DETECTION OF VIABLE *ESCHERICHIA COLI* IN ENVIRONMENTAL WATER USING A COMBINED PROPIDIUM MONOAZIDE STAINING-REAL-TIME PCR

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COMBINED PROPIDIUM MONOAZIDE STAINING-REAL-TIME PCR

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DETECTION OF VIABLE ESCHERICHIA COLI IN ENVIRONMENTAL WATER USING A COMBINED PROPIDIUM MONOAZIDE STAINING-REAL-TIME PCR

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ABSTRACT

Escherichia coli, as a principal fecal indicator bacterium, is used to monitor water quality world-wide. Real-time PCR (qPCR) is a promising way to achieve a rapid and sensitive detection of *E. coli* in water samples. The ability to detect only viable *E. coli* cells specifically provides a more accurate reflection of water quality and safety. The objective of this study was to test a new self-designed primer set targeting the *ycj*M gene of *E. coli* in a propidium monoazide (PMA)-qPCR assay, and to investigate its specificity and efficiency in detecting only viable *E. coli* in environmental water.

Specificity of the *ycj*M primer set was checked using nineteen different *E. coli* strains, including *E. coli* ATCC 25922, *E. coli* K12, *E. coli* O157 strains and strains from environmental sources, as well as *Shigella. dysenteriae*, *S. flexneri* and *S. sonnei*. It showed that *ycj*M primers could detect most of the *E. coli* strains but did not amplify any DNA from *Shigella* strains.

Four cultures of *E. coli* Ct 14, Dr 23, H 24, P 24, freshly grown separately, and then mixed together and serially diluted to 10^2-10^6 CFU/mL. Dead cell suspensions were obtained by heating at 80 °C for 20 min. After being spiked with the cell suspension, tap water and other environmental water samples, including water from Lake of the Ozarks, Missouri River and Mississippi River were filtered or centrifuged for the cell collection. Samples were then treated with PMA, followed by DNA isolation and TaqMan® real-time PCR detection.

Results showed that in pure culture, 5 μM PMA with a 10-min light exposure was efficient to inhibit the amplification of DNA from 10^5 CFU /mL dead *E. coli* cells, with a detection limit of 10^2 CFU/100mL. In tap and winter environmental water, a higher PMA concentration of 10 μM was required and as low as 10^3 CFU/100 mL viable cells could be detected, in the presence of 10^5 CFU/100 mL dead cells; in water samples collected in summer, 10^2 CFU/10 mL viable cells could be detected after a 20 μM PMA treatment, in the presence of 10^4 CFU/10 mL dead cell by this optimized PMA-qPCR assay. Significant and strong correlations were found between the PMA-qPCR and EPA Standard Method 1603, without over or underestimation of PMA-qPCR compared with Method 1603.

In conclusion, the PMA-qPCR could accurately and effectively differentiate viable *E. coli* cells from dead cells by suppressing the amplification of DNA from dead cells, but optimization steps to remove suspended solids in environmental water samples were necessary.

CHAPTER 1

INTRODUCTION

According to the World Health Organization (WHO), 1.8 billion people around the world use feces-contaminated water as drinking water (WHO 2014). Various pathogens like *Cryptosporidium*, *Campylobacter*, *Giardia*, pathogenic *E. coli*, *Shigella*, *Salmonella*, Norwalk virus, and rotavirus (Hrudey and Hrudey 2007) can get into human bodies through the fecal-oral route through water, and cause illnesses or symptoms such as: gastroenteritis, dermatitis, primary amebic meningoencephalitis, and typhoid fever (Craun and others 2005). Instead of direct testing a large variety of pathogens, which is difficult, pricey, and time-consuming, the United States Environmental Protection Agency (EPA) detects the presence of the fecal indicator bacteria (FIB) to monitor fecal contamination in water. Since they are commonly found in human and animal feces, their presence may suggest the existence of pathogenic microorganisms in streams and the possibility of health risk to human bodies (EPA 1986).

As a member of FIB, a significantly direct relationship between *E. coli* and the risk of gastrointestinal illness associated with swimming in recreational fresh water was found (EPA 2009). It is the best indicator of the microbial quality of recreational waters among all FIB (EPA), and it can be also applied on the monitoring of drinking water and food (WHO 1997; Tsen and others 1998; Wade and others 2003; Tantawiwat and others

2005; EPA 2009). Although several serotypes of *E. coli* are pathogenic (like: *E. coli* O157:H7), most of them are not (Chetta and others 2012).

It may be the strict methodological regulations and guidelines lead EPA only has approved culture-based methods for *E. coli* for routine water quality monitoring so far. However, due to their long incubation time (usually 24 h) and high dependence on bacterial metabolism (Frahm and Obst 2003), more rapid and accurate detection methods are urgently needed.

Real-time PCR is one of the molecular technologies, which can quantitatively detect the target DNA and indicate its amount by accumulating fluorescence intensity in real time. Compared with traditional culture-based methods, real-time PCR usually only needs 2 h for detection. And since it directly detects the target gene, the accuracy and specificity are generally guaranteed.

However, one major shortage of this method is that it cannot distinguish viable cells from the dead. To solve this problem, the viability dyes: propidium monoazide (PMA) and ethidium monoazide bromide (EMA) can be applied to real-time PCR assays to achieve specific monitoring of viable *E. coli* (Gensberger and others 2014). Based on the principle that only viable cell can keep the integrity of the cell membrane, the two DNA intercalation dyes can selectively bind with free DNA and DNA from dead cell whose membrane is compromised. While the signal of viable cells, including viable but not culturable (VBNC) cells will not be effect too much in certain dye concentration ranges (Fittipaldi and others 2012). Compared with EMA, PMA shows better

differentiation between viable and dead cells, and less toxic effect on live cells. Therefore, it may be a better choice when dealing with environmental samples, which commonly have complex matrices but also mixed bacterial species.

It was believed that *E. coli* could only transiently sustain its life after released through fecal droppings from its primary habitat to the external environment. While recently, "naturalized" *E. coli*, which can tolerate and maintain their populations for a long time outside their hosts, had been found in soils (tropical, temperate and frozen temperate soils), beach sands, lake and river watersheds, and aquatic periphyton communities (Ishii and others 2010; Byappanahalli and others 2012). This means even though *E. coli* cells were detected from water or food, they may not (at least all of them) come from the feces of human or warm-blood animals. Therefore, the existence of "naturalized" *E. coli* challenges the current water monitoring programs around the world. (Walk and others 2007, 2009). Lately, a new set of primers was developed to target the putative glucosyltransferase gene (*ycj*M) of *E. coli*. It was proved enteric-specific and could differentiate between "naturalized" *E. coli* strains and *E. coli* stains isolated from animal feces (Deng and others 2014).

Therefore, the objective of this research was to use PMA-real-time PCR by targeting *ycj*M gene for detection and estimation of only viable, non-"naturalized" *E. coli* in environmental waters.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Microbial contamination of water

2.1.1 Waterborne outbreaks

According to the World Health Organization (WHO), 1.8 billion people around the world use feces-contaminated water as drinking water (WHO 2014). Microbial contamination of drinking water may happen worldwide in both developing and developed countries, but its situation is vague due to the limited surveillance systems in place. Till 2003, only the United States and the United Kingdom had good quality disease surveillance and could reliably report outbreaks associated with waterborne diseases (Hunter and others 2003).

In the United States, between 2005 and 2010, a total of 293 recreational water-associated outbreaks were reported in the US nationwide, which sickened 19,701 people. Out of these, 178 people were sent to the hospital and 5 died. In recreational water (lakes, creeks and ponds), illnesses could be caused by parasites, bacteria, viruses, or toxin, which included acute gastrointestinal illness (AGI, more than 80% in all cases), dermatologic illnesses, acute respiratory illness and others. AGI was particularly associated with *Cryptosporidium* in treated recreational water (pools and hot tubs or spas) in most situations (CDC, 2008, 2011, 2014). While for drinking water, less outbreaks (89

times), but more illnesses (5780 cases) and a higher rate of death (16 cases) were stated. Etiologic agents, such as bacteria, viruses and parasites, and especially *Legionella* and *Campylobacter*, could trigger acute respiratory illness, acute gastrointestinal illness or hepatitis (CDC, 2008, 2011, 2013).

In Missouri, three community-wide waterborne outbreaks were reported since 1989. From December 15, 1989 to January 20, 1990, the first waterborne *Escherichia coli* (*E. coli*) O157:H7 infection in Missouri caused 243 people in Cabool to come down with bloody or non-bloody diarrhea, abdominal cramps or hemolytic uremic syndrome, and 4 people died. Untreated municipal water was determined to be the source of this largest *E. coli* O157:H7 outbreak in Missouri (Swerdlow and others 1992). Another outbreak associated with drinking water was witnessed in 1993 in Gideon, which included 650 cases, 15 hospitalizations and 7 deaths. This outbreak was due to *Salmonella* Typhimurium contamination of unboiled water (Angulo and others 1997). In 2005, an outbreak of cryptosporidiosis caused 56 children to come down with bloody or non-bloody diarrhea, abdominal cramps, fatigue, nausea, vomiting, fever, chills and body aches after eating at a swimming pool. This indicated that not only drinking water, but recreational water could be a vehicle for disease transmission (Turabelidze and others 2007).

2.1. 2 *E. coli* as an indicator for waterborne diseases

The illnesses or symptoms of waterborne diseases include gastroenteritis, dermatitis, primary amebic meningoencephalitis, and typhoid fever (Craun and others 2005). They can be caused by various pathogens, like *Cryptosporidium*, *Campylobacter*, *Giardia*, pathogenic *E. coli*, *Shigella*, *Salmonella*, Norwalk virus, and rotavirus (Hrudey and Hrudey 2007). These pathogens use water as a vehicle of transmission to get into human bodies through the fecal-oral route. Therefore, an elevated level of fecal pollution that may be caused by increasing human population density due to fast urban growth, or a cumulative animal density due to intensive agricultural operations, can ultimately lead to an increase of waterborne diseases (Foulds and others 2002). Extreme weather, wastewater contamination, inadequate water filtration or poor sanitation during water treatment， are also associated with waterborne outbreaks (Hrudey and Hrudey 2007). Since directly testing a large variety of pathogens will be difficult, pricey, and time-consuming, the United States Environmental Protection Agency (EPA) uses fecal indicator bacteria (FIB) to monitor fecal contamination in water (EPA 1986). Since they are commonly found in human and animal feces, their presence may suggest that pathogenic microorganisms also exist in streams and raise health risk. FIB consist of coliforms and fecal streptococci groups, including total coliforms, fecal coliforms, *E. coli*, fecal streptococci, and enterococci. Among all FIB, *E. coli* is the best indicator of recreational waters monitoring (EPA).

2.2 Characteristics of *E. coli*

Escherichia coli (*E. coli*), which belongs to the Enterobacteriaceae family, is a Gram-negative, facultatively anaerobic, rod-shaped and non-spore-forming bacterium. Among eight different strains and 171 serotypes, some *E. coli* are pathogens, including enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), adherent and invasive *E. coli* (AIEC), and uropathogenic *E. coli* (UPEC). They can cause gastroenteritis, urinary tract infections, sepsis, neonatal meningitis or other diseases in healthy humans (Kalita and others 2014). However, most *E. coli* strains are not pathogenic, although they may act as opportunistic pathogens and cause infections in immune-compromised hosts (Chetta and others 2012).

As a member of the fecal coliforms, *E. coli* is a common inhabitant of the intestinal tract of humans and warm-blooded animals. It is found abundantly in feces, and thus, can indicate sewage pollution and the possible presence of enteric pathogens in drinking water, recreational water and food (Tantawiwat and others 2005; Tsen and others 1998; Wade and others 2003) (EPA 2009, WHO 1997). As a fecal indicator bacterium (FIB), a significantly direct relationship between *E. coli* and the risk of gastrointestinal illness associated with swimming in recreational fresh water was found (EPA 2009).

2.3 Detection methods of *E. coli* in water

The quality of recreational and drinking water has a direct relationship with human health. Therefore, to achieve a better water monitoring system, a series of detection methods for *E. coli* were developed. In 1986, EPA recommended a microbiological criteria for *E. coli* to indicate the quality of fresh recreational waters, which is ≤126/100 mL (EPA 1986). So far, EPA approves only culture-based methods for *E. coli* for routine water quality monitoring. This may be due to the strict methodological regulations and guidelines that are established by US federal, state and local governments (Craun and others 2005; Frahm and Obst 2003). Culture-based methods usually require more than 24 h to confirm *E. coli* contamination, which is time-consuming (EPA 2009). In addition, a high dependence on bacterial metabolism can lead to an inaccurate estimation of *E. coli* numbers (Frahm and Obst 2003). Consequently, alternative *E. coli* detection methods based on nucleic acids, biosensors and immunological agents were developed to achieve more rapid, accurate and multiple target detection capabilities. Moreover, using molecular technologies, one can realize accurate traceability of fecal contamination, which means a direct comparison of different sources of fecal emissions can be accomplished (Farnleitner and others 2000).

2.3.1 Cultured-based methods

The media used in culture-based methods can be solid or liquid, allowing the bacteria to grow and be counted. However, culture-based detection methods have their own limitations. First, their procedures require a relatively long time (usually more than 24 h) to confirm results. Moreover, injured *E. coli* cells in water that have entered the viable but non-culturable (VBNC) state may not be detectable, due to their low metabolic activity. In addition, most culture-based methods target β-galactosidase activity to detect total coliforms and β-glucuronidase activity to detect *E. coli*. However, false-negative results due to β- glucuronidase-negative *E. coli* phenotypes, and false positive results (EPA 2010) associated with putative *E. coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Aeromonas hydrophila*, and *Enterobacter* species (Khan and others 2007) were observed. Last, but not least, evaluation from different detection methods may vary, since each has dissimilar recovery abilities for *E. coli*. For example, MI (method 1604) and mTEC (method 1103.1) had similar assessment of recreational water, while modified mTEC (method 1603) and Colilert (method 9233 B) gave statistically different results from the mTEC method (Francy and Darner 2000). In another study, membrane filtration method (9222 G) had the lowest count, compared with the multiple tube fermentation method (Method 9221 F) and the Colilert method (Method 9223 B) (Noble and others 2003; Noble and others 2004).

