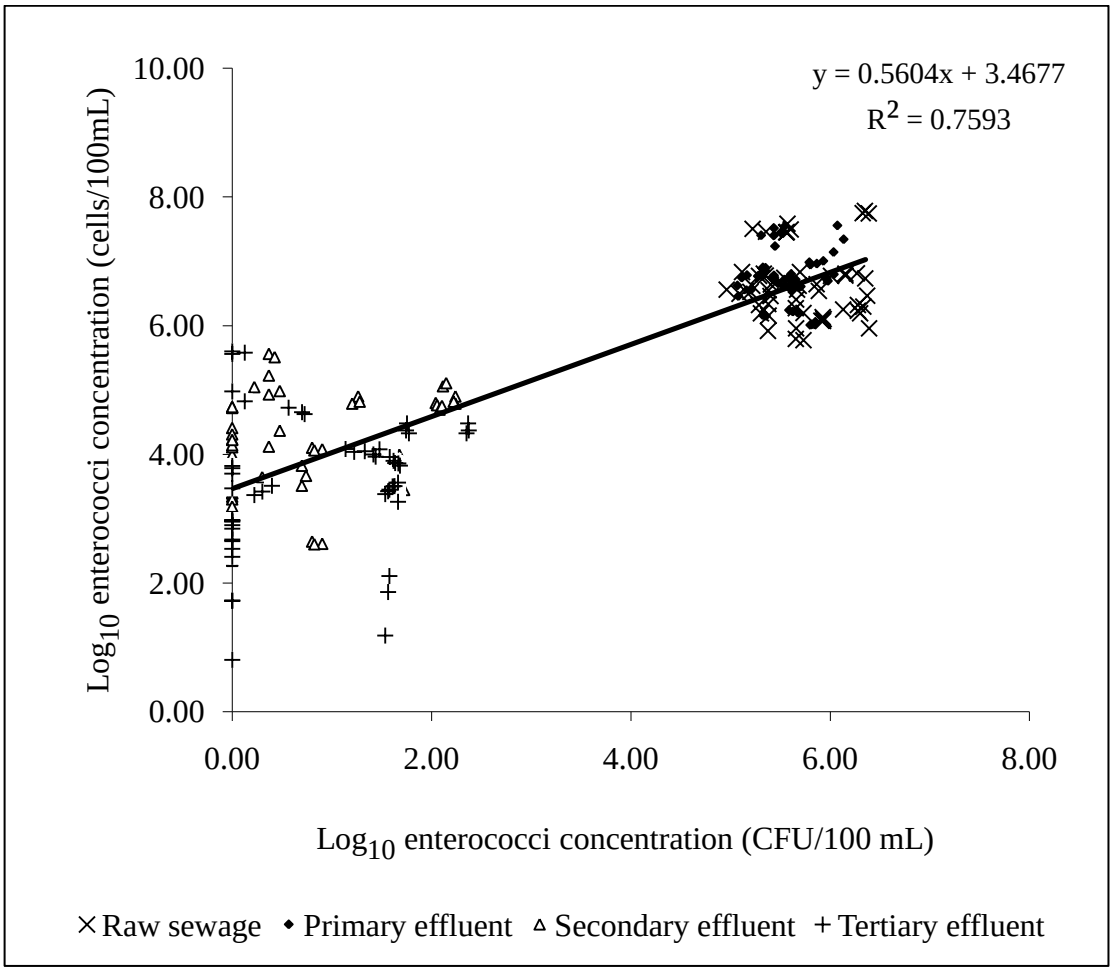
Total number of samples, n=216 (54 for each treatment)

Figure 4.11: Correlation between concentrations of *E. coli* by cultivation methods (CFU/100 mL) and qPCR (cells/100 mL) in untreated and treated data sets of wastewater samples



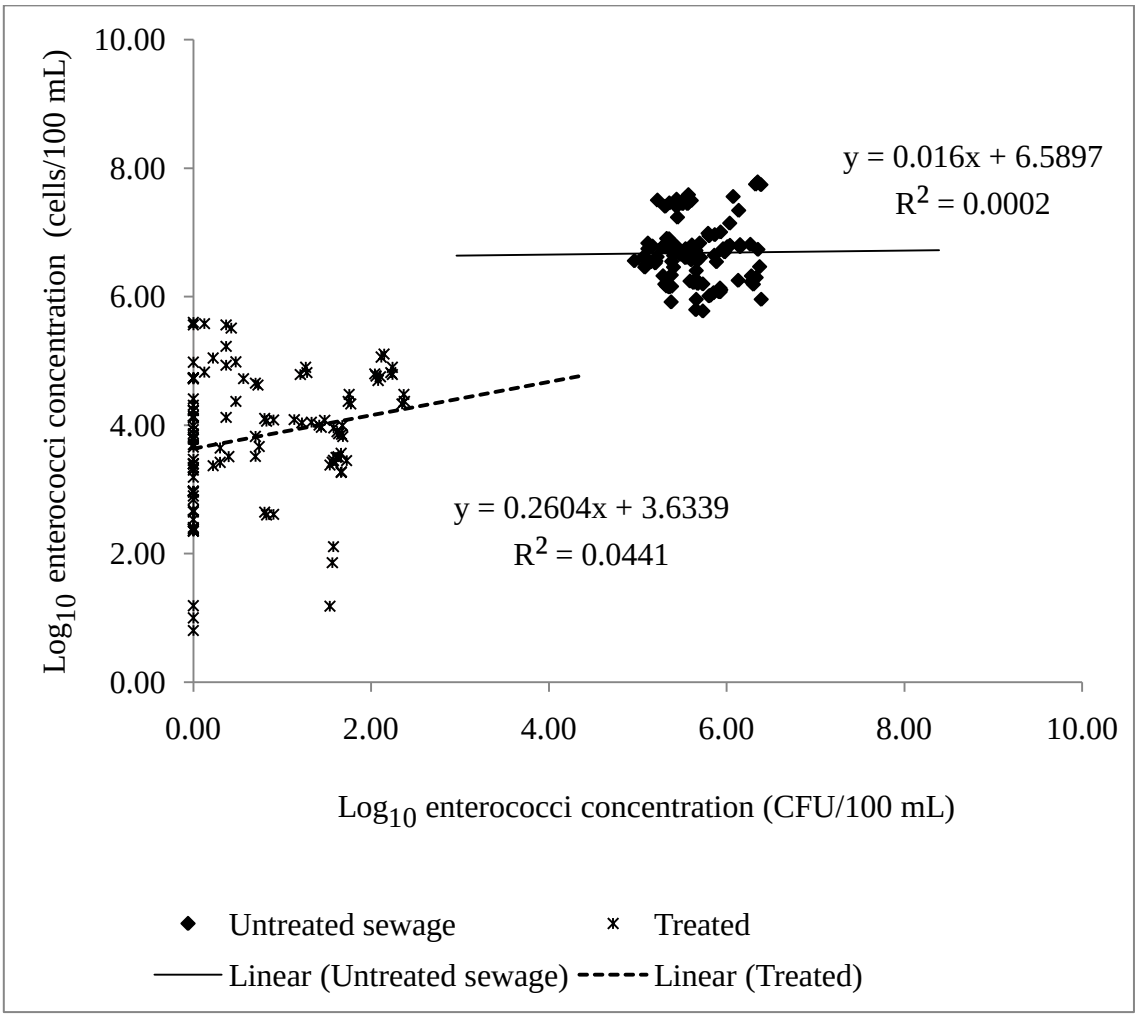
n=108 samples (raw sewage and primary effluent data combined)
n=108 samples (secondary and tertiary effluents data combined)

Figure 4.12: Correlation between concentrations of enterococci by cultivation methods (CFU/100 mL) and qPCR (cells/100 mL) in pooled data from wastewater treatment samples.



Total number of samples, N=216 (n=54 for each treatment)

Figure 4.13: Correlation between concentrations of enterococci by cultivation methods (CFU/100 mL) and qPCR (cells/100 mL) in untreated and treated data sets of wastewater samples



n=108 samples (raw sewage and primary effluent data combined)
n=108 samples (secondary and tertiary effluents data combined)

4.3.2 Septage samples

The average $\log_{10}$ transformed concentrations of *E. coli* and enterococci by cultivation methods and qPCR, and *B. thetaiotaomicron* by qPCR for a total of 48 samples (24 for each treatment) from the septage treatment plant are shown in Table 4.6.

The qPCR equivalent concentrations of *E. coli* in raw septage ranged from $6.80x10^6$ to $6.23x10^8$ cells/100mL, enterococci ranged from $3.72x10^6$ to $6.235x10^7$ cells/100mL and *B. thetaiotaomicron* were in the range of $1.19x10^7$ to $1.17x10^8$ cells/100mL. The final effluent, after the treatment had a range of concentration of $9.09x10^2$ to $5.92x10^4$ qPCR equivalent cells/100mL of *E. coli*, $4.23x10^4$ to $4.57x10^5$ cells/100mL of enterococci and $3.83x10^3$ to $3.67x10^5$ cells/100mL of *B. thetaiotaomicron* (Table 4.7). There was no significant difference between *B. thetaiotaomicron* and conventional indicators as measured by qPCR in raw septage and they were present in concentrations of $>10^7$ cells/100mL. *E. coli* and enterococci when measured by cultivation method were at least one $\log_{10}$ lower as compared to qPCR equivalent concentrations.

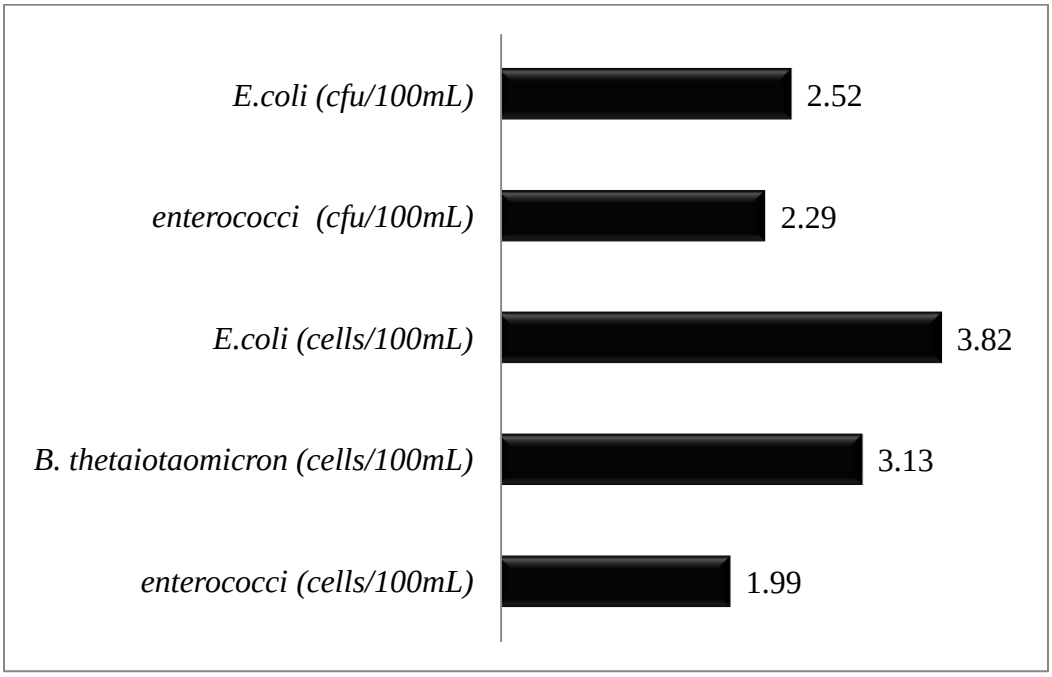
Table 4.6: Average concentrations of bacteria in raw septage and effluent by cultivation and qPCR methods.

Samples*		*E.coli* (log_{10} CFU/100mL)	enterococci (log_{10} CFU/100mL)	*E.coli* (log_{10} cells/100mL)	enterococci (log_{10} cells/100mL)	*B. thetaiotaomicron* (log_{10} cells/100mL)
Raw septage	Mean	6.47	6.36	7.33	7.31	7.55
	Std dev	0.45	0.82	0.68	0.36	0.34
Effluent	Mean	3.96	4.07	3.51	5.32	4.42
	Std dev	0.86	0.96	0.67	0.28	0.55

*Total number of samples, n=48 (24 samples for each treatment)

Following the treatment, there was a significant average $\log_{10}$ reduction in all target organisms, shown in Figure 4.14. The highest $\log_{10}$ reduction during treatment was found for *E. coli* qPCR equivalent cells with a $\log_{10}$ difference of 3.82 whereas the difference in $\log_{10}$ concentrations for *E. coli* as measured by cultivation methods was 2.52. However, this difference in $\log_{10}$ removals between both methods is due to the higher initial qPCR equivalent concentrations in raw septage. The log10 reduction for enterococci by cultivation methods was 2.29 whereas this difference was only 1.99 by qPCR. The $\log_{10}$ reduction for *B. thetaiotaomicron* was found to be 3.13

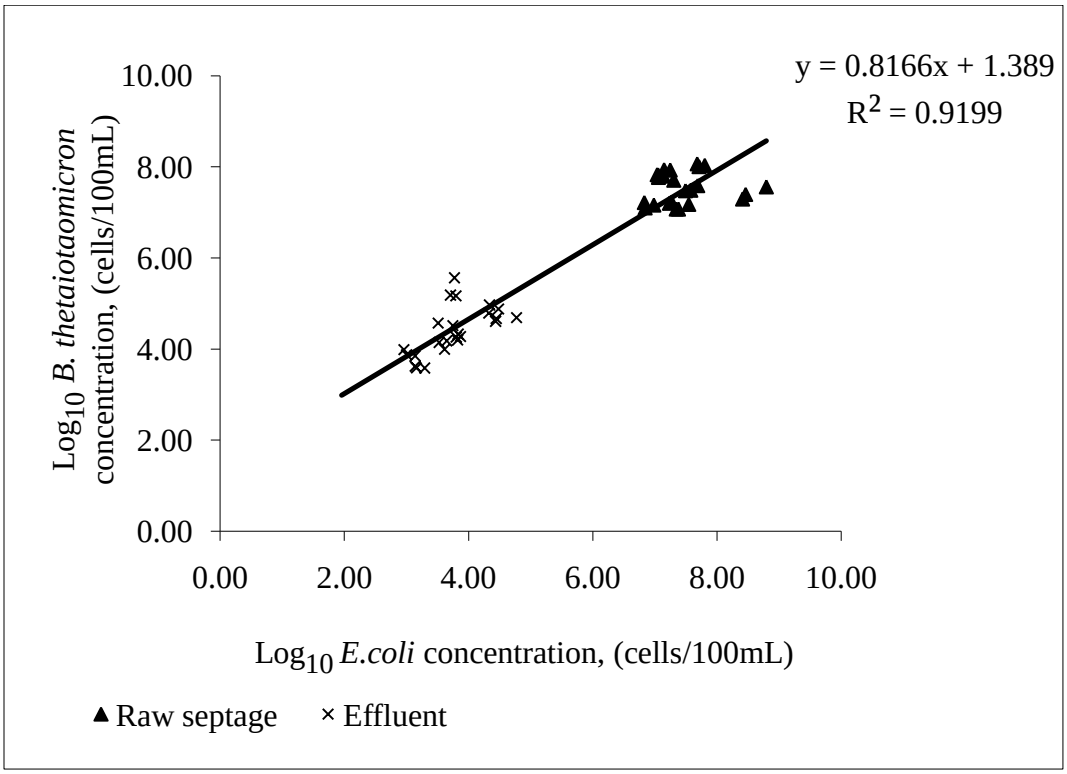
Figure 4.14: Average log reduction of *E. coli*, enterococci by cultivation and qPCR and *B. thetaiotaomicron* targets by qPCR during septage treatment process.



Number of samples, n=48 (24 samples from each treatment location)

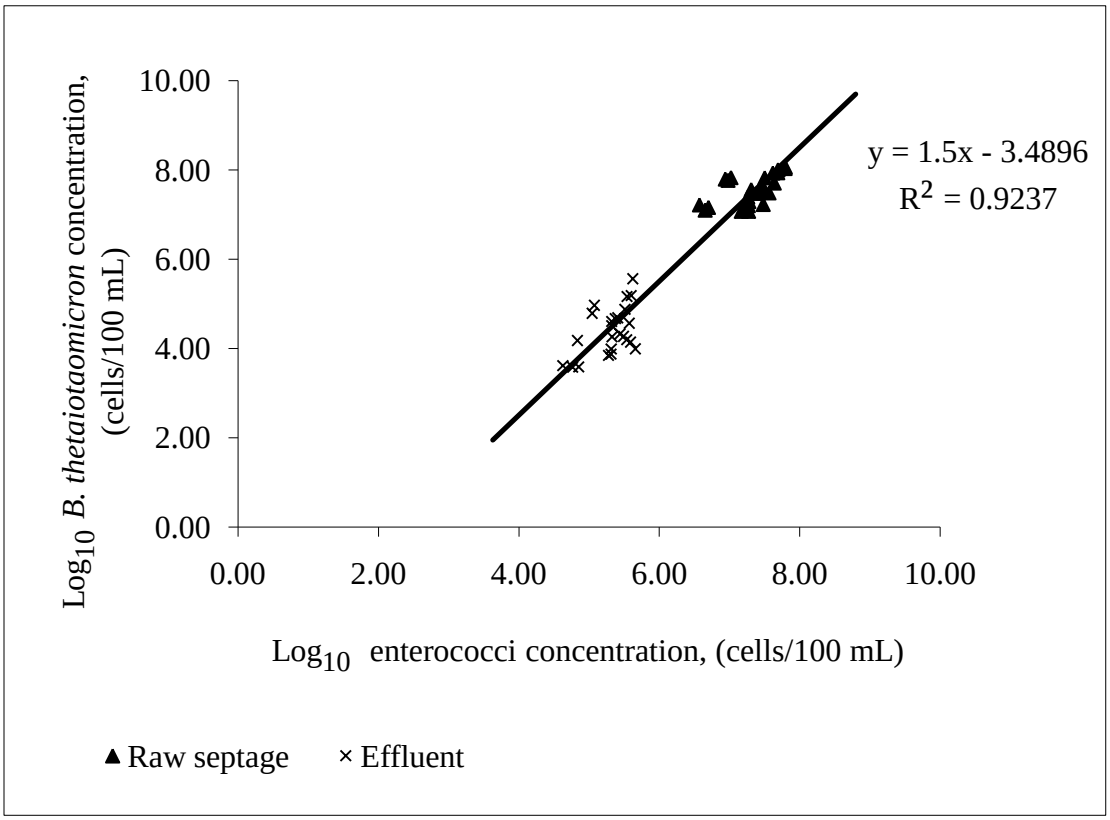
Regression analysis was used to test the strength of association between conventional indicators and *B. thetaiotaomicron* (Figures 4.15 and 4.16). The equation for each regression is displayed on each chart. *E. coli* and *Bacteroides* concentrations displayed strong correlations with R^2=0.91 and between enterococci and *Bacteroides* showed correlation with R^2=0.92; with only little scatter at the different sites.

Figure 4.15: Correlation between *E. coli* and *Bacteroides thetaiotaomicron* by qPCR in septage treatment plant.



Total number of samples, n=48 (24 samples from raw septage and 24 treated effluents)

Figure 4.16: Correlation between enterococci and *Bacteroides thetaiotaomicron* in septage treatment samples.

