

Molecular-Based Methods to Detect Viable Bacterial Pathogens in Source Waters

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Humans can be exposed to waterborne bacterial pathogens and numerous outbreaks have been reported involving these microorganisms around the world. Many different enteric pathogens can be found in source waters used for drinking water. Assessing these pathogens and their possible threat to public health has always been important. Waterborne pathogens can be difficult to detect, and despite a large variety of recognized microbial detection techniques, the cause of many outbreaks has not been unidentified. Effective and rapid pathogen detection techniques are required to achieve reliable data for microbial source water quality, outbreak investigations, and for drinking water treatment efficacy monitoring.

Bacteria have long been detected using classical culture-based methods, with the rationale that living cells are able to grow/replicate. However, many pathogenic bacteria in source waters may turn into viable but not culturable (VBNC) cells and are thus undetectable by growth-based methodologies. Alternatively, PCR-based techniques have been developed to detect both non-culturable and culturable bacteria. Yet with these techniques, post-death DNA persistency can inaccurately overestimate the number of viable cells. This problem may be circumvented by an alteration to the PCR procedure that is reported to be able to block PCR amplification of DNA that originates from dead cells. This alteration involves a chemical pre-treatment step prior to PCR using a photoreactive intercalating dye, propidium monoazide (PMA).

In this research, a successful modification was made to the PMA-PCR method that can result in substantial suppression of the PCR signal from dead cells, and provide results that can more accurately measure bacterial pathogen viability. PMA-PCR was applied to high concentrations (1×10^7 cells mL⁻¹) of heat-killed cells of *Salmonella enterica* and *Campylobacter jejuni*. Using PMA-PCR in combination with primers that amplified a relatively short fragment of the *S. enterica invA* gene (119 bp), only a 3-log reduction of the dead cell PCR signal was obtained. Similarly, for *C. jejuni* using PCR primers that amplified a relatively short fragment of DNA (174 bp of *cpn60* gene), only a 1-log reduction of the PCR signal was observed for dead cells. Therefore, PMA treatment followed by PCR amplification of short DNA fragments resulted in incomplete signal inhibition of heat killed *Salmonella* and *Campylobacter*. To further investigate how PCR conditions can affect the ability of PMA to inhibit PCR amplification, primers were then used that could amplify a larger fragment of DNA. PCR amplification of a longer DNA fragment (1614 bp of *invA* gene for *S. enterica* and 1512 bp of *cpn60* gene for *C. jejuni*) strongly suppressed the signal (7 log reduction) for both heat-killed *Salmonella* and *Campylobacter*. For UV-treated *S. enterica* and *C. jejuni*, short

amplicon PMA-PCR showed no or very low PCR signal reduction, in part due to intact membranes directly after UV irradiation. Long amplicon qPCR, however, resulted in dead cell signal removal and PMA pretreatment had no effect on PCR signal suppression.

This study used quantitative PCR and the PMA-PCR viability assays to evaluate the levels and occurrences of four groups of pathogenic bacteria in surface water samples from two locations on the Grand River, Ontario, Canada, to demonstrate the reliability of the PMA-PCR technique for the enumeration of viable cells. The bacterial groups investigated included *S. enterica*, thermophilic *Campylobacter*, *Escherichia coli* O157:H7, and *Arcobacter butzleri*. Small numbers of dead cells (not more than $0.5 \log 100 \text{ mL}^{-1}$) were present, detected as the difference between PMA-PCR and PCR without PMA treatment. In this particular river, pathogen enumeration by PCR was only slightly influenced by false positive signal detection due to the presence of dead cells or extracellular DNA and reliable bacterial pathogen detection could be attained by PCR without PMA pretreatment. Viable *A. butzleri* were detected at elevated concentrations (up to $4.8 \log$ cells per 100 mL) in the Grand River. *Arcobacter* has not been previously studied in the Grand River and this is one of the few studies that have quantitatively assessed *Arcobacter* in the environment. This suggests that additional research is required on the pathogenicity of this organism and its occurrence in water.

In the next stage of this research, both the improved viability assay (long amplicon PMA-PCR) and conventional quantitative PCR were applied to investigate the survival trends of selected enteric bacterial pathogens including *Yersinia enterocolitica*, *S. enterica*, *C. jejuni*, and *A. butzleri*. The target bacteria were inoculated into sterile or non-sterile river water to study the impact of background microbiota on cell survival. These experiments were performed at 3 different temperatures (5, 15, and 25°C) and at high/low dissolved oxygen (DO) concentrations (for *C. jejuni*, and *A. butzleri* only) to evaluate the effect of these potential environmental stresses on bacterial survival trends. The results indicated that the autochthonous microbiota in river water had a significant effect on the bacterial die-off. Although lower temperatures enhanced bacterial survival in non-sterile river water, it was found that PCR may overestimate the effect of temperature on survival and that the PCR viability assays (PMA-PCR) could more accurately measure the impact of temperature. The survival of viable *C. jejuni* was adversely affected by high DO levels only at a low temperature (5°C) and this effect was observed only when the PMA-PCR viability assay was applied. *A. butzleri* survival was not affected by water DO levels.

This research provides an improved understanding of viable/active enteric waterborne bacteria and their survival in the aquatic microcosms as well as reliable data to better elucidate the effect of

environmental factors on the occurrence of pathogenic bacteria. It can also offer valuable information for microbial risk assessments used by regulators and decision makers.

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Since my early years in school, I have always dreamed of becoming a “scientist”, and in my childhood thoughts this meant discovering something exciting in the lab while wearing a huge pair of glasses and an oversized stained white coat! I believe I have followed the dream through the years, and although I’m not there (yet!), it is highly rewarding to complete steps toward it. Here I would like to take the opportunity to thank the people who have helped me take these steps and made the research in this thesis possible.

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Dedication

This thesis is dedicated to my parents who have supported me all the way since the beginning of my studies.

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List of Acronyms

AMV-RT	Avian Myeloblastosis Virus Reverse Transcriptase
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CAT	Cefoperazone amphotericin teicoplanin (Agar)
CCDA	Charcoal Cefoperazone Deoxycholate Agar
cDNA	Complimentary DNA
CFU	Colony Forming Unit
CSM	Charcoal-Based Selective Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DO	Dissolved Oxygen
EMA	Ethidium monoazide
FRET	Fluorescence Resonance Energy Transfer
GITC	Guanidine isothiocyanate
mCCDA	Modified Charcoal Cefoperazone Deoxycholate Agar
mFC-BCIG	Modified Fecal Coliform 5-bromo-4-chloro-3-indolyl-b-D-glucuronide
mRNA	Messenger ribonucleic acid
NASBA	Nucleic Acid Sequence-based Amplification
NCBI	National Center for Biotechnology Information
NIST	National Institute of Standards and Technology
NTP	Nucleoside triphosphate
NTU	Nephelometric Turbidity Unit
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
OIE	Office International des Epizooties (World Organisation for Animal Health)
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PMA	Propidium monoazide
QMRA	Quantitative Microbial Risk Assessment
QPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
UV	Ultra Violet
VBNC	Viable but non-culturable
WHO	World Health Organization

Chapter 1

Introduction

1.1 Problem Statement and Motivation

The uncertain risk of many waterborne bacterial pathogens to human health is due to unreliable estimation (Environment Canada, 2001) and/or inconclusive outbreak investigations (National Research Council (U.S.), 1999). To address this problem, detection method improvement is crucial as well as additional surveillance investigations, to further identification of bacterial risk to public health.

Bacteria have long been detected using classical culture-based methods, based on bacterial growth on selective media, forming colonies. The colony and cell morphology and biochemical/immunological characteristics can then be studied for further identification. However, the rationale of these conventional methods is that living cells are able to grow/replicate. It is now known that “non-viability cannot be equated with non-culturability” (Moore *et al.*, 2001) and a large fraction of the total viable microbial population in an environmental sample are non-culturable (Colwell and Grimes, 2000). Therefore culture-based methods are only able to measure the number of viable, colony-forming cells and not viable-but-not culturable (VBNC) cells in water samples.

To address this problem, DNA-based methods such as polymerase-chain reaction (PCR) amplification have been developed. Although PCR was a significant step toward accurate and highly sensitive monitoring of live bacteria, it suffers from a major drawback. The detection using PCR is generally based on signals of amplified targets of nucleic acids which can originate from both live and dead cells. Therefore, it is important to develop techniques that can rapidly detect only viable cells, and mainly target only specific sub-groups that are infectious to humans.

1.2 Objectives

The overall goal of this research was to reliably evaluate the occurrence of specific pathogenic microorganisms in the aquatic environment through molecular-based methods. This key goal was achieved through the following specific objectives:

1. To develop and assess molecular-based methods for the detection of viable pathogenic bacteria.
2. To validate these detection methods and their application for environmental source water samples used for drinking water.

1.3 Thesis Organization

The thesis is composed of three main results chapters that were written in journal article format. One of these chapters has been published and the other two will be submitted to peer-reviewed journals. The overall thesis structure was formed consisting of six chapters as described below.

Following this introduction (Chapter 1), Chapter 2 provides background to introduce the subject material and additional preparatory material that was not included in the introduction to each results chapter, since they were prepared in journal manuscript format. Presented in this chapter is also an overview on microbial water safety, water safety plans, and microbial risk management as well as the importance of source water quality. Bacterial detection methods for water quality, molecular-based methods, and bacteria viability assays are briefly reviewed. Current and emerging bacteria targeted in this study and their occurrence in environmental waters are also described in this chapter.

Chapter 3 presents results of the study performed to evaluate the propidium monoazide-PCR viability assay to detect viable bacteria. It also includes the improvement made to the PMA-PCR viability assay to target long amplicon sizes. The details and results for the experiments performed on validating the improved viability method for heat-killed and UV-killed bacteria are also presented in this chapter.

The application of the PMA-PCR viability assay for viable bacterial detection in natural river water samples is documented in Chapter 4. The occurrence of selected enteric bacteria in samples collected from 2 locations on the Grand River was assessed by applying both PCR and PMA-PCR viability assays over a period of approximately 8 months. Recommendations are also made by comparing results of PCR with the PMA-PCR viability assay.

Chapter 5 focuses on the multi-parametric bacterial survival studies in natural (non-sterile) and sterile river water, which was also performed using both the PCR and the long amplicon PMA-PCR viability assays. The results and discussion related to the effect of temperature and dissolved oxygen as well as PMA pretreatment on bacteria survival trends are presented in this chapter.

Finally, Chapter 6 is dedicated to integrating the conclusions from each set of studies as well as an overall discussion and relevance of this work.

Chapter 2

Background

2.1 Microbial Safety of Drinking Water

Microbial safety of drinking water is essential at various levels including consumers, water suppliers, regulators, and public health authorities. A considerable number of reported waterborne outbreaks annually illustrate the importance of pathogenic microbial contamination transmitted through drinking water supplies (Medama *et al.*, 2003a). Waterborne pathogenic microorganisms have long been a main cause of human infection. One-third of the intestinal infections in the world are estimated to be related to waterborne diseases (Hunter, 1997). The occurrence of enteric diseases in Canada, with an incidence rate as high as 1.3 cases per person per year (Majowicz *et al.*, 2004), demonstrates the related health concerns.

Among the waterborne pathogens, those which cause bacterial enteric diseases in humans are of high concern. With water as one of their main routes of transmission (in addition to food and infected animals or humans) they can cause infection in humans through the ingestion of contaminated drinking water or through recreational use. At least 78 drinking water outbreaks were reported in Canada involving enteric bacteria between 1974 and 1996 (Todd and Chapman, 1996).

The traditional bacterial water quality indicators such as coliforms and enterococci as fecal pollution indicators were successful in improving the public health protection and drinking water safety to some extent; however, they were shown to be inadequate (Warburton, 1992; Jenkins *et al.*, 2011) and therefore waterborne illnesses may still occur by the consumption of drinking water that has been considered safe according to regulatory safety standards. Nevertheless, these traditional microbial indicators are still able to play an important role when the available resources are limited and can be helpful if properly used. Additional key approaches are needed toward improving the safety of drinking water. These approaches include: (1) Water safety plans, and (2) Risk assessment at all stages from source to tap (Medema *et al.*, 2003a). This “source to tap” or multi-barrier approach requires knowledge of source water quality, control of water treatment processes, distribution and storage system integrity, as well as appropriate monitoring and response (Figure 2.1). These elements are also able to guarantee a robust drinking water system, which is essential to provide safe drinking

water (Huck and Coffey, 2004). To protect public health through ensuring microbial drinking water safety, the above mentioned approaches need to be combined and performed properly by different responsible sectors (Figure 2.1), including water suppliers, regional/local government, water authorities, and public health authorities (Medema *et al.*, 2003a).

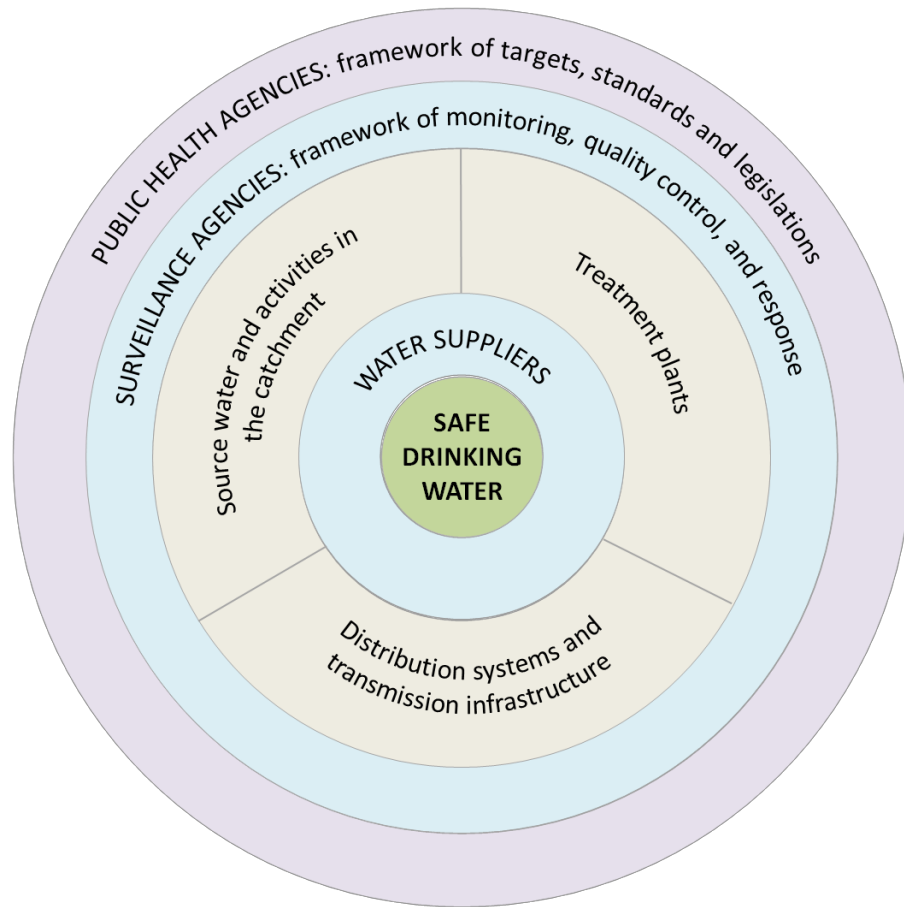


Figure 2.1 Drinking water safety from source to tap

Generally, in developed countries, water passes through many different treatment processes before it gets to the consumer, which highly reduces the risk of waterborne diseases. However, unpredictable contamination of source water and/or water treatment failure can still cause incidences of disease affecting the lives of a considerable number of people. Even in reliable water treatment systems, source water quality is of high concern due to the possibility of point sources or non-point sources of contamination. This critical role of source water was highlighted in the Clean Water Act in Ontario, Canada, which makes minimizing threats to source water a provincial priority (Ontario Ministry of Environment, 2006).

Pathogenic microorganisms including protozoa, bacteria, and viruses present in raw water are among the main targets for removal or inactivation in general water purification processes. Drinking water treatment processes are generally required to remove 99-99.99% of pathogenic microorganisms. Higher removal levels may be necessary for water supplies with high-impact microbial contamination sources. Bacteria are typically spherical (*cocci*), rod (*bacilli*), or spiral (*spirilli*) in shape and their sizes range from 0.3 to 2 μm . Bacterial pathogens are removed through conventional coagulation/filtration and chlorination as well as a number of enhanced treatment processes such as membrane filtration, ozonation, and ultraviolet (UV) disinfection. Coagulation/flocculation processes can remove between 0.2 to 2 log of bacteria, and between 2 to 3 log when combined with rapid filtration (Stanfield *et al.*, 2003). Due to their size range, bacteria are generally easier to remove through physical treatment processes such as filtration when compared to viruses; however, coagulation/flocculation processes are required to enmesh bacteria into particles large enough to be removed by conventional granular media filtration. Treatment or disinfection failure has been reported as the cause of many waterborne outbreaks (Hrudey and Hrudey, 2004). Particle removal processes or disinfection failure may occur due to fluctuations in bacterial loading as well as variability of chemical and physical factors that can affect treatment process efficiency, such as temperature, flow, turbidity, alkalinity, and pH.

2.2 Microbial Water Quality Testing

The use of traditional microbial water quality indicators (such as *Escherichia coli* and total coliforms) has improved the understanding of microbial water quality. However, a large number of waterborne gastrointestinal pathogens were discovered using pathogen testing methods, and results showed that the absence of conventionally used microbial indicators could not guarantee safe drinking water in terms of pathogenic microbial quality (Savichtcheva and Okabe, 2006). Yet, as water contains a large number of different microbial pathogens and pathogen testing methods are relatively specific, it is obviously not practical to perform so many tests.

Nevertheless, pathogen detection methods have become a valuable and necessary tool for water safety planning and risk management by providing information on microbial source water quality. This information is crucial to determine and set treatment goals, to evaluate the efficiency of current and new treatment processes, and also to initiate and evaluate corrective actions towards an improved microbial water quality, such as regulatory modifications. For example, Health Canada has developed a Drinking Water Risk Assessment Model for water treatment systems that can be applied to provide

a quantitative microbial risk assessment (QMRA) based on the occurrence of pathogens in source waters. The QMRA application however, is typically limited by a lack of data for microbial pathogen concentrations in source water, and pathogen detection methods are the key tools in filling this gap.

Traditionally, bacterial pathogen detection is performed by water sample concentration (e.g. filtration, centrifugation) followed by growing the target microorganism on selective media and counting colonies, which usually takes 1-3 days. Sometimes additional selective enrichment or treatment steps are required which further extend the analysis time. Still, these culture-based detection methods cannot assure that all the target microorganisms are taken into account, because they might have lost their ability to grow and/or reproduce (as discussed in the following section). For rapid, sensitive and specific pathogen detection, molecular technologies have been developed as discussed in detail in Section 2.4. Molecular methods have been endorsed by the World Health Organization (WHO, 2004), and have been suggested to “offer the best hope for improved and rapid detection of microbial contamination in water” (Dufour *et al.*, 2003). Although these methods are considered to be highly valuable, the viability of the detected microorganisms remains uncertain, which is known as one of the challenges with these methods (Medema *et al.*, 2003a).

2.3 Bacteria Viability and VBNC State

Bacterial viability has long been an important issue, not only from a clinical point-of-view, but also as a key factor in food and water safety. Using conventional methods, it was assumed that all viable bacteria would form detectable colonies, until 1982 when Xu *et al.* (1982) suggested a viability status referred as a ‘viable but nonculturable’ (VBNC) state. Extensive studies showed that a large number of bacteria inhabiting natural reservoirs are not capable of growing on laboratory growth media, and so are regarded as noncultivable bacteria (Oliver, 1999). It has been shown that in samples from soil and water environments, less than 1% of the total bacteria detected by direct microscopic tests are able to grow on culture media (Sardesai, 2005; Colwell *et al.*, 2000). Many variations in bacterial populations in natural environments previously described as the seasonal die-off of cells is now believed to be caused by the cells’ turning into a VBNC state (Sardesai, 2005).

The VBNC state can be defined as a failure of the bacterial cell to grow on the bacteriological media, while the cell is actually viable with a fluctuating metabolic activity (Lahtinen, 2007). This state is a kind of cell resistance to one or more environmental stresses, and once the stresses are gone the cell regains culturability (Oliver, 2005). A large number of environmentally and medically

relevant bacteria have been reported to enter the VBNC state. Around 100 species of bacteria in more than 30 genera are reported to show this physiological response (Oliver, 2006) which comprise many human pathogenic bacteria such as *Campylobacter*, *E. coli*, *Helicobacter pylori*, *Legionella pneumophila*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella*, *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Oliver, 2005).

2.4 Molecular Techniques

Molecular methods that are specific for the detection of viable cells are generally based on different signals that can be expected from a viable active cell, such as cellular integrity and activity, physiological responsiveness or metabolic activity, and the presence of nucleic acid presence (Keer and Birch, 2003). However, many existing methods for the detection of viable versus dead cells cannot be used to differentiate specific bacteria in mixed populations quantitatively, especially in environmental samples. Viable/dead staining-based detection technologies are not appropriate for analyzing different bacteria in mixed communities (Nogva *et al.*, 2003). Also, some methods of bacterial detection need a pre-enrichment step for environmental matrices, which can change natural conditions such as nutrients and biochemical interactions. These changes cause different sample characteristics from that of their natural state in the environment. However, methods that are able to detect nucleic acids to determine cell viability make it possible to evaluate environmental samples with a mixed population of bacteria, and so are the targets of this study.

2.4.1 Conventional and Quantitative PCR

The polymerase chain reaction (PCR) is a DNA amplification technique which is able to detect a target fragment of DNA even at very low quantities, and can generate a large number of a target DNA copies in a relatively short time (Toze, 1999). For this reason, it is commonly used for detecting microorganisms from environmental samples. PCR requires four components: a pair of primers, DNA polymerase, deoxyribonucleotides, and DNA template. The primers are short single-stranded pieces of DNA with a typical length of 20-30 nucleotides, each of which is synthesized to target a specific sequence on double-stranded DNA in a 5' to 3' orientation (Marmiroli *et al.*, 2007). PCR is generally performed in three steps, each at a different temperature. These three steps are then cycled. In the first step, double stranded DNA denatures into single strands at temperatures above 90°C. In the next step, the temperature is lowered to 50°C-60°C which lets the primers anneal to the target sequence or “amplicon.” The temperature is typically defined by the primer (primer annealing temperature).

Finally, at a temperature of usually 72°C the polymerase enzyme generates a copy of the amplicon. These temperature cycles are repeated several times. After 25-30 cycles, more than 10^9 - 10^{10} copies of the target DNA can be formed (Coleman and Tsongalis, 1997). This great number of target DNA fragments can then be detected using general nucleic acid detection methods (e.g. Wu *et al.*, 2006; Poltronieri *et al.*, 2008).

Quantitative PCR (qPCR) or real-time PCR has highly revolutionized the amplification methodology. QPCR makes it possible to monitor the amplification instantaneously. To detect the final amplification products by conventional PCR, the copies are labelled with DNA-binding fluorescent dyes and then detected by gel electrophoresis. Quantitative PCR, however, monitors in real-time the production of PCR amplification products. Also, using a closed system significantly reduces possible contamination (Mothershed and Whitney, 2006). In addition to two flanking primers, this method typically uses a fluorescently labeled reporter (probe) with an increasing signal directly proportional to the number of PCR products (Fratamico *et al.*, 2005). Real-time PCR results in several benefits over conventional PCR, including better sensitivity and rapidity, simplicity, reproducibility, and quantitative capacity (Yang and Rothman, 2004). Various fluorescent detection chemistries are presently used in real-time PCR analyses. The amplicon generation can be monitored instantaneously using fluorescent DNA intercalating dyes, such as SYBR-Green I, or using sequence-specific fluorescent-labelled internal DNA probes (Mackey *et al.*, 2007). Intercalating dyes attach non-specifically to double-stranded DNA produced through amplification, while probes specifically anneal within the target amplification region. Various types of probes can be used including TaqMan, fluorescence resonance energy transfer (FRET), and molecular beacons (Fratamico *et al.*, 2005). The probe produces a fluorescent signal during each amplification cycle, and so the signal increases as more target sequences are generated.

2.4.2 Nucleic Acid Targets

Nucleic-acid based methods can target different types of nucleic acids, such as DNA and various types of RNA (rRNA and mRNA). However it has been shown that the type of nucleic acid chosen as the target for molecular techniques can greatly influence the validity of its correlation with viability. This is probably because of the target gene stability. If the nucleic acids persist after cell death, it will lead to false-positive signals (Fey *et al.*, 2004). DNA was considered as an appropriate target nucleic acid, since it was assumed to degrade quickly after cell death (Jamil *et al.*, 1993). However, several studies have shown that DNA can remain intact long after cell death, and is therefore not an

appropriate target for viability detection (for example, Masters *et al.*, 1994; Young *et al.*, 2007). The half-life of DNA in non-viable bacterial cells is suggested to be dependent on environmental conditions and varies accordingly (Lindahl, 1993).

Ribosomal RNA (rRNA) has also been studied as a bacterial viability indicator, yet different studies have shown inconsistent results. rRNA has been reported to be highly correlated with viability (van der Vliet *et al.*, 1994; McKillip *et al.*, 1998), but its applicability as a viability marker has been suggested to depend on the type of applied stress (Tolker-Nielsen *et al.*, 1997; Lahtinen *et al.*, 2008). However, other studies have shown that rRNA can remain intact and be detected for longer periods of time, and therefore is considered to be an unreliable viability marker compared to mRNA (Keer and Birch, 2003). This has been demonstrated to occur in different stress situations including exposure to heat and ethanol (Uyttendaele *et al.*, 1997; Sheridan *et al.*, 1998).

2.5 Molecular Assessment of Cell Viability

2.5.1 Reverse transcriptase (RT)-PCR

mRNA has been suggested as an indicator of cell viability due to its instability and short half-life inside the cell. Therefore, even if the bacteria are inactive or in the VBNC state, some particular mRNA molecules should be produced to maintain cell viability (Sung *et al.*, 2004). Bustin (2002) suggests that the most common technique to determine or validate gene expression patterns and compare mRNA levels in diverse samples is reverse transcriptase (RT-PCR)-based methods.

RT-PCR involves the following two steps: (1) Reverse transcription, in which the target sequence of mRNA uses sequence-specific primers accompanied by the reverse transcriptase enzyme to transcribe into a complementary DNA (cDNA). (2) Amplification, when the single strand cDNA is used to form another cDNA strand using the DNA *Taq* polymerase. It is also possible that the single stranded cDNA serves directly as a substrate for PCR amplification (Keer and Birch, 2003). Using RT-PCR, it is always important to consider that any background DNA contamination can result in incorrect RNA quantification (Bustin, 2002). To solve this problem, either intron-flanking primers must be designed or the DNase enzyme should be used to remove DNA contamination (Keer and Birch, 2003).

2.5.2 NASBA

Kievits *et al.* (1991) first proposed and optimized the Nucleic Acid Sequence Based Amplification (NASBA) method for viral HIV-1 detection. As described by Rodriguez-Lazaro and Hernández (2006), the NASBA amplification method targets single-stranded RNA and utilizes three enzymes simultaneously under isothermal conditions: avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 RNA polymerase. Two specific oligonucleotide primers, complementary to sequences in the target RNA, are included in the reaction, one of which carries a recognition sequence for T7 RNA polymerase. The reaction also requires both dNTPs and NTPs.

The first primer containing the T7 RNA polymerase promoter attaches to the RNA, followed by complementary DNA (cDNA) strand production using the reverse transcriptase enzyme, forming an “RNA-DNA duplex” (Ginocchio, 2004). RNase H then identifies this duplex and digests the RNA part of the hybrid, and leaves single-stranded cDNA containing the T7 promoter sequence untouched (Loens *et al.*, 2005). The second primer then attaches to the cDNA, and the reverse transcriptase enzyme produces a double-stranded cDNA copy of the original sequence. Finally, this double-stranded DNA (with the T7 promoter sequence) is recognized and subsequently transcribed by the T7 RNA polymerase to generate great numbers of antisense RNA transcripts. The reaction continues cyclically.

Cools *et al.* (2006) describes a number of advantages of NASBA compared to PCR and RT-PCR. Since NASBA is an isothermal process, each step can proceed once the intermediary amplification products are available (Loens *et al.*, 2005). Therefore, it produces the same number of gene copies in a shorter time than RT-PCR, because the increase in each cycle is exponential in NASBA while it is binary in PCR (Chan and Fox, 1999). Compared to RT-PCR, NASBA is done at a single temperature, usually 41°C, and therefore the DNA from target bacteria does not denature and stays double-stranded, and cannot become a substrate for amplification (Edwards *et al.*, 2004). As a result, a DNase pre-treatment is not needed, unlike the RT-PCR method (Cook *et al.*, 2003). NASBA uses shorter incubation times (90-150 min) compared to RT-PCR which requires 3-5 h (Cools *et al.*, 2006).

NASBA also has a number of weaknesses. Since NASBA is an isothermal reaction, the number of cycles cannot be adjusted to control the level of the reaction (Cools *et al.*, 2006). NASBA results in less efficient amplification, if the length of the target sequence on RNA is not in the appropriate range of 120-150 nucleotides (Loens *et al.*, 2005). While using NASBA, it is more likely to result in non-

specific amplification, since the temperature should not go above 41°C to prevent enzymatic denaturation (Tai *et al.*, 2003; Cools *et al.*, 2006; Loens *et al.*, 2005).

2.5.3 EMA/PMA-PCR

Physical dissimilarities between viable and dead cells are broadly employed in both microscopy and flow cytometry (Lahtinen, 2007). Ethidium monoazide (EMA) has been used as a live/dead stain in many of these methods (Nogva *et al.*, 2003). Ethidium is a typical nucleic acid ligand, and EMA (3-amino-8-azido-5-ethyl-6-phenyl-phenanthridinium chloride) is an azide derivative of ethidium (Soejima *et al.*, 2007). EMA is able to intercalate with DNA, generating stable complexes (Figure 2.2), and is used in DNA photolabelling techniques (Reidy *et al.*, 1990).

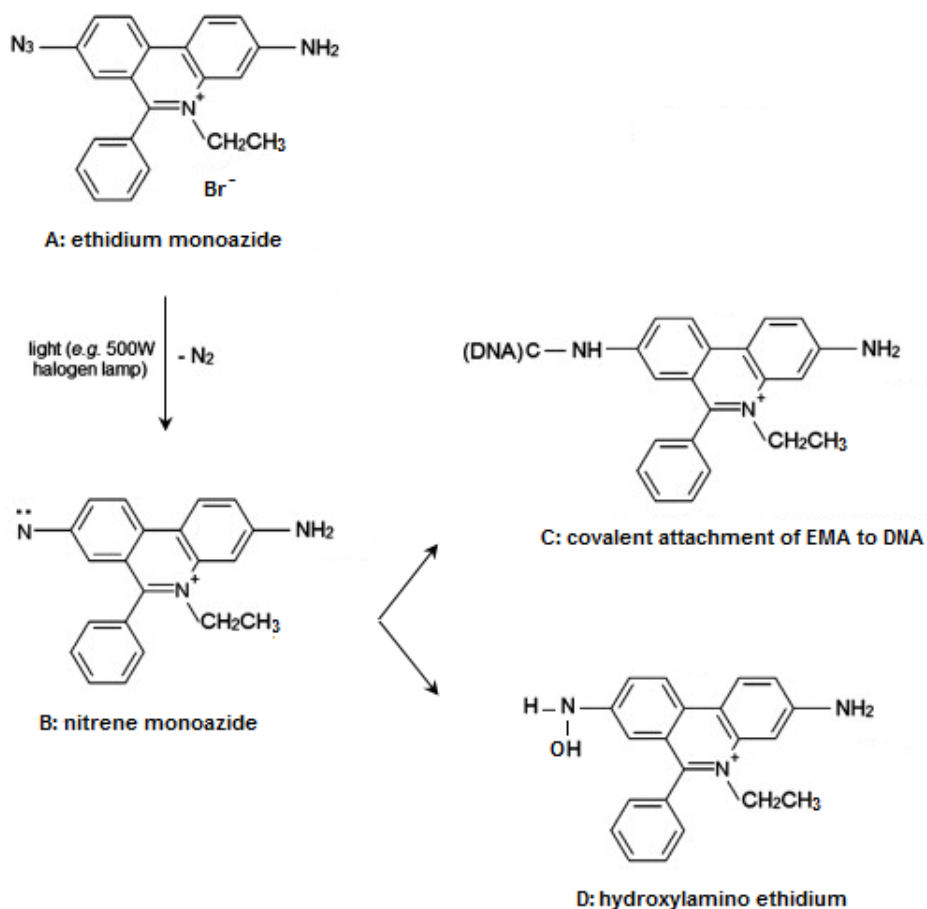


Figure 2.2 (A) EMA, a DNA intercalating agent. (B) Photoreactivation of the EMA produces an extremely reactive nitrene. (C) The reactive nitrene will form a covalent bond with DNA. (D) The unused free EMA is photolyzed and converts to hydroxylamino ethidium (HA). Figure based on Soejima *et al.* (2007).

EMA/DNA non-covalent intercalation is generally reversible. However, if EMA is exposed to low-intensity irradiation of visible light (Soejima *et al.*, 2007; Reidy *et al.*, 1990) or UV (Jepras *et al.*, 1995), it forms irreversible cross-links by covalent bond (Figure 2.2C). EMA photolysis produces an extremely reactive nitrene (Jepras *et al.*, 1995) (Figure 2.2B). This nitrene radical subsequently forms covalent bonds with DNA (Reidy *et al.*, 1990; Nogva *et al.*, 2003). The unused free EMA in the sample is photolyzed concurrently resulting in hydroxylamino ethidium (HA), which is no longer reactive and cannot form covalent bonds (Figure 2.2D) (Soejima *et al.*, 2007; Nogva *et al.*, 2003).

EMA-PCR is suggested to be a promising method for assessing cell viability, as was first described by Rudi *et al.* (2002, 2005) and Nogva *et al.* (2003). Their goal was to employ the irreversible binding of photoactivated EMA to DNA, and thus to prevent PCR amplification from dead bacteria. This goal was based on the fact that like many other nucleic acid binding dyes, EMA enters dead cells with broken membranes but not live cells (with intact membranes) (Figure 2.3). However the mechanism for the EMA exclusion from the live cells is not clear as studied by Rudi *et al.* (2005), although their results indicate it may be due to a passive exclusion mechanism and not an active efflux process.

