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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**MOLECULAR DETECTION AND ANALYSIS OF
ENTEROCOCCUS FAECALIS SURVIVAL IN
TROPICAL ENVIRONMENTAL WATERS**

**GOH SHIN GIEK
SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING
2012**

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ENTEROCOCCUS FAECALIS SURVIVAL IN
TROPICAL ENVIRONMENTAL WATERS**

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School of Civil and Environmental Engineering

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ABSTRACT

The USEPA recommends *E.coli* as indicator bacteria to indicate the presence of disease-associated pathogens in freshwaters, while enterococci are recommended as the indicator for both freshwaters and marine waters. The main limitations of current microbial water quality monitoring that depends solely on the culturing techniques are (i) lack of correlation between fecal indicator bacteria and the presence of pathogens; (ii) long incubation time (at least 18 hours) and (iii) inability to capture bacteria which are injured or in the viable but nonculturable (VBNC) state.

Enterococci which are used as fecal indicators in Singapore are also known to enter the VBNC state when subjected to environmental stresses. In this physiological state, these bacteria cannot be detected by standard culture-based methods and hence, affect the interpretation of microbial water quality measurement. This study aims to develop a sensitive and rapid method to detect all viable enterococcal cells including those in the VBNC state in tropical surface waters, and to understand their significance in surface waters.

Biochemical tests were used to identify *Enterococcus* species in Marina Reservoir catchments, Singapore. The results show that the major *Enterococcus* species is *E.faecalis* (38%), followed by *E.faecium* (29%). The enterococci selective media that is commonly used in membrane filter techniques, mE agar and EIA, were found to give 21% false positive results. *Streptococcus bovis* II 4 and *S.bovis* I were the two main species that contributed to the false positive results in the selective media. *E.faecalis* was selected as the bacteria of interest in this study due to its dominance in urban catchment waters, as well as its significant difference characterization and survival among the *Enterococcus* species.

The development of a rapid method for detection of *E.faecalis* involved both sample preparation and molecular analysis. Three different pre-concentration methods (i) centrifugation, (ii) filtration and (iii) combination of filtration and centrifugation, were examined for their recovery rate. Centrifugation was found to be the most suitable pre-concentration method for local catchment water samples. Conventional DNA extraction and commercially available DNA extraction kits (Wizzard[®] Genomic DNA Purification Kit, Promega; QIAamp[®] DNA Mini Kit, Qiagen) were evaluated according to the purity of extracted DNA and the relative recovery efficiency of extraction. Results show that the commercial kit, QIAamp[®] DNA Mini Kit (Qiagen), gave the best performance. The application of silica membranes in QIAamp[®] DNA Mini Kit was proven to enhance the DNA recovery with minimum interference of inhibitors. A new primer and probe set was designed for downstream quantitative PCR (qPCR) analysis. The newly designed primer and probe set which targets the penicillin binding protein 5 (*pbp5*) gene achieved very sensitive detection (detection limit of 1 gene copy/μl) and greatly improved the specific detection of *E.faecalis*.

Molecular methods, such as PCR and qPCR, are able to detect *E.faecalis* rapidly and specifically. However, these methods are hampered by the detection of dead cells. DNA-intercalating dyes, such as ethidium monoazide (EMA) and propidium monoazide (PMA), have been suggested to overcome this problem. A comparison between EMA and PMA in the pretreatment to bind the extracellular DNA or free DNA from dead cells was conducted. The protocols combining qPCR with EMA (EMA-qPCR) and PMA (PMA-qPCR) were developed to detect *E.faecalis* in environmental water samples. Both EMA-qPCR and PMA-qPCR successfully reduced the amplification of heat-killed cells up to 4 log units. However, EMA-qPCR gave higher amplification inhibition in viable cells compared to PMA-qPCR. PMA-qPCR reduced the amplification of heat-killed cells by approximately 4 log units with minimal effect on viable cells (difference of 0.23 log unit). PMA-qPCR treatment was optimized for application to natural environmental waters by considering cell density and turbidity. Through the optimization process, PMA concentration was reduced, resulting in cost savings and

making this pretreatment method practical for routine water quality monitoring. PMA-qPCR which targets the *pbp5* gene appears to be a promising method to enumerate viable *E.faecalis* in recreational waters by reducing false positive signals from dead cells.

The survival of *E.faecalis* under different environmental stresses (salinity, starvation and sunlight) was examined under controlled conditions in the laboratory. Different decay kinetics were observed for measurements of the bacteria using a culture-based method and a molecular technique (PMA-qPCR). The cell counts for the culture-based method were found to be generally lower than PMA-qPCR over time in the stressed microcosms. This phenomenon suggests the entry of the cells into another physiological state i.e. the viable but non-culturable (VBNC) state. The difference between the two enumeration methods (i.e. the VBNC cells) was modelled as a function of environmental variables. The decay coefficients (k values) showed a strong correlation with light irradiation. In addition, this study demonstrated the ability of *E.faecalis* to adapt to a wide range of salinity. Models were developed to predict the occurrence of VBNC cells under stressed conditions from light irradiation. These models can enhance current water quality models by providing a more accurate picture of the survival characteristics of enterococci in surface waters.

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LIST OF SYMBOLS / ABBREVIATIONS

1D PAGE	One-dimensional polyacrylamide-gel electrophoresis
2D PAGE	Two-dimensional polyacrylamide-gel electrophoresis
A _f	Accuracy factor
ANOVA	One-way analysis of variance
APHA	American Public Health Association
APZ	Acceptable prediction zone
ASW	Artificial sea water
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B _f	Bias factor
BHQ	BlackHoleQuencher
BLAST	Basic Local Alignment Search Tool
bp	Base-pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CE	Capillary electrophoresis
CFU	Colony forming unit
CIEF	Capillary isoelectric focusing
CP	Crossing point
CT	Threshold cycle
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DAPI	4'-6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
DO	Dissolved oxygen
DVC	Direct viable count
EDTA	Ethylenediaminetetraacetic acid
EMA	Ethidium monoazide

FAM	6-carboxyfluorescein
GE	Genomic equivalent
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IMS	Immunomagnetic separation
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
KCl	Potassium chloride
kDa	Kilo Dalton
MF	Membrane filter technique
MPN	Most probable number
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
N	Nitrogen
NaCl	Sodium chloride
NASBA	Nucleic acid sequence-based amplification
NNIS	National Nosocomial Infections Surveillance
NTPs	Ribonucleoside triphosphates
NTU	Nephelometric turbidity units
OD	Optical density
P	Phosphorus
PBP	Penicillin binding protein
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PE	Prediction error
PI	Propidium iodide
PMA	Propidium monoazide
qPCR	Quantitative real-time polymerase chain reaction
R ²	Coefficient of determination
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SGH	Singapore General Hospital

TAMRA	Tetramethyl-6-Carboxyrhodamine
TDS	Total dissolved solids
TGGE	Temporal temperature gradient electrophoresis
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
tRNA	Transfer ribonucleic acid
TSA	Tryptic soy agar
TSS	Total suspended solids
UDG	Uracil-DNA-glycosylase
USEPA	United States Environmental Protection Agency
UV	Ultra-violet
VBNC	Viable but nonculturable
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization

LIST OF PUBLICATIONS

Journal papers

Goh, S.G., Gin, K.Y.H. and Panda, N.R. “Predictive Models for the Occurrence of the Viable but Non-culturable (VBNC) Bacteria under Various Environmental Stresses”, Water Practice and Technology. **6**(4), doi: 10.2166/wpt.2011.076.

Goh, S.G. and Gin, K.Y.H. “Development and Evaluation of *Enterococcus Faecalis* Detection in Tropical Waters using PMA-qPCR and EMA-qPCR”, submitted to Journal of Applied Microbiology.

Gin, K.Y.H. and **Goh, S.G.** “Modeling the Effect of Light and Salinity on Viable but Non-Culturable (VBNC) *Enterococcus*”, submitted to Water Research.

Conference papers

Goh, S.G. and Gin, K.Y.H. “Quantification of Viable *Enterococcus faecalis* in Recreational Water by Propidium Monoazide Quantitative PCR” 14th International Biotechnology Symposium and Exhibition, 14-18 September 2010, Italy.

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Book chapter

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CHAPTER 1 INTRODUCTION

1.1 Overview of microbial water quality monitoring

Very often we would like to ask whether water is safe for us to carry out water activities. To determine the safety of the recreational water, one has to determine the microbial water quality, i.e. to examine whether pathogens which can cause disease are present in the water. These pathogens could be viruses, bacteria and protozoas, and pose a health risk to humans even at low concentrations. Owing to their generally low concentrations, direct detection of pathogens is difficult, impractical and expensive. As such, some bacteria that are correlated to the presence of pathogens have been suggested as indicators to measure the water quality (Tallon et al., 2005; Savichtcheva and Okabe, 2006). Indicator bacteria, such as total coliforms, fecal coliforms *E. coli* and enterococci, have been used extensively to indicate the presence of human enteric pathogens that could cause illness in exposed persons (Noble et al., 2003).

An ideal indicator to assess the water quality should (1) always be present whenever enteric pathogens are present, (2) have longer survival time than the hardiest enteric pathogen, (3) not grow in water, (4) be found in warm-blooded animal's intestines, (5) be unambiguously identified by a simple and easy test, (6) have a direct relationship to the degree of fecal pollution, and (7) be applicable for all types of water (Ohrel and Register, 2006; Gerba, 2009). Total coliforms and fecal coliforms have been used as fecal indicator bacteria since the 1920s (Ferguson and Signoretto, 2011). Both are found in lower intestines of warm-blooded animals and thus their presence indicates contamination from sewage, and possibly enteric pathogens (Ohrel and Register, 2006). Detection of total coliforms and fecal coliforms is simple and relatively cheap. However, studies showed that some species in total coliforms and fecal coliforms (i.e. *Klebsiella pneumoniae*, *Budvicia* and *Rahnella*) were of non-fecal origin, and can be found in nutrient-rich soil and

decaying plant material (Payment et al., 2003). As such, total coliforms and fecal coliforms may not be suitable as fecal indicator bacteria for microbial water quality monitoring. *E.coli*, a member of fecal coliform group, and enterococci, a member of the fecal streptococcus group, are currently the preferred bacteria indicators mainly due to the better prediction of swimming-associated gastrointestinal illness as compared to total coliforms and fecal coliforms (Cabelli et al., 1982). The United States Environmental Protection Agency (USEPA) recommends the use of *E.coli* as indicator bacteria for freshwater recreational waters, while *Enterococcus* is recommended for both freshwater and marine recreational waters (USEPA, 1986).

1.2 Limitation of current microbial water quality monitoring methods

Despite the wide use of fecal indicator bacteria in microbial water quality monitoring, the suitability of fecal indicator bacteria to represent the health risk level is still been challenged. One of the reasons is the lack of data correlating with the presence of disease-associated pathogens. With the improvement in microbiological test methods and bacterial species identification, there is more and more evidence that fecal indicator bacteria, including *E.coli* and enterococci, do not necessarily correlate well with some of the pathogens, particularly viruses (Griffin et al., 1999; Jiang et al., 2001; Nobel and Fuhrman, 2001; Miagostovich et al., 2008). There is also evidence to show that *E.coli* and enterococci are able to survive, grow and establish populations in natural environments such as freshwater lakes and streams, soils and sediments (Byappanahalli et al., 2006). Although there are several bacteria (such as *Bacteroidales* spp. and *Bacteroides thetaiotamicron*) that have been suggested as better alternatives to *E.coli* and enterococci, a comprehensive study is still required to evaluate the reliability of these alternative bacteria indicators.

The current USEPA approved method to evaluate recreational waters is the culture-based method that requires 18 to 96 hours incubation period. Several studies have shown that temporal changes in indicator bacteria levels in beach water occur on much shorter time scales (Nobel and Weisberg, 2005). Thus, these methods

could cause potential problems by not providing rapid warning for indicating public health risks. An extended incubation period is the major limiting factor for the present detection methods, which solely depend on culturing techniques. In addition, the culture-based method could possibly miss the detection of injured cells or viable but nonculturable (VBNC) cells. Generally, all current fecal indicator bacteria are known to enter the VBNC state (Oliver, 2010; Ferguson and Signoretto, 2011). Survival capability of fecal indicator bacteria is highly dependent on the bacterial species, bacterial strain and the environmental stresses encountered by the bacteria. *E.coli* has been shown to survive up to several weeks in oligotrophic media, whereas enterococci can survive for a longer period (i.e. 3 months) in oligotrophic microcosm at cold temperatures (4°C) (Lleo et al., 2001; Boaretti et al., 2003).

1.3 Research questions and objectives

The occurrence of the VBNC state in enterococci constitutes a significant health problem especially these bacteria are not detected by traditional culture-based methods which we commonly use in routine water quality monitoring. Hence, it is important to develop a sensitive and rapid method to detect all viable (including the VBNC) enterococci in environmental waters. The following are research questions to be answered in this thesis:

1. What are typical enterococcal cell density levels in Singapore catchment waters?
2. What are the main species of *Enterococcus* in Singapore catchment waters?
3. Is there any relationship between the physical environmental parameters and the occurrence of enterococcal cells?
4. How long can *Enterococcus* survive in tropical environmental waters?
5. What are the environmental factors that trigger the entrance of *Enterococcus* into the VBNC state for tropical environmental waters?

Based on the research questions, the objectives of this study are defined as following:

1. To identify the major species of *Enterococcus* in a selected Singapore catchment.
2. To investigate the VBNC triggering factors for *Enterococcus* in tropical waters.
3. To develop a sensitive and rapid real-time quantitative detection method that is able to detect the normal and VBNC enterococci in environmental waters.
4. To generate mathematical models to predict the occurrence of VBNC enterococcal cell under various environmental stresses.

1.4 Thesis outline

This thesis is presented in seven chapters with the following organization:

- a) Chapter 1 provides a general introduction and overview of current microbial water quality monitoring. Research questions and objectives are included in this chapter.
- b) Chapter 2 is the comprehensive literature review which covers the overview of enterococci and the different detection methods including the problems associated with each method. A detailed literature review on VBNC related issues and mathematical modeling of bacterial survival are discussed in this chapter.
- c) Chapter 3 describes the detection, enumeration and identification of *Enterococcus* species in local reservoir catchment waters. This chapter also explores the relationship between environmental parameters and occurrence of enterococci.
- d) Chapter 4 describes the molecular techniques detection of *Enterococcus faecalis*. Method development for both polymerase chain reaction (PCR)

and quantitative polymerase chain reaction (qPCR) analyses discussed, including the pre-concentration and DNA extraction steps prior to molecular detection. The design of a new primer and probe set which is suitable for qPCR to detect *E.faecalis*, is also presented. In addition, this chapter addresses the current problem of molecular techniques, i.e. detection of free DNA from dead cells. Pre-treatment of ethidium monoazide and propidium monoazide were optimized for the detection of *E.faecalis* in local reservoir catchment waters.

- e) Chapter 5 examines the decay kinetics of *E.faecalis* under different environmental stresses by using both culture-based method and molecular techniques. Mathematical models were developed to predict the occurrence of enterococcal VBNC cells under various environmental stresses.
- f) Chapter 6 summarizes the major conclusions in this study and suggests some recommendations for future study.

CHAPTER 2 LITERATURE REVIEW

2.1 Overview

This chapter is organized into five main sections: (1) a description of enterococci and their pathogenicity; (2) an overview of VBNC phenomenon in aquatic environments; (3) a review of current methodologies available for VBNC cells detection; (4) a discussion on the problems associated with molecular detection method; and (5) a discussion on predictive modeling of microbial water quality.

2.2 Enterococcus

2.2.1 Bacteria indicator

Enterococci are Gram positive bacteria which are currently utilized as fecal indicators in monitoring microbial water quality. The presence of enterococci indicates the presence of human pathogens due to their prolonged viability in natural environmental waters. Studies have shown a strong positive correlation between the occurrences of gastrointestinal illness and acute febrile respiratory illness with the concentration of enterococci in marine waters (WHO, 2006). The U.S Environmental Protection Agency (USEPA) has recommended a maximum geometric mean of 33 CFU/100ml and 35 CFU/100ml (USEPA, 2004) for enterococci in freshwater and marine water respectively. The enumeration of enterococci is dependent on the number of colonies forming (CFU) on designed selective media, mE agar or improved media, mEI agar.

2.2.2 Pathogenicity of enterococci

In recent years, the enterococci, which were traditionally known as the commensal bacteria inhabiting the alimentary canals of humans and animals, are now emerging as life-threatening opportunistic human pathogens (Fisher and Phillips, 2009). *E.faecalis* and *E.faecium* are the two main species in the *Enterococcus* genus that are leading causes of nosocomial infections (de Fátima Silva Lopes et al., 2005). Among them, majority of human infections are caused by *E.faecalis* while *E.faecium* has been isolated from most of the rest (Huycke et al., 1998; Giraffa, 2002; HPA, 2007). These infections include bacteremia, urinary tract infections and endocarditis. Infections from other species of *Enterococcus* are generally rare. According to statistics in the United States, a huge number of infections are associated with enterococci. There are approximately 110,000 urinary tract infections, 25,000 cases of bacteremia, 40,000 wound infections and 1,100 cases of endocarditis reported annually (Huycke et al., 1998).