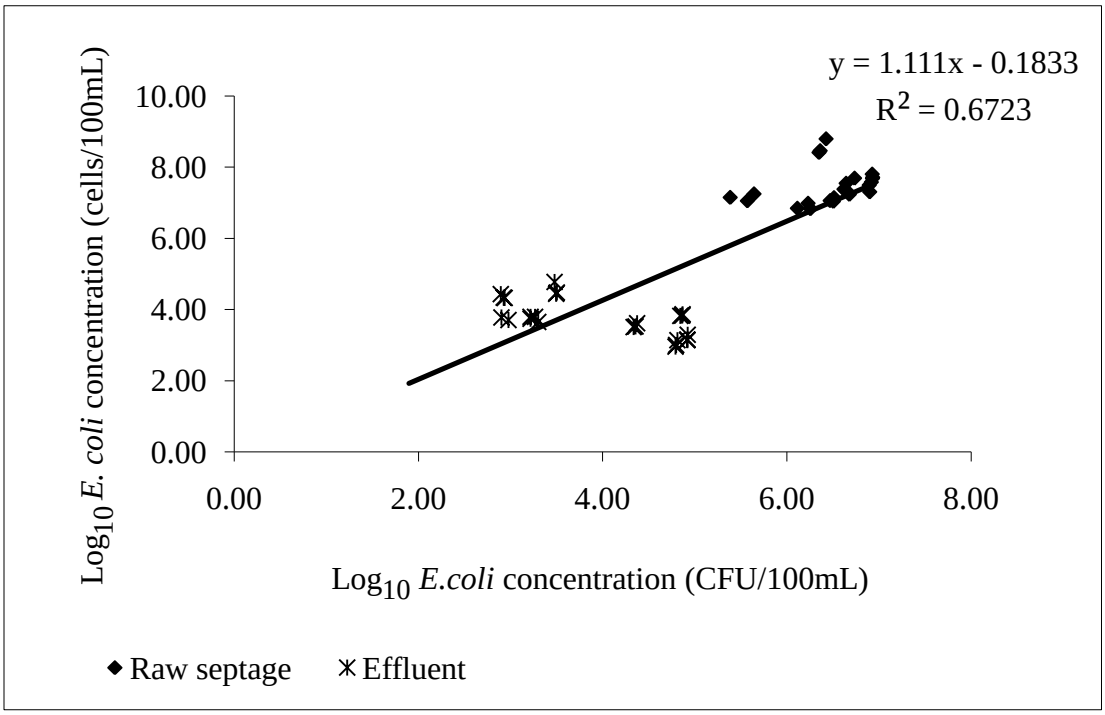


Total number of samples, n=48 (24 samples from raw septage and 24 treated effluents)

Correlation between concentrations of *E. coli* and enterococci present in pooled data from septage treatment plant samples were determined. Both the correlations were found to be significant with R^2=0.67 for *E. coli* and R^2=0.60 for enterococci. Regression analysis was used to test the strength of association between methods for *E. coli* and enterococci, as shown in Figures 4.17 and 4.19, respectively. This association was due to the similar trends associated with reduction through the treatment process which changes when raw septage and effluent samples are analyzed as two separate data sets as shown in

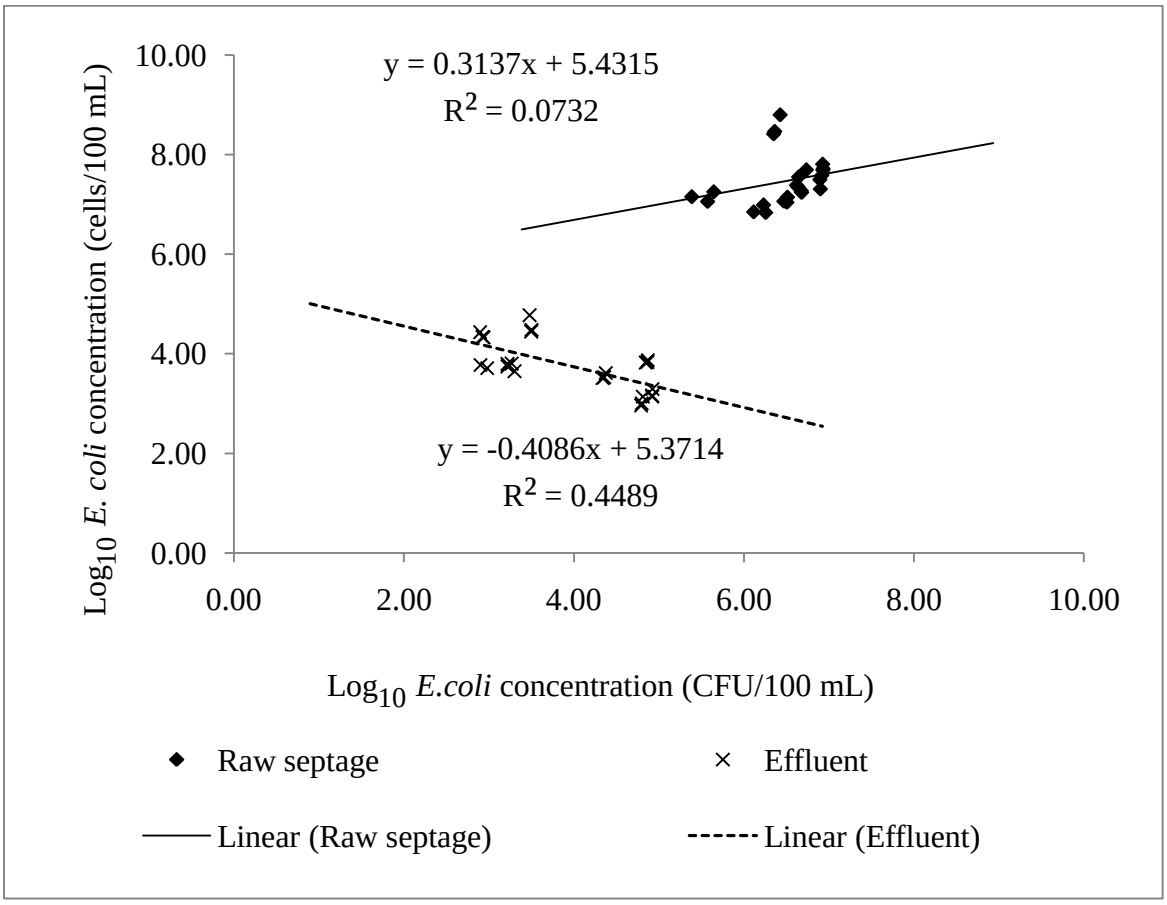
Figures, 4.18 and 4.20, there was a weak correlation between cultivation and qPCR methods for concentrations of *E. coli* with correlation coefficient, R=0.26 in raw septage and a significant correlation with R=0.66 ($p<0.05$) in effluent samples. However, for enterococci, there was no correlation between both methods in raw septage and effluent samples.

Figure 4.17: Correlation between concentrations of *E. coli* by cultivation methods (CFU/100 mL) and qPCR (cells/100 mL) in pooled data from raw septage and effluents.



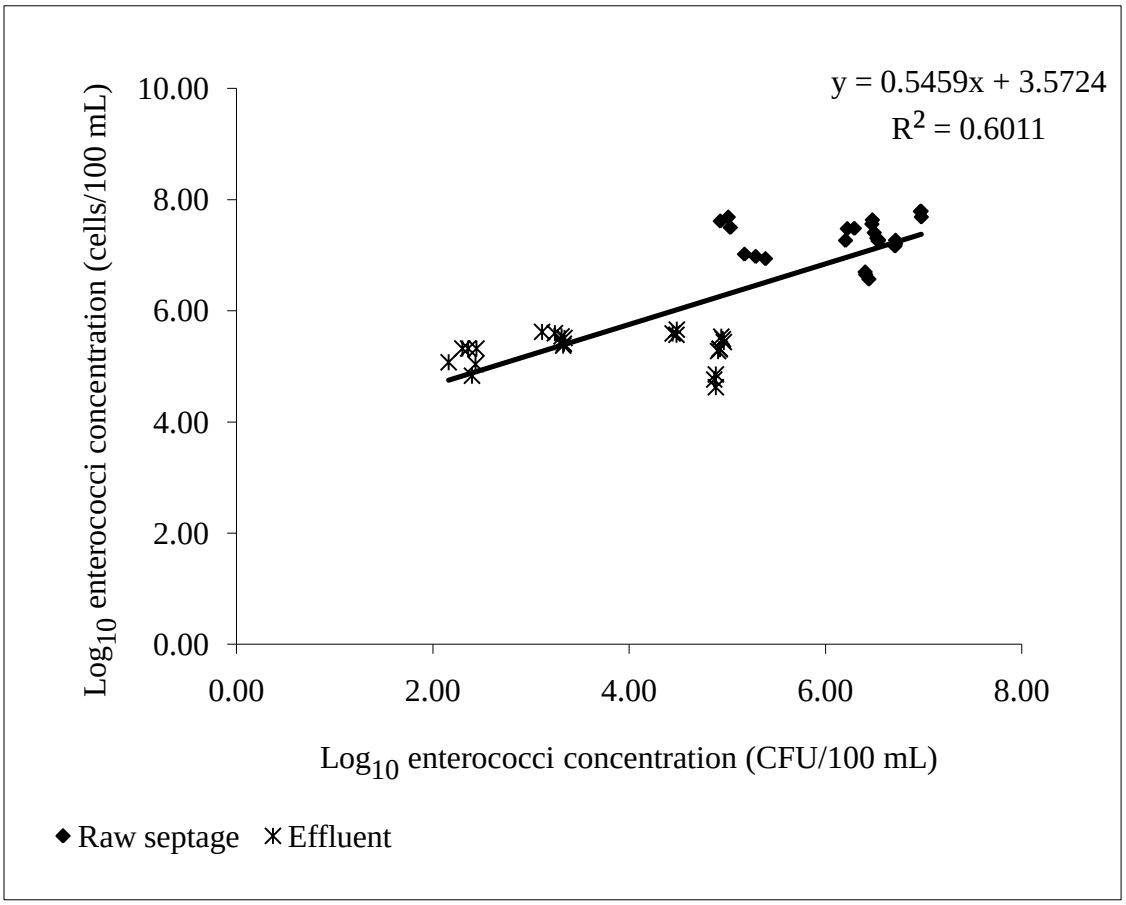
Total number of combined data from two sets, n=48 (24 samples from raw septage and 24 treated effluents)

Figure 4.18: Correlation between concentrations of *E. coli* by cultivation methods (CFU/100mL) and qPCR (cells/100mL) in two data sets from raw septage and effluents.



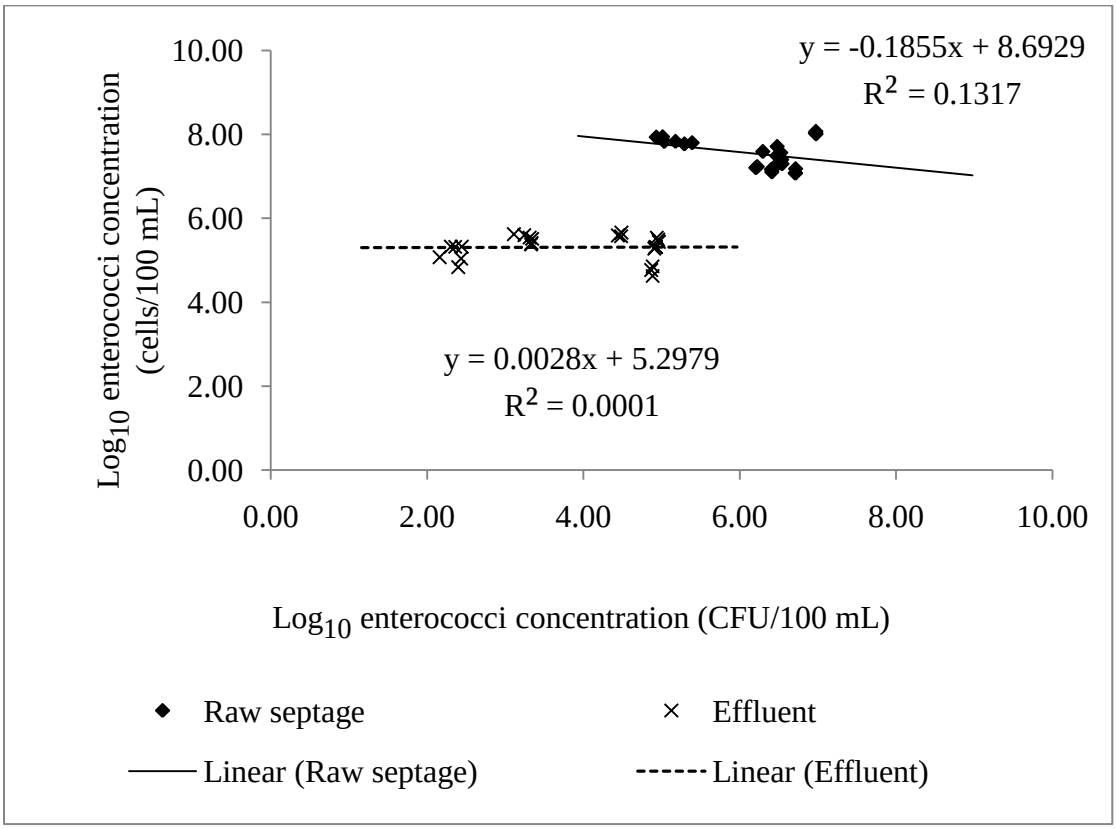
Number of samples, n=24 for each data series

Figure 4.19: Correlation between concentrations of enterococci by cultivation methods (CFU/100 mL) and qPCR (cells/100 mL) in pooled data from septage treatment samples.



Total number of combined data from two sets, n=48 (24 samples from raw septage and 24 treated effluents)

Figure 4.20: Correlation between concentrations of enterococci by cultivation methods (CFU/100mL) and qPCR (cells/100mL) in two sets of data from raw septage and effluents.



Number of samples, n=24 for each data series

4.4 Discussion

This study represents a characterization of human waste treatment using a novel MST target, *B. thetaiotaomicron* in comparison with the conventional indicators *E. coli* and enterococci. This study is the first to contrast raw sewage and septage and to study the fate of all of these bacterial molecular signals during waste treatment.

In this study, occurrence of unique human fecal specific marker, *B. thetaiotaomicron* in raw sewage and septage and its fate during their treatment process has been quantified. It was found that *B. thetaiotaomicron* qPCR equivalent cells were present in significantly higher concentrations than that of *E. coli* or enterococci in raw sewage and septage. No studies have quantified *B. thetaiotaomicron* levels in both these samples. Yampara-Iquise (2008) found a wide range of *B. thetaiotaomicron* from 6.88×10^2 to 1.07×10^9 cells in one gram of human feces, when stool samples from 10 human subjects were analyzed by qPCR targeting the α-mannanase 1-6 gene. This variation was attributed to the variation in DNA extraction efficiency.

Other human fecal MST markers such as the host-specific *Bacteroides-Prevotella* 16S rRNA gene markers have been detected in raw sewage and septage at concentrations of 10^8-10^9 cells per 100ml (Seurinck et al 2005, Silkie & Nelson 2009; Sercu et al 2009) whereas these markers were found in a wide range of concentrations from 10^5 – 10^{11} cells per gram of wet human feces (Seurinck et al, 2005; Okabe et al, 2007; Sercu et al, 2009). There is still some speculation about cross reactivity of these human specific 16S rRNA genetic markers with cat and dog feces (Kildare et al, 2007). Conversion of 16S rRNA gene copies into cell counts is not accurate as copy number of *Bacteroides-*

Prevotella 16S rRNA varies in different species of the genera. Therefore, comparison between *Bacteroides* 16S rRNA human marker and *B. thetaiotaomicron* α-mannanase 1-6 gene, where one copy of gene represents one cell, may not be the best approach for determining the sensitivity of these markers.

E. coli was found to occur at concentrations of 10^6 target gene copies per 100ml of raw sewage in this study which is almost three logs lower than the concentrations reported by another study who found 10^9 copies of *lacZ* gene targets for *E. coli* by qPCR in the same volume of raw sewage (Wery et al. 2008). But in five other studies, qPCR cell concentrations of *E. coli* or enterococci in raw sewage agree with previous reports on the concentrations of both these indicator organisms in raw sewage by qPCR assays targeting *uidA* (Table 4.7). One possibility for such high numbers of *E. coli* found by Wery et al. could be due to the cross amplification of the *E. coli lacZ* qPCR assay with other bacterial strains that harbor this gene. The specificity of the assay was tested with only four bacteria during the assay development of this PCR assay (Foulds et al. 2002).

Enterococci qPCR assays published before have mostly targeted 23S rRNA gene using the same primer sets as used in this study and have found similar concentrations of target gene copies in raw sewage. Log_{10} removal of enterococci qPCR signals across the wastewater treatment found in this study is almost similar to the study by Varma et al. (2009) (Table 4.7).

Strong correlation between conventional indicators and *B. thetaiotaomicron* markers in both sewage and septage samples was observed. This relationship started to diminish after qPCR signals were reduced following secondary treatment. Strong correlations

between *Bacteroides* 16S rRNA human markers and indicators such as total and fecal coliforms or *E. coli* have been observed before with untreated sewage samples (Savichtcheva et al. 2007; Dick and Field. 2004). This lack of correlation between facultatively anaerobic *E. coli* or enterococci and obligately anaerobic *Bacteroides sp* in treated effluent could be due to the difference in their removals by activated sludge, but more importantly a background signal of DNA is found in effluents. Summary of all studies that have monitored raw sewage using qPCR for indicators and MST is given in the Table below.

Table 4.7: Summary of all qPCR applications to monitor the wastewater and treated effluents in the literature

Treatment	Sampling	Samples, n=	*E. coli* [x] Target gene*	Enterococci [x] Target gene*	*Bacteroides* Human Marker [x] Target gene*	References
			uidA*	23S rRNA*	alpha-mannanase* [a]	
RS	Grab	54	2.88E+06	1.71E+07	1.82E+07	
PE	Grab	54	3.02E+06	2.25E+07	2.04E+07	
SE (Pre-cl)	Grab	18	1.01E+03	1.68E+05	1.84E+04	This study
SE (Post-cl)	Grab	54	1.12E+03	5.40E+04	1.55E+04	
TE	Grab	54	6.61E+02	1.56E+04	4.68E+03	
Septage	Grab	24	2.14E+06	8.17E+07	3.55E+07	
STE	Grab	24	3.24E+03	8.36E+05	2.63E+04	
					16S rRNA [b]	
RS	Grab	9		2.76E+07	1.75E+09	
PE		9	-	3.71E+07	1.59E+09	
SE (Pre-cl)		9		4.12E+05	3.47E+07	Varma et al. 2009
SE (Post-cl)		9		1.58E+05	3.17E+07	
			uidA*	23S rRNA*	16S rRNA [b]	Silkie and Nelson
RS	Grab	12	2.00E+07	1.00E+07	7.94E+08	2009
					16S rRNA [b]	
RS	Grab	3	-	-	7.8E+08	Sercu et al. 2009
Septage	Grab	3	-	-	3.9E+08	
			uidA*			
RS	Grab	5	-	-	-	Lavender and
PE	Grab	5	-	-	-	Kinzelman 2009
TE (UV disinfected)	Grab	5	-	-	-	
			2.85 [c]	2.59 [c]		

Table 4.7 (cont'd)

			lacZ*			
RS	composite	6	1.34E+09			
PE		6	1.05E+09			Wery et al.2008
SE (Pre-cl)		6	2.95E+05	-	-	
					16S rRNA[b]	
RS	Grab	12	-	-	2.10E+05	Savichtcheva et al. 2007
PE	Grab	12			2.90E+05	
					16S rRNA[b]	
RS	Grab	4	-	-	1.72E+10	Seurinck et al.2006
			uidA*			
RS	Grab	3	1.66E+07	-	-	
TE (UV disinfected)	Grab	3	6.12E+02			Lee et al. 2006

[x] qPCR target gene copies/100mL and

[a] *B. thetaiotaomicron*

[b] Order Bacteriodales

[c] Log_{10} difference between raw sewage and Tertiary effluent (Initial raw sewage or final effluent concentrations not reported)

RS-Raw sewage
PE=Primary effluent after grit removal
SE(Pre-cl)=Secondary effluent from secondary clarifier prior to chlorination
SE(Post-cl)=Secondary effluent after disinfection
TE= Teritiary effluent after filtration or UV disinfection
STE=septage treated effluent

During primary treatment in wastewater treatment process, there was no significant change in the concentrations of any of the targets. Primary treatment of sewage primarily allows heavy solids to settle down when oil, grease float to the surface which is then skimmed off and settled particles are also removed. Another study that monitored treatment process for different indicators found that enterococci concentrations were reduced by 0.4 $\log_{10}$ units during primary sedimentation step (Lucena et al, 2004). A recent case study in Netherland that monitored the changes in effluent when raw sewage was directly bypassed to further processes without primary settling observed that there was no improvement in the quality of effluent nor did it improve removal efficiencies-rates and microbiology populations when the influent was loaded with particulate matter (Puig et al, 2010).

Significant reduction in all targets (both cultivable and qPCR equivalent cells) were observed during secondary treatment process comprising of biological process (activated sludge), sedimentation and disinfection (chlorination). Most of the molecular signals were lost during secondary treatment before disinfection due to biological and sedimentation process. Reduction of bacteria during activated sludge process is due to several factors such as attachment to solids, sedimentation and subsequent removal in sludge, predation by protozoa, alterations in parameters such as pH, temperature, and competition with naturally occurring microbes. In many places, sludge is recycled by land application but this also imposes serious health risks to humans and animals due to the presence of many pathogenic microorganisms (Gerba and Smith, 2005). Based on evaluation of treatment steps in this study, it is estimated that approximately 10^4 CFU/ml of *E. coli* or enterococci could be ending up in sludge. This is a rough estimate as other

mechanisms such as predation, inactivation etc also play a role in reduction of cells. Previous sludge monitoring studies show varying concentrations of *E. coli* in sludge; 10^4 CFU/g wet weight (Payment et al, 2001) 10^5-10^6 cu/g dry weight (Pourcher et al, 2005). Results in this study suggest that qPCR equivalent cells may be found in higher concentrations in sludge as compared to cultivable cells. So, typically when qPCR is applied to monitor sludge it should detect higher concentrations of bacterial targets than cultivation methods. A previous study that monitored liquid sludge detected 3.7 log higher concentrations of *E. coli* by qPCR method than by the cultivation based method (Wery et al, 2008). The findings in this study support the previous recommendation of proper treatment of sludge for the inactivation of pathogens before land application.

It was also observed that filtration did not additionally remove any of the targets tested. The treatment plant uses rapid sand filters; slow sand filters are found to be more efficient in removing pathogens than rapid sand filters (Pundsnack, 2001). The efficiency of rapid sand filtration in physically removing microorganisms and suspended particles during waste water treatment process is enhanced when coagulants are added. One study found a positive relationship between removal of pathogens such as *Cryptosporidium* and enhanced coagulation conditions (Dugan et al. 2001). In the absence of coagulants, particle size remains small due to lack of chemical modifications, thus increasing the probability of penetration through the pores in the filter bed. As there is no floc formed in the filter due to the absence of coagulants, microorganisms are also not retained efficiently in the filters thus increasing their concentrations in final effluents (Koivunen et al. 2003).