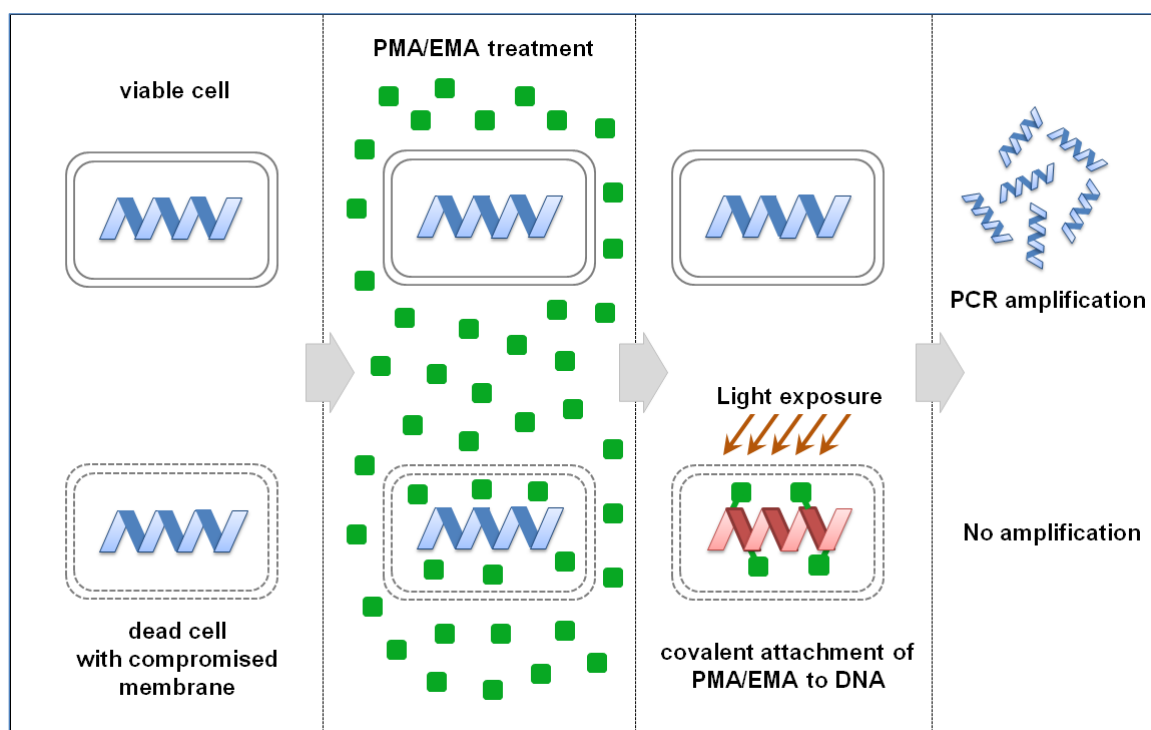


Figure 2.3 EMA/PMA enters dead cells with broken membranes but not live cells. The irreversible binding of photoactivated EMA/PMA to DNA prevents PCR amplification from dead bacteria

More recent studies (Rueckert *et al.*, 2005; Nocker and Camper, 2006; Flenka *et al.*, 2007; Pitz *et al.*, 2007; Cawthorn and Witthuhn, 2008) have shown that EMA is able to enter not only dead cells but also viable cells, yet to a lesser extent in live cells. EMA has also been suggested to have cytotoxic effects on intact live cells (Rueckert *et al.*, 2005). The level of EMA taken up by live cells is reported to vary between bacteria (Nocker *et al.*, 2006; Flenka *et al.*, 2007) and is also highly dependent on the EMA concentration in the treated sample (Rueckert *et al.*, 2005). For example, Nocker and Camper (2006) suggested that EMA can only be regarded as membrane impermeable to a number of specific bacteria which are more resistant to EMA staining, such as *Salmonella typhimurium* and *Serratia marcescens*, with no loss of DNA. However, it was considered not to be appropriate for *Pseudomonas syringae*, which can be relatively easily stained with EMA. For these reasons, EMA-PCR is considered to perform poorly as a viability assay.

Nocker *et al.* (2006) proposed the use of an alternative, propidium monoazide (PMA) (Figure 2.4). PMA is similar to propidium iodide (PI), with an extra azide group providing the cross-connecting characteristic of PMA to DNA when exposed to visible light. Nocker *et al.* (2006) found that PMA acts more selectively compared to EMA which is suggested to be due to the higher positive charge of PMA compared to EMA (Nocker and Camper, 2006; Cawthorn and Witthuhn, 2008).

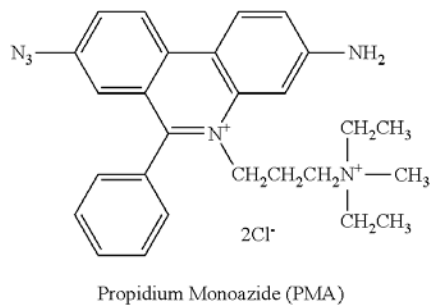


Figure 2.4 Propidium monoazide (PMA) structure

2.6 Current and emerging bacterial pathogens of concern in water

Current bacterial pathogens of concern as described by the Guidelines for Canadian Drinking Water Quality (2006) are those groups of enteric pathogens which have historically caused gastrointestinal infection and are known to be a cause of waterborne enteric illnesses. Emerging pathogenic bacteria, on the other hand, are those which are newly and progressively more recognized to be associated with waterborne diseases and include those that have recently been discovered.

Surface water pollution by bacteria is known to mainly originate from a number of contamination sources including wastewater effluents in urbanized areas, stormwater discharges (urban and agricultural runoff), livestock, and wild animals (mammals and birds). Among many of the current bacterial pathogens of concern in water, *Salmonella*, *Campylobacter*, *Yersinia*, and *E. coli* O157:H7 are the major bacterial contaminants that are commonly found in relatively high concentrations in all of the above mentioned pollution sources (Medema *et al.*, 2003b) and hence have been selected as the focus of this study. In addition, *Arcobacter* was selected for this study, as it is a recently suggested emerging waterborne bacterial pathogen (Lee *et al.*, 2012), as described in further detail below.

2.6.1 *Salmonella*

Salmonella are rod-shaped, non-spore forming, Gram-negative *Enterobacteriaceae*. They were first described by Theobald Smith and Daniel Elmer Salmon and isolated from the intestine of a pig during a study on swine fever, and was initially known as *Salmonella choleraesuis* (Ellermeier and Schlauch, 2006). The genus *Salmonella* consists of two species including *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into six subspecies (*enterica*, *salamae*, *diarizonae*, *indica*, and *houtenae*). Out of 2400 *Salmonella* serotypes that have been described, just around 50 serotypes are associated with infections in humans and warm blooded animals and are all within the subspecies *enterica* (Levantesi *et al.*, 2012). *Salmonella* is a known foodborne zoonotic bacterium and hence, several animals are reported as reservoirs, including domestic species (cattle, chickens, pigs) (Wray and Wray, 2000) and wild animals such as birds (Tizard, 2004), rodents (Healing, 1991) and hedgehogs (Handeland *et al.*, 2002).

Salmonella can be divided into the typhoidal and non-typhoidal serotypes based on their clinical syndromes. However, *Salmonella* serotypes Typhi, which are host specific (human only), are no longer common in developed countries. As such, waterborne typhoid fever incidents have become rare in developed countries due to extensive municipal water and wastewater treatment (Levantesi *et al.*, 2012). Non-typhoid *Salmonella* serotypes on the other hand are more common in industrialized countries and are found in animals as well as humans. They are able to cause acute, but usually self-limiting gastroenteritis as well as possibly more severe non-enteric diseases such as pneumonia and meningitis (Pond, 2005). The human infective dose of *Salmonella* is serotype-dependent. The non-typhoidal salmonellosis infectious dose is approximately 10^3 bacilli, which might vary between patients (Blaser and Newman, 1982).

The aquatic environment can be contaminated by *Salmonella* contamination through various routes such as wastewater effluent discharge and runoff which has been impacted by agricultural activities, livestock, and wild animals (Wray and Wray, 2000). Surface water environments are considered as natural reservoirs of *Salmonella*. *Salmonella* have been detected in low and highly impacted water sources with high detection frequencies; however, detection frequencies can vary between source waters due to variable *Salmonella* inputs into a watercourse (Thomas *et al.*, 2012) and/or environmental limitations that can affect the survival and transport of these bacteria (Lavantesi *et al.*, 2012).

Non-typhoidal *Salmonella* was the cause of 8% of drinking water outbreaks (a total of 15 outbreaks) in the US between 1971 and 2000 (Craun *et al.*, 2004). Only infrequent drinking water outbreaks caused by non-typhoidal *Salmonella* were reported in the following six years (2000-2006) (Lavantesi *et al.*, 2012). In March-April 2008, a *Salmonella* waterborne outbreak in Alamosa, Colorado resulted in more than 440 reported illnesses and one death, and was ultimately attributed to a lack of chlorination for disinfection of groundwater (Falco and Williams, 2009). This was followed by another *Salmonella* outbreak in 2010 caused by consumption of unprotected spring water (Kozlica *et al.*, 2010).

2.6.2 *Campylobacter*

Campylobacter was first discovered in 1886 while Theodor Escherich was studying infants that died from 'cholera infantum' (Skirrow and Butzler, 2000). Later, several researchers isolated campylobacters and classified them as various *Vibrio* spp. (McFayden *et al.*, 1913; King, 1957; 1962). In 1963, the genus *Campylobacter* was suggested by Sebald and Veron (1963). *Campylobacter* was considered as an animal pathogen until 1972 when it was isolated from human blood and feces (Dekyser *et al.*, 1972). Bultzer *et al.* (1973) reported *Campylobacter* spp. to be frequently associated with human diarrhea. Based on these studies, *Campylobacter* was accepted as an important pathogen for both human and animals.

Campylobacter spp. are an important cause of diarrheal illness, causing 5%–14% of diarrhea worldwide (Heymann, 2004). These organisms are one of the main bacterial causes of diarrheal illnesses in developed countries (Miller and Mandrell, 2005). Annually, *Campylobacter* infections occur in approximately 50-100 per 100,000 population, yet the true occurrence of *Campylobacter* infections is not well monitored through laboratory investigations (Percival *et al.*, 2004). 2.5 million *Campylobacter* infections are reported each year in the USA (Mead *et al.*, 2000). The threat of

Campylobacter to humans is by gastrointestinal disease caused mainly by thermophilic campylobacters such as *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari*, and *Campylobacter upsaliensis* (Koenraad *et al.*, 1997). Infection initially occurs in the gastrointestinal tract and usually causes diarrheal illness. In some cases, *Campylobacter* can become extraintestinal, particularly in individuals with underlying illness, and can result in more severe illnesses including bacteraemia, pseudoappendicitis, and Guillian-Barre syndrome (Young and Mansfield, 2005). The infectious dose of *Campylobacter* in humans is estimated to be 500-1000 organisms, depending on the intensity of the disease (Bhunia, 2008).

Campylobacter has been isolated from avian sources including domesticated and wild birds (Miller and Mandrell, 2005). *C. jejuni*, which is the most frequent cause of human *Campylobacter* infection (Lin, 2009), is found primarily in poultry. Since the optimal growth conditions are provided, it can favourably grow in intestinal mucosa, particularly the cecum of birds (Wassenaar and Newell, 2007). It has also been isolated less frequently from other non-avian species including cattle, sheep, and pigs, and wildlife including primates, ungulates, wild cats, canines, bears, ferrets, and rodents (Miller and Mandrell, 2005). *Campylobacter* can optimally grow at 42°C and generally within the range of 30-45°C (Lawley *et al.*, 2008). Therefore, it cannot reproduce when it enters the environment or at ambient temperatures in foods (van de Giessen *et al.*, 1996). Despite the above limitations, *Campylobacter* can survive in food and aqueous environments at infectious doses, resulting in outbreaks (Miller and Mandrell, 2005).

Campylobacter are microaerophilic bacteria. They are able to grow on culture media with 5–10% oxygen and 3–5% CO₂ (Wassenaar and Newell, 2007). In 1977, a selective medium for *Campylobacter* spp. was developed (Skirrow *et al.*, 1977). The development of more specific isolation techniques such as using different selective media including blood-containing media (e.g. Preston agar, Skirrow agar, Bultzer agar, Campy-cefex) and charcoal-based solid media (e.g. mCCDA, CSM, CAT), showed the real pathogenic potential of these organisms (OIE, 2008). Although *Campylobacter* is detectable using conventional culture methods (Bolton *et al.*, 1999), it is difficult to isolate in the laboratory due to its fastidiousness and stringent growth conditions (Bhunia, 2008) and also due to the fact that they are able to readily form VBNC cells in order to persist in unfavorable environments (Baffone *et al.*, 2006). This will cause underestimation in the environment and in human disease occurrences, and can cause problems with outbreak investigations.

Campylobacter spp. are commonly occurring pathogenic bacteria in the environment. They have been found in surface and sea waters, as well as in municipal sewage and non-disinfected sewage effluents (Miller and Mandrell, 2005). Unlike sewage effluent, the number of *Campylobacter* detected in fresh waters is usually low (Miller and Mandrell, 2005). *Campylobacter* spp. can live for several weeks in the viable but nonculturable state in groundwater (4°C) (Gondrosen, 1986) and in surface water (Chan *et al.*, 2001). *C. jejuni* has been found in water more than the two other predominant *Campylobacter* species, *C. coli* and *C. lari* (Percival *et al.*, 2004). However, other studies have shown that *C. lari* was the predominant strain isolated from rivers in Southern Ontario (Van Dyke *et al.*, 2008). Although foodborne causes of *Campylobacter* infection are well studied, transfer pathways of *Campylobacter* from the water to humans is less well understood, probably because of the complexity of its behavior in the aquatic environment (Schallenberg *et al.*, 2005). Some research has been done on the survival of *Campylobacter* using modeling simulations (Brown *et al.*, 2004; Skelly and Weinstein, 2003), and some on experimental simulations (Obri-Danso *et al.*, 2001; Thomas *et al.*, 1999). However, limited studies have been performed on the survival of *Campylobacter* in real environments (Korhonen and Martikainen, 1991). It was shown that *Campylobacter* cannot survive more than a few hours in unfavorable conditions, including exposure to undesirable temperatures (Fratamico *et al.*, 2005), with improved survival at low temperatures (Korhonen and Martikainen, 1991). The survival of *Campylobacter* was reported to be significantly enhanced in mixed samples with other organisms (Buswell *et al.*, 1998).

Outbreaks due to *Campylobacter* have been linked to drinking water (Vogt *et al.*, 1982; Brieseman, 1987; Sobsey, 1989; Skirrow and Blaser, 1992; Jones and Roworth, 1996; Pebody *et al.*, 1997; Clark *et al.*, 2003). *C. jejuni* has been the cause of around 90% of the *Campylobacter* outbreaks, while *C. coli* was reported to be the cause of 5-10% of the other identified cases (Miller and Mandrell, 2005). In the UK, *Campylobacter* spp. have been the major cause of outbreaks in water that has not been effectively disinfected (Duke *et al.*, 1996; Furtado *et al.*, 1998). In Canada, *Campylobacter* has been considered the cause of waterborne outbreaks, such as the Orangeville outbreak in 1985 with over 200 cases of gastroenteritis caused by *C. jejuni* contamination of unchlorinated groundwater (Hrudey *et al.*, 2002). In 2000, the waterborne outbreak in Walkerton, Ontario caused over 2000 cases of gastrointestinal illness which were known to be associated with *Campylobacter* and *E. coli* O157:H7. Although it has been described as an *E. coli* outbreak, 41% of stool samples were *Campylobacter* positive (Miller and Mandrell, 2005). This is considered the fourth largest *Campylobacter* outbreak in North America (Miller and Mandrell, 2005). In several European countries, *Campylobacter* is

regarded as the most significant bacterial cause of waterborne infections (Strenstrom *et al.*, 1994; Furtado *et al.*, 1998). Although many waterborne outbreaks of *Campylobacter* involving large numbers of people have been reported in the literature (Pebody *et al.*, 1997; Miller and Mandrell, 2005), the bacteria causing most waterborne outbreaks have seldom been isolated (Szewzyk *et al.*, 2000), likely caused by *Campylobacter* occurrence in a VBNC form (Percival *et al.*, 2004).

2.6.3 *Arcobacter*

Arcobacter is a subgroup of the *Campylobacteraceae* family, which includes *Campylobacter*, *Arcobacter*, and *Sulfurospirillum* (Fratamico *et al.*, 2005). *Arcobacter* are aerotolerant *Campylobacter*-like bacteria first isolated from aborted bovine fetuses (Ellis *et al.* 1977; Vandenberghe *et al.*, 2004). They were preliminary classified as "aerotolerant campylobacters" because of their similar morphology to campylobacters and their similar microaerobic growth conditions (Phillips and Bates, 2004). Yet they can actively grow under aerobic conditions and, unlike thermophilic pathogenic campylobacters, at temperatures lower than 30°C (Wesley *et al.*, 1995; Diergaardt *et al.*, 2003; Vandenberghe *et al.*, 2004; Phillips and Bates, 2004). Their fatty acid profile is also different from campylobacters (Wesley *et al.*, 1995). The genus *Arcobacter* was proposed by Vandamme and De Ley (1991), classifying them differently from genus *Campylobacter*. They have been reported to survive better in Brain Heart Infusion (BHI) broth at low temperatures compared to *Campylobacter* (Kjeldgaard *et al.*, 2009). Therefore, they are more likely to survive better than *Campylobacter* in the environment (Diergaardt *et al.*, 2003).

Arcobacter currently includes the species *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter nitrofigilis*, *Arcobacter skirrowii*, and *Arcobacter cibarius* (Houf *et al.*, 2005) and also a newly described species *Arcobacter halophilus* (Donachie *et al.*, 2005). *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*, (Houf *et al.*, 2005) and also *A. cibarius* (Donachie *et al.*, 2005), are considered to be infectious to animals and humans. The International Commission on Microbiological Specifications for Foods (ICMSF, 2002) has introduced *A. butzleri* as a "serious hazard to human health" (Atabay *et al.*, 2006). *Arcobacter* causes the same clinical disease symptoms and microbiological features as *C. jejuni* infection (Vandenberghe *et al.*, 2004; Phillips and Bates, 2004). Yet it has been reported to be generally more persistent and more often related to watery diarrhea and not related as much to bloody diarrhea as *C. jejuni* (Vandenberghe *et al.*, 2004).

Arcobacter spp. have been reported in the feces of animals such as cattle (Logan *et al.* 1982; Van Driessche *et al.*, 2005), swine (Suarez *et al.* 1997), and birds (Atabay *et al.*, 2008). They have also

been isolated from the intestines of healthy livestock (Lehner *et al.*, 2005). The occurrence of *Arcobacter* spp. in food is not clearly known. Poultry have been suggested as a highly prevalent reservoir of *Arcobacter* spp. (Lehner *et al.*, 2005). However, its original source is not well known (Van Driessche *et al.*, 2005). Meat is also regarded as a potential source of *Arcobacter* infection in humans (Rivas *et al.*, 2004; Ho *et al.*, 2006). In addition to its occurrence in food, *Arcobacter* isolates have also been reported from drinking water reservoirs, surface water, groundwater, and sewage (Ho *et al.*, 2006), and therefore water has the potential of transmission of human diarrhea caused by *Arcobacter* (Lehner *et al.*, 2005). *A. bultzeri* from contaminated water has been reported be the cause of more than 60% of *Arcobacter* human infections (McClure, 2002). *Arcobacter* has also the potential of forming biofilms in water distribution systems (Assanta *et al.*, 2002; Van Driessche and Houf, 2008). Therefore, they are able to survive cleaning and disinfection processes (Van Driessche and Houf, 2008).

The detection methods used for fecal samples have generally underestimated the importance and true occurrence of human infections caused by *Arcobacter* (Houf and Stephan, 2007). To date, no standardized isolation protocol has been accepted for the detection of *Arcobacter* spp. (Cervenka, 2007; Fernandez *et al.*, 2004). Numerous studies compared different culture-based protocols for *Arcobacter* detection in recent years (Phillips, 2002; Lehner *et al.*, 2005). *Arcobacter* can be aerobically cultured in an enrichment broth at 25°C. The incubation time of a targeted *Arcobacter* spp. can be relatively long (average of 4-5 days) (Snelling *et al.*, 2006). There are a number of commercially available isolation media for *Arcobacter* spp. such as cefoperazone, amphotericin B and teicoplanin agar (CAT), and charcoal cefoperazone, deoxycholate agar (CCDA) (Lehner *et al.*, 2005; Atabay *et al.*, 1998). Studies have also been performed to detect *Arcobacter* spp. using PCR-based methods (Harmon and Wesley, 1997; Houf *et al.*, 2000; Antolin *et al.*, 2001; Kabeya *et al.*, 2003; González *et al.*, 2007; Atabay *et al.*, 2008).

2.6.4 *Yersinia enterocolitica*

Yersinia enterocolitica was described more than 70 years ago (Schleifstein and Coleman, 1939), however, it only started to gain research interest about 20 years later when it was isolated from animal species and was considered as a possible cause of human infection. It has become known as an emerging food and waterborne bacterial pathogen over the past 30 years (Skovgaard, 2007) as it was increasingly found to be more related to human disease (Fukushima *et al.*; Sharma *et al.*, 2003).

Y. enterocolitica is a rod-shaped, Gram-negative, facultative anaerobe belonging to the *Enterobacteriaceae*. The genus *Yersinia* consists of 11 species (Chen *et al.*, 2010), three of which are known to be infectious to human, including: *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Y. enterocolitica* (Percival *et al.*, 2004). There are other *Y. enterocolitica*-like species (*Y. intermedia*, *Y. frederiksenii*, *Y. mollaretii*, *Y. kristensenii*, *Y. aldovae*, *Y. bercovieri*, *Y. ruckeri* and *Y. rohdei*) which are not yet well defined in terms of human pathogenicity (Sabina *et al.*, 2011). Among the species of *Yersinia*, *Y. enterocolitica* are commonly isolated from source waters (Percival *et al.*, 2004).

Y. enterocolitica can cause human infections particularly intestinal diseases with symptoms such as fever, abdominal pain, and often bloody inflammatory diarrhea, pseudoappendicitis, post-infectious extraintestinal manifestations such as joint pain (reactive arthritis), and skin rash (erythema nodosum) (Fàbrega and Vila, 2012). However, not all biotypes of *Y. enterocolitica* are pathogenic. *Y. enterocolitica* consists of six biotypes (1A, 1B, 2, 3, 4, and 5) and more than 50 distinct serotypes (Bari *et al.*, 2011) which include pathogenic strains (e.g. 4/O:3 and 2/O:9), and non-pathogenic strains (e.g. biotype 1A and *Y. enterocolitica*-like species discussed above) (Huovinen *et al.*, 2010).

Many foodborne *Yersinia* outbreaks have been reported (Health Canada, 2006). *Y. enterocolitica* infection is usually caused by consuming contaminated food such as raw or undercooked pork, meat, oysters, mussels, shrimp, blue crab, fish, salad, carrots, cabbage, celery, mushrooms, and milk (Rahman *et al.*, 2011), or untreated water (Sabina *et al.*, 2011), with food being considered as the major source of yersiniosis. Only in rare cases have pathogenic *Y. enterocolitica* been isolated from food samples, possibly due to method limitations (Rahman *et al.*, 2011). In rare cases, *Y. enterocolitica* has also been reported to transfer from person-to-person (Moriki *et al.*, 2010).

It is not yet clear whether humans act as *Y. enterocolitica* reservoirs. It has been isolated from a small percent of asymptomatic humans (Bari *et al.*, 2011; Bottone, 1997). On the other hand, a large variety of animals are considered to be a significant *Y. enterocolitica* reservoir including rabbits, livestock (e.g. pigs, poultry, sheep, cattle), cats, horses, deer, and raccoons. Among these animals, pigs are known to be the major reservoir for *Y. enterocolitica* involved in human infections (WHO, 2004; Wang *et al.*, 2010). Pathogenic and non-pathogenic *Y. enterocolitica* have been infrequently isolated from sewage (Falcão *et al.*, 2004) and drinking water (Weber *et al.*, 1981). Drinking water has been only reported to be a reservoir to non-pathogenic *Y. enterocolitica* (Aleksic and Bockemuhl, 1988; Arvanitidou *et al.*, 1994; Waage *et al.* 1999; WHO, 2004). However, several studies have shown the presence of pathogenic *Y. enterocolitica* in environmental surface water samples (e.g.

Sandery *et al.*, 1996; Falcão *et al.*, 2004; Cheyne *et al.*, 2010) indicating that pathogenic *Y. enterocolitica* can be transmitted through contaminated surface and environmental waters.

Waterborne *Yersinia* outbreaks have been reported in Europe (Eden *et al.*, 1977), Japan (Inoue *et al.*, 1988), Canada (Thompson *et al.*, 1986) and United States (Craun *et al.*, 2004), most of which were associated with *Y. enterocolitica*. *Yersinia* has also been known to survive for long times at cold water temperatures and is considered to be a psychrotrophic bacteria. Several studies have reported *Yersinia* to be able to survive for several months in low temperature waters (Harvey *et al.*, 1976; Highsmith *et al.*, 1977).

2.7 Viability assays and method selection

The PMA-PCR viability assay was selected for use in this thesis primarily because it was the most commonly applied method available in the literature at the commencement of this project (Feb, 2009). This study then improved and optimized the method further (Chapter 3). This method was selected from the previously discussed viability detection methods for a number of reasons. PMA-PCR is a method that allows the use of DNA detection for viability assessment. Post-purified DNA “robustness” makes it a more reliable detection marker, while RNA can degrade more rapidly and is prone to the risk of genomic DNA contamination. RT-PCR and NASBA are both RNA-based methods that are suggested to target mRNA to improve the reliability of viable cell detection, since mRNA is believed to degrade quickly after cell. However, mRNA is highly labile and therefore the analysis can vary between the target bacterial species, bacterial killing treatments, and different storage conditions. Moreover, with mRNA as the viability marker, there is always the chance of false negative detection in VBNC cells where some mRNAs are not necessarily expressed (Cenciarini-Borde *et al.*, 2009). Thus, the mRNA-based viability assays must target genes with continued transcription in bacteria that are in a VBNC state. For these reasons, the RNA-based methods are more time-consuming and more challenging to apply as viability assays and cannot be used by all laboratories. Since this work partly aims to provide and suggest viability assays as appropriate tools for a wide range of researchers and testing laboratories, it was decided to focus on the PMA-PCR viability assay, which turns out to be relatively quick, easy to perform, and less case-dependent.

2.8 Research gaps

This research was motivated based on the following identified research gaps.

2.8.1 Assessing PMA-PCR effectiveness as a bacterial viability assay

The application of PMA pretreatment to PCR (PMA-PCR) as a viability assay has been suggested in recent years and is gaining more attention. However, there has been a debate on the effectiveness of PMA-PCR in differentiating the live from the dead cells. Several studies have reported that although PMA-PCR was able to reduce the false positive signal from non-viable cells, the signal removal was only partially effective (Pan and Breidt, 2007; Kralik *et al.*, 2010). Further investigations were needed to assess the effectiveness of PMA pretreatment prior to PCR in targeting viable bacteria only, using appropriate experimental approaches and also by targeting specific enteric bacterial pathogens commonly found in source waters used for drinking water.

2.8.2 Improving the PMA-PCR viability assay

The PMA-PCR assay can reliably measure viable bacteria in water samples only when dead cells are not detected. The suggested PMA mechanism of action (PMA intercalation with DNA) as explained in the literature (Nogva *et al.*, 2003) was not able to explain the reasons behind partial false positive signal reduction. There was one study which suggested additional mechanisms of EMA/DNA interaction (DNA cleaving effect of EMA) (Soejima *et al.*, 2007). Additional investigations on the available knowledge of PMA/DNA interaction was thought to be able to explain the reason behind the incomplete false positive signal removal by PMA-PCR, which could lead to possible method improvement to address the issue.

Moreover, since the PMA-PCR is a viability assay based on dead cell permeability, researchers have been questioning if this viability assay is applicable to UV-killed cells (Nocker *et al.*, 2007a; Parshionikar *et al.*, 2010). This is important when applying viability enumeration assays to microbial pathogens present in natural source waters which are exposed to sunlight. Answering this question can help to better identify whether the PMA-PCR viability assay can be reliably used to detect pathogenic bacteria in natural surface waters.

2.8.3 Bacterial detection in source water using viability assays

Although PMA-PCR viability assays have been applied to detect various microorganisms, there are only a limited number of studies that have applied this viability assay to environmental water samples (Nocker *et al.*, 2007b; Nocker *et al.*, 2010). Further studies are needed to better evaluate the applicability of PMA-PCR to source water quality investigations. Based on the small number of studies available in this area, it is not clear whether dead bacteria are present in river waters and if it is

necessary to apply viability assays when measuring in bacterial pathogens in source waters used for drinking water.

Also there have been no previous studies on the correlation between pathogenic bacteria as measured using a PMA-PCR viability assay and bacterial indicators (such as total *E. coli*) in natural waters. Comparing correlation data between viable and total cells may improve the understanding on the usefulness of the bacterial indicators in evaluating the disease-causing pathogens present in surface waters.

2.8.4 *Arcobacter*: a potential waterborne emerging bacterial contaminant

Arcobacter spp. are recently gaining more attention as potential foodborne and waterborne pathogens. However, due to a small number of related studies in water, the significance of their occurrence in natural surface waters is not yet clear. Extensive studies are necessary on the occurrence and concentrations of this bacterial group in source waters and particularly on the pathogenic species such as *A. butzleri*. In parallel, it is helpful to determine the levels of viable pathogenic *Arcobacter* spp. in source waters to better identify the potential health risk associated with these bacteria.

2.8.5 Impact of environmental conditions on the survival of viable bacteria

The survival of many waterborne enteric pathogenic bacteria in surface waters and the effect of various environmental conditions have previously been studied. However, the detection of bacteria in these studies using molecular detection methods, and using molecular viability methods have not been previously done. Therefore, it is important to re-evaluate the survival trends of enteric bacteria of concern in surface waters under a number of key environmental stresses using these improved methods. For example, although the effect of temperature on bacterial survival has been extensively investigated, it is not clear whether similar survival trends can be expected under various temporal conditions if PCR methods that target viable cells are used. Additionally, there are limited studies on bacterial survival in water under a number of other environmental stresses such as dissolved oxygen and in the presence of background microflora. Therefore, improved understanding of viable bacterial survival under these conditions is needed.

2.8.6 Research goals

Based on the research needs described above, the goals of this study were to:

1. Assess the effectiveness of the PMA-PCR viability assay in removing the PCR signal originating from dead cells or extracellular DNA.
2. Improve the PMA-PCR viability assay in order to achieve reliable viable bacterial detection through reduction of false positive signal from non-viable cells or extracellular DNA.
3. Investigate the effectiveness of PMA pretreatment prior to PCR in differentiating viable and UV-killed bacterial cells and provide appropriate solutions for the reliable detection of live/UV-killed cells.
4. Apply the PMA-PCR viability assay to evaluate the levels of dead bacterial cells in natural river water and provide reliable data on the occurrence of target enteric bacterial pathogens in river water.
5. Evaluate the effect of viable bacteria detection in river water on the applicability of bacterial indicators (total *E. coli*) in bacterial water quality analyses.
6. Provide better understanding on the occurrence of *A. butzleri*, a potential emerging waterborne bacterial pathogen in river waters.
7. Provide better understanding on the effect of environmental stresses (such as temperature, background biota and DO levels) on bacterial survival trends in river water by detecting only viable bacteria.

Chapter 3

Long-amplicon propidium monoazide-PCR enumeration assay to detect viable *Campylobacter* and *Salmonella*

This chapter is based on a published article with the same title in the Journal of Applied Microbiology (August 2012), volume 113, issue 4, pages 863-873. Cited references are in the consolidated list of references at the end of the thesis.

3.1 Summary

The effect of amplicon length on the ability of propidium monoazide-PCR (PMA-PCR) to reliably quantify viable cells without interference from dead cells was tested on heat- and ultraviolet (UV)-killed *Salmonella enterica* and *Campylobacter jejuni*, two important enteric pathogens of concern in environmental, food and clinical samples. PMA treatment followed by quantitative PCR (qPCR) amplification of short DNA fragments (<200 bp) resulted in incomplete signal inhibition of heat-treated *S. enterica* (3 log reduction) and *C. jejuni* (1 log reduction), whereas PCR amplification of a long DNA fragment (1.5 and 1.6 kb) strongly suppressed the dead cell signal. PMA pretreatment of UV-irradiated cells did not affect PCR amplification, but long-amplicon PCR was shown to detect only viable cells for these samples, even without the addition of PMA. The long-amplicon PMA-PCR method was effective in targeting viable cells following heat and UV treatment and was applicable to enteric pathogens including *Salmonella* and *Campylobacter* that are difficult to enumerate using culture-based procedures. PCR amplicon length is important for effective removal of the dead cell signal in PMA pretreatment methods that target membrane-damaged cells, and also for inactivation mechanisms that cause direct DNA damage.

3.2 Introduction

The quantification of pathogenic microorganisms is important in food, environmental and clinical samples to identify their contribution to public health. The health risk caused by pathogens requires reliable and sensitive detection methods, with the additional challenge to specifically detect viable cells. Classical growth-based methods can underestimate the viable cell count. Many types of bacteria

can enter a VBNC state and are unable to grow on culture media, and yet VBNC cells can maintain metabolic activity and resuscitate to a virulent state (Colwell and Grimes, 2000). To circumvent this problem, molecular based methods such as PCR amplification have been developed. However, PCR can result in an overestimation of targeted live (active and VBNC) cells due to DNA persistency after cell death (Josephson *et al.*, 1993). One suggested approach to address this problem is to block the availability of DNA originating from dead cells for PCR amplification. This is the principal of a relatively recent approach, which applies a photoreactive dye (propidium monoazide [PMA] or ethidium monoazide [EMA]) which can enter dead cells with a broken membrane but not live cells with an intact membrane (Nogva *et al.*, 2003). The photoreactive dye forms an irreversible cross-linkage with DNA when exposed to visible light and prevents PCR amplification (Nogva *et al.*, 2003; Rudi *et al.*, 2005).

Compared with EMA, PMA was shown to be less toxic to live cells and has a higher affinity to DNA (Nocker *et al.*, 2009). PMA-PCR has been tested on a wide variety of bacteria, protozoa, viruses and fungi including pathogenic and environmental strains (e.g. Nocker *et al.*, 2007a,b; Vesper *et al.*, 2008; Brescia *et al.*, 2009; Fittipaldi *et al.*, 2010). However, some studies found that the PMA-PCR method was not fully effective at removing the signal from dead cells. Kralik *et al.* (2010) reported that not more than a 2 log decrease in PCR signal could be obtained using membrane permeable cells of *Mycobacterium avium paratuberculosis*. Pan and Breidt (2007) also showed that PMA-PCR did not always remove the signal of heat-killed *Listeria monocytogenes*. Similar results showing incomplete suppression of the dead cell signal have also been reported using EMA-PCR (e.g. Flekna *et al.*, 2007; Wagner *et al.*, 2008; Kobayashi *et al.*, 2009). In addition, the use of membrane-based viability assays with inactivation mechanisms that do not affect the cell membrane have always been questioned (Nocker *et al.*, 2007a; Parshionikar *et al.*, 2010). In many environments, bacteria can be killed by processes (e.g. ultraviolet [UV] light) that do not directly cause membrane damage but instead cause injury to the genetic material. PMA treatment, for instance, was not successful at differentiating between live and UV-killed *Escherichia coli* O157:H7 (Nocker *et al.*, 2007a).

The application of longer PCR amplicon sizes may be an effective method to improve the efficacy of the intercalating dye viability assays (EMA-PCR and PMA-PCR). Long amplicon sizes were shown to improve the effectiveness of EMA-PCR for heat-killed bacteria measured using both endpoint and quantitative PCR (qPCR) assays (Soejima *et al.*, 2008; 2011). Recent studies using PMA-

PCR have also shown that amplification product size was important when applied to heat-killed *E. coli* O157:H7 and *Enterobacter aerogenes* (Luo *et al.*, 2010) and also *Vibrio anguillarum* and *Flavobacterium psychrophilum* (Contreras *et al.*, 2011). However, there are limited studies that have quantitatively assessed the effect of amplicon size on PMA-PCR, and that have also extended the evaluation to include both heat and UV-killed cells, both of which were evaluated in the present work. PCR amplification of specific gene targets were assessed using short and long amplicon sizes to optimize the reduction of the dead cell signal, and this effect was measured using qPCR methods.