Unfortunately, *E.faecalis* and *E.faecium* are also found to be resistant to multiple antibiotics. Among the antibiotics, resistance to vancomycin is particularly of serious concern today. This is because vancomycin usually remains as the final available treatment for life-threatening infections. Thus, these infections will become more difficult to treat and subsequently can cause human mortality. Vancomycin-resistant enterococci (VRE) were first isolated in 1987 in Europe (Leclercq et al., 1988; Uttley et al., 1988). Within 10 years, these VRE spread rapidly in United States (Diekema et al., 2004) and Europe (Widmer, 2012), and also have been isolated from patients in Asia (Ko et al., 2005; Zheng et al., 2007), Australia (Bell et al., 1998) and Africa (McCarthy et al., 2000). The data obtained from the National Nosocomial Infections Surveillance (NNIS) System report revealed that there is an increasing trend of nosocomial infections caused by VRE in US hospitals from 1998 to 2003 (System, 2004).

A localized VRE outbreak has also been reported in Singapore General Hospital (SGH) in year 2004 and 2005 (Kurup et al., 2008). In the first outbreak,

two patients were detected with VRE in their blood or stool samples. Both of them died from underlying comorbidities, which included myeloid leukemia (Chlebicki et al., 2006). In addition, four other inpatients were detected positive for VRE in their stool samples. In the second outbreak, a total of 147 VRE carriers and 4 VRE related clinical cases were detected (Kurup et al., 2008). The latter four patients were suffering from carcinoma. Unfortunately, with the low immunity in these patients, three patients died due to bacteremia and another patient died due to wound infection.

2.3 Viable but nonculturable cells (VBNC)

In the natural environment, bacterial populations are often exposed to various types of stresses. Some common examples of stresses are limitation of nutrient, changes in temperature, salinity, oxygen concentration and solar illumination. To respond to these perturbations, many bacteria will employ a variety of genotypic and phenotypic mechanisms which can increase their adaptive capability to the stressed environment for survival (Colwell, 2008).

Spore formation is one of the sophisticated survival strategies used by some bacteria. This mechanism is believed to be the bacteria's genetically programmed physiological response to enhance subsistence under an environmentally stressed situation. In the spore-forming state, the bacteria's DNA is preserved in the spore, and the bacteria will remain alive but inactive. The spore will only start to germinate once the surrounding environment improves. Subsequently, the bacteria will become active again.

Non-spore-forming bacteria may adopt another mechanism to resist environmental stress, i.e. the viable but nonculturable (VBNC) state. This state is explained by the observation that a certain portion of the bacterial population seems to “disappear” from natural environments during certain seasons of the year, and only making reappearance at other times (Oliver, 1993).

2.3.1 Definition of VBNC

The “nonculturable” concept was raised since 1982, when a group of researchers found that bacteria which have been exposed to a variety of environmental stress were unable to grow in a nutrient broth under optimum conditions. However, the total cell numbers observed through microscope did not decrease with time (Xu et al., 1982).

Based on this concept, Kogure and colleagues developed a direct count method to examine whether the cells in this state were viable or dead (Kogure et al., 1979). In their study, they revealed that almost all the cells remained metabolically active, even though these cells were unable to grow in the nutrient broth or plate. Hence, they defined this physiological state as “viable but nonculturable” (VBNC). Figure 2.1 shows a typical VBNC response.

Figure 2.1 Entry of *Vibrio vulnificus* into the VBNC state on incubation at 5°C. Shown are total cell count (□), culturable counts (○), and viable counts (●) (Oliver, 2005)

Oliver et al. (1993) offered a pragmatic definition, namely, “a bacterial cell in viable but nonculturable (VBNC) state is a cell which is metabolically active, while incapable of undergoing the cellular division required for growth in/on a medium normally supporting growth of that cell” (Oliver, 1993). This VBNC

response is analogous to cell survival when the cell is faced with various environmental stresses which could be lethal to the cell. To complete this analogy, a VBNC cell should be able to emerge from this state and become culturable on routine media when inducing stresses are removed (Figure 2.2).

Figure 2.2 Effects of temperature down- and upshifts on stationary phase cells of *Vibrio vulnificus* in NSS (nine-salt solution) microcosms. Plate counts (●), Direct viable acridine orange direct counts (AODCs) (■), and total viable counts determined by the INT assay (○) (Nilsson et al., 1991).

Note. Abbreviation: INT = 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride

As of today, most microorganisms in the biosphere are still nonculturable *in vitro*. To narrow down the scope of this study, the focus will be on bacteria that are normally readily culturable using standard methods.

2.3.2 Factors that induce cells into the VBNC state

There are several reports on the inducing factors that cause bacteria to enter the VBNC state. In this study, the focus will be on VBNC cells which are induced by environmental stresses. The environmental stresses (particularly in aquatic environments) that have been shown to induce cells to the VBNC state include starvation, depletion of nutrients, variation in salinity, changes in temperature and irradiation. These environmental stresses might be lethal to normal bacteria cells. A

non-spore-forming bacterium can escape from the lethal effect by entering the VBNC state.

Starvation and depletion of nutrient

Generally, aquatic environments such as surface freshwater and marine waters contain relative low concentrations of organic matter (1 to 15mg of carbon per liter) (Roszak and Colwell, 1987) and bioavailable nutrients compared to the optimum growth conditions (Gauthier, 2000). Hence, the entry of aquatic bacteria into the VBNC state may enable these bacteria to withstand long periods of exposure in oligotrophic environments. Studies have shown that bacterial cells are able to enter the VBNC state, by setting up artificial microcosms in the laboratory to simulate an oligotrophic environment (Oliver et al., 1991; Oliver, 1993; Hartke et al., 1998; Trainor et al., 1999; Lleo et al., 2005). In addition, studies have also reported that nutrient starvation, or some other stationary-phase-induced response, may affect the kinetics of VBNC formation (Oliver et al., 1991). This concept is demonstrated in Figure 2.3 which shows the stationary growth phase of the inoculums, delaying the entry of VBNC state when the cells are exposed to a temperature downshift (Oliver et al., 1991).

Figure 2.3 Effect of growth phase on entry into the nonculturable state. Cells of *Vibrio vulnificus* C7184 were cultured in M9 broth plus glucose at room temperature to logarithmic (OD_{610} , 0.15) (●) or stationary phase (OD_{610} , 0.95) (□). Cells were inoculated into ASW + N + P microcosms held at 5°C, and samples were taken periodically for plateable cells.

To date, there is still no finding regarding the influence of variations in nutrient concentrations on the VBNC response. Different bacterial species reported different types of nutrient limitation as inducing factors to the VBNC state (Bryan et al., 1999; Trainor et al., 1999).

Osmotic stress (Salinity)

Survival of *V. parahaemolyticus* at different osmotic stresses have been tested. In a controlled experiment, it was shown that lower osmotic stress (0.5% NaCl) can induce these bacteria to enter the VBNC state in a shorter period compared with microcosms at higher osmotic stress (3% NaCl) (Wong and Wang, 2004). This finding is particularly true for *V. anguillarum* J-O-1, where the bacteria lost its culturability very quickly in freshwater (Eguchi et al., 2000). Hence, decreasing salinity is always the VBNC inducing factor, particularly for marine bacteria.

Temperature

Temperature is another factor that induces bacteria to enter the VBNC state. This is predominant in temperate climate regions where bacteria are exposed to elevated or reduced temperature. Entry of the VBNC state for some of the bacteria is induced by lowering the temperature (Lleo et al., 1998; Wong and Wang, 2004). However, bacteria such as *Pseudomonas fluorescens* was reported to enter the VBNC state when incubated at 35-37°C (Bunker et al., 2004).

Light irradiation

Bacteria that live in aquatic environments are greatly affected by both UV and visible light. Pommepuy et. al. (1996a) demonstrated the effect of natural sunlight, under normal diurnal cycles, on *E. coli* in seawater. In their study, *E. coli* entered the VBNC state when exposed to sunlight. On the other hand, when *E. coli* were kept in the dark, the *E. coli* remained culturable (Pommepuy et al., 1996a).

In conclusion, different environmental factors or the combination of various environmental factors can trigger the formation of the VBNC state in different species of bacteria.

2.3.3 Morphological and molecular changes in VBNC cells

It has been well documented that VBNC cells undergo certain morphological and molecular changes. The morphological changes include changes in cell size, shape, membrane and extracellular polysaccharide. Molecular changes in VBNC cells refer to condensation of nucleic acids and induction or expression of specific genes.

2.3.3.1 Changes in cell size and shape

Many studies have reported the reduction in cell size when cells enter the VBNC state. Two mechanisms were used to justify this size reduction phenomenon; reductive division and gradual size reduction without cell division (Byrd, 2000).

The first mechanism occurs when the cell is under stressed conditions such as starvation. In reductive division, the cell number increases with a continuous decrease in cell size. This pattern of division is suggested as a survival strategy that increases dispersal (Kalanetra et al., 2005). Bacterial species demonstrating reductive division upon starvation have been listed in Byrd's review paper (Byrd, 2000).

The second mechanism involves a reduction in cell size but no increment in cell number. Various bacteria such as *Vibrio vulnificus* (Linder and Oliver, 1989; Marco-Noales et al., 1999) and *Vibrio cholerae* (Hood et al., 1986) have been reported to undergo this mechanism. This reduction of size is always coupled with changes in cell shape. *Campylobacter coli* and *Campylobacter jejuni* have been reported to change the cell shape from spiral shape in the culturable state to coccoid

form in the VBNC state (Rollins and Colwell, 1986; Harvey and Leach, 1998; Lazaro et al., 1999; Thomas et al., 1999; Alonso et al., 2002). Paradoxically, Tholozan et al. (1999) reported different change in shape for *C. jejuni* strain 85, strain 79 and strain Bf. For *C. jejuni* strain 85, VBNC cells observed were shorter and thicker than culturable cells. However, VBNC cells of *C. jejuni* strain 79 and Bf remained as spiral shape except that they were longer (Tholozan et al., 1999). The formation of a spherical shape has also been observed in *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Helicobacter pylori*, *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* (Hood et al., 1986; Linder and Oliver, 1989; Bode et al., 1993; Benaissa et al., 1996; Jiang and Chai, 1996; Marco-Noales et al., 1999; Shleeve et al., 2002).

In contrast, there is a study that refuted the previous observation where *C. jejuni* decreased in cell size when entering the VBNC state (Tholozan et al., 1999). In this report, a notable increase in cell volume was observed with *C. jejuni* in the VBNC state. The gram positive bacteria, *E. faecalis*, has also been shown to have a slightly elongated shape in the VBNC state (Signoretto et al., 2000). It is possible that the morphological changes in terms of cell size and shape are dependent on the strain of bacteria and the induction factor to VBNC state.

2.3.3.2 Changes in membrane and extracellular polysaccharide

Shrinkage of the cytoplasmic membrane has been observed in many gram negative bacteria when they respond to stresses such as starvation. Formation of bleb-like membrane vesicles is believed as a process for cell volume adjustment (Kusters et al., 1997). Through size reduction, the cell will achieve higher surface-volume ratio, subsequently minimizing cell maintenance requirements and enhancing substrate uptake. *C. jejuni* and *V. parahaemolyticus* formed bleb-like membranes when exposed to starvation stress at low temperature (Jiang and Chai, 1996; Lazaro et al., 1999; Thomas et al., 1999). However, *E. faecalis* did not show significant shrinkage when incubated in a nutrient poor microcosm. Instead, the VBNC *E. faecalis* shows increasing resistance to mechanical disruption when

compared with exponentially growing, stationary and UV-killed cells (Signoretto et al., 2000).

In response to environmental changes, modification of the bacterial cell membrane plays a crucial role in the survival strategy. Maintenance of fluidity is important for the membrane to function under different environmental conditions. This membrane fluidity is regulated through fluctuations in the proportion of saturated-to-unsaturated fatty acid species and the chain lengths of phospholipid fatty acids (Magnuson et al., 1993). As the incubation temperature decreases, the percentage of unsaturated fatty acid in the bacterial membrane is expected to increase. *V. vulnificus*, *V. cholerae* and *E. coli* have demonstrated increasing unsaturated fatty acids in membranes of starved cells at low temperature (e.g. 5°C) (Hood et al., 1986; Linder and Oliver, 1989; Day and Oliver, 2004). On the other hand, starved cells at room temperature show increased saturated fatty acids but decreased unsaturated and hydroxyl fatty acids (Hood et al., 1986).

Another morphological change is the production of extracellular polysaccharides. An extracellular substance that adheres to the cell surface of *V. vulnificus* has been observed after long term starvation (Marco-Noales et al., 1999). A similar observation was reported in *V. parahaemolyticus* (Jiang and Chai, 1996). The production of polysaccharide is suggested as one of the starvation-survival strategies particularly for benthic microorganisms, whereby detachment of bacteria from surfaces permits scavenging for nutrients (Byrd, 2000). Nevertheless, production of extracellular polysaccharides is species variable and may not be found in other bacteria such as *Vibrio* sp strain Ant-300 and *Klebsiella pneumoniae* (Byrd, 2000).

2.3.3.3 Condensation of nucleic acids

Several journal papers have reported that an increase in VBNC cells is required to obtain a PCR result (Brauns et al., 1991; Coleman and Oliver, 1996). This problem draws an assumption that nucleic acids in VBNC cells exist in

condensed form (McDougald et al., 1998). A comprehensive study on DNA condensation was carried out by combining LIVE/DEAD® BacLight™ (Molecular Probes, Eugene/Poland, OR) staining with flow cytometry and epifluorescence microscopy (Sachidanandham et al., 2005). Two bacteria strains, namely *E.coli* and a coliform isolate H03N1, were induced to the VBNC state by saline stress in static conditions at 30°C, followed by three cycles of freezing and thawing in liquid nitrogen. From the discrepancies observed in m-FC assay and flow cytometric counts, the stressed cells were shown to enter the VBNC state. When the VBNC cells were stained with SYTO 9 and propidium iodide, a fraction of the population, were observed to produce a bright green emission from the center of the cells under an epifluorescence microscope (Figure 2.4). This observation clearly shows that SYTO 9 dye binds to the condensed DNA at the center of the VBNC cells.

Figure 2.4 *Escherichia coli* cells stained with BacLight and viewed under epifluorescence microscope using blue light excitation. (A) Normal *E.coli* stained with BacLight. (B) VBNC *E.coli* stained with BacLight. VBNC cells with intact membrane fluorescence green by SYTO 9, which binds to the condensed DNA at the center of the cells (Sachidanandham et al., 2005).

2.3.3.4 Expression of specific genes and proteins

Protein profiles and gene expression for microorganisms confronting stressed conditions were shown to be different when compared with those from exponential growing and stationary phases. The difference in protein profiles and

gene expression are very useful for studying the viability/activity of the cell and at the same time estimating the pathogenic potential of the cell.

During the VBNC state, the *rpoS* gene (encoding the stress sigma factor RpoS) and *tufA* gene (encoding the elongation factor EF-TU) in *V. vulnificus* were continuously expressed in both *in situ* and *in vitro* studies (Smith and Oliver, 2006). The virulence gene, *vvhA* (hemolysin gene) was expressed only in *V. vulnificus* strain C7184k/o but not in another two strains during the VBNC state. However, two strains (out of three strains) were successfully resuscitated from VBNC state when the temperature was upshifted to room temperature. *In-vivo* resuscitation of *V. cholerae* has been performed on iron-dextran-treated mice (Asakura et al., 2007a). This shows that the VBNC cell may initiate infections during the VBNC state or after resuscitation. In contrast, another study on *V. parahaemolyticus* reported that the virulence genes were expressed in VBNC cells but not induced in these cells after the recovery of culturability (Coutard et al., 2007).

In addition to the *tufA* gene and *rpoS* gene, which were expressed continuously in VBNC cells, the *relA* gene was reported to have higher RNA copies in VBNC *V. cholerae* when compared to starved *V. cholerae*. The authors suggested that *relA* mRNA is preserved in the VBNC state but not in the starvation state (Gonzalez-Escalona et al., 2006). An extensive study in gene expression for *Vibrio* spp. has been carried out by microarray-based analysis (Vora et al., 2005). In their study, they found that *Vibrio* spp. continued to express known toxin and virulence genes in the VBNC state (Vora et al., 2005).

Proteomic analysis revealed that different protein expression patterns were found for exponentially growing, starved and VBNC cells. Heim et al. (2002) reported two general stress proteins, namely GroEL and DnaK, were persistent but expressed at a slightly lower level in *E. faecalis* during the VBNC state. Surprisingly, an unknown protein spot was observed only in VBNC cells at molecular mass 30kDa, pI 6 (Heim et al., 2002). Penicillin binding proteins (PBPs) were reported to be maintained or even increased in VBNC *E. faecalis*. This is more

apparent in PBP5 where during the VBNC state, PBP5 continues to maintain its function and penicillin binding capability (Signoretto et al., 2000).

Changes in protein components of *Escherichia coli* O157:H7 under oxidation stress conditions were studied (Asakura et al., 2007b). The results showed an increase of approximately 2100-fold in outer membrane protein W (OmpW) during the VBNC state when compared with the level at early incubation. It was suggested that OmpW is an oxidation-responsive protein in *E. coli* and will only be expressed when the cell enters VBNC state.