Under the Clean Water Act of 1972, wastewater treatment plant discharges into streams have to be monitored to meet the permitted levels of pollutants specified in National Pollutant Discharge Elimination System (NPDES). Wastewater treatment plants use fecal coliforms to monitor the treatment efficacy. Even though *E. coli,* a fecal coliform is reduced during the treatment process, other coliforms such as *Klebsiella, Enterobacter,* and *Citrobacter* have been found to proliferate during wastewater treatment processes and in effluents which can lead to large numbers of numbers of fecal coliforms in wastewater effluents. Wastewater effluents alone may not be responsible for high fecal coliform counts in surface water and any other fecal sources located upstream of the plant could also be contributing the bacteria in to the streams.

Fecal coliforms were considered for monitoring recreational water quality after epidemiological studies conducted by U.S. Public Health Service (PHS) who found that any increase in the levels of fecal coliforms could possibly be used as a "warning signal" (Stevenson, 1953) and National Technical Advisory Committee commissioned by Federal Water Pollution Control Administration in 1968 recommended fecal coliforms to be used for monitoring recreational water quality (USEPA 1976). Later, U. S. EPA adapted *E. coli* and enterococci as new water quality criteria based on other epidemiological studies conducted in marine and fresh water beaches to find the relationship between these indicator levels and swimming related illnesses (USEPA 1986). The Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000 are currently exploring new and rapid technologies such as qPCR to monitor water quality at recreation beaches (Environmental Protection Agency, 2000).

Thus, recreational water quality regulations are improvised based on epidemiological studies or with development in technologies. However, other programs under CWA such as NPDES permits still use fecal coliforms as criteria with levels not to exceed 200 CFU/mL based on the geometric mean of five or more samples from wastewater discharges collected over a 30-day period. As USEPA is in the process of implementing new and revise water quality criteria for recreational water, changes are necessary in NPDES indicator bacteria permits for discharges from wastewater plants and other point sources.

Wade et al (2006) found a strong correlation between enterococci qPCR daily average concentrations and swimming related gastrointestinal illnesses in two of the Great Lakes beaches they studied by applying the 23S rRNA qPCR assay for enterococci. According to their study, a $\log_{10}$ increase in the daily average of qPCR cell equivalents of enterococci was associated with 1.30 (95% CI, 1.08–1.57) increase in the odds of gastrointestinal illness for any contact with water. The types of enterococci concentrations that were found at the beaches ranged from 1.90 to 2.04 $\log_{10}$ qPCR cell equivalents per 100ml. Wastewater effluents were suspected as impacting these beaches. The discharged effluents from wastewater treatment plant in this dissertation study had a $\log_{10}$ qPCR cell concentration of 3.59 per 100ml of enterococci. The *B. thetaiotaomicron* qPCR equivalent cell levels ($\log_{10}$ average of 3.67 cells/100mL) were almost in the same levels as that of enterococci in 100mL of treated final effluents. These numbers would be expected to be reduced by dilution of sewage effluent into rivers and could even be undectable levels by the time the surface water at the discharge site flows to the

recreational waters depending on the distance and the dilution between recreational beaches and wastewater treatment locations. Other factors such sunlight degradation of DNA. However, following a CSO or SSO event the levels of these signals would be very high.. High numbers of qPCR target signals detected in the effluents in this study and correlations found in the epidemiological study by Wade et al. suggest that viability may not be an issue in applying qPCR tools in monitoring water environment for both enterococci and *Bacteroides* to protect public health risk.

In conclusion, this study found that *Bacteroides thetaiotaomicron* qPCR equivalent cells were present in significantly higher concentrations than that of *E. coli* or enterococci in raw sewage ($p<0.05$) and in same concentrations in septage. High concentrations of human specific *Bacteroides thetaiotaomicron* DNA markers were present throughout the wastewater treatment processes. There was a significant correlation between this MST marker and each of the conventional indicators throughout the waste treatment process for both raw sewage and septage ($p<0.05$). Effluents discharged from wastewater treatment plants have 2-3 $\log_{10}$ of qPCR signals of both the indicators and a human marker. Overall, this study has evaluated the tools that can be efficiently used in monitoring wastewater treatment processes and has provided the scientific knowledge that may be useful as new regulations are made to protect public health.

REFERENCES

4.5 References

Avelar, K. E. S., S. R. Moraes, L. J. F. Pinto, W. Souza, R. Domingues, and M. C. D. Ferreira. 1998. Influence of stress conditions on Bacteroides fragilis survival and protein profiles. Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases **287:**399-409.

Bernhard, A. E., and K. G. Field. 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. Applied and Environmental Microbiology **66:**4571-4574.

Daly, K., and S. P. Shirazi-Beechey. 2003. Design and evaluation of group-specific oligonucleotide probes for quantitative analysis of intestinal ecosystems: their application to assessment of equine colonic microflora. Fems Microbiology Ecology **44:**243-252.

Dick, L. K., and K. G. Field. 2004. Rapid estimation of numbers of fecal Bacteroidetes by use of a quantitative PCR assay for 16S rRNA genes. Applied and Environmental Microbiology **70:**5695-5697.

Dugan N. R, Fox K. R, Owens J. H, Miltner R. J. 2000. Controlling *Cryptosporidium* oocysts using conventional treatment. JAWWA. 92:64–76.

Elmund, G. K., M. J. Allen, and E. W. Rice. 1999. Comparison of Escherichia coli, total coliform, and fecal coliform populations as indicators of wastewater treatment efficiency. Water Environment Research **71:**332-339.

Environmental Protection Agency. 2000. Improved enumeration methods for recreational water quality indicators: Enterococci and *Escherichia coli*. Washington, DC: Office of Science and Technology, EPA/821-R-97/004.

Field, K. G., A. E. Bernhard, and T. J. Brodeur. 2003. Molecular approaches to microbiological monitoring: Fecal source detection. Environmental Monitoring and Assessment **81:**313-326.

Fong, T. T., L. S. Mansfield, D. L. Wilson, D. J. Schwab, S. L. Molloy, and J. B. Rose. 2007. Massive microbiological groundwater contamination associated with a waterborne outbreak in Lake Erie, South Bass Island, Ohio. Environmental Health Perspectives **115:**856-864.

Foulds, I. V., A. Granacki, C. Xiao, U. J. Krull, A. Castle, and P. A. Horgen. 2002. Quantification of microcystin-producing cyanobacteria and *E. coli* in water by 5'-nuclease PCR. J Appl Microbiol **93**, 825–834.

Franks, A. H., H. J. M. Harmsen, G. C. Raangs, G. J. Jansen, F. Schut, and G. W. Welling. 1998. Variations of bacterial populations in human feces measured by

fluorescent in situ hybridization with group-specific 16S rRNA-Targeted oligonucleotide probes. Applied and Environmental Microbiology **64:**3336-3345.

Gerba, C. P., and J. E. Smith. 2005. Sources of pathogenic microorganisms and their fate during land application of wastes. Journal of Environmental Quality **34:**42-48.

Harmsen, H. J. M., G. C. Raangs, T. He, J. E. Degener, and G. W. Welling. 2002. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Applied and Environmental Microbiology **68:**2982-2990.

Holdeman, L. V., I. J. Good, and W. E. C. Moore. 1976. Human fecal flora:variation in bacterial composition within individuals and a possible effect of emotional stress. Applied and Environmental Microbiology **31:**359-375.

Kamel, A. H., M. A. Ali, H. G. El-Nady, S. Aho, P. Pothier, and G. Belliot. 2010. Evidence of the co-circulation of enteric viruses in sewage and in the population of Greater Cairo. Journal of Applied Microbiology **108:**1620-1629.

Kildare, B. J., C. M. Leutenegger, B. S. McSwain, D. G. Bambic, V. B. Rajal, and S. Wuertz. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. Water Research **41:**3701-3715.

Koivunen, J., A. Siitonen, and H. Heinonen-Tanski. 2003. Elimination of enteric bacteria in biological-chemical wastewater treatment and tertiary filtration units. Water Res. **37:**690-698.

Kreader, C. A. 1998. Persistence of PCR-detectable Bacteroides distasonis from human feces in river water. Applied and Environmental Microbiology **64:**4103-4105.

Lavender, J.S. and Kinzelman, J.L. 2009. A cross comparison of QPCR to agar-based or defined substrate test methods for the determination of Escherichia coli and enterococci in municipal water quality monitoring programs. Water Research 43(19), 4967-4979.

Lee, D.Y., Shannon, K. and Beaudette, L.A. 2006. Detection of bacterial pathogens oligonucleotide microarray in municipal wastewater using an ind real-time quantitative PCR. Journal of Microbiological Methods 65(3), 453-467.

Lee, D. Y., H. Lauder, H. Cruwys, P. Falletta, and L. A. Beaudette. 2008. Development and application of an oligonucleotide microarray and real-time quantitative PCR for detection of wastewater bacterial pathogens. Science of the Total Environment **398:**203-211.

Lucena, F., A. E. Duran, A. Moron, E. Calderon, C. Campos, C. Gantzer, S. Skraber, and J. Jofre. 2004. Reduction of bacterial indicators and bacteriophages infecting faecal bacteria in primary and secondary wastewater treatments. Journal of Applied Microbiology **97:**1069-1076.

Michigan Department of Environmental Quality. R 323.1062 Water quality standards. R 323.1041-323.1117

Michigan Natural Resources and Environmental Protection Act 1994. Part 4. Water Quality Standards, , PA 451

Nayak, A. K., and J. B. Bose. 2007. Detection of Helicobacter pylori in sewage and water using a new quantitative PCR method with SYBR (R) green. Journal of Applied Microbiology **103:**1931-1941.

Okabe, S., N. Okayama, O. Savichtcheva, and T. Ito. 2007. Quantification of host-specific Bacteroides-Prevotella 16S rRNA genetic markers for assessment of fecal pollution in freshwater. Applied Microbiology and Biotechnology **74:**890-901.

Payment, P., R. Plante, and P. Cejka. 2001. Removal of indicator bacteria, human enteric viruses, Giardia cysts, and Cryptosporidium oocysts at a large wastewater primary treatment facility. Canadian Journal of Microbiology **47:**188-193.

Pourcher, A. M., P. Morand, F. Picard-Bonnaud, S. Billaudel, S. Monpoeho, M. Federighi, V. Ferre, and G. Moguedet. 2005. Decrease of enteric micro-organisms from rural sewage sludge during their composting in straw mixture. Journal of Applied Microbiology **99:**528-539.

Puig, S., M. C. M. van Loosdrecht, A. G. Flameling, J. Colprim, and S. C. F. Meijer. 2010. The effect of primary sedimentation on full-scale WWTP nutrient removal performance. Water Research **44:**3375-3384.

Pundsack, J., R. Axler, R. Hicks, J. Henneck, D. Nordman, and B. McCarthy. 2001. Seasonal pathogen removal by alternative on-site wastewater treatment systems. Water Environment Research **73:**204-212.

Robertson, L. J., L. Hermansen, and B. K. Gjerde. 2006. Occurrence of Cryptosporidium oocysts and Giardia cysts in sewage in Norway. Applied and Environmental Microbiology **72:**5297-5303.

Rowan, N. J. 2004. Viable but nonculturable forms of food and waterborne bacteria: Quo Vadis? Trends in Food Science & Technology **15:**462-467.

Sadowsky, M.J., Call, D.R. and J.W. Santo Domingo. 2007. Application of microbial source tracking studies. In Microbial Source Tracking ed . Santo Domingo, J.W. and Sadowsky, M.J. 235–277 Washington, DC: ASM Press.

Savichtcheva, O., N. Okayama, and S. Okabe. 2007. Relationships between Bacteroides 16S rRNA genetic markers and presence of bacterial enteric pathogens and conventional fecal indicators. Water Research **41:**3615-3628.

Scott, T. M., J. B. Rose, T. M. Jenkins, S. R. Farrah, and J. Lukasik. 2002. Microbial source tracking: Current methodology and future directions. Applied and Environmental Microbiology **68:**5796-5803.

Sercu, B., L. C. Van De Werfhorst, J. Murray, and P. A. Holden. 2009. Storm Drains are Sources of Human Fecal Pollution during Dry Weather in Three Urban Southern California Watersheds. Environmental Science & Technology **43:**293-298.

Seurinck, S., T. Defoirdt, W. Verstraete, and S. D. Siciliano. 2005. Detection and quantification of the human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Environmental Microbiology **7:**249-259.

Shah, H. N., and D. M. Collins. 1990. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. International Journal of Systematic Bacteriology **40:**205-208.

Shanks, O. C., J. W. Santo Domingo, J. R. Lu, C. A. Kelty, and J. E. Graham. 2007. Identification of bacterial DNA markers for the detection of human fecal pollution in water. Applied and Environmental Microbiology **73:**2416-2422.

Silkie, S. S., and K. L. Nelson. 2009. Concentrations of host-specific and generic fecal markers measured by quantitative PCR in raw sewage and fresh animal feces. Water Research **43:**4860-4871.

Simpson, J. M., J. W. Santo Domingo, and D. J. Reasoner. 2002. Microbial source tracking: State of the science. Environmental Science & Technology **36:**5279-5288.

Stevenson, A.H. 1953. Studies of bathing water quality and health. *American Journal of Public Health*, 43: 529–538.

U SEPA. 1976. Quality criteria for water, 1976. US Environmental Protection Agency, Washington, DC.

USEPA. 1986. Bacteriological Water Quality Criteria for Marine and Fresh Recreational Waters. EPA-440/5-84-002. Cincinnati, OH:U.S. Environmental Protection Agency, Office of Water Regulations and Standards.

USEPA. 2002. *Enterococcus spp.* in Water by Membrane Filter using membrane Enterococcus Indoxyl-B-D-Glucoside Agar (mEI). EPA-821-R-02-022. Office of Water, Washington D.C.

USEPA. 2005**.** *Escherichia coli* (*E. coli*) in Water by Membrane Filtration using Modified membrane-Thermotolerant *Escherichia coli* Agar (modified mTEC). EPA 821-R-04-025. Office of Water, Washington D.C.

USEPA. 2007. **Report of the Experts Scientific Workshop on Critical Research Needs for the Development of New or Revised Recreational Water Quality Criteria.** Washing**ton**, DC. EPA 823-R-07-006, http://www.epa.gov/**water**science/**criteria**/recreati**on**/ (1 July 2010, date last accessed).

Varma M, Field R, Stinson M, Rukovets B, Wymer L, Haugland R. 2009. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. Water Res **43**:4790–4801.

Wade, T. J., R. L. Calderon, E. Sams, M. Beach, K. P. Brenner, A. H. Williams, and A. P. Dufour. 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspect. **114**:24-28.

WHO. 2008. Guidelines for drinking-water quality. 3rd Ed Incorporating First and Second Addenda. Geneva.

Wery, N., C. Lhoutellier, F. Ducray, J. P. Delgenes, and J. J. Godon. 2008. Behaviour of pathogenic and indicator bacteria during urban wastewater treatment and sludge composting, as revealed by quantitative PCR. Water Research **42:**53-62.

Wood, J., K. P. Scott, G. Avgustin, C. J. Newbold, and H. J. Flint. 1998. Estimation of the relative abundance of different Bacteroides and Prevotella ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. Applied and Environmental Microbiology **64:**3683-3689.

Xu, J., M. K. Bjursell, J. Himrod, S. Deng, L. K. Carmichael, H. C. Chiang, L. V. Hooper, and J. I. Gordon. 2003. A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. Science **299:**2074-2076.

Yampara-Iquise, H., G. Zheng, J. E. Jones, and C. A. Carson. 2008. Use of a Bacteroides thetaiotaomicron-specific alpha-1-6, mannanase quantitative PCR to detect human faecal pollution in water. Journal of Applied Microbiology **105:**1686-1693.

CHAPTER 5

EVALUATION OF COW SPECIFIC MICROBIAL SOURCE TRACKING MARKERS IN CATTLE MANURE

5.1 Introduction

Contamination of surface and ground water by animal wastes is a severe problem that can lead to illness and death in humans due to transmission of pathogens. There have been many waterborne disease outbreaks where animal waste has been the suspected source of the etiological agent (Table 5.1). Unlike human waste, animal waste is not treated adequately due to the cost required to treat the large amount of waste produced. United States Department of Agriculture (USDA) estimates that US produces 335 million tons of "dry matter" waste annually in farms (USDA 2006) and animal feeding operations produce 100 times more manure than sludge from human waste treatment per year (Gerba and Smith 2005). Therefore, manure is applied to fields prior to each growing season as an efficient disposal alternative due to the high costs involved in the treatment of these wastes and to utilize the high nutrient content.

Surface water or ground water can be fecally contaminated by animal waste through many routes (Figure 5.1). Fecal contamination of surface water bodies occurs through by runoff often after a storm event following manure application (Culley and Philips, 1982). Significantly high levels of bacteria in overland flow samples have been demonstrated by simulated rainfall events on a steep grazed hillside (Collins et al. 2005). Direct contamination of water by animals is another problem encountered by regions where cattle are not confined to a smaller grazing area. Elevated levels of fecal coliforms in

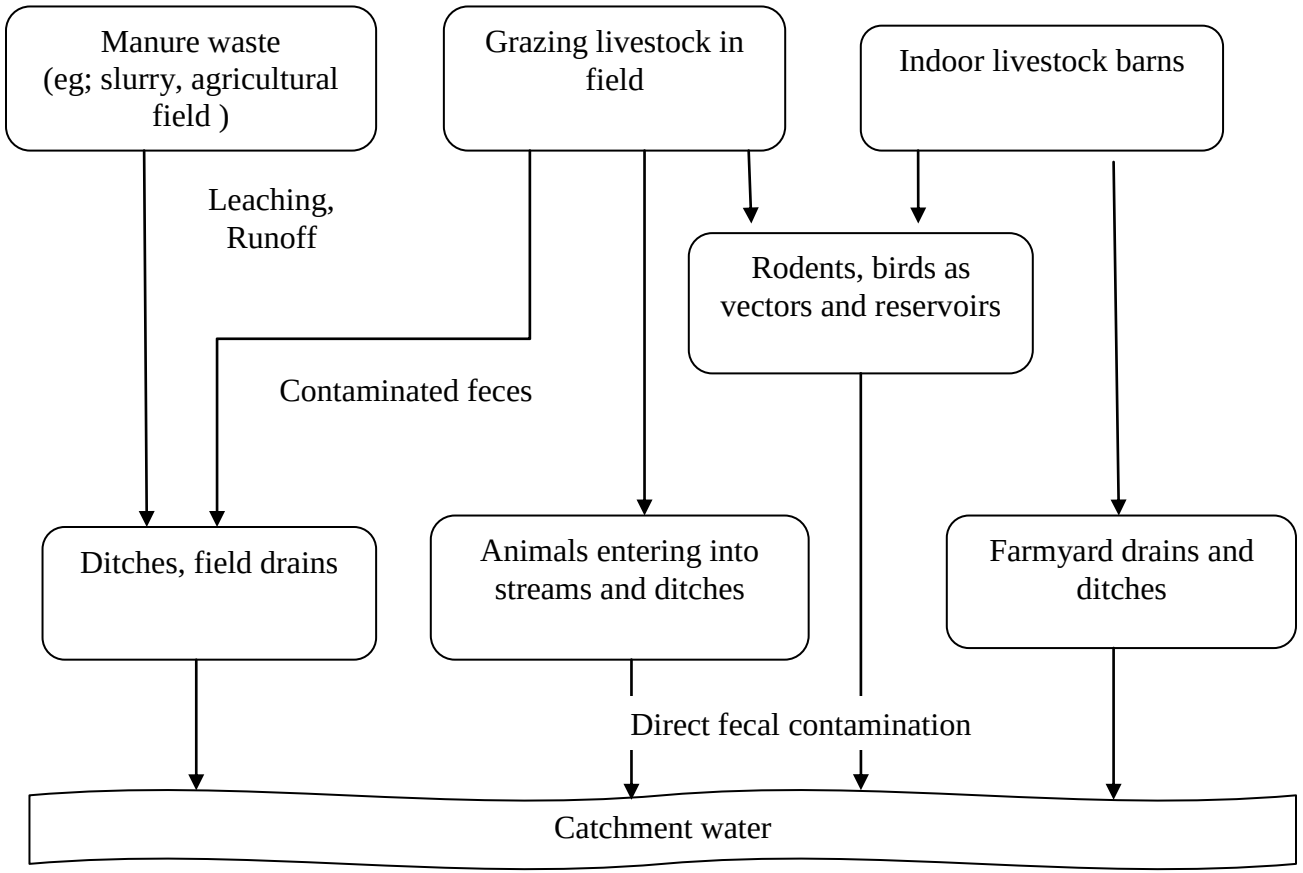
streams following grazing by livestock animals have been reported (Gary et al., 1983, Doran et al., 1981; Howell et al., 1995). The impacts of water quality by grazing cattle studied in New Zealand showed that *E. coli* counts increased up to 100 times background levels in a stream after a dairy herd had crossed over. It was estimated that around 10^{11} *E. coli* were deposited into the river during each crossing event (Davies-Colley et al. 2004). Fecal contamination of ground water by manure also occurs during which microbes are transported through pores of soil and this is influenced by factors such as soil type, bacterial shape, size, vegetative state of bacteria etc (Pachepsky et al. 2006). The outbreak in Walkerton which is listed below in Table 5.1 was suspected to be due to contamination of groundwater with manure (McQuigge 2000; Hrudey et al. 2003).