The bacteria included in this study were *Salmonella* and *Campylobacter*, which are important food and waterborne enteric pathogens. *Campylobacter* have been reported to be the most common bacteria associated with enteric disease worldwide and is transmitted mainly through contaminated food and water (Miller and Mandrell, 2005). *Campylobacter* are fastidious with stringent growth conditions and can readily turn into VBNC cells under environmental conditions (Bhunia, 2008), which can cause monitoring difficulties using conventional culture-based laboratory methods. *Salmonella* is also a common cause of food and waterborne disease and of major concern to public health throughout the world. *Salmonella* species have also been reported to enter into a VBNC state in response to environmental stresses (Oliver, 2005). Culture-dependent detection of both of these pathogens can be time consuming and their quantification can be difficult, especially for environmental samples that typically require a most-probable number method. Therefore, the development of qPCR methods that can accurately assess viable cells of these bacterial pathogens following exposure to different inactivation mechanisms are of particular importance for identifying human health risk.

3.3 Materials and Methods

3.3.1 Bacteria and Culture

Campylobacter jejuni subsp. *jejuni* ATCC 35920 and *Salmonella enterica* subsp. *enterica* ATCC 13311 were obtained from the American Type Culture Collection. Both strains were grown from long term stocks stored in 25% glycerol peptone medium at -80°C. *S. enterica* was grown on nutrient agar (BD) plates at 37°C overnight, and cells were inoculated into 100 mL of nutrient broth (BD) in a 250 mL Erlenmeyer flask. The culture was grown overnight at 37°C without shaking. One mL of the culture was harvested by centrifugation at 12,000 × g for 5 min, and the cell pellet was resuspended in 10 mL of sterile phosphate buffered saline (PBS). The *S. enterica* concentration was adjusted to 1 ×

10^7 colony-forming units (CFU) mL^{-1} by measuring optical density (OD) together with a standard curve comparing OD (600 nm) versus plate count for this strain. *C. jejuni* was grown on Mueller Hinton blood agar (BD) at 42°C under microaerophilic conditions (CampyPak Plus System; BD) for 2-3 d. Using a sterile swab, colonies were suspended in a sterile $0.2\ \mu\text{m}$ -filtered 0.85% NaCl solution. A NaCl solution was used since PBS can interfere with the BacLight viability count, as described by the manufacturer. The *C. jejuni* suspension was stained and enumerated by direct fluorescence microscopic cell count as described by Van Dyke *et al.* (2010), and the concentration adjusted to 1×10^7 CFU mL^{-1} in 0.85% NaCl.

3.3.2 Preparation of Heat- and UV-killed Cells

To prepare heat-treated cells, *S. enterica* and *C. jejuni* cell suspensions (each 1×10^7 cells mL^{-1}) were incubated at 90°C for 20 min. To prepare UV treated cells, 15 mL of *S. enterica* and *C. jejuni* (each 1×10^7 cells mL^{-1}) was transferred into 5.0 cm diameter glass Petri dishes and exposed to $50\ \text{mJ cm}^{-2}$ low pressure UV light using a collimated beam apparatus (Calgon Carbon Corp). The collimated beam apparatus was fitted with a 12W low pressure mercury lamp, and UV exposure times were calculated using the software and method described by Bolton (2002). Irradiance was measured using a radiometer (International Light Model IL1700) equipped with a SED 240 UV detector. The radiometer and probe were calibrated by International Light, according to the US National Institute of Standards and Technology (NIST) method. The solutions were mixed during the exposure time using a magnetic stir bar.

Untreated (live) control, heat treated or UV-irradiated *S. enterica* samples were enumerated by serial dilution and viable plate count on nutrient agar. *C. jejuni* samples were enumerated using the BacLight LIVE/DEAD Viability Kit (Invitrogen). Live or killed cell preparations were transferred in 0.5 mL aliquots to 1.5 mL sterile, transparent microcentrifuge tubes, and placed in the dark at room temperature. Within 30 min of heat or UV treatment, control and killed cells were incubated with and without PMA (each treatment in duplicate) as described below, followed by qPCR analysis.

3.3.3 PMA Treatment

Solid PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride) was purchased from Biotium (Inc., Hayward, CA, USA), and a 4 mM stock solution was prepared in 20% (v/v) dimethyl sulfoxide (DMSO). The stock solution was transferred to 1.5 mL light-impermeable microcentrifuge tubes and stored at -20°C .

PMA was added to *S. enterica* at a final concentration of 10 μ M and to *C. jejuni* at a final concentration of 15 μ M. Optimal PMA concentrations were previously determined by testing a range of PMA concentrations (0–100 μ M) on heat treated cells and using the same exposure conditions described below (see Appendix B). The optimal PMA concentrations resulted in a maximum reduction of the dead cell signal without affecting the live cells. PMA concentrations higher than 20 μ M were found to cause toxicity to live cells as determined by viable cell count measurements (see Appendix B). Controls were also done to confirm that DMSO at the concentrations used in this study did not affect cell viability (Appendix B). After PMA addition, the cell suspension was mixed well by vortexing, followed by incubation in the dark for 5 min with constant mixing by inversion. The sample tubes were then placed on ice to avoid excessive heating and exposed to a 500 W halogen lamp for 10 min at a distance of 20 cm with the caps open. Two hundred μ L of each duplicate sample was removed for DNA extraction and PCR as described below. Viable cell enumerations were done from one tube of each treatment. One hundred μ L of *S. enterica* sample was serially diluted in PBS, and enumerated by spread plating onto nutrient agar as described above. Five μ L of *C. jejuni* sample was enumerated using BacLight LIVE/DEAD Viability Kit (Invitrogen).

3.3.4 DNA Extraction

DNA was extracted from 200 μ L of PMA-treated and non-PMA treated (control) samples using the Qiagen DNeasy tissue kit. Cell lysis and DNA extraction was not preceded by a centrifugation step to ensure that free DNA was also measured in the samples. For this reason, the method was modified by adding 400 μ L of AL buffer to each sample, and complete cell lysis was confirmed microscopically. Following column purification (as described by the manufacturer), samples were eluted in 200 μ L of AE buffer and stored at -80°C until analysis.

3.3.5 PCR Analysis

For *S. enterica*, two sets of primers were used that targeted the *invA* gene (Table 1), including one that amplified a 119 bp gene fragment (Hoorfar *et al.*, 2000), and a set that amplified a 1614 bp gene fragment (this study). For *C. jejuni* the *cpn60* gene was targeted. Three different primers sets were used (Table 1) that could amplify a 174 bp fragment (Chaban *et al.*, 2009), an 899 bp fragment (this study) and a 1512 bp fragment (this study). The design of primers in this study was performed using Beacon Designer 7.7 software (Bio-Rad) and the sequence data was obtained from the National Center for Biotechnology Information (NCBI). Sequence alignments were carried out using ClustalW

multiple alignment programme (Thompson *et al.*, 1994) and refined using JalView alignment editor (Clamp *et al.*, 2004). Primers and probes were all obtained from Sigma-Genosys.

For all *Salmonella invA* and *Campylobacter cpn60* primer sets, qPCR amplification was performed using Ssofast EvaGreen Supermix (Bio-Rad). PCR amplification was performed using the BioRad iCycler iQ Real-Time PCR Detection System. Each 25- μ L reaction contained 10 μ L DNA template, 400 nmol of each primer, and 1 \times EvaGreen supermix. Each PCR run included duplicate standard curves (see Appendix C) and negative controls. The PCR amplification conditions for the *Salmonella invA* 119 bp gene fragment were as follows: one cycle at 95°C for 3 min; 50 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30s; and one cycle at 72°C for 10 min. Conditions for the 1614 bp primers were similar but used annealing/extension conditions of 53°C for 30 s/72°C for 1.5 min. Amplification conditions for the *Campylobacter cpn60* 174 bp primers were: one cycle at 95°C for 3 min; 50 cycles at 95°C for 30s, 62.8°C for 30 s, 72°C for 30s ; and one cycle at 72°C for 10 min. Conditions for the *cpn60* 899 bp and 1512 primers were similar but used annealing/extension conditions of 46°C for 30 s/72°C for 1.5 min and 55°C for 30 s/72°C for 1.5 min, respectively. For all the EvaGreen qPCR runs, PCR product specificity was confirmed by melting curve analysis (see Appendix C) using a ramping rate of 0.5°C/10 s from 55-95°C.

QPCR amplification products were also analyzed by agarose gel electrophoresis. Ten μ L aliquots of amplification product was mixed with DNA loading buffer (Bio-Rad) and analyzed in 1 \times TRIS-acetate-EDTA buffer (EMD, Darmstadt, Germany) on 1% (v/w) agarose gels at constant voltage (100 V). The agarose gel staining was performed in a 0.5 μ g mL⁻¹ ethidium bromide solution followed by visualization with a Bio-Rad Universal Hood II transilluminator using Quantity One 4.6.2 software. The GeneRuler 100 bp Plus DNA Ladder (Fermentas Canada Inc.) was used as a DNA marker.

Table 3.1 Target genes and primers used in this study

Species	Gene Target	Primers	Primer sequence	Product size (bp)	Reference
<i>Salmonella</i>	<i>invA</i>	StyinvA-JHO-2-left StyinvA-JHO-2-right	5'-TCGTCATTCCATTACCTACC-3' 5'-AAACGTTGAAAACTGAGGA-3'	119	Hoorfar <i>et al.</i> , 2000
<i>Salmonella</i>	<i>invA</i>	Sal-1614-F Sal-1614-R	5'-ACAGTGCTCGTTTACGACC-3' 5'-TACGCACGGAAACACGTTC-3'	1614	This study
<i>Campylobacter</i>	<i>cpn60</i>	JH0039 JH0040	5'-GAGCTTTCAAGCCCTTATATC-3' 5'-AAGAACACCGCGAAGTTTATT-3'	174	Chaban <i>et al.</i> , 2009
<i>Campylobacter</i>	<i>cpn60</i>	JH23F JH921R	5'-CAGATGAAGCAAGAAAYAAAC-3' 5'-AGTTCTTCCAAGYTCTTC-3'	899	This study
<i>Campylobacter</i>	<i>cpn60</i>	JH23F JH1534R	5'-CAGATGAAGCAAGAAAYAAAC-3' 5'-GCATRCTAGCTACWGAAAC-3'	1512	This study

3.4 Results

3.4.1 QPCR Assays

QPCR assays using the fluorescent intercalating dye EvaGreen were used for both the *Salmonella invA* and the *Campylobacter cpn60* gene targets, and all sizes of amplicons tested could reliably determine the viable cell count present in the samples (Figure 3.1A and Figure 3.2A). Each PCR assay produced standard curves with R^2 values of 0.99 or greater with slopes ranging between -3.6 and -3.9 as a measure of the PCR efficiency. DNA amplification was constantly observed for PCR reactions containing 10 cells which indicated that the detection limit was less than 10 cells per reaction. PCR products were analyzed by melt curve analysis and non-specific products were not observed. Long amplicon (1614 bp) primers for the *Salmonella invA* gene were designed in this study to be specific for the *Salmonella* genus. The 2 new sets of *Campylobacter cpn60* primers (899 bp and 1512 bp) were designed to specifically target the thermophilic species *C. jejuni*, *C. lari*, and *C. coli*, which are the strains most frequently isolated from humans. The Basic Local Alignment Search Tool (BLAST) software (Madden *et al.*, 1996) and the cpnDB chaperonin sequence database (Hill *et al.*, 2004) were used to assess the specificity of the primers, but it was not tested against a range of closely related species.

3.4.2 Effect of Amplicon Size on PMA Pre-treatment of Heat-killed Cells

In our study, high concentrations of live and heat-killed cells (1×10^7 cells mL^{-1}) with and without pretreatment with PMA were enumerated by qPCR. Following treatment of live cells with PMA, there was no bactericidal effect on either *S. enterica* or *C. jejuni* and qPCR results of live cells and live cells with PMA treatment were the same (Figure 3.1A and Figure 3.2A). After heat treatment, the viable plate count (*S. enterica*) and the viable microscopic cell count (*C. jejuni*) were below the detection level for both strains (2 cells mL^{-1}). The killed cells without PMA pretreatment showed less than 1 log reduction in qPCR signal compared with live cells, and this effect was the same for both *S. enterica* and *C. jejuni* at short and long amplicon sizes (Figure 3.1A and Figure 3.2A). However, when killed cells were treated using PMA in combination with PCR primers that amplified a relatively short fragment of the *S. enterica invA* gene (119 bp), only a 3 log reduction of the dead cell PCR signal was obtained (Figure 3.1A). For *C. jejuni*, PMA treated killed cells detected with PCR primers that amplified a short fragment of DNA (174 bp of *cpn60* gene) resulted in only a 1 log reduction of the PCR signal (Figure 3.2A).

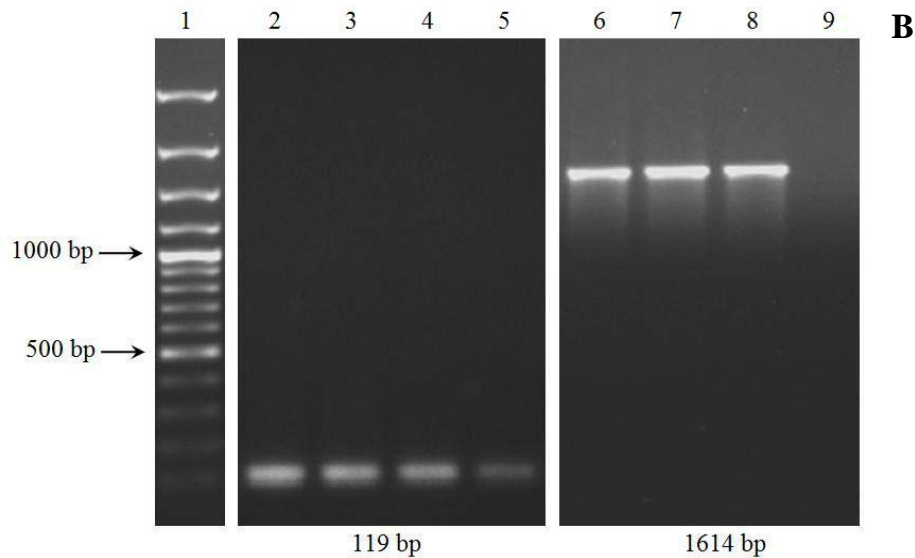
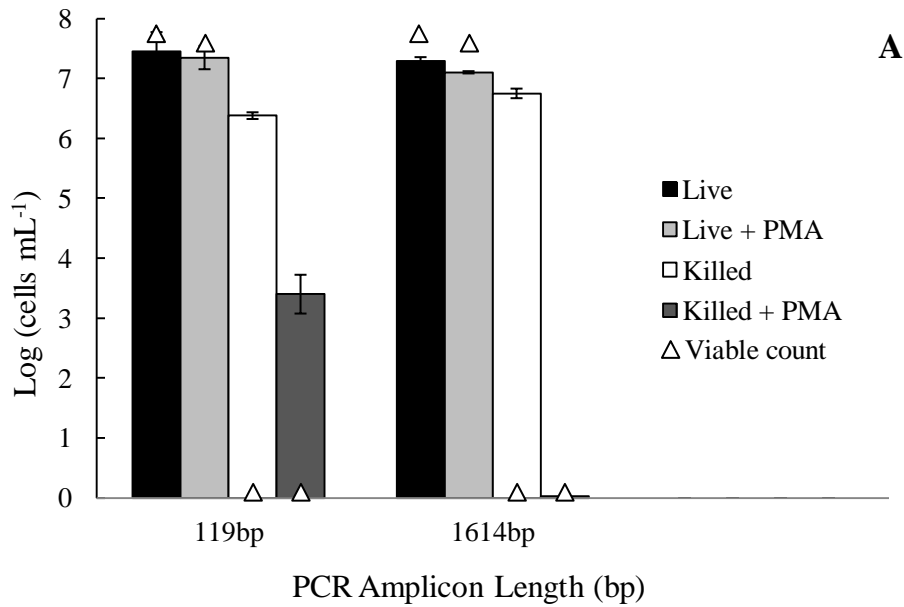


Figure 3.1 Effect of amplicon size on qPCR signal reduction of heat-killed *S. enterica*. Live and heat killed cells were treated with or without PMA, and quantitative PCR analysis was done using primers that targeted a 119 or 1614 bp fragment of the *invA* gene (A). qPCR data shows the average of duplicate data points, and error bars correspond to the range of values. Amplification products were also analyzed by gel electrophoresis (B): Lane 1, DNA Ladder; lanes 2 and 6, live cells without PMA; lanes 3 and 7, live cells with PMA; lanes 4 and 8, heat-treated cells without PMA; lanes 5 and 9, heat-treated cells with PMA.

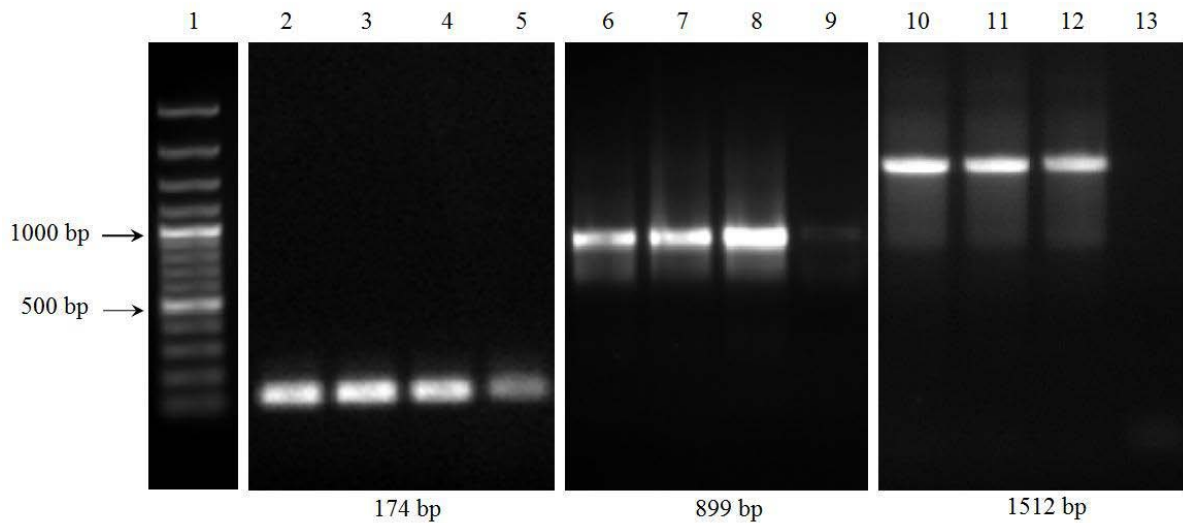
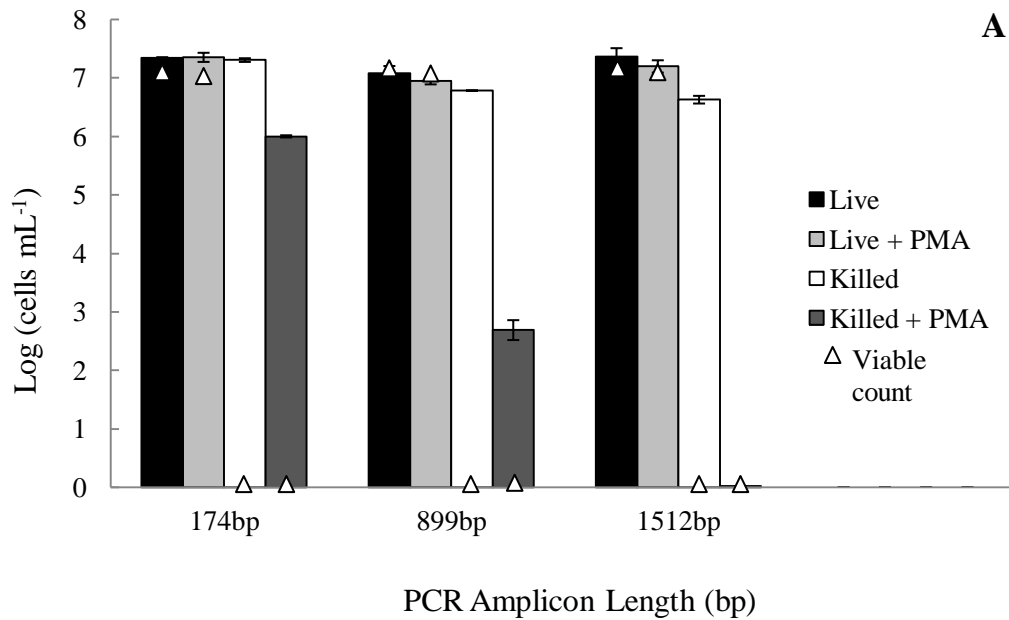


Figure 3.2 Effect of amplicon size on qPCR signal reduction of heat-killed *C. jejuni*. Live and heat killed cells were treated with or without PMA, and quantitative qPCR was done using primers that targeted a 174, 899, or 1512 bp fragment of the *cpn60* gene (A). PCR data shows the average of duplicate data points, and error bars correspond to the range of values. Amplification products were also analyzed by gel electrophoresis (B) : Lane 1, DNA Ladder; lanes 2, 6 and 8, live cells without PMA; lanes 3, 7 and 11, live cells with PMA; lanes 4, 8 and 12, heat-treated cells without PMA; lanes 5, 9 and 13, heat-treated cells with PMA.

These results show that PMA treatment followed by PCR amplification of short DNA fragments did not completely reduce the signal for heat killed *S. enterica* and *C. jejuni*. Agarose gel electrophoresis confirmed the qPCR results (Figure 3.1B and Figure 3.2B) and show that PCR primers that target short gene fragments resulted in strong bands for both live and killed cells without PMA, and apparent but somewhat fainter bands for the heat-treated with PMA samples.

Primers were then used that targeted a larger fragment size on the same gene. Results showed that PCR primers that amplified an 899 bp fragment of the *cpn60* gene for *C. jejuni* resulted in a significant improvement in reducing the false positive signal from dead cells treated with PMA, and resulted in a 4 log suppression of the PCR signal (Figure 3.2A). PCR amplification of a longer DNA fragment (1512 bp of *cpn60* gene for *C. jejuni*) strongly suppressed the signal to below the detection limit (7 log reduction; Figure 3.1A). Similarly, PCR amplification of a long fragment size (1614 bp of *invA* gene) for PMA treated *S. enterica* reduced the dead cell signal by 7 logs to below the detection level (Figure 3.2A). Agarose gel electrophoresis confirmed the qPCR results, and no band was observed for both heat-killed *S. enterica* and *C. jejuni* treated with PMA using long amplicon sizes (Figure 3.1B and Figure 3.2B).

3.4.3 Effect of Amplicon Size on PMA pre-treatment of UV-killed Cells

The ability of the PMA-PCR method to remove the false positive signal from dead cells was also tested using the same high concentrations (1×10^7 cells mL^{-1}) of UV-killed *S. enterica* and *C. jejuni*. Following irradiation by UV light at 50 mJ cm^{-2} , the viable cell count was reduced by 3.4 log for *S. enterica* (Fig. 3.3A). PCR amplification of a short gene fragment (119 bp *invA* gene) showed no signal reduction in UV-killed *S. enterica* both with and without PMA treatment (Figure 3.3A). However, with a long amplicon target (1614 bp) the decrease in PCR signal (3-3.2 log) observed for both PMA and non-PMA treated samples was similar to the decrease in the viable cell count. Similar results were observed for UV-treated *C. jejuni* (Figure 3.3B) with no decrease in the PCR signal for UV treated cells using the short amplicon size, and 3-3.2 log PCR signal reduction using the long amplicon size for both PMA and non-PMA treated cells.

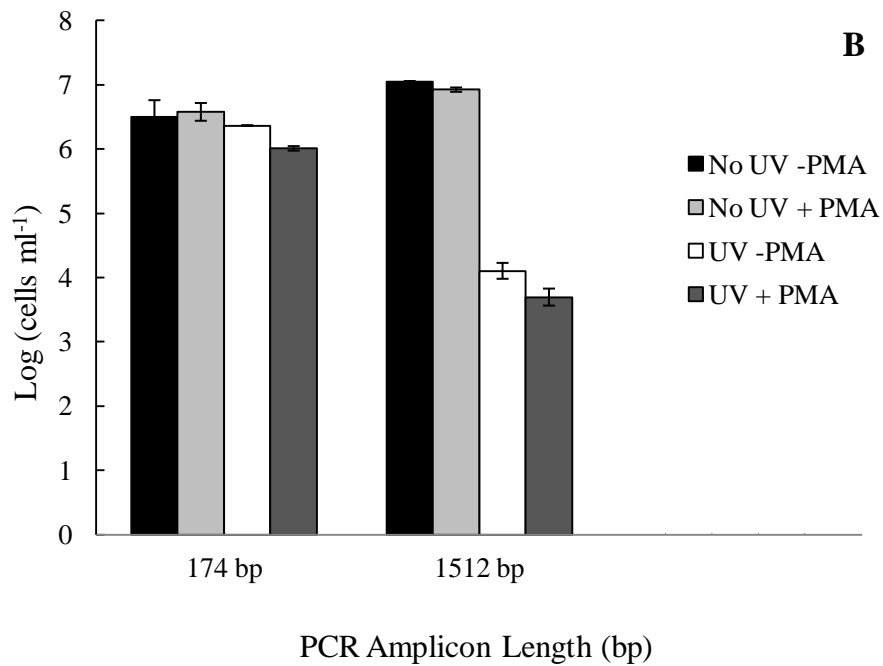
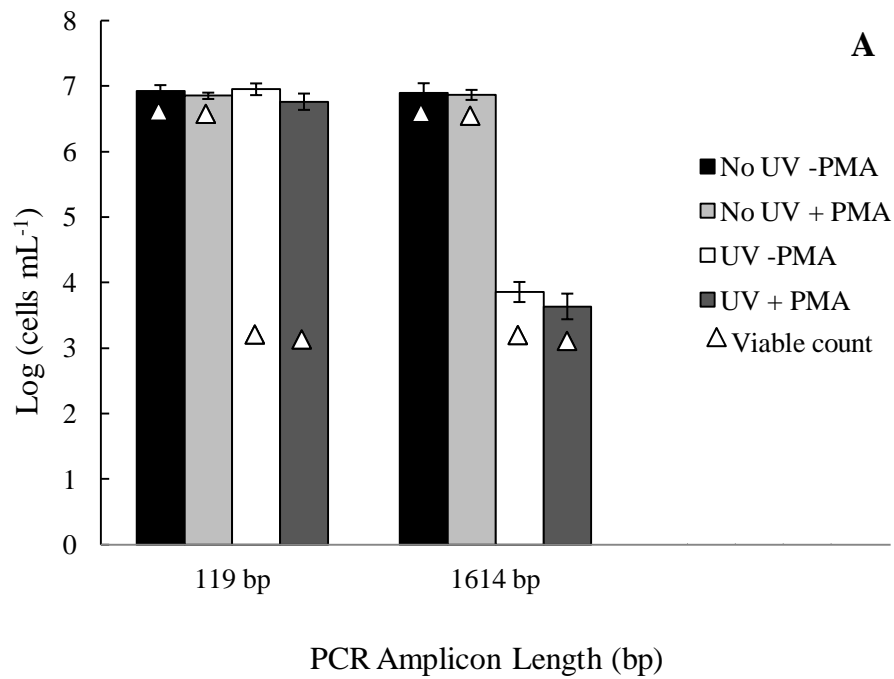


Figure 3.3 Effect of amplicon size and PMA on qPCR quantification of UV-treated or live (no UV) *S. enterica* (A) and *C. jejuni* (B). qPCR data shows the average of duplicate data points, and error bars correspond to the range of values.

3.5 Discussion

This study investigated how PCR amplicon size can affect the ability of PMA pretreatment methods to target only viable cells and remove interference from dead cells. Results showed that PMA pretreatment prior to PCR amplification can be effectively used as a viability assay for membrane damaged cells, but that PCR amplicon size plays an important role in method design. The length of the PCR product has been shown to be a critical factor when analyzing samples with high concentrations of dead bacterial cells.

In this study, heat-killed cells of *S. enterica* at an initial concentration of 1×10^7 cells mL⁻¹ were pretreated with PMA, and PCR amplification of a 119 bp *invA* gene fragment resulted in only 3 log reduction of the dead cell signal. PCR amplification of a 174 bp DNA fragment of heat-killed *C. jejuni* (1×10^7 cells mL⁻¹) reduced the unwanted PCR signal by only 1 log. An incomplete signal reduction of dead cells using the PMA-PCR method has also been reported in other studies that also targeted relatively short PCR amplicon sizes (Pan and Breidt, 2007; Kralik *et al.*, 2010).

QPCR assays normally use short amplicon sizes to guarantee method efficiency and for use with probe-based (i.e. Taqman) qPCR procedures. Relatively small amplicon sizes are widely used for quantification of both *Salmonella* (Josefsen *et al.*, 2007; Löfström *et al.*, 2009) and *Campylobacter* (Rönner and Lindmark, 2007; Josefsen *et al.*, 2010) in different applications such as clinical, food, and water quality studies. Although a 1-3 log PCR signal reduction of dead cells may be satisfactory when studying samples with low bacterial concentrations (such as surface or drinking water), incomplete suppression of the false PCR signal means that the method is not applicable to samples with higher concentrations of dead cells. High concentrations of bacterial pathogens can be present in clinical, environmental (i.e. sewage), food and laboratory studies (i.e. disinfection efficacy testing). Additionally, for unknown samples with no information on cell concentration, the results obtained using short amplicon PMA-PCR assay cannot be considered reliable in terms of live/dead cell evaluation.

To further evaluate the effect of amplicon size on the PMA-PCR method, this study evaluated longer PCR amplicons that also targeted the *invA* gene in *Salmonella* and the *cnp60* gene in *Campylobacter*. We observed that PCR amplification of a 1614 bp target for *Salmonella* and a 1512 bp target for *Campylobacter*, a PCR signal could not be detected for heat-killed cells pretreated with PMA, resulting in a 7 log suppression of the dead cell signal. This was a considerable improvement compared to the incomplete dead cell signal reduction of 1-3 log using a short amplicon size of less

than 200 bp. In our study, an intermediate amplicon size of 899 bp greatly reduced the dead cell signal from PMA treated *C. jejuni* (4.2 log), but did not completely remove the dead cell signal. PCR amplification of larger DNA fragments has been previously reported to correlate better with viable cell populations (McCarty and Atlas, 1993; Aellen *et al.*, 2006), due to general nucleic acid degradation subsequent to cell death. In terms of the intercalating dye viability assays (EMA-PCR and PMA-PCR), a number of studies have also found improved viability detection with longer PCR amplicons (Luo *et al.*, 2010; Contreras *et al.*, 2011; Soejima *et al.*, 2011). Our findings are comparable with the observations made by Luo *et al.* (2010) who assessed the PMA-PCR method on heat-killed *E. coli* O157:H7, *Enterobacter aerogenes* and *Alcaligenes faecali*. Using end-point PCR targeting the 16S rRNA gene, amplification of a 1400 bp PCR product resulted in complete band disappearance; whereas PMA had little effect on the 230 bp product.

These results show that PMA is able to inhibit PCR amplification of heat-treated cells more effectively when longer amplicons are targeted. The main mechanism of action of EMA/PMA for inhibiting PCR amplification is the formation of irreversible cross-linkages with DNA following exposure to light. The structural damage to DNA caused by cross-linkage with the dye is suggested to prevent strand elongation during PCR amplification and result in removal of the dead-cell signal (Nogva *et al.*, 2003; Rudi *et al.*, 2005). However, studies by Soejima *et al.* (2007; 2008) showed that EMA cross-linkages can cause cleavage to chromosomal double stranded DNA when exposed to visible light, which was suggested as the mechanism for PCR signal reduction. Some earlier studies have also demonstrated that ethidium is able to cleave single stranded DNA (Deniss and Morgan, 1976). Nevertheless, both of the above mechanisms may be responsible for PCR signal reduction of dead cells following treatment with PMA. Targeting longer amplicons will result in a greater chance that PMA induced damage will be encountered and interfere with the PCR amplification process. The required amplicon length can be dependent on both PMA-DNA binding and PMA-DNA cleaving characteristics, as well as the level of DNA breakage caused by mechanical shearing during DNA extraction (Contreras *et al.*, 2011).

We observed dissimilarity in the ability of PMA to suppress the PCR signal of heat-killed cells of two different types of bacteria. With short PCR amplicons of similar size (119 and 174 bp), PMA-PCR resulted in 3 log suppression for heat-killed *S. enterica* and 1 log suppression for heat-killed *C. jejuni*. This difference may be due to the selected target genes, since the *invA* gene was used for *S. enterica* and the *cpn60* gene was used for *C. jejuni*. However, Warning *et al.* (1965) reported little or

no sequence preference for propidium iodide (PI), and assuming a similar behaviour for PMA, difference in PCR signal reduction between strains is likely not due to gene sequence differences. Differences in EMA inhibitory effect have also been reported by Soejima *et al.* (2011) between five different genera of bacteria with short amplicon sizes even when the same gene was targeted, but similar to our study, these disparities were no longer present when they tested PCR amplicons over 2000 bp in size. Therefore, the benefit of using a long amplicon PCR method is that the dead cell signal is removed, and any differences in PMA-PCR induced DNA damage between strains and/or gene targets is no longer important. This results in a method that is more applicable to samples containing mixed populations of microorganisms.

In our study, relatively low PMA concentrations of 10-15 μM were used, compared to other studies that have used PMA at 50 μM (e.g. Nocker *et al.*, 2007a; Pan and Breidt, 2007). However we found that maximum reduction of the dead cell signal was achieved at 10-15 μM of PMA, and that higher concentrations did not result in improved signal suppression regardless of the amplicon length used. Contreras *et al.* (2011) suggested that with a longer PCR amplicon, lower concentrations of PMA (10 μM) are needed to result in removal of the dead cell PCR signal. Since we found that PMA concentrations greater than 20 μM resulted in cell toxicity, a combination of low PMA concentration and long amplicon size resulted in the accurate discrimination of the viable and dead cell signal.

The effect of PCR amplicon size was also evaluated on UV-treated *S. enterica* and *C. jejuni*. PMA-PCR has been previously reported not to suppress the false positive PCR signal of UV-killed cells (Nocker *et al.*, 2007a). Our results using short amplicon sizes confirmed this, and showed that no or very low PCR signal reduction was achieved for UV-killed cells of both *S. enterica* and *C. jejuni*. This was expected because the main targets of UV damage are nucleic acids, and therefore cells are not expected to have permeabilized membranes directly after UV irradiation. We observed suppression of the dead cell signal to below the detection level when long amplicon qPCR was applied, and PMA pretreatment had no effect on PCR signal suppression. Our results support previous findings that signal reduction of UV-killed cells can be dependent on the size of fragment amplified by PCR (Süss *et al.*, 2009; Rudi *et al.*, 2010). Primary structural UV-induced damage to DNA includes the photoproducts cyclobutane pyrimidine dimer (CPD) and 6-4 pyrimidine-pyrimidone (Moan, 1989) both of which can lead to transcription blockage, replication arrest, and consequently to cell death (Britt, 1995). DNA breakage has also been reported to occur in UV-induced cells, not as a direct consequence of UV irradiation but as the consequence of cellular repair

mechanisms (Bradley, 1981). Considering the mechanisms of UV damage to DNA, PCR amplification can be prevented more effectively with an increase in amplicon size, similar to the actions caused by PMA cross-linkage to membrane permeabilized cells.