Gene expression and protein profiles are currently used as the marker for cell viability/activity. However, some papers reported gene expression as the better indicator for viability/activity due to the shorter half-life in RNAs compared with proteins which are more stable (Sheridan et al., 1998; Lleo et al., 2000).

2.3.4 Significance of VBNC cells

To date, there are more than 60 types of bacteria that have been described to enter the VBNC state. Among them are human pathogens such as *Campylobacter* spp., enteropathogenic *Escherichia coli*, *Helicobacter pylori*, *Legionella pneumophila*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus* (Oliver, 2005). Unfortunately, current microbial monitoring still depends on culture-based methods, which may underestimate the bacteria population size. As a result of this, the public could be exposed to significant health risk.

2.3.4.1 The VBNC state in natural environments

There have been numerous studies conducted on the entry of bacteria into the VBNC state. Most of them were demonstrated in laboratory microcosms by modifying selected parameters such as nutrients, salinity, radiation intensity and

temperature. However, a valid study on VBNC bacteria will not be complete without an *in-situ* study.

An earlier study on *E. coli* H10407 demonstrated the entrance of these cells into the VBNC state in natural seawater at 25°C (Grimes and Colwell, 1986). A membrane chamber was used in the study to contain the cells, and at the same time allow the cells to respond to the natural environment. Viability/activity was tested by fluorescent-antibody staining. The result revealed that viable/active cells were present after 13 hours following its introduction in the seawater, even though no colonies were formed on the culture media (Grimes and Colwell, 1986).

V. vulnificus which is often found in seafood, particularly raw oysters, is capable of causing life-threatening infections in humans through ingestion. Furthermore, the contact of open wounds and *V. vulnificus* contaminated water may also cause infection. There was an outbreak of *V. vulnificus* related disease reported due to the inability to detect these bacteria especially during the winter. Hence, scientists strongly believe that *V. vulnificus* enters the VBNC state during the winter (Oliver, 1993). An *in-situ* study on *V. vulnificus* was subsequently carried out in natural estuary water during both winter and summer months. The study applied similar methods as in the Grimes et al. (1986) study. *V. vulnificus* were proved to enter the VBNC state when exposed to surrounding water at low temperature, an average of 15°C. Those cells in VBNC state were eventually resuscitated in the summer months with an average temperature of 25°C (Oliver et al., 1995). This experiment may explain why *V. vulnificus* escape being detected by culture-based method and subsequently can cause infection.

2.3.4.2 Virulence potential of VBNC pathogens

Resuscitation of VBNC pathogens poses a significant risk to public health. The pathogens which undergo resuscitation are able to return to a culturable state at a rapid rate. Many studies on pathogens have demonstrated the entry into the

nonculturable state, as well as resuscitation of the VBNC pathogens. There are several methods being used in resuscitation experiments, such as *in vitro* resuscitation by temperature upshift under laboratory control, *in vivo* resuscitation by animal passage and *in situ* resuscitation by exposing the cells to natural water at a suitable temperature.

Amongst them, *in vivo* resuscitation gave better indication of virulence potential of VBNC pathogens. By examining the survival of the animal models or colonization of pathogens in animal models, virulence effects and infection doses can be known. Resuscitation of VBNC *V. vulnificus* has been demonstrated through injection into iron loaded mice. It was proven that the injection of 0.04 CFU caused the death in the mice (Oliver and Bockian, 1995). Maintenance of virulence in *V. parahaemolyticus* was demonstrated through the mice model after ingestion (Baffone et al., 2003). The embryonated egg is another common model being used in resuscitation studies. *C. jejuni* and *C. coli* are virulent in embryonated eggs (Cappelletti et al., 1999; Chaveerach et al., 2003). Apart from animal models, the virulence effect of VBNC *V. cholerae* was tested on human volunteers and shown to be a potential risk (Colwell et al., 1996).

However, the evidence of virulence is contradictory. For example, an earlier study demonstrated the loss of virulence in the VBNC state of *V. vulnificus* (Linder and Oliver, 1989). This contradicts with a later study on *V. vulnificus* (Oliver and Bockian, 1995). The authors explained that the virulence decreases over time as the cells enter VBNC state. Hence, it may greatly reduce the infection where the previous study employed aged VBNC cells.

Pathogenicity in a host organism is a sophisticated and dynamic process. The investigations of virulence are complicated even more by the variation of virulence factors between species and the invasive processes. With the development of molecular methods, virulence potential during the VBNC state could be identified by the expression of virulence genes. Hemolysin gene (*vvhA*), a virulence gene in *V. vulnificus* was found to be expressed in the VBNC state. The gene

continues to be expressed after VBNC cells were resuscitated by temperature upshift to room temperature (Smith and Oliver, 2006). A comprehensive study on gene expression in *Vibrio* spp revealed the presence of common toxin and pathogenicity genes during the VBNC state (Vora et al., 2005). Thus, it is suggested that pathogens in the VBNC state can initiate infections. In contrast, virulence genes expressed in VBNC *V.parahaemolyticus* were not able to be expressed after the recovery of culturability (Coutard et al., 2007).

Although the issue of virulence is uncertain, there is enough evidence to suggest that a potential risk does exist and we need to better understand the dynamics of VBNC in the environment.

2.3.5 VBNC enterococcal cells

In 1998, enterococci were first shown to enter the VBNC state (Lleo et al., 1998). Various environmental stress factors were studied for different species of enterococci. Oligotrophic conditions and temperature are two common factors used to study the VBNC state in enterococci. Different enterococcal species are shown to react differently to the environmental stress factors. *E.faecalis* and *E.hirae* are reported to enter the VBNC state in approximately two weeks in laboratory microcosms with sterilized lake water at 4°C. Both *E.faecalis* and *E.hirae* are able to remain in the VBNC state for three months. However, *E.faecium* takes about four weeks to enter the VBNC state in the same laboratory microcosms but only remain in the VBNC state for two weeks (Lleo et al., 2001).

During the VBNC state, the enterococci are shown to demonstrate active metabolism. An early experiment to test the viability of enterococci was based upon the ability of the cell to uptake the labeled amino acid (³H-leucine) and incorporate it into the proteins (Lleo et al., 1998). The original viability assay, direct viable count (DVC) has also been studied to examine the metabolic activity in enterococcal VBNC (Lleo et al., 2003). In addition, a continuous detection of DNA targeting penicillin binding protein 5 (PBP5) gene was reported in *E.faecalis* during

the VBNC state. This indicates the presence of intact cells, which is one of the key techniques to assessing viability (Lleo et al., 1998). The integrity of the cell membrane can also be tested by a commercial kit, LIVE/DEAD[®] BacLight[™] (Molecular Probes, Eugene/Poland,OR) (Lleo et al., 2003).

Modifications in the *E.faecalis* cell wall have been observed during the VBNC state. The dimensions of the *E.faecalis* VBNC cells were observed to be slightly elongated, with a cell length of around $1.48 \pm 0.31\mu\text{m}$ compared to exponentially growing and stationary cells length of $1.38 \pm 0.31\mu\text{m}$ and $1.29 \pm 0.35\mu\text{m}$ respectively (Signoretto et al., 2000). These VBNC cells were shown to have twice the resistance to mechanical disruption when compared to exponentially growing, stationary and UV-killed cells. In the same study, the authors also showed that the peptidoglycan chemical composition of *E.faecalis* VBNC cells was increased from 39% in growing cells to 48% in the VBNC cells in total cross-linking (Signoretto et al., 2000). With the increase in cross-linking, a harder wall is formed. This increases the resistance to mechanical disruption.

In proteome analysis, the protein profile of *E.faecalis* VBNC cells has been shown to differ from starved and exponentially growing bacteria (Heim et al., 2002). Two proteins, namely fructose biphosphate aldolase and elongation factor Ts (EF-Ts) were observed to be overexpressed in *E.faecalis* VBNC cells. An unknown protein spot (molecular mass of 30kDa at pI 6) was observed only in the VBNC cells. In their study, the overexpressions of these two proteins were further confirmed by transcription analysis.

Gene expression has been studied using the synthesis of specific mRNAs such as *pbp5*, *fba* (fructose biphosphate aldolase), *tsf* (EF-Ts) *vanA* and *vanB* gene in enterococcal VBNC cells (Lleo et al., 2000; Heim et al., 2002; Lleo et al., 2003). The *vanA* and *vanB* genes are responsible for the virulence factor in vancomycin-resistant enterococci. Studies have reported that the *vanA* and *vanB* gene expression capability in *E.faecalis* were maintained for one month under the VBNC state. In contrast, *E.faecium* VBNC cells were observed to maintain the expression of these

genes for a shorter period of 7 days to 14 days (Lleo et al., 2003). This phenomenon indicates that the enterococcal VBNC cells can continue to pose a potential risk to human health.

In addition, *E.faecalis* VBNC cells are reported to maintain their adherence capability in human cells such as human colon carcinoma cells, girardi heart cells and epithelial cells (Pruzzo et al., 2002). Although the attachment efficiency has been reduced by about 50% to 70%, it is still possible to reactivate and initiate the full colonization process of *E.faecalis* from the VBNC state. The colonization will subsequently cause the infection.

2.3.6 Detection of VBNC cells

Since the introduction of the VBNC state in the 1980s as one of the physiological states occurring in non-spore-forming bacteria, there are numerous methods to assess the viability/activity of nonculturable cells. As VBNC cells are not able to grow in normal culture media, all viability/activity assays are growth-independent assays. However, none of these methods are universally appropriate in all cases. Each method has its advantages and its limitation. Generally, the assessment of bacterial viability/activity can be categorized into two groups, using either a direct or indirect indicators. Direct indicator methods mainly examine the metabolic activity of the cell, whereas, indirect indicator methods focus on the maintenance of cellular structures.

2.3.6.1 Direct indicators

Metabolic activities that have been used as evidence of viability/activity of the cell include substrate responsiveness, respiratory activity, protein synthesis, substrate uptake and the presence of molecules that have a high turnover rate (e.g. mRNA, ATP).

The direct viable count (DVC) assay is the original assay used to determine the VBNC condition based on substrate responsiveness (Kogure et al., 1979). In this assay, cells are incubated together with yeast extract and antibiotic (e.g. nalidixic acid, ciprofloxacin). The concept behind this is to provide nutrients for cell growth but inhibit the division process in the cell. Thus, the viability/activity can be examined microscopically based on the enlargement/elongation of cells. These enlarged/elongated cells indicate that metabolic activities are retained in the cells and the cells can potentially undergo the cell division process once the inhibitor (antibiotic) is removed. However, the DVC assay poses several limitations. One of the major disadvantages is the difficulty in differentiating the enlarged/elongated cells with normal cells. In addition, this assay highly depends on the ability of microorganisms to respond to the nutrient and antibiotic added. A limited supply of carbon source has also been reported to slow down the elongation process (Rodriguez et al., 1992). Antibiotic conditions have also not been established for all bacterial species (Barcina et al., 1995).

In addition to the assessment of substrate responsiveness in the DVC, assessment of respiratory activity is another common direct indicator assay for the VBNC cells. Respiratory activity is also known as the electron transport chain, which is closely related to the oxidative metabolism of bacteria. Tetrazolium salts such as 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) are employed as artificial electron acceptors in the assay. These artificial electron acceptors will compete with molecular oxygen in the electron transport chain. For INT, the electron transport system of respiring bacteria will reduce soluble INT to insoluble INT-formazan crystals and accumulate in metabolically active bacteria as dark red spots. The INT-formazan can be observed or enumerated by light microscopy (Zimmermann et al., 1978). CTC is introduced later to provide an advantage of simple detection due to the red fluorescence in the reduced form. Respiratory activity is indicated by CTC-formazan which can be visualized by epifluorescence microscopy (Rodriguez et al., 1992). Nevertheless, papers have shown the toxic and inhibitory effects on bacterial

metabolism of CTC in both brackish water and freshwater environments (Ullrich et al., 1996).

For rapid detection of indicator bacteria, it is suggested that fluorogenic substrates be used to demonstrate enzymatic activity in aquatic environments (Berg and Fiksdal, 1988; Fiksdal et al., 1994). Studies reported continuous production of β -D-galactosidase in the nonculturable state of bacteria (Davies et al., 1995; Nwoguh et al., 1995; Pommepuy et al., 1996b). The main drawback of this measurement is the interference of background noise such as plant and algal biomass (Tryland and Fiksdal, 1998). In addition, different bacteria show stable enzyme activity at different temperatures. Hence, enzymatic measurement is highly temperature dependent (Tryland and Fiksdal, 1998).

Another way to determine the metabolic activity of bacteria is utilizing the ability of bacteria to uptake labeled amino acid (^{35}S -labelled methionine) and incorporation of methionine into protein (Rahman et al., 1994). Briefly, the VBNC cells are incubated with ^{35}S -labelled methionine and further analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Rahman et al., 1994). Results showed that the VBNC cells of *Shigella dysenteriae* type 1 are able to retain cytopathic effect on HeLa cells which may secrete cytotoxic substance(s) when resuscitated (Rahman et al., 1994). The greatest disadvantage posed in substrate uptake measurement is the prolonged incubation time. At least 2 days is required, as the uptake of amino acid by the VBNC cells is a slow process.

Recently, viability/activity assays were developed towards the identification of the presence of specific molecules in the cell. The molecules used to represent viability/activity usually have high turnover rate such as messenger ribonucleic acid (mRNA) and adenosine triphosphate (ATP). Detection of mRNA will be discussed in section 2.3.6.3.

2.3.6.2 Indirect indicators

Indirect indicators of viability/activity assays normally examine the maintenance of cellular structure. Maintenance of cellular structure can be monitored in terms of persistence of nucleic acid, membrane integrity and membrane potential.

Nucleic acid staining is one of the earliest indirect indicators for the viability/activity assay. Acridine orange, 4'-6-diamidino-2-phenylindole (DAPI) and SYTO dyes are some of the common dyes used to indicate the presence of intact nucleic acid. Acridine orange or DAPI is used to count the total number of cells including culturable, non-culturable and dead cells. By counterstaining DAPI with CTC, enumeration of total cells and the VBNC cell can be done simultaneously (Cappelletti et al., 1997). The use of the SYTO dye is gaining more popularity due to its lower molecular weight where it can penetrate through intact cell membranes. Hence, dead cells will not be stained and can be eliminated. When compared with SYTO dyes, acridine orange requires careful control of pH in the staining assay, while DAPI has been reported to be influenced by transfer ribonucleic acid (tRNA) (Katouzian-Safadi et al., 1989) and chlorinated water (Saby et al., 1997).

Besides staining of nucleic acid, hybridization and/or polymerase chain reaction (PCR) can be applied to measure the persistence of nucleic acid. The molecular genetic detection of VBNC cells will be further discussed in section 2.3.6.3.

Presumably if a dead cell undergoes the cell lysis process, a living cell should, in contrast, maintain the integrity of its cell membrane. Thus, cell membrane integrity can be used to identify whether the cell is alive or dead. SYTO 9 (Berney et al., 2007), propidium iodide (Banning et al., 2002; Berney et al., 2007) and green fluorescent protein (Lowder et al., 2000; Janne Lehtinen, 2004) are some of the common dyes used to examine the integrity of cell membranes. Following the

staining process, flow cytometry or epifluorescence microscopy is used for visualization and enumeration. Several studies combined two dyes in order to simultaneously stain both live and dead cells (Banning et al., 2002; Janne Lehtinen, 2004). A commercial kit, LIVE/DEAD[®] BacLight[™], combines two fluorescence dye, SYTO 9 and propidium iodide to achieve viability/activity assessment (Sachidanandham et al., 2005). The green fluorescence dye, SYTO 9 can pass through the intact cell membrane and stain nucleic acids due to its low molecular weight. However, propidium iodide with higher molecular weight can penetrate into the cell to stain the nucleic acid only when the cell is lysed (damaged membrane cell). Hence, the intact and damaged cells can be differentiated through the fluorescence colour. Unfortunately, the fluorescence colour produced may not always distinctly differentiate the living or dead region. It could be in the intermediate state where a conclusive result cannot be achieved.

The presence of membrane potential is another alternative to assess viability. It has been suggested that an intact and functioning cell membrane will possess membrane potential. Energisation-sensitive probes such as oxonols and Rhodamine 123 are used as indicators of membrane potential together with flow cytometry (Lopez-Amoros et al., 1995). A limitation of this method is the inconsistency of the dyes' permeability in all bacterial species (Rice et al., 2000).

2.3.6.3 Detection of VBNC cell using molecular genetics

In recent decades, the detection of VBNC cell has shifted to molecular genetic methods. Hybridization probes and amplification of nucleic acid are two main methods involved in molecular genetic detection. Hybridization probes are single stranded nucleic acid (DNA or RNA) which are radioactively or chemically labeled. These probes are used to detect complementary target DNA or RNA in a heterogeneous mixture of nucleic acids. On the other hand, amplification of nucleic acid is achieved by the PCR method which utilises a thermostable DNA polymerase and a pair of oligonucleotide primers. PCR involves a repetition of three steps, which are denaturation, annealing and extension.

