Evaluation of different indicator microorganism enumeration protocols for water quality monitoring

by

Jing Yuan

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Animal Sciences

Auburn, Alabama
May 7, 2016

Keywords: water quality, *Escherichia coli*, Enterococci, enumeration methods

Approved by

Luxin Wang, Chair, Assistant Professor of Animal Sciences
Christy Bratcher, Associate Professor of Animal Sciences
Emefa Monu, Assistant Professor of Poultry Science
Eric Reutebuch, Director of Alabama Water Watch

Abstract

Water quality, such as drinking water quality, irrigation water quality, and recreational water quality, is important for public health. *Escherichia coli*, coliform and Enterobacteriaceae have been used as indicator organisms to monitor the potential contamination of water. Previous studies indicate that bacterial concentrations may be significantly different when sampled at different times of the day. There are also studies showing that sediments contain higher levels of indicator microorganisms than the surface water. In recent years, in addition to *E. coli* and coliform, *Enterococcus* has been proposed to be used as an indicator organism for water quality due to its ability to survive in salt water. Although the literature provides important information, no parallel comparison among those enumeration methods have been conducted.

The purposes of this study are: 1) to compare the efficiency of three enumeration methods for *E. coli*, including the mTEC membrane filter/USEPA method 1603, Coliscan® Easygel agar plates, and 3M™ Petrifilm™ method for field sampling; 2) to better understand the impact of the sampling times (morning vs. afternoon and months) and sample types (surface water vs. sediment) on water quality monitoring results; 3) to compare the efficacy of four different Enterococci enumeration protocols, including the mEI membrane filter/USEPA method 1600, the Enterolert® method, the Easygel cards™ method and the USEPA qPCR method 1611, for freshwater monitoring.

Our results show that there were no differences among the three *E.coli* enumeration methods ($P > 0.05$). Therefore, the Coliscan® Easygel agar plates method (used by the Alabama

Water Watch) was used to evaluate the impact of sampling times and sample types on the enumeration of *E. coli*. Field sampling results show that both the sampling times and sample types may impact the enumeration results ($P < 0.05$), regardless of the indicator microorganisms used. When samples were collected in the afternoon, the surface water samples contained more indicator microorganisms than samples collected in the morning. Sediments contained more indicator microorganisms than the surface water ($P < 0.05$) and impacted the surface water monitoring results. The comparison of four Enterococci enumeration protocols show that while the Easygel cards™ method has the lowest price ($1 per sample), the USEPA qPCR method 1611 ranks the highest among all tested methods based on the shorter processing time needed (~ 4 hours) and the widest detection range (2.47-8.47 log CFU/mL for surface water and 2.47-8.47 log CFU/g for sediment). Because of this, different DNA extraction methods were tested and compared to prepare samples for the qPCR protocol. Results show that, for surface water samples, the PrepMan® boiling procedure can substitute for the DNA extraction procedure used by the USEPA qPCR method 1611, however, for sediment samples, the PowerSoil® DNA Isolation Kit cannot be replaced by the PrepMan® boiling procedure. The results also show that the USEPA qPCR method 1611 is an efficient method for enumerating *Enterococcus* both in surface water and sediment.

Acknowledgments

First of all, I would like to take this opportunity to thank my mentor and major advisor, Dr. Luxin Wang, for offering me the opportunity to pursue a graduate degree at Auburn University and for supporting and teaching me during my research. The knowledge I have gained from you will be extremely beneficial in my future career. Additionally I would like to thank Mr. Eric Reutebuch, for supporting me during the field sampling trips for the project and helping me with my research. Thank you Dr. Christy Bratcher and Dr. Emefa Monu for the good advice and review of my research.

I would also like to thank Patty Tyler and my coworkers Chao Liao, Dong Han, Amanda Windham and Kayla Golson who worked with me in the lab and were always willing to give a hand for my project.

Also, I would like to extend a special thanks to Wind Creek State Park staff, Lake Watch of Lake Martin volunteer monitors and Logan Martin Lake Protection Association volunteer monitors. Without their help I could not finish this study and complete my research. Thanks for providing time and talent to make this study success.

Last but not least, I would like to thank my parents for their supporting and their constant love.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

APHA	American Public Health Association
AWW	Alabama Water Watch
CDC	Centers of Disease Control and Prevention
CFU	Colony Forming Units
DAEC	Diffuse-adherent *E. coli*
EAggEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FDA-BAM	Food and Drug Administration Bacteriological Analytical Manual
ISO	International Organization for Standardization
MPN	Most Probable Number
NORS	National Outbreak Reporting System
PCR	Polymerase Chain Reaction
RWI	Recreational Water Illnesses
SFIB	Standard Fecal Indicator Bacteria
STEC	Shiga toxin-producing *E. coli*
USEPA	United States Environmental Protection Agency

USGS	United states Geological Survey
VTEC	Verocytotoxigenic *E. coli*
WBDO	Waterborne disease outbreak
WBDOSS	Waterborne Disease and Outbreak Surveillance System
WHO	World Health Organization

CHAPTER 1. LITERATURE REVIEW

1.1 Importance of water quality

Water is an essential element in the maintenance for all life, and most living organisms can only survive for short periods without water (Tchobanoglous and others, 1985). According to the United States Geological Survey, 71% of the earth's surface is water-covered, including oceans, lakes, rivers, ponds, and streams (USGS, 2015). Human beings are made up of more than 60% water (Hall and others, 1991; Armstrong and others 1985), and apart from drinking to survive, water is used for producing food, washing and recreating (Gleick, 1993).

In recent years, waterborne diseases cause serious public health concerns in both developed and developing countries. According to the World Health Organization (WHO), about 1.1 billion people drink unsafe water (Kindhauser, 2003) and the majority of diarrheal disease (88%) is related to unsafe water, sanitation and hygiene (WHO, 2003). About 3.1% of annual deaths (1.7 million) and 3.7% of the annual health problems (54.2 million) are also related to unsafe water (Payment and others, 1997).

Waterborne disease outbreak (WBDO) statistics have been compiled in the United States since 1920 (Gorman and others, 1939). From 1937 to 1970, WBDO statistics were collected by Federal agencies and various investigators (Eliassen and others, 1948; Weibel and others, 1964), and since 1971, the United States Environmental Protection Agency (USEPA), Centers for Disease Control and Prevention (CDC) and Council of State and Territorial Epidemiologists are involved in the WBDO statistics collection (USEPA, 1971; CDC, 1974).

In the United States, between the years 1920 to 2002, there were at least 1870 outbreaks associated with drinking water, an average of 22.5 per year. The annual average number increased from 11.1 (from 1951 to 1960) to 32.4 (from 1971 to 1980). In recent decades, 207 WBDOs and 433,947 illnesses were reported (Craun and others, 2006). In developing nations, because of the higher rate of endemic (background) gastrointestinal disease and pathogen concentrations in wastewater (Martins and others, 1983; Jimenez and others, 2002), specific waterborne diseases are rarely identified. Waterborne illnesses can cause a variety of symptoms, including diarrhea and vomiting, and other symptoms such like skin, ear, respiratory, or eye problems (Modlin, 1986).

According to the World Health Organization, some 842,000 people are estimated to die each year from diarrhea as a result of unsafe drinking water and tens of millions are seriously sickened because of polluted drinking water (WHO, 2011). In both developing and developed nations drinking water microbiological contaminations are considered higher risks than chemical and physical contaminations (Craun, 1993; Downs and others, 1999). Disease-causing pathogens transmitted through drinking water are predominantly of fecal origin (Ashbolt and others, 2001; Hunter and others, 2002).

Water used for agriculture or growing crops is called irrigation, and groundwater, surface water, and human wastewater are commonly used for irrigation (Snyder and others, 2005). The quality of irrigation water may affect both crop yields and soil physical conditions (Bauder and others, 2007). Using polluted irrigation water raises obvious potential health risks for farmers and consumers and studies showed clear links between wastewater irrigation and the health of exposed farming households (Drechsel and others, 2010; Gelting and others, 2012).

Foodborne pathogens can survive in irrigation water for a long time (Allende and others, 2015). Irrigation with poor-quality water is one way that fruit and vegetables can become contaminated with foodborne pathogens (Steele and others, 2004). In the United States, irrigation water has been identified as a potential source of foodborne pathogen outbreaks. Greene and others reported an outbreak of *Salmonella* infections associated with eating tomatoes (2008). Contaminated tomatoes were traced back to the eastern shore of Virginia, where the outbreak strain was isolated from pond water used to irrigate tomato fields (Greene and others, 2008). In Sweden, there was an outbreak of *Escherichia coli* (*E. coli*) infections caused by the consumption of lettuce irrigated by water from a small stream, in which *E. coli* O157 strains was isolated (Söderström and others, 2008).

Humans also use water for recreational purposes. According to the CDC, recreational water illnesses (RWIs) may be caused by bacteria ingested or inhaled while swimming (CDC, 2012). In the United States, during 1997 to 1998, a total of seven states reported eight outbreaks of waterborne diseases, specifically gastroenteritis, associated with recreational water that affected over 1,000 individuals (Barwick and others, 2000). During 2011 to 2012, 90 recreational water–associated outbreaks were reported to CDC's Waterborne Disease and Outbreak Surveillance System (WBDOSS) through the National Outbreak Reporting System (NORS). The 90 outbreaks resulted in at least 1,788 cases, 95 hospitalizations, and one death (Hlavsa and others, 2015).

Because of the importance of water quality (including drinking water, irrigation water, and recreational water), water quality is of great importance for public health and needs to be monitored closely. According to the water pollution guide, there are 7 basic

types of water pollution, including Surface water pollution, Groundwater pollution, Microbiological pollution, Oxygen depletion pollution, Nutrient pollution, Suspended matter pollution and Chemical pollution (Knight and others, 1980).

The research of Sorvall (1971) showed that Surface water pollution is the most visible form of pollution, and includes items such as water bottles, plastic and other waste products, and Groundwater pollution is usually caused by toxic chemicals and pesticides from farming and industrial processes (Sorvall, 1971). In microbiological pollution, including bacteria, viruses and protozoa, not all microbiological pollution cases are harmful, but some are pathogenic and can cause severe diarrhea or even death. This pollution is a significant problem for humans and animals (Wilber, 1969). When oxygen levels are too low or nutrient levels are too high in water, this can cause oxygen depletion pollution and nutrient pollution (William, 1969; Mueller and others, 1996). Suspended matter pollution can harm living creatures in water by taking away nutrients and disturbing their habitat (Sorvall, 1971). Chemical pollution is caused by chemical compounds, such as heavy metals, and the toxins can accumulate and be transferred to people (Patnaik, 2010).

Traditionally, the principal reason for monitoring water quality was to verify whether the water source was suitable for an intended use. Water quality monitoring has evolved and the main purposes are various, including assessments of the current state of water quantity and quality, classification of water, support the water management (World Meteorological Organization, 2013). However, water quality can be difficult to monitor simply by looking at it because most pollutants are invisible to the naked eye (Southwest

Florida Water Management District, 2012b). Therefore, water quality indicators need to be found to help monitor water quality.

1.2 Water quality indicator microorganisms

Although water quality is often described by concentrations of different chemicals of interest (Tchobanoglous and others, 1985), this study will focus on the microorganism indicators as microbiological pollution is a significant type of water pollution. Early studies showed that people who swam in waters with a geometric mean concentration of coliform above 2300 colonies/100 mL for three days had higher illness rates (Stevenson, 1953). According to the USEPA, *E. coli* and Enterococci were recommended as indicators of recreational water quality in 1986 (USEPA 1986). In recent years, the Enterobacteriaceae family was proposed as indicator microorganisms because they may have more pathogenic bacteria and greater resistance to the environmental conditions (Ewing and others, 1973; Spector and others, 2011; Talbot and others, 1980).

1.2.1 Enterobacteriaceae

The Enterobacteriaceae family is defined by Bergey, Breed, Murrany and Hitchens 1939 as Gram-negative, non-sporogenic rods widely distributed in nature. All species can grow well on artificial media and can use glucose, forming acid or acid and visible gas (H_2 present). Characteristically, nitrites are produced from nitrates by this group of bacteria. When motile, their flagella are peritrichous. This family consists of innumerable, intergrading races, types or varieties, divisible into genera. This family frequently occurs as saprophytes causing decomposition of plant materials containing carbohydrates. (Bergey and others, 1939)

The Enterobacteriaceae family contains about 20 genera. Some strains are harmless such as the coliform group and the nonpathogenic *E. coli*, while others are important pathogens such as *Salmonella*, *Shigella*, *Klebsiella* and *Yersinia* (Jean, 1980). Members of the Enterobacteriaceae are widely distributed (Baylis and others, 2011), many of them are a normal part of the gut flora found in the intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants (Lund and others, 1988).

In addition to the fact that the Enterobacteriaceae family includes both pathogenic and non-pathogenic bacteria, studies have also shown that this family has a greater resistance to the environment (Ewing and others, 1973). For example, *Salmonella* has demonstrated a wide range of stress resistance (Spector and others, 2011), and *Klebsiella* has shown high levels of resistance to different antibiotics (Talbot and others, 1980).

1.2.2 Coliform

Coliform bacteria are members of the Enterobacteriaceae family, according to the American Public Health Association, coliform bacteria are defined as Gram-negative, rod-shaped, non-spore forming and motile or non-motile bacteria which can ferment lactose with the production of acid and gas when incubated at 35–37°C (APHA,1995). The coliform group includes *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella* (Hudson and others, 1983). They are widely found in plant material, soil and water. They are universally present in large numbers in the feces of warm-blooded animals (Cohen and others, 1972).

Even though they are generally not normal causes of serious illness, they are easy to culture and can indicate the possible presence of pathogenic bacteria, viruses and protozoans (APHA, 1995). Therefore, coliforms are commonly used as a bacterial indicator of the sanitary quality of foods and water.

1.2.3 *Escherichia coli*

E. coli is a very widely studied organism. It belongs to the coliform bacteria group, and it is defined as a Gram-negative, rod-shaped, non-spore former and facultative anaerobic bacterium (Singleton, 1999).

Most *E. coli* strains are part of the normal gut microflora and harmless to human and animal populations. They can benefit their hosts by producing vitamin K2 (Bentley and others, 1982) and by helping to keep out other pathogenic bacteria (Hudault and others, 2001; Reid and others 2001). Unfortunately, some can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (Vogt and others, 2005).

E. coli and other facultative anaerobes constitute about 0.1% of gut flora (Eckburg and others, 2005), and fecal–oral transmission is the major route through which pathogenic strains can cause disease. Cells are able to survive outside of the body for a limited amount of time, which makes them ideal indicator organisms to monitor environmental samples for potential fecal contamination (Feng and others, 2002). A growing body of research found that the environmentally persistent *E. coli* can survive for extended periods outside of a host (Ishii and others, 2008).

Six known pathogenic types associated with gastrointestinal infections have been recognized (Donnenberg, 2002), these pathogenic types including, Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Verocytotoxigenic *E. coli* (VTEC) (or Shiga toxin-producing *E. coli* (STEC) of which Enterohemorrhagic *E. coli* (EHEC) are a subgroup), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAggEC), and Diffuse-adherent *E. coli* (DAEC) (Bettelheim, 2007). The symptoms of pathogenic *E. coli* infection may include: fever, nausea, vomiting, stomach cramps, diarrhea, generalized swelling, and excessive bleeding, even death (Donnenberg, 2002).

In the past decades, there have been many illnesses and outbreaks that were related to the pathogenic types of *E. coli*. In 1982, *E. coli* O157:H7 was involved in the Michigan and Oregon outbreak associated with eating hamburger sandwiches at restaurants belonging to the same fast-food restaurant chain (Lim and others, 2010). In 2006, *E. coli* O103:H25 was involved in an outbreak in Norway (L'Abée-Lund and others, 2012). In 2011, *E. coli* O104:H4 was involved in a major outbreak, causing 53 deaths, 3,000 patients with acute gastroenteritis, and 600 others with hemolytic uremic syndrome (Hauswaldt and others, 2013). In 2015 and 2016, *E. coli* O26 was involved in a multistate outbreak linked to Chipotle Mexican grill restaurants. According to the report, 5 people were infected in three states in 2015 and a total of 55 people were infected from a total of 11 states in the larger outbreak in 2015. The majority of these cases were reported from Oregon and Washington during October 2015 (CDC, 2016). *E. coli* is specific to fecal material from humans and other warm-blooded animals. Therefore, in 1986, the United States Environmental Protection Agency (USEPA) recommended using *E. coli* as an indicator of fecal pollution for purposes of evaluating fresh water quality (USEPA, 1986a). In the state of

Alabama the regulations state that for drinking water, no *E.coli* can be detected. For non-coastal recreational water, the *E. coli* concentration should be less than 235 colonies/100 mL and the geometric mean of *E. coli* density must be less than or equal to 126 colonies/100 mL (Alabama Department of Environmental Management, 2014).

1.2.4 *Enterococcus*

Besides Enterobacteriaceae, coliforms, and *E. coli*, streptococci are also widely used as indicators of possible water contamination (USEPA, 1986a). Many group D streptococci have been reclassified and placed in the genus Enterococci, including *E. faecalis*, *E. faecium*, *E. durans*, and *E. avium* (Köhler, W, 2007).

Enterococci are Gram-positive cocci that occur singly, in pairs, or as short chains, and fit within the general definition of lactic acid bacteria (Gilmore, 2002). Enterococci are facultative anaerobes with an optimum growth temperature of 35°C and a growth range from 10 to 45°C. Some species are motile (Facklam and others, 1995). Enterococci are part of the normal intestinal microflora of humans and animals (Penas and others, 2013). Two species are common commensal organisms in the intestines of humans: *E. faecalis* and *E. faecium* (Köhler, W, 2007).

Enterococci are widely distributed in nature, and can be found in foods, plants, soil, water, human feces and animal feces (Layton and others, 2010). Usually Enterococci represent less than 1% of the environmental flora (Tendolkar and others, 2003), but they are ubiquitous in human feces and persist in the environment.

Enterococci can cause diverse opportunistic infections (Penas and others, 2013) and have the ability to survive heat treatments and adverse environmental conditions (Johnston and others, 2006; Ronconi and others, 2002). These bacteria can acquire antibiotic resistance determinants through gene transference by plasmids and transposons (Said and others, 2016). Therefore, they can also be used as indicator microorganisms to evaluate fecal contamination in the environment.

In 2004, *Enterococcus* was adopted as an indicator of human fecal pollution in water (Jin and others, 2004). Because of their ability to survive in salt water, *Enterococcus* is recommended as the best indicator of health risk in marine water used for recreation by USEPA in 2004 (USEPA, 2004).

In the state of Alabama regulations for coastal recreational waters state that the Enterococci concentration should be less than 104 colonies/100 mL, and the geometric mean Enterococci density must be less than or equal to 35 colonies/100mL (Alabama Department of Environmental Management, 2014).

1.3 Impact of sediment on water quality

Besides surface water, the influence of sediment in water quality has attracted the public's attention in recent years. Studies showed that there were significant differences in surface water and bacterial contamination of sediment at recreational waters (An and others, 2002; Jamieson and others, 2005; Garzio-Hadzick and others, 2010; Piorkowski and others, 2014). An and others (2002) found that *E. coli* concentrations in sediment were much higher compared to those in lake water (An and others, 2002). Jamieson and others (2005) found that the association of microorganisms with sediment particles is one

of the primary complicating factors in assessing microbial fate in aquatic systems (Jamieson and others, 2005). Garzio-Hadzick and others (2010) found that *E. coli* in surface waters can subsequently be deposited into sediments, and *E. coli* survived much longer in sediments than in the surface water (Garzio-Hadzick and others, 2010). Piorkowski and others (2014) found that *E. coli* concentrations in sediments were significantly different than in surface waters, and that *E. coli* can persist in sediments and can be re-suspended into surface waters which then influence water monitoring program results (Piorkowski and others, 2014).

1.4 Enumeration methods

According to the indicator microorganisms mentioned above, each of them has certain characteristics that can be used for water quality monitoring. Therefore, different methods are used to detect and enumerate the different indicator microorganisms.

1.4.1 Enterobacteriaceae

According to the International Organization for Standardization (ISO) 21528-1: 2004, Enterobacteriaceae can be detected with a pre-enrichment method, using Enterobacteriaceae enrichment (EE) broth. The enumeration is carried out by calculation of the most probable number (MPN) after incubation at 37 °C for 24 ± 2 hours. The MPN is a method that permits estimation of population density without an actual count of single cells or colonies. This method is applicable to products intended for human consumption and the feeding of animals, and environmental samples in the area of food production and food handling (ISO, 2004).

USEPA does not have a standard method to enumerate the Enterobacteriaceae in the environment. However, 3M produces 3M™ Petrifilm™ Enterobacteriaceae count (EB) plates used to enumerate the Enterobacteriaceae and this is one of the enumeration techniques studied in this research. According to the manufacturer of 3M™ Petrifilm™ EB plates, this plate is a sample-ready-culture medium system that contains modified Violet Red Bile Glucose (VRBG) nutrients, a cold-water-soluble gelling agent, and a tetrazolium indicator that facilitates colony enumeration which can identify contamination in as few as 24 hours. The Enterobacteriaceae are oxidase-negative, Gram-negative rods that ferment glucose and the colonies produce acid and/ or gas. The media system results are considered positive when there are red colonies with yellow zones, red colonies with gas bubbles, or red colonies with yellow zones and gas bubbles and these are considered to be Enterobacteriaceae. This plate is certified by the ISO 9001 for design and manufacturing as useful for the enumeration of Enterobacteriaceae in the food and beverage industries. The 3M™ Petrifilm™ EB plates have not been evaluated against other possible environmental protocols. However, this is an effective method to assess environmental samples to enumerate Enterobacteriaceae organisms (3M, 2013).