Table 5.1: Selected waterborne outbreaks where cattle manure was suspected as the source of the etiological agents

Location	Year	Pathogens	Morbidity	Mortality	References
Walkerton, Canada	2000	*E. coli* O157:H7 *Campylobacter spp.*	1346 cases	6 deaths	Health Canada, 2000
Ontario, Canada	1995	*E. coli* O157:H7	1 case	-	Jackson et al., 1998
Africa	1992	*E. coli* O157:H7	1000s of cases	some deaths[a]	Isaacson et al., 1993
Milwaukee, WI	1993	*Cryptosporidium parvum*	400 000 cases	87 deaths	Mac Kenzie et al., 1994
New York	1999	*E. coli* O157:H7 *Campylobacter* spp.	116 cases	2 deaths	CDC, 1999
UK	1989	*Cryptosporidium parvum*	516 cases	-	Richardson et al., 1991
Ayrshire, UK	1988	*Cryptosporidium parvum*	27+	-	Smith et al., 1989
Bradford, UK	1992	*Cryptosporidium parvum*	125 cases	-	Atherton, 1995
Sakai City, Japan	1995	*E. coli* O157:H7	12 680 cases	3 deaths	Hideshi et al, 1999
Cabool, MO	1990	*E. coli* O157:H7	243 cases	4 deaths	Swerdlow et al; 1992

[a] Exact number not reported

Figure 5.1: Fecal contamination of catchment water associated with the manure environment (Adapted from Hooda et al. 2000)



Fecal indicator bacteria such as *E. coli* and enterococci are very common in bovine manure and are used to monitor manure contaminated water. Even though high numbers of these bacteria in water bodies denote input of feces from warm blooded animals, it does not differentiate between the sources. Public health risks due to agriculture runoff can be addressed primarily by using Microbial Source Tracking (MST) tools that enable the differentiation of fecal inputs due to cattle from those of other animals. This will aid in development, prevention, and control strategies. One of the popular microbiological MST tools used to differentiate different animal fecal contamination from human in 1950's and 60's was the Fecal Coliform to Fecal Streptococcus (FC:FS) Ratio. Humans have excretion levels of $1.30x10^7$ CFU/g for fecal colifoms and $3.00x10^6$ CFU/g for fecal streptococci thus giving a FC:FS ratio of 4.3 whereas cows supposedly have an excretion levels of $2.30x10^5$ CFU/g for fecal colifoms and $1.30x10^6$ CFU/g for fecal streptococci thus giving a FC:FS ratio of 0.17 (Gerba 2000). However, this method was discontinued as MST tool as it was shown not to be scientifically valid. These reasons include alteration of ratio due to different die-off rates of fecal streptococci, varied bacterial concentration outside of pH range of 4.0-9.0, and unreliable ratio with pollution from several sources, among others (Meays et al. 2004).

One approach for molecular based MST for bovine specific fecal contamination has been conventional PCR, targeting *Bacteroides-Prevotella* 16S rRNA (Bernhard and Field 2000). Another method has been a quantitative approach using qPCR assays targeting 16S rRNA genes of *Bacteroides-Prevotella* group (Kildare et al. 2007; Layton et al. 2006; Okabe et al. 2007), M2 and M3 genetic markers with *Bacteroidales*-like sequences obtained by genomic fragment enrichment method (Shanks et al. 2006). Real-time

reverse transcription assays have also been used for bovine fecal source tracking, for instance, targeting F^{+}-specific RNA coliphages (Kirs and Smith 2007) and bovine enterovirus RNA (Jiménez-Clavero 2005).

Almost all the bacterial bovine specific MST assays target 16S rRNA genes from the order *Bacteriodales*. Successful development and use of these assays depend on the host-specificity of the assay because of the prevalence of this bacterial group in different animals such as horse, pigs, human, elk, gulls, dogs, and cats. Different factors such as age, diet, and climatic conditions influence the bacterial flora in rumen intestines (Gerard-Champod et al. 2009) and therefore, prevalence of different bovine specific markers was shown to vary with herds with different diet and geographical location (Shanks et al. 2006). Thus, selection of most prevalent markers is necessary to characterize bovine fecal pollution in a particular water-shed.

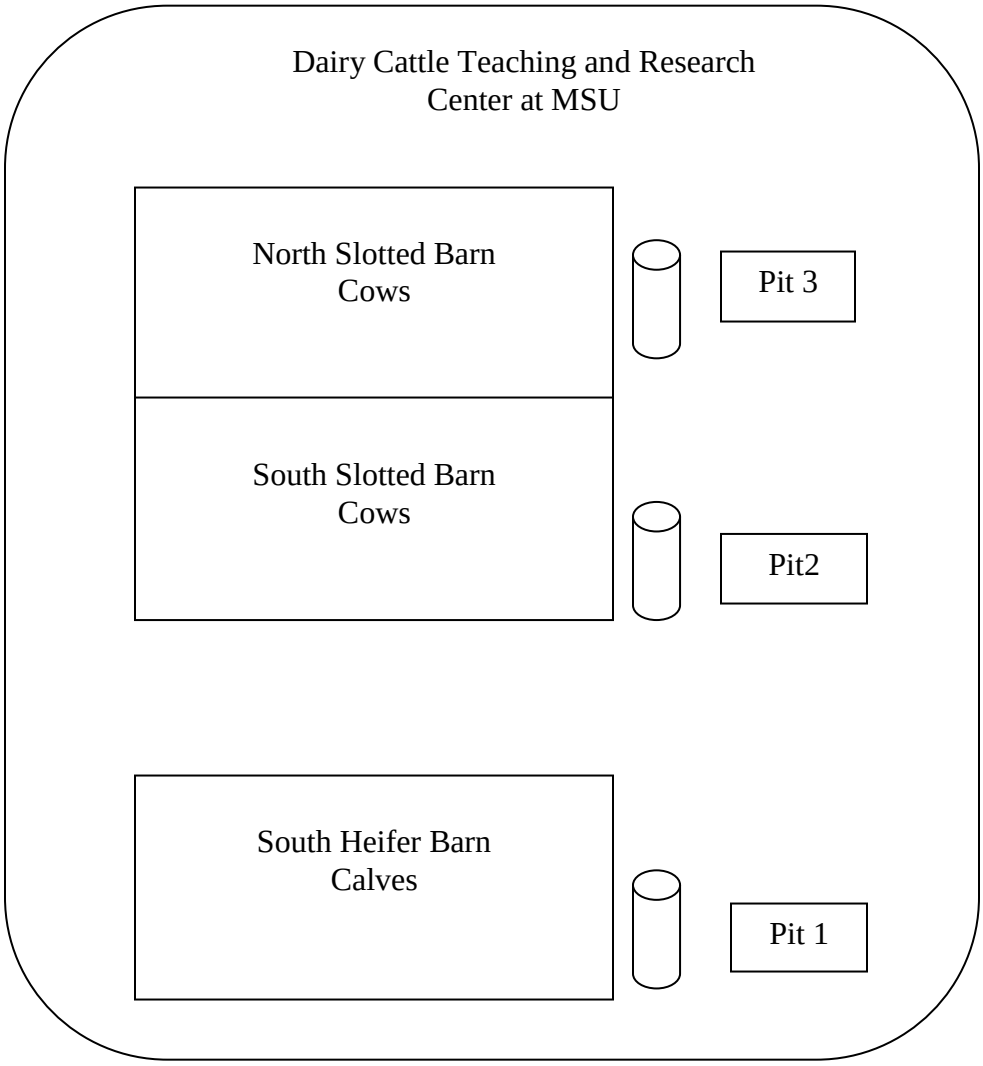
This study focused on evaluating and characterizing the concentrations of the three different cow specific markers by qPCR, namely, cow specific *Bacteroides* 16S rRNA (Bobac qPCR), M2 marker (M2 qPCR) and M3 marker (M3 qPCR) in manure pit samples collected just prior to field application. A specific goal of this study was to compare concentrations of fecal indicator bacteria such as *E. coli* and enterococci in manure pit samples and fresh cow feces by applying agar based cultivation and qPCR methods. In order to evaluate the restricted occurrence of these cow specific markers in human waste environment, qPCR assays were also applied to raw sewage and septage samples.

5.2 Materials and Method

5.2.1 Sample Collection and Processing

Fresh fecal samples were collected from individual cows during defecation at a dairy cattle farm at Michigan State University (n=10). Samples were also collected from four manure pits at Michigan State University dairy farms. Manure in these pits are emptied for land application three times during the year; Spring (around April-May), Summer (around July-August) and in Fall (around October-November). Sample collection for this study was done during the months of May and November in 2009 during the mixing of manure in these pits by agitation just prior to the field application. The four pits from which samples were collected are located in two different facilities; the Dairy Cattle Teaching and Research Center and the Beef Cattle Teaching and Research Center, are both located at MSU farms. Pit 1, Pit 2, and Pit 3 are located in the dairy cattle facility (Figure 5.2). Pit 4 is located in the beef cattle facility (not shown in the figure as the facility is a separate unit). During the month of May, samples were collected from all four pits. In the month of November, samples were collected from only three pits at the dairy cattle farm as there was no land application from the beef farm pit during that time. Five samples were collected from each pit in both seasons in specimen containers that holds approximately 125 mL of sample; thus a total of 35 samples (20 samples in May and 15 samples in November) were used for the study. Samples were transferred to laboratory on ice immediately after collection and were used for analysis immediately. Collection of raw sewage and septage samples from the respective treatment plants is described in Chapter 4 under section 4.2.1 in Materials and Methods.

Figure 5.2: Diagram representing sampling locations of manure samples.



5.2.2 Analyses

5.2.2.1 Cultivation Methods

EPA Membrane Filtration methods 1603 and 1600 were used for enumerating *E. coli* and *Enterococci* respectively (USEPA 2002; 2005). For cow fecal samples, one gram (wet weight) of fresh feces was weighed and for manure, one mL of the sample was measured. These were serially diluted (10^{-1} through 10^{-5}) with sterile Phosphate Buffered Saline (PBS). After mixing each dilution thoroughly by vortexing, membrane filtration was performed on these dilutions. One ml from these dilutions was filtered through 47 mm diameter, 0.45 µm pore size, membrane filters. *E. coli* colony forming units were enumerated after incubating filters on mTEC agar plates (Beckton Dickinson, MD USA) at 35 ± 0.5°C for 2 h, followed by incubation in a water bath at 44.5 ± 0.2°C for 22 h. Similarly, enterococci CFU were enumerated after incubating filters on mEI agar plates (Beckton Dickinson, MD USA) for 24 h at 41°C. The concentrations of *E. coli* and *Enterococci* from all tested fecal samples were reported as colony forming units (CFU) per gram and CFU per mL for manure samples.

5.2.2.2 DNA Extraction and qPCR Analyses

Two hundred milligrams (wet weight) of cow feces were weighed and placed in 2 mL microcentrifuge tubes for DNA extraction. Four hundred microliters of manure samples were transferred into 2 mL microcentrifuge tubes. DNA was extracted from both sample types with the QIAamp DNA Stool Mini Kit (Qiagen). Briefly, stool samples were mixed with lysis buffer after which the impurities such as DNA-damaging substances and PCR inhibitors were adsorbed to InhibitEX matrix. Further, DNA was purified on QIAamp

Mini spin columns (Qiagen) and extracted by adding 200 µL elution buffer, after which the DNA was stored at -20^{o}C. Sample processing and DNA extraction from raw sewage and septage samples are described in Chapter 4 under section 4.2.2.2.

The bacterial genes and amplicons used in the study for qPCR analyses are described in Table 5.2. The corresponding qPCR programs for each assay are described in Table 5.3. *E. coli* uidA and enterococci 23S qPCR assays were done using Roche LightCycler® 2.0 Instrument (Roche Applied Sciences, Indianapolis, IN) and all the three cow specific qPCR assays were performed using Roche LightCycler® 480 Instrument (Roche Applied Sciences, Indianapolis, IN). Standard curves were generated for each of these qPCR machines using the ten-fold dilutions of plasmids cloned with the DNA fragment carrying the qPCR target amplicon. DNA extracted from all fecal or manure samples were analyzed by qPCR for all targets. The reaction mixture for amplification of *E. coli uidA* gene consisted of 2 µL of the Roche FastStart LightCycler Mastermix, forward and reverse primers, Taqman probe, 3.2 mM $MgCl_2$ and nuclease free water to make up the final volume to 15 µL. The enterococci and cow specific (BoBac, M2, and M3) assays were carried out separately using 10 µL of Roche LightCycler480 Probes® Mastermix, forward and reverse primers, probe, and nuclease free water to make up the final volume to 15 µL. Five microlitres of the extracted sample were used as the template. The reaction was performed in duplicate for samples and negative controls were included. Threshold cycle temperature (Ct) was measured during each amplification and target gene concentration was analyzed automatically by absolute quantification method by the LightCycler® Software 4.0.

For each qPCR assay, one of the plasmid dilutions used for creating the standard curve was also included in the assay as a positive control. The copies of all target genes present in the sample were quantified from the standard curves obtained earlier. The copies of the corresponding genes for *E. coli* and enterococci were converted to cell equivalents and final concentrations of qPCR analyses were reported as either qPCR equivalent cells/mL or qPCR target copies/mL. In the case of three cow specific MST qPCR assays, all the final concentrations qPCR analyses were reported as copies/mL of the manure samples. These cannot be converted to cells counts because either the 16S rRNA copy number of *Bacteroides-Prevotella* group varies with different species (for Bobac assay) or these assays were designed by metagenomic approach and no cultivable cells have been identified carrying the specific gene fragment to know the copy number (for M2 and M3 assays) (Layton et al 2006; Shanks 2008).

Conventional PCR targeting CF123 cluster of *Bacteroides-Prevotella* Bovine CF123 cluster was performed in a Thermocycler using a PCR reaction mixture containing 14 μL HotStar Taq Master Mix (Qiagen; Valencia, CA), forward and reverse primers and molecular grade water to get final volume of 22 μL to which 3 μL of template DNA was added. Amplified products were detected by visualizing the PCR product by electrophoresis through 1.2% agarose gel and then exposing it to UV transilluminator. DNA from cow fecal samples that was confirmed to be positive for bovine CF123 cluster by sequencing the amplified products and BLAST analysis were used as the positive control. This was used as positive control as no pure isolates carrying this maker are available and therefore, the options were to either commercially generate plasmids carrying artificially synthesized CF123 cluster gene fragment or to use cow fecal DNA

that was positive for the same assay with the sequences verified. Molecular grade water was used as the negative control. The samples were recorded as positive when the bands corresponded to the positive control at around 525bp.

Table 5.2: Bacteria and gene targets used in the study of cow feces and manure

Targets	**Assay**	**gene**	**Amplicon size**	**Reference**
E. coli	*E. coli uidA*[a]	*uidA*	163bp	Developed in this study
Enterococcus sp	Enterococci 23S[a]	23S rRNA	91bp	Frahm & Obst (2003)
Bacteroides bovine cluster	Bobac[a]	Cow specific 16S rRNA fragment	100bp	Layton et al. (2006)
M2 cow specific marker	M2[a]	HDIG domain protein	92bp	Shanks et al. (2006)
M3 cow specific marker	M3[a]	Sialic acid-specific 9-O-acetylesterase	122bp	Shanks et al. (2006)
Bacteroides-Prevotella Bovine CF123 cluster	CF123*	Cow specific 16S rRNA CF123 cluster	525bp	Bernhard and Field. (2000)

[a]quantitative PCR assay
*conventional PCR assay

Table 5.3: Primer/ probe sequences, concentration and PCR programs used for each assay

Assay	Primer/probe	Primer/probe	PCR programs
E. coli uidA	5'-CAATGGTGATGTCAGCGTT-3'	0.5 µM	6 s at 95^{o} C
	5'-ACACTCTGTCCGGCTTTTG-3'	0.5 µM	8 s at 58^{o}C
	6FAM-TTGCAACTGGACAAGGCACCAGC-BBQ	0.2 µM	8 s at 72^{o}C
Enterococci 23S	5'-AGAAATTCCAAACGAACTTG-3'	0.5 µM	15 s at 95^{o}C
	5'-CAGTGCTCTACCTCCATCATT-3'	0.5 µM	30 s at 60^{o}C
	6FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA	0.4 µM	15 s at 72^{o}C
Bobac	GAAGRCTGAACCAGCCAAGTA	0.5 µM	10 s at 95^{o}C
	GCTTATTCATACGGTACATACAAG	0.5 µM	30 s at 57^{o}C
	6-FAM-TGAAGGATGAAGGTTCTATGGATTGTAAACTT-TAMRA	0.2 µM	6 s at 72^{o}C
M2	CGGCCAAATACTCCTGATCGT	0.5 µM	15 s at 95^{o}C
	GCTTGTTGCGTTCCTTGAGATAAT	0.5 µM	60 s at 61^{o}C
	6FAMAGGCACCTATGTCCTTTACCTCATCAACTACAGACATAMRA	0.2 µM	10 s at 72^{o}C
M3	CCTCTAATGGAAAATGGATGGTATCT	0.5 µM	10 s at 95^{o}C
	CCATACTTCGCCTGCTAATACCTT	0.5 µM	60 s at 55^{o}C
	6-FAM-TTATGCATTGAGCATCGAGGCC-TAMRA	0.2 µM	1 s at 72^{o}C
*CF123	CCAACYTTCCCGWTACTC	0.4 µM	30 s at 94 ^{o}C
	CAATCGGAGTTCTTCGTG	0.4 µM	30 s at 58 ^{o}C
			60 s at 72 ^{o}C

*Conventional PCR assay

5.2.3 Statistics

All the final concentrations in feces (n=10) and manure samples (n=35) were $\log_{10}$ transformed for normalization. In order to determine the significant differences between the concentrations of cultivable cells and qPCR equivalent cells for *E. coli* and enterococci in feces and manure samples, simple t-tests were performed at the 0.05 level of significance. Levene's test for homogeneity of variances was performed using SAS software 9.2 (SAS Inc, 2002) to determine if t-test assuming equal or unequal variances should be used for comparison, with the hypothesis of equal variance being rejected at $p<0.05$. Pearson's correlation coefficient was used to test the relationship between concentrations by cultivation and qPCR methods for *E. coli* and enterococci.

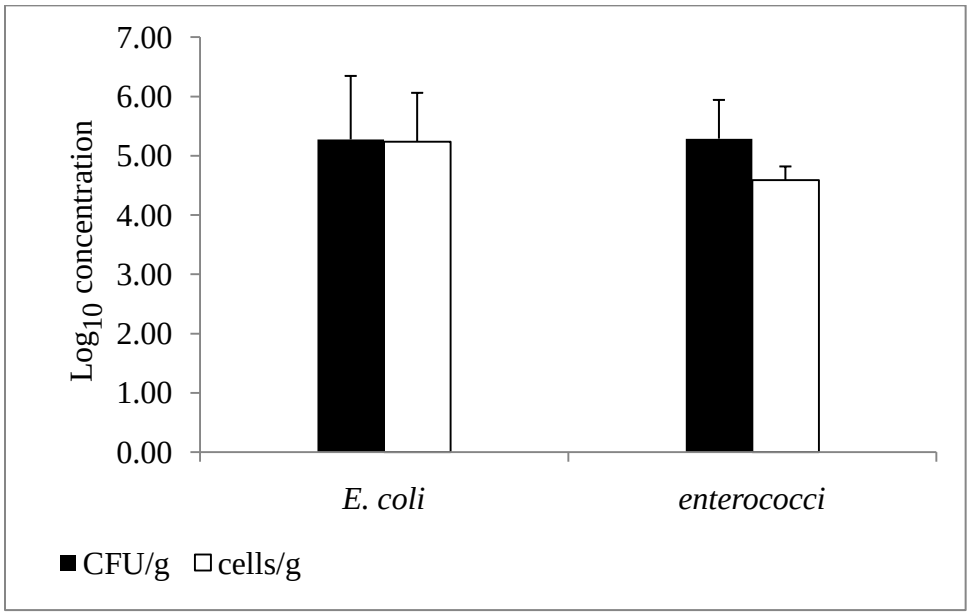
With data for manure samples from pits collected in two months, a two-way ANOVA was performed to investigate two specific questions: 1) was there any significant difference in qPCR signals for a particular target between the four pits? 2) was there any significant differences in qPCR signals for a particular target between May and November? To answer the first question, data from the four pits collected in May alone were used as data for the month of November for Pit 4 was not available. Analysis of the data for the second question was done by pooling data from Pit 1, 2 & 3 collected in May and corresponding data collected in the month of November. Pit 4 was excluded from the analysis for the same reason described above.