Table 1 Culture-based methods for coliform and *E. coli* detection in water.

Method name	Reference	Required time	Target	Result
Method 9222 G: *E. coli* Membrane Filter Partition Procedure	(National Environmental Methods Index: NEMI a)	4 h (Nutrient agar MUG); or 24 ± 2 h (EC broth -MUG)	β-galactosidase for total coliform; β-glucuronidase for *E. coli*	4-Methylumbelliferyl-β-D-glucuronide (MUG) substrate will be hydrolyzed by *E. coli*, and blue fluorescence will be observed under ultraviolet light (366 nm).
Method 9221 F: *E. coli* Procedure with multiple-tube test for total coliform	(NEMI a)	48 ± 4 h	β-glucuronidase	MUG substrate will be hydrolyzed by *E. coli*, and blue fluorescence will be observed under ultraviolet light (366 nm).
Method 9223 B: Enzyme Substrate Test	(IDEXX Laboratories, Inc. 2015, NEMI b)	24 h ± 4 h	β-galactosidase for total coliform; β-glucuronidase for *E. coli*	The yellow color of hydrolyzed *ortho*-nitrophenyl-β-galactoside (ONPG) will indicate the existence of total coliform. MUG substrate will be hydrolyzed by *E. coli*, and

				blue fluorescence will be observed under ultraviolet light (366 nm).
Colitag™ Test	(CPI International, Inc. 2013)	16 - 48 h	β-galactosidase for total coliform; β-glucuronidase for *E. coli*	Hydrolyzed ONPG's yellow color will indicate the existence of total coliform. MUG substrate will be hydrolyzed by *E. coli*, and blue fluorescence will be observed under ultraviolet light (366 nm).
Modified Colitag™ Method	(Hach Company 2015 a)	16 - 48 h	β-galactosidase for total coliform; β-glucuronidase for *E. coli*	Hydrolyzed ONPG's yellow color will indicate the existence of total coliform. MUG substrate will be hydrolyzed by *E. coli*, and blue fluorescence will be observed under ultraviolet light (366 nm).
Method 1604: membrane filtration with MI agar	(EPA 2002 a)	24 h	β-galactosidase for total coliform; β-glucuronidase for *E. coli*	*E. coli* will be blue under ambient light, because of the hydrolyzed indoxyl-β-D-glucuronide (IBDG); and also blue/green fluorescent will be observed under longwave

				ultraviolet light (366 nm), due to the breakdown of MUG.
Method 1103.1: Membrane Filtration with mTEC	(EPA 2002 b)	24 h ± 2.5 h	Urease	*E. coli* will be able to grow at elevated temperatures, and will form yellow, yellow-green, or yellow-brown colonies, because *E. coli* cannot degrade urea substrate. Non-target colonies will show other colors, like pink.
Method 1603: Membrane Filtration with Modified mTEC	(EPA 2009)	24 h ± 2.5 h	β-glucuronidase	The red/magenta colonies will indicate *E. coli*, due to the catabolism of chromogen.
The E*Colite® Test	(Charm Sciences, Inc. 2009)	28 h - 48 h	chlorine stressed *E. coli*	X-gal indicator will show indigo blue in *E. coli* positive samples
The mColiBlue24 ® Test	(Hach Company	24 h	-	*E. coli* can be distinguished by blue colonies.

	2015 b)			
Readycult® Coliforms 100	(Merck KGaA Company 2007)	24 ± 1 h	β-galactosidase for total coliform; β-glucuronidase for *E. coli*	The color of medium will turn to blue-green from its original yellow, due to the hydrolysis of X-GAL. The hydrolysis of MUG will be indicated by bright blue fluorescence under long wave (366 nm) ultraviolet (UV) light.
Chromocult® Coliform Agar	(Merck KGaA Company)	24 h	-	Tergitol® 7 in media inhibits the growth of Gram-positive bacteria. *E. coli* will from blue to violet colonies, while all non-*E. coli* coliforms produced typical pink colonies.

2.3.2 Immunoassays

Rapid immunoassay technology utilizes specific antibodies to bind with the corresponding antigens of microorganisms. Target microorganisms that are separated from complex samples, can be distinguished by enzyme conjugation, fluorescence, DNA analysis and other techniques.

2.3.2.1 Immunomagnetic separation (IMS)

IMS uses antibody-coated magnetic polystyrene beads to separate out and collect target cells from a sample matrix. With the assistance of a magnetic field, a desired bacterial population will form a bead–bacteria complex (Lee and others 2010; Lee and Deininger 2004). After separation, downstream procedures, such as polymerase chain reaction (PCR), plate counting, flow cytometry (Allegra and others 2008) or ATP bioluminescence can be applied to achieve online monitoring in the food industry (Delbeke and others 2015; Wang and others 2014), in drinking water (Deininger and Lee 2001; Delahaye and others 2003), in recreational water (Bushon and others 2009a; Griffith and others 2009) and in wastewater (Bushon and others 2009b).

The IMS/ATP method utilizes ATP from ruptured cells to quantify bacterial numbers, because luciferin can emit light under a luciferase-catalyzed oxidation. For *E. coli* detection, significant linear relationships were found between the results from IMS/ATP method and that from traditional culture-based methods in both waste and

recreational waters (Bushon and others 2009a; Bushon and others 2009b). In IMS, antibodies could be covalently linked with magnetic beads (Cov-IMS ⁄ ATP) to form a stronger bind (Lee and others 2010), or they could be coupled with magnetic nanoparticles (MNPs) to avoid pore blocking from a matrix that might contain big aggregates (Pappert and others 2010). Rapid results were achieved in 1-4 h (Bushon and others 2009a; Lee and Deininger 2004; Pappert and others 2010), and only simple equipment and assays were required by this highly repeatable method. More importantly, it can only detect viable (including viable but not culturable) cells because ATP degrades rapidly in dead cells (Bushon and others 2009b). However, false positive results would be observed due to reactions between antibodies and non-target microorganisms (Bushon and others 2009b; Pappert and others 2010).

Laczka (2011) first developed an immunosensing system that combined IMS with microfluidic and biosensor technology. *E. coli* was captured and confined in a micro-chamber incubator (a microfluidic environment), and detected by electrochemical monitoring of the activity of horseradish peroxidase (HRP). This assay took 1 h for complete detection, and could detect as low as 55 *E. coli* cells mL^{-1} in phosphate buffered saline (PBS), and 100 cells mL^{-1} in milk.

Golberg (2014) invented an all-in-one platform, called ScanDrop, to collect, detect, and identify bacteria in drinking water. This platform consisted of droplet microfluidics, a portable imaging system, cloud-based control software and data storage.

In their assay, *E. coli* in water sample were captured and then co-encapsulated in picoliter droplets with fluorescence labeled anti-*E. coli* antibodies, and finally imaged with a fluorescence microscope. Results could be acquired, compared and shared by the ‘‘cloud’’ network, to achieve a real-time water quality monitoring. However, if the number of target cells were less than 10^6 cells mL^{-1}, the probability to find droplets with encapsulated cells would be reduced due to the increase in empty droplets. Therefore, at least 8 h was needed for the entire water quality diagnostic process if the initial concentration was 3.5 CFU mL^{-1}, and most of the time of this procedure was for sample incubation.

2.3.2.2 Enzyme-linked immunosorbent assay (ELISA)/ enzyme-linked fluorescent assay (ELFA)

ELISA and ELFA are classic examples of immunoassays. Molecules that can be either antigens or antibodies can capture microorganisms in water or food. Later, secondary antibodies will also recognize target microorganisms and form colored precipitate (ELISA) or, give off fluorescence (ELFA) (EPA 2005; Lopez-Roldan and others 2013). Limited researches used this technology to detect *E. coli* in water. Pappert and others (2010) developed a sandwich chemiluminescence ELISA with nanoparticle-based IMS, to detect *E. coli* in phosphate-buffered saline (PBS). However, the detection limit of this assay was too high (more than 10^5 cells mL^{-1}) to meet the requirement of

EPA, compared with culture-based methods. In addition, the antibodies used in this research were the antibodies that were common in all members of the Enterobacteriaceae family. Thus, this might lead to a high portion of false positive results.3.3 Biosensors

Biosensors are the tools that incorporate biological (or biological derived) components (enzymes, antigens/antibodies, nucleic acids, aptamers, tissues) and physicochemical (optical, electrochemical, thermometric or magnetic) transducers or transducing microsystems (Lazcka and others 2007).

2.3.3.1 Biological recognition and immobilization

Three main classes of biological recognition elements include antibodies, nucleic acids and enzymes. The recognition and immobilization steps of these bio-molecules are critical in the design of any types of biosensors, since they give the identity and provide the specificity of biosensors.

For nucleic acid recognition, Rodríguez and Alocilja (2005) embedded single-stranded DNA as a probe to specifically detect the *uid*A gene of *E. coli* K-12. This biosensor on a platinum (Pt) electrode which electropolimerized with polypyrrole (PPY) was combined with the biochemical cyclic voltammetric technique, and could achieve a rapid hybridization with *E. coli* K-12 within 30 min. Compared with a DNA probe, a RNA probe has the advantage to distinguish viable cells from dead ones. (Baeumner and others 2003) found that after nucleic acid sequence-based amplification (NASBA), as low

as 40 CFU mL^{-1} *E. coli* could be detected by hybridization with RNA probes, and quantified by the liposome amplification in 15-20 min.

Bienzyme biosensors realize the detection of *E. coli* in water based on the organism's metabolism. A common target enzyme is β-galactosidase, which can hydrolyze 4-aminophenyl-β-D-galactopyranoside (4-APGal) into 4-aminophenol (4-AP) (Pérez and others 2001), 4-aminophenyl- β -D-galactopyranoside (4APG) into 4-AP (Nistor and others 2002), and phenyl β-D-galactopyranoside (PG) into phenol (Serra and others 2005). The activity of β-galactosidase was later indicated via the amplification of an amperometric signal. Bienzyme biosensors took 3-10 h to detect as low as 10^3 CFU mL^{-1} *E. coli* in environmental samples, although this was still too high to meet EPA requirements (Pérez and others 2001; Tang and others 2006).

2.3.3.2 Signal analysis methods

After being captured by biological recognition elements, signals from *E. coli* cells can be analyzed in optical or biochemical ways. The surface plasmon resonance (SPR) biosensor is an optical biosensor that measures changes in diffraction caused by structural variations in the vicinity of a thin film metal surface with antibodies/antigens coated on one side. Combined with microarray, SPR is able to directly and rapidly detect multiple targets over a large dynamic range, including not only *E. coli* and other bacteria, but also soluble protein toxins, spores and viruses (Marusov and others 2012). The fiber optical

biosensor belongs to the evanescent wave-based technologies, which allows the recognition and measurement of objectives at the fiber surface, with binding of fluorescently labeled antibodies/antigens. It greatly simplifies the separation and detection steps of bacteria in complex environmental samples (Noble and Weisberg 2005). The amperometric method is a widely applied electrochemical method that always combines with the biological recognition. The concentration of the analyte is indicated by the strength of the produced current at the electrode. For *E. coli* detection, the electrochemically active molecule, 4-aminophenol (4-AP) was produced under the hydrolysis by β-galactosidase, and then measured by amperometry (Nistor and others 2002; Pérez and others 2001; Serra and others 2005).

2.3.4 Nucleic acid-based methods

2.3.4.1 Fluorescent in-situ hybridization (FISH)

FISH is a cytogenetic technique that can detect and localize specific DNA or RNA sequences in microbial cells. After the cells are treated with appropriate chemical fixatives, fluorescent oligonucleotide probes can enter the cells and complementarily bind to specific target sequences. With a washing step，unbound probes are removed and stained cells can be detected via fluorescence microscopy (Lopez-Roldan and others 2013). Peptide nucleic acid (PNA) oligonucleotide is a synthetic DNA analog that has a peptide backbone. A PNA probe instead of a conventional DNA probe can result in a

stronger, more rapid and specific hybridization with nucleic acids, due to its uncharged characteristic (Bottari and others 2006; Prescott and Fricker 1999). In addition, the combination of FISH and microarray (array scanner) can achieve a multiple gene expression detection (Stender and others 2001). When this technique is applied for detecting *E. coli* in environmental water, a pretreatment revivification step was required due to a low ribosome content in the cells (Prescott and Fricker 1999), in order to enlarge and attain a better stain of the cells (Garcia-Armisen and Servais 2004; Regnault and others 2000)

2.3.4.2 Microarray

DNA microarray, also known as "microchip" or "DNA chip", is a collection of thousands of microscopic DNA probes attached to a solid surface, like glass, nylon, or silica (Noble and Weisberg 2005). In a microarray assay, target sequences of samples can specifically hybridize to surface-immobilized probes on a "chip", and be detected by chemiluminescence or fluorescence (Langer and others 2011; Noble and Weisberg 2005). DNA samples can be directly applied to a microarray after being extracted (genomic DNA microarray), or they can be amplified by polymerase chain reaction (PCR) first before the microarray application (amplified DNA microarray). However, due to their low sensitivity (100 fg of *E. coli* genomic DNA), genomic DNA microarrays may not be practical for routine monitoring of environmental samples. Therefore, a combination with

PCR amplification of the sequence of interest is a promising way to enhance their sensitivity and identification (Lee and others 2006; Maynard and others 2005). Similar to DNA microarrays, antibody microarrays use antibodies instead of nucleotides to detect target DNA. To detect viable *E. coli* in water, Langer (2011) developed a microarray immunoassay with a horseradish peroxidase-catalyzed chemiluminescence reaction. Within 67 minutes, as low as 4×10^5 cells mL^{-1} viable cells could be detected.