Polymerase chain reaction (PCR)

Since the introduction of PCR in the 1980's, several researchers have utilized this powerful amplification method in detecting VBNC cells. In the early stage, studies were focused on the DNA of VBNC cells. The persistence of intact DNA in a cell is used as one of the indicators of cell viability/activity. By targeting a specific region of the gene, the PCR method was successfully demonstrated to be a rapid, specific and sensitive method to detect VBNC cells (Bej et al., 1991a; Rahman et al., 1996; Lleo et al., 1998). However, the DNA extraction step, which releases DNA from the cell, is an essential step prior to the PCR method especially when analyzing environmental samples. The efficiency of DNA extraction highly affects the sensitivity of PCR. Unfortunately, reports show that an additional 300 times extracted DNA of *Vibrio vulnificus* in VBNC state is required to obtain an amplification signal, in comparison with the culturable cells (Brauns et al., 1991).

In addition, studies have also refuted the initial assumption that DNA would degrade rapidly once a cell dies. Cells that have been killed by boiling or UV irradiation are still able to produce amplification product (Josephson et al., 1993). It was once that DNA-based methods such as PCR are shown to be unable to distinguish whether a cell is alive or dead, and thus not suitable as viability assay (Josephson et al., 1993). Nevertheless, the introduction of additional nucleic acid pre-treatment prior DNA extraction step allows the elimination of dead cells from PCR amplification (further discussion in section 2.4.3).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Since DNA has been shown to persist even in a dead cell or killed cell, studies have attempted to solve this problem by analyzing ribonucleic acid (RNA). Among several types of RNAs, messenger RNA (mRNA) is preferred for viability/activity study (Bej et al., 1991a; Lleo et al., 2000). Detection of mRNA is suggested to provide a better indication of living cells due to its shorter half-life (a few minutes) (Ingle and Kushner, 1996).

Reverse transcription PCR (RT-PCR) is applied to detect mRNA in environmental water samples. The principle for RT-PCR is basically similar to PCR except for the additional step of reverse transcription. During reverse transcription, primers and the enzyme (reverse transcriptase) are added to generate complementary DNA (cDNA). The cDNA generated can then be used for amplification by PCR.

Numerous studies reported good correlations between cell viability and the presence of microbial mRNA. Pathogens and fecal indicators are amongst the few bacteria studied (Lleo et al., 2000; Lleo et al., 2003; Coutard et al., 2005; Smith and Oliver, 2006). Recently, another study has revealed that vancomycin resistance still maintains the VBNC state by exploring the mRNA of the *vanA* and *vanB* genes (Lleo et al., 2003).

The greatest disadvantage of RT-PCR is that no quantification is allowed. This is also an inherent weakness of PCR where both PCR and RT-PCR can only perform detection qualitatively.

Quantitative real-time PCR and quantitative real-time RT-PCR

Quantitative real-time PCR (qPCR) and quantitative real-time RT-PCR (RT-qPCR) offer a great advantage to real-time/in-situ monitoring of DNA/RNA amplification. These methods can also overcome the main limitation of conventional PCR or RT-PCR by enabling both detection and quantification of the targeted DNA/RNA sequence. Furthermore, the real-time method can eliminate post amplification processes such as gel electrophoresis.

Studies reported that qPCR are able to achieve sensitive, specific and rapid detection and quantification of the VBNC cells (Campbell and Wright, 2003; Wery et al., 2006). However, RT-qPCR is gaining more popularity than real-time PCR based on studies that have shown mRNA as a better indicator for viability/activity (Bleve et al., 2003; Gonzalez-Escalona et al., 2006; Coutard et al., 2007; Asakura et al., 2007a). Owing to the fluctuation of mRNA expression when the bacteria

encounter stresses and starvation, the expression of mRNA is perhaps the best tool to understand how the bacteria adapt to the stressed conditions (Takayama and Kjelleberg, 2000). However, mRNA may not be suitable for accurate quantification of cell number due to the lack of stability in mRNA stability at different physiological state, as well as stressed conditions. Although qPCR and RT-qPCR can deliver advantages in detecting and quantifying VBNC cells, these techniques require expensive instruments and specialized skills.

Nucleic acid sequence-based amplification (NASBA)

Another alternative for RT-PCR is the nucleic acid sequence-based amplification (NASBA) which was invented in 1991 (Compton, 1991). NASBA is an isothermal amplification method used to amplify RNA. Three enzymes namely, avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H and T7 RNA polymerase are used in the RNA amplification process. Together with these enzymes, two oligonucleotide primers, deoxyribonucleoside triphosphates (dNTPs) and ribonucleoside triphosphates (NTPs) are added for the amplification reaction. The whole amplification process is performed at a single temperature, 41°C. This eliminates the need of expensive thermocycling apparatus.

NASBA has been recommended as a viability/activity assay. This is because the NASBA method has proven to amplify exclusively RNA and not be interfered by background DNA contamination (Simpkins et al., 2000; Min and Baeumner, 2002). As NASBA is performed at a consistent temperature of 41°C, genomic DNA will not become a template for amplification and will remain in the double stranded form (Cook, 2003). In addition, NASBA was found to be more sensitive compared with RT-PCR (Birch et al., 2001).

Surprisingly, a study from Rodríguez-Lázaro et al. (2004) has demonstrated that DNA contamination is not strictly eliminated in NASBA, but instead can be amplified. Hence, it is important to verify that the original signal is indeed RNA, prior to the NASBA reaction. Enzymatic (RNase, DNase) treatment to the extracted

nucleic acids were suggested for the verification purpose (Rodríguez-Làzaro et al., 2004).

As for the viability issue, although studies have suggested mRNA as an indicator for viability (Bej et al., 1991a; Lleo et al., 2000), there are still papers reporting that NASBA has detected the persistence of mRNA even after 30 hours of heat-killing (Birch et al., 2001). However, there are several factors that may affect the persistence of mRNA. These factors include species of bacteria (Rodríguez-Làzaro et al., 2004), environmental conditions (Sheridan et al., 1999), physiological state such as VBNC state (Lleo et al., 2000), the method of cell death (Sheridan et al., 1998), and region of the transcript targeted (Norton and Batt, 1999).

Despite those advantages brought by NASBA, this method is currently not as popular as the PCR method. NASBA has been used widely in clinical samples but not in environmental samples. One of the reasons could be due to its complicated reaction which involves three enzymes. Furthermore, extensive studies on potential inhibitors to NASBA have not yet been carried out.

Microarray-based analysis

Microarray-based analysis is the enhancement of the traditional hybridization method. Microarrays contain sequence-specific probes (e.g. known sequence of PCR products or oligonucleotides) that are attached to the substrate (e.g. glass slide or membrane) in a lattice pattern (Schena, 2000). When microarrays are exposed to targets, only the correct target will hybridize to the complementary probes. Depending on the probes being used, the targets could be PCR products, genomic DNA, plasmid DNA, cDNA, total RNA or oligonucleotides. Usually, targets will incorporate a fluorescent label to allow subsequent fluorescence-based detection.

Most of the molecular methods such as PCR or real-time PCR, work with limited genes in an analysis. This shortcoming of low throughput limits a researcher to have an overall picture of the gene's function. Thus, monitoring of diverse genes

in an environment becomes tedious and challenging. In contrast, microarrays are fabricated with thousands of spots which allow massive parallel gene expression studies and parallel detection of various microorganisms.

Microarray-based analysis has been applied successfully in analyzing global gene expression in pure culture (Schena et al., 1995; Schena et al., 1996; Gao et al., 2004). However, direct application of microarrays in the environment faces various challenges in term of sensitivity, specificity and quantification (Wu et al., 2001; Rhee et al., 2004; Tiquia et al., 2004). To overcome the limitation of low sensitivity, microarrays are usually coupled with PCR. The combination of microarrays and PCR also allows identification of the PCR product without any further confirmation such as sequencing, Southern blots or dot-blots (Call et al., 2003).

Very recently, the combination of RT-PCR and an electronic microarray has been used to detect VBNC *Escherichia coli* O157:H7 in drinking water and river water (Liu et al., 2008). With some modification of the extraction procedure and concentration step, the authors reported that the combination of RT-PCR and electronic microarray was able to increase the detection sensitivity significantly. They demonstrated a detection of as few as 50 VBNC cells in 1 liter of river water. Thus, they declared their method as the best limit of detection for the VBNC cells in environmental water samples to date.

An extensive study on the detection of VBNC *Vibrio* spp. has been carried out by combining RT-PCR/90-plex PCR amplification with microarray hybridization (Vora et al., 2005). In this paper, the authors reported that *Vibrio* spp. in VBNC state continues to express known toxin and virulence-associated genes. It is noteworthy that VBNC *Vibrio* spp can initiate infection once the cells are resuscitated.

Proteome analysis

Generally, proteome analysis involves the identification of the expressed protein by a genome or tissue at a certain point in time (Pennington et al., 1997;

Williams and Hochstrasser, 1997). A common technique for the separation of protein is either one-dimensional polyacrylamide-gel electrophoresis (1D PAGE) or two-dimensional polyacrylamide-gel electrophoresis (2D PAGE). In 1D PAGE, proteins are separated according to molecular weight. In 2D PAGE, proteins are separated by isoelectric focusing (IEF) based on an isoelectric point (the point where the sample charge is the same as surrounding pH) followed by molecular weight. As the 2D PAGE system works in two dimensions, it gives higher separation capability and allows thousands of proteins to be resolved. Thus, 2D PAGE is preferred compared with 1D PAGE. Even though 2D PAGE resolves thousands of proteins, it is still not sensitive enough to detect most proteins in the cell. Moreover, high throughput analysis of proteomes is time consuming and not advisable. This is due to the fact that each spot from 2D PAGE have to be individually extracted, digested and examined. High-performance liquid chromatography (HPLC), capillary isoelectric focusing (CIEF), capillary electrophoresis (CE) and multidimensional chromatography are among the alternative methods for 2D PAGE (Washburn and Yates, 2000).

Subsequent to protein separation, the resolved proteins can be identified by mass spectrometry (MS). Besides identification, MS offers advantages of characterization and quantification of proteins (Domon and Aebersold, 2006). Combination of 2D-PAGE and MS is currently widely used in proteome analysis. Nonetheless, the main drawback is the requirement of an additional step in transferring resolved protein to MS for identification (Washburn and Yates, 2000).

One of the VBNC cell characteristics is the maintenance of metabolic activities even though the cell is unable to be cultured. However, these metabolism pathways are highly dependent on the stresses faced by the cell. In recent years, studies show that different metabolic pathways will consequently change the protein profile (Heim et al., 2002; Cuny et al., 2007; Asakura et al., 2007b; Muela et al., 2008). Heim et.al. (2002) reported the difference in protein profile in *Enterococcus faecalis* in the VBNC state, starvation state and exponentially growing states. The authors confirmed that when *E.faecalis* responds to multiple environmental stresses,

the VBNC state is a distinct physiological phase within the life cycle (Heim et al., 2002).

2.4 Problems associated with PCR

PCR has been developed as one of the most powerful tools for modern biological analysis. Its high sensitivity and specificity make this technique widely used in biological-related industries, such as clinical, food, forensic, as well as environmental. However, there are problems which may occur with PCR. Owing to the extremely sensitive detection in PCR which allows detection of only a few molecules of DNA, PCR is easily interfered by surrounding contaminants. Carryover contamination is one of the major potential sources that give false-positive detection in PCR. Another problem associated with PCR is inhibition, where inhibition can cause failure or reduced sensitivity in the PCR reaction. The third problem encountered in PCR is the detection of dead cells. The detection of dead cells will in return give false-positive results, misleading the interpretation. These three main problems associated with PCR together with some prevention approaches will be discussed in this section. The discussion will focus on the problems that arise from the application of PCR in environmental microbiology.

2.4.1 Contamination

Due to its extreme sensitivity, PCR is especially susceptible to contamination. The sources of PCR contamination are very broad. The contaminant could be from the amplicon from previous PCR, carryover from other samples, reagents, clothes, and even neighbouring laboratories (Borst et al., 2004). In PCR, each reaction will produce millions of target nucleic acid sequences. Thus, even a small aerosol will contain an abundance of target sequence generated during PCR. The pipette is one of the most common equipment that can trap nucleic acids through aerosol and subsequently, the contamination will be carried over to the next experiment. In addition, PCR contamination may come from reagents where the

reagents are usually claimed to be free from contamination by the supplier. The most commonly reported reagent that is contaminated with DNA is *Taq* DNA polymerase (Rand and Houck, 1990; Mühl et al., 2010). However, the source of contamination in *Taq* DNA polymerase is still unknown (Rand and Houck, 1990). Other PCR reagents that were found to be contaminated with DNA have been discussed extensively in a review paper by Borst et al. (2004).

Generally, there are two main approaches used to overcome the carryover contamination of PCR. The first approach is through the setup of barriers between the PCR amplification area and reagent preparation area. A strict separation of working areas for sample preparation, PCR setup, PCR amplification and analysis of PCR products is encouraged to reduce the carryover contamination. Another physical barrier to reduce contamination is through the use of aerosol-resistant pipette tips. It is recommended to always keep the reagents and samples in closed tubes, and open and close the sample tubes carefully if needed, to reduce the chances of contamination. A good laboratory practice, such as wearing clean lab coat and gloves, should always be practiced when working with PCR. In addition to the physical barrier, a chemical barrier which uses 10% sodium hypochlorite solution (bleach) followed by removal of bleach with ethanol is also suggested (Aslanzadeh, 2004). The bleach will cause damage to nucleic acid, thus preventing the reamplification of contaminants in subsequent PCR. DNA AWAY (Molecular Bio Product) and DNAZapTM (Ambion) are some of the commercially available cleaners to degrade contaminating DNA.

The second approach is modification of PCR or sterilization. The most simple and inexpensive method is through ultra-violet (UV) light irradiation. UV light can induce thymidine dimer and other covalent modification in DNA and thus the contaminating DNA will not be amplified in the subsequent PCR (Aslanzadeh, 2004). Gamma radiation has been studied to inactivate contaminating DNA prior to PCR (Deragon et al., 1990). Shaw et al. (2008) studied three different radiations (i.e. UV, gamma and beta) and examined the potential of these radiations to degrade contaminating DNA (Shaw et al., 2008). In their study, gamma radiation showed

the most successful treatment while UV radiation was the least effective treatment (Shaw et al., 2008). Nevertheless, application of these radiations for decontamination purposes should be used with caution. Excessive light radiation may have deleterious effects on the *Taq* polymerase and primers, and subsequently reduce the sensitivity of PCR (Meier et al., 1993; Pao et al., 1993; Harris and Hartley, 2003).

Pretreatment prior to PCR with the enzyme uracil-DNA-glycosylase (UDG) is another way to combat carryover contamination of previous amplification products (Hartley and Rashtchian, 1993). In this pretreatment, the dTTP in the dNTP mix need to be replaced with dUTP. This is to ensure the amplification products contain only uracil bases instead of thymine bases. If the amplicon from previous PCR becomes the source of contamination, the addition of UDG in the subsequent PCR will remove the uracil residual and create abasic sites in the amplicon. The amplicon with abasic sites will not be amplified and thus can avoid the carryover contamination. Currently, UDG or UND (UDG enzyme for *E.coli*) has been incorporated into commercial PCR kits. Since UDG works with uracil bases which are the substitution of thymine bases, the UDG approach works particularly well with thymine-rich amplification but has reduced activity with G+C-rich targets (Aslanzadeh, 2004).

Modification to the amplicon after PCR with furocoumarins (i.e. psoralen and isopsoralen) offers another alternative to inactivate the nucleic acids, and subsequently prevent carryover contamination (Aslanzadeh, 2004; Borst et al., 2004). The furocoumarin compounds will form covalent binding to nucleic acids when irradiated with long-wavelength of UV light (320-400nm)(Borst et al., 2004). However, this method increases individual risk to the exposure of carcinogenic compounds. In addition to the prevention approaches discussed in this section, there are some other contamination preventing measurements discussed in other reviews (Hartley and Rashtchian, 1993; Aslanzadeh, 2004; Borst et al., 2004; James, 2010).

2.4.2 Inhibition

Inhibition has been long recognized as one of the main problems encountered in PCR (Wintzingerode et al., 1997; Juen and Traugott, 2006; Banu et al., 2010). The success of PCR is always obstructed by the presence of inhibitors. Although there are many studies which describe various methods to remove the inhibition impact from inhibitors, there is unfortunately no single method suitable for universal application. The main reason is the extremely wide nature of inhibitors which varies from one sample to another. Common inhibitors found in environmental samples include humic acids, phenolic compounds and heavy metals. Laboratory items such as glove powder (de Lomas et al., 1992), plasticware (Tamariz et al., 2006), nitrocellulose filter and cellulose acetate filter (Bej et al., 1991b) are also potential inhibitors. In addition, materials and reagents that are used during DNA purification (i.e. NaCl, KCl, EDTA, SDS, ethanol, isopropanol and phenol) may have an inhibitory effect on PCR if applied in excessive amount (Davalieva and Efremov, 2010). A comprehensive summary of inhibitors together with facilitators in environmental samples has been discussed in Wilson's review paper (Wilson, 1997).

There are numerous approaches to overcome PCR inhibition. The most common method is DNA purification. Conventional DNA purification that involves phenol-chloroform was found to have a higher chance of inhibition, mainly due to the residual of phenol from the purification process (Bickley and Hopkins, 1999). A wide variety of commercial available kits have been introduced to extract DNA and remove inhibitors in parallel. Studies have also compared the efficiency of the commercial extraction methods in removing inhibition (Holland et al., 2000; Jiang et al., 2005; van Tongeren et al., 2011). Nevertheless, different types of sample will contain different inhibitors, and the choice of inhibitor control is highly dependent on the sample type. As such, careful selection of the DNA purification method that best suits the samples is important.