1.4.2 Coliform

Usually the coliform are enumerated by using Violet Red Bile Agar (VRBA). According to USEPA, there are two methods to enumerate total coliforms in water, one is a multiple tube fermentation technique (USEPA, 1986b), and another is a membrane – filter technique (USEPA, 1986c). The first method is similar to the Enterobacteriaceae MPN method, and based on the second method, 3M has produced a product that can effectively detect coliforms, called 3M™ Petrifilm™ Coliform count (CC) plates. This

sample-ready to use plate contains Violet Red Bile (VRB) nutrients, a cold-water-soluble gelling agent, and a tetrazolium indicator that facilitates colony enumeration. The top film traps gas produced by the lactose fermenting coliforms and the results can be received in 24 hours. Based on the U.S. Food and Drug Administration Bacteriological Analytical Manual (FDA-BAM) definition, coliforms are Gram-negative rods and can produce acid and gas from lactose during metabolic fermentation, therefore gas trapped around red colonies are considered to be coliforms. ISO 9001 has certified 3M™ Petrifilm™ Coliform count (CC) plates for enumeration of total coliforms (3M, 2014).

1.4.3 *E. coli*

As *E. coli* belongs to the coliform group, and it is much more widely used for water quality monitoring, a great number of protocols have been developed to test for the presence of *E. coli* specifically. The USEPA approved standard method to enumerate *E. coli* in fresh water is a membrane filter (MF) procedure using membrane-Thermotolerant *Escherichia coli* agar (Modified mTEC) (USEPA, 2009). The commercially available plates are produced by 3M and called 3M™ Petrifilm™ *E. coli*/Coliform count (EC) plates (3M, 2010). Micrology Laboratories has also developed a detection method called Coliscan® Easygel agar plates, which has been used by Alabama Water Watch Program (AWW) for several years (Micrology Laboratories, 2008).

1.4.3.1 The mTEC membrane filter/USEPA method 1603 (USEPA method 1603, 2009)

USEPA method 1603 is a membrane filter (MF) procedure using a selective and differential medium, membrane-Thermotolerant *Escherichia coli* agar (Modified mTEC).

The sample is first filtered through a membrane which retains the bacteria on the filter. After filtration, the membrane is placed on the selective and differential modified medium. This modified medium contains a chromogen (5-bromo-6-chloro-3-indolyl-β-D-glucuronide). Based on the characteristics of *E. coli*, this chromogen can be catabolized to glucuronic acid and a red- or magenta-colored compound because *E. coli* produces the enzyme β-D-glucuronidase. Therefore, red or magenta colonies on the plate are considered as *E. coli*.

1.4.3.2 3M™ Petrifilm™ *E.coli*/Coliform count (EC) plates (3M, 2010)

3M™ Petrifilm™ *E.coli*/Coliform count (EC) plates are also a sample-ready-culture-medium system, which contains Violet Red Bile (VRB) nutrients, a cold-water-soluble gelling agent, an indicator of glucuronidase activity, 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (BCIG), and a tetrazolium indicator that facilitates colony enumeration. This test produces results in 24 hours. Most *E. coli* (about 97%) produce β-glucuronidase, which produces a blue precipitate associated with the colony, and about 95% of *E. coli* produce gas, therefore, blue to red-blue colonies associated with entrapped gas on the 3M™ Petrifilm™ *E.coli*/Coliform count (EC) plates are considered to be *E. coli*. And similarly with 3M™ Petrifilm™ Coliform count (CC) plates, red colonies with gas trapped around them are considered to be coliform. This method is certified to ISO 9001 for food and beverage, but there is no document for environmental testing.

1.4.3.3 Coliscan® Easygel agar plates (Micrology Laboratories, 2008)

The Coliscan® Easygel agar plates method is a patented formulation for water testing, this method includes a media bottle and pre-treated petri dishes. The media in the

bottle contains two sugars linked with a dye. One sugar linked to a dye can turn the colony a pink color when acted on by the enzyme β-galactosidase, which is produced by coliforms including *E. coli*. Another sugar linked to a different dye can produce a blue-green colony when acted on by the enzyme β-glucuronidase, which is produced by *E. coli* only. Because *E. coli* can work with both sugars, the combination of these two dyes shows a purple color, while the coliform only colonies show a pink color. The pre-treated petri dish contains a special formulation which solidifies the media. This method is used by the AWW volunteers because it is easy to use and volunteers can easily be trained.

1.4.4 *Enterococcus*

Because *Enterococcus* is important as an indicator of fecal pollution, a great deal of effort has gone into developing methods for detection of enterococcus in the environment. USEPA has two approved methods, one is membrane filter (MF) using membrane-Enterococcus Indoxyl-β-D-Glucoside agar (mEI) (USEPA, 2006), and another is a quantitative polymerase chain reaction (qPCR) procedure (USEPA, 2012). IDEXX Laboratory defined-substrate assays Enterolert® and Enterolert®-E are also approved for the detection of *Enterococcus* in water in the United States (US) and the European Union (EU), respectively (IDEXX Laboratories Inc., 2004). A new method produced by Micrology Laboratories called Easygel cards™ can also enumerate *Enterococcus* in water samples (Micrology Laboratories, 2014).

1.4.4.1 The mEI membrane filter/ USEPA method 1600 (USEPA method 1600, 2006)

USEPA method 1600 is similar to method 1603 because it is also a membrane filtration (MF) procedure, but uses membrane-Enterococcus Indoxyl-β-D-Glucoside agar

(mEI). The sample is also filtered through a membrane and placed onto an mEI plate. This mEI medium contains a reduced amount of triphenyltetrazaolium chloride (TTC) and a substrate, indoxyl-β-D-glucoside. Based on the characteristics of *Enterococcus*, colonies with blue halos are considered to be *Enterococcus*.

1.4.4.2 Enterolert® (IDEXX Laboratories Inc., 2004)

Enterolert® is a method based on IDEXX's patented Defined Substrate Technology® (DST®) that can detect *Enterococcus* in fresh and marine water within 24 hours. The medium contains a DST® nutrient indicator that fluoresces when *Enterococcus* utilizes the β-glucosidase enzyme to metabolize it, and the positive result causes a blue fluorescence under long-wave ultraviolet light (365 nm).

1.4.4.3 Easygel cards™ method (Micrology Laboratories, 2014)

The Easygel cards™ is a new product produced by Micrology Laboratories. It is easy to use and the procedure is similar to that used by 3M™ Petrifilm™. However, this method needs to be tested and verified, to determine if it is comparable to the mEI membrane filter/USEPA method 1600.

1.4.4.4 Molecular method – PCR

In addition to the traditional plating method, the recently developed polymerase chain reaction (PCR) method, which is based on the enzymatic amplification of specific DNA sequences, has transformed the way people think about the application of molecular biology and molecular biotechnologies (Erlich and others, 1989).

1.4.4.4.1 PCR

The polymerase chain reaction (PCR) is a technique used in molecular biology for amplifying DNA sequences in vitro by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase (Erlich and others, 1989). Based on this technique, thousands to millions of copies of a particular DNA sequence can be generated.

This technique was first conceived in 1971, Khorana and others proposed an idea for replicating a part of duplex DNA by using two DNA synthesis primers. In 1983, Kary Mullis of Cetus Corporation and others invented the polymerase chain reaction (PCR) and they used the Klenow Fragment of *E. coli* DNA polymerase I (Pol I), which has a lot of drawbacks. Some problems include that this enzyme cannot withstand rapid heating and cooling and the extension temperature is around 37°C. In 1988, Saiki and others isolated a thermostable DNA polymerase (*Taq* DNA polymerase) from an aquatic thermophilic bacilli (*Thermus aquaticus*) found in hot springs in Yellowstone National Park (Saiki and others, 1988). Because *Taq* DNA polymerase has high heat resistance and has greatly improve the specificity of the amplified fragment and the efficiency of amplification, this enzyme is widely used now (McPherson and others, 2006). Besides the *Taq* DNA polymerase, there is another significant advance that has been developed, a thermal cycler. The earliest thermal cyclers were designed for use with the Pol I. However, Pol I needed be added every cycle, and the machine was based on an automated pipettor, with open reaction tubes. After the wide use of *Taq* DNA polymerase began, thermal cyclers were greatly simplified (Bartlett and others, 2003).

The principle of PCR is similar to the DNA replication process in vivo, including three basic reaction steps: denaturation, annealing and extension. For the first denaturation step, the double-stranded template DNA is denatured by heating to a high temperature (about 93 to 94°C) to separate it into single strands. After heat denaturation, the temperature is rapidly cooled to the specific annealing temperature to allow the oligonucleotide primers to hybridize to the template, this step is called annealing. The extension step is usually set to 72°C for efficient DNA synthesis by DNA polymerase (McPherson and others, 2006).

PCR is now a common and indispensable technique widely used in medical and biological research labs for a variety of applications, including selective DNA isolation, amplification and quantification of DNA as well as disease diagnosis.

1.4.4.4.2 DNA extraction

A basic PCR set up requires several components and reagents, including DNA a template, primers, *Taq* polymerase, deoxynucleoside triphosphates, buffer solutions etc. (Sambrook and other, 2001). The basic component is the DNA template which contains the target region that is needed to be amplified. DNA is an abbreviation for deoxyribonucleic acid, which is a molecule that carries most of the genetic instructions of all known living organisms and many viruses.

There are many different methods and technologies available for DNA extraction. In general, all methods including disruption and lysis of material, removal of proteins and other contaminants, and recovery of the DNA. To disrupt and lyse the material one can use a physical or chemical method, while removal of proteins and other contaminants are

usually achieved by digestion with proteinase K or other enzymes. The recovery of DNA is usually accomplished with precipitation by ethanol or isopropanol (Innis and others, 1990).

In this research, Enterococci strains were used, and because of the differences between sample types, three DNA extraction procedures were involved. The first procedure was the PrepMan® boiling procedure and this procedure was applied both for surface water samples and sediment samples. The major step was boiling. By doing this, cells can be disrupted and proteins and other contaminants can be denaturized (Wang and others, 2007). The second procedure applied in this study was the DNA extraction procedure based on USEPA method 1611. A physical bead beating step was used to disrupt the cells, and a specific buffer, AE buffer, was made to elute the DNA (USEPA, 2012). The third procedure of DNA extraction involved was the PowerSoil® DNA Isolation Kit. This kit also uses a physical bead beating method to disrupt the cells, and a prepared solution that can lyse cells, remove inhibitors, bind DNA, and elute the DNA (MO BIO Laboratories, 2013).

1.4.4.4.3 Real-time PCR (qPCR)

The real-time PCR (also known as qPCR) is a laboratory technique based on regular PCR, which can monitor the amplification of a targeted DNA molecule during the PCR (Logan and others, 2009). There are two common methods for the detection of PCR products in real-time PCR. The first method uses non-specific fluorescent dyes, such as SYBR® Green-based detection dye. Another method uses sequence specific DNA probes labelled with a fluorescent reporter, such as TaqMan®-based detection probe. For the

real-time PCR with non-specific fluorescent dyes as reporters, the dye binds to all double-stranded DNA in PCR, and cause fluorescence. The DNA products are then measured at the end of each cycle (Ponchel and others, 2003). For the real-time PCR with a specific probe as fluorescent reporter, the probe has a reporter and a quencher. When the probe is intact, the reporter and quencher are both active, and no fluoresce can be detected. When amplifing a DNA strand, the quencher is inactive and the reporter shows fluorescence. Therefore, the signal is detected at the beginning of each cycle (Ponchel and others, 2003). Compared with the non-specific fluorescent dyes as a reporter, the probe is more sensitive, and can prevent the false positive results caused by primer dimer. The direct comparison between SYBR® Green and TaqMan® probe method is shown in Figure 1 and Table 1.

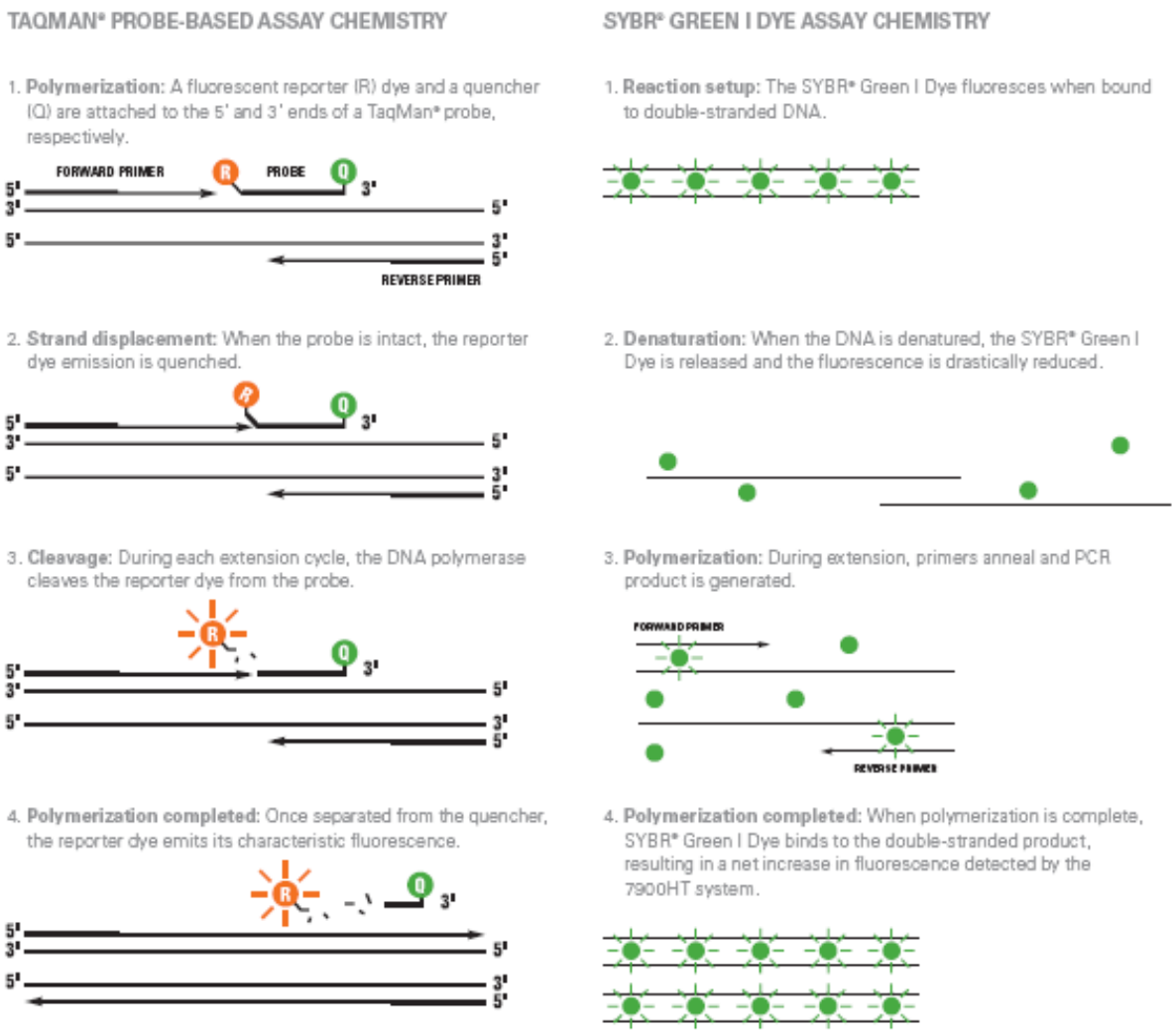

Figure 1. Comparison of TaqMan®- and SYBR®-Green based detection workflows (Adapted from BioSythesis.com)

Table 1. Comparison of TaqMan®- and SYBR®-Green based detection (Adapted from BioSythesis.com)

	SYBR®-Green based detection	TaqMan®-based detection
Chemistry Overview	Uses a highly specific, double-stranded DNA binding dye - SYBR Green I dye to detect PCR product as it accumulates during PCR cycles	Uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles
Specificity	Detect all amplified double-stranded DNA, including non-specific reaction products, such as primer dimer	Only detect specific amplification products
Applications	• One-step RT-PCR for RNA quantitation • Two-step RT-PCR for RNA quantitation • DNA/cDNA quantitation	• One-step RT-PCR for RNA quantitation • Two-step RT-PCR for RNA quantitation • DNA/cDNA quantitation - Allelic Discrimination - Plus/Minus assays using an internal positive control (IPC)
Advantages	• Enables monitoring the amplification of any double-stranded DNA sequence. • No probes are required, which reduces your assay setup and running costs. • Multiple dyes can bind to a single amplified molecule, increasing sensitivity for detecting amplification products.	• Specific hybridization between probe and target is required to generate fluorescent signal, significantly reducing background and false positives. • One can label probes with different, distinguishable reporter dyes, which allows one to amplify two distinct sequences in one reaction tube. • Post-PCR processing is eliminated, which reduces assay labor and material costs.
Disadvantages	SYBR Green based detection may generate false positive signals	Different probes need to be synthesized for each unique target sequence

1.5 Summary

According to the information above, water quality has been important for public health. Water, including drinking water, irrigation water, and recreational water, needs to be closely monitored. Four different microorganism families and groups have been proposed to be used as indicator microorganisms and different enumeration methods have been developed to monitoring their concentrations.

Currently, there are three methods commonly used to enumerate *E. coli*, including the mTEC membrane filtration/USEPA Method 1603, Coliscan® Easygel agar plates and 3M™ Petrifilm™ methods. Previous studies have shown that these methods can be used as enumeration procedures for *E. coli* in water (Stepenuck and others, 2011; Pecher and others, 2012). Stepenuck and others (2011) found that when monitoring surface water, both 3M™ Petrifilm™ and Coliscan® Easygel agar plates method had a similar overall accuracy of predicting whether a sample exceeded or fell below the 235 CFU/100mL for recreational water (Stepenuck and others, 2011). Pecher and others (2012) also found that both methods were equally accurate in detecting *E. coli* (Pecher and others, 2012). However, there was no research available to compare all three of these methods for use in water monitoring programs.

There are four methods commonly used to enumerate *Enterococcus*, including the membrane filter/USEPA method 1600, the Enterolert® method, the Easygel cards™ method and the USEPA qPCR method 1611. Previous studies compared two different methods to enumerate *Enterococcus* in water (Ferguson and others, 2013; Noble and others, 2010; Kinzelman and others, 2003; Haugland and others, 2005). Ferguson and

others (2013) focused on the species distribution of *Enterococcus*, and found that the mEI membrane filter/USEPA method 1600 and the Enterolert® generally yielded the same species of *Enterococcus*. There were some differences that were mostly related to a preferential culturing of *E. faecalis* by Enterolert® in marine and spiked samples ($P > 0.05$) but Enterolert® was more selective for *E. faecalis* in wastewater samples (Ferguson and others, 2013). Kinzelman and others (2003) showed that there were drawbacks when using Enterolert® besides the lack of correlation between methodologies and included the inability to re-culture and verify isolates as *Enterococcus*. There were also advantages, such as time consumption was decreased, ease of use and minimal technical training required of personnel in using this system (Kinzelman and others, 2003). A study by Noble and others (2010) found that there was a level of agreement of 88% between the *Enterococcus* qPCR method and the mEI membrane filter/USEPA method 1600 and a 94% level of agreement between the mEI membrane filter/USEPA method 1600 and Enterolert® (Noble and others, 2010). Haugland and others (2005) found a significant positive correlation between qPCR and the mEI membrane filter/USEPA method 1600 to enumerate the concentration of *Enterococcus*, which indicated that the qPCR has the potential to supplement or replace the mEI membrane filter/USEPA method 1600 as a means of assessing the levels of fecal contamination at freshwater recreational beaches (Haugland and others, 2005). Given that *Enterococcus* has several advantages of being used as a new indicator microorganism, there is an urgent need to compare all of the currently available methods for *Enterococcus*.

1.6 References

Alabama Department of Environmental Management. 2014. 2014 Alabama Integrated Water Quality Monitoring and Assessment Report.

Allende, A. and Monaghan, J. 2015. Irrigation Water Quality for Leafy Crops: A Perspective of Risks and Potential Solutions. Int. J. Environ. Res. Public Health. 12(7):7457-7477.

American Public Health Association. 1995. Standard Methods for the Examination of Water and Wastewater (19th ed.), American Public Health Association. Washington, D. C., USA.

An, Y. J., Kampbell, D. H., and Breidenbach, G. P. 2002. *Escherichia coli* and total coliforms in water and sediments at lake marinas. Environ. Pollut. 120:771-778.

Armstrong, K. F., and Jackson, M. S. 1985. Anatomy & Physiology for Nurses. (9th ed.). ISBN 0-7020-0737-4.

Ashbolt, N. J. 2004. Microbial contamination of drinking water and disease outcomes in developing regions. Toxicology. 198: 229-238.

Barwick, R. S., Levy, D. A., Craun, G. F., Beach, M. J., and Calderon, R. L. 2000. Surveillance of waterborne-disease outbreak—United States, 1997–1998. Morb. Mortal. Wkly. Rep. CDC Surveill. Summ. 49:1–21.

Bauder, A., Waskom, R. M., and Davis, J. G. 2007. Irrigation Water Quality Criteria. Colorado State University Extension. No. 0506.