This study highlights that amplicon length was an important factor in signal reduction of killed cells using PMA-PCR. Targeting relatively longer DNA fragments in PCR amplification can significantly improve the effectiveness of PMA-PCR method in terms of viable cell detection. This method is particularly important for live/dead cell discrimination when higher concentrations of bacteria are present. Additionally, our results reveal that long amplicon PCR can be used for viable cell determination of UV-killed cells. In a mixed population of bacteria exposed to unknown stresses, including environmental samples that will be exposed to natural sunlight, this method will be better able to target viable cells, regardless of the mechanism causing cell inactivation. This viability technique can also be a useful tool for reliable evaluation of enteric pathogens including *Campylobacter* and *Salmonella*. Therefore, long amplicon PMA-PCR is a valuable technique that can provide reliable quantification of viable microorganisms from a variety of sample types.

3.6 Conclusions

Based on the results of this study the following conclusions were made:

- PMA pretreatment combined with PCR is able to block the amplification of DNA originating from heat-killed intact cells or extracellular DNA, and therefore can be applied as an effective viability assay to avoid a false positive PCR signal.
- PMA pretreatment followed by a PCR assay targeting relatively short amplicons is only able to partially remove the false positive detection of dead cells. Therefore, the short amplicon PMA-PCR viability assay can only be effectively used when low concentrations of bacteria are being detected.
- PMA pretreatment when combined with a long amplicon PCR can fully avert the amplification of unwanted DNA from non-viable bacterial sources and thus can confidently be applied as a viability assay to samples with high bacterial concentrations.
- The effect of PCR amplicon length on the ability of the PMA-PCR viability assay to differentiate live and dead cells may vary between bacterial species.

- Long amplicon quantitative PCR was able to effectively differentiate UV-killed and live bacterial cells and PMA pretreatment had no effect on PCR signal suppression of UV-treated cells.
- Long amplicon PMA-PCR can be reliably used for bacterial viability detection in samples with mixed bacterial populations and following exposure to unidentified environmental stresses such as heat and natural sunlight.

Chapter 4

Application of Propidium Monoazide-PCR Assay to Detect Viable Bacterial Pathogens in River Water

This chapter is based on an article of the same title to be submitted for publication in a scientific journal. Cited references are in the consolidated list of references at the end of the thesis.

4.1 Summary

To better understand the occurrence and risk of waterborne bacterial pathogens, reliable enumeration methods are needed. In this study, a cell viability assay was applied to measure bacterial enteric pathogens at two locations in a river in southern Ontario, Canada. Pathogen concentrations were measured using both quantitative propidium monoazide (PMA)-PCR and quantitative PCR without pretreatment to evaluate and compare viable and total (live and dead) cells. The pathogens evaluated were *Salmonella enterica*, thermophilic *Campylobacter*, and *Escherichia coli* O157:H7. A suspected enteric pathogen, *Arcobacter butzleri*, was also investigated. The study showed that dead cells were present in few samples and the difference between total and viable concentrations for each pathogen group was always less than 0.5 log. *S. enterica*, *Campylobacter*, and *E. coli* O157:H7 were detected infrequently or at low concentrations at one sample location, but were measured at higher concentrations at a second sampling location. *A. butzleri* was detected at concentrations 2-3 log higher compared with the other pathogen groups at both sampling locations in the study. Results from this study show that in certain situations, PCR without PMA pretreatment can provide reliable enumeration data for bacterial pathogens in river water with little influence from dead cells.

4.2 Introduction

Waterborne enteric pathogens have been detected in surface waters used for drinking water and have long been an important cause of human infection. Reliable methods of bacterial enumeration and detection in water are among the key elements in monitoring and health-related risk evaluation associated with microbial contamination. Many different pathogenic microorganisms can be found in source waters used for drinking water, and outbreaks have been reported that were caused by

pathogenic *Escherichia coli*, *Campylobacter*, *Salmonella*, and several other bacterial pathogens (Hrudey and Hrudey, 2004). Therefore, assessing and monitoring these pathogens and their possible threat to public health is important for safe drinking water.

Culture-based methods have conventionally been used to detect bacteria in environmental water samples. However, many waterborne bacteria have been shown to enter a viable but non-culturable (VBNC) state in the environment, without losing their virulence (Oliver, 2010). These VBNC bacteria may fail to grow and can be incorrectly reported as absent when using culture methods. PCR methods can detect bacteria in their VBNC state since they target nucleic acids, and can therefore address the underestimation resulting from culture-based methods. For example, *Campylobacter* in river water have been detected at higher frequencies using molecular methods compared with culture-based methods (Rothrock *et al.*, 2009; Van Dyke *et al.*, 2010). However, since PCR targets the genetic material of the cells, it may detect extracellular DNA and DNA originating from dead cells which can lead to an overestimation of the presence of pathogenic bacteria.

Propidium monoazide-PCR (PMA-PCR) has been used as a viability assay to achieve a reliable count of live bacteria that can potentially be a threat for human health (Nocker *et al.*, 2006). PMA pretreatment inhibits the amplification of extracellular DNA and DNA from dead (membrane permeable) cells and has been tested on a wide range of microorganisms including bacteria (e.g. Nocker *et al.*, 2006; Pan and Breidt, 2007; Cawthorn and Witthuhn, 2008; Banihashemi *et al.*, 2012), fungi (Vesper *et al.*, 2008), viruses (Fittipaldi *et al.*, 2010), and protozoa (Brescia *et al.*, 2009). However, only a limited number of studies have applied PMA-PCR for the detection of viable microorganisms in environmental samples including surface water (Nocker *et al.*, 2007b; Nocker *et al.*, 2010), and wastewater (Varma *et al.*, 2009; Nocker *et al.*, 2007b). In particular, there are few studies that have quantitatively assessed microbial concentrations in the environment using PMA-PCR. Therefore more information is needed on the usefulness of the PMA-PCR viability assay for microbial enumeration in the environment.

In this study, the detection of four different bacterial groups in river water was compared using quantitative PCR and a PMA-PCR viability assay. Included in this study were *Salmonella enterica*, thermophilic *Campylobacter*, *E. coli* O157:H7, and *Arcobacter butzleri*, which are all food and waterborne enteric pathogens. *Salmonella*, pathogenic *E. coli*, and *Campylobacter* are among the top clinically reported enteric bacterial groups in Canada (Public Health Agency of Canada, 2009). VBNC *E. coli* O157:H7 have been reported in river and drinking water (Liu *et al.*, 2008). *Salmonella*

have similarly been reported to enter a VBNC state (Oliver, 2005). *Campylobacter* in particular can readily enter a VBNC state under environmental stresses (Bhunia, 2008).

Arcobacter has been identified in recent years as a potential foodborne and waterborne pathogen, and was suspected to be the cause of a river water outbreak in Slovenia (Kopilovic *et al.*, 2008) and a ground water outbreak in Idaho, USA (Rice *et al.*, 1999). There have been limited studies on *Arcobacter* spp., which were previously known as aerotolerant *Campylobacter* and have only recently been classified as a separate genus (Vandamme *et al.*, 1991). Currently *Arcobacter* contains 12 species, among which three main subspecies including *A. butzleri*, *A. cryaerophilus*, and *A. skirrow* have been more frequently associated with human gastrointestinal disease and are most commonly isolated from a variety of sources (Collado and Figueras, 2011). *Arcobacter* has been reported to occur in a VBNC state (Fera *et al.*, 2008). Water has been suggested as one of the major routes of exposure for *Arcobacter* (Miller *et al.*, 2007) and therefore it is important to determine the presence and level of these bacteria in environmental waters. Since *A. butzleri* is the species with the highest prevalence in human infections and is most frequently isolated from water, it was selected as a target for this work.

In this study, concentrations of bacterial pathogens in river water were compared using quantitative PCR with and without PMA pretreatment to determine if the PMA technique can improve PCR by measuring only viable cells. This study also looks at the potential correlation between viable and total pathogen concentrations and the water quality indicators total *E. coli* and turbidity. Accurate pathogen enumeration that includes a measure of cell viability may be important when assessing the relationship between pathogens and water quality parameters and this may be why conventional indicators such as total *E. coli* count are often not correlated to pathogens in water.

4.3 Materials and Methods

4.3.1 Sample Collection

River water samples were collected from two locations in the central area of the Grand River watershed located in southwestern Ontario, Canada (see Appendix A). The sampling locations were located 15 km apart in an urbanized area in Kitchener-Waterloo, Ontario. Location #2 is more heavily influenced by municipal wastewater effluents, urban runoff, wild bird populations, and upstream agricultural activities.

Thirteen samples were collected at each location every other week or following rain events, when possible, over a period of eight months (May-December 2011). At each location, 3 L of water were collected in 1-L sterile polypropylene wide-mouth bottles (VWR). Samples were collected from the river about 2-3 m away from the edge and in a fast-flowing area about 10-20 cm below the water surface. Samples were immediately placed on ice and transported to the lab for processing. Samples were then kept at 4°C and analyzed within 24 h of collection. The sampling bottles used for location #2 (which were more affected by wastewater) contained 0.5 mL of 0.1 N sodium thiosulfate to neutralize any chlorine which may be present.

4.3.2 Water Quality Analysis

Temperature was recorded on site immediately after sampling. The turbidity was measured using a Hach 2100P portable turbidimeter (Hach, Mississauga, Ontario). Water samples were analyzed for total *E. coli* concentrations using the membrane filtration mFC-BCIG agar method (Ciebin *et al.*, 1995). Flow rate data for the Grand River was obtained from the Water Survey of Canada (<http://www.wateroffice.ec.gc.ca>) from a gauging station at location #1.

4.3.3 PMA Treatment and DNA Extraction

Two-L of each sample were placed in Nalgene 500 mL centrifuge bottles and concentrated by centrifugation at $17,000 \times g$ for 40 min. Five mL of supernatant were left in the bottle to resuspend the pellet and removed. The bottle was then rinsed with 5 mL of phosphate buffer saline (PBS), pooled with the resuspended pellet and transferred to several 1.5 mL microcentrifuge tubes. The tubes were centrifuged at $12,000 \times g$ for 5 min, and the pellets were resuspended in PBS and pooled for a final volume of 800 μ L for each sample. One sample taken at location #2 (October 26) had unusually high solids content, and therefore the final pellet suspension was diluted by 1:50 in PBS. Each 800 μ L of concentrated sample was divided into two 400- μ L sub-samples and placed in separate microcentrifuge tubes, one of which was treated with PMA as described below (to measure viable cells) and the other untreated (to measure live and dead cells).

Solid PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride) was purchased from Biotium Inc. (Hayward, CA, USA), and a 4 mM stock solution was prepared in 20% (v/v) dimethyl sulfoxide (DMSO). The stock solution was transferred to 1.5 mL light-impermeable microcentrifuge tubes and stored at -20°C. PMA was added to 400 μ L of each treated sample at a final concentration of 15 μ M. After PMA addition, the cell suspension was mixed

well by vortexing, followed by incubation in the dark for 5 min with constant mixing by inversion. The sample tubes were then placed on ice to avoid excessive heating and exposed to a 500 W halogen lamp for 10 min at a distance of 20 cm with the caps open.

All the tubes (with and without PMA treatment) were then centrifuged at $12,000 \times g$ for 5 min and the supernatant was discarded. The pellets were resuspended in 1 mL of guanidium thiocyanate (GITC) buffer [5 mol L⁻¹ GITC, 0.1 mol L⁻¹ ethylenediaminetetraacetic acid (EDTA; pH 8.0), 5 g L⁻¹ N-laurylsarcosine] and transferred to a -80°C freezer until DNA extraction. After thawing, the samples were mixed for 2 h at room temperature followed by DNA purification using the Qiagen DNeasy tissue kit (Mississauga, ON, Canada) as described by Cheyne *et al.* (2010). Columns were eluted in 200 µL of AE buffer and stored at -80°C until analysis.

4.3.4 Quantitative PCR

Concentrated DNA ($5000 \times$) from the river samples was tested for the presence of four bacterial pathogen groups by quantitative PCR (qPCR) using primer and probe sequences that were specific for *E. coli* O157:H7 (Ram and Shankar, 2005), thermophilic *Campylobacter (jejuni/coli/lari)* (Van Dyke *et al.*, 2010), *S. enterica* (Hoorfar *et al.*, 2000), and *A. butzleri* (Brightwell *et al.*, 2007). The specificity of the primers for the target groups was confirmed using the Basic Local Alignment Search Tool (BLAST) software (Madden *et al.*, 1996). For all PCR assays, 50 µL reaction volumes were used and contained 10 µL of concentrated DNA, 300 nM of each primer, 100 nM of probe, 1 buffer, 3.5 mM MgCl₂, 1.25 U iTaq polymerase (Bio-Rad), 20 µg of bovine serum albumin (Sigma-Aldrich) and 200 µM dNTPs (Sigma-Aldrich). PCR amplification conditions for all assays were as follows: one cycle at 95°C for 3 min; 50 cycles at 95°C for 15 s, 60°C for 30 s, 72°C for 30 s; and one cycle at 72°C for 10 min. Primers and probes were obtained from Sigma-Genosys (Mississauga, ON, Canada). The Bio-Rad iCycler iQ Real-Time PCR Detection System was used for PCR analysis and each run included duplicate standard curves and negative controls. Each sample was analyzed by PCR in triplicate. Approximately 25% of samples were checked for the presence of PCR inhibitors using an external *luxB* amplification method as described by Cheyne *et al.* (2010), and PCR inhibition was not detected in any samples from either of the locations (see Appendix D).

Standard curves were prepared for each qPCR assay using DNA extracted from pure cultures of bacteria. The strains used for this study were as follows: *E. coli* O157:H7 ATCC 43895, *S. enterica* ATCC 13311, *C. jejuni* ATCC 33291, and *A. butzleri* ATCC 49616. All strains were grown from long term stocks stored in 25% glycerol peptone medium at -80°C. The preparation of *C. jejuni*

standard curves was described by Van Dyke *et al.* (2010). *E. coli*, *S. enterica*, and *A. butzleri* were grown for 24 h in nutrient broth (BD) at 37°C. The broth cultures were stained and enumerated by direct fluorescence microscopic cell count as described by Van Dyke *et al.* (2010). DNA extraction was performed using the Qiagen Dneasy tissue kit. Purified DNA was then serially diluted in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and stored at -80°C. Each PCR assay produced standard curves with R^2 values of 0.99 or greater and with slopes ranging between -3.6 and -4.0 as a measure of the PCR efficiency. DNA amplification was always observed for PCR reactions containing 10 cells, which indicated that the detection limit was less than 10 cells per reaction.

A control experiment was performed to evaluate the overall bacterial recovery from river water by the centrifugation process in combination with DNA extraction. Two-L of river water from the Grand River taken at a location upstream of the two sampling locations (#1 and #2) was inoculated with *S. enterica* to a final concentration of approximately 4×10^2 cells mL⁻¹. *S. enterica* inoculum was prepared as explained previously for the PCR standard curve preparation. The sample was then centrifuged and processed for DNA extraction and enumeration using the *Salmonella* PCR assay as described above. The concentration of *S. enterica* in the prepared inoculum was evaluated by plate counts performed on the *S. enterica* original stock suspension.

A second control experiment was performed to evaluate the effectiveness of PMA at the concentrations used in this study (15 µM) on river water. Three-L of river water was collected from the Grand River and concentrated as described previously. The concentrated river water sample (5000 ×) was then inoculated with purified *C. jejuni* DNA (prepared as described for the standard curve preparation) to a final concentration equivalent to 2×10^6 cells mL⁻¹. The sample was then treated with PMA, and DNA was purified from both PMA treated and non-treated samples and tested by PCR as described above.

4.3.5 Statistical Analysis

Spearman's rank correlation coefficient ($P < 0.05$ and $P < 0.01$; two tailed) was used to determine the significant correlations between viable and total *Campylobacter*, *Salmonella*, *E. coli* O157:H7, and *Arcobacter* prevalence in river water samples, and the water quality parameters (turbidity, temperature, and total *E. coli*). Triplicate qPCR data were plotted as the average and confidence interval at a level of 95% (two-sided, α). Confidence intervals were used to determine the significance of untreated compared to PMA-treated PCR measurements.

4.4 Results and Discussion

4.4.1 Sample Location and Water Quality

Samples in this study were collected from the Grand River watershed, which covers an area of approximately 7000 km² and is located in southern Ontario, Canada. The Grand River is used as a source of drinking water by several communities as well as for recreational purposes. The microbial quality of the Grand River water can be influenced by various sources. The watershed is extensively farmed with high agricultural activities and livestock (e.g. poultry, swine, and cattle) (Dorner *et al.*, 2007) and is also influenced by wildlife. It is highly urbanized in the central areas of the watershed, and is potentially affected by wastewater treatment plant discharges (Dorner *et al.*, 2007).

Water samples were analyzed for a number of water quality parameters including water temperature, turbidity as a general indicator of water quality due to surface water and sediment runoff, and total *E. coli* which is a fecal contamination indicator. The Grand River experienced higher and more variable flow rates during spring (May to June) and fall (October to December), likely due to more frequent and heavy rain events. In summer, however, the minimal base flow rate was dominant (Figure 4.1). The turbidity at location #1 ranged from 4.6 to 21.5 NTU with a median of 11.7 NTU, while at location #2 it ranged from 5.6 to 17.0 NTU with a median of 7.8 NTU. The turbidity for both sampling locations was affected by rain events in the fall (October 26 and December 16) (Figure 4.2). Although the turbidity was generally higher at location #1, the total *E. coli* was higher at location #2. Total *E. coli* at location #1 ranged from 2.9×10^1 to 1.9×10^3 CFU 100 mL⁻¹ with a median of 7.5×10^1 CFU 100 mL⁻¹, while it ranged from 6.5×10^3 to 1.4×10^5 CFU 100 mL⁻¹ with a median of 1.7×10^4 CFU 100 mL⁻¹ at location #2. Higher levels of total *E. coli* at location #2 may be due to relatively undiluted contributions from upstream sources including urban development and runoff, wastewater discharges, and upstream livestock and agricultural activities (Cooke, 2006). Wildlife including wild birds has also been suggested to influence the microbial water quality in this area (Van Dyke *et al.*, 2010). There were no temperature differences between the two sampling locations through summer and fall (Figure 4.2).

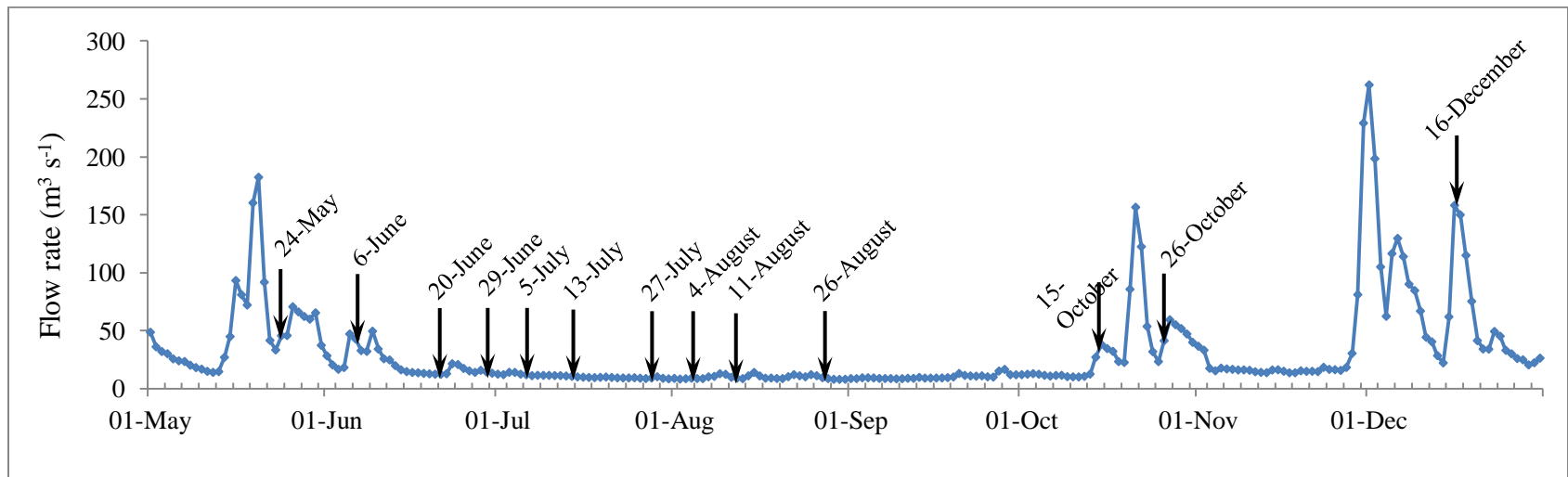


Figure 4.1 Flow rate hydrograph for the Grand River close to sampling location #1 (data provided by Water Survey of Canada, <http://www.wateroffice.ec.gc.ca>). (↓): sampling dates.

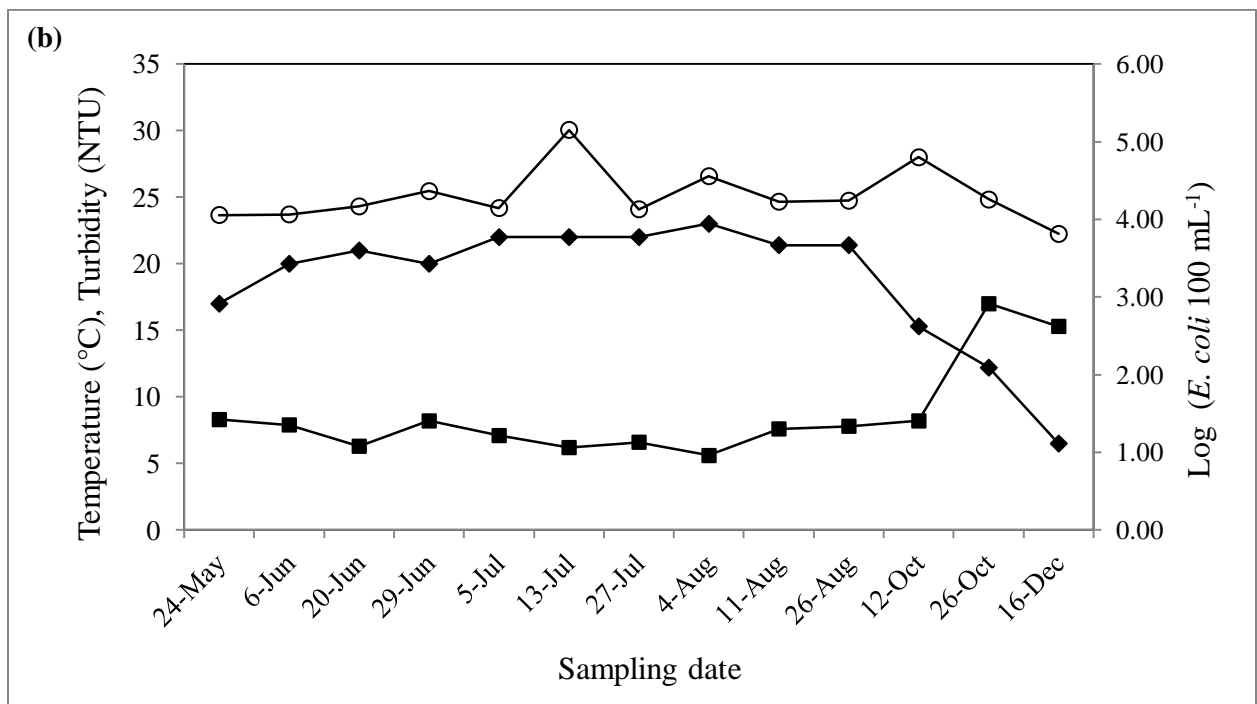
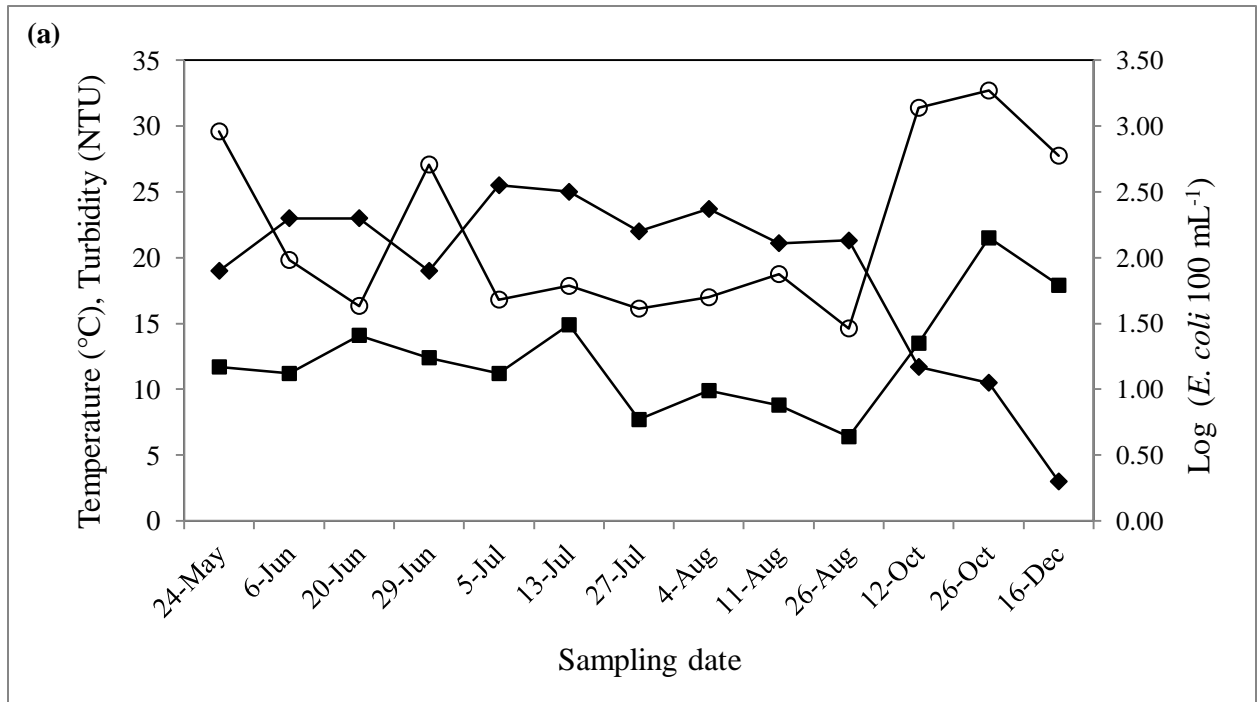


Figure 4.2 Water quality parameters including temperature (◆), turbidity (■), and total *E. coli* (○) for 13 samples collected at location #1 (a) and location #2 (b) in the Grand River over a period of 8 months.

4.4.2 Comparing the Detection of Total and Viable Bacterial Pathogens in River Water

The method used to concentrate cells and extract DNA from river water was assessed by conducting a control experiment using river water spiked with a pure culture of *S. enterica*. This control for the centrifugation process in combination with DNA extraction and PCR detection resulted in 63% recovery (see Appendix E). These recovery results are similar to others that have used ultrafiltration (40-80% recovery; Holowecky *et al.*, 2009) and centrifugation (75% recovery of *E. coli* from surface water; Courtois *et al.*, 2012). The effectiveness of PMA in suppressing dead cells or extracellular DNA in concentrated river water samples was also tested in a separate control experiment (see Appendix F). PMA was able to effectively suppress the signal from purified *C. jejuni* DNA to below the background level. This result shows that PMA effectiveness was not impeded by materials from the river water.

The PMA-PCR viability method was then applied to river water samples to evaluate the levels of naturally occurring *S. enterica*, thermophilic *Campylobacter*, *E. coli* O157:H7, and *A. butzleri*, and the results were compared to those using PCR without pretreatment. Results showed that at sampling location #1, *S. enterica* concentrations were usually below the method detection limit (2 cells 100 mL⁻¹) for both total and viable cells, except for two samples taken on June 29 and August 4, which had concentrations of 3-4 cells 100 mL⁻¹ (Fig. 4.3). Similarly, there were very low or non-detectable concentrations of *E. coli* O157: H7 at sample location #1, which was detected twice in the summer (June 6 and August 11) and three times during the fall and early winter (on October 15, October 26 and December 16), with a maximum value of 2×10^1 cells 100 mL⁻¹. Due to infrequent detection and low concentrations, no differences were observed between PMA-treated and untreated *S. enterica* and *E. coli* O157:H7 at this location. Thermophilic *Campylobacter* (*jejuni*, *lari*, and *coli*) were detected in 10 out of 13 sampling dates (all except for May 24, July 5, and December 16) at location #1, although at relatively low concentrations with a maximum value of 5.3×10^1 cells 100 mL⁻¹. There were no statistically significant differences between total (no PMA) and viable (PMA-treated) samples for thermophilic *Campylobacter*.

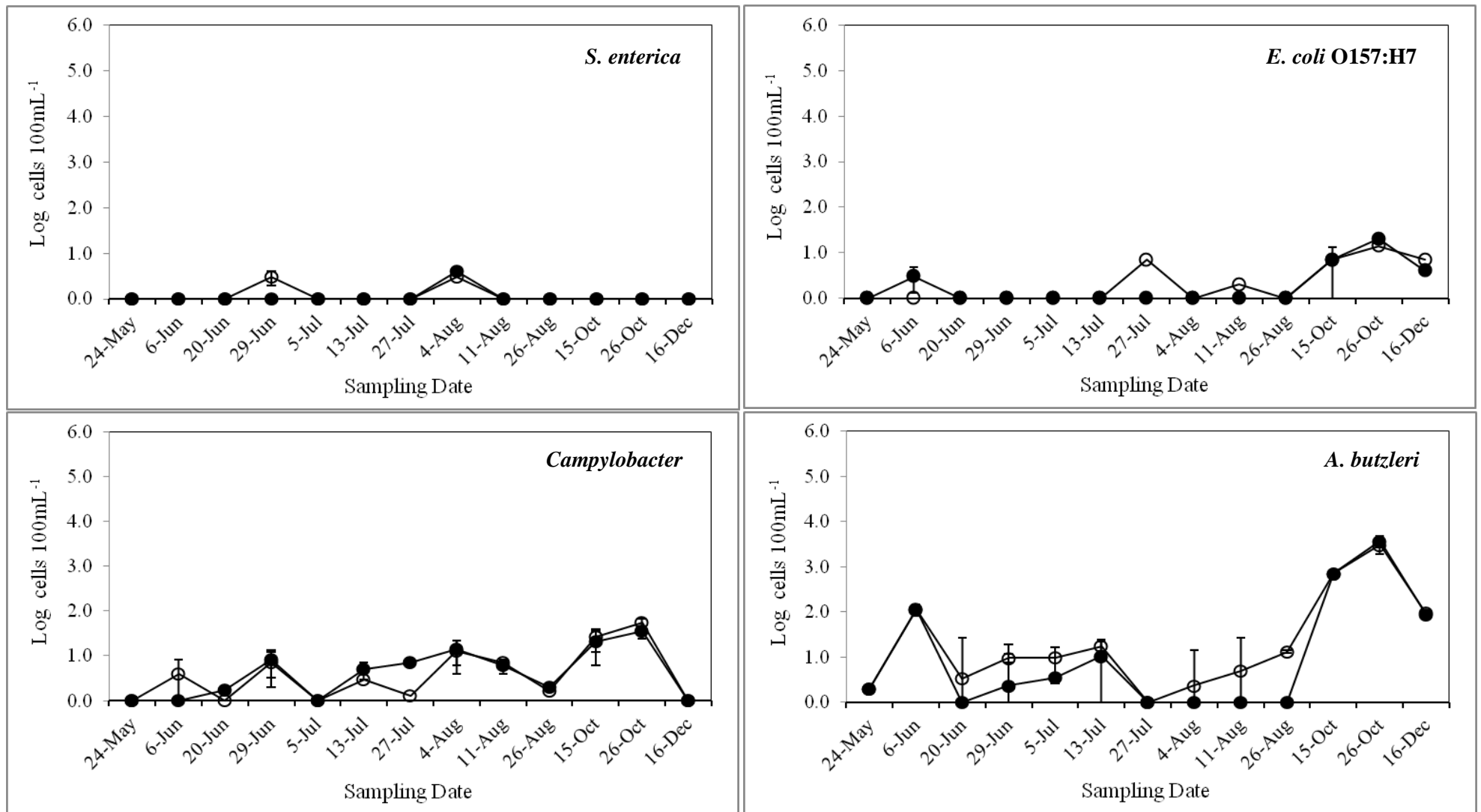


Figure 4.3 River water samples collected at location #1 over a period of 8 months and tested for *S. enterica*, *E. coli* O157:H7, thermophilic *Campylobacter*, and *A. butzleri* with PCR (●) and PMA-PCR (○) assays. Each data point shows the average of triplicate qPCR data, and error bars correspond to the range of values.

The concentrations of *A. butzleri* at location #1 detected by the qPCR assay were relatively high compared to the other bacteria studied. *A. butzleri* was detected in 12 of 13 samples (not detected on July 27) and typically at low concentrations of below 20 cells 100 mL⁻¹ particularly during summer. However elevated levels of *A. butzleri* were observed in samples taken after heavy rains on June 6, October 15, October 25 and December 16 at concentrations of 1.1×10^2 , 7.2×10^2 , 3.7×10^3 and 8.9×10^1 cells 100 mL⁻¹ in untreated samples, and 1.1×10^2 , 7.1×10^2 , 3.0×10^3 and 9.4×10^1 cells 100 mL⁻¹ in PMA-treated samples respectively. Again, there was no statically significant difference between total (no PMA) and viable (PMA-treated) *A. butzleri* at sample location #1.

The concentrations of bacteria for all four pathogen groups were higher at location #2 compared to location #1. *S. enterica*, *Campylobacter* and *E. coli* O157: H7 were detected in each sample collected from location #2 (Fig. 4.4). The concentration of *S. enterica* ranged from below the detection level to 1.7×10^2 cells 100 mL⁻¹ in untreated (total cell) samples and to 1.1×10^2 cells 100 mL⁻¹ in PMA treated (viable cell) samples. PMA treatment did not affect the *S. enterica* concentration at most sampling dates, except for those taken in August (4, 11, 26), in which a signal reduction of up to 0.5 log was observed. Samples collected in August also had the highest *S. enterica* concentrations. At location #2, *E. coli* O157:H7 was detected at concentrations that ranged from 2 to 1.9×10^3 cells 100 mL⁻¹ for untreated and 2 to 1.3×10^3 cells 100 mL⁻¹ for PMA-treated samples. The highest levels of *E. coli* O157:H7 were on July 27 and October 26. In the samples collected on August 4 and October 26, PMA treatment resulted in a statistically significant PCR signal reduction of 0.2 log, showing that a low levels of dead *E. coli* O157:H7 cells were present. *Campylobacter* concentrations ranged from 2 to 7.0×10^2 cells 100 mL⁻¹ for untreated and 2 to 2.5×10^2 cells 100 mL⁻¹ for PMA-treated samples. The sample collected on October 26 had the highest concentration of total (live and dead) *Campylobacter* with 7.0×10^2 cells 100 mL⁻¹, and dead *Campylobacter* cells were also present in the sample (0.5 log signal reduction with PMA). A significant PCR signal reduction (0.2 log) for PMA treated samples was also observed on June 6 and June 20, showing the presence of low concentrations of dead cells. *E. coli* O157:H7 and *Campylobacter* followed similar trends with the highest concentrations in late July and mid-late October.