2.3.4.3 Nucleic acid sequence based amplification (NASBA)

NASBA is similar to PCR. However, it is an isothermal technology that can achieve qualitative or quantitative detection of RNA or DNA. In this method, three enzymes are used, including AMV reverse transcriptase to synthesize cDNA, RNase H to degrade the RNA in the RNA-DNA heteroduplex, and T7 RNA polymerase to synthesize RNA from the T7 promoter (bioMérieux Inc.). When amplicons are produced, stem-loop structure probes called molecular beacons hybridize to the target gene and emit a fluorescent signal (Eurogentec Inc.). This method is accurate, sensitive and rapid. For example, 1 viable *E. coli* in 100 mL water can be detected within 3- 4 h. It has comparable results with the culture method for tap water, treated sewage and surface water (Heijnen and Medema 2009).

2.3.4.4 Polymerase chain reaction (PCR)

The PCR is a molecular biological technique that can rapidly achieve the amplification of target DNA sequences in-vitro. In this procedure, double-stranded DNA is firstly denatured under high temperature. A pair of short oligonucleotides called primers then matches and binds to ends of the DNA regions of interest, and causes the target gene to be amplified by DNA polymerase. Consequently, newly generated DNA increases in number exponentially with the repeating of thermal cycles (Lopez-Roldan and others 2013). DNA products can be detected and identified by various techniques, like gel electrophoresis, Southern blot (conventional PCR), or probes (quantitative PCR) (EPA 2005).

Table 2 Detection of *E. coli* In water by PCR technology.

Type of water	Type of PCR	Target gene	Limit of detection (LOD)	Reference
Potable water	Conventional PCR with gel electrophoresis and Southern blotting	*uid*A, *uid*R *uid* gene - encoding β-D-glucuronidase (GUD)	10 fg of genomic DNA by targeting *uid*R gene	(Bej and others 1991)
River water	Conventional PCR with gel electrophoresis Southern blotting	*uid*A, *uid*R	Not tested	(Iqbal and others 1997)
Tap, underground and pond water	Conventional PCR with gel electrophoresis	16S rRNA gene (V_3 and V_6 regions)	1 CFU/100 mL of water with 8 h enrichment	(Tsen and others 1998)
River water	PCR with denaturing- gradient gel electrophoresis (DGGE)	*uid*A gene	Not tested	(Farnleitner and others 2000)
River, pond, and lake water	qPCR with TaqMan probe	*lac*Z gene - encoding β-galactosidase	3 copies mL^{-1}	(Foulds and others 2002)
Drinking water	qPCR with TaqMan probe	*uid*A	10^3 CFU mL^{-1} in pure	(Frahm and Obst 2003)

			culture	
Drinking water	Multiplex PCR with gel electrophoresis	*lac*Z, *uid*A	100 pg for DNA from *E. coli* pure culture; 1 CFU mL^{-1} for *E. coli* cells with 6 h enrichment	(Tantawiwat and others 2005)
Agricultural and river water	qPCR with SYBR Green	16S rRNA-ITS-23S rRNA gene - Internal transcribed spacer (ITS) region that lies between the 16S–23S rRNA subunit	1 cell mL^{-1} in laboratory water; and 10 cell mL^{-1} in agricultural water	(Khan and others 2007)
Drinking and beach water	Conventional PCR with gel electrophoresis	16S rRNA-ITS-23S rRNA gene, 16S rRNA gene, *uid*A gene, *pho*E gene - encoding outer membrane protein *Pho*E;	Not tested	(Maheux and others 2009; Paradis and others 2005)

		tuf gene - encoding the elongation factor Tu gene		
Rainwater	qPCR with TaqMan probe	23S rRNA gene	10^2 CFU 100 mL^{-1}	(Ahmed and others 2012)
Wastewater	qPCR with SYBR Green	*Cad*C gene - encoding a regulatory protein that stabilizes DNA during the transcription process; *HNS* gene - encoding the allantoin transporter; *Allan* gene - encoding a transcriptional activator	Around one genome equivalent (5.06 fg)	(Chetta and others 2012)
Fresh water	PCR with Scorpion® probe	*uid*A gene	Not tested	(Krometis and others 2013)
Recreational water	Molecular enrichment PCR	23S rDNA	Not tested	(Valeriani and others 2014)

In conventional PCR, amplicons can be detected by running an agarose gel or using radiolabeled gene probes. They can only show the quality of DNA products, although the quantity could be approximately told by the strength of the signal (Iqbal and others 1997). In addition, samples from the environment (like polluted river water) could cause non-specific bands to form on agarose gels (Iqbal and others 1997).

After 2000, more rapid, specific and quantitative PCR methods were applied for the detection of *E. coli* in water. Common techniques to detect and measure PCR products are by the use of fluorogenic and fluorescent probes (fluorescent resonance energy transfer-FRET systems), including SYBR® Green Ⅰ, TaqMan™ probe and Scorpion® probe. SYBR® Green Ⅰ is an asymmetrical cyanine dye, which can emit fluorescence after it non-specifically binds to double-stranded DNA (SYBR Green® Ⅱ is for RNA). Since the bound SYBR® Green Ⅰ is 1,000-fold more fluorescent than the unbound one, it can measure the total amount of DNA products. Also, the specificity of products can be identified by a subsequent melting curve step. A TaqMan™ probe consists of a specific oligonucleotide targeting the DNA of interest, a reporter flourophore (like FAM, VIC, and TAMRA) at one end, and a quencher at the other. During an annealing step in each PCR cycle, the probe which is hybridized to the corresponding template will be cleaved and release a fluorescent emission under the exonuclease activity of *Taq* polymerase. Unlike TaqMan™ probes, Scorpion® probes are actually a probe-primer complex that includes a specific hairpin stem-loop probe, a flourophore and a quencher at each end and also one primer. In the initial PCR cycles, the primer hybridizes to the target DNA and begins extension. After one cycle of PCR

extension is complete, the complementary probe will bind to the newly synthesized target region (Botes and others 2013). Once two ends of this stem-loop structure separate apart, the fluorescence spectra can directly reflect the original amount of target cells. Since this type of probe does not depend on an enzymatic cleavage as TaqMan™, it can lead to an even more rapid detection (Noble and others 2010). The quantification cycle (*Cq* value) indicates the required number of cycles for the fluorescence signal to cross through a threshold. A higher initial DNA concentration sample can be detected in a shorter time and correspondingly has a smaller *Cq*.

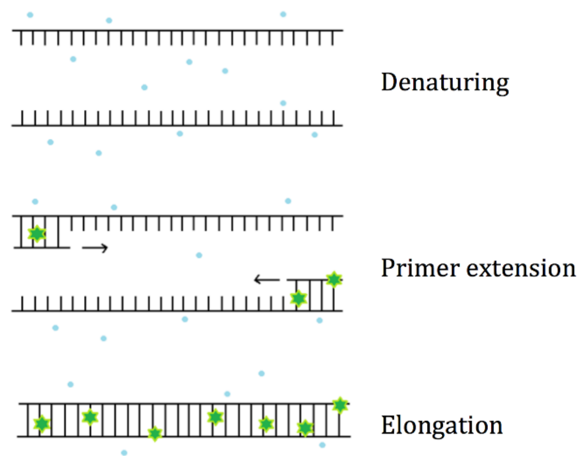

Figure 1 Detection mechanism of SYBR® Green I (Adapted from Botes and others 2013)

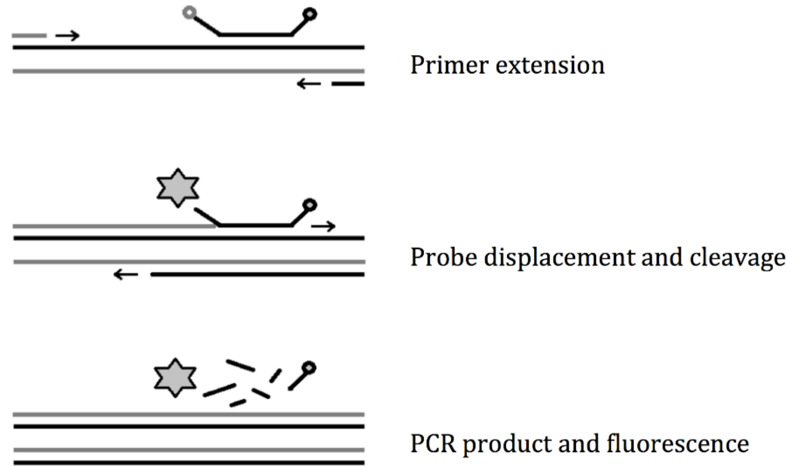

Figure 2 Detection mechanism of the TaqMan™ probe (Adapted from Botes and others 2013)

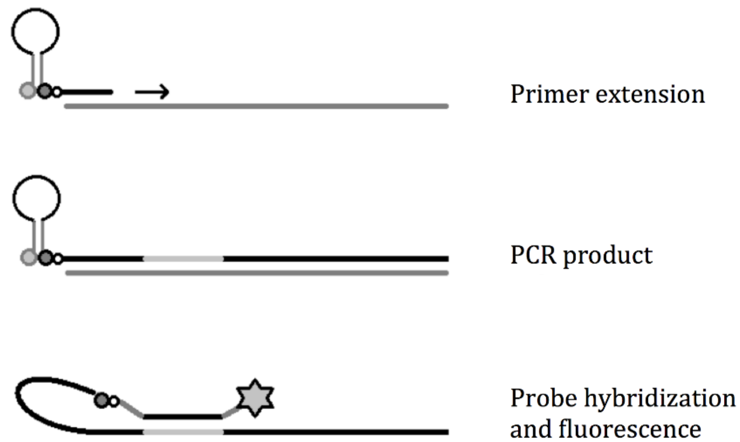

Figure 3 Detection mechanism of Scorpion® probe (Adapted from Whitcombe and others 1999)

PCR can rapidly identify *E. coli* and other microorganisms in water, and it also can be used to trace the organism back to the contamination source (Noble and others 2006). The most widely applied target gene is the *uid*A gene, which encodes the activity of β-D-glucuronidase. It is also the target to prove the presence of *E. coli* by the EPA culture-based methods. Other target genes include *uid*R, *lac*Z, *tuf*, *pho*E, *Cad*C, *HNS*,

Allan, 16S rRNA, 23S rRNA, and 16S rRNA-ITS-23 S rRNA. Although all of these target genes were designed for indicating the existence of *E. coli*, they showed different ubiquity, specificity, and productivity. It has been reported that primers targeting the *lac*Z gene could amplify DNA from coliform bacteria other than *E. coli* (Khan and others 2007). Primers targeting the 16S-ITS-23S gene region occur in many other Enterobacteriaceae species. Primers targeting the *tuf* gene could amplify all *Shigella* DNA, and primers targeting 16S rRNA, *pho*E, and *tuf* genes amplified *Escherichia fergusonii* as their unspecific target (Maheux and others 2009). Among all these primers, primer sets targeting the *uid*A gene were 100% specific for *E. coli*, which might not cause false positive results. However, it was found that sometimes they did not detect some *E. coli* isolates and would lead to false negative results (Khan and others 2007). Interestingly, using *uid*A-targeted primers could yield more products compared to the *uid*R primers, although both target the same gene (Iqbal and others 1997).

2.4 Considerations of PCR detection for *E. coli* in environmental samples

2.4.1 DNA extraction methods

Based on the EPA criteria for drinking, surface and waste water, the common volume of water sample in research is 100 mL or even larger (Ahmed and others 2012; Frahm and Obst 2003; Gensberger and others 2014), while small sample volumes were also observed for some studies of turbid wastewater (Foulds and others 2002; Li and others 2014). To concentrate *E. coli* cells, centrifugation, membrane filtration and other methods may be applied on large volume water samples (Mendes Silva and Domingues

2015). Membrane filtration is an efficient method that can collect *E. coli* cells on a membrane while allowing the water to pass through. However, this technique is limited due to clogging of the filter by colloidal and suspended particulate materials (Mendes Silva and Domingues 2015; Mull and Hill 2009) (EPA 2009). A pre-filtration step can assist to remove large particles and algae from water samples. Filtration with a 20 μm pore size nylon filter improved the efficiency of the following filtration step to concentrate target *E. coli* without causing the loss of cells (Lee and Deininger 2004).

Centrifugation is a good alternative to process turbid water samples, and potentially increase the detection limit (Mull and Hill 2009). While during a centrifugation procedure, suspended contaminants will be concentrated and form pellets along with bacterial cells, and may affect the following cell procedure, like DNA extraction and other cell treatments. To achieve a better concentration and recovery of cells, Mull and Hill (2009) used hollow-fiber ultrafiltration to pretreat surface water. Consequently, about 50 EHEC O157:H7 cells could be detected by real-time PCR from a 40-liter surface water sample.

The extraction method of DNA or RNA is critical to ensure sensitivity of both conventional and quantitative real-time PCR (Foulds and others 2002; Kim and others 2013). In environmental water, suspended contaminants can disturb DNA extraction, diminish the recovery of target nucleic acids, and inhibit polymerase activity, including sediments, cellular debris, heavy metals, humic acids and phenolic compounds. This underestimation will be more significant for low target microbial concentration samples. Since many of these inhibitors have similar solubility properties to those of the nucleic acids, a purification step is needed to avoid their concentration along with that of nucleic

acids (Botes and others 2013; Krometis and others 2013; Mendes Silva and Domingues 2015). It was proved that using phenol–chloroform (Tsai and Olson 1991), EDTA (Foulds and others 2002), $AlNH_4(SO_4)_2$ (Braid and others 2003) or polymeric absorbent Superlite™ DAX-8 (Schriewer and others 2011) could successfully remove those PCR inhibitors during nucleic acid extraction(Botes and others 2013). Commercial DNA isolation kits are also available for the DNA extraction from bacteria in the environmental water to avoid subsequent inhibitions, like FastDNA™ Spin Kit for Soil (MP Biomedicals, USA), and PowerWater® DNA isolation Kit (MO BIO, USA).