The simplest way to overcome PCR inhibition is to dilute the sample. Through dilution, the negative impact from inhibitors can be reduced. At the same time, the number of target DNA is also reduced. This method is particularly efficient for the sample with sufficient target DNA even after dilution. For instance, a study showed that the 5-fold dilution of extracted DNA gave 83.3% removal of inhibition, however, 44.4% removal of inhibition was obtained with addition of bovine serum albumin (BSA) (Banu et al., 2010). Although BSA does not provide satisfactory results in Banu et al.'s study, BSA has shown to be an alternative to relief interference of inhibitors in PCR (Kreader, 1996; Guy et al., 2003; Jiang et al., 2005; Wang et al., 2007). BSA was reported to reduce inhibition from humic acids for at least 100-fold more than the inhibitory limit (0.1ng/μl), with minimal cost involved (Kreader, 1996).

In addition, inhibition of PCR could be due to the inactivation of thermostable DNA polymerase by inhibitors. The most widely used DNA polymerase, *Taq* polymerase from *Thermus aquaticus*, is reported to be inhibited by humic acid concentrations of less than 10ng/100μl (Tsai and Olson, 1992). Different inhibitory susceptibilities for humic acids were observed in *Taq* polymerase from different commercial sources. The resistance to humic acids between three commercially available *Taq* polymerases can be as high as 8-fold in difference (Tebbe and Vahjen, 1993). In addition to humic acids, other inhibitors such as phenol, feces, K ion, Na ion and Mg ion may contribute to the inhibition effect when analyzing environmental water samples. An extensive study comparing different DNA polymerases showed that the choice of DNA polymerases can lead to different impacts on inhibitory resistance (Abu Al-Soud and Radstrom, 1998; Davalieva and Efremov, 2010).

Another approach to overcome inhibition is through the physical removal of inhibitors. Immunomagnetic separation (IMS) method uses magnetic force to separate target bacterial cells which have been previously captured by antibody-coated magnetic beads. This method is able to capture target bacterial cells in the presence of other particles within a relatively short time, typically less than an hour

(Bushon et al., 2009). Since the IMS technique is based on antibody-antigen interactions, the specific capture of target cells can eliminate the concentration of inhibitory substances in the water samples (Fontaine and Guillot, 2003; Dudak and Boyaci, 2008). However, the main limitation is the inability of antibodies to capture all strains of target bacteria.

Recently, Schriewer et al. (2011) used an insoluble polymer, Supelite™ DAX-8 as adsorbent to remove humic acids from the water samples prior to qPCR. The authors compared both DAX-8 and BSA methods, and found overall performance of DAX-8 is better than BSA in terms of inhibition removal and qPCR amplification efficiency (Schriewer et al., 2011). Nevertheless, additional treatment of DAX-8 can lead to the loss of DNA, and the magnitude of DNA loss was proportional to the amount of DAX-8 added in the treatment (Schriewer et al., 2011).

2.4.3 Detection of dead cells

Molecular methods, such as PCR and real-time or quantitative PCR (qPCR), are able to detect target organisms rapidly and specifically. However, these methods are hampered by the detection of dead cells (Young et al., 2007; Walters et al., 2009). Studies have shown that the DNA from dead cells may persist over a prolonged period (Josephson et al., 1993). In analyzing viable cells, detection of RNA instead of DNA has been proposed to detect viability, due to RNA's shorter half-life (e.g. minutes). On the other hand, this advantage could be the greatest challenge in its application, especially for environmental samples where it is hard to process within minutes. In addition, RNA is prone to degrade with RNA inhibitors or RNA-degrading enzymes (e.g. RNase). The RNA expression level is also highly dependent on the physiological state of the organism. As a result, reproducibility is generally difficult to achieve. In recent years, the DNA-intercalating dyes, such as ethidium monoazide (EMA) and propidium monoazide (PMA) have been used to overcome the problem of detecting DNA from dead cells.

The principle of EMA and PMA is based on the integrity of cell membranes. These DNA-intercalating dyes are able to penetrate only into membrane-compromised cells (dead cells). Once the dye enters the cell, it will bind to the DNA and a covalent bond will be formed via the photoactivation process. This is an irreversible modification of the DNA and will not be amplified during the PCR amplification. The excess dye will be inactive under exposure of strong light.

2.4.3.1 Application of EMA/PMA in bacteria

EMA is a dye introduced to bind DNA from membrane-compromised cells prior to the introduction of molecular techniques (e.g. PCR or qPCR) (Rudi et al., 2002). This dye has been widely used to discriminate the dead cells of human pathogens, bacteria indicators, protozoans, and fungi. EMA pretreatment has been applied in environmental water samples (Pisz et al., 2007; Inoue et al., 2008; Delgado-Viscogliosi et al., 2009; Gedalanga and Olson, 2009; Chen and Chang, 2010) and biofilm samples (Nocker and Camper, 2006) to distinguish dead cells from viable cells. For example, when aquatic samples were spiked with heat-killed *E. coli* and pretreated with 10 $\mu\text{g}.\text{ml}^{-1}$ of EMA, a 4 logs unit reduction of DNA amplification was achieved (Inoue et al., 2008). This simple and rapid pretreatment has great potential to be incorporated into PCR or qPCR analysis to reduce the false positive detection of dead cells. However, some studies have also reported the penetration of EMA into viable cells (Rueckert et al., 2005; Nocker et al., 2006; Flekna et al., 2007; Kobayashi et al., 2009). Table 2.1 shows the application of EMA in different bacteria species as well as the EMA treatment conditions. Some studies showed the success of EMA in differentiating dead cells from viable cells, while others showed the negative impact of EMA on viable cells. It appears that the success of EMA pretreatment depends on the bacteria species, EMA concentration, treatment conditions, bacteria concentration and water matrix.

An alternative DNA-intercalating dye, PMA, was introduced to overcome the penetration of EMA through intact cell membranes (Table 2.2). PMA was shown to work better when compared with EMA due to its higher molecular charge

(two positive charges in PMA compared to one positive charge in EMA) (Nocker et al., 2006). The higher molecular charge provides greater impermeability through intact cell membranes, thus achieving better selective staining of dead cells. PMA pretreatment has been applied to different bacteria before DNA extraction including those from culture (Nocker et al., 2007) as well as environmental samples (Nocker et al., 2009; Varma et al., 2009; Bae and Wuertz, 2009a; Bae and Wuertz, 2009b). Most studies report that PMA pretreatment can achieve significant signal reduction for dead cells, without interfering the signals from viable cells (Nocker et al., 2009; Bae and Wuertz, 2009a; Bae and Wuertz, 2009b; Kralik et al., 2010). In terms of environmental application, a recent study showed that PMA successfully inhibited the amplification of dead *Salmonella* DNA in turbid creek water (43 NTU) (Nocker et al., 2009).

In contrast, some studies have reported that high levels of total suspended solid can affect the suppression of DNA amplification from dead cells (Brescia et al., 2009; Varma et al., 2009; Bae and Wuertz, 2009a). This could be due to insufficient light penetration into the samples, which would interfere with photoactivation to create covalent bonds between the dye and DNA. In addition, insufficient light could also affect the inactivation of excess dye which will subsequently bind to the released DNA from intact cells, after the DNA extraction. Thus, it is recommended to expose samples to strong light and to provide continuous mixing so that all cells are exposed to light. Another suggestion is to employ other trigger factors besides light, such as changes in pH or temperature for the crosslinking event (Nocker and Camper, 2009).

While PMA was generally shown to be the better stain for application with dead cells, EMA was found to perform better for heat-killed (45 - 95°C for 5 min) *Legionella pneumophila* (Lee and Levin, 2009). In this study, the concentrations for EMA and PMA were 1.0 µg.ml⁻¹ and 3.0 µg.ml⁻¹ respectively. A possible reason why EMA showed better performance when compared to PMA could be that the lower molecular charge of EMA allows penetration into membrane-compromised cells more readily. In addition, Rudi et al. (2005) suggested that EMA may have a

higher chance to be excluded from penetrating viable cells due to its passive diffusion. This can be achieved by using a low concentration of EMA, lower light exposure (500 W) and longer exposure period (10 min). In summary, different studies employ varying concentrations and conditions for EMA or PMA and thus, further work is needed to optimize the best conditions for this pretreatment method.

Table 2.1 Application of EMA pretreatment for detecting viable microorganisms

Target organisms (sample matrices)	Cell inactivation mechanisms	EMA conc. ($\mu\text{g}.\text{ml}^{-1}$)	Light exposure (power, W; duration, min; distance from samples, cm)	Effectiveness	Reference
<i>E. coli</i> O157:H7, <i>Salmonella enterica</i> serovar Typhimurium (drinking water biofilm)	Heat (72°C for 30s to 15min); Isopropanol (70% for 10min)	100	650; 1; 20	- Minimal signal reduction of 2.8(± 0.2) cycles in EMA treated viable cells; - Great signal reduction of 11.8(± 0.2) cycles in EMA treated heat-killed cells	Nocker and Camper (2006)
<i>E. coli</i> LKI gfp+ (soil, elemental sulfur, river biofilm)	Ethanol (70%, 20min)	90 (soil); 100 (sulfur and biofilm samples)	500; 1; 20	- Suppress 99.99% and 100% of non-viable cells in soil samples and elemental sulfur samples, respectively	Pisz et al. (2007)
<i>Campylobacter jejuni</i> , <i>Listeria monocytogenes</i>	<i>C. jejuni</i> : heat (54°C, 10min); <i>L. monocytogenes</i> : heat (70°C, 20min)	100	650; 2; 20	Poor indicator for cell viability	Flekna et al. (2007)
<i>Vibrio vulnificus</i>	Heat (100°C, 5min); Gamma irradiation	0.5	500; 15; 15	- Total elimination of heat- killed cells; - Less discrimination of gamma- irradiation-killed cells	Lee and Levin (2008)

Target organisms (sample matrices)	Cell inactivation mechanisms	EMA conc. ($\mu\text{g}.\text{ml}^{-1}$)	Light exposure (power, W; duration, min; distance from samples, cm)	Effectiveness	Reference
<i>E. coli</i> (pond water)	Heat (90°C, 10min)	10	650; 2; 20	Reduction of 4 orders of magnitude for heat-killed samples	Inoue et al. (2008)
<i>E. coli</i> (creek water, secondary effluent)	Heat (100°C, 10min)	7.5	150; 1-10; 20	- Average reduction of 5.14 logs in heat-killed cells; - Effective enumeration of viable cells in water samples with turbidity less than 10 NTU	Gedalanga and Olson (2009)
<i>Legionella pneumophila</i> (domestic hot-water)	Heat (100°C, 15min; 70°C, 1-5 hr); Chlorine (0.2– 1.6 $\text{mg}.\text{l}^{-1}$); Glutaraldehyde (500 $\text{mg}.\text{l}^{-1}$)	2.5	500; 15; ND	- Mean reduction of 3.36 logs in heat-killed cells; - Mean reduction of 0.19 logs in viable cells	Delgado-Viscogliosi et al. (2009)
<i>Legionella pneumophila</i> (cooling tower water)	Heat (70°C, 30min)	2.3	500; 20; 15	- Yield similar result in viable cells numbers as determined by BacLight kit; - EMA performed better compared to PMA in heat-killed cells	Chen and Chang (2010)

Table 2.2 Application of PMA pretreatment for detecting viable microorganisms

Target organisms (sample matrices)	Cell inactivation mechanisms	PMA conc. (μM)	Light exposure (power, W; duration, min; distance from samples, cm)	Effectiveness	Reference
<i>Salmonella enterica</i> serovar <i>Typhimurium</i> , <i>L.monocytogenes</i> , <i>E.coli</i> 0157:H7	Disinfection (Hypochlorite, Benzalkonium); UV; heat	50	650; 2; 20	- Hypochlorite and benzalkonium disinfection gave significant signal reduction; - No significant difference between samples treated with and without PMA in UV-killed cells	Nocker et al. (2007)
<i>Pseudomonas aeruginosa</i> , <i>Listeria monocytogenes</i> , <i>Salmonella typhimurium</i> , <i>Serratia marcescens</i> , <i>Escherichia coli</i> O15:H7 (Turbid creek water, 43 NTU)	Isopropanol (10min)	100	650; 2; 20	- Inhibited amplification of dead cells in the background of mixture of culture; - Dead <i>Salmonella</i> in environmental water (10^8 cfu per sample) were inhibit from amplification	Nocker et al. (2009)
<i>Cryptosporidium</i> (wastewater, surface water)	Heat (70°C, 30min)	150	800; 2; 20	- Failed to eliminate the detection of heat-killed cells in raw wastewater samples	Brescia et al. (2009)

Target organisms (sample matrices)	Cell inactivation mechanisms	PMA conc. (μ M)	Light exposure (power, W; duration, min; distance from samples, cm)	Effectiveness	Reference
<i>Bacteroidales</i> (wastewater)	Heat (80°C, 20min)	100	600; 2; 20	- Higher solids concentration affected the suppression of PCR amplification in heat-treated cells; - Signal reduction of 2.5 logs between influent and effluent samples treated with PMA	Bae and Wuertz (2009)
<i>Bacteroidales</i> , <i>Enterococcus</i> (mixed stool samples in seawater)	Sunlight and dark conditions	100	600; 2; 20	- PMA treatment provided useful estimation of ages of fecal contamination from different animal sources	Bae and Wuertz (2009)
<i>Enterococcus</i> , <i>Bacteroidales</i> (secondary effluent)	Heat (85°C, 15min)	100	650; 2; ND	- Signal reduction up to 3 to 4 logs in qPCR with PMA treatment compare to qPCR without PMA treatment; - Higher TSS interfered the effectiveness of PMA	Varma et al. (2009)

Note. ND, not determine

2.5 Mathematical modeling of bacterial survival

The occurrence of bacteria, particularly pathogens, in natural waters poses a serious health risk. However, due to the difficulties associated with pathogens detection in natural waters, the risk assessments often rely on the monitoring of bacteria indicators, such as *E.coli* and enterococci. In order to examine the occurrence of these bacteria, laborious and time-consuming tests are required. Moreover, the results are usually obtained at a much later stage, for instance, the second day upon collection of sample. As such, modeling the survival of bacteria in natural aquatic environments is an additional tool that can help in assessing water quality rapidly, as well as provide information on the potential health risk brought by the pathogens. In addition, models also serve to integrate the large variability in environmental conditions such as temperature, salinity and light (Hipsey et al., 2008). Through models, the dominant processes (i.e. advection, mixing, mortality and sedimentation) that control organism dynamics can be estimated (Hipsey et al., 2008). Ultimately, an early warning system that is based on modeling can be used to warn the public of the health risk at an early stage. Through mathematical models, the responses of bacteria to particular environmental stresses can be described and quantified, which is useful for decision making.

2.5.1 Type of models

Mathematical models can be presented in two main categories, namely (1) empirical or mechanistic and (2) deterministic or stochastic.

Empirical models are the models that describe the observed data by using statistical fitted equations and expressed in simple mathematical relationships. In empirical models, the knowledge of actual mechanisms and individual constituent parts is not required. As a result, empirical models tend to have large standard errors of prediction and are most reliable when applied within the range of observations

used to develop the model (Ji, 2007). Some examples of empirical models include polynomial models, artificial neural networks and square-root models.

The mechanistic models are the models that describe the theoretical basis of the mechanisms in a system. In hydrodynamics and water quality processes modeling, mechanistic models are developed based on the definable and observable parameters in physical, chemical and biological mechanisms that govern the water systems. Hence, the extrapolation from mechanistic models usually gives higher confidence levels and is capable of better representing water systems that are being studied. However, in most of the cases, hydrodynamics and water quality processes modeling cannot be developed based on mechanistic models solely, but integrated with empirical models. For instance, the turbulence models used in hydrology models are highly dependent on the empirical formulas (Ji, 2007). In microbiology, mechanistic models are developed based on the understanding of underlying biochemical processes and intercellular processes that are controlling cellular behaviour (Devlieghere et al., 2009). Nevertheless, due to the complexity of microbial physiology and the current limited knowledge on actual microbial interactions with the environment, the mechanistic models are difficult to develop (Kilcast and Subramaniam, 2000; Devlieghere et al., 2009). Therefore, both mechanistic and empirical models usually work together in water quality modeling.

Another way to classify the mathematical models is based on the type of outcome the models predict. Deterministic models ignore random variation and each component of input will give an exact prediction by mathematical equations. In contrast, stochastic models allow random and probabilistic elements in the relationship between two or more variables (Ji, 2007). Due to the statistical nature of stochastic models, these models will produce the prediction according to the statistical distribution of possible outcomes.

These two categories are complementary to each other. For instance, an empirical model can be either deterministic such as kinetic models or stochastic such as probabilistic models, which uses different approaches for modeling. Kinetic

models record the dynamic changes over time and are usually used to predict the survival of bacteria in natural environments. Different kinetic models may need to be developed to examine the effects from various environmental factors. The main advantage of the kinetic model is that more information can be extracted from the model, particularly the dynamic changes associated with different conditions at each time interval. However, the drawback of the kinetic model is the requirement of a huge data set in order to generate this model.