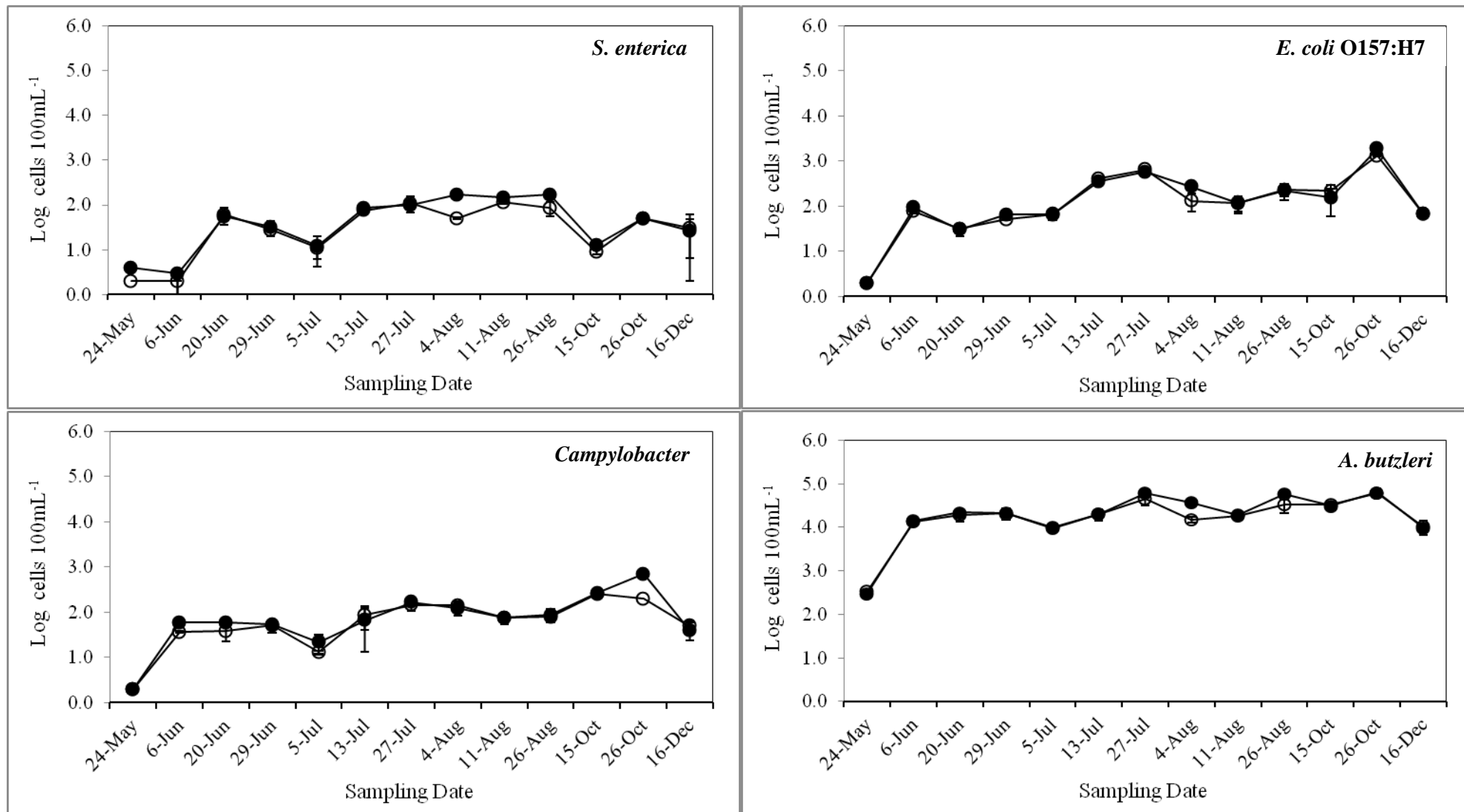


Figure 4.4 River water samples collected at location #2 over a period of 8 months and tested for *S. enterica*, *E. coli* O157:H7, thermophilic *Campylobacter*, and *A. butzleri* with PCR (●) and PMA-PCR (○) assays. Each data point shows the average of triplicate qPCR data, and error bars correspond to the range of values.

A. butzleri was also detected at higher concentrations at sampling location #2 compared with location #1. In addition, this organism was detected at higher levels compared with the other pathogen groups. The concentration of *A. butzleri* ranged from 3.4×10^2 - 6.2×10^4 cells 100 mL^{-1} and it was detected on all 13 sampling dates. The total cell concentrations (without PMA treatment) were the highest during the summer and fall months, in particular on July 27, August 26, and October 26. Similar to *S. enterica*, PMA treatment was able to suppress the small (0.5 log and 0.3 log) false positive signal caused by dead *A. butzleri* in samples taken in August (4 and 26).

A. butzleri and *S. enterica* followed a similar trend at sampling location #2 during August in that non-viable cells of both groups were mostly observed during this month. More dead cells of *S. enterica* and *A. butzleri* during the high temperature months might suggest that these two groups of bacteria are relatively heat-intolerant. In manure samples, *Salmonella* has been shown to survive for shorter times in summer compared to the winter/spring (Mannion *et al.*, 2007; Placha *et al.*, 2001). Pathogenic *Arcobacter* has also been reported to survive better at lower temperatures (D'sa and Harrison, 2005) and was detected less frequently during summer in estuarine waters (Fera *et al.*, 2010).

Our results show that for the bacterial pathogens tested, little or no difference was observed between PMA-PCR and PCR without PMA treatment. At location #1, the pathogens were either below or close to the detection limit, and therefore any differences between the two methods could not be measured. However results from location #2 in which the pathogens were detected at higher concentrations show that there were few dead cells (or extracellular DNA) present in samples taken from Grand River. Dead cells were present in only a small number of samples and the difference between total and viable cell concentrations were never more than 0.5 log 100 mL^{-1} . An explanation for this can be that dead bacteria are a source of nutrients for microorganisms (Nielsen *et al.*, 2007) and in an environment with high microbial activity such as the Grand River, lytic enzymes and nucleases are readily available leading to rapid degradation of cellular materials including DNA. Nuclease presence and activity have been reported in water and sediments (Bazelyan and Ayzatullin, 1979). Additionally, extracellular DNA release from dead cells could bind to absorbent surfaces (e.g. clays) of river sediments (Nielsen *et al.*, 2007) and therefore be removed from surface water. Similar results were observed by Varma *et al.* (2009) who found little difference between PMA-PCR and PCR enumeration of *Enterococci* in wastewater effluents; where less than one log difference was observed. These results suggest that application of conventional qPCR alone may provide reliable

data on viable cell numbers in river water and that more complicated/time consuming methods may not always be necessary.

The results from this study showed the presence of relatively high levels of viable *A. butzleri* at both sampling locations in the river. Recent studies suggest that water may be one of the main exposure routes of *Arcobacter* diarrheal infection (Lehner *et al.*, 2005). *Arcobacter* has been previously isolated from river water (Collado *et al.*, 2010), groundwater (Fera *et al.*, 2008), wastewater (González *et al.*, 2007), and sludge (Stampi *et al.*, 1999). High concentrations of *Arcobacter* have also been reported in stream water where it was detected at $> 10^5$ MPN 100 mL⁻¹ (Collado *et al.*, 2008). Lee *et al.* (2012) has also reported elevated levels of total *Arcobacter* in Lake Erie, in which water samples tested by qPCR at four different beach locations detected total *Arcobacter* concentrations greater than 10^4 cells 100 mL⁻¹. Our study on the Grand River, which flows into Lake Erie, seems to be in-line with these results and also showed high *Arcobacter* concentrations.

The high concentrations of *A. butzleri* in the Grand River may be due to upstream influences including agricultural activities and wildlife. Animal livestock (cattle, poultry, and swine) have been recognized to be a significant reservoir of *Arcobacter* spp. (Kabeya *et al.*, 2003; Chinivasagam *et al.*, 2007; Van Driessch *et al.*, 2003). Chinivasagan *et al.* (2007) detected high levels of *Arcobacter* ranging 10^5 - 10^8 MPN 100 mL⁻¹ in piggery effluent. Wild birds were shown to be reservoirs of *A. butzleri* in Southern Chile, where 6.6%, 13.3%, and 40% of samples collected from sparrows, pelicans, and ducks, respectively, were positive for *A. butzleri* (Fernandez *et al.*, 2007). Pejchalova *et al.* (2006) also reported that samples taken from a pond populated with aquatic wild birds contained *A. butzleri*. *Arcobacter* has also been detected in environments influenced by sewage and wastewater (Collado *et al.*, 2008). Stampi *et al.* (1999) reported *A. butzleri* were present at high levels in a municipal wastewater treatment plant, where more than 80% of activated sludge samples were positive, and the concentration of *A. butzleri* increased by two-fold through the activated sludge process. Snaidr *et al.* (1997) also found *Arcobacter* in activated sludge samples. *Arcobacter* has not been previously studied in the Grand River and this is one of the few studies that have quantitatively assessed *Arcobacter* in the environment. However, until the health effects of *Arcobacter* have been quantified, the risk caused by this organism in water remains unknown.

4.4.3 Correlation between Bacterial Pathogens and Water Quality Parameters

To assess the relationship between total and viable pathogens and water quality parameters, Spearman ranked correlation tests were done using data from sample location #2 only. Correlation tests were not performed using data at location #1 because of the low pathogen occurrence and concentrations measured. *Campylobacter* correlated with the total *E. coli* at a level of 95% ($P = 0.05$), with correlation coefficient values for total (untreated) and viable (PMA-treated) of 0.56 and 0.67, respectively (Table 4.1), but no significant correlation at the 99% level was observed. *A. butzleri* was also significantly correlated with total *E. coli* (0.54) for untreated and PMA treated samples at a level of 90% ($P = 0.1$).

Table 4.1 Correlation (Spearman’s rank correlation coefficient) of *E. coli* O157:H7, *S. enterica*, thermophilic *Campylobacter*, and *A. butzleri* concentrations (determined by quantitative PCR) with turbidity, temperature, and total *E. coli* indicator count at sample location #2. Analysis was done using data for total (untreated) or viable (PMA treated) cells. Only significant correlations are shown.

Bacteria	Turbidity	Temperature	Total <i>E. coli</i>
<i>Escherichia coli</i> O157:H7 (total)			
<i>Escherichia coli</i> O157:H7 (viable)			
<i>Salmonella enterica</i> (total)	-0.56*	0.57*	
<i>Salmonella enterica</i> (viable)			
Thermophilic <i>Campylobacter</i> (total)			0.56*
Thermophilic <i>Campylobacter</i> (viable)			0.67*
<i>Arcobacter butzleri</i> (total)			0.54**
<i>Arcobacter butzleri</i> (viable)			0.54**

*Significant correlation ($P < 0.05$; two-tailed).

**Significant correlation ($P < 0.1$; two-tailed).

These results are in agreement with another study on *Arcobacter* where high fecal contamination was reported to correlate with the presence of *Arcobacter* (Collado *et al.*, 2008). *E. coli* O157:H7 were not correlated with the total *E. coli*. Total *E. coli* has been previously reported to be a poor indicator for *E. coli* O157: H7 due to differences in their survival in surface waters (Jenkins *et al.*, 2011). There was also no correlation between *S. enterica* and total *E. coli*, but *S. enterica* was significantly correlated with turbidity (-0.56) and with temperature (0.57), indicating higher concentrations during the summer months or following increased surface runoff. However, because of

the short period of this study, we were not able to fully study the seasonal effects on bacterial concentrations. Overall, our results show that PMA pre-treatment was able to result in a slightly higher correlation between *Campylobacter* and total *E. coli* only. However, since this study showed either no or very small differences between total and viable pathogen concentrations, it is expected that the viable cell method would not appreciably affect the correlation analysis with water quality indicators.

4.5 Conclusions

Based on the results of this study the following conclusions were made:

- The PMA-PCR viability assay was able to show that only low concentrations of dead cells and extracellular DNA were present in river water samples tested in this study.
- PCR without pretreatment may provide reliable results for bacterial pathogen detection in the Grand River water with little influence of false positive signal detection due to the presence of dead cells.
- The results of this study demonstrate that the pathogen concentrations were different at the two sample locations in the river, showing that microbial concentrations can vary in the river depending on various factors that can affect the river water quality including localized and upstream activities.
- For the period of our river water study, the total *E. coli* correlated with both total and viable thermophilic *Campylobacter* and *A. butzleri*. However, no correlation was observed between the total *E. coli* and *S. enterica* and *E. coli* O157:H7.
- The PMA pretreatment viability assay did not affect the bacterial correlation analysis with water quality indicator (total *E. coli*).
- High concentrations of *A. butzleri* were detected in river water samples collected from the Grand River, and therefore, further studies are needed to better elucidate its occurrence in river water and their potential health risk.

Chapter 5

An Improved Understanding of Bacterial Survival in River Water: Application of Long Amplicon PMA-PCR Viability Assay

This chapter is based on an article of the same title to be submitted for publication in a scientific journal. Cited references are in the consolidated list of references at the end of the thesis.

5.1 Summary

Survival trends of enteric waterborne pathogens including *Yersinia enterocolitica*, *Salmonella enterica*, and *Campylobacter jejuni* were studied in sterile and natural (non-sterile) river water at different temperatures for a period of 28 days. The effects of temperature and dissolved oxygen (DO) levels on the survival of *C. jejuni* and *Arcobacter butzleri* were also investigated. To improve the accuracy of the results, a molecular viability assay, namely long amplicon PMA-PCR, was used to evaluate the cell concentrations to measure only viable cells, and results with and without PMA were compared. Total and viable bacterial survival was improved by up to 4 log cells mL⁻¹ for *Y. enterocolitica* and 4.5 log cells mL⁻¹ for *S. enterica* in sterile river water compared to natural river water. In non-sterile river water, low temperature significantly improved the survival of all four target bacteria (*Y. enterocolitica*, *S. enterica*, *C. jejuni*, and *A. butzleri*). Viable *A. butzleri* survival was not affected by river water DO levels at any of the incubation temperatures. Viable *C. jejuni*, however, showed sensitivity to high DO levels only at the lower temperatures (5°C), where up to a 2 log cells mL⁻¹ difference was observed at high versus low DO levels in natural river water, but only in PMA treated samples. Results show that accurate assessment using PMA-PCR analysis can provide more reliable data on viable/active enteric survival in aquatic microcosms and allows for assessment of pathogen survival in the environment.

5.2 Introduction

Disease causing microorganisms can enter surface waters through many different routes such as livestock, wildlife, and wastewater effluents. Enteric pathogenic bacteria are one of the main groups of microorganisms that can be present in source waters used for drinking water and therefore are of

concern for public health. Most enteric pathogens are not able to grow and replicate in water; however, they can survive for considerably long times in aquatic environments including surface waters and therefore water can act as a route of their transmission.

Bacterial survival patterns in surface waters can be affected by many different environmental stresses, such as temperature (Czajkowska *et al.*, 2005; Wang and Doyle, 1998), sunlight (UV) (Davies and Evison, 1991), oxygen concentration (Allen *et al.*, 1951), microbial community properties (Ducluzeau *et al.*, 1976), and chemical properties such as pH, salinity, and organic matter. To study the survival patterns and trends of waterborne disease-causing bacteria it is important to apply reliable enumeration methods. Many waterborne bacterial pathogens are able to enter a viable but non-culturable (VBNC) state. Most bacterial survival studies in water have been performed using culture-based methods, and therefore the non-culturability of bacteria due to environmental stresses were not taken into account (e.g. González *et al.*, 2012; Guillou *et al.*, 2008; Kot *et al.*, 2005; Cools *et al.*, 2003; Meckes *et al.*, 2003; Terzieva *et al.*, 1991). Therefore, culture-based methods may not provide a realistic evaluation of their survival and longevity.

The application of PCR-based methods has been suggested to address the issue of non-culturability of bacteria, yet PCR cannot assure that only viable cells are detected. PCR-based methods are not able to differentiate between live and dead cells or extracellular DNA that may be present in environmental aquatic systems. A recently developed viability assay is the propidium monoazide-PCR (PMA-PCR) method. PMA is applied as a pre-treatment to PCR and is able to selectively enter dead cells with injured membranes. PMA when exposed to visible light forms cross-linkages with any accessible DNA, including the DNA inside the dead cells, and this prevents PCR amplification (Nocker *et al.*, 2006). As shown in a previous study (Banihashemi *et al.*, 2012), PMA pretreatment combined with long amplicon PCR can ensure that the false positive signal originating from dead cells or extracellular DNA is not detected.

The present study aims to evaluate the survival trends and longevity of enteric bacteria of concern in environmental water, with a particular focus on PCR detection of dead/live bacteria and the use of PMA to assess cell viability, and also the effect of naturally occurring microflora on pathogen survival. Together with the above mentioned factors, this study also evaluated the effect of temperature and dissolved oxygen (DO) levels on bacterial survival. The effect of water microflora on bacterial survival is not often considered, and many bacterial survival studies in water have been done using filtered or sterile samples (Cook *et al.*, 2007; Moreno *et al.*, 2004; Obiri-Danso *et al.*, 2001;

McElwain, 2002; Wcisło and Chróst, 2000; Buswell *et al.*, 1998; Rollins *et al.*, 1986). For example, Moreno *et al.* (2004) studied the survival and culturability of *Arcobacter* in chlorinated and non-chlorinated drinking water, but the water was filtered and therefore the inoculated *Arcobacter butzleri* survival was studied only in the absence of background flora. McElwain (2002) also studied the survival of *A. butzleri* in filter-sterilized groundwater. Few studies have assessed the survival trends of bacteria in water with the presence of background bacteria (i.e. Meckes *et al.*, 2003). Most surface waters contain active bacterial communities that can affect the longevity of bacterial pathogens in water through processes including substrate competition, antagonism, and predation (Marino *et al.*, 1991). For this reason, it is important to consider the effect of background microflora when studying the bacterial survival in the environment.

The target bacteria in this study are important enteric pathogens in water, including *Yersinia*, *Salmonella*, *Campylobacter*, and *Arcobacter*. *Yersinia* is a waterborne pathogenic bacterium that can survive for long times, particularly in cold water (Colin, 2006). *Salmonella* has historically been an important cause of waterborne gastrointestinal illness. Although waterborne *Salmonella* gastroenteritis outbreaks are currently rare (Hrudey and Hrudey, 2004), it has been reported to be the cause of recent outbreaks (Berg, 2008 and Kozlica *et al.*, 2010) and is still considered a health threat (Levantesi *et al.*, 2012). *Campylobacter* has also been reported as one the causes of two waterborne outbreaks in Ontario including Walkerton (Hrudey *et al.*, 2002) and Orangeville (Millson *et al.*, 1991). *Campylobacter jejuni* infection has been recognized as the most common cause of waterborne Guillain-Barré Syndrome, a post-infectious rare acute polyneuropathy (Mishu *et al.*, 1993). *Campylobacter* is also recognized for readily forming a VBNC state, and therefore it is a challenge to enumerate. *Arcobacter* has been recently reported as a possible health threat in source waters (Lee *et al.*, 2012). In a previous study (Chapter 4) high levels of viable *A. butzleri* were detected in water samples from the Grand River in Ontario, Canada.

A better understanding of environmental conditions on the survival patterns of these important enteric pathogens in the aquatic environment can offer valuable information that can be effectively used with respect to regulatory issues and decision-making. It can also provide information on longevity in water following point or non-point source contamination, and provide improved data for health risk assessments, and the possible occurrence at drinking water treatment plants.

5.3 Materials and Methods

5.3.1 Bacterial Cultures

Salmonella enterica subsp. *enterica* ATCC 13311, *Yersinia enterocolitica* ATCC 9610, *C. jejuni* ATCC 35920, and *A. butzleri* ATCC 49616 were obtained from the American Type Culture Collection. *S. enterica*, *Y. enterocolitica*, and *A. butzleri* were grown on nutrient agar (BD) plates at 37°C overnight and cells from each of the plates were inoculated separately into 100 mL of nutrient broth (BD) in 250 mL Erlenmeyer flasks. The cultures were grown overnight at 37°C without shaking. One mL of each culture was harvested by centrifugation at $12,000 \times g$ for 5 min, and the cell pellet was resuspended in 10 mL of sterile phosphate buffered saline (PBS). *C. jejuni* was grown on Mueller Hinton blood agar (BD) at 42°C under microaerophilic conditions (CampyPak Plus System; BD) for 2-3 d. Using a sterile swab, colonies were suspended in 10 mL of sterile phosphate buffered saline (PBS). The cell concentration of each strain was then adjusted to 1×10^7 colony-forming units (CFU) mL⁻¹ using PBS and was measured by direct microscopic cell count (Van Dyke *et al.*, 2010).

5.3.2 Surface water collection and inoculation

To study the effect of water microflora and temperature on the survival of target bacteria, a 10 L sample of river water was collected on 26th August, 2011 from a location on the Grand River north of Waterloo, Ontario, Canada. Samples were taken 2-3 m from the river's edge and 10-20 cm below the surface in a fast-flowing area. The sample was then immediately placed on ice and transported to the laboratory for processing. Turbidity of the water sample was 10.2 NTU with a temperature of 18.5°C at the time of collection. Samples were then kept at 4°C and used within 24 h of collection. Five L of this sample was autoclaved at 121°C for 1 h and the remaining 5 L was not autoclaved. Nine 500-mL samples of (each) non-autoclaved and autoclaved river water were transferred into 1 L sterile polypropylene bottles and inoculated separately to achieve a final concentration of 1×10^6 cells mL⁻¹ with *S. enterica*, *Y. enterocolitica*, and *C. jejuni*. One bottle of each strain in sterile or non-sterile water was then incubated in 5°C, 15°C, and 25°C for 28 days. At each sampling date, one 30 mL sample was taken from each 1 L bottle and transferred to a 50-mL sterile centrifuge tube. The samples were then centrifuged at $9,000 \times g$ for 20 min. The supernatant from each tube was removed, each pellet was resuspended in 1 mL sterile PBS and transferred to a 1.5-mL microcentrifuge tube. The samples were centrifuged again at $12,000 \times g$ for 5 min to achieve a final concentrated pellet for each sample. The pellet from each tube was then resuspended in 400 µL of sterile PBS. Each 400 µL

sample was divided into two 200 μL samples, one of which was treated with PMA and the other with no PMA treatment as described below.

To study the effect of temperature and DO concentration on survival of *A. butzleri* and *C. jejuni*, a 4 L sample of river water was collected at the same sampling location on the Grand River on 9th October, 2011. The sampled water turbidity was 8.4 NTU and the temperature was 10.6°C at the time of sampling. The samples were stored at 4°C overnight before the experiment was started. This experiment was done using non-sterile water only. Two L of the river water was transferred to 2 separate 5-L carboys. The water in one carboy was sparged with helium to reduce the oxygen level. Helium can be effectively used for deoxygenation of water (Degenhardt *et al.*, 2004). It is an inert gas and that is only slightly soluble in water (Avranas *et al.*, 2006) and therefore it is not expected to affect the water characteristics. Oxygen level reduction was done by inserting a tube to the bottom of the carboy, and helium was sparged at a rate of 1 L h⁻¹ for 1 h at room temperature. The other carboy was left unsparged at room temperature. Thirty mL samples of helium-injected and control river water were transferred into 50 mL centrifuge tubes carefully without shaking. Water samples were then inoculated separately to a final concentration of 1×10^6 cells mL⁻¹ with either *C. jejuni* or *A. butzleri*. The headspace of the bottles that contained helium-injected water was purged using helium for 1 min to evacuate the air before they were capped. Non-inoculated samples of each water type (helium injected and control) were also prepared in the same way (30 mL samples in 50 mL tubes) in order to measure DO during the experiment and to avoid bacterial contamination of the equipment. All tubes were then incubated at 5°C, 15°C, and 25°C for 28 days. At each sampling time, one 50-mL tube containing 30 mL sample was sacrificed for further analysis. Samples were concentrated as described in the previous experiment: briefly, the 30 mL samples were centrifuged and resuspended in 400 μL PBS and then divided into two 200 μL samples, followed by PMA treatment. Also, one 50-mL tube containing 30 mL of non-inoculated water (helium sparged and control) was sacrificed to measure the DO at each sampling time. Duplicate measurements of DO levels were carried out using a VWR SympHony (SP70D) DO meter.

5.3.3 PMA Treatment

Solid PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride) was purchased from Biotium Inc. (Hayward, CA, USA), and a 4 mM stock solution was prepared in 20% (v/v) dimethyl sulfoxide (DMSO). The stock solution was transferred to 1.5 mL light-impermeable microcentrifuge tubes and stored at -20°C.

PMA was added to one of the 200 μL sub-samples of concentrated water a final concentration of 15 μM PMA. Optimal PMA concentrations were previously determined (Appendix A), by testing a range of PMA concentrations (0–100 μM) on heat treated cells and using the same exposure conditions described below. After PMA addition, the cell suspension was mixed well by vortexing, followed by incubation in the dark for 5 min with constant mixing by inversion. The sample tubes were then placed on ice to avoid excessive heating and exposed to a 500 W halogen lamp for 10 min at a distance of 20 cm with the caps open. All the tubes (with and without PMA treatment) were then centrifuged at $12,000 \times g$ for 5 min and supernatant was discarded. The pellets were resuspended in 1 mL guanidium thiocyanate (GITC) buffer [5 mol L^{-1} GITC, 0.1 mol L^{-1} EDTA (ethylenediaminetetraacetic acid) (pH 8.0), 5 g L^{-1} N-laurylsarcosine] and transferred to a -80°C freezer before DNA extraction.

5.3.4 DNA Extraction

Concentrated river water samples suspended in guanidine isothiocyanate (GITC) extraction buffer were mixed by inversion for 1 h at room temperature followed by purification using the Qiagen DNeasy tissue kit (Mississauga, ON, Canada) as described by Cheyne *et al.* (2009). After column purification (as described by the manufacturer), samples were eluted in 100 μL of AE buffer and stored at -80°C until analysis.

5.3.5 Quantitative PCR

One set of primers for each of the four bacteria, *S. enterica*, *Y. enterocolitica*, *C. jejuni*, and *A. butzleri* were used (Table 5.1). Primers for *S. enterica* and *C. jejuni* were designed and applied in a previous study (Chapter 3; Banihashemi *et al.*, 2012) and primers were designed in the current study for *Y. enterocolitica* and *A. butzleri* as listed in Table 5.1. The design of primers in this study was performed using Beacon Designer 7.7 software (Bio-Rad) and the sequence data was obtained from the National Center for Biotechnology Information (NCBI). Sequence alignments were carried out using ClustalW multiple alignment programme (Thompson *et al.*, 1994) and refined using JalView alignment editor (Clamp *et al.*, 2004). Primers and probes were all obtained from Sigma-Genosys. qPCR amplification was performed using Ssofast EvaGreen Supermix (Bio-Rad) and the BioRad iCycler iQ Real-Time PCR Detection System.

Table 5.1 Target genes and primers used in this study

Species	Gene Target	Primers	Primer sequence	Product size (bp)	Reference
<i>Campylobacter jejuni</i>	<i>cpn60</i>	JH23F JH1534R	5'-CAGATGAAGCAAGAAAYAAAC-3' 5'-GCATRCTAGCTACWGAAAC-3'	1512	Banihashemi <i>et al.</i> , 2012
<i>Salmonella enterica</i>	<i>invA</i>	Sal-1614-F Sal-1614-R	5'-ACAGTGCTCGTTTACGACC-3' 5'-TACGCACGGAAACACGTTC-3'	1614	Banihashemi <i>et al.</i> , 2012
<i>Yersinia enterocolitica</i>	<i>16S rRNA</i>	Yers-1213-F Yers-1213-R	5'-GGGAAGTAGTTTACTACTTTGCC-3' 5'-TGTGGTCCGCTTGCTCTC-3'	1213	This study
<i>Arcobacter butzleri</i>	<i>rpoB</i>	Arco-1415-F Arco-1415-R	5'-ACGAAGAATGTCTCTGGAAGCTC-3' 5'-GGAAGTTATGTTACTCCTGGAATG-3'	1415	This study

Each 50- μ L reaction contained 20 μ L of DNA template, 400 nmol of each primer, and 1 \times EvaGreen supermix. The PCR amplification conditions for the *Salmonella invA* 1614 bp gene fragment were as follows: one cycle at 95°C for 3 min; 50 cycles at 95°C for 30 s, 53°C for 30 s, 72°C for 1.5 min; and one cycle at 72°C for 10 min. Amplification conditions for the other three primers were the same except the annealing temperature was 46°C for the *C. jejuni cpn60* 1512 bp primers, and 58°C for both *Y. enterocolitica* 1213 bp primers and *A. butzleri rpoB* 1415 bp primers. PCR product specificity was confirmed by melting curve analysis using a ramping rate of 0.5°C/10 s from 55-95°C.

Each sample was analyzed by PCR in duplicate. Each PCR run included duplicate standard curves and negative controls. The standard curves were produced for each of the PCR assays using DNA extracted from pure cultures of each of the target bacteria. *Y. enterocolitica* ATCC 9610, *S. enterica* ATCC 13311, *C. jejuni* ATCC 33291, and *A. butzleri* ATCC 49616 were grown from long term stocks stored in 25% glycerol peptone medium at -80°C. *C. jejuni* standard curves were prepared as described by Van Dyke *et al.* (2010). *Y. enterocolitica*, *S. enterica*, *A. butzleri* were grown overnight at 37°C in nutrient broth (BD) followed by staining and direct fluorescence microscopic cell count enumeration as described by Van Dyke *et al.* (2010). The Qiagen Dneasy tissue kit was then used for DNA purification and the final standard solutions were prepared by serial dilution in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The solutions were then stored at -80°C.

5.3.6 Data Analysis

Bacterial PCR concentrations were log transformed and graphs of log cells mL⁻¹ were plotted against incubation time (day). Multivariate ANOVA statistical analysis of bacterial concentrations was used to determine if there was a significant difference in survival between temperatures. Any statistical probability equal to or less than 0.05 was considered significant. For this analysis the entire data sets were used i.e. the results from all sampling days. Strictly speaking, each of these datasets exhibits serial correlation, because the results at one sampling time are influenced by the previous results. To simplify the analysis and because trends were evident by visual inspection of the data, serial correlation was not taken into account in performing the statistical analysis. A more rigorous analysis addressing serial correlation could be performed, however it is not expected that it would alter the basic conclusions reached. Multi-level factorial analysis was performed to look at the significance of the studied parameters as well as the the interactions of three parameters including temperature, pretreatment with and without PMA, and incubation time using Expert-Design 8.0.7.1 statistical

analysis software. Duplicate qPCR data were plotted as the average and confidence intervals (95%, two-sided) were used to determine the significance of compared PCR measurements.

5.4 Results and Discussion

One of the ultimate purposes of source water protection is to ensure that the water quality at the intake of drinking water treatment plants, when combined with appropriate design for treatment process, can guarantee a safe drinking water. Therefore, regardless of the source of bacterial contamination, it is important to better understand the fate of pathogenic bacteria from the contamination source to a drinking water intake. Most human pathogenic bacteria are not able to grow in surface water due to several environmental limitations such as temperature, oxygen levels, restrictive bacterial interactions, and available nutrients, and many of these factors may also affect their survival in water. Therefore, a better understanding of the survival patterns of pathogenic bacteria under environmental stresses can be helpful to evaluate bacterial water quality when it arrives at a drinking water intake. Although the source of most water outbreaks is uncertain, many occurred when high concentrations of pathogenic bacteria entered surface waters or wells. To plan precautionary acts in such situations, it is critical to better understand the bacterial survival trends particularly when elevated levels of bacterial contamination enter source waters. Survival characteristics and trends of four enteric pathogenic bacteria, including *S. enterica*, *Y. enterocolitica*, *C. jejuni*, and *A. butzleri*, were studied in river water. This study used a long amplicon PMA-PCR viability assay which is able to provide an accurate evaluation of live bacteria. The survival study for these bacteria also considered the effect of natural background microflora on bacterial survival.

Water samples used in this study were collected from the Grand River, which is used as a drinking water source. The Grand River drainage area covers approximately 6,800 km² from its origin at the highest point in the Dundalk highlands flowing 280 km south to Lake Erie at Port Maitland (GRCA, 2008). Grand River water quality is affected by various activities along the river as it flows from north to south, including the central area of the Grand River Watershed with intensive farming in the rural areas and dense urbanization (Guelph, Brantford, and Waterloo Region), as well as wildlife habitats (e.g. wild bird populations). The Grand River water quality is also affected by wastewater discharges to the river and its tributaries from approximately 29 wastewater treatment plants (GRCA, 2008). Microbial water quality of the Grand River and its tributaries has been the focus of a number of studies including investigations on the occurrence of pathogens such as *E. coli*, *Campylobacter*

(Van Dyke *et al.*, 2010), *Yersinia* (Cheyne *et al.*, 2010), *Salmonella* (Thomas *et al.*, 2012), *Cryptosporidium*, and *Giardia* (Van Dyke *et al.*, 2013).

5.4.1 Survival of inoculated bacteria in sterile and non-sterile river water at various temperatures

S. enterica, *Y. enterocolitica*, and *C. jejuni* were each inoculated into separate samples of sterile and non-sterile river water at a final concentration of 1×10^6 cells mL⁻¹ followed by incubation at three different temperatures (5°C, 15°C, and 25°C) and their survival was monitored for 28 days (Figure 5.1, Figure 5.2, and Figure 5.3). The sterile river water was prepared by autoclaving to ensure inactivation of all microbiota. While it is possible that heating the samples may have caused some changes in the water, this method was selected to ensure inactivation of all microbial groups (including viruses). In addition, due to the high level of particles in the river water, large volume filtration to ensure a sterile water would be difficult. The three incubation temperatures were chosen to cover the range of water temperatures that occur in the river throughout the year.

Effect of water microflora on the survival of bacteria

For *S. enterica* and *Y. enterocolitica*, survival was affected by the presence of a background microbial community. Bacterial persistence was considerably improved in the absence of background microorganisms at all three temperatures, and this effect was apparent for both viable (with PMA pretreatment) and total (without PMA pretreatment) bacteria. In sterile river water, viable *Y. enterocolitica* incubated at 5°C decreased by only 2 log after 28 days, but in non-sterile river water it decreased by more than 5 log. The difference between survival in sterile and non-sterile water was less at higher temperature. For *S. enterica*, the difference in survival of viable cells in sterile water compared with non-sterile water was 2 to 3 log after 28 days incubation at each temperature (Figure 5.2). The overall difference between survival in sterile and non-sterile water when total bacteria (live and dead cells) were measured was similar; however in some cases such as *Y. enterocolitica* incubated at 25°C, a higher difference was observed.

For *C. jejuni*, the concentration of viable cells in sterile water also remained high at 5°C, and decreased by 3.3 log after 28 days. Similar to *Y. enterocolitica*, the cell concentration decreased more rapidly at higher temperatures (Figure 5.3). For *C. jejuni* inoculated into non-sterile water in experiment #1, there was an error made during the DNA extraction for a number of the samples.