Baylis, C., Uyttendaele, M., Joosten, H., and Davies, A. 2011. The Enterobacteriaceae and Their Significance to the Food Industry. International Life Sciences.

Bentley, R., and Meganathan, R. 1982. Biosynthesis of vitamin K (menaquinone) in bacteria. Microbiol. Rev. 46(3):241–280.

Bergey, D. H., Breed, R. S., Murrray, E. G. D., and Hitchens, A. P. 1939. Bergey's Manual of determinative bacteriology (5th ed). ISBN 978-0683006032.

Bettelheim, K. A. 2007. The Non-O157 Shiga-Toxigenic (Verocytotoxigenic) *Escherichia coli*; Under-Rated Pathogens. Microbiology. 33:67–87.

Center of Disease Control and Prevention. 1974. Foodborne and Waterborne Disease Outbreaks, Annual Summary 1973. U.S. Dept. of Health and Human Services, Atlanta, GA.

Center of Disease Control and Prevention. 2012. Recreational Water Illnesses (RWIs). U.S. Dept. of Health and Human Services, Atlanta, GA.

Centers of Disease Control and Prevention. 2014. General Information of *E. coli*. U.S. Dept. of Health and Human Services, Atlanta, GA.

Center of Disease Control and Prevention. 2016. Investigates Multistate Outbreak of *E. coli* O26 Infections Linked to Chipotle Mexican Grill Restaurants. U.S. Dept. of Health and Human Services, Atlanta, GA.

Cohen, J., and Shuval, H. 1972. Coliforms, fecal coliforms and fecal streptococci as indicators of water pollution. Water, Air, & Soil Pollution. 2(1973):85-95.

Craun, G. F. 1993. Safety of water disinfection: balancing chemical and microbial risks. ISBN:0944398111.

Craun, M. F. Craun, G. F., Calderon, R. L., and Beach, M. J. 2006. Waterborne outbreaks reported in the United States. J. Water Health. 4(2):19-30.

Dewaal, C. S., Robert, N., Wimer, J., and Tian X. A. 2010. A Comparison of the Burden of Foodborne and Waterborne Diseases in Three World Regions, 2008. Food Protect. Trends. 30(8):483-490.

Drechsel, P., Scott, C. A., Raschid-Sally, L., Redwood, M., and Bahri, A. 2010. Wastewater Irrigation and Health. ISBN 978-1-84407-795-3.

Donnenberg, M. S. 2002. *Escherichia coli*: Virulence Mechanisms of a Versatile Pathogen. ISBN 0-12-220751-3

Downs, T. J., Cifuentes-Garcia, E., and Suffet, I. M. 1999. Risk screening for exposure to groundwater pollution in a wastewater irrigation district of the Mexico City region. Environ. Health Perspect. 107(7):553–561.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., and Relman, D. A. 2005. Diversity of the human intestinal microbial flora. Science. 308(5728):1635–1638.

Eliassen, R. and Cummings, R. H. 1948. Analysis of waterborne outbreaks, 1938–45. J. Am. Water Resour. Asso. 40(5):509-528.

Erlich, H. A., Kazazian, H. H., and Gibbs, R. A. 1989. Current communication in molecular biology. Polymerase Chain Reaction. ISBN: 0-87969-329-0.

Ewing, W. H. and Davis, B. R. 1973. Media and Tests for Differentiation of Enterobacteriaceae. U.S. Department of Health, Education, and Welfare/ Public Health Service.

Facklam, R. R. and Elliott, J. A. 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. Clin. Microbiol. Rev. 8:479-495.

Feng, P., Weagant, S., and Grant, M. 2002. Enumeration of *Escherichia coli* and the Coliform Bacteria. FDA/Center for Food Safety & Applied Nutrition. Silver Spring. MD. USA

Ferguson, D. M., Griffith, J. F., McGee, C. D., and Weisberg, S. B. 2013. Comparison of *Enterococcus* Species Diversity in Marine Water and Wastewater Using Enterolert® and EPA Method 1600. J. Environ. Public Health. 2013:848049.

Garzio-Hadzick, A., Shelton, D. R., Hill, R. L., Pachepsky, Y. A., Guber, A. K., and Rowland, R. 2010. Survival of manure-borne *E. coli* in streambed sediment: effects of temperature and sediment properties. Water Res. 44(9):2753-2762.

Gelting, R. J., Delea, K., and Medlin, E. 2012. A conceptual framework to evaluate the outcomes and impacts of water safety plans. J. Water, Sanitation and Hygiene for Development. 2(2):103-111.

Gilmore, M. S. 2002. The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance. ISBN 978-1-55581-234-8.

Gleick, P. H. 1993. Water in Crisis: A Guide to the World's Freshwater Resources. ISBN 0-19-507627-3.

Griffin, P. M. and Tauxe, R. V. 1991. The Epidemiology of Infections Caused by *Escherichia coli* O157: H7, Other Enterohemorrhagic *E. coli*, and the Associated Hemolytic Uremic Syndrome. Epidemiol. Rev. 13(1):60-98.

Gorman, A. E. and Wolman, A. 1939. Waterborne outbreaks in the United States and Canada and their significance. J. Am. Water Resour. Asso. 31(S):225-373.

Greene, S. K., Daly, E. R., Talbot, E. A., Demma, L. J., Holzbauer, N., Patel, N. J., Hill, T. A., Walderhaug, M. A., Hoekstra, R., Lynch, M. F., and Painter, J. A. 2008. Recurrent multistate outbreak of Salmonella Newport associated with tomatoes from contaminated fields, 2005. Epidemiol. Infect. 136:157–165.

Hall, J. E. and Guyton, A. C. 1991. Textbook of Medical Physiology (8th ed.). ISBN 0-7216-3994-1.

Haugland, R. A., Siefring, S. C., Wymer, L. J., Brenner, K. P., and Dufour, A. P. 2005. Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res. 39(4):559-568.

Hauswaldt, S., Nitschke, M., Sayk, F., Solbach, W., and Knobloch, J. K. 2013. Lessons Learned From Outbreaks of Shiga Toxin Producing *Escherichia coli*. Curr. Infect. Dis. Rep. 15(1):4–9.

Hlavsa, M. C., Roberts, V. A., Kahler, A. M., Hilborn, E. D., Mecher, T. R., and Beach, M. J. 2015. Outbreaks of Illness Associated with Recreational Water — United States, 2011–2012. Morb. Mortal. Wkly. Rep. CDC Surveill. Summ. 64(24):668-672.

Hudault, S., Guignot, J., and Servin, A. L. 2001. *Escherichia coli* strains colonizing the gastrointestinal tract protect germ-free mice against *Salmonella typhimurium* infection. Gut. 49(1):47–55.

Hudson, L. D., Hankins, J. W., and Battaglia, M. 1983. Coliforms in a water distribution system: a remedial approach. Am. Water. Works Assoc. 75:564-8.

Hunter, P. R., Waite, M., and Ronchi, E. 2002. Drinking Water and Infectious Disease: Establishing the Links. ISBN: 978-0849312595.

IDEXX Laboratories, Inc. 2004. Enterolert® Test Kit Manufacturing. Westbrook, ME, USA.

Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. 1990. PCR Protocols: A Guide to Methods and Applications. ISBN: 0-12-372181-4.

International Organization for Standardization. 2004. Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of Enterobacteriaceae. ISO. Geneva, Switzerland.

Ishii, S., and Sadowsky, M. J. 2008. *Escherichia coli* in the Environment: Implications for Water Quality and Human Health. Microbes. Environ. 23(2):101–108.

Jamieson, R., Joy, D. M., Lee, H., Kostaschuk, R., and Gordon, R. 2005. Transport and deposition of sediment-associated *Escherichia coli* in natural streams. Water Res. 39(12):2665–2675.

Jean, F. M. 1980. Biochemical Tests for Identification of Medical Bacteria (3rd ed.). ISBN 0-6830-5318-3.

Jimenez, B., Maya, C., Sanchez, E., Romero, A., Lira, L., and Barrios, J. A. 2002. Comparison of the quantity and quality of the microbiological content of sludge in countries with low and high content of pathogens. Water Sci. Tech. 46(10):17–24.

Jin, G., Jeng, H. W., Bradford, H., and Englande, A. J. 2004. Comparison of *E. coli*, enterococci, and fecal coliform as indicators for brackish water quality assessment. Water Environ. Res. 76(3):245–255.

Johnston, L. M., Jaykus, L. A., Moll, D., Anciso, J., Mora, B., and Moe, C. L. 2006. A field study of microbiological quality of fresh produce of domestic and Mexican origin. Int. J. Food Microbiol. 112:83–95.

Kindhauser, M. K., 2003. Global defense against the infectious disease threat. Communicable Diseases 2002. World Health Organization, Geneva.

Kinzelman, J., Ng, C., Jackson, E., Gradus, S., and Bagley, R. 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(1):92–96.

Knight, A. W. and Simmons, M. A. 1980. Water pollution: a guide to information sources. ISBN: 978-0810313460.

Köhler, W. 2007. The present state of species within the genera *Streptococcus* and *Enterococcus*. Int. J. Med. Microbiol. 297(3):133–150.

Layton, B. A., Walters, S. P., Lam, L. H., and Boehm, A. B. 2010. *Enterococcus* species distribution among human and animal hosts using multiplex PCR. J. Appl. Microbiol.109(2):539–574.

L'Abée-Lund, T. M., Jørgensen, H. J., O'Sullivan, K., Bohlin, J., Ligård, G., Granum, P. E., Lindbäck, T. 2012. The Highly Virulent 2006 Norwegian EHEC O103:H25 Outbreak Strain Is Related to the 2011 German O104:H4 Outbreak Strain. PLoS ONE. 7(3): e31413.

Lim, J. Y., Jang, W. Y., and Carolyn, J. H. 2010. A brief overview of *Escherichia coli* O157:H7 and its Plasmid O157. J. Microbiol. Biotechnol. 20(1):5–14.

Logan, J., Edwards, K., and Saunders, N. 2009. Real-Time PCR: Current Technology and Applications. ISBN: 978-1-904455-39-4.

Lund, B. M., Sussman, M., Jones, D., and Stringer, M. F. 1988. Enterobacteriaceae in the environment and as pathogens. ISBN: 0267-4440.

Martins, M. T., Soares, L. A., Marques, E., Molina, A. G. 1983. Human enteric viruses isolated from influents of sewage treatment plants in Sao Paulo, Brazil. Water Sci. Tech. 15(5):69–73

McPherson, M. J. and Møller, S. G. 2006. PCR (2nd ed.). ISBN: 0-4153-5547-8.

Micrology Laboratories. 2008. Detection of waterborne coliforms and *E. coli* with Coliscan® Easygel. Micrology Laboratories, Goshen, IN. USA.

Micrology Laboratories. 2014. Now You Have A Better Choice Now You Have A Better Choice with Easygel Cards™. Micrology Laboratories, Goshen, IN. USA.

MO BIO Laboratories. 2013. PowerSoil® DNA Isolation Kit Instruction Manual. MO BIO Laboratories. Carlsbad, CA. USA.

Modlin, J. F. 1986. Perinatal Echovirus Infection: Insights from a Literature Review of 61 cases of serious infection and 16 outbreaks in nurseries. Rev. Infect. Dis. 8(6):918-926.

Mueller, D. K., Helsel, D. R., and Kidd, M. A. 1996. Nutrients in the nation's waters: too much of a good thing? U.S. Geological Survey, Information Services. Montgomery, AL. USA.

Noble, R. T., Blackwood, A. D., Griffith, J. F., McGee, C. D., and Weisberg, S. B. 2010. Comparison of Rapid Quantitative PCR-Based and Conventional Culture-Based Methods for Enumeration of *Enterococcus* spp. and *Escherichia coli* in Recreational Waters. Appl. Environ. Microbiol. 76(22):7437–7443.

Patnaik, P. 2010. Handbook of environmental analysis: chemical pollutants in air, water, soil, and solid wastes. ISBN: 978-1420065817.

Payment, P., Siemiatychi, J., Richardson, L., Renaud, G., Franco, E., and Prévost, M. 1997. A prospective epidemiological study of gastrointestinal health effects due to the consumption of drinking water. Int. J. Environ. Health Res. 7:5–31.

Pecher, W., and Kemp, S. 2012. Monitoring of *Escherichia coli* in Jones Falls, MD: a comparison of methods. Research Pape.

Penas, P. P., Mayer, M. P., Gomes, B. P., Endo, M., Pignatari, A. C., and Bauab, K. C. 2013. Analysis of genetic lineages and their correlation with virulence genes in *Enterococcus faecalis* clinical isolates from root canal and systemic infections. J. Endod. 39:858–886.

Piorkowski, G. S., Jamieson, R. C., Hansen, L. T., Bezanson, G. S., and Yost, C. K. 2014. Characterizing spatial structure of sediment *E. coli* populations to inform sampling design. Environ. Monit. Assess. 186(1):277–291.

Ponchel, F., Toomes, C., Bransfield, K., Leong, F. T., Douglas, S. H., Field, S. L., Bell, S. M., Combaret, V., Puisieux, A., Mighell, A. J. 2003. Real-time PCR based on SYBR-Green I fluorescence: An alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. BMC Biotechnol. 3: 18.

Reid, G., Howard, J., and Gan, B. S. 2001. Can bacterial interference prevent infection? Trends. Microbiol. 9(9):424–428.

Ronconi, M. C., Merino, L. A. and Fernández, G. 2002. Detection of *Enterococcus* with high-level aminoglycoside and glycopeptide resistance in Lactuca sativa (lettuce). Enferm. Infecc. Microbiol. Clin. 20:380–383.

Said, L. B., Klibi, N., Dziri, R., Borgo, F., Boudabous, A. B., Slama, K. B., and Torres, C. 2016. Prevalence, antimicrobial resistance and genetic lineages of *Enterococcus*

spp. From vegetable food, soil and irrigation water in farm environments in Tunisia. J. Sci. Food Agric. 96:1627-1633.

Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K., and Erlich, H. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 239 (4839): 487–491.

Sambrook, J. and Russel, D. W. 2001. Molecular Cloning: A Laboratory Manual (3rd ed.). ISBN 0-879-69576-5.

Singleton, P. 1999. Bacteria in Biology, Biotechnology and Medicine (5th ed.). ISBN 0-471-98880-4.

Snyder, R. L. and Melo-Abreu, J. P. 2005. Frost protection: fundamentals, practice, and economics. ISBN: 92-5-105328-6.

Spector, M. P. and Kenyon, W. J. 2011. Resistance and survival strategies of Salmonella enterica to environmental stresses. Food Research International. 45(2):455-481.

Steele, M. and Odumeru, J. 2004. Irrigation Water as Source of Foodborne Pathogens on Fruit and Vegetables. J. Food Prot. 67(12):2839-49.

Stepenuck, K. F., Wolfson, L. G., Liukkonen, B. W., Iles, J. M., and Grant, T. S. 2011. Volunteer monitoring of *E. coli* in streams of the upper Midwestern United States: A comparison of methods. Environ. Monit. Assess. 174(1-4):625-633.

Stevenson, A. H. 1953. Studies of Bathing Water Quality and Health. Am. J. Public Health Nations Health. 43:529–538.

Sorvall, V. 1971. Water pollution: our troubled waters. ISBN: 978-0883010402.

Southwest Florida Water Management District. 2012 a. The importance of water monitoring. Brooksville. FL. USA.

Southwest Florida Water Management District. 2012 b. About water quality. Brooksville. FL. USA.

Söderström, A., Österberg, P., Lindqvist, A., Jönsson, B., Lindberg, A., Blide-Ulander, S., Welinder-Olsson, C., Löfdahl, S., Kaijser, B., De Jong, B., Kuhlmann-Berenzon, S., and Boqvist, S. 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. Foodborne Pathog. Dis. 5:339–348.

Talbot, H. W., Yamamoto, D. K., Smith, M. W., and Seidler, R. J. 1980. Antibiotic resistance and its transfer among clinical and nonclinical *Klebsiella* strains in botanical environments. Appl. Environ. Microbiol. 39(1):97-104.

Tchobanoglous, G., and Schroeder, E. D. 1985. Water Quality: Characteristics, modeling, modification. ISBN 0-201-05433-7.

Tendolkar, P. M., Baghdayan, A. S., and Shankar, N. 2003. Pathogenic enterococci: new developments in the 21st century. Cell Mol. Life Sci. 60(12):2622–2636.

United States Environmental Protection Agency. 1971. Tabulation of 128 Waterborne Disease Outbreaks Known to Have Occurred in the United States, 1961–70. Water Supply Program, Cincinnati, OH.

United States Environmental Protection Agency. 1986a. Ambient water quality criteria for bacteria–1986. Washington, D. C.: United States Environmental Protection Agency: Office of Water.

United States Environmental Protection Agency. 1986b. Method 9131: Total coliform: multiple tube fermentation technique. Washington, D. C.: United States Environmental Protection Agency: Office of Water.

United States Environmental Protection Agency. 1986c. Method 9132: Total coliform: membrane-filter technique. Washington, D. C.: United States Environmental Protection Agency: Office of Water.

United States Environmental Protection Agency. 2004. Final Water Quality Standards Bacteria Rule for Coastal and Great Lakes Recreation Waters. Washington, D. C.: United States Environmental Protection Agency: Office of Water.

United States Environmental Protection Agency. 2006. Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-beta-D-Glucoside Agar (mEI). Washington, D. C.: United States Environmental Protection Agency: Office of Water.

United States Environmental Protection Agency. 2009. *Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using Modified membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC). Washington, D. C.: United States Environmental Protection Agency: Office of Water.

United States Environmental Protection Agency. 2012. Method 1611: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay. Washington, D. C.: United States Environmental Protection Agency: Office of Water.

United States Geological Survey. 2015. How much water is there on, in, and above the Earth? United States Geological Survey. Montgomery, AL. USA.

VanDerslice, J. and Briscoe, J. 1995. Environmental interventions in developing countries—interactions and their implications. Am. J. Epidemiol. 141(2):135–144.

Vogt, R. L., and Dippold, L. 2005. *Escherichia coli* O157:H7 outbreak associated with consumption of ground beef, June–July 2002. Public Health Rep. 120(2):174–178.

Wang, L., Li, Y., and Mustapha, A. 2007. Rapid and simultaneous quantitation of *Escherichia coli* O157: H7, *Salmonella*, and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation. J. Food Prot. 70(6):1366-1372.

Weibel, S. R., Dixon, F. R., Weidner, R. B., and McCabe, L. J. 1964. Waterborne disease outbreaks, 1946–60. J. Am. Water Resour. Asso. 56(8):947-958.

World Meteorological Organization. 2013. Planning of Water Quality Monitoring systems. Genève, Switzerland.

World Health Organization. 2003. Quantifying selected major risks to health. The World Health Report 2002. World Health Organization, Geneva.

World Health Organization. 2004. Guidelines for Drinking-water Quality: Recommendations, Volume 1. ISBN 92-4-154638-7.

World Health Organization. 2005. Nutrients in drinking water. ISBN 92-4-159398-9.

World Health Organization. 2011. Drinking Water Equity, Safety and Sustainability: Thematic report on drinking water. ISBN 978-92-806-4613-9.

Wilber, C. G. 1969. The biological aspects of water pollution. ISBN: 978-0398020620.

William, W. 1971. Oxygen regeneration of polluted rivers: the Passaic River. Washington, D. C.: United States Environmental Protection Agency: Office of Water.

3M. 2010. 3M™ Petrifilm™ *E. coli*/Coliform count (EC) plates manufacturer. Cartersville, GA. USA

3M. 2013. 3M™ Petrifilm™ Enterobacteriaceae count (EB) plates manufacturer. Cartersville, GA. USA.

3M. 2014. 3M™ Petrifilm™ Coliform count (CC) plates manufacturer. Cartersville, GA. USA

CHAPTER 2. IMPACTS OF SAMPLING METHODS, SAMPLE TIME, AND SAMPLING TYPES ON INDICATOR MICROORGANISM ENUMERATION

2.1 Abstract

Water quality, such as drinking water quality, irrigation water quality, and recreational water quality, is important for public health. *Escherichia coli*, coliform and Enterobacteriaceae have been used as indicator organisms to monitor the potential fecal contamination of water. Previous studies indicate that bacterial concentrations may be significantly different when sampled at different times of the day. There were also studies showing that sediments contain higher levels of indicator microorganisms than the surface water. This chapter looks at the impact of sampling time (morning vs. afternoon) and sample types (sediment vs. surface water) on water quality monitoring results and determines the most accurate and useful methods of testing for bacterial contamination in water and sediment samples using a parallel comparison between different sampling and plating methods. Artificial inoculation samples were first used to compare the efficiency of three methods for *E. coli* enumeration. Results showed there were no differences of the efficiency among Coliscan® Easygel agar plates, the mTEC membrane filtration/USEPA Method 1603 and 3M™ Petrifilm™ methods ($P > 0.05$). Both the 3M™ Petrifilm™ methods and Coliscan® Easygel agar plates method (used by the Alabama Water Watch (AWW)) were used to evaluate the impact of sampling time and sample types on the enumeration of *E. coli*, coliform and Enterobacteriaceae. Results showed that both the sampling time and sample types impacted the enumeration results ($P < 0.05$) regardless of the indicator microorganisms used. When samples were collected in the afternoon, the surface water samples contained more indicator microorganisms than samples collected

in the morning. Sediments contained more indicator microorganisms than the surface water ($P < 0.05$). This may related to the human and animal activities around the area.