5.3. Results

5.3.1 *E. coli* and Enterococci Cultivation Versus qPCR

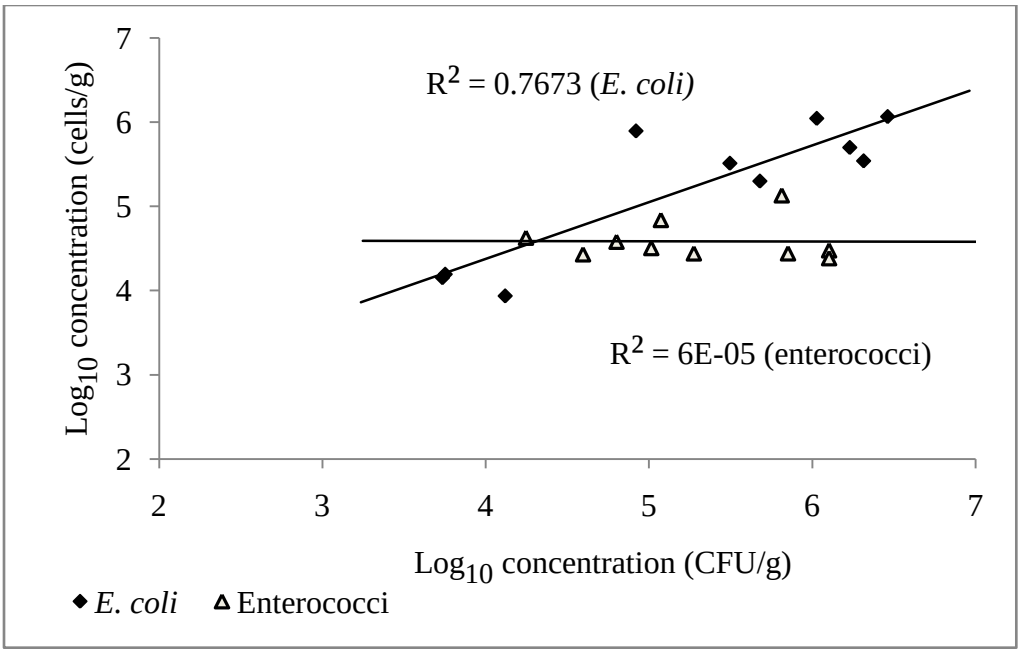
Fresh cow fecal samples and manure samples from pits were collected from MSU dairy farm. The mean concentration of cultivable *E. coli* in fresh fecal samples was found to be 8.41×10^5 CFU/gram and the mean qPCR equivalent cell concentration in these samples was 7.97×10^5 cells/gram. The mean concentration of *Enterococcus* spp. by cultivation method in cow feces was found to be 4.80×10^5 CFU/gram and the mean qPCR equivalent cell concentration in these samples was 4.69×10^4 cells/gram (Figure 5.3). There was no statistically significant difference between $\log_{10}$ transformed concentrations of *E.coli* by cultivation and qPCR in cow feces whereas the difference between concentrations of enterococci by both the methods was found to be significant ($p<0.05$). A significant ($p<0.05$) positive correlation was found between *E. coli* cultivable counts and qPCR equivalent cells with correlation coefficient, $R = 0.87$ and coefficient of determination, $R^2 = 0.76$. There was no correlation between concentrations of enterococci by cultivation and qPCR methods with $R=0.007$ (Figure 5.4).

Figure 5.3: Average *E. coli* concentrations in cow feces by cultivation (CFU/g) and qPCR (cells/g) methods



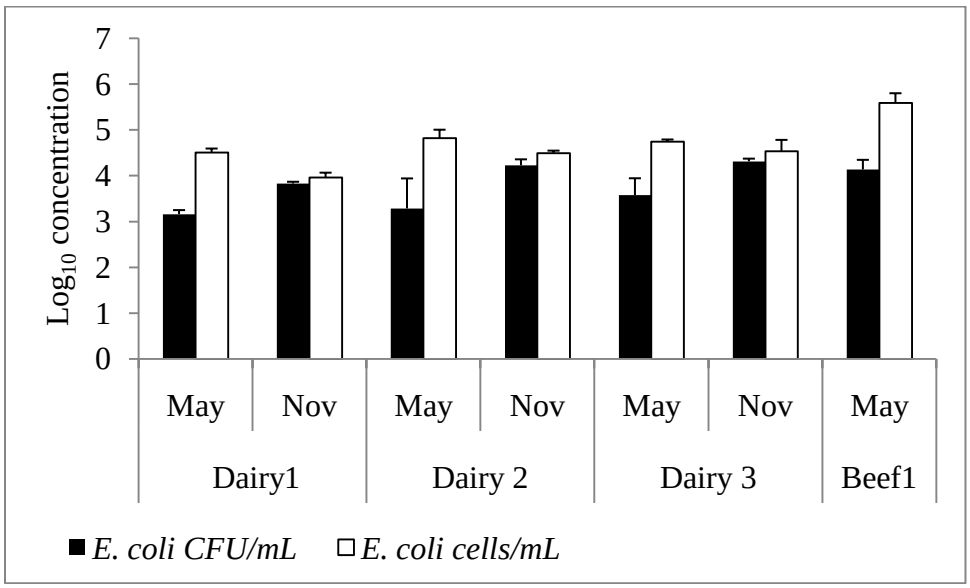
Number of cow fecal samples, n=10

Figure 5.4: Correlation between concentrations by cultivation methods (CFU/g) and qPCR (cells/g) in cow feces for *E. coli* and enterococci



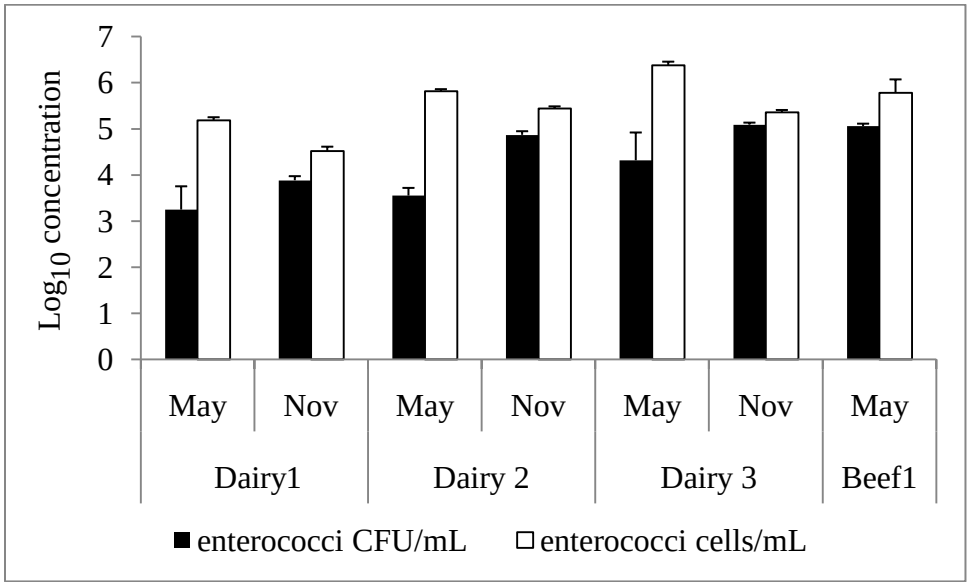
For manure samples, the concentrations of *E. coli* ranged from $3.67x10^2$ CFU/mL to $2.40x10^4$ CFU/mL by cultivation methods whereas by qPCR, the range was from $6.68x10^3$ to $5.92x10^5$ qPCR equivalent cells/mL. Concentations of enterococci in these samples ranged from $3.33x10^2$ to $1.40x10^5$ CFU/mL by cultivation and $2.37x10^4$ to $3.07x10^6$ qPCR equivalent cells by qPCR method. The qPCR equivalent concentrations of *E. coli* were significantly higher than cultivable cells in all manure samples collected in months of May and November except in dairy pit 3 samples collected in November. For enterococci, qPCR method showed significantly higher concentrations than those by cultivation methods in samples from all pits ($p<0.05$). The difference between CFU and qPCR equivalent cells was higher in May than in those samples collected in November. (Figures 5.5 and 5.6). There was no correlation found between cultivable counts and qPCR equivalent cells for both *E. coli* and enterococci in manure samples (Figure 5.7).

Figure 5.5: Average $\log_{10}$ transformed *E. coli* concentrations from manure pits by cultivation and qPCR methods



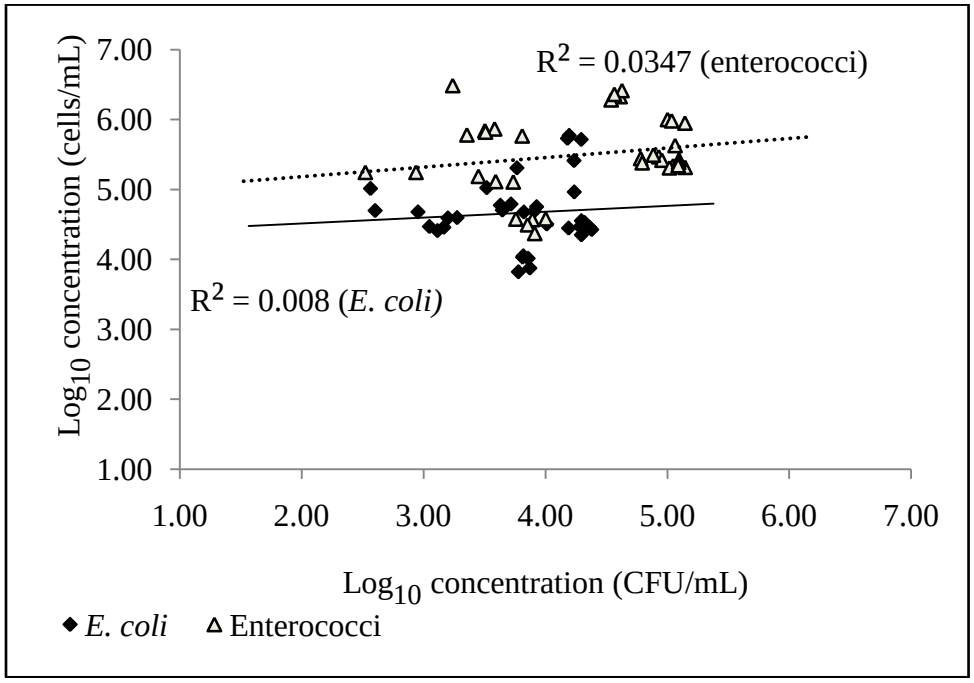
Total number of samples, n=35

Figure 5.6: Average log_{10} transformed enterococci concentrations from manure pits by cultivation and qPCR methods



Total number of samples, n=35

Figure 5.7: Correlation between concentrations by cultivation methods (CFU/mL) and qPCR (cells/mL) manure pit samples for *E. coli* and enterococci



*Total number of samples, n=35
*Data from three dairy pits and one beef pit

5.3.2 Detection of qPCR target copies of fecal indicator bacteria and Cow specific markers in manure pits

Manure pit samples were tested for two fecal indicators *E. coli uidA*, Enterococci 23S rRNA and three cow specific markers Bobac, M2, and M3 using qPCR assays, respectively. Final concentrations for these targets are all reported as copies/mL.Average $\log_{10}$ concentrations of indicators and cow-specific markers in manure samples from each pit collected in May and November are shown in Table 5.4.

Concentrations of *E. coli* qPCR target copies ranged from $6.68x10^3$ to $5.92x10^5$ copies/mL while *Enterococcus sp* ranged from $9.48x10^4$ to $1.23x10^7$ copies/mL. Bobac assay targets ranged from $9.60x10^5$ to $4.03x10^7$ copies/mL, M3 ranged from $2.60x10^6$ to $1.84x10^7$ copies/mL, and M2 ranged from $3.42x10^4$ to $1.34x10^6$ copies/mL.

The concentrations in manure from four pits were compared only in May. There was no specific pattern observed in the occurrence of qPCR signals among the four pits for all the targets. Means of each target, expressed as copies/mL, were compared in all four pits as in Figure 5.8. *E. coli* qPCR target copies/mL were found to be significantly higher in Beef Pit1 than compared to other pits located in dairy cattle farm. Highest concentrations of enterococci were found to be in Dairy Pit 3 among all the four pits ($p<0.05$). Bobac genes were detected at significantly higher target copies than rest of the qPCR targets in all four pits and were found to be significantly consistent in all the pits ($p<0.05$). Among all the cow-specific markers, M2 was found to be occurring at significantly lower concentrations in all the pits ($p<0.05$).

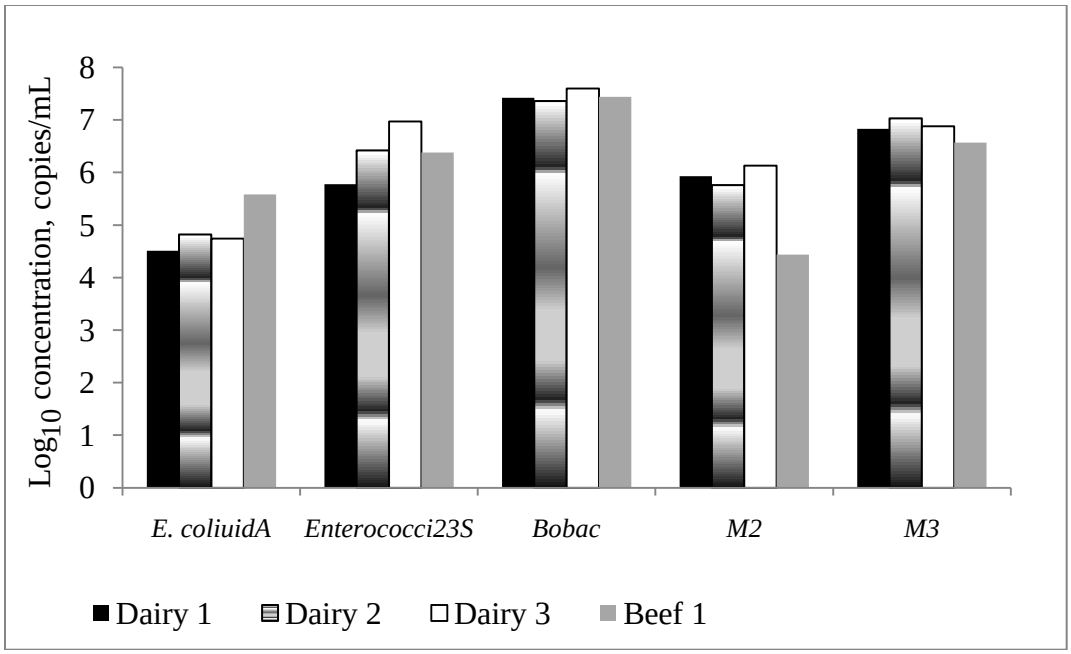
Comparison of means of qPCR target copies using pooled data from dairy pits 1, 2 and 3 between May and November was conducted and as shown in Figure 5.9, there were significant differences between the signals in both the months for all the targets. The average qPCR target copies/mL were significantly higher in May than November for all the targets tested ($p<0.05$).

Table 5.4: Average $\log_{10}$ concentrations of qPCR target copies/mL of fecal indicator bacteria and cow specific markers from manure pits

	Dairy Pit 1		Dairy Pit 2		Dairy Pit 3		Beef Pit 1	
Assays	May	Nov	May	Nov	May	Nov	May	Nov
E. coli uidA	4.51 (0.08)	3.96 (0.10)	4.82 (0.18)	4.49 (0.05)	4.74 (0.05)	4.53 (0.24)	5.58 (0.21)	ND
Enterococci 23S	5.78 (0.07)	5.12 (0.09)	6.41 (0.04)	6.04 (0.04)	6.97 (0.08)	5.96 (0.05)	6.38 (0.29)	ND
Bobac	7.41 (0.04)	6.09 (0.13)	7.56 (0.08)	5.99 (0.09)	7.60 (0.06)	5.98 (0.06)	7.44 (0.25)	ND
M2	5.93 (0.10)	5.83 (0.42)	5.76 (0.07)	5.11 (0.11)	6.12 (0.07)	5.47 (0.11)	4.44 (0.37)	ND
M3	6.83 (0.03)	6.50 (0.17)	7.03 (0.44)	6.41 (0.06)	6.89 (0.06)	6.56 (0.02)	6.57 (0.23)	ND

ND- Not determined
Nov- November
Numbers in parenthesis represents standard deviation for 5 replicates
*Dairy Pit 1= Calves from dairy farm
*Dairy Pit 2= Cows from dairy farm
*Dairy Pit 3= Cows from dairy farm
*Beef Pit 1= Cows from beef farm

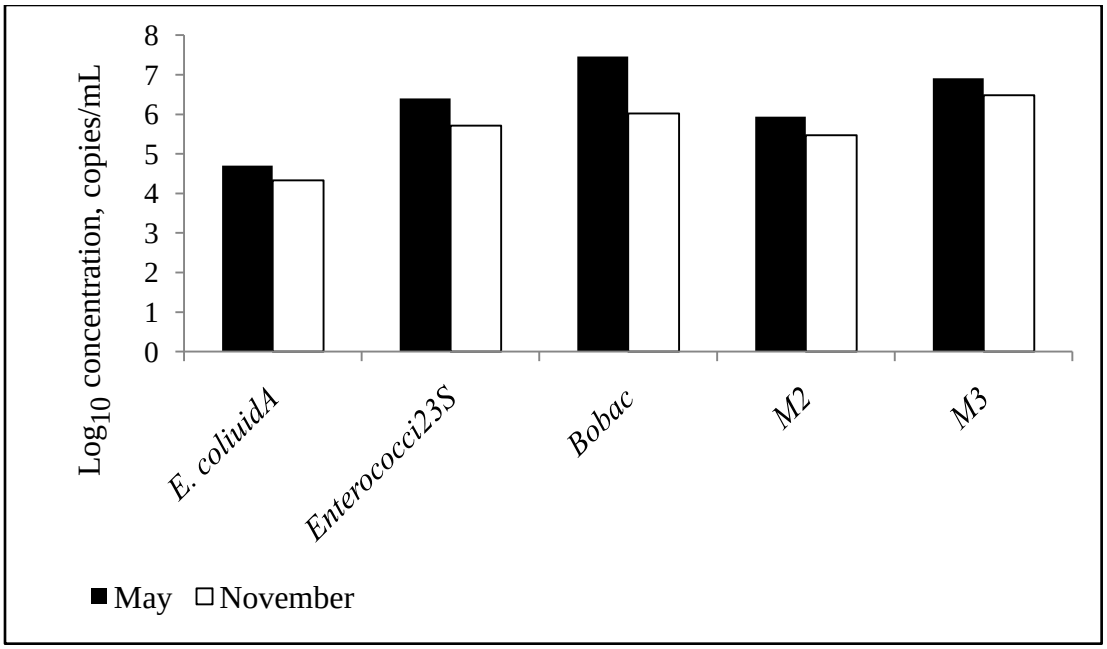
Figure 5.8: Comparison of qPCR copies signals for each target assay among the four pits



Assays	Pit 1*	Pit 2*	Pit 3*	Pit 4*
E. coliuidA	4.51a	4.82b	4.74ab	5.58
Enterococci23S	5.78	6.42c	6.97	6.38c
Bobac	7.42d	7.36d	7.6d	7.44d
M2	5.93ef	5.76e	6.13f	4.44
M3	6.83gh	7.03g	6.88g	6.57h

*Means followed by the same letters across the rows are not significantly different from each other.
*Pit 1= Calves from dairy farm
*Pit 2= Cows from dairy farm
*Pit 3= Cows from dairy farm
*Pit 4= Cows from beef farm

Figure 5.9: Comparison of qPCR copies signals for each target assay between May and November



Targets	May*	November*
E. coliuidA	4.7	4.33
Enterococci23S	6.4	5.71
Bobac	7.46	6.02
M2	5.94	5.47
M3	6.91	6.48

*Means followed by the same alphabet represents no significant difference between them

In order to evaluate the occurrence of these markers in human fecal environment, cow specific qPCR assays and CF 123 cluster conventional PCR assay were applied in raw sewage samples (n=18) and septage samples (n=8) collected for a previous study. There was no M2, M3 and CF 123 gene targets detected in any raw sewage and septage samples tested. However, Bobac gene targets were in concentrations ranging from $7.40x10^{2}$ to $2.52x10^{6}$ copies/mL (Table 5.5).

Table 5.5: Occurrence of cow specific markers in raw sewage and raw septage

Samples	M3 assay	M2 assay	Bobac assay(copies/mL)	CF123 cluster*
Raw sewage 1	-	-	3.33E+05	-
Raw sewage 2	-	-	3.39E+05	-
Raw sewage 3	-	-	4.08E+05	-
Raw sewage 4	-	-	1.36E+06	-
Raw sewage 5	-	-	3.74E+05	-
Raw sewage 6	-	-	2.42E+04	-
Raw sewage 7	-	-	9.84E+05	-
Raw sewage 8	-	-	5.60E+05	-
Raw sewage 9	-	-	5.32E+05	-
Raw sewage 10	-	-	4.92E+05	-
Raw sewage 11	-	-	3.07E+05	-
Raw sewage 12	-	-	1.85E+06	-
Raw sewage 13	-	-	1.37E+06	-
Raw sewage 14	-	-	3.40E+05	-
Raw sewage 15	-	-	1.23E+06	-
Raw sewage 16	-	-	6.72E+05	-
Raw sewage 17	-	-	1.43E+06	-
Raw sewage 18	-	-	1.12E+06	-
septage 1	-	-	1.31E+05	-
septage 2	-	-	-	-
septage 3	-	-	-	-
septage 4	-	-	-	-
septage 5	-	-	7.40E+02	-
septage 6	-	-	-	-
septage 7	-	-	2.52E+06	-
septage 8	-	-	-	-

*conventional PCR assay

5.4 Discussion

Land application of animal manure for nutrient recycling is a common fertilization practice in many parts of the world. In order to reduce the high numbers of bacteria that are present in fresh feces, manure is stored for long periods in pits or lagoons before it is applied in land. It has been suggested that most of the microorganisms will be killed during storage due to the changes in the conditions once outside the host gut (Michino *et al.* 2005). However, bacterial concentrations may not be reduced sufficiently and may change to a different physiological state such as viable but non-culturable (VBNC) where many pathogenic bacteria will not be culturable in routine growth media, but will retain their virulence (Lleo *et al.* 2000; Baffone *et al.* 2003). Bacteria in the land applied manure may also survive for longer periods as they attach to the dry matter or soil particles which enhance protection from dessication (Oliver et al. 2006). This increases the risks of contaminating surface and groundwater resources, thus making it a significant source of non-point pollution.