2.4.2 Viability of cells

Compared with traditional culture-based methods, PCR achieves a rapid, accurate, specific and multiplex detection for *E. coli* in water samples. However, one major shortage of this method arises when distinguishing viable from dead cells (Gensberger and others 2013). False positive results may cause unnecessary alarms and even recalls of water by public health, water, and wastewater agencies (Gedalanga and Olson 2009).

Fortunately, two nucleic acid stains, propidium monoazide (PMA) and ethidium monoazide bromide (EMA), can be added to a PCR assay to achieve specific monitoring of viable bacteria, including *E. coli* (Gensberger and others 2014). The two DNA intercalation dyes can selectively modify free DNA and DNA from dead cells whose membrane is compromised, while still allowing the detection of viable (including VBNC state) cells. This is very important when compared with culture-based methods, because it is found that various bacteria, including some pathogens, can enter a VBNC state due to environmental stresses and starvation (Li and others 2014; Yokomachi and Yaguchi 2012). EMA and PMA can help reduce signals from dead cells because membrane

integrity indicates cell viability. However, some researches claim that respiration, substrate uptake and conversion, and other cellular criteria are recommended to indicate metabolic activities of viable cells (Elizaquível and others 2014; Nocker and others 2011; Nocker and others 2007). Moreover, PMA and EMA cannot be applied to non-viable cells with intact membranes but destroyed DNA is caused by some disinfection methods, like UV radiation (Li and others 2014).

PMA is a fluorescent nucleic acid stain that is identical to propidium iodide (PI), but with an additional azido group. Its selectivity for DNA from dead cells is based on compromised membrane integrity. It is believed that only viable cells have an intact membrane, which can provide a barrier for the entrance of PMA. On the contrary, once a compromised (dead) cell's membrane is penetrated, PMA binds to double-stranded DNA with high affinity. The photoreactive azido group converts into a highly reactive nitrene radical upon photolysis, and forms a stable covalent nitrogen-carbon bond. After this irreversible modification, cross-linked DNA of dead cells cannot be amplified by a subsequent PCR procedure, in theory. During light exposure, unbound excess PMA loses its activity by reacting with water molecules. Therefore, although DNA from viable cells is exposed in the following extraction step, it cannot be modified by the inactive PMA dye (Fittipaldi and others 2012; Nocker and others 2010).

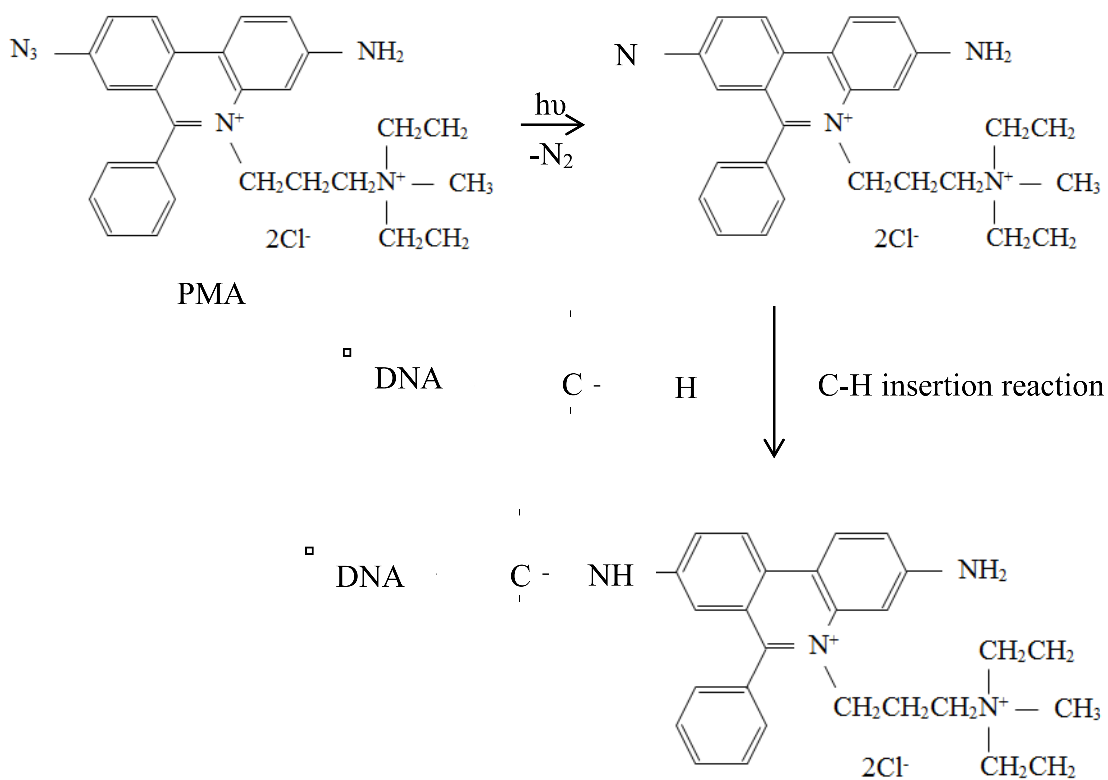

Figure 4 Mechanism of PMA and DNA binding (Adapted from Venkateswaran K J and Mohapatra B, 2012)

EMA is another membrane impermeant dye, which is also widely applied in molecular biotechnology to differentiate between live and dead cells. Similarly to PMA, this fluorescent nucleic acid stain can also intercalate double-stranded DNA after photolysis (Fittipaldi and others 2012). Although the mode of signal suppression of dead cells caused by EMA or PMA is still not completely clear, one hypothesis claims that the suppression may be caused by DNA fragmentation (Soejima and others 2007).

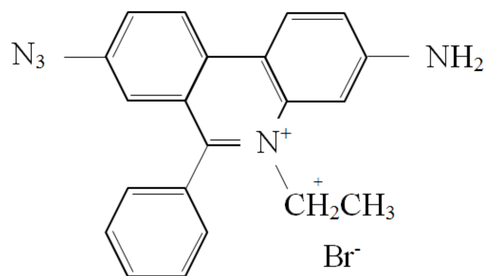

Figure 5 Structure of EMA (adapted from Venkateswaran K J and Mohapatra B, 2012)

Although these two photoreactive dyes are believed to have an almost identical intercalation for double-stranded nucleic acid, their suppression effect is diverse from each other. In researches, EMA was proved to have a better efficiency to suppress a dead cell's signal in the same conditions. However, using EMA one might underestimate the amount of live cells, since high concentrations of EMA could penetrate their membrane despite their integrity (Cawthorn and Witthuhn 2008). Unlike EMA, PMA showed a better differentiation between viable and dead cells. The increased specificity was most probably due to the two positive charges of the molecule, compared with one charge on EMA (Nocker and others 2006). However, the signal reduction for live cells is species-specific as the microscopic examination indicated. Therefore, using PMA may slightly also lead to a decreased signal of treated viable cells. When dealing with environmental samples, PMA may be a better choice. Not only because complex matrices required higher concentrations of dye since their suspended solids may prevent light penetration into samples, but also because mixed species could receive a more homogeneous treatment by PMA (Fittipaldi and others 2012). In summary, EMA has a greater extent of suppression for the signal of dead cells, but also lead to a lower signal of live cells. On the other hand, PMA causes mild and uniform effect on different species, while false

positive result from dead cells may be its limitation. Therefore, different considerations should be taken when choosing between these two dyes.

2.4.3 "Naturalized" *E. coli*

Until recently, "naturalized" *E. coli* had been found in soils (tropical, temperate and frozen temperate soils), beach sands, lake and river watersheds, and aquatic periphyton communities (Byappanahalli and others 2012; Ishii and others 2010). This so-called "naturalized" *E. coli* referred to an environmental clade of *E. coli* that persisted and reproduced outside of the intestinal tracts of warm-blood animals (Deng and others 2014; Perchec-Merien and Lewis 2013). Because of this, environmental *E. coli* may not solely represent fecal pollution accurately any more, and current water monitoring programs by United States, Australia, the European Union, and the World Health Organization face challenges (Walk and others 2009).

It was believed that once *E. coli* is released through fecal droppings from its primary habitat to the external environment, it could only transiently sustain its life due to low nutrients and other factors. However, under certain circumstances, several *E. coli* strains could tolerate and maintain their populations long enough to become "naturalized" to the environment as the secondary habitat (Ishii and Sadowsky 2008; Walk and others 2007). Although the original source(s) was still unknown (Byappanahalli and others 2012), as high as 4.2×10^{5} CFU g^{-1} soil for 1 month (Ishii and others 2006), and as long as two months of survival of "naturalized" *E. coli* were reported (Ishii and others 2010). By point mutations and recombinations, "naturalized" *E. coli* strains change slightly to adapt environment. The more adapted these *E. coli*, the more differences will emerge

between them and commensal *E. coli* (Perchec-Merien and Lewis 2013). Recently, a new set of primers was developed, which targeted the putative glucosyltransferase gene (*ycj*M) of *E. coli*. Results for PCR assays indicated that this primer set was enteric-specific and could differentiate between "naturalized" *E. coli* strains and *E. coli* stains isolated from animal feces (Deng and others 2014). Therefore, improving current *E. coli* monitoring procedures for fecal contamination can be realized in the near future.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains

Fecal *E. coli* strains used in this study were provided by Dr. C.A. Carson (College of Veterinary Medicine, University of Missouri, Columbia, MO) and were isolated from humans and other warm-blood animals. Other bacterial strains were from the Food Microbiology Laboratory of the University of Missouri, Columbia, MO. Cultures were grown in Difco™ tryptic soy broth (Becton, Dickinson and Company, Sparks, MD, USA) overnight at $37^{o}C$ (~10^{9} CFU mL^{-1}).

3.2 Water sample collection

Tap water was obtained from the Food Microbiology Laboratory, Food Science Program, University of Missouri, Columbia, MO. Environmental waters were collected from multiple sources around mid-Missouri, including the Missouri River at Katfish Katy Campgrounds, Columbia, MO (February 2015), Missouri River at Frontier Park, St Charles, MO (July 2015), Lake of the Ozarks at Bridgeport Boat Rentals, Osage Beach, MO (February 2015), Lake of the Ozarks at public beach #1 in Lake of the Ozarks State Park, Kaiser, MO (July 2015), and Mississippi River at St Louis Riverfront Trail**,** St Louis, MO (July 2015). Samples (6-12 inches below the water surface) were collected by hand with examination gloves into sterile glass jars and transported to the lab in coolers before processing.

3.3 Sample preparation of viable and dead cells

Each overnight culture of *E. coli* strains, Ct 14, Dr 23, H 24, P 24 (250 μL), was transferred and mixed to make a final sample volume of 1.0 mL in a 1.7-mL microcentrifuge tube. Cell pellets were collected by centrifugation at 12,000 ×*g* for 5 min, then re-suspended and serially diluted in 1 mL of 0.1% Bacto™ peptone (Becton, Dickinson and Company) water. To obtain dead cells, re-suspended cell pellets were heated at 80°C for 25 min before performing serial dilutions. Using plate count agar (PCA; Becton, Dickinson and Company), the viability of cells was checked by plate-counting.

3.4 PMA treatment

PMA (Biotium, Inc., Hayward, CA, USA) was dissolved in nuclease-free water to attain a 20-mM stock solution and stored at -20°C in the dark for future use. A working solution (2 mM) for experiments was prepared by diluting from the stock solution. Varying amounts of PMA were added to 1 mL of samples in 1.7-mL light transparent microcentrifuge tubes depending on the experiment. The sample tubes were incubated in the dark at room temperature for 5 min, and exposed to a 650-W halogen light (INTHL1300; Miniphoto Ltd., Co. Dublin, Ireland), at an approximate 15 cm-distance, During the photo-induced crosslinking step, tubes were horizontally laid on the surface of chipped ice that was covered with aluminum foil, to avoid excessive heat. After the light exposure, cell pellets were harvested by centrifugation at 12,000 ×*g* for 5 min, and then re-suspended, washed with 1 mL 0.1% Bacto™ peptone water and centrifuged again under the same centrifugation conditions. To figure out the influence of PMA exposure

time, each 1.0 mL of viable/dead *E. coli* cell sample (10^5 CFU/mL) was mixed with PMA separately to make a final concentration of 10 µM, followed by light exposure at different lengths of time: 0, 1, 2, 5, 10, 20 min.

For the influence of PMA concentration on amplification of DNA from viable and dead cells，1.0 mL of freshly prepared viable and dead cells (10^5 CFU/mL) was treated with PMA separately at varying amounts: 0, 2, 5, 10, 20, and 50 µM. A subsequent light exposure step lasted for 10 min.

3.5 DNA extraction

Diluted cell suspensions of overnight pure cultures were centrifuged at 12,000 × *g* for 5 min, and the genomic DNA was isolated using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's directions. The genomic DNA of *E. coli* cells that were artificially inoculated into environmental waters, was extracted by using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to that manufacturer's directions.

3.6 Quantitative PCR (qPCR)

The sequences of the primers, probes and internal amplification control (IAC) are shown in Table 3. SYBR Green qPCR was used for testing the specificity of the *uid*A, *tuf* and *ycj*M primers, while other detections were done using TaqMan® qPCR. Using the Basic Local Alignment Search Tool (BLAST) program, the specificity of the *ycj*M probe was validated. According to BLAST, no DNA from *Shigella* and only that from *E. coli*

strains could be amplified and detected by using the *ycj*M primer set and the *ycj*M probe in TaqMan®PCR assay.