A probability model predicts the possibility of an event that may occur within a certain time frame. In the context of bacterial survival, the probability model is usually used to estimate the likelihood of the presence of pathogens in certain environmental conditions. The main reason that pathogens survival is often modeled as a probability model is due to the limited information available for pathogens. Although probability models provide limited information on the survival rates of pathogens, this model could be useful for public health agencies to identify the health risk associated with the occurrence of pathogens. Subsequently, appropriate action such as temporary closure of beach/recreational water, or adequate treatment can be taken to reduce the health risk to the public.

2.5.2 Procedures for models development

Development of the models involves four main stages: (1) planning; (2) data collection and analysis; (3) mathematical description; and (4) model validation (Devlieghere et al., 2009). In this section, the discussion will focus on the development of a model to predict bacterial survival.

At the initial planning stage, it is very important to have clear objectives for the models development. Apart from the application of the models, the key controlling factors need to be identified. For instance, there could be numerous factors (i.e. nutrients, temperature, salinity, pH and sunlight) that affect the survival of bacteria in natural environments (Troussellier et al., 1998; Mika et al., 2009). However, it is not practical to develop a model with too many independent variables.

Furthermore, the additional independent variables can cause increases in labour and cost. As a result, performing preliminary experiments are always encouraged.

The second stage involves data collection and analysis. Since empirical models are developed from observed data, adequate and sufficient data are particularly important for the model development. The results from different data collection methods may give different observed data. In microbiology, most enumerations are done by examining the viability of microorganism through the growth of colonies. As described in section 2.3, there is a possibility that a microorganism can enter a dormant stage, or the VBNC stage, when the microorganism encounters stresses from the environment. Thus, enumeration of microorganisms through the culture-based method may not be suitable for certain objectives of the model. In addition, the number of samples (data collected) is crucial for empirical models. The sample size should be sufficient and adequate to represent the whole population, as well as to draw a statistically significant conclusion. For kinetic models, the optimum time interval for data collection is important to ensure that dynamic changes in the system are being captured.

In the development of models, the data collected are translated into mathematical equations to produce a model. Usually, the data will be fitted into several existing models followed by checking the goodness of fit. A first-order reaction is the typical model used to describe the decay kinetics of a bacterium in an environmental stressed condition. In the first-order reaction, a plot of the cell concentration versus time on semi-log plot will give a linear relationship. Owing to the simplicity of the first-order reaction, this model has often been applied in water quality modeling to predict the decay of the bacteria in environment. For practical application, first-order decay rate can be easily expressed in T_{90} , which is the time required for the bacterial concentration to decrease by one log unit. Nevertheless, studies have shown that the bacterial die-off in environmental waters does not exactly follow first-order kinetics, as a lag phase should also be incorporated into the model (González, 1995). A biphasic pattern of decay kinetics with a relative high first-order rate constant during the first phase and a lower constant rate in the

second phase were reported for fecal bacteria in surface waters (Hellweger et al., 2009). It was suggested that this phenomenon is attributed to population heterogeneity which consists of two different subpopulations, normal cells and persistent cells (Kussell et al., 2005; Hellweger et al., 2009). In addition to log-linear models, non-linear models such as the logistic model and Gompertz model, are also used to describe bacterial survival in the environment (Whiting, 1993; González, 1995). Once the bacterial survival curves are generated, regression analyses are carried out to draw the relationship between the bacterial survival and environmental factors. Linear regression and nonlinear regression are the two main regression analysis techniques to fit the data in a line and a curve, respectively. The simple linear regression that defines the values for the slope and intercept, is useful when there is only a dependent variable and an independent variable involved. Nonlinear regressions such as exponential curve or quadratic curve are some of the choices for those data that do not fit into the linear regression. Multiple regression which allows the investigation of relationship between multiple independent variables and a dependent variable is frequently employed to study the influence of various environmental factors in bacterial survival.

The final stage of model development is the validation of models. Through the validation process, models will be evaluated for their ability to give an accurate prediction. The coefficient of determination (R^2) is one of the simple methods to evaluate a model's predictive ability. However, R^2 may be able to describe how well the model fits with the data but is unable to provide information on the quality of fit, whether there is bias to overestimation or underestimation. On the other hand, graphical residual analysis evaluates the performance of a model by plotting the difference between observed data against the corresponding predicted values. Through graphical presentation, more information can be obtained from the graph. For example, if the residuals appear randomly scattered around zero, it means that the model fits the data well. In contrast, if the residuals have a systematic pattern, this indicates that the model is poorly fitted to the data. Bias factor and accuracy factor are two indices proposed by Ross (1996) to examine the performance of

models. Direction in the bias factor gives the information on the over or under-prediction of a model without plotting a graph.

2.5.3 Current microbial water quality models

There are many water quality models available to simulate watershed hydrology and the subsequent pollutant effects on receiving water quality. However, very few of them have included all functions such as hydrologic simulation, pollutant loading simulation, hydraulic routing, in-stream water quality, as well as microbial water quality. Hence, careful selection of an appropriate model, or combination of models is important to achieve the prediction objective with the available data. This review will focus on the models that are commonly used for microbial water quality prediction.

Microbial water quality is currently based on the concentration of fecal indicator bacteria (i.e. *E.coli* or enterococci) to indicate the presence of pathogens. The water bodies that have exceeded of maximum allowable concentration of fecal indicator bacteria are not suitable for recreational use and may cause human health risk. As such, restoring the microbial water quality in these waters requires identification of the sources and quantification of the loading rates from the sources. Models have been developed to simulate the survival of bacteria from source to receiving waters. Simple models such as mass balance (budget) models (Petersen et al., 2009) and statistical models (Dilks et al., 1992) were developed to predict the sources and effects of fecal contamination. Due to the empirical nature in statistical models, these models do not provide insight into the physical processes (Desai et al., 2011). Meanwhile, most of the budget models can only represent an average scenario of the modeled system and do not account for temporal variations in water quality (Desai et al., 2011). However, with a long term average study or careful consideration of temporal varying forcing conditions, a budget model can be improved through a dynamic budget model approach (Spillman et al., 2007).

Sophisticated models such as the Hydrologic Simulation Program in Fortran (HSPF) (Bicknell et al., 2001), Soil Water and Assessment Tool (SWAT) (Sadeghi and Arnold, 2002), Storm Water Management Model (SWMM) (Rossman, 2004) and Water Quality Analysis Simulation Program (WASP) (Di Toro et al., 1983; Ambrose et al., 1988) that incorporate spatial and temporal variability have been developed for water quality modeling. HSPF and SWAT are commonly used to model the fate and transport of bacteria in urbanized watersheds and agricultural watersheds respectively. These two models have been widely used for the development of total maximum daily load (TMDL) to describe the pollutant limits that a water body can receive while still meeting the water quality standards in the United States (Benham et al., 2006; Chin et al., 2009; Petersen et al., 2011; Russo et al., 2011). In HSPF, bacteria die-off rate on the land surface is simulated by limiting the amount of accumulated bacteria, whereas in-stream die-off is modeled by a first-order decay function. SWAT uses the first-order Chick's law to simulate the bacteria die-off rate under all conditions. One of the advantages in HSPF is that allows non-zero bacteria concentrations in the subsurface inflow to the stream and also allows estimation of resuspended bacterial loads from the streambed (Jones et al., 2007; Chin et al., 2009). However, SWAT offers an advantage of tracking both persistent bacteria and less-persistent bacteria upon deposition on the ground or stream. Separate die-off rates can be specified for different types of pathogens. This is particularly useful when persistent protozoans (e.g. *Cryptosporidium*) could eventually become the dominant species over time and pose a different health risk compared to less-persistent bacteria (e.g. *E.coli*). Comparing the application of both HSPF and SWAT models, the SWAT model was shown to give better performance in predicting the bacteria concentration (Benham et al., 2006; Chin et al., 2009). This could be due to the separate die-off rates assigned to two subpopulations of bacteria. On the other hand, HSPF has been used to model the fate and transport of bacteria by incorporating the microbial-sediment association (Russo et al., 2011).

Although the HSPF model has been used to simulate runoff in urbanized watersheds (Desai et al., 2011; Petersen et al., 2011), SWMM was found to perform better in simulating urban runoff as compared to HSPF (CDM, 2003). This is

because urban infrastructures such as drains, detention basins, sewers and related flow controls can be modeled using SWMM (Jones et al., 2007). However, SWMM does not provide in-stream water quality simulation (Jones et al., 2007). This limitation is often overcome through the integration with WASP where WASP is a well-established water quality model that has incorporated transport and reaction kinetics of pollutants (e.g. bacteria). Little manipulation is required to integrate both SWMM and WASP. In addition, both SWMM and WASP require relatively less data for model development as compared to HSPF and SWAT. This has subsequently made the development of SWMM and WASP more cost effective.

All these models (i.e. HSPF, SWAT, SWMM, WASP) are supported by the U.S. Environmental Protection Agency (EPA) through their Better Assessment Science Integrating Point and Nonpoint Sources (BASINS) system. With a series of underlying models that can be incorporated into BASINS, this system provides additional in-stream modeling such as more finely discretized stream reaches (Bossis, 2011). In addition, the integration of BASINS with geographical information system (GIS) which provides landscape information analysis, has made this modeling tool more unique and powerful. Nevertheless, the BASINS model is very complex and involves many parameters.

The common limitation faced by the above models is the estimation of reduction in microbial concentration. First-order decay is most often used to express the bacterial die-off. However, many studies have shown that bacterial survival dynamics may not follow exactly the first-order reaction (González, 1995; Kussell et al., 2005; Hellweger et al., 2009). Moreover, the decay rates are highly dependent on the bacterial species and environmental conditions. Temperature is the most common environmental variable that is used to modify die-off rates in these models. Other environmental factors such as sunlight irradiation (Carvalho et al., 2006), salinity (Wong and Wang, 2004), dissolved oxygen levels (Ballesté and Blanch, 2010) and pH (Parhad and Rao, 1974) have shown to affect bacterial survival. A comprehensive process-based model has been developed to describe a wide range of microbial behavior from a large body of literature (Hipsey et al., 2008). This

model has significantly improved the previous studies through the incorporation of natural mortality of microorganisms under various environmental factors (i.e. salinity, pH, temperature, sunlight irradiation and dissolved oxygen levels). In addition, this study also included the growth term in the model to allow the simulation of microorganisms growth, particularly in warm and nutrient rich environment. Although there is evidence of the growth of coliforms and enterococci in environmental waters, however, limited quantitative information is available to estimate the growth of these bacteria (Camper et al., 1991; Desmarais et al., 2002). Similar to grazing and/or predation of microorganisms, the predation pressures in different aquatic environments are difficult to predict. The prediction of actual number of microorganisms became more complicated with recent findings on the occurrence of VBNC cells in stressed environments (Colwell, 2009; Oliver, 2010). Hence, there is a need to deepen our understanding of the growth, predation and VBNC parameters in order to better capture the actual number of viable microorganisms in natural environments.

2.6 Summary of literature review

Studies revealed that many human pathogens are able to enter the VBNC state during stressed conditions and eventually resuscitate upon removal of the stresses. Maintenance of virulence by VBNC pathogens has also being reported. Therefore, pathogens in the VBNC state will still pose a significant public health threat. The scenario becomes worst when bacteria indicators such as *E. coli* and *E. faecalis* are also able to enter the VBNC state when confronted with unfavorable conditions. The reliability of routine water quality monitoring that employs culture-based method is therefore doubtful and uncertain. Thus, a better understanding of the factors triggering the entry of bacteria indicators into the VBNC state, particularly in tropical regions, is of critical importance.

Over the past two decades, VBNC related studies were mainly focused on the factors inducing VBNC, morphological changes during the VBNC state and evidence of the VBNC state in bacteria. Environmental factors which induce the

VBNC state in bacteria have been studied extensively. These environmental stresses include starvation, depletion of nutrient, variation in salinity, changes in temperature and radiation. Generally, the combination of various stresses leads to entrance into the VBNC state. However, different bacteria may respond differently to the environmental stresses. For example *Vibrio parahaemolyticus* enters the VBNC state when it is exposed to lower temperature. On the other hand, *Pseudomonas fluorescens* has preference to survive at low temperature conditions, and will only enter the VBNC state when exposed to 37°C. As bacteria enter the VBNC state, changes in size, shape, membrane and extracellular polysaccharide have been observed. The morphological changes in the VBNC state are dependent on the strain of bacteria and also the induction factor. Evidence of the VBNC state in bacteria was proved through a wide range of viability/activity tests. Basically, these viability/activity tests can be categorized into two main groups, direct indicators and indirect indicators. Direct indicators test the metabolic activities in the cell. In contrast, indirect indicators examine the maintenance of cellular structures, e.g. persistence of nucleic acid, membrane integrity or membrane potential.

In recent years, studies have focused on molecular changes in the VBNC bacteria. Genes are shown to be continuously expressed during the VBNC state, but at different rates. Some specific genes are expressed exclusively during the VBNC state. Similar findings are found in the protein profile. With more understanding of the gene expression and protein profile, several molecular detection methods have been developed. PCR and qPCR were used to detect and/or quantify DNA, while RT-PCR, RT-qPCR and NASBA were used to study RNA gene expression. Microarray-based analysis offers a great advantage to simultaneously detect various microorganisms. Alternatively, proteomic analysis is also another promising new direction for VBNC studies. Since the introduction of EMA and PMA pre-treatment to eliminate the influence of dead cell in PCR, detection of VBNC cells based on DNA is gaining more popular. The main reason is the relative consistent DNA copy number in different physiological states as compared to highly variable RNA gene expression in a cell under different physiological states (Takayama and Kjelleberg,

2000). As such, this study will explore the possibility of molecular methods which target the DNA gene, such as qPCR, for more accurate and rapid quantification of VBNC cells.

The greatest challenge in the study of VBNC cells is the grey definition of live, dead, injured and VBNC cells. Conventional laboratory methods often rely on the ability of a cell to grow on appropriate nutrient media. However, there is strong evidence to show that in certain circumstances, a cell is able to maintain its metabolic activity and membrane integrity although no growth has been observed in the appropriate nutrient media (Lleo et al., 1998; Colwell, 2009). There are also strong debates on whether a VBNC state is a survival physiological state or the transition state towards the death phase (Kell et al., 1998; Bogosian and Bourneuf, 2001)? How can the injured cells and the VBNC cells be differentiated? What is the cut-off point to define the live and dead status of a cell?

The delay in obtaining bacteria measurements is one of the major problems in current microbial water quality monitoring that uses culture-based methods. This is mainly due to the long incubation time (at least 18 hours) required for the culture-based method. In addition, studies have shown the much shorter time scales of temporal changes in indicator bacteria levels in beach water (Nobel and Weisberg, 2005). Thus, mathematical modeling of bacterial survival is an important tool to predict the occurrence of bacteria or pathogens at more refined temporal and spatial scales. Through the predictive models, an early warning of health risk can be delivered to the public. Unfortunately, the microbial decay rates that are usually applied in water quality models rely solely on the culture-based method. Owing to the significant health risk impact of VBNC cells, an important objective in this study is to develop a mathematical model which can predict the occurrence of VBNC cells in the tropical aquatic environment. This mathematical model will further polish the current water quality models, which often underestimate the health risk impact of VBNC cells in natural environments.

CHAPTER 3 IDENTIFICATION OF ENTEROCOCCUS SPECIES IN THE CATCHMENT WATERS OF MARINA RESERVOIR

3.1 Introduction

Fecal coliforms have long been used as the microbial indicator to evaluate the quality of recreational waters. However, *E.coli* and enterococci have been shown to give stronger correlation with swimming-associated gastrointestinal illness compared with fecal coliforms. In the latest US Environmental Protection Agency (USEPA) guideline, both *E.coli* and enterococci have been recommended as better indicators of fecal contamination than coliform or fecal coliforms for freshwater beaches, while it has been suggested that enterococci are the most suitable indicator for marine waters (USEPA, 2004).

Currently, there are two common methods applied to detect and enumerate enterococci; the membrane filter (MF) technique and the defined substrate based detection method (EnterolertTM). The MF technique is the most common detection and enumeration method used by government agencies to monitor environmental water quality and was introduced in the late 1950s. The introduction of MF gave an alternative to the Most Probable Number (MPN) procedure for microbiological analysis of water samples. Compared to the MPN method, MF offers the advantage of isolating discrete colonies of bacteria. Moreover, MF permits the analysis of a larger number of samples in a shorter time. The EnterolertTM method is based on the principle that the β -glucosidase enzyme from enterococci are capable of metabolizing the nutrient indicator, 4-methyl-umbelliferyl β -D-glucoside. Once a nutrient indicator is metabolized, methylumbelliferylone will form from the 4-methyl-umbelliferyl β -D-glucoside which initiates a fluorescence signal. The fluorescence signal can be visualized under UV light at 366nm wave length. The advantages and disadvantages for MF and EnterolertTM are summarized in Table 3.1

Table 3.1 Comparison between the MF and Enterolert™ methods

	MF method	Enterolert™
Principle	<ul style="list-style-type: none"> ▪ Retain bacteria on the membrane ▪ Selective isolation medium (mE agar) followed by substrate medium (EIA) 	<ul style="list-style-type: none"> ▪ Utilize nutrient indicator substrate (4-methylumbelliferone-β-D-glucoside)
Result presented in	CFU/100ml	MPN/100ml
Advantages	<ul style="list-style-type: none"> ▪ More than 100ml samples can be tested ▪ Effective & acceptable technique ▪ Allows the isolation & enumeration of microorganisms 	<ul style="list-style-type: none"> ▪ Relatively simple ▪ Rapid method ▪ Economical (low equipment cost)
Disadvantages	<ul style="list-style-type: none"> ▪ Time consuming ▪ Not suitable for water sample with high turbidity 	<ul style="list-style-type: none"> ▪ Upper limit detection of 2419MPN/100ml (without dilution of water sample)

Nowadays, there is a wide variety of culture media available for use in the examination of enterococci. However, most of these culture media are only capable of identification at the genus level. Conventional identification of species relies mainly on the reaction of bacteria towards different biochemical tests. Unfortunately, some of the *Enterococcus* species (i.e. *E.gallinarum*, *E.casseliflavus* and *E.raffinosis*) are proposed to be environmental strains due to their association with plants and soils (Pinto et al., 1999). As such, their value as fecal indicators needs more investigation (Leclerc et al., 1996) and the detection of different species is important to give a better indication of health risk.