2.2 Introduction

Water contamination can pose a serious threat to public health because of the high numbers of intestinal pathogens (Stevens and others, 2009). Water quality monitoring based on the application of standard fecal indicator bacteria (SFIB) has contributed to a fundamental improvement in water quality since the end of the 19th century (Tallon and others, 2005). Water quality monitoring of microbial pollution is based on the selective cultivation of SFIB, including *Escherichia coli* and intestinal Enterococci (International Organization of Standardisation, 2005).

Escherichia coli (*E. coli*) is a Gram-negative, rod-shaped, non-spore former, and facultative anaerobic bacteria (Singleton, 1999). Most *E. coli* strains are harmless to human and animal populations, however, some can cause serious diarrhea, respiratory illness and pneumonia or other illness (Vogt and others, 2005). Although *E. coli* is commonly found in the lower intestine of warm-blooded organisms, the types of *E. coli* that can cause human illness can be transmitted through contaminated water or food, or through contact with animals or persons (Centers of Disease Control and Prevention, 2014). This transmission fit the fecal–oral route, which is a route of transmission of a disease, when pathogens in fecal particles passing from one host are introduced into the oral cavity of another host (Cellini and others, 1999). Some kinds of *E. coli* are used as markers for water contamination (CDC, 2014). According to the Centers for Disease

Control and Prevention (CDC), the infection by *E. coli* may start by swallowing lake water while swimming or touching the environment in petting zoos (CDC, 2014).

Coliforms are a group of bacteria found in plant material, soil and water (Cohen and others, 1972). According to the American Public Health Association, the definition of coliform is that it is a group of bacteria that are Gram-negative, rod-shaped, non-spore forming and motile or non-motile bacteria which can ferment lactose with the production of acid and gas when incubated at 35–37°C (APHA, 1995). The coliform includes *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella* (Hudson and others, 1983).

The Enterobacteriaceae are a large family of Gram-negative bacteria, contains about 20 genera, including coliform group, *Salmonella*, *Shigella*, *Klebsiella* and *Yersinia* (Jean, 1980). Members of the Enterobacteriaceae are widely distributed (Baylis and others, 2011), many of them are a normal part of the gut flora found in the intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants (Lund and others, 1988).

According to the United States Environmental Protection Agency (USEPA), *E. coli* has been the USEPA recommended indicator of recreational water quality (USEPA, 1986). Ashbolt and others (2001) recommended that the coliforms should also be used as indicators for recreational water quality monitoring (Ashbolt and others, 2001). Because the large amount of pathogenic bacteria the Enterobacteriaceae family included and greater resistance to the environment (Ewing and others, 1973), Enterobacteriaceae family is also proposed as an indicator to evaluated microbial contamination in environment.

To determine the presence of *E. coli*, based on the USEPA method 1603 (USEPA, 2009), the Modified membrane-Thermotolerant *Escherichia coli* agar (Modified mTEC) is used to enumerate *E. coli* in water. In addition, there are other methods, which need less training and have simpler sample processing procedures for *E. coli* enumeration, such as, the Coliscan® Easygel agar plates (including media bottle and pre-treated petri dishes) used by the Alabama Water Watch Program (AWW) and the commercial 3M™ Petrifilm™. Previous studies showed that these methods can be used as enumeration procedures for *E. coli* in water (Stepenuck and others, 2011; Pecher and others, 2012). Stepenuck and others (2011) found that when monitoring surface water, both 3M™ Petrifilm™ and Coliscan® Easygel agar plates method had a similar overall accuracy of predicting whether a sample exceeded or fell below the 235 CFU/100ml for recreational water (Stepenuck and others, 2011). Pecher and others (2012) also found that both methods were equally accurate in detecting *E. coli* (Pecher and others, 2012).

However, there is no study available to compare these methods to each other to be able to give recommendations while monitoring water quality in the field. Previous studies showed that sampling time can affect water monitoring results. Barbe and others (1995) found that fecal coliform levels were significantly different when sampling the water at different seasons (Barbe and others, 1995). Ikonen and others (2013) found significant differences in *E. coli* concentrations when measured at the same site but at different time of day; and that *E. coli* levels in the water directly correlated with activity in the water, UV absorbance and turbidity (Ikonen and others, 2013).

Recent research showed that significant differences existed between bacterial contamination in surface water and the sediment. An and others (2002) found that *E. coli*

concentrations in sediment were much higher compared to those in lake water (An and others, 2002). Garzio-Hadzick and others (2010) found that *E. coli* in surface waters can subsequently be deposited into sediments, and fecal material and *E. coli* survived much longer in sediments than in the surface water (Garzio-Hadzick and others, 2010). Piorkowski and others (2014) found that *E. coli* concentrations in sediments were different than concentrations found in water with *E. coli* persisting in sediments and becoming re-suspended into surface water thereby influencing water monitoring programs (Piorkowski and others, 2014).

A better evaluation of three *E. coli* enumeration procedures is needed. In addition, the relationship between sampling time (morning vs. afternoon and seasons), the sampling type (surface water vs. sediment) and the bacterial enumeration results should be researched. Based on this, the objectives of this study were, 1, comparing three different methods to better enumerate *E. coli* in water; and 2, testing if sampling time (morning vs. afternoon and seasons) and the sampling type (surface water vs. sediment) changes the *E. coli*/ coliform/ Enterobacteriaceae enumeration results.

2.3 Material & Methods

2.3.1 Bacterial strain and inoculation procedures

Escherichia coli strain ATCC® 11775™, purchased from American Type Culture Collection (Manassas, VA, USA), was used in this study. The liquid *E. coli* culture was prepared by transferring 100 µl of thawed stock culture into 10 mL of Tryptone Soy Broth (TSB) (BBL/Difco Laboratories, Sparks, MD, USA) and incubated at 37°C ± 0.5°C for 18 to 24 hours. The next day the culture was washed by centrifugation at 4,000

rpm for 5 min (Eppendorf, Hauppauge, NY, USA) and the cell pellet was re-suspended in 10 mL of MilliQ water. The washed cells were diluted and then 1mL of the diluted cells was inoculated into 9mL of autoclaved lake water. The triplicate inoculated lake water samples were then used for the following parallel comparison of three *E. coli* enumeration methods. The three methods used in this study included the mTEC membrane filter/USEPA method 1603 (USEPA method 1603, United States Environmental Protection Agency), the Coliscan® Easygel agar plate method (Micrology laboratories) and the 3M™ Petrifilm™ method (3M, Atlanta, GA, USA).

The concentration of the inoculated *E. coli* cells was determined by plating the appropriate serial dilutions onto Trypticase soy agar (TSA plates) (BBL/Difco Laboratories, Sparks, MD, USA) and colonies were enumerated after incubating at 37°C ± 0.5°C for 24 hours.

2.3.2 Field sample collections and preparation

Samples were collected from Lake Martin and Lake Logan Martin (Alabama, USA) one day every month from May 2014 to October 2014. On each sampling day, every lake was sampled in the morning and in the afternoon. At each location, 3 surface water samples and 3 sediment samples were collected. Surface water samples were collected by using a sterile 50mL conical tube (VWR® International, Radnor, PA, USA) and placed immediately in a cooler with ice. The samples were plated within three hours and incubated in a portable incubator.

Sediment samples were collected by using sterile Telescopic Dippers (Ben Meadows® Company, Janesville, WI, USA) between 5cm and 20cm deep from the

sediment surface. The sediment samples were placed into a 100mL screw-top container (VWR® International, Radnor, PA, USA) and kept on ice. Twenty five grams of each sediment sample were homogenized with 100mL of autoclaved MilliQ water in a Whirl-Pak® filter bag (Nasco, Fort Atkinson, WI, USA) for 2 min by hand before plating.

The concentrations of *E. coli* present in field samples were enumerated with the Coliscan® Easygel agar plate (Micrology Laboratories, Goshen, IN, USA) and the coliform bacteria and Enterobacteriaceae were enumerated using the 3M™ Petrifilm™ method (3M, Atlanta, GA, USA).

2.3.3 Enumeration of *E. coli* by the mTEC membrane filter/USEPA method 1603

The modified mTEC agar (modified membrane - Thermotolerant *Escherichia coli* agar) (BBL/Difco Laboratories, Sparks, MD, USA) was prepared following the manufacturer's manual. A sterile 0.45µm pore size gridded membrane filter funnel (Pall Corporation, Port Washington, NY, USA) was placed onto the filter funnel manifold (Pall Corporation, Port Washington, NY, USA) connected to a vacuum. Three artificially inoculated water samples of 50mLs each were filtered through 0.45µm pore size gridded membrane filters. After filtration, each membrane was removed with sterile forceps and placed on mTEC agar according to USEPA method 1603. The plates were incubated at 35°C ± 0.5°C for 2 ± 0.5 hours to resuscitate injured or stressed bacteria, and then placed into a Whirl-Pak® bag, sealed and incubated at 44.5°C ± 0.2°C water bath for 22 ± 2 hours. The red or magenta colonies (suspected *E. coli* colonies) on modified mTEC agar were counted.

2.3.4 Enumeration of *E. coli* by Coliscan® Easygel agar plates

Three artificially inoculated water samples or field samples of 1mL each were dispensed using sterile pipettes into three Coliscan® Easygel media bottles. The media in the bottles contains two sugars linked with different dyes. One sugar linked to one dye can turn the colony a pink color when acted on by the enzyme β-galactosidase, which is produced by coliforms including *E. coli*. Another sugar linked to a different dye produces a blue-green color when acted on by the enzyme β-glucuronidase, which is produced by *E. coli* only. Because *E. coli* can work with both sugars, the combination of these two dyes shows a purple color, while the coliform colony only shows the pink color. The bottles were kept on ice no longer than 3 hours before pouring the plates, and the pour plated samples were incubated at 29-37°C for 30-48 hours. The purple colonies (suspect *E. coli* colonies) on the Coliscan® Easygel agar plates were counted.

2.3.5 Enumeration of *E. coli,* coliform and Enterobacteriaceae by 3M™ Petrifilm™

Three 1mL amounts of artificially inoculated water samples were plated onto 3M™ Petrifilm™ *E.coli*/Coliform count plates and three 1mL amounts of field collected water samples were plated onto 3M™ Petrifilm™ Coliform count plates. The same was done with 3M™ Petrifilm™ Enterobacteriaceae count plates. All of the petrifilms were incubated at 37°C ± 0.5°C for 24 ± 2 hours. The blue colonies with associated gas bubbles on the 3M™ Petrifilm™ *E.coli* count plates were counted as *E. coli*. The red colonies with associated gas bubbles on the 3M™ Petrifilm™ Coliform count plates were counted as coliform. The colonies with associated gas bubbles on the 3M™ Petrifilm™ Enterobacteriaceae count plates were counted as Enterobacteriaceae.

2.3.6 Statistical analysis

Statistical analysis of the artificial inoculated sample results was performed to answer if a difference existed among the two membrane filtration methods, the Coliscan® Easygel Agar Plates method and the 3M™ Petrifilm™ methods. The statistical analysis of the field sampling results was performed to answer two questions: (i) whether the sampling time (morning vs. afternoon and seasons) impacted the *E. coli*/coliform/Enterobacteriaceae enumeration results, and (ii) whether the sample type (surface water vs. sediment) impacted the *E. coli*/coliform/Enterobacteriaceae enumeration results.

Triplicates were done for all experiments and the means and standard deviations were calculated. The *P*-value was tested by ANOVA using Univariate GLM of SPSS 21.0. A *P* value of less than 0.05 was considered significant.

2.4 Results

The purpose of doing an artificial comparison was to tell if there were differences among these three methods when analyzing a known concentration of *E. coli*. The results are shown in Table 2. By using the TSA plate, which is the basic laboratory method used to enumerate concentrations of *E. coli*, the results showed 9.14 ± 0.106 log CFU/mL. Compared to the TSA basic method, the *E. coli* concentrations measured by the mTEC membrane filter/USEPA method 1603, Coliscan® Easygel agar plates method and 3M™ Petrifilm™ were 9.12 ± 0.122, 8.79 ± 0.28 and 8.97 ± 0.035 log CFU/mL respectively. Table 3 shows the analysis of variance among those methods. The table indicates that there were no significant differences ($P = 0.101 > 0.05$) among the three methods tested.

Because there were no differences, the Coliscan® Easygel agar plate method was used as the standard procedure to enumerate *E. coli* for the field samples and the 3M™ Petrifilm™ Coliform and Enterobacteriaceae count plates were used to enumerate coliform and Enterobacteriaceae during the field sampling from the two lakes.

Figure 2 shows the *E. coli* concentrations of water samples collected from Lake Martin and Lake Logan Martin at different sampling times. As shown in Figure 2 A, *E. coli* was only detected in afternoon samples in May but in both morning and afternoon samples in June and August from Lake Martin. The results of June showed that *E. coli* concentrations in the morning were higher than in the afternoon, and August showed that mornings and afternoons were not different. Figure 2 B indicates that September was the only month where no *E. coli* was detected in either morning or afternoon samples, while in July and August there were no *E. coli* in the morning samples collected from Lake Logan Martin. The results from May showed that *E. coli* concentrations in the morning were lower than in the afternoon sample, while June and October showed the same concentrations in the morning and afternoon from Lake Logan Martin.

Figure 3shows the coliform concentrations in water samples collected from Lake Martin and Lake Logan Martin at different sampling times. Coliform, unlike *E. coli*, can be detected every month at every time period. At Lake Martin (Figure 3A), coliform concentrations in September and October were lower in the morning than in the afternoon, except in July where the morning and afternoon samples were not different. Other months showed the opposite result. At Lake Logan Martin (Figure 3B), May, June and August had lower coliform concentrations in the morning than the afternoon, while July sampling

showed no differences between the morning and afternoon. September and October had higher concentrations in the afternoon than the morning samples.

Figure 4 shows Enterobacteriaceae concentrations in water samples collected from Lake Martin and Lake Logan Martin at different sample times. At Lake Martin (Figure 4A), May was the only month where no Enterobacteriaceae was detected, and only samples from the morning of October indicated the presence of Enterobacteriaceae. June samples had the same Enterobacteriaceae concentrations for morning and afternoon samples, all other months showed mornings had a higher concentration than afternoons. Lake Logan Martin (Figure 4B) did not have Enterobacteriaceae in samples from the morning of May and the afternoon of October. The July results showed morning and afternoon having same concentration. June and August both showed the afternoon had higher concentrations than the morning, while September showed the opposite. Figures 2 to 4 revealed that there were significant differences ($P< 0.05$) between different sampling times, morning and afternoon and different months.

Figure 5 has the coliform concentration comparison between surface water samples and sediment samples collected from Lake Martin and Lake Logan Martin. The result of Lake Martin (Figure 5A) and Lake Logan Martin (Figure 5B) both showed that the sediment samples have much higher concentrations than surface water samples.

Figure 6 has the Enterobacteriaceae concentration comparison between surface water samples and sediment samples collected from Lake Martin and Lake Logan Martin. Lake Martin (Figure 6A) results show that the surface water samples from May did not have Enterobacteriaceae, and all other months showed that the concentration of

Enterobacteriaceae in the sediment samples were much higher than surface water samples. Lake Logan Martin (Figure 6B) results did not detect Enterobacteriaceae in surface water samples. June, July and August samples had higher concentrations of Enterobacteriaceae in the sediment samples than in the surface water samples. September and October had higher surface water concentrations than the sediment samples. Figures 5 and 6 revealed that there were also significant differences ($P< 0.05$) between surface water and sediment.

2.5 Discussion

For the artificial experiment, results showed that there was not a significant difference ($P = 0.101$) among the mTEC membrane filte/USEPA method 1603, Coliscan® Easygel agar plates and 3M™ Petrifilm™ methods. Previous studies showed similar results when comparing the Coliscan® Easygel agar plates with the 3M™ Petrifilm™ method (Beloti and others, 2003). According to AWW, Coliscan® Easygel agar plates method was chosen to be the bacteriological monitoring method used because it was easy to use and less training was required.

Based on Figures 2 to 4, different sample collection seasons impacted the number of the *E.coli*, coliform and Enterobacteriaceae in lake water. In addition, significant differences were observed ($P < 0.05$) between the morning samples and afternoon samples. Barbe and others (1995) and Ikonen and others (2013) also found similar results for sampling at different seasons and at different times of day (Barbe and others, 1995; Ikonen and others, 2013).

On the basis of Figures 5 and 6, there were also significant differences ($P < 0.05$) in coliform and Enterobacteriaceae concentrations between surface water and sediment

samples. The sediment samples had much higher indicator microorganism concentrations than surface water samples. Recent research also shared similar results, which was that the concentration of *E. coli* in sediment was much higher than those in surface water (An and others, 2002).

The theory that was offered as the cause of differences between sampling times of day may be related to the usage of public recreational lakes. Piorkowski and others (2014) found that *E. coli* can persist in sediments and can be re-suspended into surface water which then influences water monitoring programs (Piorkowski and others, 2014). During our field trip, there were people swimming in the noon of July and August at Lake Martin and Lake Logan Martin. The results from Lake Logan Martin confirmed that the bacterial concentrations may be influenced by activities in the lake, such as swimming and boating, which cause re-suspension the bacteria into surface water and cause the differences between sampling times of day. However, the results of Lake Martin did not agree with this theory. This may be because at the Lake Martin, there seem to be many more birds (geese), around the lake swimming/sampling area (visual observations), and the birds feces may cause these result. Feeding of birds at Lake Logan Martin was highly discouraged by signage while at Lake Martin, campers were seen feeding the geese in the swimming/sampling area.

2.6 References

American Public Health Association. 1995. Standard Methods for the Examination of Water and Wastewater (19th ed.), APHA, Washington, D. C., USA.

An, Y. J., Kampbell, D. H., and Breidenbach, G. P. 2002. *Escherichia coli* and total coliforms in water and sediments at lake marinas. Environ. Pollut. 120:771-778.

Ashbolt, N. J. 2004. Microbial contamination of drinking water and disease outcomes in developing regions. Toxicology. 198: 229-238.

Barbe, D. E., and Francis, J. C. 1995. An analysis of seasonal fecal coliform levels in the Tchefuncte River. J. Am. Water Resour. Asso. 31(1):141–146.

Baylis, C., Uyttendaele, M., Joosten, H., and Davies, A. 2011. The Enterobacteriaceae and Their Significance to the Food Industry. International Life Sciences.

Beloti, V., Souza, J. A., Aguiar, M., Barros, F., Nero, L. A., Mattos, M. R., Gusmão, V. V., and Moraes, L. B. 2003. Evaluation of Petrifilm™ EC and HS for total 11 coliforms and *Escherichia coli* enumeration in water. Braz. J. Microbiol. 34(4):301-304.

Cellini, L., Dainelli, B., Angelucci, D., Grossi, L., Bartolomeo, S. D., Campli, E. D., and Marzio, L.1999. Evidence for an oral-faecal transmission of Helicobacter pylori infection in an experimental murine model. APMIS. 107(5):477–484.

Centers of Disease Control and Prevention. 2014. General Information of *E. coli*. U.S. Dept. of Health and Human Services, Atlanta, GA.

Cohen, J., and Shuval, H. 1972. Coliforms, fecal coliforms and fecal streptococci as indicators of water pollution. Water, Air, & Soil Pollution. 2(1973):85-95.

Ewing, W. H. and Davis, B. R. 1973. Media and Tests for Differentiation of Enterobacteriaceae. U.S. Department of Health, Education, and Welfare/ Public Health Service.

Garzio-Hadzick, A., Shelton, D. R., Hill, R. L., Pachepsky, Y. A., Guber, A. K., and Rowland, R. 2010. Survival of manure-borne *E. coli* in streambed sediment: effects of temperature and sediment properties. Water Res. 44(9):2753-2762.

Hudson, L. D., Hankins, J. W., and Battaglia, M. 1983. Coliforms in a water distribution system: a remedial approach. Am. Water. Works Assoc. 75:564-8.

Ikonen, J., Pitkänen, T., and Miettinen, I. T. 2013. Suitability of Optical, Physical and Chemical Measurements for Detection of Changes in Bacterial Drinking Water Quality. Int. J. Environ. Res. Public Health. 10(11):5349-5363.

International Organization of Standardisation. 2005. Water Quality — General Guidance on the Enumeration of Micro-organisms by Culture (ISO 8199:2005). ISO, Geneva, Switzerland.

Jean, F. M. 1980. Biochemical Tests for Identification of Medical Bacteria (3rd ed.). ISBN 0-6830-5318-3.

Lund, B. M., Sussman, M., Jones, D., and Stringer, M. F. 1988. Enterobacteriaceae in the environment and as pathogens. ISBN 0267-4440.

Micrology Laboratories. 2008. Detection of waterborne coliforms and *E. coli* with Coliscan® Easygel. Micrology Laboratories, Goshen, IN. USA.

Micrology Laboratories. 2014. Now You Have A Better Choice Now You Have A Better Choice with Easygel Cards™. Micrology Laboratories, Goshen, IN. USA.

Pecher, W., and Kemp, S. 2012. Monitoring of *Escherichia coli* in Jones Falls, MD: a comparison of methods. Research Paper.

Piorkowski, G. S., Jamieson, R. C., Hansen, L. T., Bezanson, G. S., and Yost, C. K. 2014. Characterizing spatial structure of sediment *E. coli* populations to inform sampling design. Environ. Monit. Assess. 186(1):277–291.

Singleton, P. 1999. Bacteria in Biology, Biotechnology and Medicine (5th ed.). ISBN 0-471-98880-4.