All qPCR reactions were performed in a Roche LightCycler® 96 System (Roche Applied Science, Basel, Switzerland). For SYBR® Green qPCR, each 10 µL reaction mixture contained 5 µL SYBR® Select Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µM primers (Integrated DNA Technologies, Inc., Coralville, Iowa, USA), and 15 ng of bacterial DNA. Nuclease-free water (Qiagen, Hilden, Germany) was used to adjust the total reaction volume. The program included: 50 ^{o}C for 2 min and 95 ^{o}C for 13 min as the initial denaturation step, and 40 cycles, including: 95 ^{o}C for 30 s as the denaturation step (for all three target genes), 56 ^{o}C (for *tuf* gene) or 60 ^{o}C (for the *uid*A and *ycj*M genes) for 1 min as the annealing step, and 72 ^{o}C for 35 s (for all the three genes) as the elongation step. The reaction mixture of TaqMan™ qPCR was 20 µL, which contained 10 µL of USB® VeriQuest™ Probe qPCR Master Mix (2×), No Reference Dye (Affymetrix, Inc., Santa Clara, CA, USA), 1 µM *ycj*M primers and 0.5 µM pUC19 primers (Integrated DNA Technologies, Inc., Coralville, Iowa, USA), 0.15 µM *ycj*M probe (Applied Biosystems, Foster City, CA, USA), 0.1 µM pUC19 probe (Integrated DNA Technologies, Inc., Coralville, Iowa, USA), 5 pg pUC19 (Thermo Fisher Scientific Inc., Waltham, MA, USA), and 2 µL of DNA sample. The cycling parameters were: 95 ^{o}C for 13 min, followed by 40 cycles of 95 ^{o}C for 30 s, and 56 ^{o}C for 1 min.

In every PCR run, a method blank control (water sample without *E. coli* inoculation during DNA extraction), IAC control (no target DNA, but including IAC and other reagents for PCR assay), no template control (no target DNA template, no IAC, but including all other reagents for PCR assay) were included as routine quality controls to

ensure that no carryover contamination had occurred during the entire detection procedure

Table 3 Target genes, primers and probes used in this assay.

Target	Primer or probe	Sequence	Product length (bp)	Reference
*uid*A	Forward primer	5’-CGGAAGCAACGCGTAAACTC-3’	67	(Azzari and others 2008)
	Reverse primer	5’-GCGTCGCAGAACATTACATTGA-3’		
tuf	TEcol553	5’-TGGGAAGCGAAAATCCTG-3’	220	(Maheux and others 2009)
	TEcol754	5’-CAGTACAGGTAGACTTCTG-3’		
*ycj*M	ycjM_EF	5’-CAG GAA GGT GCA TTA GTA AAC TGG-3’	155	(Deng and others 2014)
	ycjM_ER	5’-CTT AAC AAA ATC GCA TGG GC-3’		
	Probe	VIC-CGTACCGTCGGGATTA-MGB NFQ		
IAC (pUC19)	Forward primer	5’-GCA GCC ACT GGT AAC AGG AT-3’	118	(Liu and Mustapha 2014)
	Reverse primer	5’-GCA GAG CGC AGA TAC CAA AT-3’		
	Probe	5′-56FAM-AGAGCGAGGTATGTAGGCG G-3BHQ_1-3′		

3.7 Application of PMA-qPCR to viable *E. coli* cells

Samples were prepared by spiking 1 mL of viable *E. coli cells* (10^2 to 10^6 CFU mL^{-1}) into 100 mL peptone water. Each sample was fully shaken and filtered through a 0.45 µm, 47 mm white gridded filter membrane (Merck Millipore Ltd., County Cork, Ireland), and membranes were transferred into a 50 mL centrifuge tube (BioExpress, Kaysville, UT, USA). Residues on the membranes were washed off with 25 mL peptone water by pipetting, and were collected again at the bottom of centrifuge tubes by centrifugation at 8 000 $\times$g for 25 min. Pellets were re-suspended in 1 mL peptone water and transferred into a 1.7 mL microcentrifuge tube. For PMA staining, 2.5 µL of PMA working solution (2 mM) was applied to make the final concentration of PMA 5µM. PMA treatment, DNA isolation and qPCR steps were conducted as described earlier.

3.8 Application of PMA-qPCR to artificially contaminated waters with mixed viable and dead *E. coli* cells

Environmental water samples were autoclaved and spiked with freshly prepared *E. coli* cells. Due to differences in water turbidity, different volumes and different treatments were applied on different environmental water samples. For tap and environmental water collected in winter, 100 mL samples were well shaken and filtered through a 0.45 µm, 47 mm white gridded filter membrane. After filtration, membranes were transferred into 50 mL centrifuge tubes and residues on the membranes were washed off with 25 mL peptone water by pipetting. The pellets were collected by centrifugation at 8 000 $\times g$ for 25 min, and re-suspended in 1 mL of 0.1% peptone water. Samples were transferred into 1.7 mL microcentrifuge tubes, followed by centrifugation at 2,000 $\times g$ for 1 min to get rid of suspended particles. Supernatants were treated

with 10 μM PMA. For environmental water collected in the summer months, 10 mL sample was used, following several centrifugation steps. First, samples in 50 mL centrifuge tubes, were centrifuged at 2,000 $\times g$ for 1 min. Supernatants were transferred to new 50 mL centrifuge tubes and centrifuged again at 8,000 $\times g$ for 10 min. Pellets were re-suspended in 1 mL peptone water and transferred into 1.7 mL microcentrifuge tubes. Samples were centrifuged at 2000 $\times g$ for 1.5 min, and the supernatants treated with 20 μM PMA by adding 10 μL of 2 mM PMA. The following DNA extraction and qPCR steps were conducted as mentioned previously.

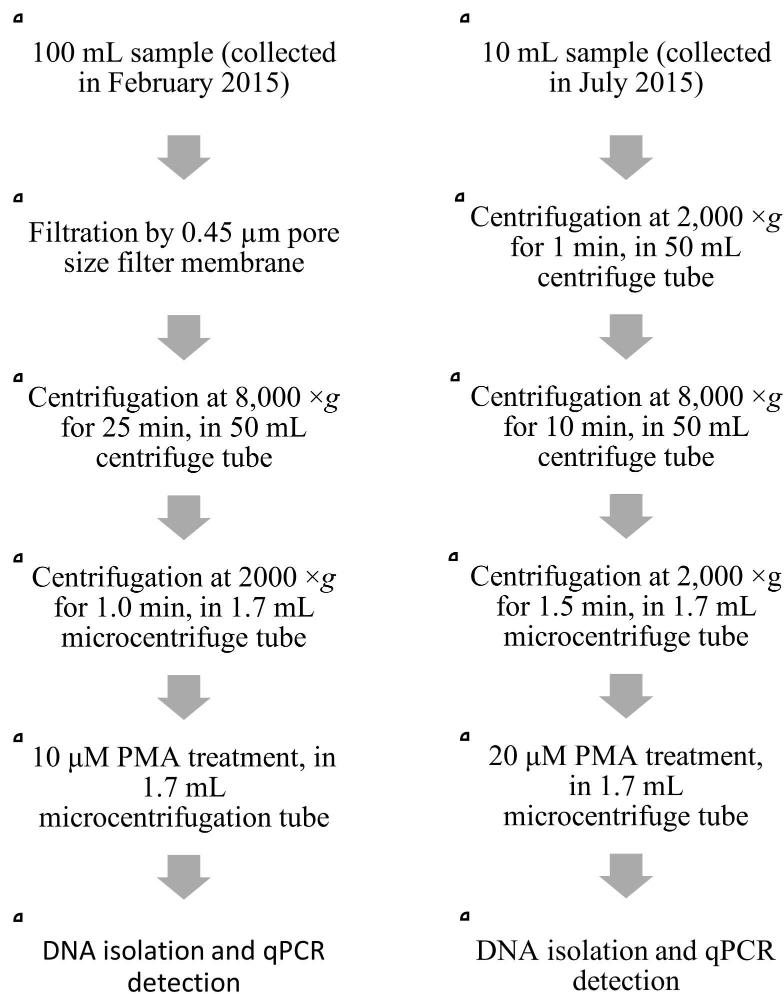

Figure 6 Flow chart of water sample processing

3.8.1 Application of PMA-qPCR among different types of water with mixed viable and dead *E. coli* cells

For each type of water, three kinds of samples were prepared. The first sample was inoculated with 1 mL of 10^5 CFU mL^{-1} live cells only, and treated with 10 μM PMA after the second centrifugation; the second one was prepared by adding 1 mL of 10^5 CFU mL^{-1} dead *E. coli* cells and 1 mL of 10^5 CFU mL^{-1} viable *E. coli* cells simultaneously, and treated by PMA with the same concentration in the next step; the third sample contained both 1 mL of 10^5 CFU mL^{-1} viable and 1 mL of 10^5 CFU mL^{-1} dead *E. coli* cells, but was not treated with PMA.

3.8.2 Comparison of qPCR and PMA-qPCR among different types of water with a mixture of viable and dead *E. coli* cells (different viable cell concentrations)

For each type of water, three sets of samples were prepared, including: 1) water samples spiked with 1 mL of live cells only at different concentrations and treated with PMA after collecting cells; 2) water samples prepared by inoculating with 1 mL of 10^5 CFU mL^{-1} dead *E. coli* cells and 1 mL of viable *E. coli* cells (various concentrations) simultaneously, and treated by PMA; 3) samples with the same bacterial content as the second set but without PMA treatment.

3.8.3 Correlation of PMA-qPCR and culture-based method (EPA Method 1603) for detection of viable *E. coli* cells in artificially contaminated waters

Water samples were artificially contaminated by with various concentrations of viable *E. coli* cells. Two equivalent water samples with the same volume and bacterial concentration were processed and the bacterial count was determined by the PMA-qPCR and a culture-based method, respectively. For the culture-based method, the procedure followed the protocol of the United States Environmental Protection Agency (EPA) Method 1603: *Escherichia coli* (*E. coli*)

in Water by Membrane Filtration Using Modified Membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC) (EPA 2009).

3.9 Statistical analysis

All experiments were repeated twice. Means for duplicate log *E. coli* aerobic plate counts were plotted against means for triplicate quantification cycle (Cq) values obtained by qPCR or PMA-qPCR. Tukey's test was applied by the SPSS software (IBM, Armonk, NY, USA) to evaluate the effect of light exposure time, concentration of PMA staining on dead and viable cells, and PMA treatment on different types of water, separately. Two-way ANOVA was applied by the SPSS software to analyze the significant differences among different water types and three sets of samples in each type of water. A significance level of 0.05 was used when comparing differences. Regression lines were performed by Microsoft Excel software (Microsoft Corporation, Redmond, Washington, USA). To evaluate the linear correlation between PMA-qPCR and EPA Method 1603, regression was also analyzed by using Minitab 16 software (Minitab Inc. State College, PA, USA).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Specificity of *ycj*M primers

The specificity of the *ycj*M primers for non-pathogenic *Escherichia coli*, fecal *E. coli*, pathogenic *E. coli* O157:H7 and closely related *Shigella* strains was analyzed by SYBR®Green qPCR. Fecal *E. coli* included *E. coli* strains isolated from chiken (C), cattle (CT), duck (D), deer (DR), dog (DG), goat (GT), goose (G), human (H), pig (P), and raccoon (R). Using *tuf* and *uid*A primers, false positive results were obtained when three *Shigella* strains were tested. Conversely, with the *ycj*M primer set, only DNA from *E. coli* (except *E. coli* D8) and not that from *Shigella* was amplified. One explanation of why *E. coli* D 8 was *ycj*M negative, was this strain might transmit from environment to animal's intestinal tract, and then be isolated from animal's feces.

Table 4 Specificity of ycjM in *E. coli* and *Shigella* strains.

Bacterial strain	PCR amplification [a]		
	tuf	*uid*A	*ycj*M
E. coli ATCC 25922	+	+	+
E.coli K12 ATCC 23716	+	+	+
E. coli C17	+	+	+
E. coli CT 23	+	+	+
E. coli D 8	+	+	-
E. coli D15	+	+	+
E. coli DG 13	+	+	+
E. coli DR 23	+	+	+

E. coli G 17	+	+	+
E. coli GT 4	+	+	+
E. coli H 24	+	+	+
E. coli P 24	+	+	+
E. coli R8	+	+	+
E. coli O157:H7 505B	+	+	+
E. coli O157:H7 C7927	+	+	+
E. coli O157:H7 G3510	+	+	+
Shigella dysenteriae 29028	+	+	-
Shigella flexneri 25929	+	+	-
Shigella sonnei	+	+	-

[a] Positive (+) or negative (-)

In the application of PCR to detect *E. coli*, various target genes have been used in other researches, including *uid*A, *uid*R, *lac*Z, *tuf*, *pho*E, *Cad*C, *HNS*, *Allan*, 16S rRNA, 23S rRNA, and 16S rRNA-ITS-23S rRNA (Ahmed and others 2012; Bej and others 1991; Chetta and others 2012; Farnleitner and others 2000; Foulds and others 2002; Frahm and Obst 2003; Maheux and others 2009; Tsen and others 1998). These target genes showed different ubiquity, specificity or productivity, even though they were all designed for detecting the existence of *E. coli* (Khan and others 2007; Maheux and others 2009). Among these target genes, *uid*A was the most widely utilized. It encodes the activity of β-D-glucuronidase, and is also the official target gene to confirm the presence of *E. coli* (EPA 2009). However, it was found that sometimes *uid*A primers

did not detect some *E. coli* isolates, which might lead to false negative results (Khan and others 2007). In addition, like primers targeting *tuf*, *pho*E, 16S rRNA and 16S rRNA-ITS-23S rRNA, *uid*A primers showed non-specificity because they could not differentiate *E. coli* from *Shigella* strains, and might overestimate the amount of *E. coli* cells (Maheux and others 2009). On the contrary, the primers of *ycj*M used in this research did not amplify any DNA from *Shigella* stains. Moreover, they were designed to target the putative glucosyltransferase gene (*ycj*M) of *E. coli*, and could differentiate between *E. coli* stains that exist in animal feces from "naturalized" *E. coli* strains that exist in the environment (Deng and others 2014). Thus, *ycj*M primers may be a better choice to monitor fecal contamination, especially for environmental samples.