In recent years, the occurrence of viable but nonculturable (VBNC) bacteria in environmental waters has drawn considerable attention from researchers, as well as from the public. The situation is made worst when studies showed the ability of enterococci to enter the VBNC state (Lleo et al., 1998; Lleo et al., 2005). The implication is that the reliability of the conventional culture-based method to identify the microbial risk using enterococci comes into question.

From previous studies, different enterococcus species were shown to have different survival ability and different times to enter the VBNC state (Lleo et al., 2001; Lleo et al., 2005). In one study, the enterococcus species were divided into three main groups based on the duration required to enter the nonculturable state in oligotrophic microcosms which were maintained at 4°C under direct illumination (Lleo et al., 2005). For instance, the first group which consisted of *E.faecalis*, *E.hirae*, *E. gallinarum* and *E.casseliflavus* reached the nonculturable state in 20-30 days, *E.avium*, *E.maleodoratus*, *E.pseudoavium*, *E.muntzii* and *E.raffinosis* in the second group were found to lose their culturability in 40-45 days while more than 50 days were required for the third group (*E.faecium*, *E.flavescens* and *E.durans*) to reach nonculturable state (Lleo et al., 2005). Since enterococcus is currently the main fecal indicator for fecal contamination, the difference in survivability and the entry into VBNC state for each species is important in evaluating the environmental water quality.

Owing to the significant difference in the characterization and survival of each species, species identification could play a better role as fecal indicator and give a good indication of the possible occurrence of VBNC enterococcal cells in Singapore waters. Hence, *Enterococcus* species identification is one of the main tasks in this study and will be discussed in this chapter. In addition, this study also compares both MF and Enterolert™ methods for the samples collected from tropical environment waters. The correlation between the occurrence of enterococci and environmental parameters will be investigated.

3.2 Study site

The water samples for the field study were collected from Marina Reservoir and its catchment. Marina Reservoir was selected for this study due to the fact that this reservoir will eventually become one of Singapore's biggest drinking-water sources in the future. Marina Reservoir is situated near the heart of Singapore City. The water from the upstream catchments flows through urbanized areas and subsequently feeds into Marina Reservoir via Singapore River, Geylang River, Kallang River, Rochor Canal and Stamford Canal (Figure 3.1). The total catchment area for Marina Reservoir is around 10,000 hectares. Apart from serving as a reservoir for potable water usage, Marina Reservoir is also proposed as a recreational reservoir where various water recreation activities are carried out. Major international water sport events, international water activities and even river tour cruises utilize this reservoir. Thus, its aesthetic quality and cleanliness play important roles in projecting Singapore's image as an International Water Leader.

Figure 3.1 Marina Reservoir catchment plan (Daryl, 2008)

3.3 Materials and Methods

3.3.1 Water sampling

Water samples were collected from five locations at Marina Reservoir catchments (Figure 3.1). Water samplings were carried out in between June 2008 to July 2009. Water samples were collected according to the recommended procedure in Section 9060 of *Standard Methods for the Examination of Water and Wastewater* (APHA, 1998). In brief, 1-liter water samples (in duplicate) and 100-mL water samples (in duplicate) were collected in pre-sterilized bottles. The water samples were kept in an ice box during transit to the laboratory. All samples were analyzed for microbiological examination within 6 hours from collection.

3.3.2 Water quality analysis

Water quality parameters examined on-site include temperature, salinity, conductivity and total dissolved solid (TDS). These parameters were measured by YSI probe (model number: 6600). Dissolved oxygen was measured on site with YSI model 52 (YSI probe 5739). All parameters were measured twice to get the average reading. Duplicate samples were collected for all water quality analyses in the laboratory.

Turbidity and pH were measured in the laboratory with a turbidity meter (Euteoh Instruments, TN-100) and Thermo pH meter (Orion 555A) respectively. Total suspended solids (TSS) was measured according to APHA 2540D (APHA, 1998). Briefly, the water sample was filtered through pre-treated (washed and dried) 0.45 μ m glass-fiber filter. The filter with retained solids was dried to a constant weight at 103°C to 105°C. The increase in weight of the filter represents the total suspended solids (APHA, 1998). The water samples were kept in 4°C and analyzed on the same day of sampling date. It is not advisable to keep the samples more than 24 hours to avoid microbiological decomposition of solids (APHA, 1998).

Total organic carbon (TOC) was analyzed by a TOC analyzer (Shimadzu TOC-V_{CSH}) according to manufacturer's instruction and APHA 5310B (APHA, 1998). In general, the TOC test measures the production of carbon dioxide that is released from the oxidation of organic material through combustion at high temperature. Sample pre-treatment was carried out by adding two drops of concentrated HNO₃ to remove the inorganic carbonates in the water. The samples were preserved not more than 28 days in 4°C with minimal exposure to light and atmosphere.

Total nitrogen (TN) was analyzed by Flow-injection analysis (FIA, Lachat QuikChem 8500) which complied with USEPA method 353.2. Prior to the Flow-injection analysis, the samples were digested with persulfate to oxidize all forms of nitrogen to nitrate according to APHA 4500-N C (APHA, 1998; Gross and Boyd, 1998). The samples were kept in -20°C freezer until analysis.

Total phosphorus (TP) was measured by the ascorbic acid method according to APHA 4500-P E (APHA, 1998). Due to the existence of phosphorus in several distinct forms in natural waters, a digestion step is required to convert various form of phosphate-phosphorus to the orthophosphate form. The digestion method for TP was similar to TN (Gross and Boyd, 1998).

Standards were generated for each TOC, TN and TP tests. The concentration of TOC, TN or TP was determined by comparison with calibration results obtained from standards of varying concentration.

3.3.3 Detection and quantification of enterococci in environmental water samples

3.3.3.1 Membrane filter technique

The enumeration procedure for enterococci was performed according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1998). Two culture media, namely mE agar (Difco, Detroit, MI) and EIA substrate (Difco, Detroit, MI) were used to perform enumeration in MF. The addition of nalidixic acid (Sigma-Aldrich, St. Louis, MO) and triphenyltetrazolium chloride (Difco, Detroit, MI) in the mE agar allows isolation and differentiation of enterococci from other bacteria. EIA substrate that contains esculin will be hydrolyzed by the enterococci to form a reddish brown to black precipitate on the underside of the filter. The water samples were filtered through a 0.45µm membrane. After filtration, the membrane was transferred onto the mE agar and incubated for 48 hours at 41°C. This was then followed by transferring the membrane filter onto the EIA substrate for the *in situ* substrate test to identify the colonies which were capable of hydrolyzing esculin. A quick incubation was carried out for 20 minutes at 41°C before the hydrolyzed colonies were examined. Dark red colonies coupled with dark precipitates were scored as enterococci.

It is recommended that verification tests should be done on the colonies formed in MF to eliminate false positive results. Figure 3.2 shows the flow chart for the series of verification tests. First, pick single colony that formed on the membrane and streak for isolation on brain-heart infusion agar (BHIA) plate. Repeat isolation for at least five colonies from each membrane. The BHIA plates with streaked colonies were incubated at 35°C for 24 to 48 hours. The well-isolated colony was subjected to several verification tests which include the growth test on bile esculin agar and brain-heart infusion broth with 6.5% NaCl, catalase test with hydrogen peroxide, and morphological test with gram staining. The media preparations for verification test were described in Appendix.

Figure 3.2 Series of verification tests summarized from APHA (APHA, 1998)

Enumeration was done by counting the colonies on the membrane filter with 20 to 80 colonies but not more than 200 colonies. The equation to compute the enterococci density is shown in Equation 3.1, whereas Equation 3.2 gives the confirmed enterococci count.

$$\frac{\text{Presumptive count}}{100\text{ml}} = \frac{\text{Number of colonies on membrane filter} \times 100}{\text{Volume of sample filtered (ml)}} \quad (3.1)$$

$$\text{Confirmed enterococci} = \text{Presumptive enterococci} \times \text{Proportion of the isolates that are catalase-negative} \quad (3.2)$$

3.3.3.2 Defined substrate based detection method (Enterolert™)

The Enterolert™ procedure was performed according to manufacturer's instructions. In brief, one pack of Enterolert™ powder reagent was added to a sterile, transparent and non-fluorescing vessel containing 100ml of water sample (or 10 times dilution of the water sample). Subsequently, the powder was shaken and dissolved. The mixture was then poured into a Quanti-Tray and mechanically sealed. The sealed tray was incubated at $41^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 hours. After incubation, the test results were read in a dark environment by placing the tray within 5 inches under a 6-watt, 365 nm, UV light. Any blue fluorescence well was counted as a positive result as it indicates the presence of enterococci. The number of positive wells was then compared with a MPN table or IDEXX calculator to determine the enterococcal density per 100ml of water sample. The dilution factor was also considered in the calculation. Wells with no fluorescence were considered as negative results, i.e. enterococci are absent.

3.3.4 Species identification

In this study, a commercial biochemical test kit, API 20 Strep (bioMérieux, France) was utilized to identify the enterococcus species. A total of 20 biochemical tests have been combined into API 20 Strep to enable group or species identification. Basically, API 20 Strep strip contains 20 microtubes with dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars (Table 3.2). After incubating for 24 – 28 hours, identification was then conducted based on numerical analysis upon the visual interpretation of color changes.

Table 3.2 Tests and reactions used in API 20 Strep (bioMérieux, France)

Abbreviations	Tests	Reactions
VP	Pyruvate	Acetoin production
HIP	Hippurate	Hydrolysis
ESC	Aesculin	β glucosidase
PYRA	Pyrrolidonyl-2-naphthylamide	Pyrrolidonylarylamidase
α GAL	6 Br 2 naphthyl α D galactopyronoside	α galactosidase
β GUR	Naphthol ABSI β D glucuronate	β glucuronidase
β GAL	2 naphthyl β D galactopyronoside	β galactosidase
PAL	2 naphthylphosphate	Alkaline phosphatase
LAP	L-leucyl 2 naphthylamide	Leucinearylamidase
ADH	Arginine	Arginine dehydrolase
RIB	Ribose	Acidification
ARA	L-arabinose	Acidification
MAN	Mannitol	Acidification
SOR	Sorbitol	Acidification
LAC	Lactose	Acidification
TRE	Trehalose	Acidification
INU	Inulin	Acidification
RAF	Raffinose	Acidification
AMD	Starch	Acidification
GLYG	Glycogen	Acidification

Species identification of *Enterococcus* spp. was performed through API 20 Strep (bioMérieux, France). The isolated colony from EIA or mEI agar was subcultured onto Columbia blood agar. After 24 hours incubation in anaerobic conditions at $36^{\circ}\text{C} \pm 2^{\circ}\text{C}$, the haemolysis reaction was recorded. The colonies on the Columbia blood agar were then transferred into the API Suspension Medium provided. Next, approximately 100 μ l of this suspension was transferred to the cupules (from VP to ADH) on the strep. The rest of the suspension (approximately 500 μ l) was transferred into the culturing media (API GP Medium) provided. The new suspension in the culturing media was transferred to the remainder cupules (from RIB to GLYG). The strep was then incubated at $36^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 4 hours. After 4 hours of incubation, one drop of 40% potassium hydroxide and one drop of 6% alpha naphthol in ethyl alcohol were added to the VP cupule. Two drops of ninhydrin were also added to the HIP cupule after that. Cupules PYRA, α GAL, β GUR, β GAL, PAL and LAP were added with one drop each of reagent ZYM A and ZYM B. Then, the strep was incubated 10 minutes under the light. The results were obtained by observing the colour changes and comparing it with the

manufacturers' guide sheet. A further incubation to 24 hours at $36^{\circ}\text{C} \pm 2^{\circ}\text{C}$ was required to obtain the final reading. APIWEB Software (bioMérieux, France) was used to analyze the reading from all tests in API 20 Strep and further identified the bacterial isolates up to species level.

Enterococcus species identification was performed on the water samples collected from Marina Reservoir's catchments (i.e. Geylang River, Kallang River, Rochor Canal, Stamford Canal and Singapore River). The bacteria were grown on the selective agar (mE agar and EIA) and subsequently isolated for the biochemical test for the identification of the enterococcus species. Ten colonies formed on the agar were isolated from each station for identification purposes.

3.3.5 Statistical analysis

Preliminary analysis was carried out to examine the distribution of data before the selection of the statistical analysis method. The non-parametric statistical method was selected to test the correlations between the enterococcus enumeration methods (i.e. MF and EnterolertTM) and environmental parameters. The relationships between environmental parameters and estimated enterococci counts were determined by Spearman's correlation coefficient.

The paired t-test was used to compare the means between two different methods (MF and EnterolertTM) in an identical sample. The strength and significance of the relationship between two methods was examined by Pearson's r correlation coefficient. All statistical analyses were done by SPSS software (Version 14.0, SPSS Inc.).

3.4 Results and Discussion

3.4.1 Environmental parameters

The values for 12 different environmental parameters, including 2 enterococci enumeration methods, are presented in Table 3.3. As Singapore is located in the equatorial region, there is little difference in water temperature throughout the whole year. A drastic change in salinity and conductivity levels were found in water samples collected from all catchments in January 2009. This phenomenon may be attributed to the backflow of existing seawater in Marina Reservoir upon the closure of Marina Barrage. Since the closure of Marina Barrage in October 2008, the desalting process began through gradual and natural replacement by rainwater. In May 2009 to June 2009, salinity levels obtained from the five catchments were generally lower than 0.7 ppt. Other interesting environmental parameters were turbidity and TSS. Wide variability in turbidity and TSS were observed at different catchments. Stamford Canal and Rochor Canal were the two main catchments with higher turbidity (mean = 25.0 NTU and 37.8 NTU, respectively) and TSS level (mean = 18.9 mg/L and 28.9 mg/L, respectively) due to nearby construction activities. The water pH in all catchments was found to be more alkaline, ranging from 7.4 to 9.1 with the average pH of 8.01. DO solubility levels obtained from Marina Reservoir catchments showed a wide range, from 3.4mg/L to 12.8mg/L. It has been showed that DO solubility correlates with temperature (Badran, 2001). However, with an average annual temperature difference of approximately 7°C in Singapore (http://app2.nea.gov.sg/weather_statistic.aspx), high fluctuation in DO solubility levels should not be expected, unless under other external influences. Spearman's correlation analysis showed that in addition to temperature ($R = -0.435$), turbidity is another environmental factor that has inverse correlation with DO solubility level ($R = -0.464$) (Table 3.4). The particles present in turbid water will absorb heat from sunlight and increase the water temperature. Subsequently, this may lower the DO solubility level in waters. In addition, the inverse correlation between DO solubility and turbidity may be because of high organic content in waters, which is subsequently decomposed by bacteria consuming oxygen in the process. As for nutrient levels in the Marina Reservoir

catchment, TP and TN were within the range of 0.01mg/L to 0.27mg/L and 0.43mg/L to 1.95mg/L, respectively. TOC, ranging from 2.32mg/L to 16.30mg/L, was found to be correlated with pH and TN ($R = 0.45$ and $R = 0.544$, respectively).