Stepenuck, K. F., Wolfson, L. G., Liukkonen, B. W., Iles, J. M., and Grant, T. S. 2011. Volunteer monitoring of *E. coli* in streams of the upper Midwestern United States: A comparison of methods. Environ. Monit. Assess. 174(1-4):625-633.

Stevens, G., Mascarenhas, M., and Mathers, C. 2009. Global health risks: progress and challenges Bull. World Health Organ. 87(9):646.

Tallon, P., Magajna, B., Lofranco, C., and Leung, K. T. 2005. Microbial indicators of faecal contamination in water: a current perspective. Water, Air, & Soil Pollution. 166(1):139–166.

United States Environmental Protection Agency. 1986. Ambient water quality criteria for bacteria–1986. Washington, D. C.: United States Environmental Protection Agency: Office of Water.

United States Environmental Protection Agency. 2009. *Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using Modified membrane-Thermotolerant Escherichia coli Agar (Modified mTEC). Washington, D. C.: United States Environmental Protection Agency: Office of Water.

Vogt, R. L., and Dippold, L. 2005. *Escherichia coli* O157:H7 outbreak associated with consumption of ground beef, June–July 2002. Public Health Rep. 120(2):174–178.

Table 2.*E. coli* concentration in inoculated lake water enumerated by different methods

	TSA	The mTEC membrane filter/USEPA method 1603	Coliscan® Easygel Agar Plates	3M™ Petrifilm™
E. coli concentration (log CFU/mL)	9.14 ± 0.105	9.12 ± 0.121	8.79 ± 0.288	8.97± 0.036

*The information showed in Table 2 was genomic mean ± standard deviation.

Table 3.Analysis of variance among different enumeration methods from Table 2

	Sum of squares	*df*	Mean Square	*F*	*P*
Between Groups	0.239	3	0.80	2.915	0.101
Within Groups	0.219	8	0.27		
Total	0.458	11			

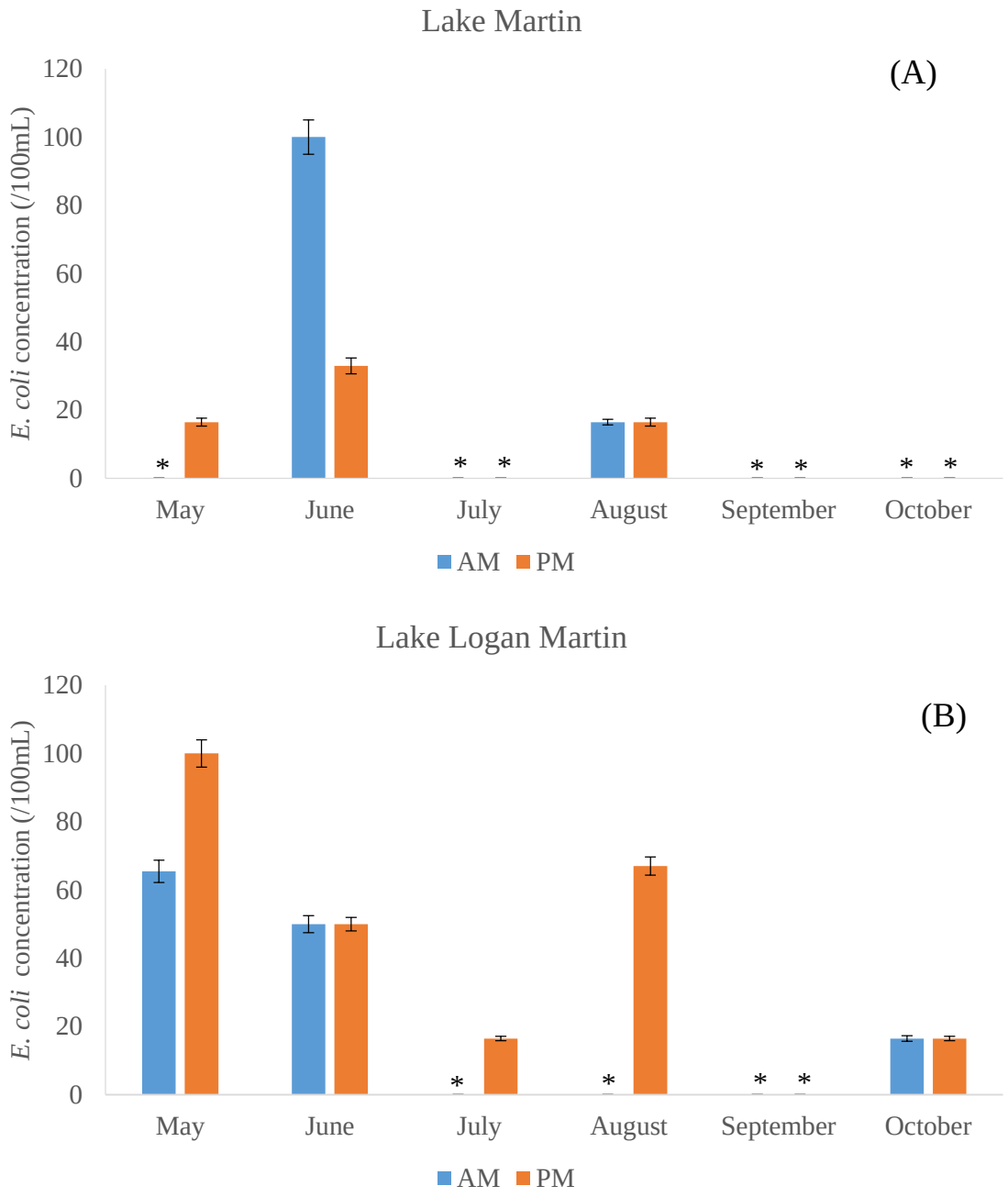

Figure 2. *E. coli* concentrations in water samples collected from Lake Martin and Lake Logan Martin at different sampling times. Error bars stand for the standard deviation. * refers to the *E. coli* concentrations are under the limit of enumeration range.

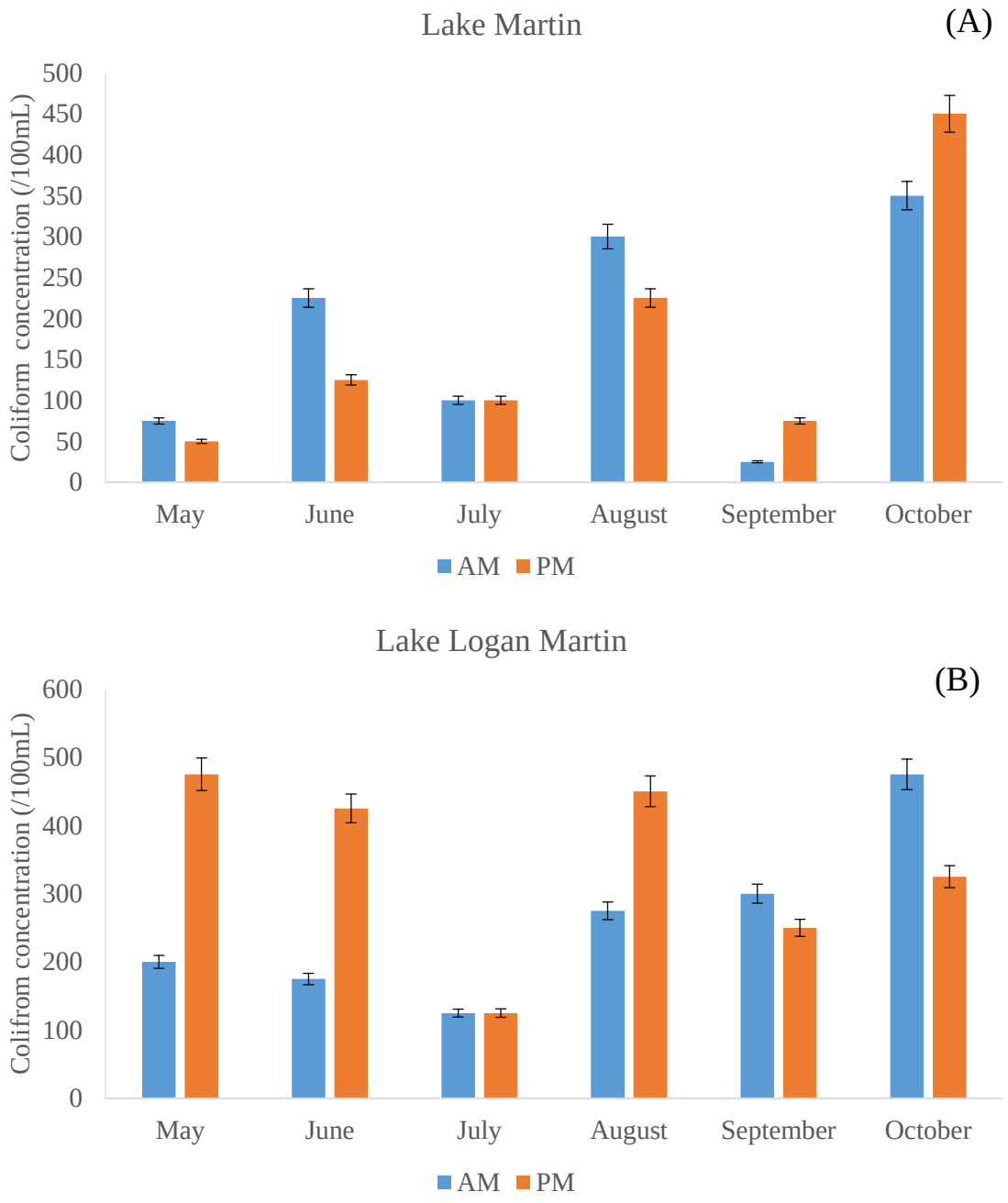

Figure 3. Coliform concentrations in water samples collected from Lake Martin and Lake Logan Martin at different sampling times. Error bars stand for the standard deviation.

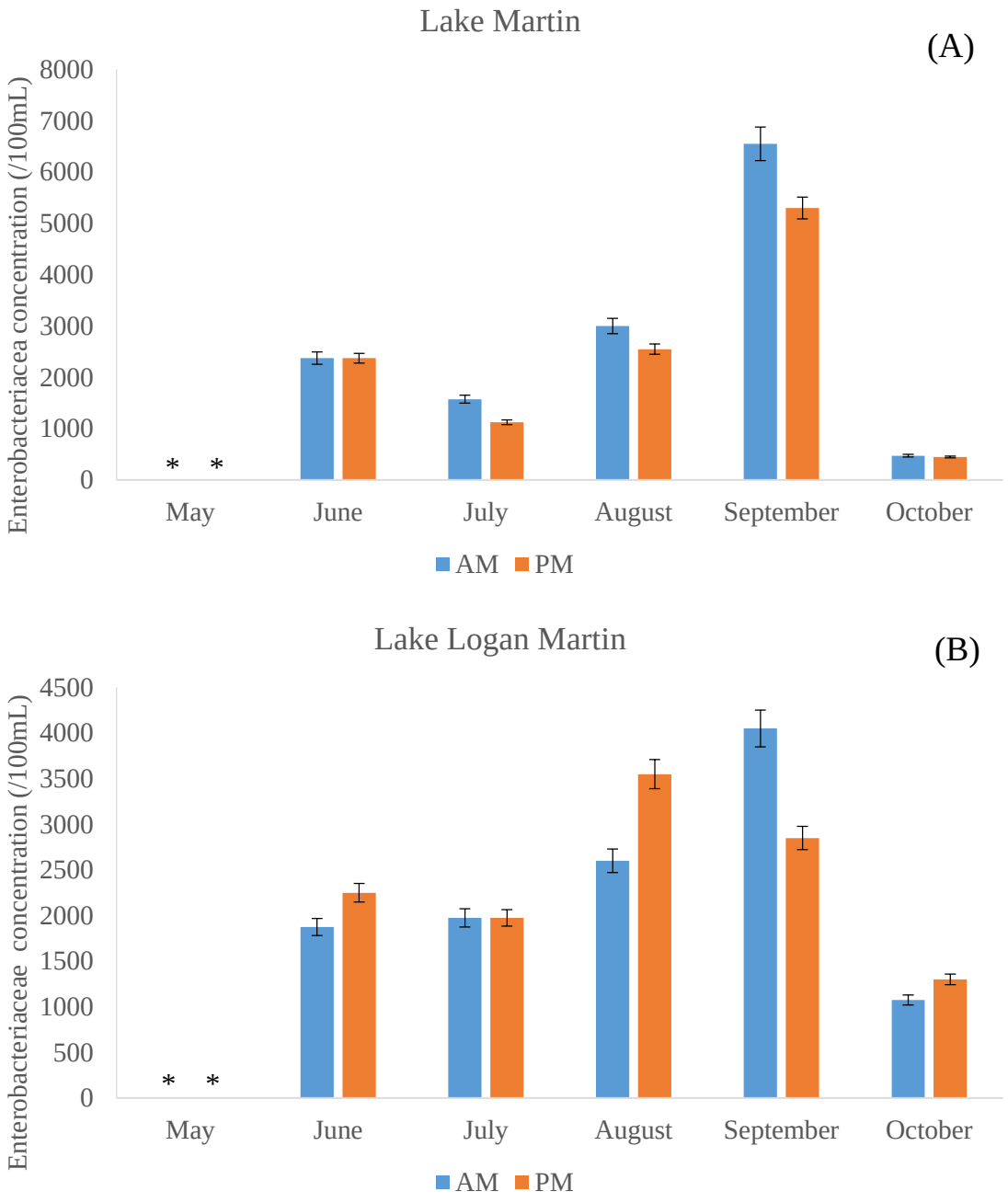

Figure 4. Enterobacteriaceae concentrations in water samples collected from Lake Martin and Lake Logan Martin at different sampling times. Error bars stand for the standard deviation. * refers to the Enterobacteriaceae concentrations are under the limit of enumeration range.

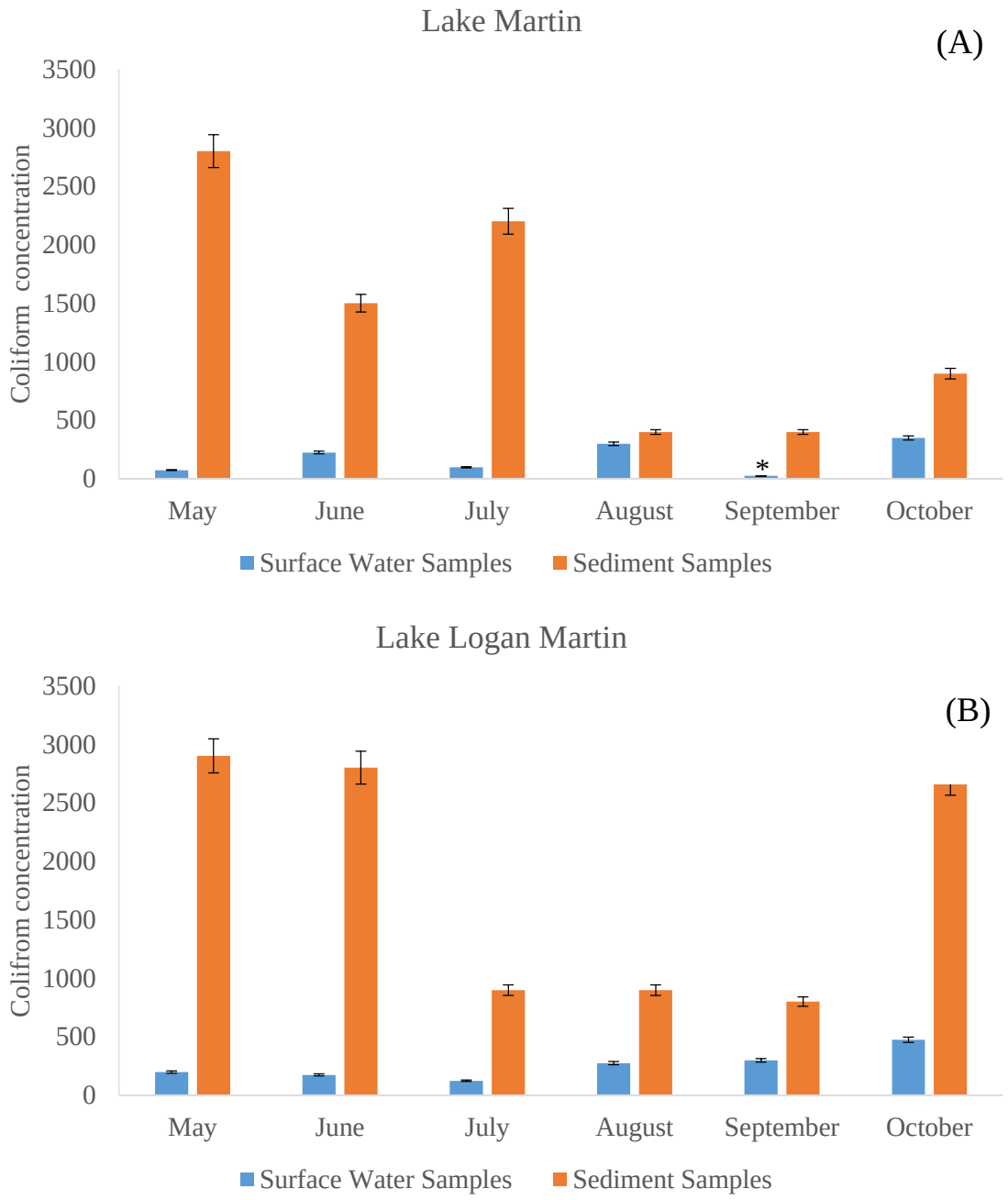

Figure 5. Comparison of coliform concentrations between surface water samples (/100mL) and sediment (/100g) samples collected from Lake Martin and Lake Logan Martin. Error bars stand for the standard deviation. * refers to the coliform concentrations are under the limit of enumeration range.

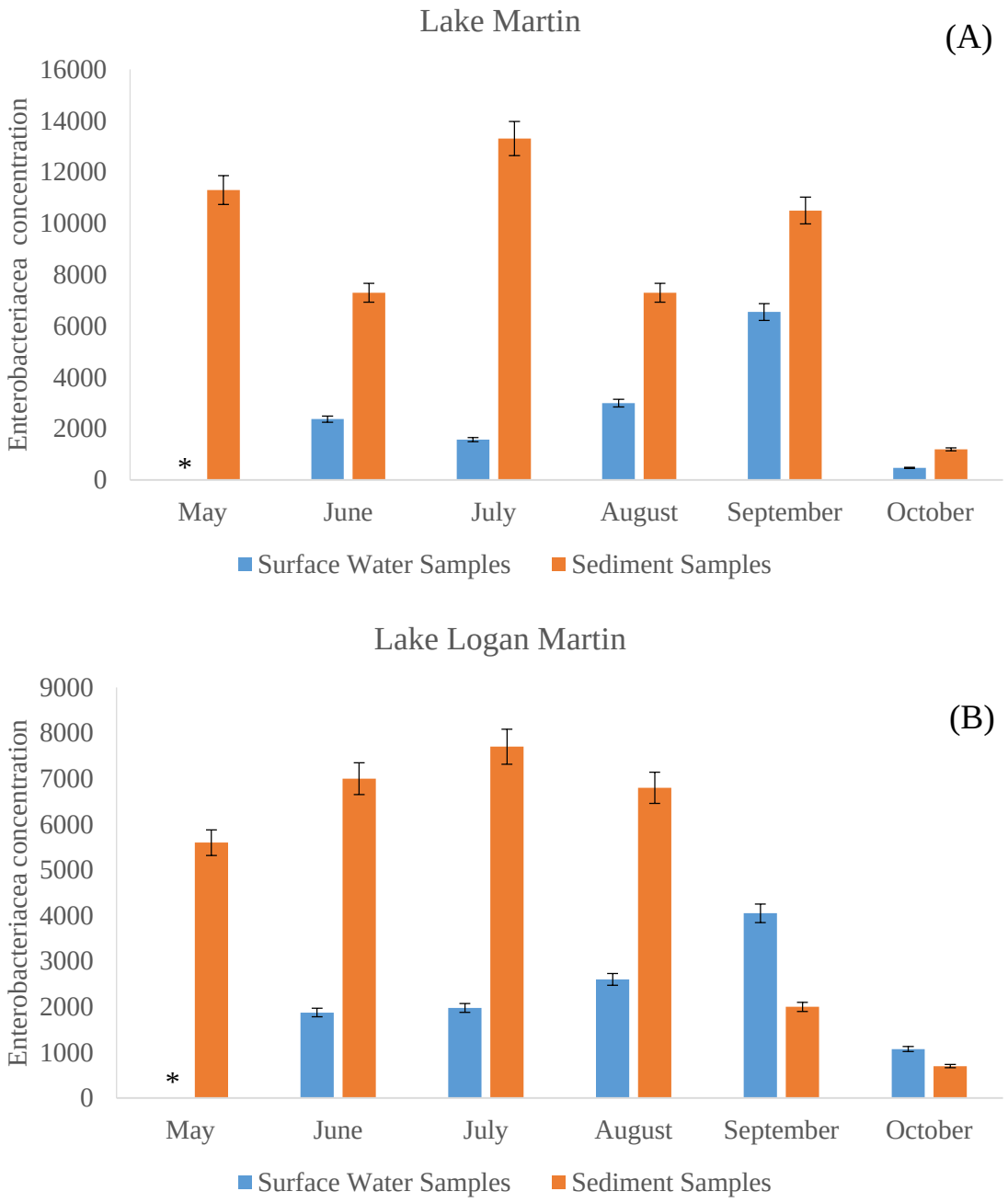

Figure 6. Comparison of Enterobacteriaceae concentrations between surface water samples (/100mL) and sediment (/100g) samples collected from Lake Martin and Lake Logan Martin. Error bars stand for the standard deviation. * refers to the Enterobacteriaceae concentrations are under the limit of enumeration range.