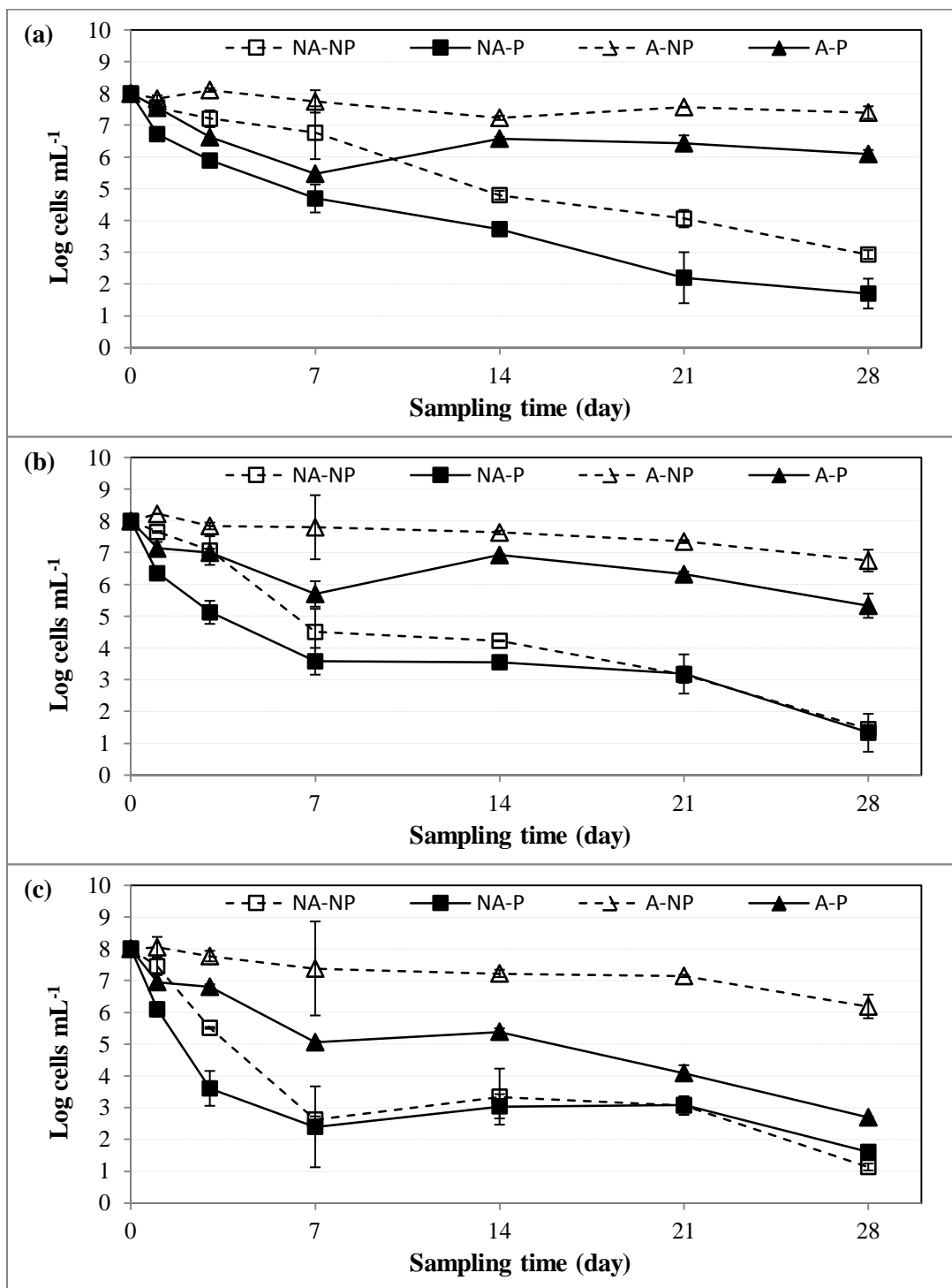


Figure 5.1 Survival of *Y. enterocolitica* in autoclaved (A) river water (▲, △) and non -autoclaved (NA) river water (■, □) incubated at 5°C (a), 15°C (b), or 25°C (c). Cell concentrations were measured by quantitative PCR without PMA (NP) (- - -) and with PMA (P) (---). Error bars represent the 95% confidence intervals of duplicate qPCR measurements made for each sample.

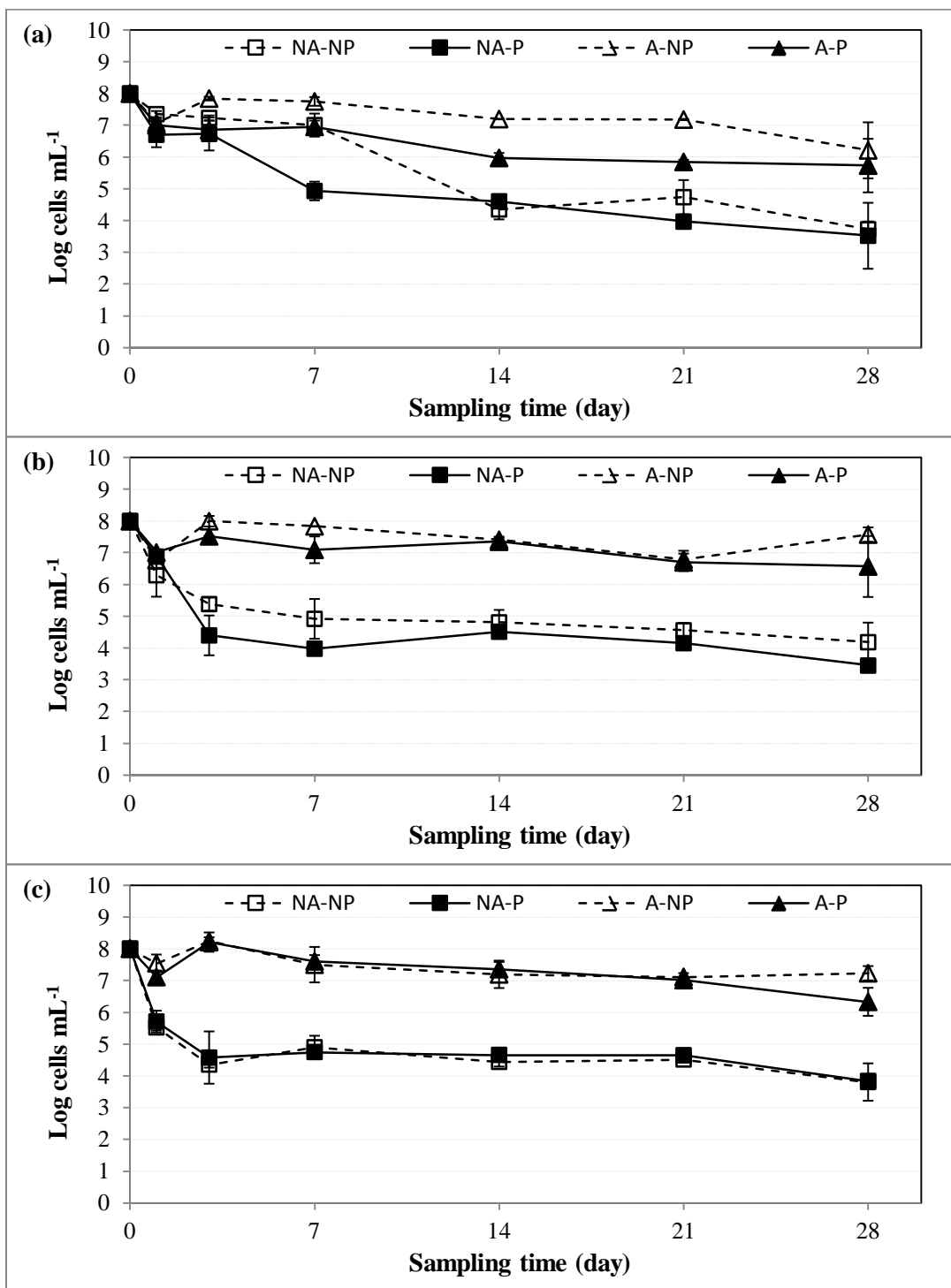


Figure 5.2 Survival of *S. enterica* in autoclaved (A) river water (▲,△) and non -autoclaved (NA) river water (■,□) incubated at 5°C (a), 15°C (b), or 25°C (c). Cell concentrations were measured by quantitative PCR without PMA (NP) (- - -) and with PMA (P) (---). Error bars represent the 95% confidence intervals of duplicate qPCR measurements made for each sample.

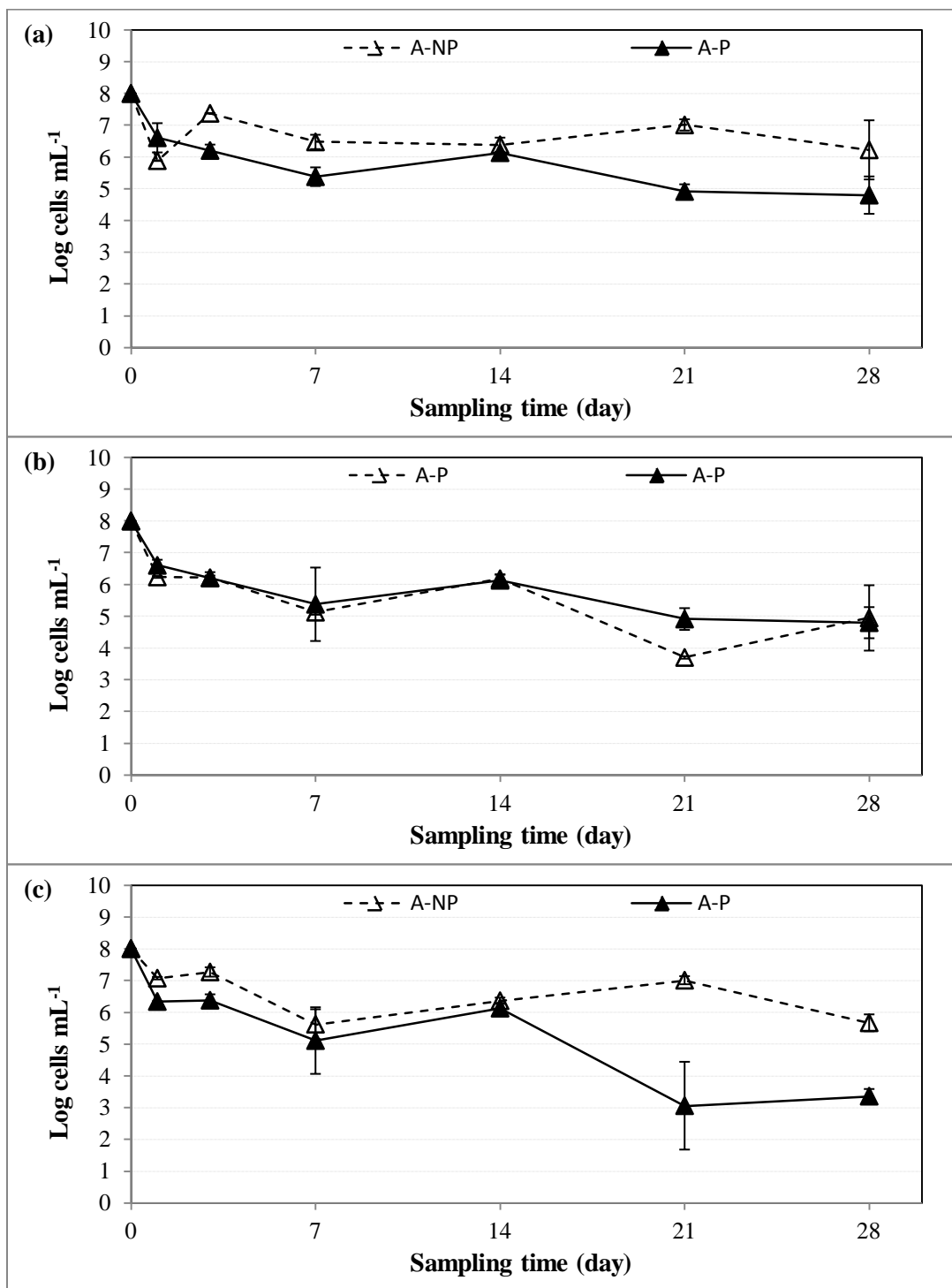


Figure 5.3 Survival of *C. jejuni* in autoclaved (A) river water ($\blacktriangle, \triangle$) incubated at 5°C (a), 15°C (b), or 25°C (c). Cell concentrations were measured by quantitative PCR without PMA (NP) (- - -) and with PMA (P) (---). Error bars represent the 95% confidence intervals of duplicate qPCR measurements made for each sample.

Therefore the data from these treatments could not be used, and only *C. jejuni* survival in sterile river water is presented (Figure 5.3). Therefore a direct comparison between survival in sterile and non-sterile water could not be made for this strain. However, *C. jejuni* survival in sterile water was higher than survival in non-sterile water measured in a subsequent experiment (Figure 5.7).

These results are similar to other studies, including Ramalho *et al.* (2001) who also showed that the survival of *S. enterica* and *Y. enterocolitica* was negatively affected by autochthonous flora present in bottled water. Unlike our results, this study found that the cell concentration declined more rapidly in both sterile and non-sterile water, although this may be because survival was monitored using plate counts. In another study by Kersters *et al.* (1996), *Aeromonas hydrophila* survival in filtered and autoclaved water was compared with unfiltered water, and this was tested for both surface and groundwater. This study found that cell survival was improved by the removal of background microflora, with a difference of up to 5 log cells mL⁻¹ over 16 days as determined by viable plate count. Similarly, Flint *et al.* (1987) used a viable plate count to demonstrate better *E. coli* survival in river water that was both filtered and autoclaved compared to 0.45 µm-filtered (not autoclaved), and survival was even lower in untreated river water. They concluded that *E. coli* survival was more inhibited when incubated in river water in the presence of higher levels of biotic components. These results suggest that bacterial survival in natural aquatic microcosms can be inhibited by the presence of microbial biota, possibly through amensalism, parasitism, and competition (Kersters *et al.*, 1996). On the other hand, a number of studies have suggested otherwise. Kerr *et al.* (1999) showed that *E. coli* O157:H7, when incubated at 15°C for 70 days, survived better in natural compared to sterile (both non-distilled and distilled) mineral water. Tatchou-Nyamsi-Königet *et al.* (2006) observed no difference in *C. jejuni* survival in filtered and non-filtered natural mineral water at 25°C. Korzniewska *et al.* (2005) also observed only a slight difference in *E. coli* and *A. hydrophila* survival in filtered and unfiltered mineral water. However, these studies that compared filtered and unfiltered water might have been affected by the presence of autochthonous viruses or bacteria that passed through the membrane filter. It has also been suggested that autochthonous bacteria may adversely affect the survival of only certain types of bacteria. For example, Marinero *et al.* (2004) showed that while autochthonous microflora did not affect the survival of *E. coli* and *Enterobacter cloacae*, it negatively affected the survival of *Klebsiella pneumoniae* in mineral water.

Apparently, the effect of background microflora on bacteria survival in water is not fully understood due to the diversity of published results. A large majority of these survival studies applied

culture-based counts which can under-estimate the true viable cell count. Also, by using molecular detection methods (i.e. PCR) results can be overestimated. The application of a PMA-PCR viability assay in this study can provide a more accurate evaluation on the effect of background biota on the survival of target bacteria because it is not affected by VBNC cells, and also by excluding the false positive signal from dead cells. The difference in survival between viable (with PMA) and total (no PMA) bacterial cells was clearly evident in our results. For example, PCR measurement (no-PMA) showed a 4.6 log cells mL⁻¹ difference in *Y. enterocolitica* concentration in sterile compared with non-sterile river water after 28 days of incubation at 25°C (Figure 5.1c), but by using PMA-PCR only a 1 log cells mL⁻¹ difference was observed. Our results demonstrate that the adverse effect of background biota on bacterial survival might, in some cases, be overestimated if conventional PCR is used. Therefore, applying bacterial viability assays as done in this study, can provide reliable survival evaluations in sterile versus non-sterile river water.

Effect of temperature on survival trends

Incubation of each strain at three different temperatures (5°C, 15°C, and 25°C) in natural river water (non-sterile) shows that temperature affected the survival trends and that lower temperatures improved survival of viable cells for *Y. enterocolitica*, and *S. enterica*. Higher concentrations of viable *Y. enterocolitica* were detected at lower temperatures, and by three days *Y. enterocolitica* were measured at a level of 6.0, 5.2 and 3.8 log cells mL⁻¹ at 5, 15 and 25°C, respectively. Temperature continued to affect the decrease in *Y. enterocolitica* cell count until a 6.5 log decrease was reached for all temperatures at day 28. For viable *S. enterica*, survival differences were observed after only one day of incubation where a 1-1.2 log cells mL⁻¹ decrease was observed at 5 and 15°C compared with 2.2 log cells mL⁻¹ at 25°C. By day three, similar viable *S. enterica* concentrations were measured at 15 and 25°C, but the count remained higher at 5°C and did not match the counts at 15 and 25°C (3.5-4 log cells mL⁻¹) until day 28.

In sterile water, survival of viable (PMA-treated) bacteria was only somewhat affected by incubation temperature. Over the first two weeks, viable *Y. enterocolitica* survival was not significantly affected by temperature. However, an improved survival was observed at lower temperatures beyond 2 weeks. After 28 days, *Y. enterocolitica* maintained a level of 6 log cells mL⁻¹ at 5°C, 5 log cells mL⁻¹ at 15°C, but reduced down to 2.7 log cells mL⁻¹ at 25°C. This effect of temperature was not observed for viable *S. enterica* survival in sterile water which remained relatively stable during the 28 days of the experiment at 6.5-7 log cells mL⁻¹. Viable *C. jejuni* survival

in sterile river water was affected by temperature only after 2 weeks. After 28 days of incubation, viable *C. jejuni* was reduced down to 4.4 log cells mL⁻¹ at 15°C and 5°C compared to 3.2 log cells mL⁻¹ at 25°C. Interestingly, temperature did not affect the survival of total (live and dead) bacteria in sterile (no-PMA) river water inoculated with any of the three bacteria (*Y. enterocolitica*, *S. enterica*, and *C. jejuni*).

To further evaluate the effect of temperature and PMA treatment on survival trends in river water, a statistical comparison using a multivariate ANOVA was performed between each pair of temperatures (5°C and 15°C, 5°C and 25°C, 15°C and 25°C) in non-sterile river water only, and this analysis was repeated for both viable (PMA) and total (no PMA) results. This statistical analysis showed that both viable (with PMA) and total (no PMA) bacteria persistence is significantly affected by temperature, as the calculated F_{observed} values for almost all paired comparisons (between each of the two temperatures) exceeded the F_{critical} value of 4.75 (Table 5.2 and Table 5.3). Total *Y. enterocolitica* measurements (no-PMA treatment) showed a high F_{observed} values for all paired comparisons (Table 5.2) showing that the effect of temperature on improved survival of total *Y. enterocolitica* was statistically significant. Viable *Y. enterocolitica* (as measured by PMA-PCR viability assay) is also able to survive better at lower temperatures, but the F_{observed} values were much lower than for total *Y. enterocolitica* at each temperature range. Similarly, for *S. enterica* (Table 5.3) statistical analysis shows that viable cell survival is less affected (smaller F_{observed} values) by incubation temperature compared to the survival of total *S. enterica*, and in particular there is no significant difference in the survival of viable *S. enterica* at 15 and 25°C. Overall results of the statistical analysis shows that the survival differences between temperatures were considerably less for viable compared to total *Y. enterocolitica* and *S. enterica* cells. Although this should be further studied, these results suggest that the effect of temperature on the survival of bacteria may be over-estimated using some methods that cannot differentiate viable and dead cells.

Table 5.2 F-values by two-way ANOVA analysis to test the significance of temperature effect on *Y. enterocolitica* survival in non-autoclaved water. The F_{critical} value at 95% confidence is 4.75.

Sample type	Between 5°C and 15°C	Between 5°C and 25°C	Between 15°C and 25°C	Significance of temperature (paired comparison)
Non-autoclaved viable (with PMA)	5.62	30.20	13.15	Significant
Non-autoclaved total (no-PMA)	62.27	220.65	54.40	Highly significant

Table 5.3 F-values by two-way ANOVA analysis to test the significance of temperature effect on *S. enterica* survival in non-autoclaved water. The $F_{critical}$ value at 95% confidence is 4.75.

Sample type	Between 5°C and 15°C	Between 5°C and 25°C	Between 15°C and 25°C	Significance of temperature (paired comparison)
Non-autoclaved viable (with PMA)	17.09	8.40	1.75	Significant (except for 15°C vs 25°C)
Non-autoclaved total (no-PMA)	33.21	162.05	13.36	Highly significant

Another observation on bacterial survival in natural (non-sterile) river water was that the differences between total (no PMA) and viable (with PMA) cell concentrations were affected by both temperature and incubation time. Incubation time is the duration that the bacteria have been under environmental stresses, and also the age of the bacterial cells (assuming that the pathogens do not multiply in the river water). Therefore, a statistical factorial analysis of three-factors (temperature, PMA vs no-PMA, and sampling date) was performed to further study the interaction of these variables, and this was done only for bacteria incubated in natural (non-sterile) river water. Figure 5.4 shows that for *Y. enterocolitica* in non-sterile river water at the low temperature (5°C), the total cell count (no-PMA) was noticeably higher than the viable cells count (with PMA), indicating that dead cells and extracellular DNA from dead cells were observed at each sampling time during this survival study (Figure 5.4). At a higher temperature (15°C), the total cell count was higher than the viable cell count only until day 14, and on days 21 and 28 there was no difference between the two detection methods. At 25°C, a difference between live and dead *Y. enterocolitica* cells was only evident during the first three days of the survival study.

For *S. enterica* (Figure 5.5), dead cells were present at higher concentrations at lower temperatures (5°C) and during the early sampling dates; however, the effect was much less than what was observed for *Y. enterocolitica*. This suggests that it is more important to apply a viability assay when studying *Yersinia* survival at lower temperatures, and also generally during the early stages of the survival study.

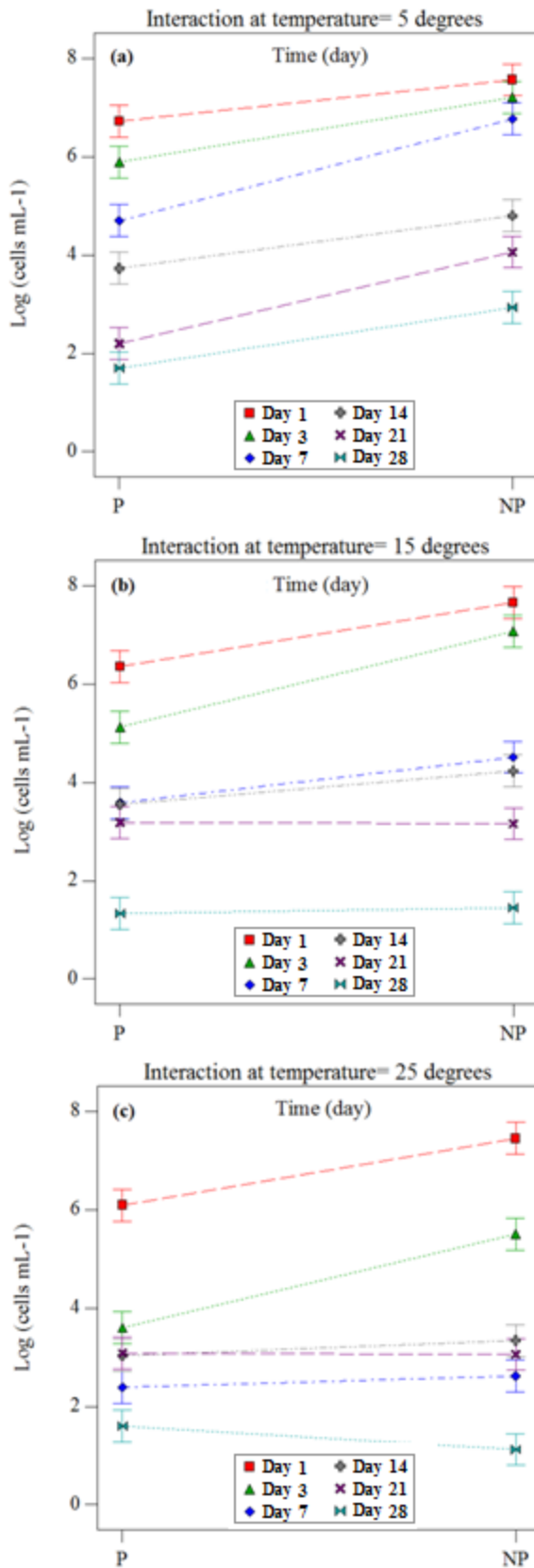


Figure 5.4 Interaction graphs produced by factorial analysis on the effect of temperature, PMA pretreatment, and incubation time on the survival *Y. enterocolitica* in non-sterile (natural) river water. P: PCR with PMA pretreatment; NP: PCR without PMA pretreatment. Incubation temperatures were 5°C (a), 15°C (b), 25°C (c). Error bars correspond to standard errors.

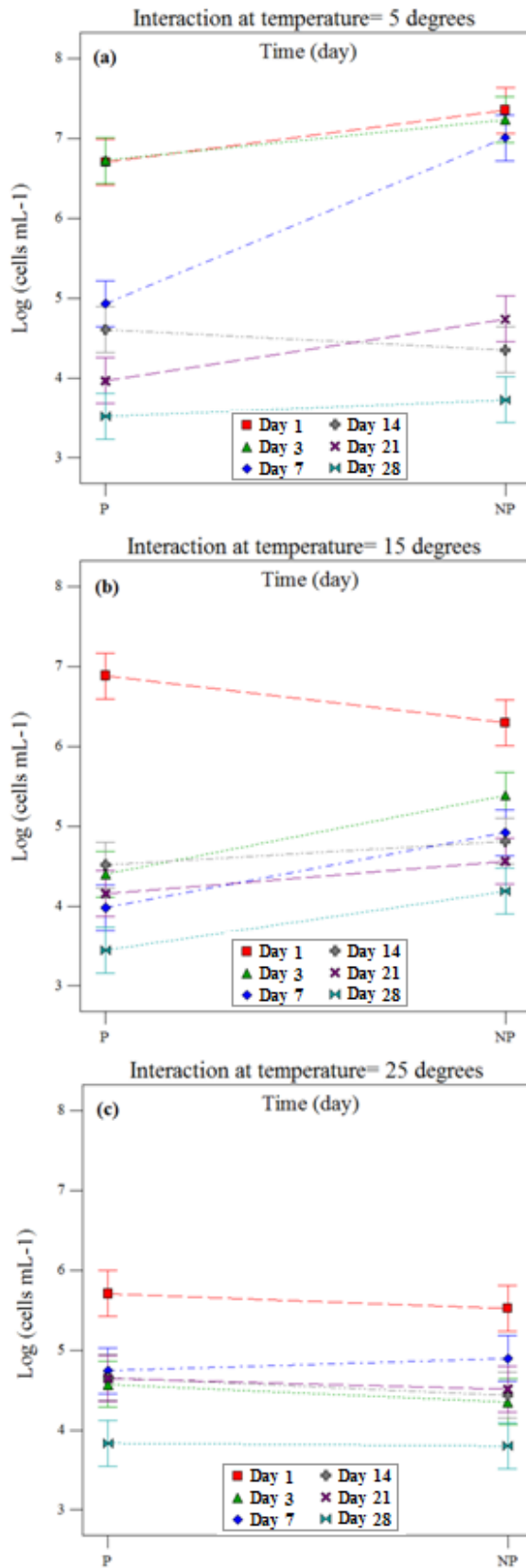


Figure 5.5 Interaction graphs produced by factorial analysis on the effect of temperature, PMA pretreatment, and incubation time on the survival *S. enterica* in non-sterile (natural) river water. P: PCR with PMA pretreatment; NP: PCR without PMA pretreatment. Incubation temperatures were 5°C (a), 15°C (b), 25°C (c). Error bars correspond to standard errors.

Our results show that bacterial survival was more significantly affected by temperature in non-sterile river water than in sterile river water. Therefore, the cell reduction rate was related to the activity of background microflora and that this activity was increased at higher temperatures. Background microflora may include grazing protozoan and bacterial predators that are able to prey on the target bacteria, and they are reported to be more active at higher temperatures (McCambridge and McMeekin, 1980; Sherr *et al.*, 1988). Predatory effects are suggested to be optimal at 15-20°C for grazing protozoa (McCambridge and McMeekin, 1980). Also, non-sterile river water with high microbial activity (i.e. Grand River water) can contain lytic enzymes and nucleases that can be responsible for dead cell and DNA degradation (Bazelyan and Ayzatullin, 1979). This extracellular enzymatic activity could be adversely affected by low temperatures (Deming and Baross, 2005), and may be the reason for slower cell and DNA degradation at lower temperatures (i.e. 5°C and 15°C vs 25°C) that was generally observed for the bacteria tested. Also, at higher temperatures the cell wall may be more fragile while interacting with extracellular enzymes (Baatout *et al.*, 2005). The additive effects of higher microflora activity may be the reason why total (live + dead) cell reduction was more affected by high temperature. Therefore, measuring viable cells is needed to avoid overestimating the effect of temperature on cell reduction.

Many other researchers observed similar survival trends related to temperature. Buswell *et al.* (1998) also showed differences in survival of several strains of *C. jejuni*. Inoculated *C. jejuni* in sterile tap water survived longer at lower temperatures (4°C and 10°C) than at higher temperatures (22°C and 37°C). Terzieva *et al.* (1991) also observed better survival for strains of *C. jejuni* and *Y. enterocolitica* at low temperatures. After 2 weeks of incubation a strain of *C. jejuni* decreased by 4 log cells mL⁻¹ (initial 10⁸ cell mL⁻¹) at 6°C compared to 6 log cells mL⁻¹ at 16°C. Similarly, a strain of *Y. enterocolitica* decreased by 2 log cells mL⁻¹ (initial 10⁸ cell mL⁻¹) at 6°C compared to 4 log cells mL⁻¹ at 16°C. Meckes *et al.* (2003) studied the survival of *E. coli* and *Salmonella* spp. in egg wash water (with no sterilization) at 3 temperatures (5, 15, and 25°C). Using plate counts they observed improved survival for both *E. coli* and *Salmonella* spp. at lower temperatures (5°C >15°C >25°C), with this results being similar to those of our study for that of total (live and dead) bacteria.

5.4.2 Effect of DO and temperature on survival of inoculated *C. jejuni* and *A. butzleri* in river water

Low levels of DO in water can occur in a variety of environmental source waters. River water can be affected by waste/sewage discharge originating from various sources such as municipal, agricultural,

and/or industrial effluents, which can lead to low DO concentrations in water bodies. For example, low DO levels may occur through thermal pollution from industrial sources as well as increased nutrient input from agricultural or industrial discharges, causing eutrophication. Additionally, at high temperatures during the summer, DO levels usually abate for several reasons such as increased levels of plant and animal activities, decreases in the amount of gas dissolved, and more importantly, declined flow rates.

Campylobacter species are mostly considered as microaerophilic bacteria, generally known to require low levels of oxygen (3-15%) (Chynoweth and Thom, 1998). The genus *Arcobacter* was previously categorized and misidentified as aerotolerant *Campylobacter*. The *Arcobacter* are aerotolerant and are able to grow at lower temperatures (below 37°C) making them distinct from the genus *Campylobacter* (D'sa, 2002). However, there are also reports that *Campylobacter* can grow under aerobic conditions which suggested that *C. jejuni* is able to develop aerotolerance (Engvall *et al.*, 2002). Therefore, for both *Campylobacter* and *Arcobacter*, low DO levels in water can potentially be considered as an environmental constraint which might affect their survivability in aquatic microcosms.

High concentrations of *A. butzleri* were detected in our previous study in samples collected from the Grand River (Chapter 4), which is used as a drinking water source. *A. butzleri* is recently gaining more attention in aquatic environments and only a few studies have looked at its survival in tap (Moreno *et al.*, 2004) or synthetic environmental water (Van Driessche and Houf, 2008). In this study the effect of DO levels and temperature on *A. butzleri* and *C. jejuni* survival trends in river water were compared. Non-sterile river was used to perform the study under realistic conditions and in the presence of background microflora based on the results obtained from the previous survival study.

Effect of DO on survival trends

River water with low DO levels was prepared by purging samples with helium, and these were compared with a control water that was not treated (high DO). Low DO levels were measured in a range of 2.3-3.7 mg L⁻¹ and high DO levels had a range of 8.1-9.3 mg L⁻¹ (see Appendix G). There was some increase in oxygen levels in low DO samples over time, but this increase was small (1.4 mg L⁻¹). Results showed that low levels of oxygen slowed the die-off of viable (PMA-treated) *C. jejuni* at low temperature (5°C), starting at day 7 and continuing to day 28, where viable *C. jejuni* survived by

up to 1.3 log cells mL⁻¹ better at low compared to high levels of DO when incubated for longer than 3 days (Figure 5.a). Total *C. jejuni* survival, however, was not affected by the level of DO in water at 5°C. At higher temperatures (15 and 25°C) there was no difference in survival of viable or total *C. jejuni* at low and high DO (Figure 5.6b and Figure 5.c). These results align with the findings of Buswell *et al.* (1998), where no difference was observed between inoculated *Campylobacter jejuni* strains in autoclaved water when incubated under aerobic and anaerobic conditions at a temperature of 37°C. At a lower temperature (10°C), they found the average survival times were longer under anaerobic conditions, although the effect of anaerobiosis was not statistically significant. Similarly, Rollins *et al.* (1986) showed declined *C. jejuni* survival in shaken (aerated) compared to statically incubated (non-aerated) inoculated water.

The total and the viable *Arcobacter* survival were not affected by the DO levels in river water at any of the tested temperatures (Figure 5.). *Arcobacter* spp. are able to grow both in the presence of atmospheric oxygen (aerobic conditions) and under microaerophilic conditions (requiring 3-10% oxygen) (Vandamme *et al.*, 1992). This behaviour is likely because *Arcobacter* produces a large number of respiration-associated proteins which makes it capable of growing and surviving under a wide range of oxygen concentrations (Miller *et al.*, 2007). This can possibly explain the similar *A. butzleri* survival trends in high and low DO river water that was observed in this study, although the role of oxygen on the long-term survival of *Arcobacter* spp. has not been previously studied. *C. jejuni* spp. are known as microaerophiles and although they require oxygen to grow, the atmospheric levels of oxygen are considered lethal to these cells due to oxygen metabolism toxic products. Although new findings suggest that *C. jejuni* contains a wide range of oxidative stress resistance proteins (Atack and Kelly, 2009), they are not as effective as in aerobic bacteria. These proteins are able to eradicate reactive oxygen species (ROS) to some extent and partly avoid associated damage to the bacterial cells (Bui *et al.*, 2012). As observed in this study, high DO adversely affected the *C. jejuni* survival in river compared to low DO levels; these oxygen stress resistance systems may be the reason why the differences were not large.