Due to the advantages of the field of Microbial Source Tracking (MST), new bovine markers have been developed as potential candidates to study impacts of animal manure on water quality (Bernhard and Field, 2000; Layton et al. 2006). *Bacteroides* bovine cluster 16S rRNA markers (Bobac), M2 (M2) and M3 markers (M3) were selected as cow specific markers in this study due to their reported host specificity. These markers were found to have 100% specificity when assessed during development of these assays (Shanks et al. 2008; Layton et al; 2006). Previous studies used these markers to monitor fresh feces from different cow herd populations or to assess watersheds for any bovine fecal contamination; however this is the first study to address the quantification of these

markers in manure pits. Conventional indicators *E. coli* and enterococci were also included in the study due to their current regulatory status in water quality monitoring. These fecal indicator bacteria have also been used to study the fate and transport of manure borne pathogens in the environment (Thurston et al. 2005; Thiagarajan et al. 2007).

Methodologically, cultivation methods and qPCR were both used and compared for detection of *E. coli* and enterococci. Application of qPCR tools in such complex matrix needs evaluation due to the coextraction of PCR inhibitory substances during DNA extraction (Uwatoka et al.1996). Comparable qPCR equivalent cells and CFU observed in this study for *E. coli* in fresh feces is in agreement with a recent study that has compared qPCR with cultivation based methods for *E. coli* and enterococci for fresh feces (Klein et al. 2010). Although the qPCR assay for their study also targeted *uidA* gene, Brilliance *E. coli*/Coliform Selective Agar was used for plate counts. Even though there was a significant correlation between both methods in this study, Klein et al found greater correlation with a correlation coefficient (R) of 0.95.

Concentrations of enterococci in fresh feces detected by qPCR were lower than CFUs cultivated on mEI agar media. This could be due to several reasons. Loss of DNA during extraction using manual kits is one possibility. Variability in total DNA yield with different DNA extraction methods has been reported previously (Yu and Morrison, 2004). An automated DNA extraction method was evaluated in this research to see the possibility of improving the concentrations of enterococci from cow feces but concentrations of enterococci were significantly higher in manual extraction kit (results described in Chapter 3). Another possibility is the variation in the copy number of

enterococci 23S rRNA gene between two and six copies among the enterococcal species (Oana et al. 2002) that could result in bias in the conversion of gene copy numbers to cell eauivalents. Standardization of enterococci qPCR targeting 23S rRNA gene was done using *E. fecalis in* several studies including this research, therefore copy number to cell conversions were done based on the four copies of the gene present in the species (Haughland et al. 2005; Wade et al. 2005). However, depending on the species abundance in a particular sample the accuracy of quantification could be affected and this quantification bias is being evaluated by USEPA as part of qPCR method development for monitoring recreational water quality (http://www.epa.gov/waterscience/methods/method/biological/rapid1.pdf)

Another possibility in the lower qPCR signals as compared to CFU could be due to variation in the sequences of 23S rRNA gene between the different enterococci species. A highly conserved central loop of domain V of 23S RNA has been found to have sequences varying in different enteroccocal species that included isolates of *E. faecium, E. faecalis, E. durans, E. hirae, E. gallinarum, E. casseliflavus, E. avium* and percentage of homology based on these sequences varied between 91.4 to 100%. It is possible that in bovine intestine, the heterogeneity in sequences of enterococcal species is contributing to the low signals by qPCR as compared to CFU (Tsiodras et al. 2000). These results suggest that there is still a gap in the knowledge about ecology of enterococci in bovine intestines. Even though specificity has been tested for 23S rRNA assay for enterococci previously (Frahm and Obst 2003), there is no assurance that this assay is detecting all species of enterococci present in cow feces.

A 6% high false positive associated with mEI media could also result in high cultivable levels of enterococci in mEI than compared to qPCR levels. There is no literature for evaluation of this medium for growing enterococci from cow fecal samples and with the diversity of the bacteria as well as complexity of the fecal matrix, detection of false positives in mEI cannot be ruled out.

In manure pits, where manure is stored for months before land application, die-off of bacteria is a natural phenomenon and it is not surprising to see higher concentrations of qPCR equivalent cells than cultivable counts for both *E. coli* and enterococci. However, there is also a possibility that some of these cells are transformed into viable but non cultivable state in manure and there is not much evidence in the literarature regarding this. It has been demonstrated that bacteria such as *E. coli* O157:H7 may become viable but nonculturable during conditions such as changes in temperature, available nutrients, or pH (Juhna et al. 2007; Kolling et al. 2001. Mizunoe et al. 1999). But the results indicate that there are some cultivable cells still present in the manure that are being applied on the fields, suggesting that pathogens that survive aging process of manure in pits can also be transported to the soil during land manure application. Based on the scheduled removal of manure from the pits sampled in this study, samples obtained in November had been aged in the pits only for approximately two to three months, whereas samples collected during May had been in the pit for five to six months. Higher concentrations (atleast one $\log_{10}$ higher) of cultivable cells in samples from November compared to May indicate that longer storage time in pits may be required to reduce the risks caused by pathogens in land applied manure. Log reduction of cultivable bacteria

during storage of manure pits have been found to be enhanced by addition of urea and ammonia (Ottoson et al. 2008).

Any individual *Bacteroides* bovine cluster isolates carrying cow specific 16S rRNA fragments, targeted in CF123 conventional PCR or Bobac assay, have not been cultivated yet and M2 and M3 cow specific markers have been originally designed by a metagenomic approach; therefore there is not much known about factors that influence prevalence of these markers in different bovine populations. A recent study that has quantified these three cow-specific markers found that *Bacteroides* bovine cluster markers were more abundant in the feces collected from 11 different bovine populations as compared to M2 and M3 cow specific markers (Shanks et al. 2010). This is consistent with the results in all manure pits in this study with Bobac occurring in higher concentrations than M2 or M3 markers in May and in November.

Variation in the concentrations of these markers could be attributed to either the variation in the densities of these targets in the cow intestines or it may be due to the difference in copy number of these target genes in the cells. Higher concentrations of Bobac markers could be due to the abundance of 16S rRNA copies which has been shown to vary between different bacteria and some bacterial genome may have upto 15 copies of this gene (Klappenbach et al. 2001). For M2 and M3 markers, there is no knowledge about the exact copy number of target genes (HDIG domain protein, Sialic acid-specific 9-O-acetylesterase) present in a cell. Functional annotation of these target genes indicate the involvement of these genes in energy metabolism and electron transport (Shanks et al. 2006) and based on the assumption that most functional genes

may have one copy of gene in a cell, M2 and M3 target genes may be one copy per cell; thus being detected in lower concentrations as compared to the Bobac markers.

Discharge of cattle farm waste into municipal sewers is possible; therefore raw sewage samples sometimes test positive for cow specific MST markers (Wolfe et al. 2010). Raw sewage and septage samples collected for a previous study were therefore monitored for these cow specific markers as well as for *Bacteroides-Prevotella* bovine CF123 cluster marker. High concentrations of Bobac markers were detected in all 18 sewage samples and three out of 8 septage samples, when the rest of the cow specific markers were absent in these. This indicates the cross specificity of Bobac assay with other host feces. This cross specificity was further confirmed by performing the assay on four human fecal samples which all tested positive for the assay (results not shown).

Conventional PCR assay for *Bacteroides-Prevotella* bovine CF123 cluster showed positive amplification in all pit samples tested (data not shown). The conventional PCR assay for CF 123 cluster has previously been used for feces from ruminants but was not found in raw sewage (Bernhard and Field 2000) which agrees with the findings in the current study. Shanks et al. (2010) reported 85% prevalence of *Bacteroides-Prevotella* bovine CF 123 cluster marker in 247 fecal samples collected from 11 herd populations. However, they also found this marker to be positive in feces from dog, chicken, duck and pig.

In conclusion, it was found in this study that qPCR equivalent cells were either same or less than agar based cultivation based methods for *E. coli* and enterococci in cow feces but qPCR equivalent cells were higher in manure samples for both of these indicators. Thus, there is die-off during storage that is resulting in the detection of qPCR signals than

cultivable cells. M2 and M3 cow specific markers were found consistently in manure samples tested that were collected just prior to the field application. These markers could be used as MST tools to monitor agricultural pollution in water bodies. Among these two markers, M3 marker is recommended for MST application as it was found to occur in in significantly higher concentrations than M2 markers ($p<0.05$) in all the manure pits and this may need further evaluation in other geographical setting or with other cow populations. This study does not recommend using Bobac assay for MST due to its lack of specificity and results obtained using this assay could be misleading the source of fecal contamination. Overall, this study has demonstrated that cow specific markers such as M2 and M3 can be used as a tracer to study the transport and fate of molecular signals from manure applied land without getting any amplification from naturally occurring organisms present in soil. This study also recommends the need to address the issues such as inefficiency in DNA extraction, inability to distinguish dead and live cells etc associated with application of qPCR tools in complex environmental samples.

REFERENCES

5.5 References

Atherton, F., C. P. S. Newman, and D. P. Casemore. 1995. An outbreak of waterborne cryptosporidiosis associated with a public water-supply in the uK. Epidemiology and Infection **115:**123-131.

Baffone, W., B. Citterio, E. Vittoria, A. Casaroli, R. Campana, L. Falzano, and G. Donelli. 2003. Retention of virulence in viable but non-culturable Vibrio spp. International Journal of Food Microbiology **89:**31-39.

Bernhard, A. E., and K. G. Field. 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. Applied and Environmental Microbiology **66:**4571-4574.

CDC. 1999. Outbreak of *Escherichia coli* O157:H7 and *Campylobacter* among attendees of the Washington County Fair-New York, 1999. MMWR Morb Mortal Wkly Rep:803-805.

Collins, R., S. Elliott, and R. Adams. 2005. Overland flow delivery of faecal bacteria to a headwater pastoral stream. Journal of Applied Microbiology **99:**126-132.

Culley, J. L. B., and P. A. Phillips. 1982. Bacteriological quality of surface and subsurface runoff from manured sandy clay loam soil. Journal of Environmental Quality **11:**155-158.

Davies-Colley, R. J., J. W. Nagels, R. A. Smith, R. G. Young, and C. J. Phillips. 2004. Water quality impact of a dairy cow herd crossing a stream. New Zealand Journal of Marine and Freshwater Research **38:**569-576.

Doran, J. W., J. S. Schepers, and N. P. Swanson. 1981. Chemical and bacteriological quality of pasture runoff. Journal of Soil and Water Conservation **36:**166-171.

Frahm, E., and U. Obst. 2003. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of Enterococcus spp. and Escherichia coli in water samples. Journal of Microbiological Methods **52:**123-131.

Gary, H. L., S. R. Johnson, and S. L. Ponce. 1983. Cattle grazing impact on surface-water quality in a colorado front range stream. Journal of Soil and Water Conservation **38:**124-128.

Gerard-Champod, M., S. Blanquet-Diot, L. Mazuranok, and M. Alric. 2009. Enumeration of some cultivable bacterial groups and characterization of some abiotic variables in the jejunoileal content of Prim'Holstein veal calves. Journal of Animal Science **87:**1664-1675.

Gerba, C. P., and J. E. Smith. 2005. Sources of pathogenic microorganisms and their fate during land application of wastes. Journal of Environmental Quality **34:**42-48.

Health Canada. 2000**.** Waterborne outbreak of gastroenteritis associated with a contaminated municipal water supply, Walkerton, Ontario, May–June. *Can Commun Dis Rep* 2000;26 (20).

Hooda, P. S., A. C. Edwards, H. A. Anderson, and A. Miller. 2000. A review of water quality concerns in livestock farming areas. Science of the Total Environment **250:**143-167.

Howell, J. M., M. S. Coyne, and P. Cornelius. 1995. Fecal bacteria in agricultural waters of the bluegrass region of kentucky. Journal of Environmental Quality **24:**411-419.

Hrudey, S. E., P. Payment, P. M. Huck, R. W. Gillham, and E. J. Hrudey. 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. Water Science and Technology **47:**7-14.

Hutchison, M. L., L. D. Walters, T. Moore, D. J. I. Thomas, and S. M. Avery. 2005. Fate of pathogens present in livestock wastes spread onto fescue plots. Applied and Environmental Microbiology **71:**691-696.

Isaacson, M., P. H. Canter, P. Effler, L. Arntzen, P. Bomans, and R. Heenan. 1993. Hemorrhagic colitis epidemic in africa. Lancet **341:**961-961.

Jackson, S. G., R. B. Goodbrand, R. P. Johnson, V. G. Odorico, D. Alves, K. Rahn, J. B. Wilson, M. K. Welch, and R. Khakhria. 1998. Escherichia coli O157 : H7 diarrhoea associated with well water and infected cattle on an Ontario farm. Epidemiology and Infection **120:**17-20.

Jimenez-Clavero, M. A., E. Escribano-Romero, C. Mansilla, N. Gomez, L. Cordoba, N. Roblas, F. Ponz, V. Ley, and J. C. Saiz. 2005. Survey of bovine enterovirus in biological and environmental samples by a highly sensitive real-time reverse transcription-PCR. Applied and Environmental Microbiology **71:**3536-3543.

Juhna, T., D. Birzniece, and J. Rubulis. 2007. Effect of phosphorus on survival of Escherichia coli in drinking water biofilms. Applied and Environmental Microbiology **73:**3755-3758.

Katharine, G. F., and M. Samadpour. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Research **41:**3517-3538.

Kildare, B. J., C. M. Leutenegger, B. S. McSwain, D. G. Bambic, V. B. Rajal,

and S. Wuertz. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. Water Research **41:**3701-3715.

Kirs, M., and D. C. Smith. 2007. Multiplex quantitative real-time reverse transcriptase PCR for F+-specific RNA coliphages: a method for use in microbial source tracking. Applied and Environmental Microbiology **73:**808-814.

Klappenbach, J. A., P. R. Saxman, J. R. Cole, and T. M. Schmidt. 2001. rrndb: the Ribosomal RNA Operon Copy Number Database. Nucleic Acids Research **29:**181-184.

Klein, M., L. Brown, B. van den Akker, G. M. Peters, R. M. Stuetz, and D. J. Roser. 2010. Monitoring bacterial indicators and pathogens in cattle feedlot waste by real-time PCR. Water Research **44:**1381-1388.

Kolling, G. L., and K. R. Matthews. 2001. Examination of recovery in vitro and in vivo of nonculturable Escherichia coli O157 : H7. Applied and Environmental Microbiology **67:**3928

Layton, A., L. McKay, D. Williams, V. Garrett, R. Gentry, and G. Sayler. 2006. Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Applied and Environmental Microbiology **72:**4214-4224.

Lleo, M. D., S. Pierobon, M. C. Tafi, C. Signoretto, and P. Canepari. 2000. mRNA detection by reverse transcription-PCR for monitoring viability over time in an Enterococcus faecalis viable but nonculturable population maintained in a laboratory microcosm. Applied and Environmental Microbiology **66:**4564-4567.

Mackenzie, W. R., N. J. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addiss, K. R. Fox, J. B. Rose, and J. P. Davis. 1994. A massive outbreak in milwaukee of cryptosporidium infection transmitted through the public water-supply. New England Journal of Medicine **331:**161-167.

Meays, C. L., K. Broersma, R. Nordin, and A. Mazumder. 2004. Source tracking fecal bacteria in water: a critical review of current methods. Journal of Environmental Management **73:**71-79.

Michino, H., K. Araki, S. Minami, S. Takaya, N. Sakai, M. Miyazaki, A. Ono, and H. Yanagawa. 1999. Massive outbreak of Escherichia coli O157 : H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. American Journal of Epidemiology **150:**787-796.

Mizunoe, Y., S. N. Wai, A. Takade, and S. Yoshida. 1999. Restoration of culturability of starvation-stressed and low-temperature-stressed Escherichia coli

O157 cells by using H2O2-degrading compounds. Archives of Microbiology **172:**63-67.

Nicholson, F. A., S. J. Groves, and B. J. Chambers. 2005. Pathogen survival during livestock manure storage and following land application. Bioresource Technology **96:**135-143.

Okabe, S., N. Okayama, O. Savichtcheva, and T. Ito. 2007. Quantification of host-specific Bacteroides-Prevotella 16S rRNA genetic markers for assessment of fecal pollution in freshwater. Applied Microbiology and Biotechnology **74:**890-901.

Oliver, D. M., P. M. Haygarth, C. D. Clegg, and L. Heathwaite. 2006. Differential E-coli die-off patterns associated with agricultural matrices. Environmental Science & Technology **40:**5710-5716.

Ottoson, J., A. Nordin, D. von Rosen, and B. Vinneras. 2008. Salmonella reduction in manure by the addition of urea and ammonia. Bioresource Technology **99:**1610-1615.

Pachepsky, Y. A., A. M. Sadeghi, S. A. Bradford, D. R. Shelton, A. K. Guber, and T. Dao. 2006. Transport and fate of manure-borne pathogens: Modeling perspective. Agricultural Water Management **86:**81-92.

Richardson, A. J., R. A. Frankenberg, A. C. Buck, J. B. Selkon, J. S. Colbourne, J. W. Parsons, and R. T. Mayonwhite. 1991. An outbreak of waterborne cryptosporidiosis in swindon and oxfordshire. Epidemiology and Infection **107:**485-495.

Shanks, O. C., E. Atikovic, A. D. Blackwood, J. R. Lu, R. T. Noble, J. S. Domingo, S. Seifring, M. Sivaganesan, and R. A. Haugland. 2008. Quantitative PCR for detection and enumeration of genetic markers of bovine fecal pollution. Applied and Environmental Microbiology **74:**745-752.

Shanks, O. C., K. White, C. A. Kelty, S. Hayes, M. Sivaganesan, M. Jenkins, M. Varma, and R. A. Haugland. 2010. Performance Assessment PCR-Based Assays Targeting Bacteroidales Genetic Markers of Bovine Fecal Pollution. Applied and Environmental Microbiology **76:**1359-1366.

Smith, H. V., W. J. Patterson, R. Hardie, L. A. Greene, C. Benton, W. Tulloch, R. A. Gilmour, R. W. A. Girdwood, J. C. M. Sharp, and G. I. Forbes. 1989. An outbreak of waterborne cryptosporidiosis caused by posttreatment contamination. Epidemiology and Infection **103:**703-715.

Swerdlow, D. L., B. A. Woodruff, R. C. Brady, P. M. Griffin, S. Tippen, H. D. Donnell, E. Geldreich, B. J. Payne, A. Meyer, J. G. Wells, K. D. Greene, M. Bright, N. H. Bean, and P. A. Blake. 1992. A waterborne outbreak in Missouri of

Escherichia-coli-O157:H7 associated with bloody diarrhea and death. Annals of Internal Medicine **117:**812-819.

Thiagarajan, A., R. Gordon, A. Madani, and G. W. Stratton. 2007. Discharge of Escherichia coli from agricultural surface and subsurface drainage water: Tillage effects. Water Air and Soil Pollution **182:**3-12.

Tsiodras, S., H. S. Gold, E. P. G. Coakley, C. Wennersten, R. C. Moellering, and G. M. Eliopoulos. 2000. Diversity of domain V of 23S rRNA gene sequence in different Enterococcus species. Journal of Clinical Microbiology **38:**3991-3993.

USDA Agricultural Research Service. *"FY-2005 Annual Report Manure and Byproduct Utilization*, 31 May 2006

Uwatoko, K., M. Sunairi, A. Yamamoto, M. Nakajima, and K. Yamaura. 1996. Rapid and efficient method to eliminate substances inhibitory to the polymerase chain reaction from animal fecal samples. Veterinary Microbiology **52:**73-79.

Wolf, S., J. Hewitt, and G. E. Greening. 2010. Viral Multiplex Quantitative PCR Assays for Tracking Sources of Fecal Contamination. Applied and Environmental Microbiology **76:**1388-1394.

Yu, Z. T., and M. Morrison. 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques **36:**808-+.

CHAPTER 6

QUANTITATIVE PCR FOR DETECTION OF *E. COLI* O157:H7 SPECIFIC *eae* AND *rfbE* GENES IN MANURE AND SEWAGE

6.1 Introduction

E. coli, one of the most genetically diverse groups of bacteria, is a common inhabitant in the intestines of warm blooded animals. However, there are some strains that are pathogenic and can cause illnesses. Based on the mechanism of pathogenicity, there are five classes of diarrheagenic *E. coli* which are as follows: 1) enterotoxigenic or ETEC, 2) enteroinvasive or EIEC, 3) Shiga toxin-producing *E. coli*, which has a subgroup called enterohemorrhagic or EHEC, 4) enteropathogenic or EPEC, 5) enteroaggregative or EAEC. Each group carries virulence factors that are responsible for their respective pathogenicity. ETEC have genes that code for heat-labile (LT) or heat-stable (HT) toxins. EIEC invade and multiply within the epithelium of colon and have plasmid encoded genes responsible for virulence factors. STEC carry genes that code for Shiga-like toxins, which are subdivided into two major classes, stx1 and stx2. A subgroup of shiga toxin carrying *E. coli*, known as EHEC, additionally has a Locus of Enterocyte Effacement or LEE. LEE, a pathogenicity island that confers to EHEC additional virulence genes such as *eae* which codes for intimin, and *Tir* which regulates the translocated intimin receptor. EPEC harbors LEE but do not have *stx* genes (genes that attach and efface (A/E) causing lesions in the intestinal epithelium. EAEC have virulence factors that enable them to cause auto agglutination on the surface of intestinal mucosa enhancing mucosa production.

E. coli O157, one of the most important members of the EHEC group, is transmitted through human and animal fecal sources (Muniesa *et al,* 2006). The O157: H7 refers to the specific markers found on the surface of the bacterium. The pathogenicicty of this strain is due to the large quantities of powerful verotoxins or Shiga-like toxins (stx1 and stx 2). They cause hemorrhagic colitis (inflammation of the intestinal wall) and the toxins cause damage to endothelial cells in the kidneys, thus inhibiting the organs' ability to function (Ludwig *et al*. 2002). Young children and the elderly can develop Hemolytic Uremic Syndrome (HUS) as a result of exposure to *E. coli* O157:H7, a condition that can lead to serious kidney damage and even death (Fitzpatrick, 1999).