4.2 Effect of light exposure time of PMA treatment on qPCR

To explicate the effect of the light exposure time of PMA treatment on the amplification of DNA from pure viable and dead *E. coli* cells, aliquots of 10^5 CFU mL^{-1} viable and dead *E. coli* cell suspensions were separately treated with a concentration of 5 μM PMA, followed by light exposures of 0, 1, 2, 5, 10, 20 min. An aliquot of viable and an aliquot of dead *E. coli* cell suspensions (10^5 CFU/mL each) without PMA treatment were set up as control group, respectively.

Light exposure is a critical factor for PMA treatment because it does not only activate the dye to crosslink with nucleic acids, but it also inactivates any excess dye from forming hydroxyl amines. For *E. coli* in environmental samples, the exposure time usually ranges from 3 to 10 min (Gensberger and others 2014; Gensberger and others 2013; Li and others 2014; Luo and others 2010; Shi and others 2011). Since bacterial species, light sources (halogen or LED), distances of the sample from the light source, sample containers, sample turbidities and other factors

(Fittipaldi and others 2012; Yokomachi and Yaguchi 2012) might affect the light exposure time of PMA treatment, it was specifically optimized for this study.

No significant differences among the Cq values for viable cells with or without PMA treatment were observed (Figure 7), meaning that a light exposure time of as long as 20 min did not reduce DNA amplification of viable *E. coli* cells. On the other hand, for heat-killed cells, the Cq values increased with the extension of light exposure time from 1 to 10 min, which illustrated the growing inhibition of the qPCR amplification of dead cells by PMA treatment. After 10 min, the amplification of target DNA from most of log 5 CFU dead cells was inhibited, since the inhibition curve approached Cq 35 and flattened out. Therefore, an exposure time of 10 min was selected for further use in this study.

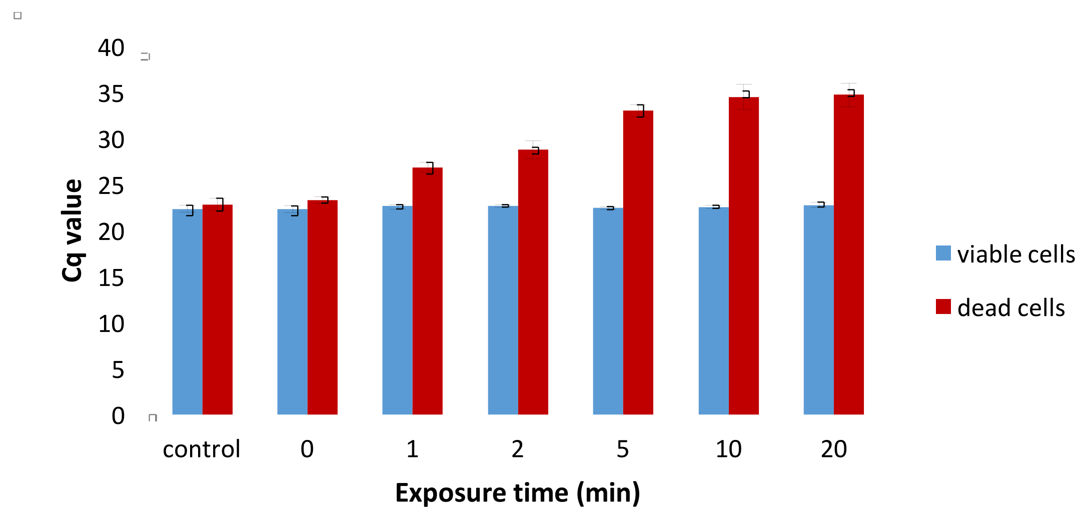

Figure 7 Influence of light exposure time of PMA treatment on inhibition of PCR amplification of DNA from 10^5 CFU mL^{-1} of viable/heat-treated *E. coli* cells.

4.3 Influence of PMA concentration on qPCR

To estimate the effect of PMA on viable and heat-killed cells, various amounts of PMA were added to 10^5 CFU mL^{-1} viable and dead *E. coli* cell suspensions separately to reach the final

PMA concentrations of 2, 5, 10, 20, 50 μM. DNA samples extracted from viable and dead cell suspensions without PMA treatment were treated as the control groups.

The results of the effect of PMA concentration on viable and dead cells are shown in Figure 8. For dead cells, the increasing Cq values of dead cells from the 0 to 5 μM PMA treatment indicated a continual decrease of DNA amplification. From the inhibition curve, the Cq value from dead cells was closed to 35 and no significant increases was yielded starting at the concentration of 5 μM, which meant that a minimal concentration of 5 μM PMA was needed to stain as much as 10^5 CFU mL^{-1} dead cells. On the other hand, no significant increase in Cq value was observed for viable cells when they were treated with no more than 20 μM PMA. Thus, it issuggested that the membranes of live *E. coli* cells could successfully prevent the penetration of PMA up to a concentration of 20 μM. The 50 μM PMA treatment showed cytotoxic effects on viable cells, since the Cq values of viable cells treated with 50 μM PMA were slightly higher than the Cq values of other viable cell samples treated with lower PMA concentrations. The results indicated that PMA at high concentrations might inhibit DNA amplification of viable cells, which would result in the underestimation of the amount of total viable *E. coli* cells. This phenomenon was consistent with the conclusion of Fittipaldi and others (2012) that the cytotoxicity of PMA was concentration-dependent, and would have a significant effect on live cells at high concentrations (≥ 100 μM).

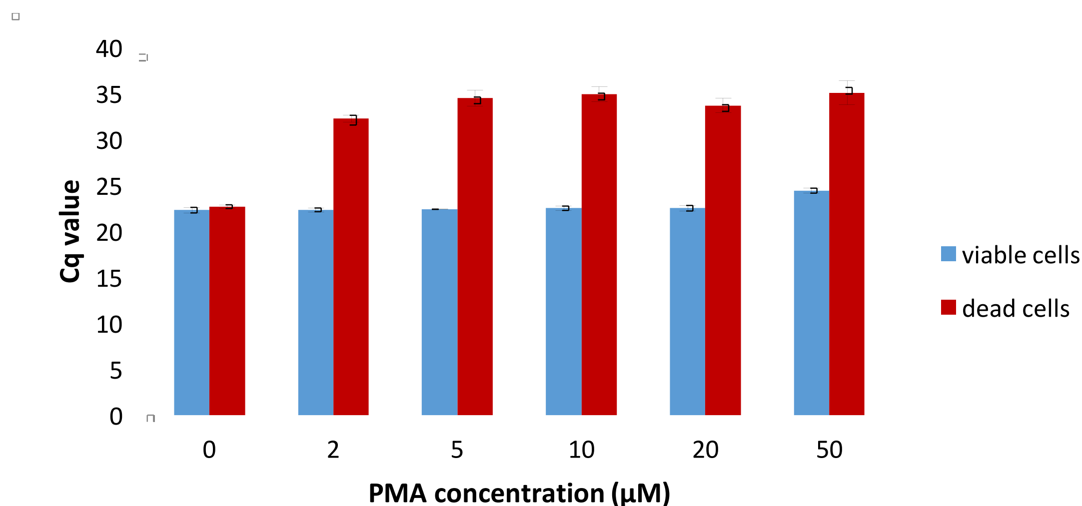

Figure 8 Influence of PMA concentration on inhibition of PCR amplification of DNA from 10^5 CFU mL^{-1} of viable/heat-treated *E. coli* cells.

According to the results above, 5 µM of PMA could efficiently inhibit the DNA amplification of log 5 CFU mL^{-1} dead cells, without causing an underestimation of viable cells counts. However, considering that environmental water samples have higher turbidity and might affect the efficiency of the PMA treatment, the final PMA concentration of 10 or 20 µM was applied for subsequent experiments, based on different turbidity levels of water samples (details were mentioned in the Materials and Methods chapter). A similar trend of PMA usage amount was also described in other researches: an amount of 10 µM PMA was used by Gensberger (2014) to treat *E. coli* in drinking water and process water samples, and higher amounts of 50 µM (Yokomachi and Yaguchi 2012), 100 µM (Li and others 2014; Taskin and others 2011), or even 200 µM were used to treat wastewater, sewage sludge and biosolids (Van Frankenhuyzen and others 2013).

4.4 Application of PMA-qPCR to viable *E. coli* cells

One hundred milliliters of peptone water sample containing viable *E. coli* cells of various concentrations, were filtered, centrifuged, and treated with PMA. Based on the previous experiment, 5 µM PMA was efficient to inhibit the DNA amplification of 10^5 CFU mL^{-1} dead cells. Therefore, 5 µM PMA was used in this experiment.

No significant differences were found between the Cq values of the PMA treated and untreated groups, and the detection ranges of both qPCR and PMA-qPCR were from 10^2 to 10^6 CFU /100 mL (Figure 9). These results demonstrated that 5 µM PMA treatment did not have a significantly negative effect on detection of viable *E. coli* by qPCR.

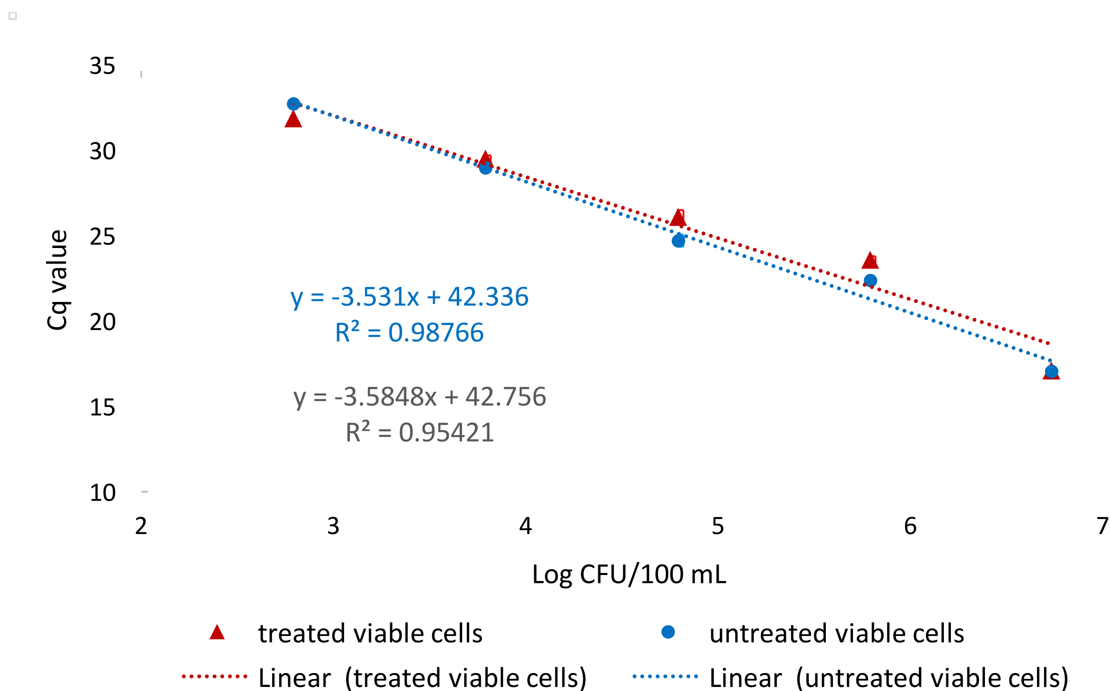

Figure 9 Standard curve for detection of viable E. coli cells by qPCR and PMA-qPCR.

The detection limit in this assay was 10^2 CFU/100 mL peptone water. However, it was still unsatisfactory, since the criterion of *E. coli* in recreational water is 126 CFU in 100 mL fresh recreational waters (EPA 1986) and it is 0 CFU/100 mL in drinking water (EPA). (Tantawiwat

and others (2005) successfully detected *E. coli* at concentrations of 10^0-10^1 CFU/mL of spiked water after 6 h of enrichment. Therefore, to increase the sensitivity of detection, a pre-enrichment step might be needed when the initial number of bacteria is low.

4.5 Application of PMA-qPCR to artificially contaminated tap and environmental waters with mixed viable and dead *E. coli* cells

4.5.1 Application of PMA-qPCR among different types of water with viable or mixed viable and dead *E. coli* cells

One milliliter of 10^5 CFU/mL viable cells or 1 mL dead (10^5 CFU/mL) and 1 mL viable (10^5 CFU/mL) cells mixture were respectively spiked into 100 mL autoclaved water samples, including tap water, Lake of the Ozarks water (collected in February 2015) and Missouri River water (collected in February 2015). Cell pellets were collected by filtration through a 0.45 μm filter membrane. After the washing and centrifugation steps, cells were treated with 10 μM PMA.

Significant differences in Cq values were found among three types of water (data not shown). This might be due to different turbidities of these three water samples, or other soluble components in them, such as heavy metals, humic acids and phenolic compounds (Botes and others 2013; Krometis and others 2013; Mendes Silva and Domingues 2015), that affected the detection of this PMA-qPCR assay.

However, among all three water types, lower Cq values of the untreated dead/viable *E. coli* mixture groups indicated that 10^5 CFU mL^{-1} dead cells caused a significant false positive PCR signal, when compared with the treated groups (Figure 10). It showed that dead cells in a water sample would lead to an overestimation of *E. coli* population by PCR detection. The Cq values of treated dead/viable *E. coli* cells mixture were not significantly different from the Cq

values of treated viable cells alone, which meant that the PMA treatment efficiently removed the false positive signal from DNA of at least 10^5 CFU mL^{-1} dead cells. For lake water, the Cq value of treated *E. coli* cell mixture was in between the Cq value of treated viable cells alone and the Cq value of the untreated cell mixture. This might be due to the fact that fine suspended solids in lake water still remained in the sample during the photo-activation step and triggered the inefficiency of the PMA treatment. Considering that the inefficiency of the PMA treatment might be caused by the large amount of suspended solids in environmental samples, a higher PMA concentration or smaller sample volume would be applied for future studies for more turbid water samples.