CHAPTER 3. COMPARISON OF DIFFERENT ENUMERATION PROTOCOLS FOR ENTEROCOCCI IN WATER AND SEDIMENT

3.1 Abstract

Escherichia coli is used as an indicator organism to monitor water quality. In recent years, it has been proposed that Enterococci can also be used as an indicator organism for water quality monitoring of fresh water sources. Enterococci are part of the normal flora of the gastrointestinal tracts of humans and animals and have been used as an indicator microorganism for sea water quality. The purpose of this study was to compare the efficacy of different Enterococci enumeration protocols for freshwater monitoring. The protocols used in this study included the membrane filter/USEPA method 1600 (United States Environmental Protection Agency), the Enterolert® method, the Easygel cards™ method and the USEPA qPCR method 1611. Different DNA extraction methods were also tested and compared. Although the Easygel cards™ method has the lowest price ($1 per sample), the USEPA qPCR method 1611 ranks highest among all of the tested methods based on the shortest processing time needed (~ 4 hours) and the widest detection range (2.47-8.47 log CFU/mL). For fresh water samples, the USEPA qPCR method 1611 DNA extraction procedure requires more training than the PrepMan® boiling procedure, and the PrepMan® boiling procedure showed a similar DNA extraction efficiency ($P>0.05$) for water samples compared to the USEPA qPCR method 1611 DNA extraction procedure. Therefore, the USEPA qPCR method 1611 DNA extraction can be replaced by the PrepMan® boiling procedure for fresh water samples. Besides the fresh water sample, the sediment samples can also be enumerated using the USEPA qPCR method 1611. Unlike the fresh water sample, the PowerSoil® DNA

Isolation Kit cannot be replaced by the PrepMan® boiling procedure due to the significant differences of DNA quantity and quality between two procedures ($P< 0.05$).

3.2 Introduction

Enterococcus is a facultative anaerobic, Gram-positive coccus, a large genus of lactic acid bacteria which belongs to human and animal gastrointestinal flora and is widely-distributed in the environment (Gilmore, 2002). Two species are common commensal organisms in the intestines of humans: *E. faecalis* and *E. faecium* (Köhler, 2007). Usually *Enterococcus* represent less than 1% of the flora (Tendolkar and others, 2003), but they are ubiquitous in human feces and persist in the environment.

Since 2004, *Enterococcus* has been adopted by the Federal Register as an indicator of human fecal pollution in water (Federal Register, 2004; Jin and others, 2004). USEPA also recommends using *Enterococcus* as the indicator for all recreational marine waters (USEPA, 2004). In Hawaii and most of the United States, the limit of *Enterococcus* for marine waters off of the beaches is a geometric mean of 35 colony-forming units (CFU)/100 mL and a single sample maximum limit of 104 CFU/100mL (Hawaii State Department of Health, 2012).

Because of the importance of *Enterococcus* as a water quality indicator, great efforts have been made in the development of methods for enumerating *Enterococcus* in water samples. Based on the USEPA method 1600 (USEPA, 2006), membrane-*Enterococcus* Indoxyl-β-D-Glucoside Agar (mEI) is used to enumerate *Enterococcus* in water. USEPA method 1611 (USEPA, 2012) uses a quantitative polymerase chain reaction (qPCR) procedure to enumerate *Enterococcus*. In addition, there are other

methods that can be used to detect *Enterococcus*, including a commercial method called Enterolert® (IDEXX Laboratories, 2014), and Easygel cards™ produced by Micrology Laboratories (Micrology Laboratories, 2014).

Previous studies compared two different methods to enumerate *Enterococcus* in water (Ferguson and others, 2013; Kinzelman and others, 2003; Noble and others, 2010; Haugland and others, 2005). Ferguson and others (2013) focused on the species distribution of *Enterococcus*, and found that the mEI membrane filter/USEPA method 1600 and the Enterolert® generally yielded the same species of *Enterococcus*. There were some differences that were mostly related to a preferential culturing of *E. faecalis* by Enterolert® in marine and spiked samples ($P > 0.05$) but Enterolert® was more selective for *E. faecalis* in wastewater samples (Ferguson and others, 2013). Kinzelman and others (2003) showed that there were drawbacks when using Enterolert® besides the lack of correlation between methodologies and included the inability to re-culture and verify isolates as *Enterococcus*. There were also advantages, such as time consumption was decreased, ease of use and minimal technical training required of personnel in using this system (Kinzelman and others, 2003). A study by Noble and others (2010) found that there was a level of agreement of 88% between the *Enterococcus* qPCR method and the mEI membrane filter/USEPA method 1600 and a 94% level of agreement between the mEI membrane filter/USEPA method 1600 and Enterolert® (Noble and others, 2010). Haugland and others (2005) found a significant positive correlation between qPCR and the mEI membrane filter/USEPA method 1600 to enumerate the concentration of *Enterococcus*, which indicated that the qPCR has the potential to supplement or replace

the mEI membrane filter/USEPA method 1600 as a means of assessing the levels of fecal contamination at freshwater recreational beaches (Haugland and others, 2005).

The objectives of this study include, first, to better understand the advantages and disadvantages of each enumeration method for *Enterococcus* and provide proven technical information for water quality monitoring of freshwater sources. The second objective is to focus on the qPCR method and determine if the standard DNA extraction method can be replaced by other simplified methods. The third objective is to understand whether the qPCR method can enumerate *Enterococcus* both in surface water and sediment from freshwater sources.

3.3 Material & Methods

3.3.1 Bacterial strains and growth conditions

Enterococcus faecalis strain ATCC® 29212™ from Dr. Yucheng Feng (Auburn University, Auburn, AL, USA) was used in this study. The strain was prepared by transferring 100 µl of frozen stock culture into 10 ml of Tryptone Soy broth (TSB) (BBL/Difco Laboratories, Sparks, MD, USA) and incubated at 41°C ± 0.5°C for 18 to 24 hours. Numbers of colony forming units (CFU) were determined by plating the appropriate serial dilutions onto Trypticase soy agar (TSA) (BBL/Difco Laboratories, Sparks, MD, USA) and enumerating the colonies after incubating the plates at 41°C ± 0.5°C for 24 hours.

3.3.2 Artificial inoculation

The overnight fresh culture was washed by centrifugation at 4,000 rpm for 5 min (Eppendorf, Hauppauge, NY, USA) and the cell pellet was re-suspended in 10 ml of

MilliQ water. The washed cells were then diluted. One milliliter of each dilution was inoculated into 9mL of autoclaved lake water. To prepare the artificially inoculated sediment samples, 10ml of each dilution was inoculated into 10 grams of autoclaved lake sediment. To enumerate the cells artificially inoculated into the sediment sample, for every 10 grams of inoculated sediment sample, 100 mL of autoclaved MilliQ water was added in a Whirl-Pak® filter bag together with the sediment (Nasco, Fort Atkinson, WI, USA) and homogenized by hand for 2 minutes. The homogenized samples were analyzed using different protocols.

Both the artificially inoculated water and sediment samples were triplicated and used for enumerating the *Enterococcus* by four different methods, including the mEI membrane filter/USEPA method 1600 (USEPA, Washington, D. C., USA), Enterolert® (IDEXX Laboratories, Inc. Westbrook, ME, USA), Easygel cards™ (Micrology Laboratories, Goshen, IN, USA) and the USEPA qPCR method 1611.

3.3.3 Enumeration of *Enterococcus* by the mEI membrane filter/USEPA method 1600

The mEI plates (membrane – *Enterococcus* Indoxyl-β-D-Glucoside Agar) (BBL/Difco Laboratories, Sparks, MD, USA) were prepared following the manufacturer's manual. A sterile 0.45µm pore size gridded membrane filter funnel (Pall Corporation, Port Washington, NY, USA) was first placed onto the filter funnel manifold (Pall Corporation, Port Washington, NY, USA) and was connected to a vacuum. Three milliliters, ten milliliters, and thirty milliliters of each inoculated water sample were filtered through three 0.45µm pore size gridded membrane filters, respectively. For every inoculated sediment sample, three replicates of 50mL of sediment suspension were used

for filtration. After filtration, each membrane was placed onto a mEI agar plate by a sterile forceps according to the mEI membrane filter/USEPA method 1600. Plates were incubated at 41°C ± 0.5°C for 24 ± 2 hours. Colonies with blue haloes on mEI plates were counted as *Enterococcus*. The suspect colonies were collected for Gram staining and transferred onto Bile Esculin agar (BEA) (BBL/Difco Laboratories, Sparks, MD, USA) for confirmation.

3.3.4 Enumeration of *Enterococcus* by Enterolert®

Three 100mL subsamples of each artificially inoculated water samples were mixed with three packs of Enterolert® reagent. The mixture was poured into a Quanti-Tray*/2000 (IDEXX Laboratories, Inc. Westbrook, ME, USA) according to the manufacturer's instructions. Quanti-Trays were sealed by an IDEXX Quanti-Tray* Sealer and the sealed tray was incubated at 41°C ± 0.5°C for 24 ± 2 hours. The number of wells producing fluorescence under UV light at 365nm within 5 inches of the tray in a dark environment was recorded. The *Enterococcus* was calculated according to the manufacturer – provided Most Probable Number (MPN) table.

3.3.5 Enumeration of *Enterococcus* by Easygel cards™

Three 1mL subsamples of each artificially inoculated water samples were plated onto Easygel cards™, and incubated at 41°C ± 0.5°C for 24 ± 2 hours. The blue colonies on the Easygel cards™ were counted as *Enterococcus*.

3.3.6 DNA extraction and measurement

Three DNA extraction methods were applied in this study. The first method was the PrepMan® boiling procedure following the protocol used by Wang and others (2007).

One milliliter of each water sample or 0.25 grams of each sediment sample were transferred into 1.5mL tubes (VWR® International, Radnor, PA, USA) and centrifuged at 12000 rpm for 2 min at 4°C. After centrifugation, the supernatant was removed and the pellet was re-suspended in 100 µL of the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA). Samples were boiled for 10 min, and then centrifuged at 12000rpm for 1 min at 4°C. The supernatant was collected for a DNA template.

The second DNA extraction method followed USEPA method 1611. 10mM of Tris-Cl and 0.5mM of EDTA (Ethylenediaminetetraacetic acid) was mixed and adjusted to pH 9.0 to make AE buffer. This buffer was used to elute DNA from the membrane and allowed long term storage of DNA at refrigeration or freezing temperatures. One hundred milliliter of artificially inoculated fresh water sample was filtered through a 0.45 µm pore size gridded membrane filter, and the filter was transferred into a labeled extraction tube with glass beads. Five hundred and ninety microliters of the AE buffer were added into each tube, the tube was placed in the Mini-Bead-Beater-16 (BioSpec Products, Inc., Bartlesville, OK, USA) for 60 seconds at the maximum rate and then centrifuged at 12000 rpm for 1 min. Four hundred microliters of the supernatant were transferred to a new tube and centrifuged again for 5 min, and 350µL of supernatant was collected for a DNA template.

The third DNA extraction method was for sediment samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, CA, USA). Two hundred and fifty milligrams of each sediment sample was transferred into one PowerBead tube together with 60µL of Solution C1, the tube was vortexed briefly and

centrifuged at 10,000g for 30 seconds at room temperature. The supernatant was transferred and mixed with 250µL of Solution C2 in a new tube. After a 5 min incubation at 4°C, the tube was centrifuged at 10,000g for 1 min at room temperature, and 600µL of supernatant was mixed with 200µL of Solution C3 in a new tube. The mixture was incubated again at 4°C for 5 min, then centrifuged at 10,000g for 1 min. Seven hundred and fifty microliters of supernatant was then transferred out and mixed with 1200µL of Solution C4 in a new tube. The mixture was loaded through one Spin filter three times and centrifuged at 10,000g for 1 min. After that, Solution C5 and Solution C6 were used to wash the DNA template. Five hundred microliters of Solution C5 were added and centrifuged at 10,000g for 1 min to wash the filter. Next, 100µL of Solution C6 was used to wash the filter again and centrifuged at 10,000g for 30 seconds to elute the DNA template into new tubes.

DNA quantity and quality were measured by a NanoVue Plus Spectrophotometer (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Usually, the most common DNA purity calculation is the ratio of the absorbance at 260nm divided by the reading at 280nm (Promega Corporation 2014).

3.3.7 The USEPA qPCR method 1611

The primers used in this study followed USEPA method 1611 using the forward primer of: 5'-GAGAAATTCCAAACGAACTTG-3' and the reverse primer of: 5'-CAGTGCTCTACCTCCATCATT-3'. Amplification reaction mixtures (25µL) contained template (5µL), SYBR Green SuperMix Low ROX (10µL) (Quanta Biosciences, Inc., Gaithersburg, MD, USA), forward and reverse primers (0.5µL for each one) and MilliQ

water (9µL). The reaction was carried out on the ABI 7500 (Applied Biosystem, CITY, CA, USA). Before amplification, the mixtures were heated to 50°C for 2 min and at 95°C for 10 min. The amplification program was 45 cycles of 95°C for 15 seconds and 60°C for 1 min. Standard curves were built by constructing regression lines with the X-axis being Log CFU/mL or Log CFU/g and the Y-axis being Ct values.

3.3.8 Statistical Analysis

All experiments were done in triplicate and the means and standard deviations were calculated. The *P*-value was tested by ANOVA using Univariate GLM of SPSS 21.0. A *P* value of less than 0.05 was considered significant.

3.4 Results

To better compare the four methods discussed: the mEI membrane filter/USEPA method 1600, the Enterolert®, the Easygel cards™ and the USEPA qPCR method 1611, price, time consumption, and accurate detection ranges were observed during the innoculation experiment and shown in Table 4. As the table shows, the Easygel cards™ method was the cheapest at about $1 per sample to enumerate *Enterococcus* in water, while the mEI membrane filter/USEPA method 1600, was the most expensive one at about $16 per sample. The Enterolert® and USEPA qPCR method 1611 cost about $10 and $4 to $6 per sample respectively. The USEPA qPCR method 1611 only needed 4 hours to get results while the other three methods all need an overnight incubation to enumerate *Enterococcus*. The accurate estimation range of Enterolert® was 0 to 2419.6 CFU/100mL, the mEI membrane filter/USEPA method 1600 and the Easygel cards™ accurate enumeration range were both between 1.34 and 2.34 log CFU/mL or 1.34 and

2.34 log CFU/g while the USEPA qPCR method 1611 had the widest detection range, from 2.47 to 8.47 log CFU/mL or 2.47 to 8.47 log CFU/g for *Enterococcus* enumeration.

Based on this result, more experiments were done using the USEPA qPCR method 1611. The first step was to find the best DNA extraction procedure. For the fresh water samples, USEPA method 1611 and the PrepMan® boiling procedures were compared. The DNA concentration is shown in Table 5 and Table 6 has the ANOVA of these two different procedures. As Table 5 shows, at the same *Enterococcus* inoculation level, these two procedures have similar results, and the *P*-value shown in Table 6 indicates that there was no significant difference ($P > 0.05$) between these two procedures. The DNA quality is shown in Table 7, and Table 8 contains the ANOVA of the DNA quality of the two methods. Table 7 shows that both methods can give the ratio of A260/A280 between 1.7 and 2.0, indicating that the DNA has good quality. Table 8 revealed that there were no significant differences ($P >0.05$) between these two procedure. This indicates that the PrepMan® boiling procedure was interchangeable with the USEPA method 1611 DNA extraction procedure.

For sediment samples, the PowerSoil® DNA Isolation Kit and the PrepMan® boiling procedures were compared. The DNA concentration is shown in Table 9, and Table 10 has the ANOVA of the two different procedures. As Table 9 shows, the PowerSoil® DNA Isolation Kit recovered a much higher concentration of the DNA template than the PrepMan® boiling procedure did while the *P*-value shown in Table 10 indicates that there was a significant difference ($P < 0.05$) between these two procedures. The DNA quality is shown in Table 11 and Table 12 has the results of the ANOVA of the two procedures showing the DNA quality. Table 11 shows that the PowerSoil® DNA

Isolation Kit gave the ratio of A260/A280 results between 1.7 and 2.0 indicating the DNA had good quality, while the PrepMan® boiling procedure did not produce a good quality ratio. Table 12 revealed that there were significant differences ($P < 0.05$) between these two procedures, which mean the PrepMan® boiling procedure was not interchangeable with the PowerSoil® DNA Isolation Kit.

For further study, standard curves of the USEPA qPCR method 1611 using different DNA templates were done, and are shown in Figures 7 and 8. Figure 7 shows the standard curve of water samples when using different DNA extraction procedures did not impact the standard curve. The DNA template extracted using the PrepMan® boiling procedure was more linear (R^2) than the DNA template extracted using the USEPA method 1611. Sediment samples had a low quantity and quality of DNA using the PrepMan® boiling procedure, therefore the Ct-values were almost undetectable and no standard curve could be made.

3.5 Discussion

Based on information shown in Table 4, the Easygel® cards™ method was the cheapest (~ $1 per sample) method used to enumerate *Enterococcus* in fresh water, while the mEI membrane filter/USEPA method 1600 was the most expensive (~ $16 per sample). The USEPA qPCR method 1611 took the least time consumption to produce results (~ 4 hour) while the other three methods needed an overnight incubation for *Enterococcus* enumeration. The USEPA qPCR method 1611 also had the widest detection range (2.47-8.47 log CFU/mL or 2.47-8.47 log CFU/g) compared to the other methods discussed.

According to the data analysis of DNA extraction procedure, there were no significant differences ($P > 0.05$) between USEPA method 1611 procedure and the PrepMan® boiling procedure when comparing the efficiency of these two methods of DNA extraction from fresh water samples. The PrepMan® boiling procedure can replace the USEPA method 1611 procedure for surface water samples. However, there were significant differences ($P < 0.05$) between the PowerSoil® DNA Isolation Kit and the PrepMan® boiling procedures when comparing their efficiency for extracting DNA from sediment samples. This indicates that the PowerSoil® DNA Isolation Kit cannot be replaced by the PrepMan® boiling procedure and the PowerSoil® DNA Isolation Kit is better when preparing DNA samples from sediments for the USEPA qPCR method 1611 to enumerate *Enterococcus*.

3.6 References

Federal Register. 2004. Water Quality Standards for Coastal and Great Lakes Recreation Waters; Final Rule 2004. 69(220):67218–67243.

Ferguson, D. M., Griffith, J. F., McGee, C. D., and Weisberg, S. B. 2013. Comparison of *Enterococcus* Species Diversity in Marine Water and Wastewater Using Enterolert® and EPA Method 1600. J. Environ. Public Health. 2013:848049.

Gilmore, M. S. 2002. The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance. ISBN 978-1-55581-234-8.

Haugland, R. A., Siefring, S. C., Wymer, L. J., Brenner, K. P., and Dufour, A. P. 2005. Comparison of *Enterococcus* measurements in freshwater at two recreational

beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res. 39(4):559-568.

Hawaii State Department of Health. 2012. State of Hawaii Water Quality Monitoring and Assessment Report. The Hawaii Department of Health, Honolulu, Hawaii. USA.

IDEXX Laboratories, Inc. 2004. Enterolert® Test Kit Manufacturer. Westbrook, ME, USA.

Jin, G., Jeng, H. W., Bradford, H., and Englande, A. J. 2004. Comparison of *E. coli*, enterococci, and fecal coliform as indicators for brackish water quality assessment. Water Environ. Res. 76(3):245–255.

Kinzelman, J., Ng, C., Jackson, E., Gradus, S., and Bagley, R. 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(1):92–96.

Köhler, W. 2007. The present state of species within the genera *Streptococcus* and *Enterococcus*. Int. J. Med. Microbiol. 297(3):133–150.

Micrology Laboratories. 2014. Now You Have A Better Choice Now You Have A Better Choice with Easygel CardsTM. Micrology Laboratories, Goshen, IN. USA.

MO BIO Laboratories. 2013. PowerSoil® DNA Isolation Kit Instruction Manual. MO BIO Laboratories. Carlsbad, CA. USA.

Noble, R. T., Blackwood, A. D., Griffith, J. F., McGee, C. D., and Weisberg, S. B. 2010. Comparison of Rapid Quantitative PCR-Based and Conventional Culture-Based

Methods for Enumeration of *Enterococcus* spp. and *Escherichia coli* in Recreational Waters. Appl. Environ. Microbiol. 76(22):7437–7443.

Tendolkar, P. M., Baghdayan, A. S., and Shankar, N. 2003. Pathogenic enterococci: new developments in the 21st century. Cell Mol. Life Sci. 60(12):2622–2636.

United States Environmental Protection Agency. 2004. Final Water Quality Standards Bacteria Rule for Coastal and Great Lakes Recreation Waters. Washington, D. C.: United States Environmental Protection Agency: Office of Water.

United States Environmental Protection Agency. 2006. Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-beta-D-Glucoside Agar (mEI). Washington, D. C.: United States Environmental Protection Agency: Office of Water

United States Environmental Protection Agency. 2012. Method 1611: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay. Washington, D. C.: United States Environmental Protection Agency: Office of Water.

Wang, L., Li, Y., and Mustapha, A. 2007. Rapid and simultaneous quantitation of *Escherichia coli* O157: H7, *Salmonella*, and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation. J. Food Prot. 70(6):1366-1372.