Though this bacterium was discovered in 1977, no infection by *E. coli* O157 was reported in humans until 1982 (Su and Brandt, 1995). *E. coli* O157 was first isolated from two separate outbreaks of gastrointestinal illness in 1982, one in Oregon and one in Michigan, characterized by severe crampy abdominal pain, starting with a watery diarrhea that is followed by grossly bloody diarrhea, and little or no fever. Both the outbreaks was traced back to eating sandwiches with contaminated beef at restaurants belonging to the same fast-food chain (Riley et al; 1983). Food and waterborne transmission are common.

There were 31 waterborne outbreaks of *E. coli* O157 reported from the years 1982-2000 in United States and 21 were associated with recreational water and 10 with drinking water through contaminated local well water systems, municipal water supply systems, and spring water, residential faucet water, and ice that may have been cross contaminated (Rangel et al. 2005).

Cattle feces are a major reservoir of *E. coli* O157 (Al-Saigh *et al*, 2004; Bach *et al* 2004; Chapman *et al*, 1993; LeJeune *et al*, 2001; Meng *et al*. 2001; Shere *et al.* 2002) where they inhabit the intestine along with other microorganisms without actually causing any disease. The bacteria have also been isolated from sheep, dogs, goats, deer, horses and seagulls (Karch *et al*. 1999, Kudva *et al*, 1996). *E. coli* O157 outbreaks with ground beef as a transmission route were reported to be high in summer months; 71% occurred from May to August and this could be correlated with seasonal influence on prevalence rate in cattle which is high in summer and early fall (Hancock et al, 2001). This pathogen is able to survive for extended periods of time in manure (Franz *et al*, 2005; Kudva *et al* 1998; Wang *et al* 1996). Several studies have found them to survive well in low pH and low temperature for up to 56 days (Miller and Kaspar 1994; Conner and Kotrola 1995; Benjamin and Datta 1995). One mesocosm survival study found that O157 strain was reduced by 90% in wastewater from lagoons within 10 days and no cultivable colonies were observed after four weeks of the study (Ravva et al. 2006).

Differentiation of *E. coli* O157 from other members of the family Enterobacteriaceae in cultivation based methods are based on their specific biochemical characteristics; lack of β-glucuronidase activity and negative D-sorbitol fermentation within 24 h at 37°C (Vernozy-Rozand 1999). Detection of *E. coli* O157 has been performed most often by using a differential media, sorbitol MacConkey agar where *E. coli* O157 colonies appear as white colonies due to their inability to ferment sorbitol, while other members of the family Enterobacteriaceae appear as pink colonies. Another cultivation media used for detection of this pathogen is Rainbow AgarO157, where β-glucuronidase negative strains such as *E. coli* O157 appear as black or gray colonies and other strains of *E. coli* that are

positive for this enzyme appear as purple, violet or blue colonies in this media (Radu et al. 2000).

As with many other cultivation based methods, these methods take at least 24 hours to obtain the results. Since Polymerase Chain Reaction (PCR) is able to circumvent this issue by enabling detection of specific bacteria within a few hours, variants of PCR such as multiplex PCR, qPCR have been developed to detect and quantify *E. coli* O157 from various matrices. All of the qPCR assays in the literature for *E. coli* O157 are multiplexed, that is more than one gene is targeted in the same assay. This has proven to be a very useful method to discriminate between multiple closely related *E. coli* types. Since probes are labeled with different dyes that are detected at different wavelengths, multiplex assay targeting different genes of same organism or different organisms can be performed with in one tube. This method offers specificity and is cheaper as compared to singleplex PCR as reagent use will be reduced when multiple reactions are run in the same tube (Henegariu et al.1997). However, standardization of the assay may be more complex than for the singleplex PCR.

Multiplex qPCR assays for detection of *E. coli* O157 have also been developed (Ibekwe et al. 2002; Sharma and Dean-Nystrom 2003; Grant, 2008 ; Oberst et al. 2003) One qPCR protocol targeted stx1 and stx 2 simultaneously after a 24 h enrichment in fresh produce spiked with two strains of *E. coli* O157:H7 (Grant, 2008). Another multiplex PCR assay targeted three genes in the same assay, *stx1*, *stx 2* and *eae* genes to quantify *E. coli* O157:H7 in soil, manure cow fecal samples, and dairy wastewater. Specificity testing used 9 different strains of *E. coli* and *E. coli* O157:H7 and other strains such as O78:H16, O55: NM, O101, O116:H27, O91:H21. All non-O157:H7 strains had

either one or the other genes out of these three genes tested, while *E. coli* O157:H7 were positive for all the three genes (Ibekwe et al. 2002). Another used target gene for detection of *E. coli* O157:H7 included *rfbE* gene that encodes O antigen for O157 (Sharma 2006). Sequences specific to *E. coli* O157:H7 have been utilized to develop real-time PCR assay in combination with testing of viablility by reverse transcription of mRNA from O157:H7 cells (Sharma 2006). Multiplex real-time PCR assays targeting *rfbE* and *eae* genes specific for *E. coli* O157 were evaluated and to test for any amplification produced from non-*E. coli* O157 strains due to the presence of one or the other genes, these assays were tested for their specificity as a singleplex real-time PCR also (Sharma 2006).. Out of the 38 *E. coli* isolates tested, *eae* O157 assay showed positive amplification for three isolates of O157 strains, two isolates of O55:H7 and also showed a weak amplification signal one of the isolates of O111:H2. The *rfbE* specific primer sets amplified all three O157:H7 isolates but showed positive signals O149: NM and O157:H43. Several studies have used several combinations of stx1, stx2, eae, rfbE genes have been used to quantify *E. coli* O157 in food and water samples (Oberst et al. 2003; Childs et al.2006; Mull and Hill 2010; Bai et al.2010).

In this study, environmental samples were monitored using qPCR assays for detection of *E. coli* O157. But one of the problems is that there is no consistent or consensus on how to measure *E. coli* O157 in environmental samples. Therefore, the goal of this study was to examine the best method and gene targets with some modifications to characterize the manure and human waste environments using primer sets that targeted O157 specific sequences from *rfbE* and *eae* genes. Since the primary goal of this study was specific detection of *E. coli* O157 and since various combinations of stx1 and stx2 genes may be

present in other Shiga toxin producing *E. coli* serotypes (Nataro and Kaper 1998), assays targeting these two genes were not included in this study as quantification of toxin genes were not the goal of the study.

6.2 Materials and Method

6.2.1 Environmental samples

Manure from beef and dairy cattle manure pits (n=35), raw sewage collected from wastewater treatment facility at East Lansing, Michigan (n=18), and raw septage (n=8) collected from a septage treatment plant at Charveloix, Michigan were used in this study. Collection of samples, sample processing and DNA extraction are described in Sections 5.2.1 and 5.2.2, respectively. Sample processing and DNA extraction from raw sewage and septage samples are described in Chapter 4 under section 4.2.2.2.

6.2.2 Bacterial strains

ATCC strain of *E. coli* O157:H7 (ATCC 43895) was used for preparing standard curves. All the non-ATCC *E. coli* strains used in this study and hosts from where they were isolated are given in Table 6.1. These strains were provided by Microbial Evolution Laboratory, National Food Safety and Toxicology Center, at Michigan State University. All the bacterial strains were grown in Trypticase Soy Broth at 37^{o}C for 18-24 hours. DNA was extracted from these cultures using QIAmp DNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Table 6.1: *E. coli* strains used for specificity testing of *E. coli* O157 qPCR assays

***E. coli* seroptypes**	**Host**
O26:H11	human
O55:H7	meat
O111:H8	human
O76:H19	human
O103:H2	human
O45:H2	human
O113:H21	human
O104:H21	human
O91:H7	human
O146:H21	human
O103:H6	human
O15:H27	human
O125:HNM	human
O156:H21	cow
O5:HN	cow
OX03:HNM	cow
O149:HNM	cow
O128:H2	human
O157:H7	hamburger
O157:H7	human
O157:H7	human
O157:H7	human
O157:H7	human
O157:H7	human
O157:H7	human

*All isolates were obtained from Microbial Evolution Laboratory, National Food Safety and Toxicology Center, at Michigan State University

6.2.3 qPCR Assay standardization and analysis

This study addressed two genes. The standardization of *eae* and *rfbE* gene for *E. coli* was optimized by using the primers and probes described in Table 6.2. A series of tenfold dilutions of DNA extracted from ATCC strain of *E. coli* O157:H7 ranging from 10^6 to 10^1 were prepared and used as templates to prepare the standard curves. The reaction mix for amplification of *eae* gene consisted of 10 µL of the LightCycler 480 Probes Mastermix (Roche, Indianapolis, IN), 0.5 µM of each of forward and reverse primers, 0.2 µM of the probe, 1.6 µM $MgCl_2$ and nuclease free water to make up the final volume to 20 µL. The reaction mix for amplification of *rfbE* gene consisted of 10 µL of the LightCycler 480 Probes Mastermix (Roche, Indianapolis, IN), 0.5 µM of each of forward and reverse primers, 0.4 µM of the probe, 1.6 µM $MgCl_2$ and nuclease free water to make up the final volume to 20 µL.

The assays were carried out in LightCycler 480® (Roche, Indianapolis, IN) through the following temperature profiles; initial denaturation at 10 minutes at 95^{o}C followed by 50 cycles of denaturation for 20 seconds at 95^{o}C; annealing for 60 seconds at 60^{o}C and extension at 72 ^{o}C for 15 seconds. Triplicate analysis was done for each dilution and negative controls. The crossing point (Cp) of each PCR reaction was automatically determined by the LightCycler® Software 4.0 and final concentrations of *eae* and *rfbE* genes were analyzed by absolute quantification method. qPCR analysis for DNA extracts from *E. coli* isolates, manure, raw sewage and septage samples were carried out using the

same reaction mix and run conditions as described above for the standards. PCR-grade water was used as negative control and one of the dilutions were included as a positive control in each run.

Table 6.2: Primers and Probes used in detection of *E. coli O157*

Target genes	Sequences	Amplicon size	Reference
eae_{O157}	FP 5'GTAAGTTACACTATAAAAGCACCGTCG3' RP5'TCTGTGTGGATGGTAATAAATTTTTG3' Probe 5'HEX-AAATGGACATAGCATCAGCATAATAGGCTTGCT-BHQ1a-5HEX	106	Sharma et al. (2006)
$rfbE_{O157}$	FP 5'TCAAAAGGAAACTATATTCAGAAGTTTGA3' RP5'CGATATACCTAACGCTAACAAAGCTAA3' Probe 5'Cy5-AATAAATTTGCGGAACAAAACCATGTGCAA-BHQ2a-Cy5	129	Sharma et al. (2006)

6.3 Results

A qPCR was performed on DNA extracts for all *E. coli* O157:H7 and non-O157 serotype pure cultures. As shown in Table 6.3, there were 7 *E. coli* O157: H7 and 20 non-O157 serotypes tested. Twenty five out of 27 serotypes tested positive for one gene or the other. All seven *E. coli* O157 serotypes showed amplification at a very early Ct (typically around 11.00) for both the genes. Nine out of 20 non-O157 serotypes amplified at Ct of above 34 for both the genes. Serotypes O146:H21 and O128:H2 were negative for both the genes. Eight out of 20 serotypes were positive for just *eae*, whereas only one serotype, O118:H16 was positive for just *rfbE* gene. Among the 20 non-O157 strains, O55:H7 showed amplification at Ct of 17.51 for eae gene and O149:HNM showed amplification at a Ct of 17.66 for *rfbE* gene.

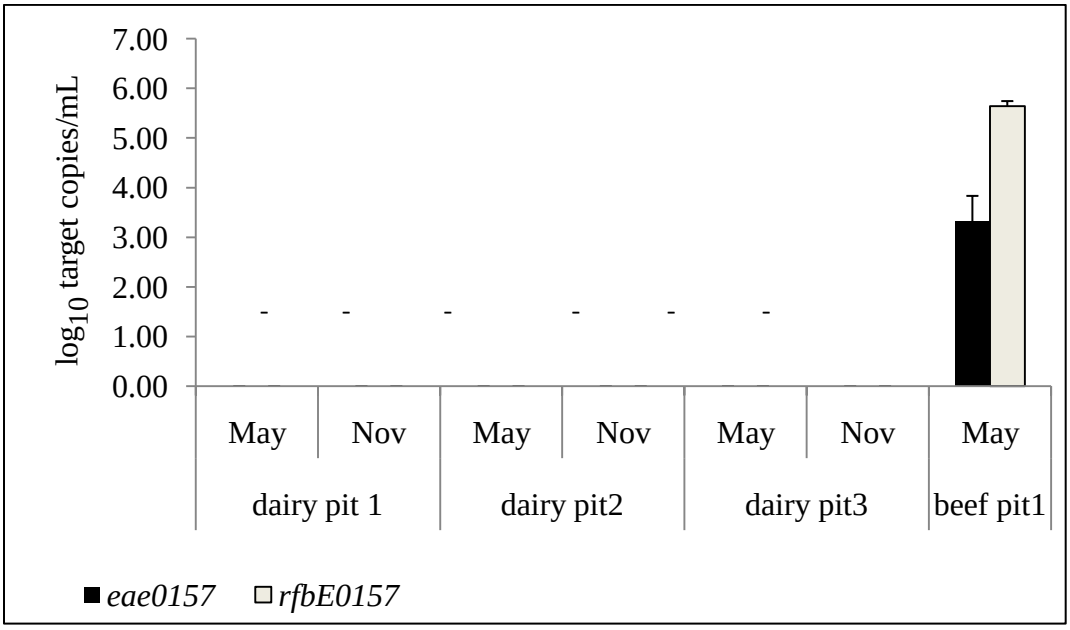
Table 6.3: Assay specificity with *E. coli* O157 and non-O157 serotypes

***E. coli* seroptypes**	**Cycle threshold**	
	eae	*rfbE*
OX03:HNM*	39.03	29.24
O91:H7	35.24	
O103:H6	35.9	
O146:H21		
O104:H21	37.43	
O113:H21	37.74	39.69
O149:HNM*	39.44	17.66
O128:H2*		
O25:Hneg	38.04	39.42
O15:H27	34.39	
O125:HNM	34.75	38.07
O156:H21*	35.77	
O5:HN*	37.69	
O76:H19	35.85	38.51
O111:H8	39.1	
O118:H16		37.03
O55:H7	17.51	
O45:H2	40.07	39.26
O103:H2	38.36	38.31
O26:H11	38.53	36.84
O157:H7	11.43	10.82
O157:H7	11.23	10.76
O157:H7	12.66	12.72
O157:H7	11.86	11.56
O157:H7	11.38	11.74
O157:H7	11.5	12.05
O157:H7	11.26	12.43

All serotypes are pathogenic except those marked with *
*No data available about pathogenicity

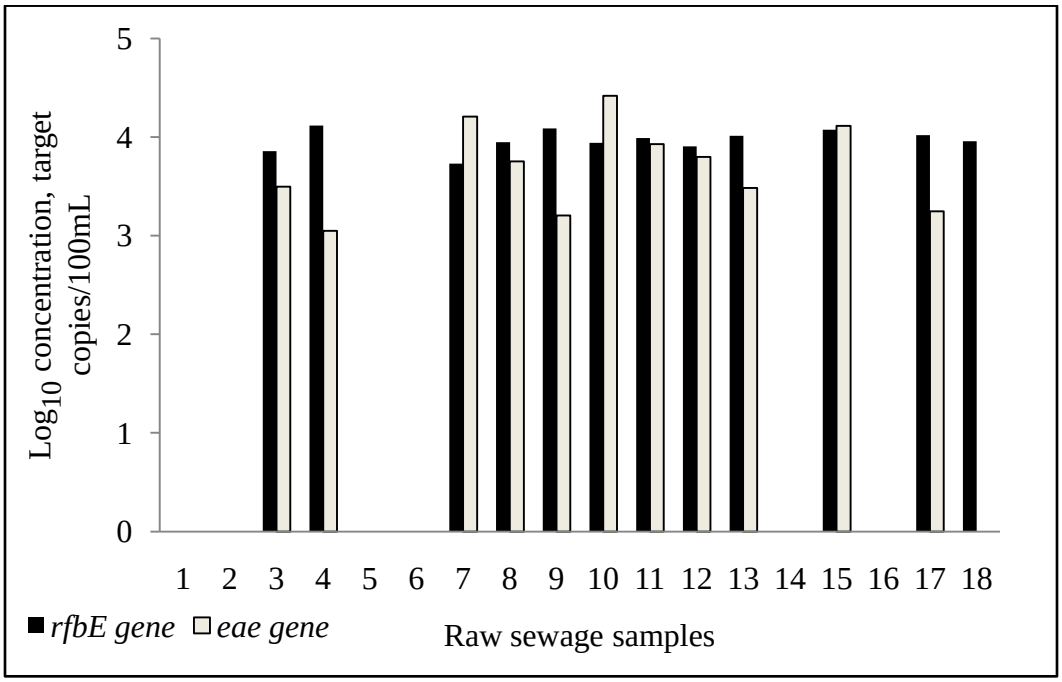
Occurrence of *E. coli* O157:H7 specific *eae* and *rfbE* genes were quantified in manure, raw sewage and raw septage samples. In manure samples, *rfbE* and *eae* genes were detected only in the pit from the beef cattle farm and in none of the samples from pits in dairy cattle farm showed amplification. Average concentration of O157 specific *eae* gene detected in five samples collected from beef cattle farm were 3.25×10^3 copies/mL and that of *rfbE* genes were 4.50×10^5 copies/mL (Figure 6.1). Cycles of threshhold for corresponding copies of reaction for these eae positive signals was around 38.80 and for *rfbE* positive signals, it was 33.70

Figure 6.1: Occurrence of *E. coli* O157:H7 specific *eae* and *rfbE* genes in manure samples



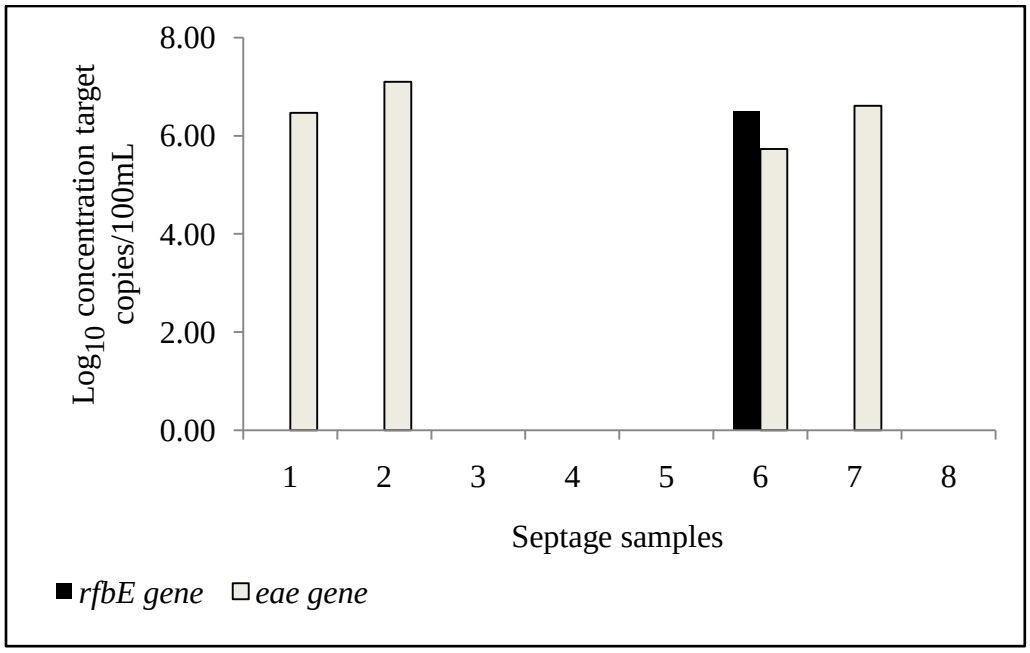
Out of 18 raw sewage samples tested, *rfbE* gene was detected in 11 sewage samples ranging from $1.35x10^5$ to $3.28x10^5$ copies/100 mL. The *eae* gene was detected in 10 of the 11 sewage samples positive for *rfbE* gene ranging from $2.81x10^4$ to $6.56x10^5$ copies/100mL.

Figure 6.2: Occurrence of *E. coli* O157:H7 specific *eae* and *rfbE* genes in sewage samples



Out of 8 septage samples tested only one sample showed amplification for the *rfbE* gene and four samples showed amplification for *eae* gene. The *rfbE* gene was present in the concentration of $3.10x10^6$ copies/100 mL in the one sample whereas the *eae* genes were detected in the concentrations of $5.39x10^5$ to $1.26x10^7$ copies/100 mL.

Figure 6.3: Occurrence of *E. coli* O157:H7 specific *eae* and *rfbE* genes in septage samples



6.4 Discussion

In this study, qPCR assays for *E. coli* O157 were applied to detect any *E.coli* O157 specific *eae* and *rfbE* genes present in manure, raw sewage and septage samples. These genes were selected due to their reported specificity (Sharma 2006).

E. coli O157 has been reported to be detected in cow manure environment (Omisakin et al. 2003; LeJeune et al. 2004; Kobayashi et al. 2003). The O157 specific target genes were detected only in the manure samples from beef cattle pit in this study and were not detected in any of the manure samples from pits in dairy cattle farm. One hypothesis for this finding is that difference in the diet supplied for both cattle groups may influence the colonization of cattle intestine with O157 (Callaway et al. 2009).