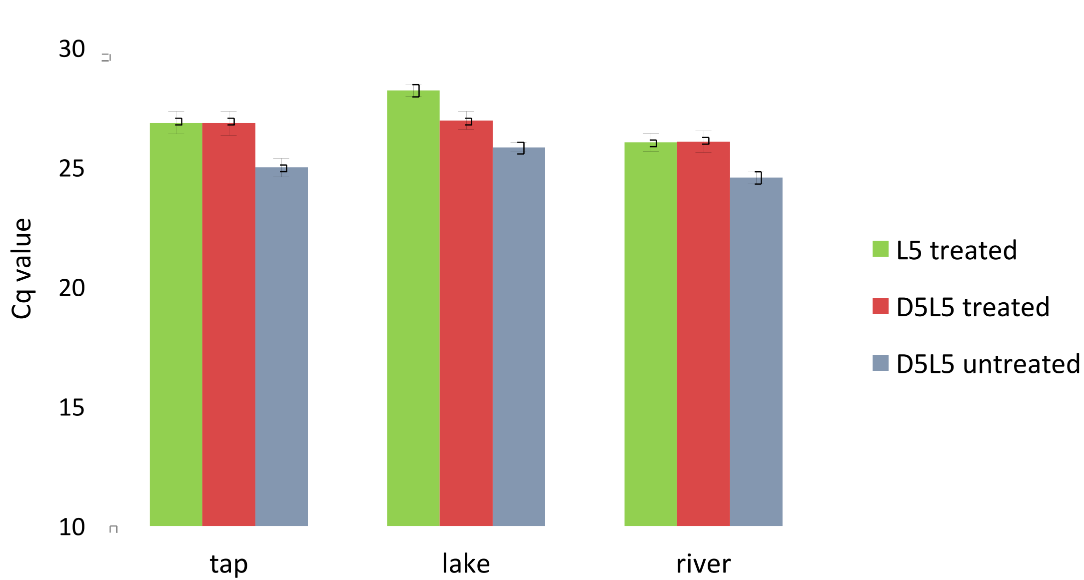

Figure 10 Influence of PMA treatment on inhibition of PCR amplification of *E. coli* DNA in tap and environmental water (L5 treated: sample with 10^5 CFU mL^{-1} live cells alone, treated with PMA; D5L5 treated: sample with mixture of 10^5 CFU mL^{-1} live cells and log 5 dead cells, PMA treated; D5L5 untreated: sample with mixture of 10^5 CFU mL^{-1} live cells and log 5 dead cells, not treated with PMA.)

4.5.2 Application of PMA-qPCR on different types of artificially contaminated water with mixed dead and *E. coli* cells (different viable cell concentrations)

One milliliter of 10^5 CFU/mL dead cells and 1 mL viable cells with various concentrations (10^3-10^6 CFU/mL) were respectively spiked into 100 mL autoclaved tap water, Lake of the Ozarks water collected in February 2015, and 10 mL of Lake of the Ozarks water collected in July 2015. Details of cell collection and PMA treatment were mentioned in the Materials and Methods.

As Figure 11 shows, no significant differences of Cq values were found between the treated dead and viable mixture group and the treated viable cells alone group, although the slopes of their standard curves were a little different ($y = -3.2947x + 45.787$; $R^2 = 0.98415$ for the treated mixed cell group, and $y = -2.7478x + 42.868$; $R^2 = 0.99465$ for the treated viable cells group). On the other hand, the Cq values of untreated dead and viable mixture group were significantly different from other two groups. This indicated that in tap water, PMA treatment could efficiently detect viable cells even in the presence of log 5 CFU/100 mL dead cells.

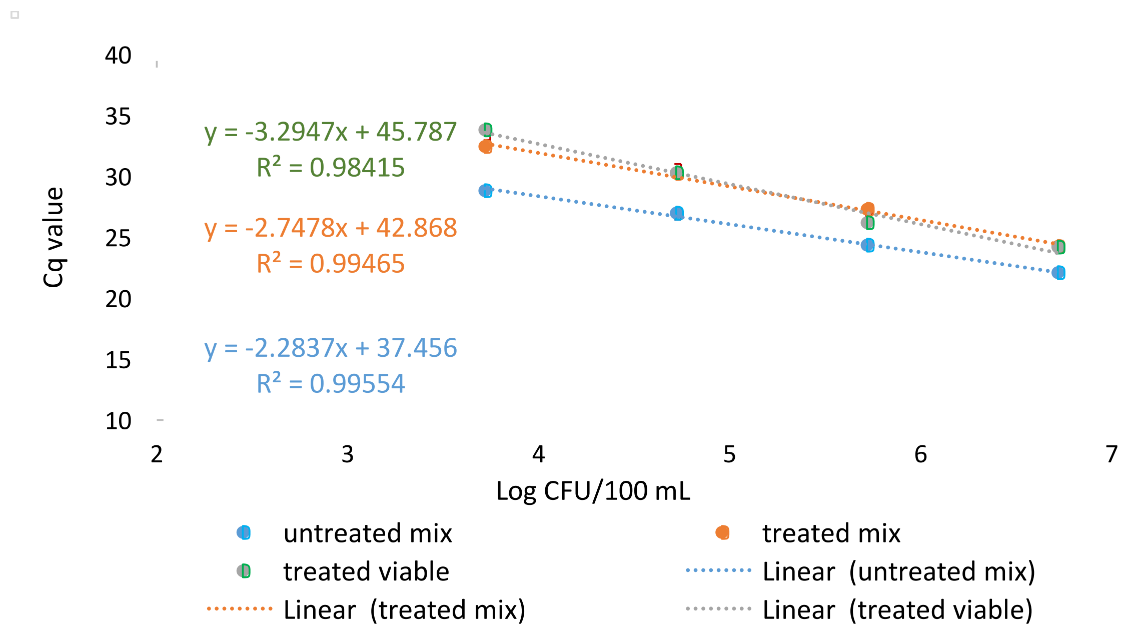

Figure 11 Relationship between the log number of viable *E. coli* cells and mean Cq value from qPCR and PMA–qPCR amplification in tap water. Untreated mix group: 10^5 CFU dead cells and viable cells with various concentrations (10^3-10^6 CFU/mL), not treated with PMA; treated mix group: 10^5 CFU dead cells and viable cells with various concentrations (10^3-10^6 CFU/mL), treated with PMA; treated viable group: viable cells only with various concentrations (10^3-10^6 CFU/mL), treated with PMA.

In this research, up to log 5 CFU/mL dead cells did not mask the detection of log 3 – log 6 CFU/mL viable cells when they were mixed together in tap water. These results were consistent with the investigation of Kantonale Laboratorium (2009) in that the number of dead bacteria should not exceed that of live cells by a factor of 100 for PMA-qPCR, and free DNA could not be greater than 4×10^5 in copy numbers. Similar results were claimed by Yang (2011) that the ratio between viable and dead cells of *E. coli* should be greater than 1%, or else dead cells could be only partially inhibited by PMA. Yokomachi and Yaguchi (2012) found that the ratio could not even be less than 10% in order for viable *E. coli* cell counts to have a linear

relationship with the Cq values from PMA-PCR. On the other hand, Fittipaldi (2012) indicated that the ratio of viable to dead cells could be as low as 0.1%, without the impact of detecting viable ones. The presence of dead cells can affect the efficiency of EMA/PMA treatment, especially when viable cells are in much lower concentrations. This means that when a high concentration of dead cells is present, the Cq values of live and dead mixture cells decrease compared with that of viable cells alone. One possible explanation of this phenomenon is that a larger amount of dead cells may have a higher capacity of taking up the dye, causing less available dye molecules per cell to bind with their DNA (Fittipaldi and others 2012).

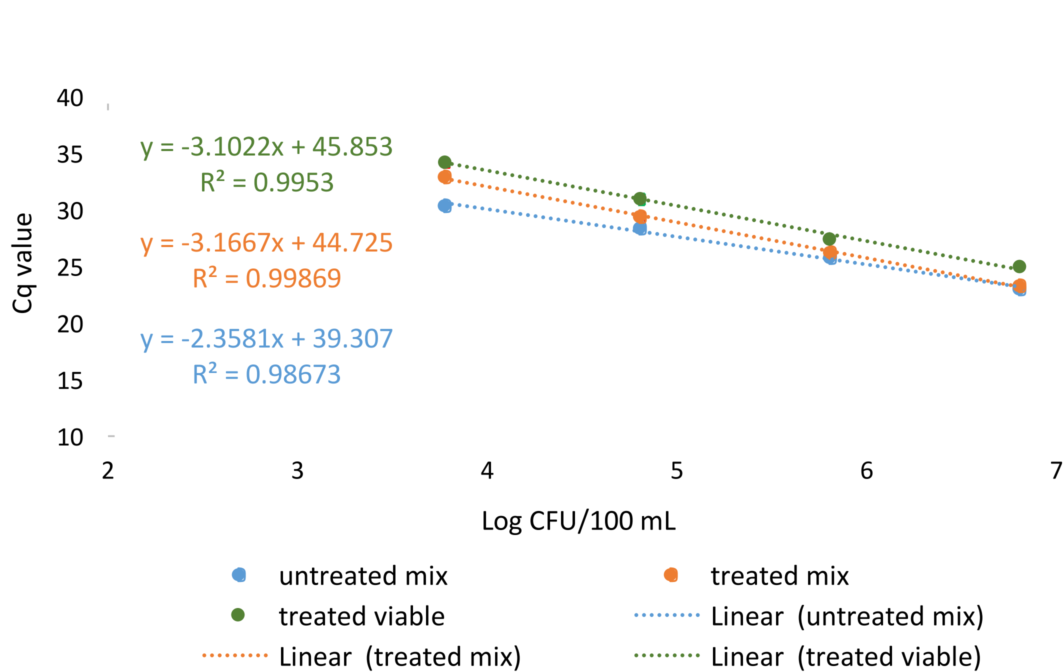

Figure 4 Relationship between the number of viable *E. coli* cells and mean Cq value from qPCR and PMA–qPCR amplification of spiked Lake of the Ozarks water collected in February 2015. Untreated mix group: 10^5 CFU dead cells and viable cells with various concentrations (10^3-10^6 CFU/mL), untreated with PMA; treated mix group: 10^5 CFU dead cells and viable cells with

various concentrations (10^3-10^6 CFU/mL) treated with PMA; treated viable group: viable cells only with various concentrations (10^3-10^6 CFU/mL) treated with PMA.

For Lake of the Ozarks water collected in winter (Figure 12), Cq values of the treated dead and viable cell mixture groups were lower than that of treated viable cells alone, but higher than that of the untreated mixture groups, demonstrating that PMA treatment did inhibit the amplification of DNA from dead *E. coli* cells, but it was not efficient to differentiate viable cells from dead cells completely. This inefficiency of PMA treatment might possibly be due to heavily suspended solids in Lake of the Ozarks samples. In complex environmental samples, many factors, such as pH, salt concentration, and water turbidity, might have adverse effects on EMA/PMA-PCR. The number of nephelometric turbidity units (NTU) of environmental water varies, depending on the suspended materials, such as soil particles (clay, silt, and sand), algae, plankton, and microorganisms. With the increase in NTU, the environmental sample contains more suspended solids and becomes darker (EPA), preventing light from penetrating into samples and finally inhibiting the crosslinking of viability dyes (Li and others 2014). Unfortunately, only quite clear water samples do not prevent DNA binding by PMA. For example, when the turbidity of the sample was less than 10 NTU, viability dyes could successfully reduce the signal from dead cells (Gedalanga and Olson 2009; Luo and others 2010). Similar results were also obtained from *E. coli* in opaque samples, such as milk or meat homogenates (Yang and others 2011). Bae (2009) stated that PMA-qPCR could only function properly at relatively high solid concentrations (TSS =1,000 mg/L) after optimization steps. To reduce the inhibition of solids, samples may be diluted to 1–2 NTU before the dye treatment (Fittipaldi and others 2012). However, this can result in a low concentration of viable cells, which may lead to false negative or false positive results. In this research, after filtration and the

first centrifugation step, water samples were concentrated from 100 mL to 1 mL, which resulted in a dramatically increased turbidity. Although after the second centrifugation step, most of the suspended solids were removed, some fine sized particles still existed in the supernatant and prevented full light penetration during the photo-activation step. Since suspended solids have a similar physical behavior as that of bacteria in water, it is challenging to remove all of them without causing a loss of bacterial count. Therefore, solutions to optimization are still much needed to effectively apply PMA-qPCR on turbid and complicated environmental or waste water samples.

During the summer months, environmental water becomes more turbid due to rain and flood. To reduce the negative effect of relatively high solids concentration, a volume of 10 mL was chosen for Lake of the Ozarks water collected in the summer. Several centrifugation steps were applied to remove suspended solids as much as possible before treating the sample with PMA. Compared with tap water and Lake of the Ozarks water collected in winter, a higher PMA concentration (20 μM) was used for this type of water.