Table 4.Prices, time consumption, and accurate detection range of four Enterococci enumeration methods

Method	Price ($/Sample)	Time	Accurate Enumeration Range
Enterolert®	10	24 ± 2 hours	0-2419.6CFU/100mL (estimation)
The mEI membrane filter/USEPA method 1600	16	24 ± 2 hours	1.34-2.34 log CFU/mL or 1.34-2.34 log CFU/g
Easygel cards™	1	24 ± 2 hours	1.34-2.34 log CFU/mL or 1.34-2.34 log CFU/g
The USEPA qPCR method 1611	4-6*	4 hours	2.47-8.47 log CFU/mL or 2.47-8.47 log CFU/g

* The different DNA extraction procedures have different prices.

Table 5. Water sample DNA concentration (ng/µL) extracted by different methods

Methods	*Enterococcus* Inoculation Levels (log CFU/mL)							
	1	2	3	4	5	6	7	8
The USEPA qPCR method 1611 DNA extraction procedure	20.7 ± 10.07	80.5 ± 9.19	110.3 ± 6.66	171.0 ± 20.07	290.7 ± 11.06	407.3 ± 60.50	466.0 ± 54.78	597.7 ± 62.98
The PrepMan® boiling procedure	21.3 ± 4.93	79.5 ± 4.95	113.0 ± 8.72	175.3 ± 19.22	295.7 ± 12.01	409.0 ± 64.71	461.3 ± 47.82	602.7 ± 69.83

*The information showed in Table 5 was genomic mean ± standard deviation.

Table 6.Analysis of variance of water sample DNA concentration (ng/µL) extracted by different procedures

Enterococcus Inoculation Levels (log CFU/mL)		Sum of squares	*df*	Mean Square	*F*	*P*
	Between Groups	0.667	1	0.667	0.011	0.923
1	Within Groups	251.333	4	62.833		
	Total	252.000	5			
	Between Groups	1.000	1	1.00	0.018	0.905
2	Within Groups	109.000	2	54.500		
	Total	110.000	3			
	Between Groups	10.667	1	10.667	0.177	0.695
3	Within Groups	240.667	4	60.167		
	Total	251.333	5			
	Between Groups	28.167	1	28.167	0.073	0.800
4	Within Groups	1544.667	4	386.167		
	Total	1572.833	5			
	Between Groups	37.500	1	37.500	0.281	0.624
5	Within Groups	533.333	4	133.333		
	Total	570.833	5			
	Between Groups	4.167	1	4.167	0.001	0.976
6	Within Groups	15696.667	4	3924.167		
	Total	15700.833	5			
	Between Groups	32.667	1	32.667	0.012	0.917
7	Within Groups	10574.667	4	2643.667		
	Total	10607.333	5			
	Between Groups	37.500	1	37.500	0.008	0.931
8	Within Groups	17685.333	4	4421.333		
	Total	17722.833	5			

Table 7. Water sample DNA quality (A260/A280) extracted by different methods

Methods	*Enterococcus* Inoculation Levels (log CFU/mL)							
	1	2	3	4	5	6	7	8
The USEPA qPCR method 1611 DNA extraction procedure	1.704 ± 0.0093	1.717 ± 0.0085	1.757 ± 0.0246	1.774 ± 0.0114	1.793 ± 0.0086	1.818 ± 0.0065	1.859 ± 0.0157	1.953 ± 0.0115
The PrepMan® boiling procedure	1.716 ± 0.0125	1.725 ± 0.0093	1.747 ± 0.0120	1.779 ± 0.0174	1.807 ± 0.0157	1.825 ± 0.0215	1.839 ± 0.0142	1.958 ± 0.0221

*The information showed in Table 7 was genomic mean ± standard deviation.

Table 8. Analysis of variance of water sample DNA quality (A260/A280) extracted by different procedures

Enterococcus Inoculation Levels (log CFU/mL)		Sum of squares	*df*	Mean Square	*F*	*P*
	Between Groups	0.000	1	0.000	1.678	0.265
1	Within Groups	0.000	4	0.000		
	Total	0.001	5			
	Between Groups	0.000	1	0.000	1.414	0.300
2	Within Groups	0.000	4	0.000		
	Total	0.000	5			
	Between Groups	0.000	1	0.000	0.375	0.573
3	Within Groups	0.001	4	0.000		
	Total	0.002	5			
	Between Groups	0.000	1	0.000	0.198	0.679
4	Within Groups	0.001	4	0.000		
	Total	0.001	5			
	Between Groups	0.000	1	0.000	1.663	0.267
5	Within Groups	0.001	4	0.000		
	Total	0.001	5			
	Between Groups	0.000	1	0.000	0.290	0.619
6	Within Groups	0.001	4	0.000		
	Total	0.001	5			
	Between Groups	0.001	1	0.001	2.582	0.183
7	Within Groups	0.001	4	0.000		
	Total	0.001	5			
	Between Groups	0.000	1	0.000	0.120	0.746
8	Within Groups	0.001	4	0.000		
	Total	0.001	5			

Table 9. Sediment sample DNA concentration (ng/μL) extracted by different methods

Methods	*Enterococcus* Inoculation Levels (log CFU/mL)							
	1	2	3	4	5	6	7	8
The PowerSoil® DNA Isolation Kit	63.3 ± 9.29[a]	143.0 ± 16.65[a]	213.7 ± 7.64[a]	305.0 ± 9.17[a]	427.7 ± 23.46[a]	503.3 ± 8.74[a]	592.3 ± 18.18[a]	669.0 ± 32.42[a]
The PrepMan® boiling procedure	\	12.3 ± 5.86[b]	26.7 ± 6.51[b]	53.0 ± 5.57[b]	77.7 ± 6.11[b]	98.7 ± 8.02[b]	119.0 ± 7.21[b]	144.0 ± 12.00[b]

*The information showed in Table 9 was genomic mean ± standard deviation.

[a,b]Indicate significant differences between groups based on statistical analyses ($P < 0.05$)

Table 10. Analysis of variance of sediment sample DNA concentration (ng/µL) extracted by different procedures

Enterococcus Inoculation Levels (log CFU/mL)		Sum of squares	*df*	Mean Square	*F*	*P*
1	Between Groups	23064.000	1	23064.000	148.004	0.000
	Within Groups	623.333	4	155.833		
	Total	23687.333	5			
2	Between Groups	52453.500	1	52453.500	1042.123	0.000
	Within Groups	201.333	4	50.333		
	Total	52654.833	5			
3	Between Groups	95256.000	1	95256.000	1656.626	0.000
	Within Groups	230.000	4	57.500		
	Total	95486.000	5			
4	Between Groups	183750.000	1	183750.000	625.355	0.000
	Within Groups	1175.333	4	293.833		
	Total	184925.333	5			
5	Between Groups	245632.667	1	245632.667	3492.408	0.000
	Within Groups	281.333	4	70.333		
	Total	245914.000	5			
6	Between Groups	336066.667	1	336066.667	1757.977	0.000
	Within Groups	764.667	4	191.167		
	Total	336831.333	5			
7	Between Groups	413437.500	1	413437.500	691.946	0.000
	Within Groups	2390.000	4	597.500		
	Total	415827.500	5			
8	Between Groups	23064.000	1	23064.000	148.004	0.000
	Within Groups	623.333	4	155.833		
	Total	23687.333	5			

Table 11. Sediment sample DNA quality (A260/A280) extracted by different methods

Methods	*Enterococcus* Inoculation Levels (log CFU/mL)							
	1	2	3	4	5	6	7	8
The PowerSoil® DNA Isolation Kit	1.717 ± 0.0182[a]	1.748 ± 0.0108[a]	1.764 ± 0.0127[a]	1.787 ± 0.0076[a]	1.811 ± 0.0095[a]	1.835 ± 0.0091[a]	1.868 ± 0.0096[a]	1.921 ± 0.0237[a]
The PrepMan® boiling procedure	\	0.906 ± 0.0685[b]	0.987 ± 0.0265[b]	0.877 ± 0.1090[b]	1.114 ± 0.0872[b]	0.935 ± 0.0513[b]	1.382 ± 0.1465[b]	1.377 ± 0.0255[b]

*The information showed in Table 11 was genomic mean ± standard deviation.

[a,b]Indicate significant differences between groups based on statistical analyses ($P < 0.05$)

Table 12. Analysis of variance of sediment sample DNA quality (A260/A280) extracted by different procedures

Enterococcus Inoculation Levels (log CFU/mL)		Sum of squares	*df*	Mean Square	*F*	*P*
1	Between Groups	4.420	1	4.420	26763.370	0.000
	Within Groups	0.001	4	0.000		
	Total	4.421	5			
2	Between Groups	1.052	1	1.052	436.991	0.000
	Within Groups	0.010	4	0.002		
	Total	1.061	5			
3	Between Groups	0.906	1	0.906	2102.135	0.000
	Within Groups	0.002	4	0.000		
	Total	0.908	5			
4	Between Groups	1.244	1	1.244	208.306	0.000
	Within Groups	0.024	4	0.006		
	Total	1.268	5			
5	Between Groups	0.729	1	0.729	189.605	0.000
	Within Groups	0.015	4	0.004		
	Total	0.744	5			
6	Between Groups	1.215	1	1.215	895.797	0.000
	Within Groups	0.005	4	0.001		
	Total	1.220	5			
7	Between Groups	0.353	1	0.353	32.790	0.005
	Within Groups	0.043	4	0.011		
	Total	0.396	5			
8	Between Groups	0.443	1	0.443	730.813	0.000
	Within Groups	0.002	4	0.001		
	Total	0.446	5			

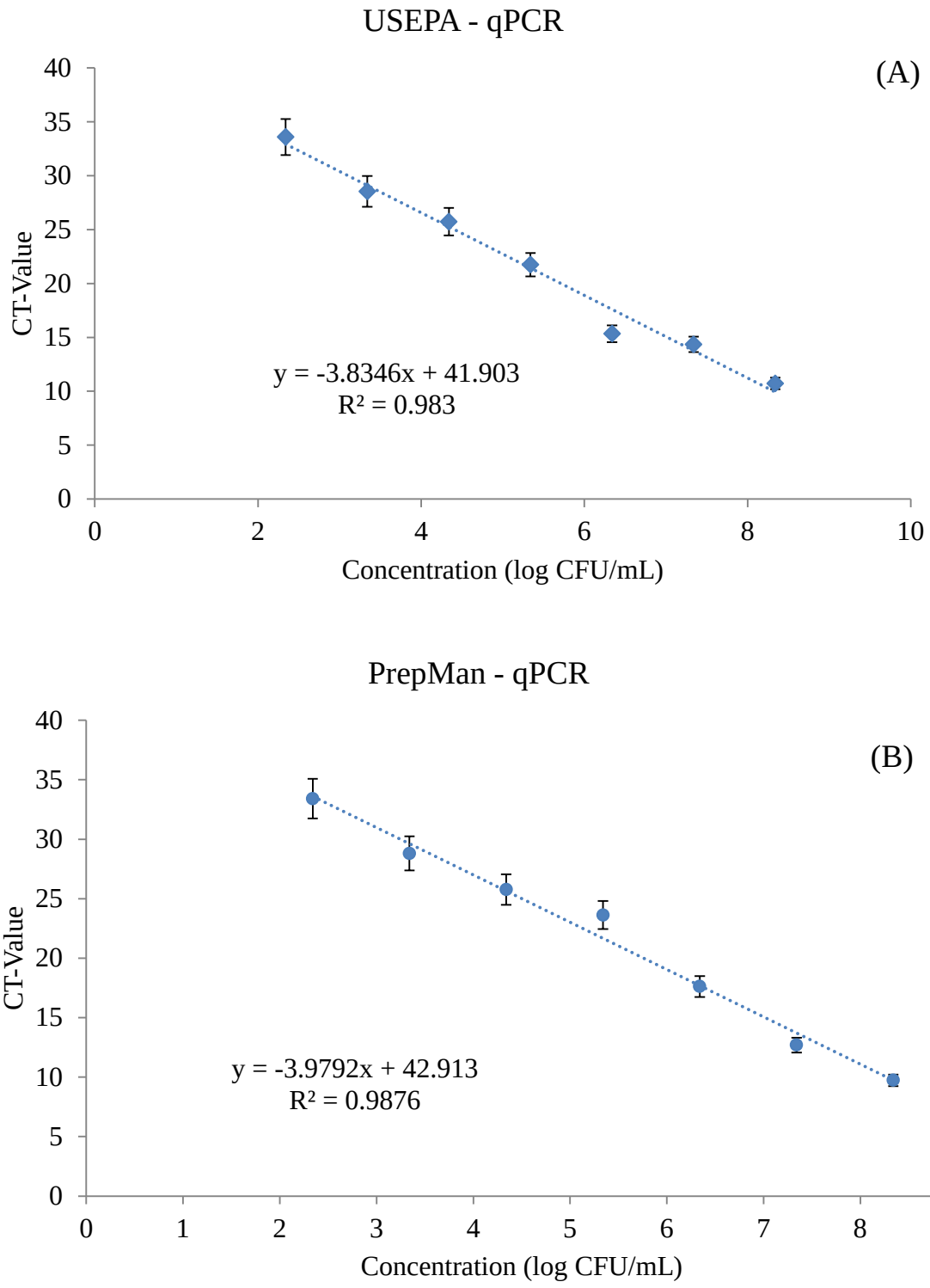

Figure 7. Standard curves of qPCR for water samples

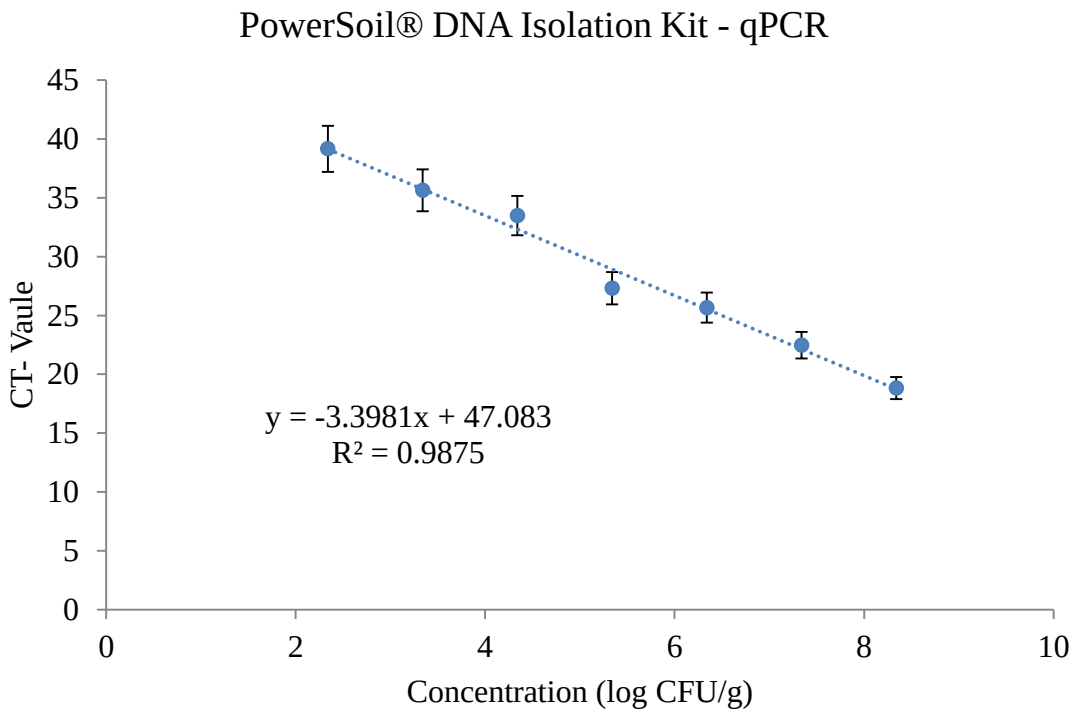

Figure 8. Standard curve of qPCR for sediment samples

CHAPTER 4. CONCLUSION

In recent years, water quality issues have caught more and more attention from people because they are important for maintaining human health. According to the AWW program, water monitoring includes chemistry monitoring (water pH, total hardness, total alkalinity, air temperature and water temperature, dissolved oxygen and turbidity), bacteriological monitoring and stream biomonitoring (AWW). When studying bacteriological monitoring, *Escherichia coli*, coliform, Enterobacteriaceae and *Enterococcus* may all serve as indicators of water quality monitoring.

When using *Escherichia coli* in these laboratory studies where fresh water samples were sterilized and inoculated artificially to compare the membrane filter/USEPA method 1603, Coliscan® Easygel agar plates and 3M™ Petrifilm™ methods, there were no significant differences among them. These methods can be used for field sampling, and can be chosen based on the field sampling requirements.

Using *Enterococcus* in these laboratory studies as in the *E.coli* study above to compare the mEI membrane filter/USEPA method 1600, the Enterolert® method, the Easygel cards™ method and the USEPA qPCR method 1611 has shown that there were differences among these methods based on the price, time expenditure, and enumeration range. Results showed that, when comparing the price, the cheapest method is the Easygel cards™ method while the most expensive method is the the mEI membrane filter/USEPA method 1600. When comparing the time expenditure, the USEPA qPCR method 1611 only takes around 4 hours to enumerate *Enterococcus*, while the other methods all need an overnight incubation period of at least 12 hours. Comparing the

methods by the enumeration range, Enterolert® has the narrowest range, while the USEPA qPCR method 1611 has the widest detection range. Based on the DNA extraction with the USEPA qPCR method 1611, the PrepMan® boiling procedure can substitute for the USEPA method 1611 procedure for extracting DNA samples from surface water, but it is not interchangeable with the PowerSoil® DNA Isolation Kit when extracting DNA from sediment samples.

When the time of day for field sampling of the swimming sites were compared, the sampling times (morning vs. afternoon) and sampling types (surface water vs. sediment) both impacted the bacterial concentrations significantly. Sediment has much higher bacterial concentrations than surface water and the high concentrations of indicator microorganisms in sediment may be one of the reasons for the differences seen in the indicator microorganism enumeration results of the surface water at different sampling times because of disturbances of sediment at different times of day.

By comparing the advantages and disadvantages of different indicator microorganism enumeration methods and thoroughly investigating the impact of sampling time and sample types generated on water monitoring results, solid information about the criteria for selecting specific indicator monitoring protocol was generated and all of this information will help with the future development of water monitoring plans for fresh water quality control.