Manure samples from beef and cattle dairy pits, had similar levels of generic *E .coli* using qPCR for the *uidA* gene, present in concentrations of 10^5 cells/100ml. The same concentration of copies of *rfbE* genes were detected in the same samples, thus suggesting that the detection of *rfbE* genes lack specificity.

In this study, high numbers of O157 specific *eae* and *rfbE* genes were detected in some of the raw sewage samples tested. In the past, *E. coli* O157 has been detected in raw sewage using immunomagnetic absorbent assay combined with cultivation method (Sahlström et al. 2004) or qualitatively by conventional PCR (Grant et al. 1996). But what was surprising in this study was that the concentrations of *E. coli* O157 specific genes were only one log lower than the concentrations of generic *E. coli* detected in the same volume of raw sewage using the qPCR assay that targeted *uidA* gene. In septage samples, the concentrations of generic *E. coli* present were around 10^7-10^8 cells/100 mL and O157 specific *eae* genes were at concentrations of 10^5-

10^7 copies or 10^6 copies of *rfbE* genes in the same volume of sewage as with the manure this suggests that there could be some cross amplification in the samples using these assays.

Specificity of the assay performed in this study showed weak amplification for many non-O157 strains tested, questioning the reliability of these genes for specific detection of *E. coli* O157. The cross reaction with *E. coli* O55:H7 by O157 specific *eae* qPCR was found during the development of assay (Sharma et al. 2006) and was also found in this study. *E.coli* O55: H7 has been suggested to be genetically related and part of an initial lineage for *E. coli* O157 and therefore many assays that target O157 may also amplify O55: H7 (Zhou et al. 2010). There is also a speculation of cross reactivity of any O157 specific assays with other serotypes that possess homologs of the particular gene targets (Sharma et al. 2006).

The qPCR assay development and evaluation using multiple gene targets was carried out using seeded *E. coli* O157 or using isolates of non O157 serotypes for specificity testing (Ibekwe et al. 2003; Jothikumar and Griffiths 2002; Liu et al. 2010). When applied in environmental samples, these assays may express cross specificity with hundreds of serotypes of *E. coli* or other bacteria that have homologs of genes. Thus quantification of *E. coli* O157 with multiple qPCR assays targeting these genes may not be best approach if specific detection and quantification of *E. coli* O157 is the goal. The results of this study suggest that any specificity of reported *E. coli* O157 assays need further evaluation against many non-O157 serotypes before it is applied for detection of O157 in environmental samples. Confirmation of the O157 specific positive amplicons should be done by sequencing or by melting curve analysis. Though expensive, this approach may help to ascertain specificity of the assay.

REFERENCES

6.5 References

Al-Saigh, H., C. Zweifel, J. Blanco, J. E. Blanco, M. Blanco, M. A. Usera, and R. Stephan. 2004. Fecal shedding of Escherichia coli O157, Salmonella, and Campylobacter in Swiss cattle at slaughter. Journal of Food Protection **67:**679-684.

Bach, S. J., T. A. McAllister, G. J. Mears, and K. S. Schwartzkopf-Genswein. 2004. Long-haul transport and lack of preconditioning increases fecal shedding of Escherichia coli and Escherichia coli O157 : H7 by calves. Journal of Food Protection **67:**672-678.

Bai, J. F., X. R. Shi, and T. G. Nagaraja. 2010. A multiplex PCR procedure for the detection of six major virulence genes in Escherichia coli O157:H7. Journal of Microbiological Methods **82:**85-89.

Benjamin, M. M., and A. R. Datta. 1995. Acid tolerance of enterohemorrhagic escherichia-coli. Applied and Environmental Microbiology **61:**1669-1672.

Callaway, T. R., M. A. Carr, T. S. Edrington, R. C. Anderson, and D. J. Nisbet. 2009. Diet, Escherichia coli O157:H7, and Cattle: A Review After 10 Years. Current Issues in Molecular Biology **11:**67-79.

Chapman, P. A., D. J. Wright, P. Norman, J. Fox, and E. Crick. 1993. Cattle as a possible source of verocytotoxin-producing escherichia-coli-o157 infections in man. Epidemiology and Infection **111:**439-447.

Childs, K. D., C. A. Simpson, W. Warren-Serna, G. Bellenger, B. Centrella, R. A. Bowling, J. Ruby, J. Stefanek, D. J. Vote, T. Choat, J. A. Scanga, J. N. Sofos, G. C. Smith, and K. E. Belk. 2006. Molecular characterization of Escherichia coli O157 : H7 hide contamination routes: Feedlot to harvest. Journal of Food Protection **69:**1240-1247.

Conner, D. E., and J. S. Kotrola. 1995. Growth and survival of escherichia-coli o257-h7 under acidic conditions. Applied and Environmental Microbiology **61:**382-385.

Fitzpatrick M. 1999. Haemolytic uremic syndrome and *E. coli* O157. *BMJ* **318**:684-685.

Franz, E., A. D. van Diepeningen, O. J. de Vos, and A. H. C. van Bruggen. 2005. Effects of cattle feeding regimen and soil management type on the fate of Escherichia coli O157 : H7 and Salmonella enterica serovar typhimurium in manure, manure-amended soil, and lettuce. Applied and Environmental Microbiology **71:**6165-6174.

Grant, S. B., C. P. Pendroy, C. L. Mayer, J. K. Bellin, and C. J. Palmer. 1996. Prevalence of enterohemorrhagic Escherichia coli in raw and treated municipal sewage. Applied and Environmental Microbiology **62:**3466-3469.

Hancock, D., T. Besser, J. Lejeune, M. Davis, and D. Rice. 2001. The control of VTEC in the animal reservoir. International Journal of Food Microbiology **66:**71-78.

Henegariu, O., N. A. Heerema, S. R. Dlouhy, G. H. Vance, and P. H. Vogt. 1997. Multiplex PCR: Critical parameters and step-by-step protocol. Biotechniques **23:**504-511.

Ibekwe, A. M., P. M. Watt, C. M. Grieve, V. K. Sharma, and S. R. Lyons. 2002. Multiplex fluorogenic real-time PCR for detection and quantification of Escherichia coli O157 : H7 in dairy wastewater wetlands. Applied and Environmental Microbiology **68:**4853-4862.

Karch, H., M. Bielaszewska, M. Bitzan, and H. Schmidt. 1999. Epidemiology and diagnosis of shiga toxin-producing Escherichia coli infections. Diagnostic Microbiology and Infectious Disease **34:**229-243.

Kobayashi, H., A. Miura, H. Hayashi, T. Ogawa, T. Endo, E. Hata, M. Eguchi, and K. Yamamoto. 2003. Prevalence and characteristics of eae-positive Escherichia coli from healthy cattle in Japan. Applied and Environmental Microbiology **69:**5690-5692.

Kudva, I. T., K. Blanch, and C. J. Hovde. 1998. Analysis of Escherichia coli O157 : H7 survival in ovine or bovine manure and manure slurry. Applied and Environmental Microbiology **64:**3166-3174.

Kudva, I. T., P. G. Hatfield, and C. J. Hovde. 1996. Escherichia coli O157:H7 in microbial flora of sheep. Journal of Clinical Microbiology **34:**431-433.

LeJeune, J. T., T. E. Besser, and D. D. Hancock. 2001. Cattle water troughs as reservoirs of Escherichia coli O157. Applied and Environmental Microbiology **67:**3053-3057.

LeJeune, J. T., T. E. Besser, D. H. Rice, J. L. Berg, R. P. Stilborn, and D. D. Hancock. 2004. Longitudinal study of fecal shedding of Escherichia coli O157 : H7 in feedlot cattle: Predominance and persistence of specific clonal types despite massive cattle population turnover. Applied and Environmental Microbiology **70:**377-384.

Ludwig, K., V. Sarkim, M. Bitzan, M. A. Karmali, C. Bobrowski, H. Ruder, R. Laufs, I. Sobottka, M. Petric, H. Karch, and D. E. Mulller-Wiefel. 2002. Shiga toxin-producing Escherichia coli infection and antibodies against Stx2 and Stx1 in household contacts of children with enteropathic hemolytic-uremic syndrome. Journal of Clinical Microbiology **40:**1773-1782.

Meng, J., Doyle, M.P., Zhao, T. and Zhao, S. 2001. Enterohemorrhagic *Escherichia coli*. In *Food Microbiology: Fundamentals and Frontiers*, 2nd edn. ed. Doyle, M.P. Beuchat, L.R. and Montville, T.J. pp. 193– 213. Washington, DC: ASM Press.

Miller, L. G., and C. W. Kaspar. 1994. Escherichia-coli o157-h7 acid tolerance and survival in apple cider. Journal of Food Protection **57:**460-464.

Mull, B. and Hill, V. R. 2009. Recovery and detection of Escherichia coli O157:H7 in surface water, using ultrafiltration and real-time PCR Appl. Environ. Microbiol. **75**: 3593–3597

Muniesa, M., J. Jofre, C. Garcia-Aljaro, and A. R. Blanch. 2006. Occurrence of Escherichia coli O157 : H7 and other enterohemorrhagic Escherichia coli in the environment. Environmental Science & Technology **40:**7141-7149.

Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic Escherichia coli. Clinical Microbiology Reviews **11:**142-201..

Oberst, R. D., M. P. Hays, L. K. Bohra, R. K. Phebus, and J. M. Sargeant. 2003. Detection of Escherichia coli O157 : H7 in cattle feces using a polymerase chain reaction-based fluorogenic 5 ' nuclease (TaqMan (R)) detection assay after secondary enrichment. Journal of Veterinary Diagnostic Investigation **15:**543-552.

Omisakin, F., M. MacRae, I. D. Ogden, and N. J. C. Strachan. 2003. Concentration and prevalence of Escherichia coli O157 in cattle feces at slaughter. Applied and Environmental Microbiology **69:**2444-2447.

Omisakin, F., M. MacRae, I. D. Ogden, and N. J. Strachan. 2003. Concentration and prevalence of *Escherichia coli* O157 in cattle feces at slaughter. Appl. Environ. Microbiol. 69:2444-2447

Radu, S., Rusul, G., Ling, O.W., Purwati, E., Mustakim, M. and Lihan, S. 2000. Rapid isolation and detection of Escherichia coli O157:H7 by use of rainbow agar O157 and PCR assay. South East Asian Journal of Tropical Medicine and Public Health 31, 77–79.

Rangel, J. M., P. H. Sparling, C. Crowe, P. M. Griffin, and D. L.Swerdlow. 2005. Epidemiology of *Escherichia coli* 0157:H7 outbreaks,United States, 1982-2002. Emerg Infect Dis 11:603-9.

Ravva, S. V., C. Z. Sarreal, B. Duffy, and L. H. Stanker. 2006. Survival of Escherichia coli O157 : H7 in wastewater from dairy lagoons. Journal of Applied Microbiology **101:**891-902.

Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen. 1983. Hemorrhagic colitis associated with a rare escherichia-coli serotype. New England Journal of Medicine **308:**681-685.

Sahlstrom, L., A. Aspan, E. Bagge, M. L. Danielsson-Tham, and A. Albihn. 2004. Bacterial pathogen incidences in sludge from Swedish sewage treatment plants. Water Research **38:**1989-1994.

Sharma, V. K. 2006. Real-time reverse transcription-multiplex PCR for simultaneous and

specific detection of rfbE and eae genes of Escherichia coli O157 : H7. Molecular and Cellular Probes **20:**298-306.

Sharma, V. K., and E. A. Dean-Nystrom. 2003. Detection of enterohemorrhagic Escherichia coli O157 : H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins. Veterinary Microbiology **93:**247-260.

Shere, J. A., C. W. Kaspar, K. J. Bartlett, S. E. Linden, B. Norell, S. Francey, and D. M. Schaefer. 2002. Shedding of Escherichia coli O157 : H7 in dairy cattle housed in a confined environment following waterborne inoculation. Applied and Environmental Microbiology **68:**1947-1954.

Su, C. Y., and L. J. Brandt. 1995. *Escherichia coli* O157:H7 infection in humans. Annals of Internal Medicine **123:**698-714.

Vernozy-Rozand, C. 1999. Verotoxin-producing Escherichia coli (VTEC) and Escherichia coli O157 : H7 in medicine and food industry. Annales De Biologie Clinique **57:**507-515.

Wang, R. F., W. W. Cao, and C. E. Cerniglia. 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Applied and Environmental Microbiology **62:**1242-1247.

Zhou, Z. M., X. M. Li, B. Liu, L. Beutin, J. G. Xu, Y. Ren, L. Feng, R. T. Lan, P. R. Reeves, and L. Wang. 2010. Derivation of *Escherichia coli* O157:H7 from Its O55:H7 Precursor. Plos One **5:** e8700.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Recent advances in MST field have detected targets that can be used for detection of fecal contamination as well as to determine the host of origin (human versus bovine) and source (sewage versus manure). The primary goals of this research were to investigate the prevalence and concentrations of human and cow specific MST markers in comparison with *E. coli* and enterococci in both the human and cow waste environments and their fate during wastewater and septage treatments. This study explored human and cattle waste environment for the following targets: *E. coli* Colony Forming Units (CFU), enterococci CFUs, *E. coli* qPCR equivalent cells and enterococci qPCR equivalent cells; and MST markers human specific α- mannannase *B.thetaiotaomicron,* Bobac 16S rRNA bovine cluster markers, M2 cow specific and M3 cow specific markers and pathogenic *E. coli* O157 specific gene targets.

This study is the first to evaluate the MST markers in comparison with two regulatory conventional indicators, *E. coli* and enterococci using both conventional and qPCR tools. Septage, which is a non-point source of human fecal contamination, has not been monitored before for the levels of cultivable cells and qPCR signals of indicators. This study has made the first attempt to understand the fate of these signals during septage treatment. Evaluation of cow specific markers and indicators in manure used for land application has also not been published before.

The major conclusions from this work are:

- Evaluation of methods found that 1) quantitative results are preferable for measuring fecal contamination in waterbodies as there is background DNA signal with quantitative measures

one can follow the change in the signal, 2) an automated method can be used for DNA extraction from human raw waste and treated effluent samples after an external lysis step but the instrument used in this study is not suitable for recovery from cow fecal samples, 3) the EMA-qPCR method applied to distinguish live and dead cells in chlorinated effluents is not valid.

- In characterizing human sewage environment, it was found that human marker, *B. thetaiotaomicron* qPCR equivalent cells were present in significantly higher concentrations than that of *E. coli* or enterococci in raw sewage and septage. However, the fate of this target qPCR signal was similar to *E. coli* and enterococci DNA during wastewater and septage treatment. A significant correlation was found between this MST marker and each of the conventional indicators throughout the waste treatment process for both raw sewage and septage. It was observed that around 10^2 to 10^3 qPCR equivalent cells/100mL of these targets are being discharged through the final effluents into surface waters.
- Non-point sources such as manure applied to land had cultivable *E. coli* and enterococci cells in the concentrations of 10^5CFU/100 mL. Cow specific markers, M3 were found at concentrations of 10^8 copies/100 mL and M2 at levels of 10^7 copies/100 mL in the manure applied in the land. Another cow marker, *Bacteroides* bovine cluster was detected in human sewage environment and should not be used for source tracking cow fecal contamination.
- Comparison of qPCR with cultivation methods performed with all the samples tested in this research suggested that qPCR could be used in the environmental monitoring to obtain quicker results.

The concentrations of indicators and MST markers quantified in the samples collected in this study are summarized in Table 7.1 and Table 7.2, respectively.

Table 7.1: Average concentrations of the indicator organisms in the samples monitored in this research

Samples	n	*E. coli* CFU/100mL	Enterococci CFU//100mL	*E. coli* qPCR cells/100mL	EnterococciqPCR cells/100mL
Raw sewage	54	1.62E+06	5.25E+05	2.88E+06	4.27E+06
Primary effluent	54	1.48E+06	3.80E+05	3.02E+06	5.62E+06
Secondary effluent (Prechlorination)	18	3.47E+03	2.29E+03	1.00E+03	4.19E+04
Secondary effluent (Post-chlorination)	54	1.02E+01	4.37E+00	1.12E+03	1.35E+04
Tertiary effluent	54	1.10E+01	5.62E+00	6.61E+02	3.89E+03
Raw septage	24	2.95E+06	2.29E+06	2.14E+07	2.04E+07
Treated septage effluent	24	9.12E+03	1.17E+04	3.24E+03	2.09E+05
Cow manure	35	6.14E+05	1.94E+06	4.61E+06	3.14E+07
Cow feces*	10	8.41E+05	4.80E+05	7.97E+05	4.69E+04

*Units for CFU= CFU/g
*Units for qPCR= cells/g

Table 7.2: Average concentrations of the human and cow specific MST markers in the samples monitored in this research

	n	*B.thetaiotaomicron*[a] Copies/100mL	Bobac* Copies/100mL	M2* Copies/100mL	M3* Copies/100mL
Raw sewage	54	1.82E+07	2.65E+06	Neg	Neg
Primary effluent	54	2.04E+07	ND	ND	ND
Secondary effluent (Prechlorination)	18	1.82E+04	ND	ND	ND
Secondary effluent (Post-chlorination)	54	1.55E+04	ND	ND	ND
Tertiary effluent	54	4.68E+03	ND	ND	ND
Raw septage	24	3.55E+07	6.82E+07	Neg	Neg
Treated septage effluent	24	2.63E+04	ND	ND	ND
Cow manure	35	ND	2.15E+09	6.19E+07	6.65E+08
Cow feces	10	ND	ND	ND	ND

[a]Human specific marker
*Cow specific marker
ND-Not Determined
Neg-Negative

Method development included development of a new qPCR for *E. coli* targeting *uidA* gene. Compared to the cultivation methods for the detection of *E. coli* such as cultivation on mTEC, enzyme based colilert assay, qPCR was rapid, and produced sensitive results. An automated method was used for extracting DNA from environmental samples due to its reproducibility, efficiency in reducing the technical variability, and lower risks of cross contamination. Significantly higher ($p<0.05$) DNA can be recovered using autoextraction compared to manual extraction kits from raw sewage and effluents from wastewater treatment process when tested for *E. coli* qPCR concentrations. The machine was not found to be satisfactory with DNA extraction from cow feces. New automation is needed for environmental samples as molecular methods are being applied widely in this field and DNA extraction plays a key role in any molecular investigation.

One major disadvantage of qPCR method is the inability to differentiate live and dead cells. This study attempted to address the viability issue by treatment of samples with Ethidium monoazide dye prior to DNA extraction. The method did not work with chlorine disinfected samples even though it was found to distinguish heat-killed cells. To understand the mechanism of chlorine disinfection on cultivable cells or DNA during wastewater treatment, chlorine dosage studies using pre-chlorinated secondary effluent seeded with viable bacterial cells in combination with EMA or other efficient dyes are recommended. Modifications in the sample treatment with dye may also improve the efficiency of the method but requires detailed evaluation.

A new human marker *B. thetaiotaomicron,* α- mannanase gene qPCR assay was found in sewage 100% of the time the samples were tested and was reduced by secondary treatment process to 3 $\log_{10}$ units. This marker was found to be abundant in septage also with concentrations around 10^7 cells/100mL which got reduced to 10^4 cells following the treatment by lime stabilization process. These markers were found to correlate significantly ($p<0.05$) with each of the conventional indicators, *E. coli* and enterococci throughout the wastewater treatment process.

Two new cow markers M2 and M3 were found in all manure samples tested and should be explored further for MST applications. This study recommends M3 marker due to its occurrence in higher concentrations in manure samples tested. However, the prevalence of these two markers may vary in different cow herds and in different cow populations and should be tested further in other locations. The manure samples that were mixed and applied in the agricultural lands had cow specific markers occurring at concentrations of 10^4-10^7 qPCR target copies per mL and conventional indicators occurring in the concentrations of 10^3 to 10^7 qPCR target copies

per mL. Occurrence of higher levels of cultivable cells of *E. coli* and enterococci in samples collected after a longer storage period of around six months than in samples after a storage period of two-three months suggested the importance of longer storage to reduce the pathogen levels in manure before the land application.

One of the specific goals of this research was to monitor all these waste environments for the waterborne pathogen, *E. coli* O157 using published qPCR assays reported to be specific for this serotype of *E. coli. E. coli* O157 specific *eae* and *rfbE* genes were detected in beef manure pits, and also in human sewage and septage environments. But they were found at the same levels as that of generic *E. coli* target gene that were evaluated in the same samples. There are issues about cross specificity associated with the *E. coli* O157 specific *eae* and *rfbE* qPCR assays when they are applied in the environment due to the genetic variability of *E. coli* serotypes. Further evaluation and specificity testing for these *E. coli* O157 assays will be done in future to address the concerns found in this research.

In conclusion, qPCR assay targeting α-mannanase 1-6 gene of *B. thetaiotaomicron* was found to be present in similar levels as that of *E. coli* and enterococci in human waste environment and appears to be a reliable source tracking indicator for predicting human fecal contamination. This study suggests that this marker be included in a tool-box approach to monitor water bodies and to quantify human fecal contamination in the water bodies. These human specific qPCR signals can be used to track the fate of bacterial signals once discharged into the surface waters through effluents and thus avoid interferences from any non-human fecal contamination. M3 cow specific marker is suggested to be a reliable marker as compared to M2 marker based on this study and recommended for source tracking of agricultural pollution in

water bodies. Occurrence of these markers in high concentrations in the manure applied in the field can be utilized in designing run-off studies from manure applied land.