Similar to tap water and Lake of the Ozarks lake water collected in February, the Cq value of untreated viable/dead cell mixture in Ozarks lake water collected in July was closer to that of treated viable cells when the concentration of viable cells increased. It might be because with the appearance of more viable cells, the less impact dead cells would have on the decrease in the Cq value (Figure 13). A good consistency was found between PMA treated groups even with the presence of 10^4 CFU/10 mL dead cells, which meant that the PMA treatment efficiently inhibited all significant amplification of dead *E. coli* cells even in the presence of suspended solids and background microflora. This was similar to tap water but different from Lake of the Ozarks water collected in February. Because a smaller amount of sample was chosen and more

pre-treatment steps were applied for the Lake of the Ozarks water collected in summer (July 2015), suspended solids were better removed from this environmental water sample, which allowed for a better performance of the PMA during the photo-activation step. For Lake of the Ozarks water collected in February, as low as 10^3 CFU/100 mL viable *E. coli* cells were detected. As low as 10^2 CFU/10 mL *E. coli* cells in Lake of the Ozarks water collected in July could be detected by PMA-qPCR. It indicated that in the practical application of PMA-qPCR, a smaller volume (like 10 mL in this research) can be used to have a better PMA treatment, without reducing its accuracy.

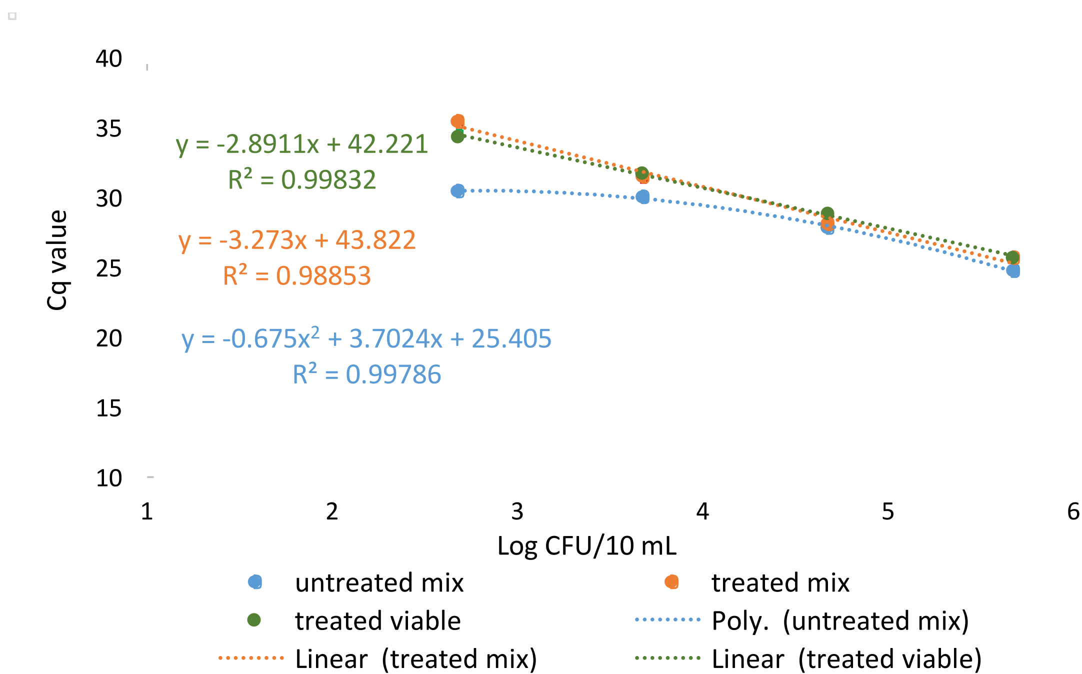

Figure 13 Relationship between the log number of viable *E. coli* cells and mean Cq value from qPCR and PMA–qPCR amplification of Lake of the Ozarks water collected in July 2015. Untreated mix group: 10^5 CFU dead cells and viable cells at various concentrations (10^3-10^6 CFU/mL), not treated with PMA; treated mix group: 10^5 CFU dead cells and viable cells at various concentrations (10^3-10^6 CFU/mL), treated with PMA; treated viable group: viable cells only at various concentrations (10^3-10^6 CFU/mL), treated with PMA.

4.5.3 Correlation of PMA-qPCR and culture-based method (EPA Method 1603) for detection of mixed viable and dead *E. coli* cells in different types of artificially contaminated waters

To determine the correlation between PMA-qPCR and EPA Standard Method 1603, different types of water samples were artificially contaminated with various concentrations of viable *E. coli* cells (process details were mentioned in the Materials and Methods). Bacterial counts were calculated from Cq values that were indicated by PMA-PCR, using regression equation from the previous standard curve for tap water (Figure 14).

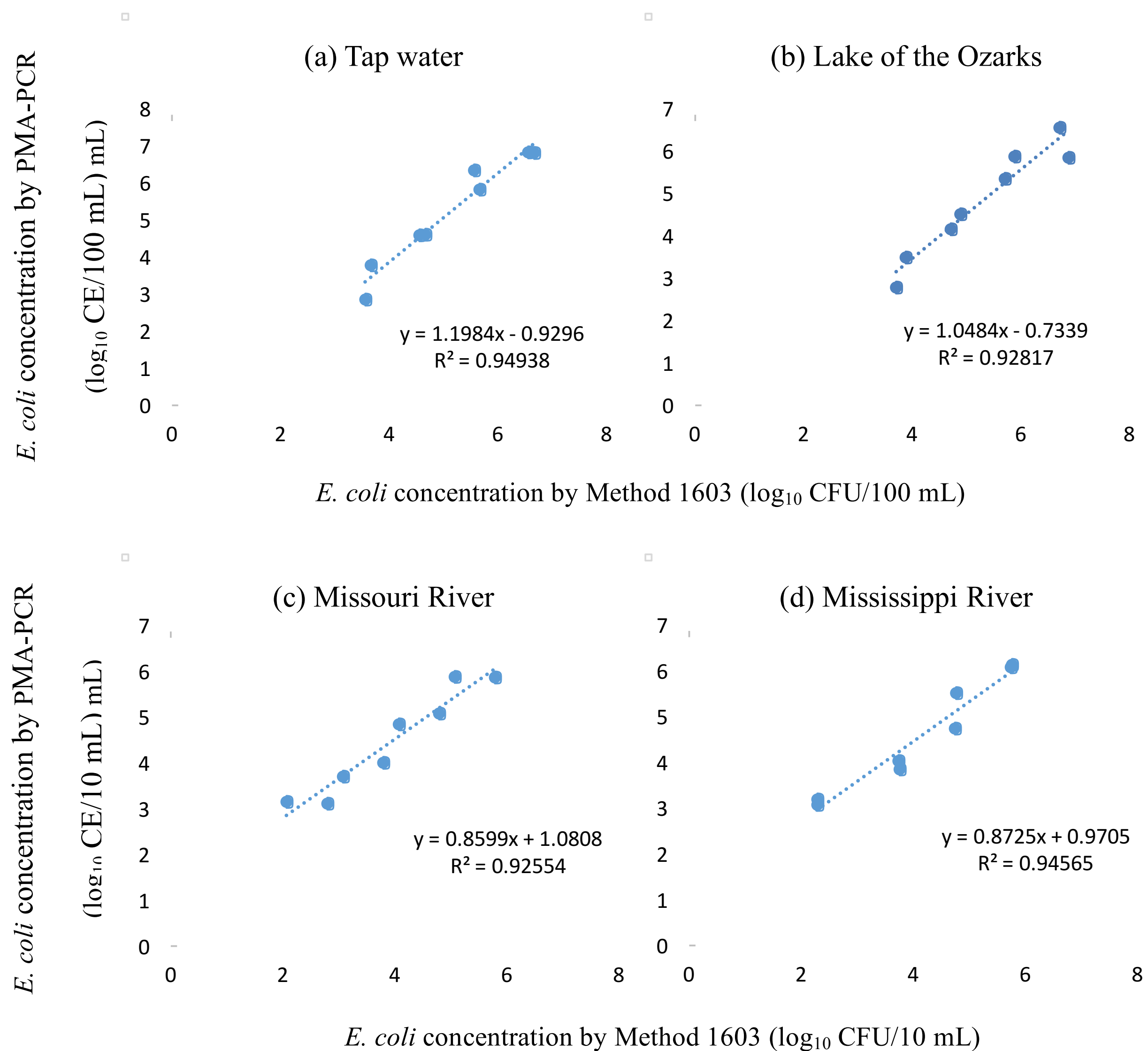

Figure 14 Correlations between results detected by PMA-PCR and EPA Method 1603 for (a) tap water, (b) Lake of the Ozarks water collected in February 2015, (c) Missouri River water collected in July 2015, and (d) Mississippi River water collected in July 2015. For (a) and (b), x-axis: *E. coli* concentration by Method 1603 (log_{10} CFU/100 mL), and y-axis: *E. coli* concentration by PMA-PCR (log_{10} CE/100 mL); for (d) and (c), x-axis: *E. coli* concentration by Method 1603 (log_{10} CFU/10 mL), and y-axis: *E. coli* concentration by PMA-PCR (log_{10} CE/10 mL). CE: cells equivalent.

A strong and significant correlation was found among all four types of water tested, as their R^2s were all greater than 90% (Figure 14). For all water types, intercept parameter was very close to zero in the statistical sense. Thus, only the slope of each regression equation was used for comparing the bacterial counts detected by both PMA-PCR and Method 1603. Since their slopes were all close to 1, no under or over estimation would be obtained by PMA-PCR in relation to the results of EPA Method 1603. Some researchers also claimed that *E. coli* qPCR had up to a 98% agreement with culture-based assays for drinking water and environmental water (Frahm and Obst 2003; Noble and others 2010). However, Noble and others (2010) found that qPCR underestimated the total count of *E. coli* in recreational water, which might be caused by the loss of *E. coli* cells at the processing steps or false positive results of standard Colilert-18 tests. Other studies showed that qPCR could detect significantly higher numbers of *E. coli* in water than culture-based methods (Khan and others 2007; Ahmed and others 2012). One explanation was that qPCR could detect DNA from both viable and nonviable cells (Ahmed and others 2012). In addition, viable but non-culturable (VBNC), or free-phase

cells could also be detected by qPCR, especially for cells in adverse environmental conditions (Noble and Weisberg 2005; Krometis and others 2013). Moreover, qPCR could achieve the detection of β-glucuronidase-negative *E. coli* isolates. Therefore, it may overcome some potential problems of most EPA approved culture-based methods for *E. coli* detection, including Method 1603 and Colilert test, since they are depended on detecting the activity of β-glucuronidase (Bej and others 1991).

CHAPTER 5

CONCLUSIONS

In this study, PMA treatment combined with qPCR was tested on dead, viable and a mixture of dead and viable *E. coli* cells, to achieve only viable cell DNA detection in various water samples.

To detect *E. coli* as a fecal indicator of water quality, the *ycj*M primer set was chosen in this study since it could differentiate *E. coli* strains that exist in the feces of warm-blood animals from "naturalized" *E. coli* strains that are found in the environment (Deng and others 2014). The specificity of the *ycj*M primers showed that they could detect most of the *E. coli* strains isolated from animal feces but did not amplify any DNA from *Shigella* strains. Light exposure time was also investigated in this study, and it was found that the fluorescent signals of dead cells became unnoticeable when the light exposure was equivalent to or longer than 10 min, while the amplification of DNA from viable cells was not affected. The usage of PMA on samples was also optimized. Results showed that in order to prevent false-positive results from dead *E. coli* cells, at least 5 μM PMA was required. No significant inhibition of PMA treatment was observed on viable *E. coli* cells when the PMA concentration was less than 20 μM. However, when the concentration of PMA increased to 50 μM, a slightly inhibitory effect was noticed on the detection of viable cells. Therefore, considering that environmental water samples are turbid and complex, 20 μM was applied on them for PMA treatment. The presence of dead cells decreased the Cq values when they were mixed together with viable cells, which led to an overestimation of live *E. coli* cell amounts. This study found that 20 μM

PMA was efficient to inhibit all signals from dead cells while not affecting the amplification of DNA from live cells in the cell mixture as long as the ratio of dead to viable cells was as high as 100.

In 100 mL peptone water, the detection range of this PMA-qPCR assay was log 2 to log 6 CFU/ 100 mL. However, for tap and environmental water, only as low as log 3 CFU/100 or log 2 CFU/10 mL *E. coli* cells could be detected. Heavily suspended solids in environmental water dramatically blocked the photo-active crosslinking activity of PMA during the light exposure step. To solve this problem, a pre-centrifugation step or smaller sample volumes were applied before the PMA treatment, and a higher concentration of PMA was used to treat *E. coli* cells. However, after optimization of the cell collection, some fine sized particles still remained in the samples which caused an inefficient PMA treatment. In 100 mL of Lake of the Ozarks water collected in winter, Cq values from the dead and viable cell mixture group with PMA treatment were different from those of the viable cells alone group, and false-positive signals were shown in the PMA-treated mixture group. When a smaller sample volume, *viz*. 10 mL was applied on the Lake of the Ozarks water collected in the summer, a good differentiation was attained between live and dead cells by the PMA treatment. Therefore, to achieve an ideal PMA treatment on complex environmental water samples, optimization of PMA treatment and pre-processing of water samples to remove suspended solids are highly recommended. The correlation of PMA-qPCR and EPA standard method – Method 1603 was studied, and a strong correlation was found among all four types of water: tap water, Lake of the Ozarks water, Missouri River water and Mississippi River water. Moreover, no over or underestimation of PMA-qPCR was found when comparing these with the

culture-based method: Method 1603. This meant that the PMA-qPCR could estimate the count of *E. coli* as accurately as the standard method, but in a relatively shorter time.

Further studies should still focus on the pre-treatment of water sample, to removed suspended solids efficiently while does not cause significant loss of bacterial cells. Otherwise, subsequent PMA treatment could work improperly, even incapably. In order to have a better suppression of DNA amplification by PMA, bigger amplicon size of the target gene could be designed. Since PMA covalently crosslink to DNA in a certain stoichiometry, longer targeted region makes more possibility of DNA modification by viability dyes, a more significant reduction of signal from membrane-compromised cells would be achieved (Fittipaldi and others 2012).

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