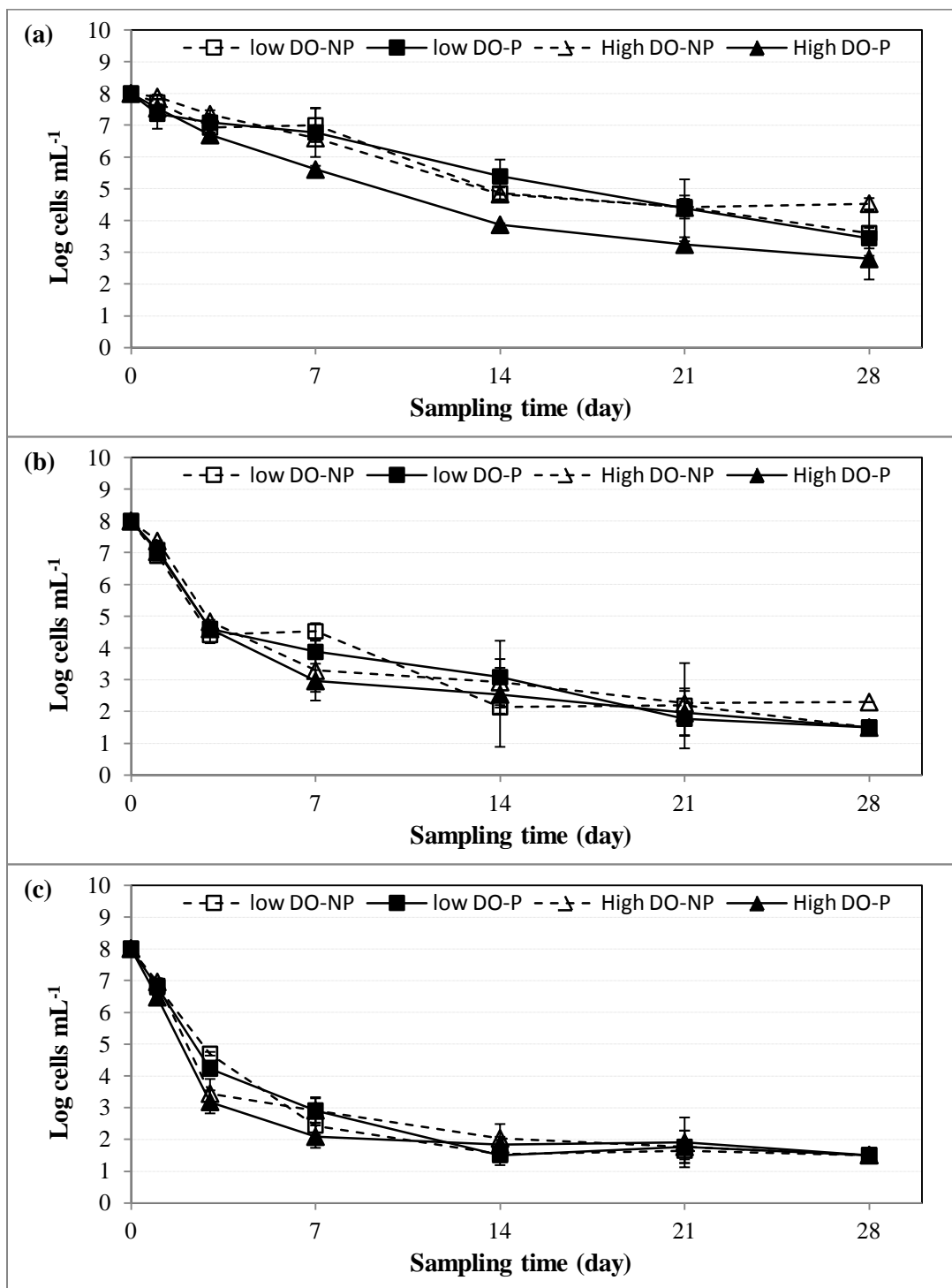


Figure 5.6 *C. jejuni* survival in non-sterile river water containing high DO (▲) or low DO (■) levels and incubated at 5°C (a), 15°C (b), and 25°C (c). Cell concentrations were measured by quantitative PCR without PMA (NP) (- - -) and with PMA (P) (---). Error bars represent the 95% confidence intervals of duplicate qPCR measurements made for each sample.

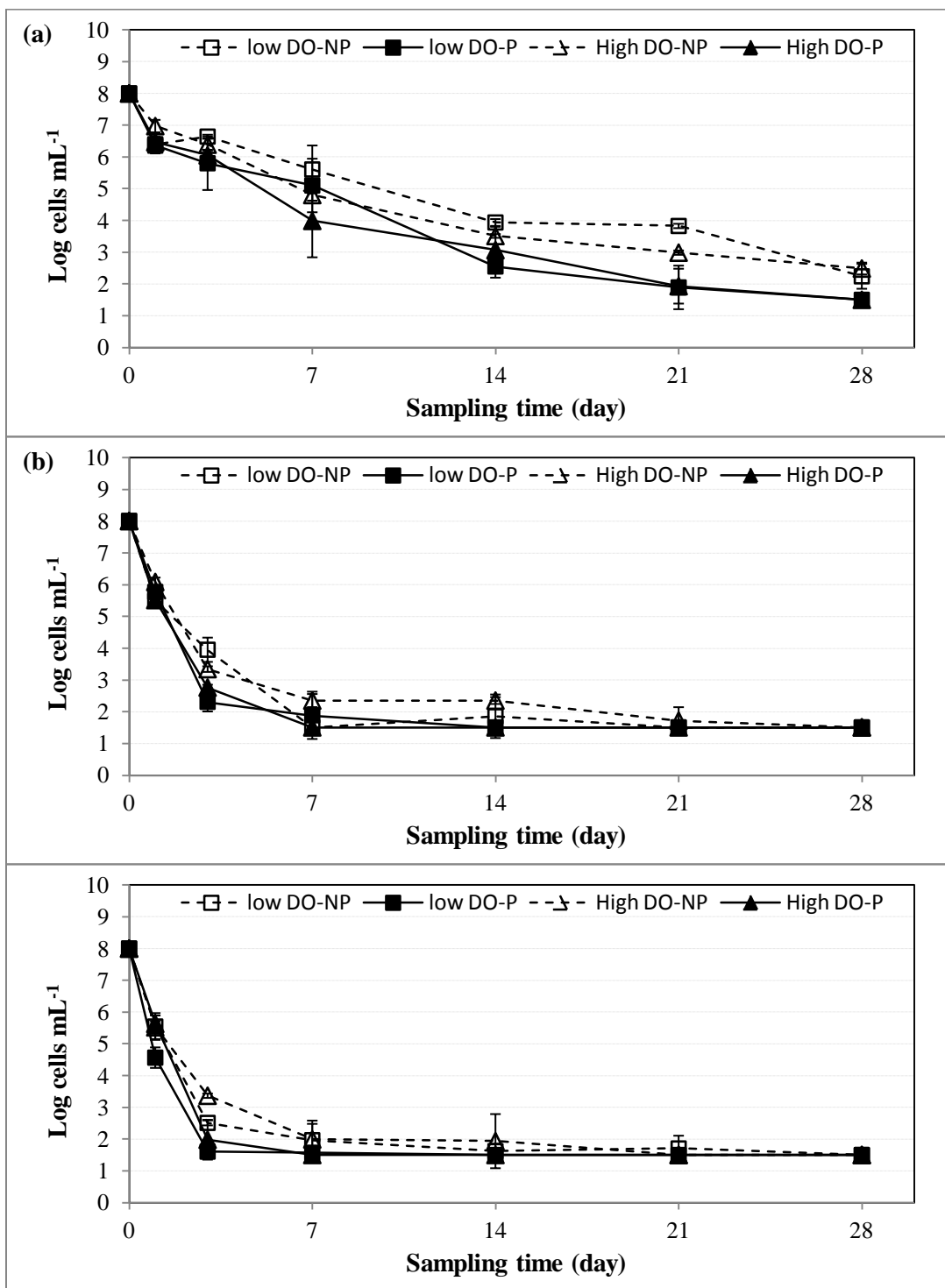


Figure 5.7 *A. butzleri* survival in non-sterile river water containing high DO (▲) or low DO (■) levels and incubated at 5°C (a), 15°C (b), and 25°C (c). Cell concentrations were measured by quantitative PCR without PMA (NP) (- - -) and with PMA (P) (---). Error bars represent the 95% confidence intervals of duplicate qPCR measurements made for each sample.

Effect of temperature on survival trends

Both *Campylobacter jejuni* and *Arcobacter butzleri* survived better at lower temperatures at both low and high DO levels (Figure 5. and Figure 5.). However, *Arcobacter* die-off was faster compared to *Campylobacter*. *Arcobacter* levels decreased more than 6.5 log cells mL⁻¹ (below the detection limit [1.5 log cells mL⁻¹]) in less than 3 weeks at 5°C and in less than a week at both 15°C and 25°C and (Figure 5.). *Campylobacter* decreased by more than 3.5 log cells mL⁻¹ at 5°C, 5.5 log cells mL⁻¹ at 15°C in three weeks, and more than 6.0 log cells mL⁻¹ at 25°C in less than only 2 weeks (Figure 5.). The most considerable effect of temperature on *C. jejuni* survival was observed during the first 3 days where viable cells decreased to 6.7-7.2 log cells mL⁻¹ (high DO - low DO) at 5° C, compared to 4.5 log cells mL⁻¹ at 15° C and 3.1-4.2 log cells mL⁻¹ (high DO - low DO) at 25° C.

PMA treatment demonstrated that dead *C. jejuni* cells were present in high DO river water at 5°C beyond three days of incubation, where a difference of up to 1.8 log cells mL⁻¹ was observed between PMA and no-PMA treated samples. Therefore, PMA-PCR at this low temperature was able to measure that *C. jejuni* survived differently in high and low DO river water, while PCR without PMA pretreatment did not show such differences (Figure 5.a). There was no difference between total and viable *C. jejuni* concentrations at higher temperatures (15 and 25°C). Similarly, *A. butzleri* survival showed up to 2 log cell mL⁻¹ difference at 5°C with and without PMA treatment starting on day 3. However, unlike *C. jejuni*, these differences were observed for *A. butzleri* in both high and low DO river water. At higher temperatures, the concentrations of total and viable *A. butzleri* differed only on particular sampling dates (day 3 at 15°C, and day 1 and day 3 at 25°C). These results suggest that particularly at low temperatures the PMA-PCR viability assay is able to more reliably evaluate the effect of DO levels of river water on *C. jejuni* and *A. butzleri* survival.

The effect of temperature on the survival of *Arcobacter* in water was in general agreement with a previous study by Van Driessche and Houf (2008) where several species of *Arcobacter* survived better in unchlorinated drinking water at lower temperatures (4°C and 7°C) compared with 20°C. Their results showed that after 28 days, approximately 75% of initial *A. butzleri* survived at 4°C and 7°C, compared to 50% at 20°C. However, in their study, *A. butzleri* was able to survive considerably longer at all temperatures compared to our observations. This may be due to the differences in water type, such as the number and nature of the background flora. In other words, the presence of a higher number of natural bacterial background in our study could have reduced the survival of *Arcobacter*, and could explain the faster decrease in *Arcobacter* levels.

5.5 Conclusions

- The presence of natural background autochthonous microbiota in river water highly accelerated the die-off of the target bacterial pathogens studied.
- The application of the PMA-PCR viability assay was able to avoid the over-estimation of the effect of background biota on bacterial survival.
- The bacterial survival (both live and total for all four strains) was significantly enhanced at lower temperatures in natural (non-sterile) river water, especially during the first week.
- Temperature decreases most significantly improved the survival of *A. butzleri* and *C. jejuni*, followed by *Y. enterocolitica* and *S. enterica*.
- Survival of viable (PMA-treated) bacteria was only slightly affected by incubation temperature in sterile water.
- The application of bacterial PCR detection, which cannot differentiate between viable and dead cells, may over-estimate the effect of temperature on the survival of bacteria. This can be avoided by using viability assays such as PMA-PCR.
- High DO levels adversely affected the survival of viable *C. jejuni* only at low temperature (5°C) and only when the PMA-PCR viability assay was applied. *A. butzleri* survival was not affected by water DO levels, which can be explained by its aerotolerance properties unlike the microaerophilic *C. jejuni*.
- The PMA-PCR viability assay was able to more accurately measure the effect of DO levels on bacterial survival.
- The application of a viability assay in our survival studies was able to provide a better understanding of the survival of target waterborne bacteria in river water and provide a more realistic evaluation of the significance of environmental stresses on bacteria in surface waters.

Chapter 6

Overall Discussion and Future Directions

6.1 Overall Discussion and Relevance

6.1.1 Source Water Protection and Public Health Concerns

Pathogenic microbiological contaminants are the source of the most significant risk to public health from source and drinking water. For example, the Guidelines for Canadian Drinking Water Quality (Health Canada, 2012) provided by Federal-Provincial-Territorial Committee on Drinking Water has set water quality “benchmarks” as part of a multi-barrier approach where water quality is tested at the source, water treatment facility, and distribution system (source to tap) to ensure that the water is safe to drink for the consumer. This risk management system is emphasized in terms of pathogenic microbiological contaminants.

Drinking water source protection is perhaps the most challenging step of the multi-barrier approach to safe drinking water. The Ontario Clean Water Act (Ontario Ministry of Environment, 2008) provides a list of the pathogenic contamination sources of surface and ground water such as:

- Application (to land), management, storage and handling of agricultural source materials
- Application of non-agricultural source materials
- Domestic sewage (e.g. wastewater treatment plant effluent)
- Industrial sewage
- Waste management and disposal
- Livestock grazing and pasturing (land use)

These potential pathogen sources suggest that drinking water sources located or potentially exposed to any of the above should be studied and monitored sufficiently to ensure a minimum public health risk. However, any microbial water quality assessment plan requires a key tool: a microbial detection technique. Although proper assessment plans are critical, it is also important to ensure that these assessments use data that have been measured by reliable techniques, in order to provide an accurate evaluation of possible microbial contamination.

Microbial detection techniques and their ability to accurately target and detect the active pathogens in source waters have caused uncertainties in microbial evaluations. For the most part, this is due to the inability of methods to differentiate live and dead microorganisms, which has brought doubt to the output of studies that aim to target pathogenic populations present in source waters. PCR-based methods have substantially assisted in addressing the issue of non-culturable but still pathogenic microbial populations in environmental waters; these methods are able to ensure that there is no ignored threat in terms of pathogen detection. But then it poses the question: are we being too conservative? PCR-based methods detect any genetic material emanating from any source, including extracellular DNA or DNA originating from inactive/dead cells. There have been obvious and substantial differences between the outputs of culture-based vs. PCR-based methods for microbial pathogen detection in environmental water, but the actual pathogen count likely lies somewhere between these two levels. An ideal method would be able to distinguish between live active cells and all other DNA/dead cell interferences. Therefore, viability detection methods can clarify these uncertainties and answer a number of crucial questions, such as:

- Are the data provided by the detection methods regarding pathogen occurrence in water bodies realistic?
- Are the data provided by the detection methods realistic regarding the numbers of pathogens that different pollution sources contribute into the surface waters used for drinking water? In other words, if a PCR-based method is used, aren't we significantly over-reporting the discharged numbers of live active pathogens?
- Microbial water quality assessment of drinking water sources still relies on culture-based detection of bacterial indicators. To what extent are these bacterial indicators able to predict the presence of live/active pathogens in source waters?
- Microbial pathogens are exposed to several environmental stresses, which can be fatal. How realistic is our understanding of viable pathogen survival trends and resistance to these stresses in an aquatic environment, and particularly in surface waters used for drinking water.
- How do wet-weather events affect pathogens levels and what causes peak pathogen concentrations.

In the present study, important improvements were made to a PMA-PCR viability method and applied to address a number of major uncertainties as discussed above.

6.1.2 Method Improvement and Validation

The first step toward applying a pathogen detection method is to select and evaluate the method in order to ensure its applicability and accuracy. Therefore an extensive background study was performed to gather the most highly recommended viability methods in the literature. Since the aim of this study was to apply the viability method to actual environmental water where mixed microbial populations exist, one of my main criteria of the investigation was to select appropriate viability methods for these kinds of water samples. The proposed methods included propidium monoazide (PMA)-PCR as a DNA-based method, and also RNA-based methods including nucleic acid sequence based amplification (NASBA) and the RNA reverse transcriptase method. The PMA-PCR DNA-based method was eventually selected to be evaluated because RNA-based methods are more time-consuming, and RNA purity and integrity is more challenging to maintain compared to DNA-based methods. In addition, it has been reported that RNA and DNA detection provide similar results when targeting viable microbial populations in water (Kahlisch *et al.*, 2012). Therefore, the RNA-based methods were to be considered only if the PMA-PCR method was not successful.

PMA-PCR live/dead cell differentiation, as discussed in Chapter 2 is based on membrane permeability. Therefore, in theory, cells with broken or compromised membrane are considered to be dead inactive cells. The controls included in our experiments (ie. BacLight testing) as well as reports of other studies (Kahlisch *et al.*, 2012) confirm that membrane injury of microbial cells can be regarded as a reliable criterion for dead/damaged cells. At the time of commencement of this study, PMA-PCR was a recently suggested method with only a few research publications regarding the method details and optimization (e.g. Nocker *et al.*, 2006). Results in Chapter 3 show that investigations to apply the method and evaluate it as suggested in the available literature were not completely successful, and false positive PCR signals were still being observed for 3 to 4 log of the heat-killed bacteria even after PMA treatment.

Preliminary work with long amplicon PCR in combination with PMA pre-treatment was promising and therefore new sets of primers were designed to target long fragments of DNA to further improve the method and provide particular PCR-based methods to target bacteria in this study. Application of long amplicon PCR in combination with PMA also showed that UV-killed cells could be effectively excluded in the viability test, which was one of the concerns of using a membrane-based viability assay. The long amplicon PMA-PCR (LA-PMA-PCR) method was demonstrated to be an effective viability detection technique for bacterial pathogens. Having a powerful and effective bacterial

viability detection tool made it possible to apply and eventually answer the several questions and uncertainties (as discussed above).

6.1.3 Bacterial Occurrence in Surface Waters

The application of PMA-PCR to the samples collected from two locations at the Grand River (Chapter 4) did not show significant differences between no PMA and PMA-treated samples which suggests that injured/dead bacterial cells or extracellular DNA were not present or present only at low concentrations. The observed low differences between no PMA and PMA-treated samples were mostly found at location #2, which is located just downstream of a wastewater treatment plant discharge with relatively high bacterial concentrations. These results suggest that inactive/dead bacterial cells or extracellular DNA may not necessarily be of concern in surface waters, and less complicated detection methods (i.e. PCR without pretreatment) may still be able to provide realistic information regarding active pathogenic bacteria in Grand River water. Grand River water can be considered as typical river water used for drinking water purposes and these results may infer similar live/dead cell occurrence and conditions in other surface waters. However, surface water specifications, bacterial contamination sources, river characteristics (e.g. river bed sediments), weather and seasonal conditions, and bacterial communities and concentrations can vary between surface waters, and it is possible that these dissimilarities can affect the live/dead cell ratios.

In terms of the target bacterial occurrence in the Grand River, the results showed that although high concentrations of viable bacterial pathogens were detected downstream of one of the contamination sources (wastewater treatment plant discharge), concentrations at the drinking water treatment intake 15 km downstream, are much lower. Conditions in the Grand River water between these two locations seem to act effectively to inactivate/remove or disperse bacterial contamination.

6.1.4 *Arcobacter*: Is it an Unstudied Threat?

High concentrations of live *Arcobacter butzleri* at both sampling locations on the Grand River suggest a need for further studies regarding the threat and emergence of these bacteria. Very limited information is available on their occurrence and waterborne health risk due to very few relevant studies. More importantly, the source and transmission routes of these bacteria need to be better studied. Other studies have shown that *Arcobacter* have been frequently detected in pathogen contamination sources related to livestock, wildlife and wastewater (as discussed in Chapter 4) which makes these species an important candidate for additional research.

6.1.5 Bacterial Indicators and Viable Pathogen Occurrence in Surface Waters

In general, few or no differences between the concentrations of total and live target bacteria observed in this study meant that the application of the viability detection method did not affect the correlation with the bacterial indicator (*E. coli*). Furthermore, the results from this study emphasized that although *E. coli* indicator concentrations might correlate well with the occurrence of some groups of pathogenic bacteria (e.g. *Campylobacter* and *Arcobacter* in this study) it was unable to predict the occurrence of some others (e.g. *E. coli* O157:H7 and *S. enterica*). These results also suggest that while *E. coli* can be used as a general indicator of fecal contamination (pathogen presence in water), it may still not correlate with pathogen concentrations. Although similar findings have been observed, it is still not well addressed in drinking water guidelines or everyday operational decision-making.

6.1.6 Viable Bacteria Survival in Natural Aquatic Environment

Pathogenic bacterial contamination in surface waters can originate from various pollution sources as discussed at the beginning of this chapter. One of the main factors that can facilitate the understanding of the occurrence of these pathogens in surface water is to study their survival patterns, particularly under conditions that most influence their survival and transport in natural aquatic environments. In other words, it is important to answer the question of how these bacteria survive under environmental stresses in source waters used for drinking water supplies.

Obviously applying a viability assay (i.e. LA-PMA-PCR) can provide a more accurate survival pattern assessment. The results from the study presented in Chapter 5 were able to show that the application of the LA-PMA-PCR viability assay can improve the knowledge of survival patterns of pathogenic bacteria in surface waters under important environmental conditions.

Pathogens in surface water are affected by factors such as: distance and time of travel from discharge, temperature, UV light, and background flora. As was shown in Chapter 5, targeting viable active cells was able to more clearly show that survival patterns can vary between different bacterial pathogens under a range of temperatures and in the presence of background microorganisms present in river water. This can be important when studying how pathogens are able to survive under particular transport or environmental conditions. The viability assay was also able to clarify that although lower temperature waters are considered better environments for pathogenic bacteria to survive, the effect may be different from those studies which apply conventional culture-based bacterial detection methods (e.g. Meckes *et al.*, 2003; Terzieva *et al.*, 1991).

In general, more accurate information on bacterial survival trends can better influence regulatory decision-making. Improved knowledge on the survival capabilities of pathogenic bacteria in surface waters can improve our understanding regarding the effect of seasons on the survival of pathogens in water after point or non-point source contamination (ie. manure spreading, wastewater discharges or spills, storm runoff), or if pathogenic bacteria discharged into the surface water survive long enough to reach a water treatment plant intake.

6.1.7 General Relevance

The data from this study provides key information on pathogens in surface water and promotes the understanding and characterization of source water quality. Results of this work will ultimately provide information that can be used by water protection authorities (i.e. municipalities) to identify source water quality issues, categorize public health threats and provide protective water safety plans. High concentrations of *Arcobacter butzleri* detected in samples from the Grand River (as discussed in Chapter 4) are an example of an issue that warrants further investigation and can be considered as a possible emerging bacterial threat from surface water.

Source water quality is also a major element used in performance measures for public health risk management. As an additional element of this health risk management, optimal and effective water treatment processes can also benefit from the results of this study, since source water quality and its characteristics prescribe the type and level of treatment necessary to produce a safe drinking water. The microbial viability assay suggested in this study can be applied to a broad range of environmental investigations, from ecological to more applied engineering issues, including water/wastewater treatment. By utilizing this viability assay, treatment processes can be more accurately evaluated in terms of their effectiveness in pathogen removal. This can include disease-causing bacteria as well as viruses and protozoa. Additionally, one of the recent approaches toward risk management of pathogen threats which is gaining more attention is the application of microbial risk assessment models (Health Canada, 2010). Similar to any other model, input data (i.e. source water microbial quality and concentrations) plays a vital role in its ability to generate representative and accurate output assessments. The input data to these models related to commonly used indicator pathogens (e.g. *Campylobacter*) is usually not available or is not accurate due to detection method uncertainties. Therefore, not only can the data from this study offer more reliable input for these models, but also the bacterial viability assay that was developed can be used as a powerful tool to provide more accurate data in any source water that is used for drinking water.

6.2 Future Directions

Based on the contributions of this thesis, further identification of gaps in the literature, and critical review of current state-of-the-art research, here we name a number of notable areas of research together with ideas and visions for future research directions:

- Extend the application of PMA-PCR viability assay to study other types of bacterial pathogens, and also different strains within each group.
- Further evaluate the occurrence of human pathogenic *Arcobacter* strains in natural river waters and perform appropriate studies to better understand the potential health threats related to these bacteria.
- Apply the LA-PMA-PCR as a reliable tool to evaluate the effectiveness of treatment processes in removing pathogenic bacteria.
- Design LA-PMA-PCR viability assay methods which are able to perform more reliable studies on virus or protozoa occurrence/removal.
- Design LA-PMA-PCR assays which are able to target virulence genes of waterborne enteric pathogens to assess both pathogen viability and pathogenicity.
- Extend the application of LA-PMA-PCR to investigate the presence and survival trends of bacteria in other surface water pathways or reservoirs, such as soil, and river bed sediments.

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Appendices

Appendix A

Grand River Watershed and River Water Sampling Locations



Figure A.1 Grand River and river water sampling location (http://www.chrs.ca/Rivers/Grand/Grand-M_e.php)

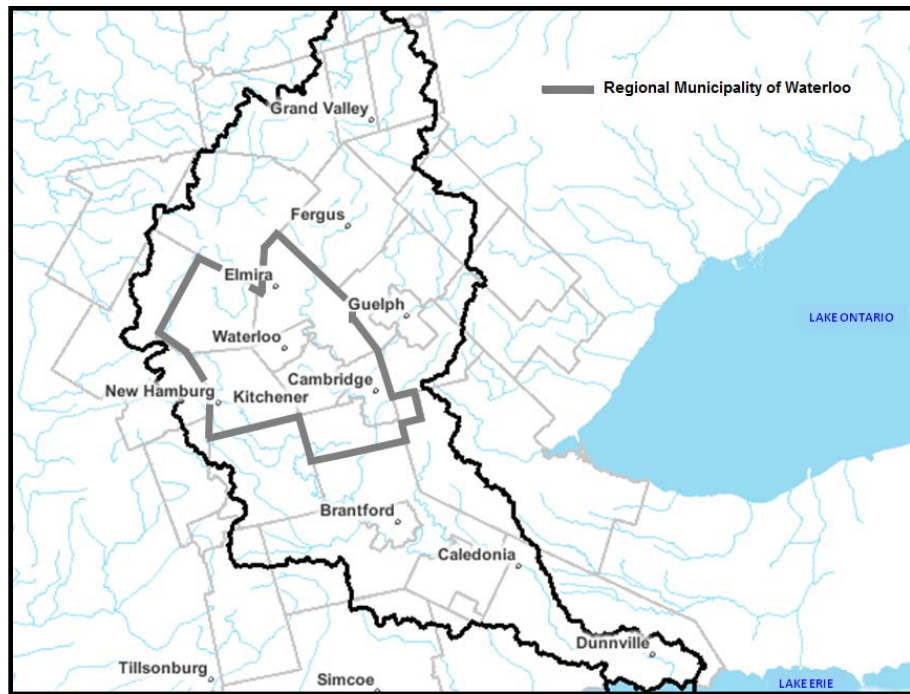


Figure A.2 Grand River watershed and the Region Municipality of Waterloo (GRCA, produced using information under License with the Grand River Conservation Authority © Grand River Conservation Authority, 2013. <http://www.grims.grandriver.ca/>). The Region Municipality of Waterloo boundary line is added to the map by the author.

Appendix B

Propidium monoazide optimal concentration

This appendix presents the results for the experiments performed to initially optimize the PMA concentration by applying a range of concentrations of PMA to *Salmonella enterica* and *Campylobacter jejuni*. The concentrations for further experiments were evaluated defined by:

- Measuring the lethal effects of different levels of PMA on live cells (the optimal PMA concentration should not be toxic to live cells). Also, evaluating the effectiveness of various concentrations of PMA in removing the false positive signal from dead cells.
- Ensuring that the solvent used to dissolve PMA (DMSO) did not cause cytotoxic effects at the concentrations used

PMA concentration

The PMA concentration was initially optimized for *S. enterica*. The optimal PMA concentration was then examined later through a smaller control experiment for *C. jejuni*. *S. enterica* ATCC 13311 and *C. jejuni* ATCC 35920 cultures were prepared as described in section 3.2.1. The *S. enterica* suspension was then adjusted to final concentration of 5×10^6 cell mL⁻¹. For *C. jejuni*, the cell suspension was adjusted to a final concentration of 1×10^8 cell mL⁻¹. Heat-killed *S. enterica* and *C. jejuni* samples were prepared by incubation at 90°C for 20 min. A series of live (no heat treatment) and heat-killed samples of 500 µl were then prepared in microcentrifuge tubes.

PMA was added to *S. enterica* samples at final concentrations of 0, 10, 20, 50, and 100 µM. Each concentration was tested in duplicate. The *S. enterica* cell suspensions were mixed and incubated followed by light exposure as described in section 3.2.3. Four hundred µL of each sample was removed for DNA extraction, and the remaining 100 µL was serially diluted in PBS and used for plating enumeration on nutrient agar. DNA extraction of 400 µl samples of live and heat-killed cells was performed as described in Section 3.2.4 using Qiagen Dneasy tissue kit with a small modification. Since the samples were of a larger volume, 800 µl of AL buffer was added to each sample followed by column purification. Samples were then eluted in 100 µl of AE buffer and stored at -80°C until analysis. For the subsequent testing PMA concentrations on *C. jejuni*, the PMA test range was reduced (0-30µM) and PMA was added to *C. jejuni* samples at final concentrations of 0,

10, 15, 20, 25 and 30 μM . *C. jejuni* DNA purification was performed similarly to *S. enterica* but viable plate count was not done for *C. jejuni*.

qPCR amplification was performed using primers, probes, and PCR conditions as described in section 4.3.4 for both *S. enterica* (Hoorfar *et al.*, 2000) and *C. jejuni* (Van Dyke *et al.*, 2010) quantification. For all PCR assays, 25 μL reaction volumes were used and contained 10 μL of concentrated DNA, 300 nM of each primer, 100 nM of probe, 1 \times buffer, 3.5 mM MgCl_2 , 1.25 U iTaq polymerase (Bio-Rad), 20 μg of bovine serum albumin (Sigma-Aldrich) and 200 μM dNTPs (Sigma-Aldrich).

The results showed that for *S. enterica*, 10 μM PMA was not toxic to live cells and no significant improvement in PMA effectiveness is observed at higher concentrations (Figure B.1). For *C. jejuni*, no significant improvement in PMA effectiveness is observed at PMA concentrations higher than 15 μM (Figure B.2).

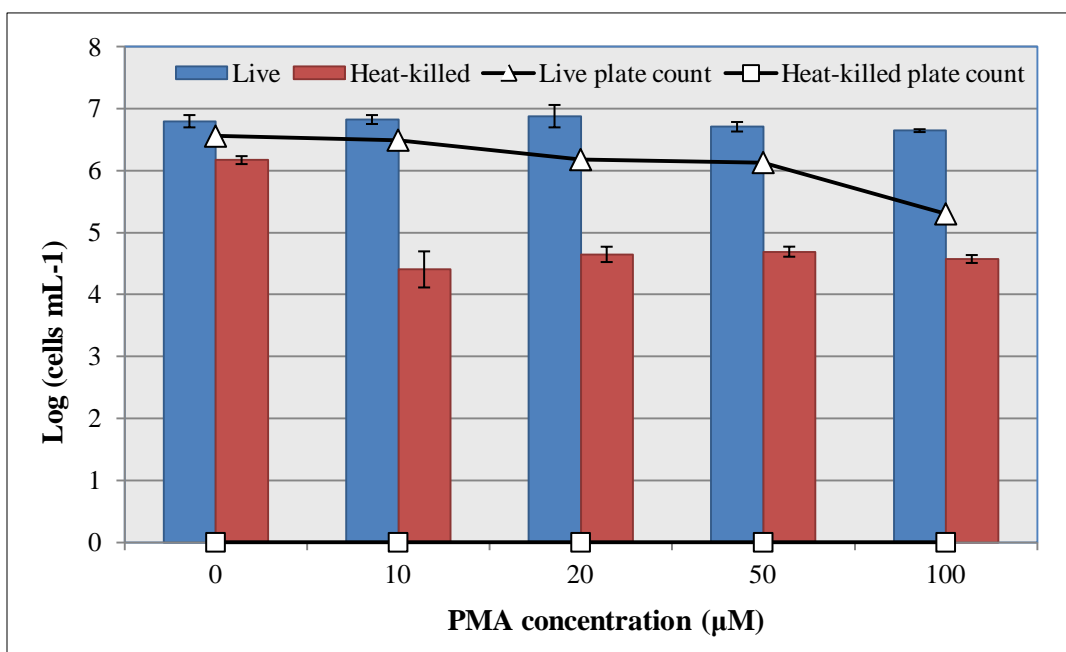


Figure B.1 Optimizing PMA concentration for *S. enterica*; 10 μM PMA is not toxic to live *S. enterica* and no significant improvement in PMA effectiveness is observed at higher PMA concentrations.

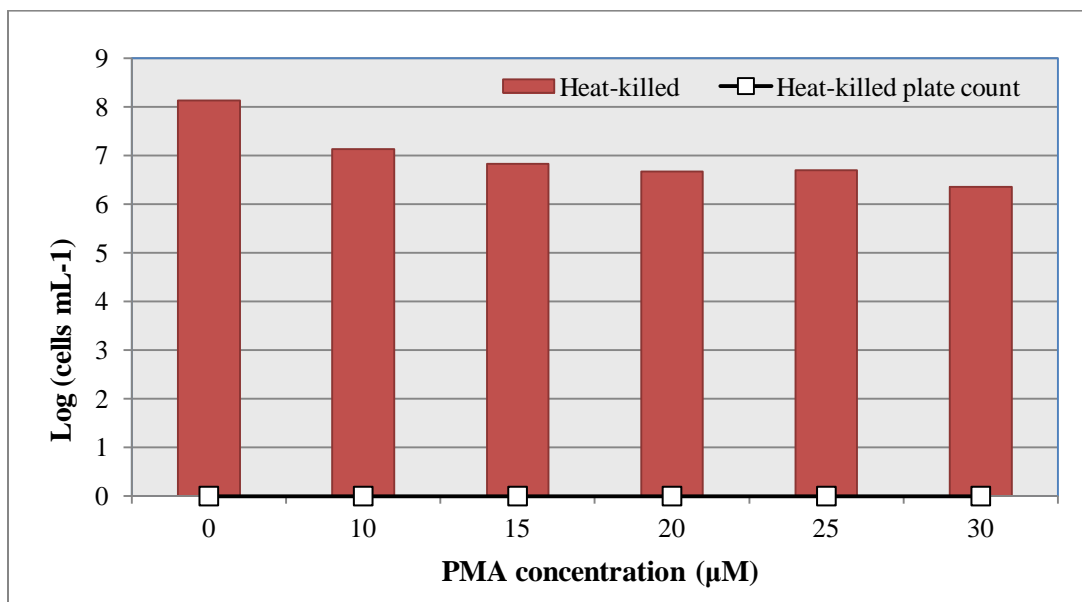


Figure B.2 Optimizing PMA concentration for *C. jejuni*; no significant improvement in PMA effectiveness is observed at higher PMA concentrations than 15 µM.

Evaluation of the cytotoxic potential of dimethylsulfoxide (DMSO)

DMSO was used as a PMA solvent. At certain concentrations, DMSO can result in cell membrane permeabilization; however, this requires DMSO concentrations greater than 1% v/v (Gurtovenko and Anwar, 2007). The final concentrations of DMSO used in this study were substantially lower (up to 0.15% v/v), and this concentration has been reported to have no cytotoxic effect in terms of cell viability and/or growth for *E. coli* (Markarian *et al.*, 2002).

However, to ensure that DMSO caused no effect on the bacteria tested, a control experiment was performed. An *S. enterica* culture was prepared and the concentration adjusted to 1×10^7 CFU mL⁻¹ in 0.85% NaCl as described in Section 3.3.1. Five hundred µL aliquots of the *S. enterica* suspensions were transferred to 1.5 mL sterile microcentrifuge tubes. DMSO (without PMA) was added to *S. enterica* at a final DMSO concentration of (0.15% v/v final concentration), and this was tested in duplicate. A control sample (tested once) was incubated without DMSO. The samples were then mixed for 5 min followed by incubated for 10 min at room temperature. The samples were then serially diluted in PBS, and enumerated by spread plating onto nutrient agar. Results showed that the DMSO solvent when added at the highest concentration used in the PMA experiments did not have

any toxic affect on *S. enterica* (Table B.1). These results align with DMSO suggested non-cytotoxic concentrations in the literature as discussed above (Markarian *et al.*, 2002).

Table B.1. Control to evaluate the potential cytotoxicity of DMSO on *S. enterica* at the highest concentration used in the PMA experiments.