IDEXX Quanti-Tray®/2000 MPN Table

(per100ml)

#Large Wells Positive	#Small																							
0	<1	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0	14.1	15.1	16.1	17.1	18.1	19.1	20.2	21.2	22.2	23.3	24.3
1	1.0	2.0	3.0	4.0	5.0	6.0	7.1	8.1	9.1	10.1	11.1	12.1	13.2	14.2	15.2	16.2	17.3	18.3	19.3	20.4	21.4	22.4	23.5	24.5	25.6
2	2.0	3.0	4.1	5.1	6.1	7.1	8.1	9.2	10.2	11.2	12.2	13.3	14.3	15.4	16.4	17.4	18.5	19.5	20.6	21.6	22.7	23.7	24.8	25.8	26.9
3	3.1	4.1	5.1	6.1	7.2	8.2	9.2	10.3	11.3	12.4	13.4	14.5	15.5	16.5	17.6	18.6	19.7	20.8	21.8	22.9	23.9	25.0	26.1	27.1	28.2
4	4.1	5.2	6.2	7.2	8.3	9.3	10.4	11.4	12.5	13.5	14.6	15.6	16.7	17.8	18.8	19.9	21.0	22.0	23.1	24.2	25.3	26.3	27.4	28.5	29.6
5	5.2	6.3	7.3	8.4	9.4	10.5	11.5	12.6	13.7	14.7	15.8	16.9	17.9	19.0	20.1	21.2	22.2	23.3	24.4	25.5	26.6	27.7	28.8	29.9	31.0
6	6.3	7.4	8.4	9.5	10.6	11.6	12.7	13.8	14.9	16.0	17.0	18.1	19.2	20.3	21.4	22.5	23.6	24.7	25.8	26.9	28.0	29.1	30.2	31.3	32.4
7	7.5	8.5	9.6	10.7	11.8	12.8	13.9	15.0	16.1	17.2	18.3	19.4	20.5	21.6	22.7	23.8	24.9	26.0	27.1	28.3	29.4	30.5	31.6	32.8	33.9
8	8.6	9.7	10.8	11.9	13.0	14.1	15.2	16.3	17.4	18.5	19.6	20.7	21.8	22.9	24.1	25.2	26.3	27.4	28.6	29.7	30.8	32.0	33.1	34.3	35.4
9	9.8	10.9	12.0	13.1	14.2	15.3	16.4	17.6	18.7	19.8	20.9	22.0	23.2	24.3	25.4	26.6	27.7	28.9	30.0	31.2	32.3	33.5	34.6	35.8	37.0
10	11.0	12.1	13.2	14.4	15.5	16.6	17.7	18.9	20.0	21.1	22.3	23.4	24.6	25.7	26.9	28.0	29.2	30.3	31.5	32.7	33.8	35.0	36.2	37.4	38.6
11	12.2	13.4	14.5	15.6	16.8	17.9	19.1	20.2	21.4	22.5	23.7	24.8	26.0	27.2	28.3	29.5	30.7	31.9	33.0	34.2	35.4	36.6	37.8	39.0	40.2
12	13.5	14.6	15.8	16.9	18.1	19.3	20.4	21.6	22.8	23.9	25.1	26.3	27.5	28.6	29.8	31.0	32.2	33.4	34.6	35.8	37.0	38.2	39.5	40.7	41.9
13	14.8	16.0	17.1	18.3	19.5	20.6	21.8	23.0	24.2	25.4	26.6	27.8	29.0	30.2	31.4	32.6	33.8	35.0	36.2	37.5	38.7	39.9	41.2	42.4	43.6
14	16.1	17.3	18.5	19.7	20.9	22.1	23.3	24.5	25.7	26.9	28.1	29.3	30.5	31.7	33.0	34.2	35.4	36.7	37.9	39.1	40.4	41.6	42.9	44.2	45.4
15	17.5	18.7	19.9	21.1	22.3	23.5	24.7	25.9	27.2	28.4	29.6	30.9	32.1	33.3	34.6	35.8	37.1	38.4	39.6	40.9	42.2	43.4	44.7	46.0	47.3
16	18.9	20.1	21.3	22.6	23.8	25.0	26.2	27.5	28.7	30.0	31.2	32.5	33.7	35.0	36.3	37.5	38.8	40.1	41.4	42.7	44.0	45.3	46.6	47.9	49.2
17	20.3	21.6	22.8	24.1	25.3	26.6	27.8	29.1	30.3	31.6	32.9	34.1	35.4	36.7	38.0	39.3	40.6	41.9	43.2	44.5	45.9	47.2	48.5	49.8	51.2
18	21.8	23.1	24.3	25.6	26.9	28.1	29.4	30.7	32.0	33.3	34.6	35.9	37.2	38.5	39.8	41.1	42.4	43.8	45.1	46.5	47.8	49.2	50.5	51.9	53.2
19	23.3	24.6	25.9	27.2	28.5	29.8	31.1	32.4	33.7	35.0	36.3	37.6	39.0	40.3	41.6	43.0	44.3	45.7	47.1	48.4	49.8	51.2	52.6	54.0	55.4
20	24.9	26.2	27.5	28.8	30.1	31.5	32.8	34.1	35.4	36.8	38.1	39.5	40.8	42.2	43.6	44.9	46.3	47.7	49.1	50.5	51.9	53.3	54.7	56.1	57.6
21	26.5	27.9	29.2	30.5	31.8	33.2	34.5	35.9	37.3	38.6	40.0	41.4	42.8	44.1	45.5	46.9	48.4	49.8	51.2	52.6	54.1	55.5	56.9	58.4	59.9
22	28.2	29.5	30.9	32.3	33.6	35.0	36.4	37.7	39.1	40.5	41.9	43.3	44.8	46.2	47.6	49.0	50.5	51.9	53.4	54.8	56.3	57.8	59.3	60.8	62.3
23	29.9	31.3	32.7	34.1	35.5	36.8	38.3	39.7	41.1	42.5	43.9	45.4	46.8	48.3	49.7	51.2	52.7	54.2	55.6	57.1	58.6	60.2	61.7	63.2	64.7
24	31.7	33.1	34.5	35.9	37.3	38.8	40.2	41.7	43.1	44.6	46.0	47.5	49.0	50.5	52.0	53.5	55.0	56.5	58.0	59.5	61.1	62.6	64.2	65.8	67.3
25	33.6	35.0	36.4	37.9	39.3	40.8	42.2	43.7	45.2	46.7	48.2	49.7	51.2	52.7	54.3	55.8	57.3	58.9	60.5	62.0	63.6	65.2	66.8	68.4	70.0
26	35.5	36.9	38.4	39.9	41.4	42.8	44.3	45.9	47.4	48.9	50.4	52.0	53.5	55.1	56.7	58.2	59.8	61.4	63.0	64.7	66.3	67.9	69.6	71.2	72.9
27	37.4	38.9	40.4	42.0	43.5	45.0	46.5	48.1	49.6	51.2	52.8	54.4	56.0	57.6	59.2	60.8	62.4	64.1	65.7	67.4	69.1	70.8	72.5	74.2	75.9
28	39.5	41.0	42.6	44.1	45.7	47.3	48.8	50.4	52.0	53.6	55.2	56.9	58.5	60.2	61.8	63.5	65.2	66.9	68.6	70.3	72.0	73.7	75.5	77.3	79.0
29	41.7	43.2	44.8	46.4	48.0	49.6	51.2	52.8	54.5	56.1	57.8	59.5	61.2	62.9	64.6	66.3	68.0	69.8	71.5	73.3	75.1	76.9	78.7	80.5	82.4
30	43.9	45.5	47.1	48.7	50.4	52.0	53.7	55.4	57.1	58.8	60.5	62.2	64.0	65.7	67.5	69.3	71.0	72.9	74.7	76.5	78.3	80.2	82.1	84.0	85.9
31	46.2	47.9	49.5	51.2	52.9	54.6	56.3	58.1	59.8	61.6	63.3	65.1	66.9	68.7	70.5	72.4	74.2	76.1	78.0	79.9	81.8	83.7	85.7	87.6	89.6
32	48.7	50.4	52.1	53.8	55.6	57.3	59.1	60.9	62.7	64.5	66.3	68.2	70.0	71.9	73.8	75.7	77.6	79.5	81.5	83.5	85.4	87.5	89.5	91.5	93.6
33	51.2	53.0	54.8	56.5	58.3	60.2	62.0	63.8	65.7	67.6	69.5	71.4	73.3	75.2	77.2	79.2	81.2	83.2	85.2	87.3	89.3	91.4	93.6	95.7	97.8
34	53.9	55.7	57.6	59.4	61.3	63.1	65.0	67.0	68.9	70.8	72.8	74.8	76.8	78.8	80.8	82.9	85.0	87.1	89.2	91.4	93.5	95.7	97.9	100.2	102.4
35	56.8	58.6	60.5	62.4	64.4	66.3	68.3	70.3	72.3	74.3	76.3	78.4	80.5	82.6	84.7	86.9	89.1	91.3	93.5	95.7	98.0	100.3	102.6	105.0	107.3
36	59.8	61.7	63.7	65.7	67.7	69.7	71.7	73.8	75.9	78.0	80.1	82.3	84.5	86.7	88.9	91.2	93.5	95.8	98.1	100.5	102.9	105.3	107.7	110.2	112.7
37	62.9	65.0	67.0	69.1	71.2	73.3	75.4	77.6	79.8	82.0	84.2	86.5	88.8	91.1	93.4	95.8	98.2	100.6	103.1	105.6	108.1	110.7	113.3	115.9	118.6
38	66.3	68.4	70.6	72.7	74.9	77.1	79.4	81.6	83.9	86.2	88.6	91.0	93.4	95.8	98.3	100.8	103.4	105.9	108.6	111.2	113.9	116.6	119.4	122.2	125.0
39	70.0	72.2	74.4	76.7	78.9	81.3	83.6	86.0	88.4	90.9	93.4	95.9	98.4	101.0	103.6	106.3	109.0	111.8	114.6	117.4	120.3	123.2	126.1	129.2	132.2
40	73.8	76.2	78.5	80.9	83.3	85.7	88.2	90.8	93.3	95.9	98.5	101.2	103.9	106.7	109.5	112.4	115.3	118.2	121.2	124.3	127.4	130.5	133.7	137.0	140.3
41	78.0	80.5	83.0	85.5	88.0	90.6	93.3	95.9	98.7	101.4	104.3	107.1	110.0	113.0	116.0	119.1	122.2	125.4	128.7	132.0	135.4	138.8	142.3	145.9	149.5
42	82.6	85.2	87.8	90.5	93.2	96.0	98.8	101.7	104.6	107.6	110.6	113.7	116.9	120.1	123.4	126.7	130.1	133.6	137.2	140.8	144.5	148.3	152.2	156.1	160.2
43	87.6	90.4	93.2	96.0	99.0	101.9	105.0	108.1	111.2	114.5	117.8	121.1	124.6	128.1	131.7	135.4	139.1	143.0	147.0	151.0	155.2	159.4	163.8	168.2	172.8
44	93.1	96.1	99.1	102.2	105.4	108.6	111.9	115.3	118.7	122.3	125.9	129.6	133.4	137.4	141.4	145.5	149.7	154.1	158.5	163.1	167.9	172.7	177.7	182.9	188.2
45	99.3	102.5	105.8	109.2	112.6	116.2	119.8	123.6	127.4	131.4	135.4	139.6	143.9	148.3	152.9	157.6	162.4	167.4	172.6	178.0	183.5	189.2	195.1	201.2	207.5
46	106.3	109.8	113.4	117.2	121.0	125.0	129.1	133.3	137.6	142.1	146.7	151.5	156.5	161.6	167.0	172.5	178.2	184.2	190.4	196.8	203.5	210.5	217.8	225.4	233.3
47	114.3	118.3	122.4	126.6	130.9	135.4	140.1	145.0	150.0	155.3	160.7	166.4	172.3	178.5	185.0	191.8	198.9	206.4	214.2	222.4	231.0	240.0	249.5	259.5	270.0
48	123.9	128.4	133.1	137.9	143.0	148.3	153.9	159.7	165.8	172.2	178.9	186.0	193.5	201.4	209.8	218.7	228.2	238.2	248.9	260.3	272.3	285.1	298.7	313.0	328.2
49	135.5	140.8	146.4	152.3	158.5	165.0	172.0	179.3	187.2	195.6	204.6	214.3	224.7	235.9	248.1	261.3	275.5	290.9	307.6	325.5	344.8	365.4	387.3	410.6	435.2

IDEXX Quanti-Tray®/2000 MPN Table

(per100ml)

#Large Wells Positive	#Small																							
0	25.3	26.4	27.4	28.4	29.5	30.5	31.5	32.6	33.6	34.7	35.7	36.8	37.8	38.9	40.0	41.0	42.1	43.1	44.2	45.3	46.3	47.4	48.5	49.5
1	26.6	27.7	28.7	29.8	30.8	31.9	32.9	34.0	35.0	36.1	37.2	38.2	39.3	40.4	41.4	42.5	43.6	44.7	45.7	46.8	47.9	49.0	50.1	51.2
2	27.9	29.0	30.0	31.1	32.2	33.2	34.3	35.4	36.5	37.5	38.6	39.7	40.8	41.9	43.0	44.0	45.1	46.2	47.3	48.4	49.5	50.6	51.7	52.8
3	29.3	30.4	31.4	32.5	33.6	34.7	35.8	36.8	37.9	39.0	40.1	41.2	42.3	43.4	44.5	45.6	46.7	47.8	48.9	50.0	51.2	52.3	53.4	54.5
4	30.7	31.8	32.8	33.9	35.0	36.1	37.2	38.3	39.4	40.5	41.6	42.8	43.9	45.0	46.1	47.2	48.3	49.5	50.6	51.7	52.9	54.0	55.1	56.3
5	32.1	33.2	34.3	35.4	36.5	37.6	38.7	39.9	41.0	42.1	43.2	44.4	45.5	46.6	47.7	48.9	50.0	51.2	52.3	53.5	54.6	55.8	56.9	58.1
6	33.5	34.7	35.8	36.9	38.0	39.2	40.3	41.4	42.6	43.7	44.8	46.0	47.1	48.3	49.4	50.6	51.7	52.9	54.1	55.2	56.4	57.6	58.7	59.9
7	35.0	36.2	37.3	38.4	39.6	40.7	41.9	43.0	44.2	45.3	46.5	47.7	48.8	50.0	51.2	52.3	53.5	54.7	55.9	57.1	58.3	59.4	60.6	61.8
8	36.6	37.7	38.9	40.0	41.2	42.3	43.5	44.7	45.9	47.0	48.2	49.4	50.6	51.8	53.0	54.1	55.3	56.5	57.7	59.0	60.2	61.4	62.6	63.8
9	38.1	39.3	40.5	41.6	42.8	44.0	45.2	46.4	47.6	48.8	50.0	51.2	52.4	53.6	54.8	56.0	57.2	58.4	59.7	60.9	62.1	63.4	64.6	65.8
10	39.7	40.9	42.1	43.3	44.5	45.7	46.9	48.1	49.3	50.6	51.8	53.0	54.2	55.5	56.7	57.9	59.2	60.4	61.7	62.9	64.2	65.4	66.7	67.9
11	41.4	42.6	43.8	45.0	46.3	47.5	48.7	49.9	51.2	52.4	53.7	54.9	56.1	57.4	58.6	59.9	61.2	62.4	63.7	65.0	66.3	67.5	68.8	70.1
12	43.1	44.3	45.6	46.8	48.1	49.3	50.6	51.8	53.1	54.3	55.6	56.8	58.1	59.4	60.7	62.0	63.2	64.5	65.8	67.1	68.4	69.7	71.0	72.4
13	44.9	46.1	47.4	48.6	49.9	51.2	52.5	53.7	55.0	56.3	57.6	58.9	60.2	61.5	62.8	64.1	65.4	66.7	68.0	69.3	70.7	72.0	73.3	74.7
14	46.7	48.0	49.3	50.5	51.8	53.1	54.4	55.7	57.0	58.3	59.6	60.9	62.3	63.6	64.9	66.3	67.6	68.9	70.3	71.6	73.0	74.4	75.7	77.1
15	48.6	49.9	51.2	52.5	53.8	55.1	56.4	57.8	59.1	60.4	61.8	63.1	64.5	65.8	67.2	68.5	69.9	71.3	72.6	74.0	75.4	76.8	78.2	79.6
16	50.5	51.8	53.2	54.5	55.8	57.2	58.5	59.9	61.2	62.6	64.0	65.3	66.7	68.1	69.5	70.9	72.3	73.7	75.1	76.5	77.9	79.3	80.8	82.2
17	52.5	53.9	55.2	56.6	58.0	59.3	60.7	62.1	63.5	64.9	66.3	67.7	69.1	70.5	71.9	73.3	74.8	76.2	77.6	79.1	80.5	82.0	83.5	84.9
18	54.6	56.0	57.4	58.8	60.2	61.6	63.0	64.4	65.8	67.2	68.6	70.1	71.5	73.0	74.4	75.9	77.3	78.8	80.3	81.8	83.3	84.8	86.3	87.8
19	56.8	58.2	59.6	61.0	62.4	63.9	65.3	66.8	68.2	69.7	71.1	72.6	74.1	75.5	77.0	78.5	80.0	81.5	83.1	84.6	86.1	87.6	89.2	90.7
20	59.0	60.4	61.9	63.3	64.8	66.3	67.7	69.2	70.7	72.2	73.7	75.2	76.7	78.2	79.8	81.3	82.8	84.4	85.9	87.5	89.1	90.7	92.2	93.8
21	61.3	62.8	64.3	65.8	67.3	68.8	70.3	71.8	73.3	74.9	76.4	77.9	79.5	81.1	82.6	84.2	85.8	87.4	89.0	90.6	92.2	93.8	95.4	97.1
22	63.8	65.3	66.8	68.3	69.8	71.4	72.9	74.5	76.1	77.6	79.2	80.8	82.4	84.0	85.6	87.2	88.9	90.5	92.1	93.8	95.5	97.1	98.8	100.5
23	66.3	67.8	69.4	71.0	72.5	74.1	75.7	77.3	78.9	80.5	82.2	83.8	85.4	87.1	88.7	90.4	92.1	93.8	95.5	97.2	98.9	100.6	102.4	104.1
24	68.9	70.5	72.1	73.7	75.3	77.0	78.6	80.3	81.9	83.6	85.2	86.9	88.6	90.3	92.0	93.8	95.5	97.2	99.0	100.7	102.5	104.3	106.1	107.9
25	71.7	73.3	75.0	76.6	78.3	80.0	81.7	83.3	85.1	86.8	88.5	90.2	92.0	93.7	95.5	97.3	99.1	100.9	102.7	104.5	106.3	108.2	110.0	111.9
26	74.6	76.3	78.0	79.7	81.4	83.1	84.8	86.6	88.4	90.1	91.9	93.7	95.5	97.3	99.2	101.0	102.9	104.7	106.6	108.5	110.4	112.3	114.2	116.2
27	77.6	79.4	81.1	82.9	84.6	86.4	88.2	90.0	91.9	93.7	95.5	97.4	99.3	101.2	103.1	105.0	106.9	108.8	110.8	112.7	114.7	116.7	118.7	120.7
28	80.8	82.6	84.4	86.3	88.1	89.9	91.8	93.7	95.6	97.5	99.4	101.3	103.3	105.2	107.2	109.2	111.2	113.2	115.2	117.3	119.3	121.4	123.5	125.6
29	84.2	86.1	87.9	89.8	91.7	93.7	95.6	97.5	99.5	101.5	103.5	105.5	107.5	109.5	111.6	113.7	115.7	117.8	120.0	122.1	124.2	126.4	128.6	130.8
30	87.8	89.7	91.7	93.6	95.6	97.6	99.6	101.6	103.7	105.7	107.8	109.9	112.0	114.2	116.3	118.5	120.6	122.8	125.1	127.3	129.5	131.8	134.1	136.4
31	91.6	93.6	95.6	97.7	99.7	101.8	103.9	106.0	108.2	110.3	112.5	114.7	116.9	119.1	121.4	123.6	125.9	128.2	130.5	132.9	135.3	137.7	140.1	142.5
32	95.7	97.8	99.9	102.0	104.2	106.3	108.5	110.7	113.0	115.2	117.5	119.8	122.1	124.5	126.8	129.2	131.6	134.0	136.5	139.0	141.5	144.0	146.6	149.1
33	100.0	102.2	104.4	106.6	108.9	111.2	113.5	115.8	118.2	120.5	122.9	125.4	127.8	130.3	132.8	135.3	137.8	140.4	143.0	145.6	148.3	150.9	153.7	156.4
34	104.7	107.0	109.3	111.7	114.0	116.4	118.9	121.3	123.8	126.3	128.8	131.4	134.0	136.6	139.2	141.9	144.6	147.4	150.1	152.9	155.7	158.6	161.5	164.4
35	109.7	112.2	114.6	117.1	119.6	122.2	124.7	127.3	129.9	132.6	135.3	138.0	140.8	143.6	146.4	149.2	152.1	155.0	158.0	161.0	164.0	167.1	170.2	173.3
36	115.2	117.8	120.4	123.0	125.7	128.4	131.1	133.9	136.7	139.5	142.4	145.3	148.3	151.3	154.3	157.3	160.5	163.6	166.8	170.0	173.3	176.6	179.9	183.3
37	121.3	124.0	126.8	129.6	132.4	135.3	138.2	141.2	144.2	147.3	150.3	153.5	156.7	159.9	163.1	166.5	169.8	173.2	176.7	180.2	183.7	187.3	191.0	194.7
38	127.9	130.8	133.8	136.8	139.9	143.0	146.2	149.4	152.6	155.9	159.2	162.6	166.1	169.6	173.2	176.8	180.4	184.2	188.0	191.8	195.7	199.7	203.7	207.7
39	135.3	138.5	141.7	145.0	148.3	151.7	155.1	158.6	162.1	165.7	169.4	173.1	176.9	180.7	184.7	188.7	192.7	196.8	201.0	205.3	209.6	214.0	218.5	223.0
40	143.7	147.1	150.6	154.2	157.8	161.5	165.3	169.1	173.0	177.0	181.1	185.2	189.4	193.7	198.1	202.5	207.1	211.7	216.4	221.1	226.0	231.0	236.0	241.1
41	153.2	157.0	160.9	164.8	168.9	173.0	177.2	181.5	185.8	190.3	194.8	199.5	204.2	209.1	214.0	219.1	224.2	229.4	234.8	240.2	245.8	251.5	257.2	263.1
42	164.3	168.6	172.9	177.3	181.9	186.5	191.3	196.1	201.1	206.2	211.4	216.7	222.2	227.7	233.4	239.2	245.2	251.3	257.5	263.8	270.3	276.9	283.6	290.5
43	177.5	182.3	187.3	192.4	197.6	202.9	208.4	214.0	219.8	225.8	231.8	238.1	244.5	251.0	257.7	264.6	271.7	278.9	286.3	293.8	301.5	309.4	317.4	325.7
44	193.6	199.3	205.1	211.0	217.2	223.5	230.0	236.7	243.6	250.8	258.1	265.6	273.3	281.2	289.4	297.8	306.3	315.1	324.1	333.3	342.8	352.4	362.3	372.4
45	214.1	220.9	227.9	235.2	242.7	250.4	258.4	266.7	275.3	284.1	293.3	302.6	312.3	322.3	332.5	343.0	353.8	364.9	376.2	387.9	399.8	412.0	424.5	437.4
46	241.5	250.0	258.9	268.2	277.8	287.8	298.1	308.8	319.9	331.4	343.3	355.5	368.1	381.1	394.5	408.3	422.5	437.1	452.0	467.4	483.3	499.6	516.3	533.5
47	280.9	292.4	304.4	316.9	330.0	343.6	357.8	372.5	387.7	403.4	419.8	436.6	454.1	472.1	490.7	509.9	529.8	550.4	571.7	593.8	616.7	640.5	665.3	691.0
48	344.1	360.9	378.4	396.8	416.0	436.0	456.9	478.6	501.2	524.7	549.3	574.8	601.5	629.4	658.6	689.3	721.5	755.6	791.5	829.7	870.4	913.9	960.6	1011.2
49	461.1	488.4	517.2	547.5	579.4	613.1	648.8	686.7	727.0	770.1	816.4	866.4	920.8	980.4	1046.2	1119.9	1203.3	1299.7	1413.6	1553.1	1732.9	1986.3	2419.6	>2419.6

09-63235-01