No DMSO (CFU/mL)	With DMSO (CFU/mL) Rep.1	With DMSO (CFU/mL) Rep.2
1.00×10^7	1.15×10^7	9.3×10^6

Appendix C

qPCR standard and melt curves for long amplicon primer sets designed in the current study

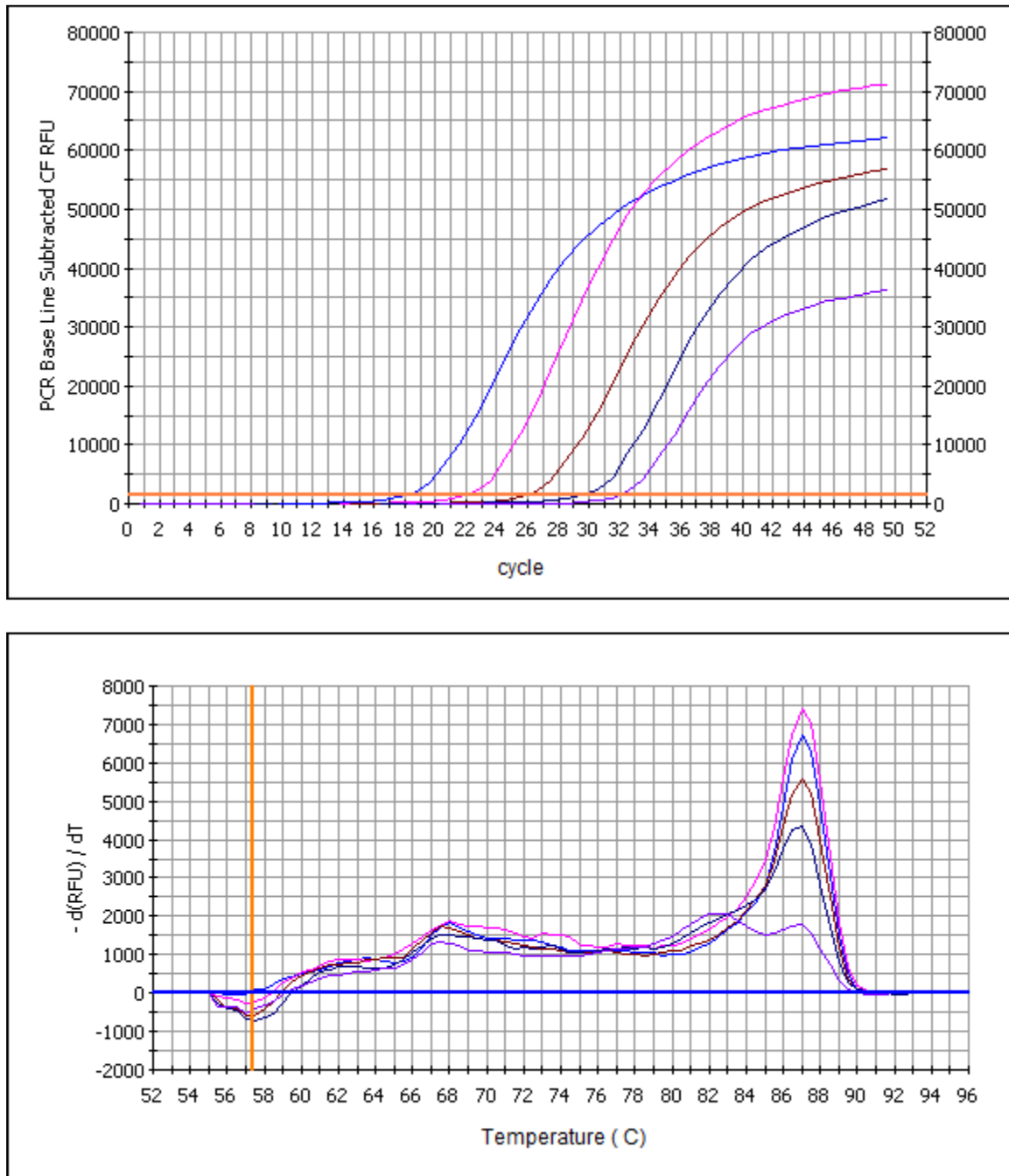


Figure C.1 Standards and corresponding melting curves for *Salmonella invA* long amplicon (1614 bp) PCR assay (see Table 3.1). Standards curves of DNA amplification (shown from left to right) included standard DNA samples with *S. enterica* ATCC 13311 concentrations of 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 and 1×10^3 cells mL⁻¹.

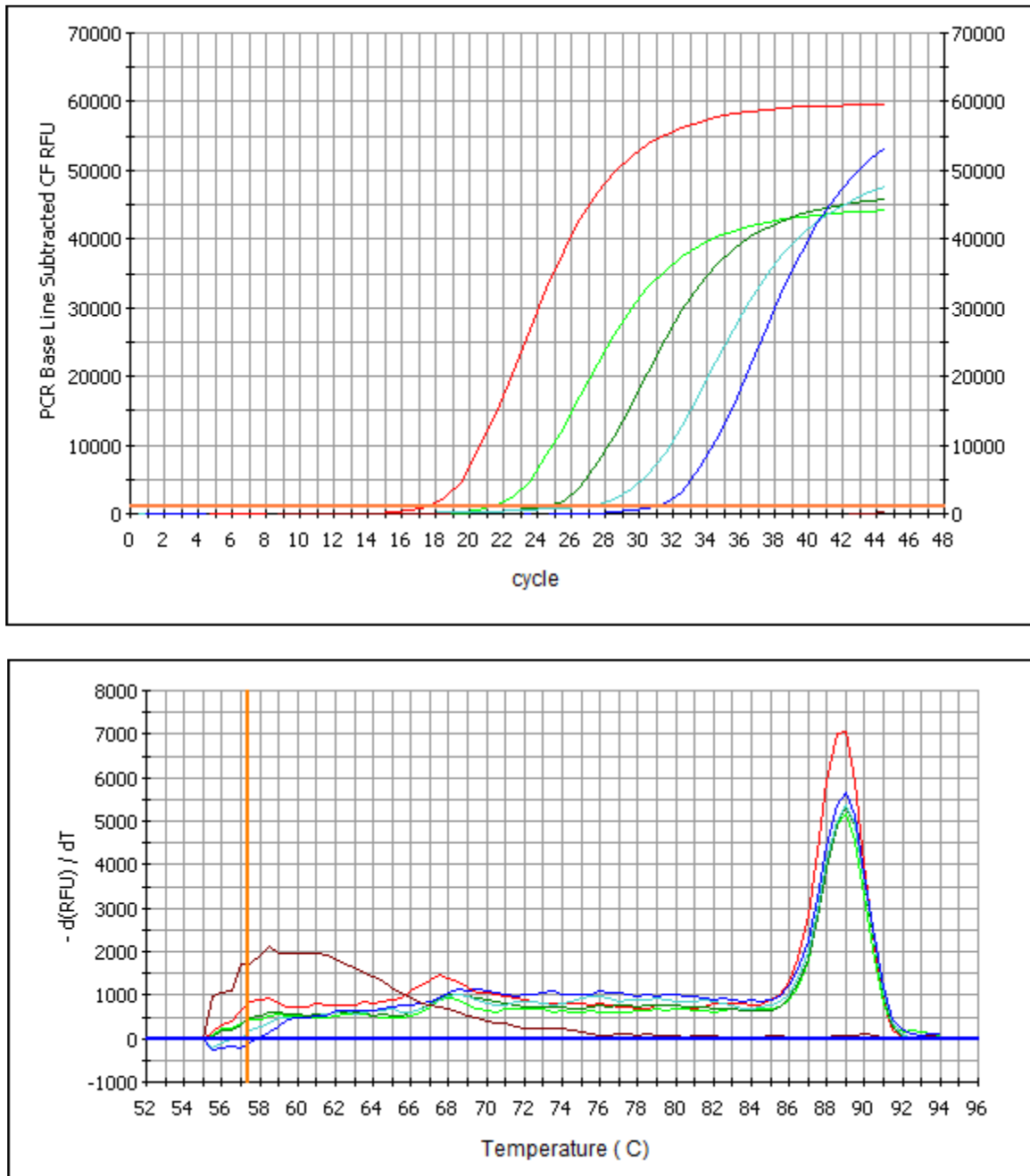


Figure C.2 Standard and corresponding melting curves for *Yersinia enterocolitica* 16S rRNA long amplicon (1213 bp) PCR assay (see Table 5.1). Standards curves of DNA amplification (shown from left to right) included standard DNA samples with *Y. enterocolitica* ATCC 9610 concentrations of 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 and 1×10^3 cells mL⁻¹.

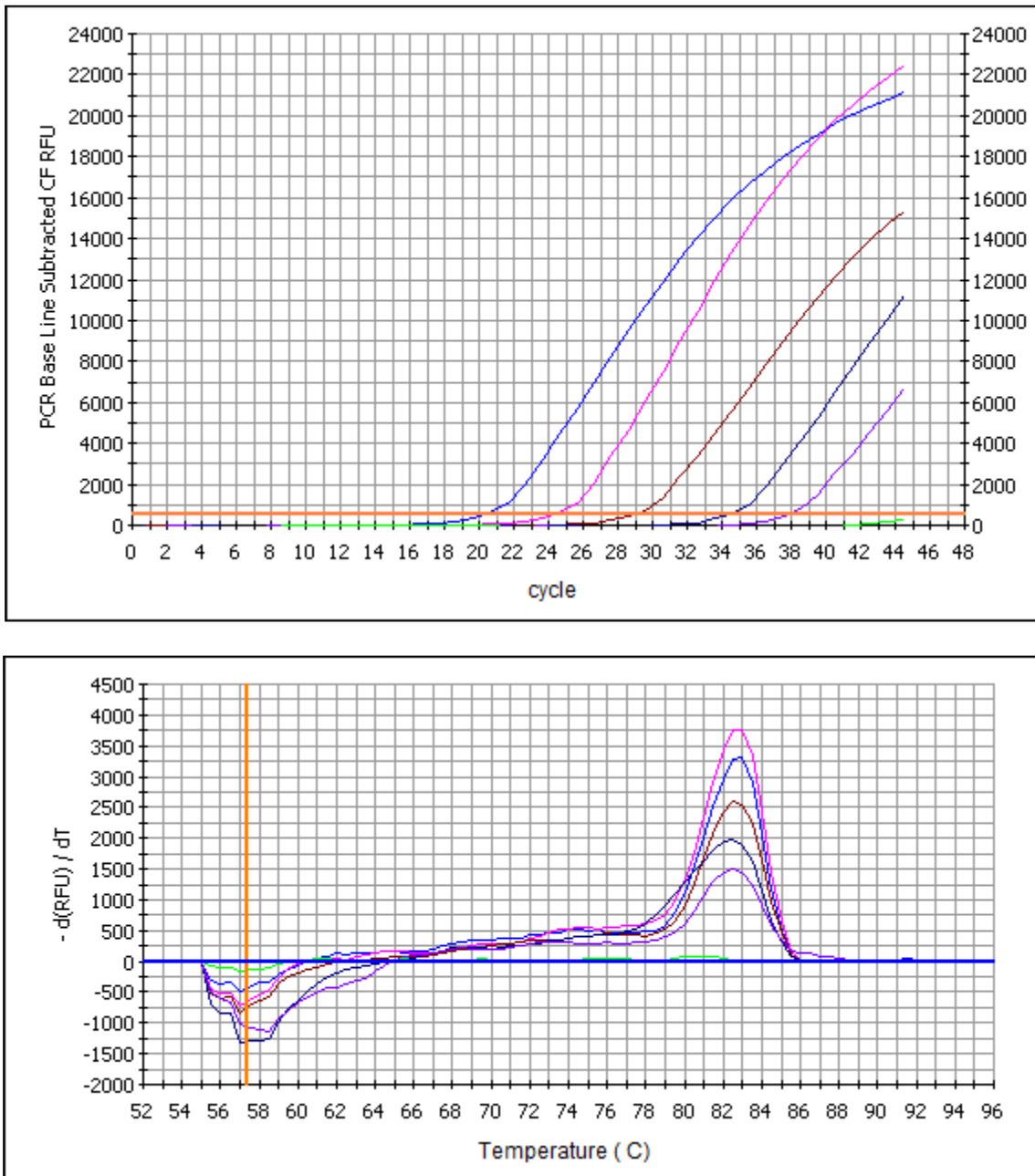


Figure C.3 Standard and corresponding melting curves for *Campylobacter cpn60* 899 bp amplicon PCR assay (see Table 3.1). Standards curves of DNA amplification (shown from left to right) included standard DNA samples with *C. jejuni* ATCC 33291 concentrations of 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 and 1×10^3 cells mL⁻¹.

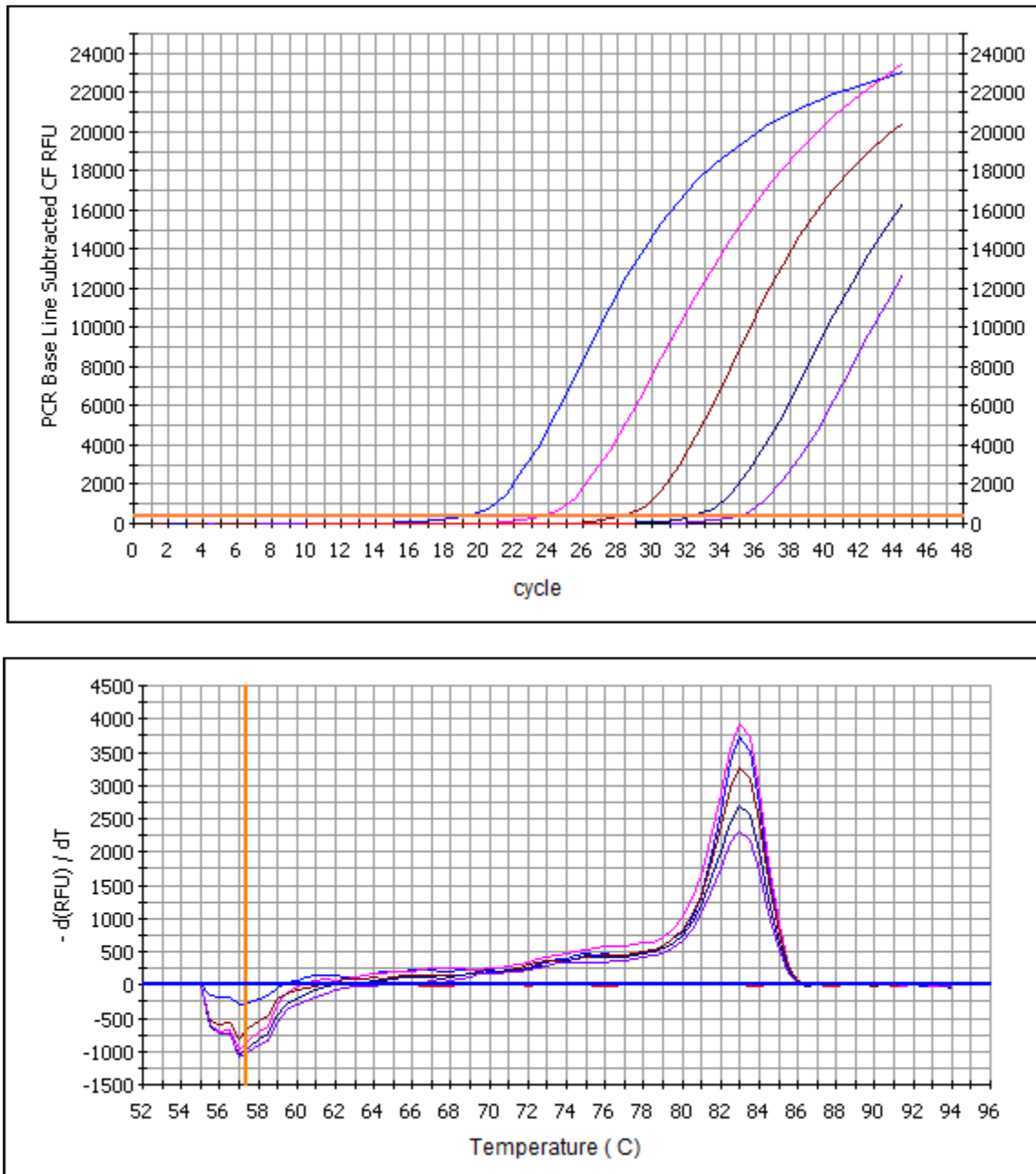


Figure C.4 Standard and corresponding melting curves for *Campylobacter cpn60* long amplicon (1512 bp) PCR assay (see Table 3.1). Standards curves of DNA amplification (shown from left to right) included standard DNA samples with *C. jejuni* ATCC 33291 concentrations of 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 and 1×10^3 cells mL⁻¹.

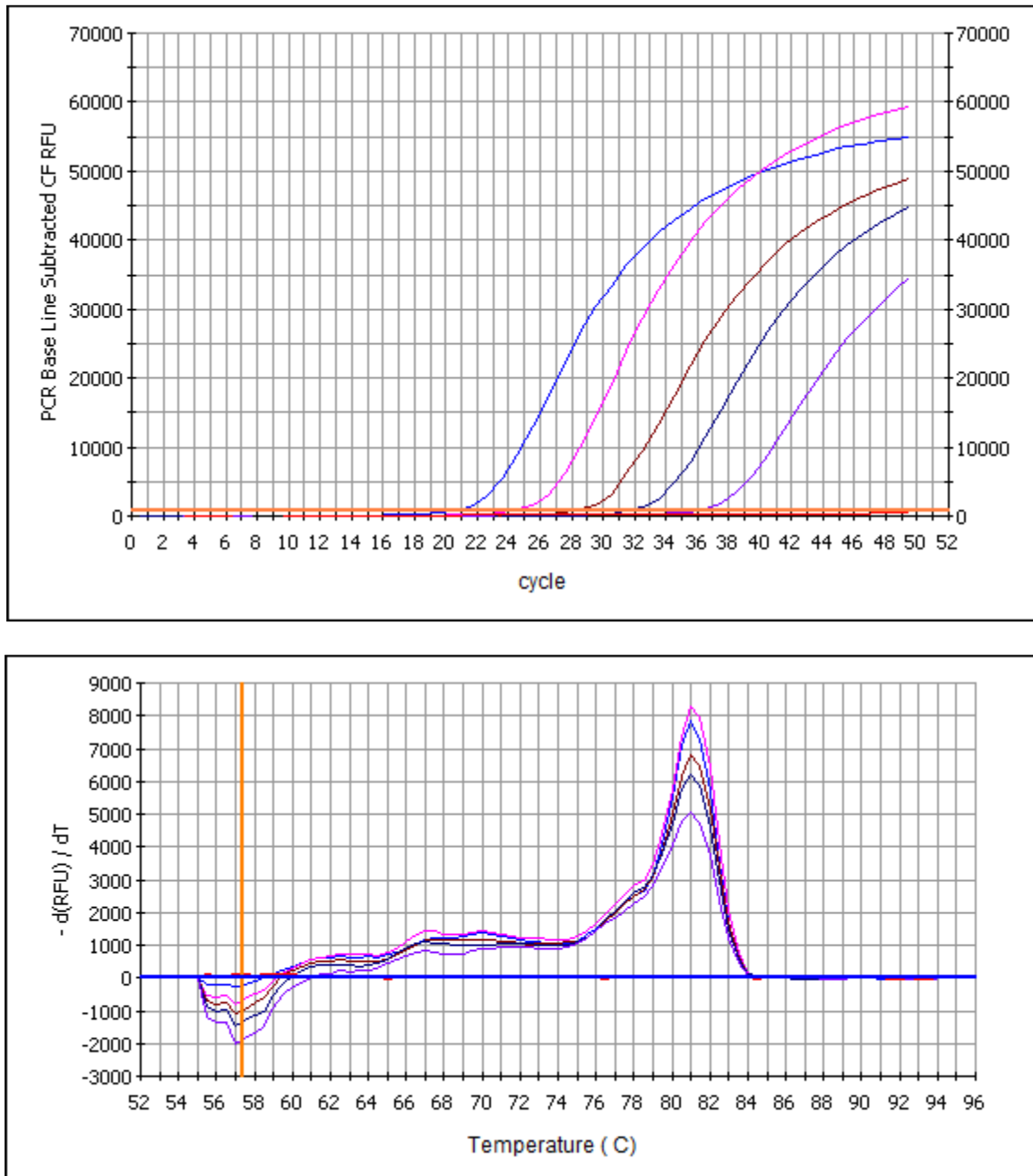


Figure C.5 Standard and corresponding melting curves for *Arcobacter butzleri* long amplicon (1415bp) PCR assay (see Table 5.1). Standards curves of DNA amplification (shown from left to right) included standard DNA samples with *A. butzleri* ATCC 49616 concentrations of 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 and 1×10^3 cells mL^{-1} .

Appendix D

PCR interference check for presence of PCR inhibitors

PCR inhibitors may be present in DNA samples extracted from concentrated river water. Therefore the possible interference of these inhibitors in PCR reactions on river water samples was evaluated for approximately 30% of the samples collected at four sampling dates (Table E.1) including those collected from both locations #1 and #2.

For the interference check on the extracted DNA samples from river water, a *luxB* PCR assay was used as an external control reaction. This PCR assay applies a *Pseudomonas aeruginosa* strain (known as *P. aeruginosa* UG2Lr) which is synthetically marked with the *luxB* gene from *Vibrio harveyi* (Flemming *et al.*, 1994). Since *V. harveyi* is a marine bacteria, *luxB* gene cannot be found in river water and therefore can be considered as a proper target for this control test. To perform the test, DNA template from concentrated river water is added to the *luxB* PCR reactions and tested along with controls containing no river water DNA template. The presence of interfering inhibitors is diagnosed if the *luxB* PCR signal from samples containing the river water template is weaker than that of controls.

The primers and probe were same as those used by Cheyne *et al.* (2010). Fifty μL reaction volumes were used and contained 10 μl of *luxB* DNA template, 300 nM of each primer, 100 nM of probe, 1 \times buffer, 3.5 mM MgCl_2 , 1.25 U iTaq polymerase (Bio-Rad), 20 μg of bovine serum albumin (Sigma-Aldrich), 200 μM dNTPs (Sigma-Aldrich), and 10 μl of concentrated DNA extracted from river water. For inhibition test reactions, 10 μl of *luxB* DNA template added was equivalent to final concentration of 10^5 cells mL^{-1} . PCR amplification conditions were as follows: one cycle at 95°C for 3 min; 40 cycles at 95°C for 15 s, 60°C for 30 s, 72°C for 30 s; and one cycle at 72°C for 10 min.

In the absence of inhibitors, the signal from inhibition test samples is expected to be comparable to the 1×10^5 cells mL^{-1} standard in terms of signal and intensity. The obtained signals for four tested sampling dates are presented in Table E.1. The results showed that inhibitor levels are not different from the control (no river water DNA). A sample set of PCR curves is shown in Figure E.1 which shows that neither of the samples from location #1 or location #2 contains inhibitors at significant levels.

Table E.1 PCR interference check results based on *luxB* amplification (log cells mL⁻¹)

Sampling date (sample number)	<i>lux B</i> (log cells mL ⁻¹)	
	Location #1 (Water treatment Intake)	Location #2 (wastewater discharge)
Control (no river water DNA)	5.00	5.00
24 May, 2010 (#1)	5.04	4.98
6 June, 2010 (#2)	4.99	5.06
27 July, 2010 (#7)	5.27	5.02
16 Dec, 2010 (#13)	5.32	5.09

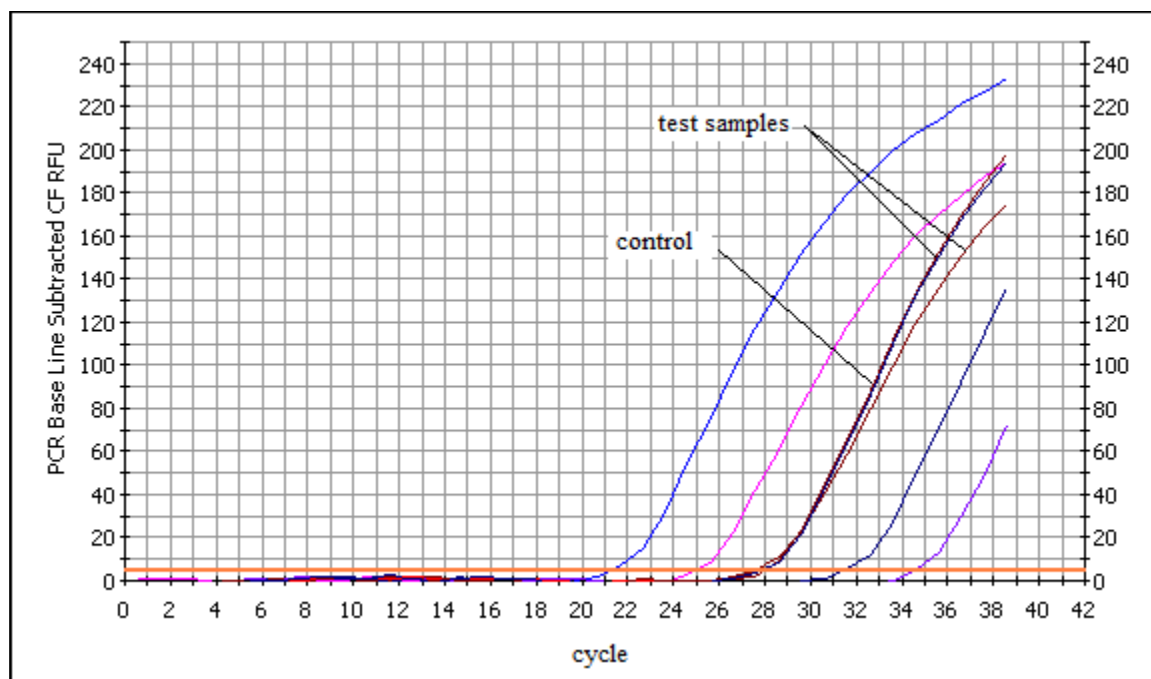


Figure E.1 PCR curves, including standard curves (1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , and 1×10^3 cells mL⁻¹, left to right) and test samples curves (containing DNA extracted from river water, May 2010, location #2) matching the 1×10^5 cells mL⁻¹ standard curve.

Appendix E

Bacterial recovery test for centrifugation in combination with DNA extraction

This recovery test was performed to evaluate the level of bacteria recovery from environmental river water samples as performed in Chapter 4. The recovery method included centrifugation followed by DNA extraction.

Five hundred μL of an *S. enterica* cell suspension prepared as described in section 3.3.1, was inoculated into the 2-L sample of river collected from a location #1. The 2-L sample was then placed in Nalgene 500 mL centrifuge bottles and concentrated by centrifugation as described in Section 4.3.3 to a final volume of 800 μL . The 800 μL concentrated sample was then divided into two 400- μL sub-samples which were used as duplicate. The samples were then centrifuged and the pellets were resuspended in 1 mL of GITC. The samples were then mixed for 2 h at room temperature followed by DNA purification using the Qiagen DNeasy tissue kit. Columns were then eluted in 400 μL of AE buffer followed by PCR as presented in Section 4.3.4.

S. enterica cell suspension was serially diluted in PBS, and enumerated by spread plating onto nutrient agar. The plate count of the *S. enterica* cell suspension was evaluated 1.6×10^6 cells mL^{-1} ; therefore the final concentration of *S. enterica* in the 2L inoculum was calculated as 4×10^2 cells mL^{-1} . Based on this final concentration, the expected *S. enterica* PCR count in the tested duplicate samples was 1×10^6 cells mL^{-1} in case of an ideal recovery of 100%. The average PCR count for recovered *S. enterica* (in concentrated sample) was measured as 6.26×10^5 cells mL^{-1} . Therefore, the achieved recovery by centrifugation followed by DNA extraction was approximately 63%.

Appendix F

PMA effectiveness test in concentrated natural river water samples

Environmental water samples may contain impurities (such as turbidity and high concentrations of extracellular DNA) which may interfere with the PMA effectiveness in removing the false positive signal from target dead cells and extracellular DNA. Therefore a control test was performed prior to the experiments on river water samples (Chapter 4). To assess the possible effect of natural river water impurities on PMA, a PMA effectiveness test was performed.

A 3.5-L river water sample (turbidity = 12.6 NTU) was collected from location #1 in 1-L sterile polypropylene wide-mouth bottles (VWR) and transported to the lab for processing as described in Section 4.3.1. Three-L of the sample was then placed in Nalgene 500 mL centrifuge bottles and concentrated by centrifugation as described in Section 4.3.3. The pellet was then resuspended in PBS and pooled for a final volume of 1200 μL concentrated river water samples. The 1200 μL of concentrated sample was divided into three 400- μL sub-samples and placed in separate microcentrifuge tubes. Two of the sub-samples were inoculated with purified *C. jejuni* DNA to a final concentration equivalent to 2×10^6 cells mL^{-1} one of which was treated with 15 μM PMA (to measure viable cells) and the other untreated (to measure live and dead cells). The PMA pre-treatment was performed as described in Section 4.3.3. One sub-sample was used as a background control with no DNA inoculation or PMA treatment. The full samples were then used for DNA purification (not pre-centrifuged to save the inoculated DNA) by adding 800 μL GITC to each sample followed by column purification using the Qiagen Dneasy tissue kit. Columns were then eluted in 200 μL of AE buffer followed by PCR analysis as described in Section 4.3.4.

The results showed that PMA was able to remove the signal from purified *C. jejuni* DNA to below the background level (Figure F.1). This result shows that PMA effectiveness at the concentration tested was not affected by materials from the river water, and therefore PMA at the applied concentration (15 μM) can be efficiently used for the river water experiments presented in Chapter 4.

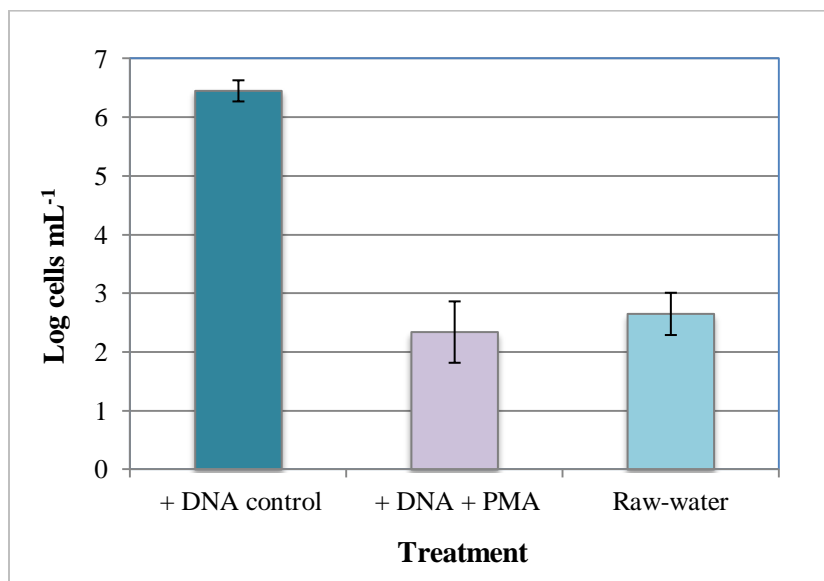


Figure F.1 PMA effectiveness test in concentrated natural river water samples. PMA Concentrated river water inoculated with purified DNA without PMA treatment (■), concentrated river water inoculated with purified DNA with PMA treatment (■), and concentrated river water with no DNA inoculation (■).

Appendix G

Dissolved oxygen (DO) levels during survival experiments of low DO vs high DO water samples

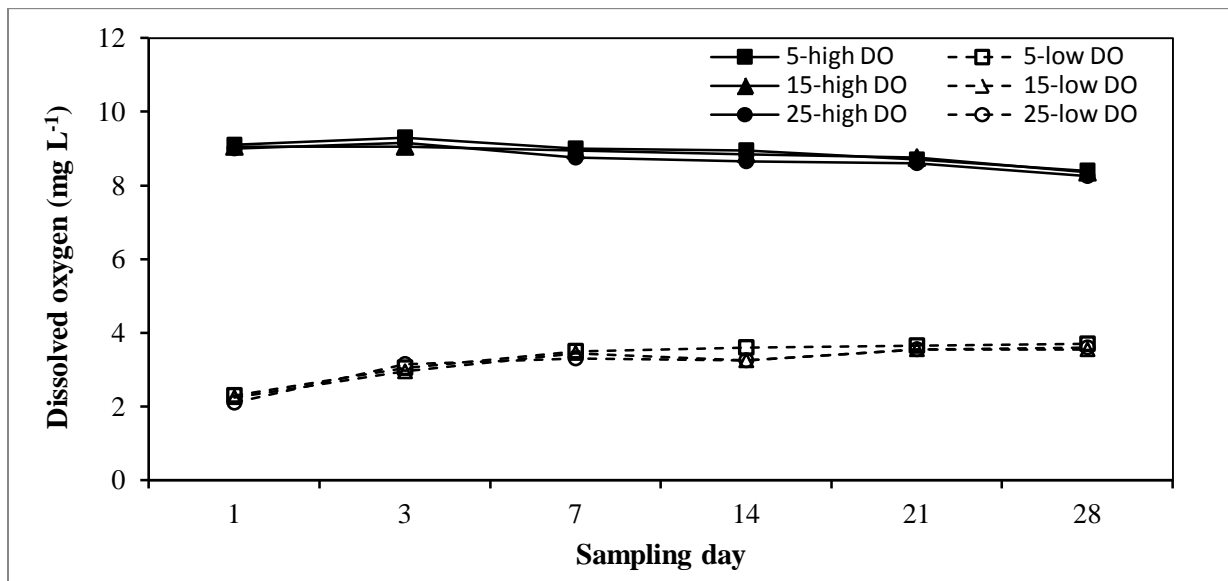


Figure G. 1 Dissolved oxygen (DO) levels of controls during survival experiments of low DO vs high DO water samples (Chapter 5).

Appendix H

PCR efficiency: short amplicon vs. long amplicon PMA-PCR

The application of long amplicons for qPCR analysis is known to reduce the PCR performance and efficiency, and using short amplicon lengths of up to 300bp is always recommended. In this research a number of long-amplicon PMA-PCR assays were applied (Chapter 3 and Chapter 5). Therefore, to evaluate the performance of these short and long amplicon PMA-PCR assays, two efficiency parameters from representative performed PCR assays were compared between short and long amplicon PMA-PCR assays applied in this research (for *C. jejuni* and *S. enterica*). Also these parameters were checked for long amplicon PMA-PCR for both *Y. enterocolitica* and *A. butzleri* to ensure acceptable PCR performances (short amplicon PMA-PCR assays were not used in this study, see Chapter 5).

These two efficiency parameters includes (1) standard curve correlation coefficient (R^2 value), and (2) PCR efficiency (E%). The R^2 value requires to be > 0.98 and an acceptable PCR efficiency should lie between 90-110% (pfaffl *et al.*, 2009). As the table below presents, using a long amplicon PMA-PCR did not adversely affect the PCR performance in terms of efficiency, compared to the short amplicon PCR assays (*C. jejuni* and *S. enterica*). Also, the R^2 values and the PCR efficiency lies in the acceptable range for the long amplicon PCR assays for both *Y. enterocolitica* and *A. butzleri*.

Table H. 1 PCR performance parameters for representative long and short amplicon PMA-PCR assays applied in this research

Target Bacteria	Amplicon length (bp)	Standard curve correlation coefficient	PCR Efficiency (E%)
<i>Campylobacter jejuni</i>	174	1.00	89.6
	1512	0.994	92.3
<i>Salmonella enterica</i>	116	0.998	92.2
	1614	0.994	108.7
<i>Yersinia enterocolitica</i>	1213	0.993	106.0
<i>Arcobacter butzleri</i>	1415	0.988	90.2