Table 3.3 Environmental water parameters in Marina Reservoir catchments

Parameters	Minimum	Maximum	Mean	Standard deviation
Temperature (°C)	26.1	30.4	28.5	1.3
Conductivity ($\mu\text{S}/\text{cm}$)	153.9	32935.0	5180.4	10185.6
Salinity (ppt)	0.1	22.5	3.2	6.6
pH	7.4	9.1	8.0	0.4
Turbidity (NTU)	6.0	98.8	21.2	20.4
TSS (mg/L)	8.8	67.5	19.1	12.8
DO (mg/L)	3.4	12.8	9.4	2.9
TOC (mg/L)	2.3	16.3	6.1	2.6
TP (mg/L)	0.01	0.27	0.08	0.06
TN (mg/L)	0.43	1.95	1.05	0.43
MF (CFU/100ml)	11	8110	1920	2371
Enterolert (MPN/100ml)	10	6088	1210	1520

Table 3.4 Spearman's correlation coefficient matrices for different environmental variables collected from Marina Reservoir catchments

	Temp.	Cond.	Salinity	pH	Turbidity	TSS	DO	TOC	TP	TN	MF
Cond.	0.138										
Salinity	0.142	0.944**									
pH	0.277	-0.084	-0.193								
Turbidity	0.021	-0.521*	-0.454*	0.124							
TSS	0.223	0.073	0.189	0.245	0.377						
DO	-0.435*	0.322	0.229	-0.068	-0.464*	-0.196					
TOC	0.109	-0.065	-0.076	0.450*	0.110	0.214	0.182				
TP	0.482*	0.367	0.278	0.287	-0.094	0.244	-0.139	0.163			
TN	0.278	-0.149	-0.181	0.715**	0.324	0.475*	-0.093	0.544**	0.312		
MF	-0.366	-0.358	-0.416*	0.140	0.526**	0.025	-0.062	0.194	-0.314	0.139	
Enterolert	-0.099	-0.336	-0.388	0.177	0.633**	0.215	-0.299	0.130	-0.135	0.222	0.772**

* correlation is significant at the 0.05 level (2-tailed)

** correlation is significant at the 0.01 level (2-tailed)

3.4.2 Microbiological analyses

Enterococci were detected in five catchments using both MF and EnterolertTM methods. Enterococci counts using MF ranged from 10.8 CFU/100ml to 8110 CFU/100ml (Table 3.3), while these ranged from 10 MPN/100ml to 6088 MPN/100ml using EnterolertTM method. Although MF and EnterolertTM method gave results in CFU/100ml and MPN/100ml respectively, the quantifications are functionally equivalent (Chua et al., 2010). According to the USEPA guideline, the maximum allowable density of enterococci for a single sample for infrequently used full body contact recreational freshwater is 151 CFU/100ml (USEPA, 1986). However, most of the water samples collected in Marina Reservoir catchments exceeded the standard guideline value, with a few exceptions.

Figure 3.3 shows the enterococci count by the two different methods for each sub-catchment and presented as box plots. Using the MF method, the highest enterococci count was observed in water samples collected from Stamford Canal. This is followed by Rochor Canal. Using the EnterolertTM method, both Stamford Canal and Rochor Canal gave similar values for the median of enterococci counts, except for an extreme outlier for a water sample from Stamford Canal (Figure 3.3). This study suggests that the high enterococci concentrations in Stamford Canal and Rochor Canal are related to turbidity in both canals. The particles in turbid water can act as shields for bacteria from sunlight irradiation, thereby reducing the impact of sunlight inactivation on enterococci. Enterococci cell concentrations in Geylang River were the lowest with an average concentration of 317 CFU/100ml and 124 MPN/100ml, by MF and EnterolertTM method respectively. In this study, the MF method generally gave higher counts than EnterolertTM. This is in agreement with Adcock and Saint (2001) who reported that EnterolertTM significantly underestimated enterococci cell concentrations as compared to the MF method with m-Enterococcus media.

Figure 3.3 Box plots of enterococci cell concentration enumerated by (A) MF method, and (B) EnterolertTM method. The thick horizontal line in the box indicates the median value. The top and bottom of the box show the 75th and 25th percentile values, while the vertical lines extending from the box represent the largest and smallest values. The outlier with more than 1.5 box-lengths and 3 box-lengths are indicated by (o) and (*), respectively.

Spearman's correlation analysis was used to identify the correlation between enterococci and environmental parameters. The analysis showed that turbidity is the main environmental factor that has significant correlation with both MF and EnterolertTM methods with Spearman's correlation coefficient of 0.526 and 0.633, respectively (Table 3.4). This is in agreement with Suter et.al.'s (2011) study where they showed that over half of the enterococci ($52.9\% \pm 20.9\%$) were associated with

particles and suggested that the microenvironment around particles may encourage the settling of enterococci and subsequently favor longer persistence of enterococci (Suter et al., 2011). Studies also showed that turbidity could be auto-correlating in response to rainfall events which mobilize catchment sediment and bacteria resuspension from sediment at the same time (Reeves et al 2004). In addition, considerable attachment of bacteria to particles was found in turbid water environments (Pommepuy et al., 1992). The attachment to particles can increase the sedimentation rate as well as provide shading to bacteria from sunlight inactivation. In this study, salinity also showed good correlation with the MF method but was not correlated with the EnterolertTM method. It is suspected that the higher salinity could impose more stress to enterococci survival, subsequently inducing cells into the dormant or viable but non-culturable (VBNC) state (Lleo et al., 2005). As such, cells may not be able to grow on the highly selective media (i.e. mE agar or mEI agar) in the MF method. An inverse correlation was observed between salinity and turbidity. This could be due to the dilution and flushing effect from rainfall where the rainfall runoff dilutes the water salinity level and flushes sediment from the bottom.

3.4.2.1 Comparison between MF and EnterolertTM detection method

A total of 40 samples were tested using both methods and were compared statistically. The paired t-test was selected to test the mean difference between these two methods. From the statistical analysis, there was no statistically significant difference between the means of the two methods ($t < t_{crit}$; $P = 0.511$). However, the data was widely scattered around the linearly regressed trend line ($R^2 = 0.60$) (Figure 3.4).

Figure 3.4 Correlation between EnterolertTM and MF

The correlation between MF and EnterolertTM found in this study is closed to Kinzelman et al. (2003) and Budnick et al. (1996) studies where they found weak correlations ($R^2 = 0.62$ and 0.57 , respectively) between the MF and EnterolertTM in freshwater samples. However, both methods were well correlated in another study on marine water samples which was reported to have an R^2 of 0.98 (Budnick et al., 1996). A comparison between EnterolertTM and MF methods in this study and other studies are summarized in Table 3.5.

Table 3.5 Evaluation of Enterolert™ and MF methods

Paper	No. of samples			R ² *		
	Freshwater	Marine water	Total	Freshwater	Marine water	Total
(Budnick et al., 1996)	43	95	138	0.57	0.98	0.94
(Abbott et al., 1998)	N.D	N.D	343**	N.D	N.D	0.86**
(Kinzelman et al., 2003)	124	-	124	0.62	-	0.62
(Valente et al., 2010)	-	46	46	-	0.67	0.67
This study	40	-	40	0.60	-	0.60

Notes:

N.D – not determined

* - indicates the correlation coefficient which quantifies the degree of linear association between two variables

** - samples collected from various sites including recreational bathing water, tidal lagoons, water from marinas, untreated sewage effluent outfalls, and marine water samples from stormwater-drainage sites

For species identification purposes, MF is preferred as MF facilitates a good basis for isolation of colonies which can represent a single cell species.

3.4.3 Biochemical identification of enterococcus species

Screening of enterococcus species in the waters within Marina Reservoir's catchments was carried out in five main sub-catchments, namely Geylang River, Kallang River, Rochor Canal, Stamford Canal and Singapore River. The samples were collected on September 2008, May 2009 and June 2009 respectively. Figure 3.5 shows the biochemical tests in cupules. The reactions in each cupule were scored according to changes in colour, following manufacturer's instruction.

Figure 3.5 Biochemical test by API 20 Strep (bioMérieux, France)

Table 3.6 shows the percentage of distribution for the two main enterococcus species (i.e. *E.faecalis* and *E.faecium*), other enterococcus species (i.e. *E.durans*, *E.hirae*, *E. E.avium* and *E.gallinarum*) and non-enterococcus species (i.e. *Streptococcus bovis*). From the biochemical identification of enterococcus species, there is no obvious trend for the species distribution in the catchments for the different sampling dates. However, in most of the samples, *E.faecalis* and *E.faecium* were shown to be dominant species with a proportion of 60% or higher. The average percentage of *E.faecalis* was found to be consistently higher than *E.faecium* in most of the catchments except for Kallang River where both *E.faecalis* and *E.faecium* were of equal proportion (36.7%) on average.

The biochemical results obtained were quite surprising as the majority of the isolated colonies from Rochor Canal collected in September 2008 and May 2009 were non-enterococcus species. 70% of the isolates from Rochor Canal collected in September 2008 were identified as non-enterococcus species, while in May 2009 about 60% of the isolates were found to be non-enterococcus species. As for water samples collected from Singapore River in the same period, approximately 40% of the isolates were identified as non-enterococcus species. Among the non-enterococcus species, *Streptococcus bovis* II 4 and *S.bovis* I were dominant in the isolated colonies, at 50% and 30% respectively. This is similar to Ferguson et al.'s

study where it was found that non-enterococcus species such as *S.bovis* and other *Streptococcus* spp. could appear identically to enterococci on mEI agar (Ferguson et al., 2005).

Table 3.6 Percentage distribution of enterococcus species in different water catchments

Location	Sampling date	<i>Percentage distribution</i>			
		<i>E.faecalis</i>	<i>E.faecium</i>	Other enterococcus	Non-enterococcus
Geylang	Sept'08	50	30	10	10
	May'09	30	40	30	0
	June'09	40	40	0	20
	Average	40.0	36.7	13.3	10.0
Kallang	Sept'08	30	40	20	10
	May'09	30	30	20	20
	June'09	50	40	10	0
	Average	36.7	36.7	16.7	10.0
Rochor	Sept'08	20	10	0	70
	May'09	20	20	0	60
	June'09	40	40	0	20
	Average	26.7	23.3	0	50.0
Stamford	Sept'08	40	40	10	10
	May'09	40	20	20	20
	June'09	70	20	10	0
	Average	50.0	26.7	13.3	10.0
Singapore	Sept'08	60	20	20	0
	May'09	20	20	20	40
	June'09	30	30	10	30
	Average	36.7	23.3	16.7	23.3

The microorganisms from *Streptococcus bovis* group isolated from Rochor Canal can cause bacteremia and infective endocarditis. From the serological classification, both enterococcus species and *S.bovis* group are from the Lancefield group D. Hence, microorganisms from the *S.bovis* group have often been misidentified as enterococci due to the sharing of a similar group D antigen. Nevertheless, enterococcus species can be differentiated from *S.bovis* group by their

heat resistivity at 60°C for 30 minutes, pyrrolidonyl arylamidase (PYR)-positive and 6.5% NaCl-positive characteristics. On the other hand, *S.bovis* microorganisms group give negative reactions in PYR and 6.5% NaCl tests (HPA, 2007).

In short, the dominant species isolated from EIA medium from the Marina Reservoir catchment was identified as *E.faecalis* (38%) (Figure 3.6). This was followed by *E.faecium* which scored 29% in the total species isolated. Other enterococcus species (12%) such as *E.duran* and *E.avium* were minority species in the water catchment. About 21% of the total species isolates were identified as non-enterococcus species, including *Streptococcus bovis* II 4 and *S.bovis* I.

Figure 3.6 Pie chart showing the overall species identification

The results using API 20 test kit show that a number of isolates (21%) which were previously identified as enterococcus on mE agar and EIA were in fact identified as species other than enterococcus. This is consistent with a study conducted in southern California where it was found that a percentage of about 11.4% to 25.5% of the isolated colonies on mEI agar were non-enterococcus species although they were presumptively identified as enterococcus (Ferguson et al., 2005). The false-positive rate obtained from the study conducted by Ferguson et al. and

this study are much higher than the false-positives reported by the USEPA (USEPA, 2002). Based on the USEPA report, approximately 10% and 6% of the false-positive detection are to be expected for mE agar and mEI agar respectively.

3.5 Conclusions

From the field study, significant correlations were found between MF and salinity ($R = -0.416$), and MF and turbidity ($R = 0.526$), whereas, EnterolertTM only correlated with turbidity ($R = 0.633$). Enumeration of enterococci cell concentration with both MF and EnterolertTM method revealed that most of the water samples collected from Marina Reservoir catchments exceeded the USEPA guideline value for non-contact recreational water.

Comparison between MF and EnterolertTM method showed relatively low correlation ($R^2 = 0.6$) in this study as compared with Budnick et al. (1996) and Abbott et al. (1998) where they obtained higher correlation in marine water samples (i.e. $R^2 = 0.98$ and $R^2 = 0.86$ respectively). Currently, EnterolertTM is an approved method for enterococcus detection in marine water analysis. EnterolertTM offers great advantages such as ease of application and shorter incubation period. However, further study should examine the reliability of EnterolertTM for freshwater analysis.

Although the MF method has been used for decades to enumerate enterococci in either freshwaters or marine waters, the isolated colonies on selective media (mE agar and EIA) have been shown to demonstrate false-positive results. In this study, 21% of the isolated colonies on selective media were reported as false-positive detection after examination with biochemical tests. *Streptococcus bovis* was the main species that contributed to the false-positive results in the MF method. Thus, additional tests such as PYR, heat resistant, 6.5% NaCl, catalase, bile aesculin hydrolysis are required for enterococcus genus identification.

Enterococcus species identification from biochemical tests showed that the major enterococcal species found in Marina Reservoir's catchments is *E. faecalis*.

This species is also known to cause bacteremia, urinary tract infections and endocarditis and is thus considered as an opportunist pathogen (Murray, 1990). However, *E.faecalis* has been demonstrated to enter the VBNC state in stressed environmental conditions (Lleo et al., 1998; Lleo et al., 2005). The bacteria in this physiological state can therefore escape from culture-based detection. Hence, this study will focus on the method development for the detection and understanding of VBNC state for this species (discussed in Chapter 4).

CHAPTER 4 DEVELOPMENT OF MOLECULAR TECHNIQUES FOR DETECTION OF VIABLE *ENTEROCOCCUS FAECALIS*

4.1 Introduction

In our earlier studies, the water samples collected from local reservoir catchments revealed that the dominant species among enterococcus is *E.faecalis* (Chapter 3). This part of study aims to develop a sensitive and rapid method for the detection of all viable *E.faecalis* including the VBNC *E.faecalis* cells. Culture-based method is the most common method to enumerate the concentration of bacteria indicators. However, conventional culture-based method always relies on long culturing incubation time (i.e. 18-96 hours). In addition, it is difficult to detect and enumerate *E.faecalis* at species level by using culture-based method. Thus, molecular-based methods such as PCR or qPCR become suitable alternatives which offer high sensitivity and specificity detection, at the same time can give early warning of potential disease outbreaks. Studies also have shown that culture-based method could possibly miss the detection of injured cells or VBNC cells (Colwell, 2008; Oliver, 2010). Molecular-based methods which are independent of culturing techniques may overcome this problem.

Although molecular-based methods offer great advantages, however, there are three main limiting factors: (i) low sample volume is used in molecular-based method, thus requiring pre-concentration of larger volume of water prior to sample analysis, particularly for those samples with relatively low densities of bacteria, (ii) the presence of interfering substances in environmental waters which can inhibit molecular-based methods and (iii) the persistence of free DNA (DNA released from dead cells) which will subsequently give false positive signals in molecular-based methods. This study aims to develop a rapid and sensitive molecular method which can overcome these three major limitations for the detection of all viable *E.faecalis*, including the VBNC *E.faecalis*.

To overcome the problem of low number of target microorganisms in water samples, several concentration methods (i.e. centrifugation and membrane filtration) will be evaluated and optimized for concentrating *E.faecalis* in environmental water samples. The impact of interfering substances (also known as inhibitors) to molecular-based methods can be removed through the purification steps in DNA extraction, addition of bovine serum albumin (BSA) or dilution method. This study will examine and evaluate the purity of extracted DNA and the relative recovery efficiency of extraction with different DNA extraction methods.

Another major drawback of DNA-based molecular methods is the inability to differentiate between viable and dead cells. Studies have shown that free DNA (DNA released from dead cells) persists in the water over a long period (i.e. days) (Young et al., 2007; Walters et al., 2009). Under such circumstances, higher false-positive results have been found in the molecular detection methods that are targeting the DNA. DNA-intercalating dyes such as ethidium monoazide (EMA) or propidium monoazide (PMA) have been applied to eliminate free DNA prior to the downstream molecular analysis. The fundamental concept of EMA and PMA pretreatment is based on the covalent binding of EMA or PMA with the DNA. EMA or PMA will penetrate into dead cells with compromised membranes. In the membrane-compromised cell, EMA or PMA will intercalate with DNA to form a stable covalent bond upon strong photolysis. This permanent DNA modification will not be amplified in the downstream PCR analysis. Previous studies have reported convincing results with respect to the reduction in free DNA in various sample matrices (Nogva et al., 2003; Rudi et al., 2005; Nocker and Camper, 2006; Bae and Wuertz, 2009a; Bae and Wuertz, 2009b). However, it is still a challenging task to develop an effective treatment to reduce free DNA in natural environmental water matrices as there are a number of environmental factors which complicates the issue. For example, turbidity is one of the major parameters that affects the effectiveness of EMA or PMA treatment (Gedalanga and Olson, 2009).

The VBNC state and dormant state are some of the terminologies used to describe viable cells which have lost their cultivating ability. Studies have reported

that cells entering this physiological state still maintain their virulent effects (Oliver and Bockian, 1995; Du et al., 2007). The conventional detection method is based on cultivation of bacteria on selective agar (i.e. mE agar and EIA or mEI agar). However, enterococci have been reported to enter the VBNC state when facing unfavourable conditions (Lleo et al., 1998; Lleo et al., 2001; Signoretto et al., 2004; Lleo et al., 2005). In this physiological state, the cells lose their culturability but maintain their metabolic activities. Thus, these cells may escape detection using the culture based method. Meanwhile, the non-EMA or PMA based molecular methods (i.e. qPCR) are constrained because they also detect free DNA. As a result, there is a need to develop a method in between qPCR and culture-based methods, such as EMA or PMA pretreatment with qPCR which can potentially be used for the detection of viable cells including the VBNC cells.

This study will evaluate the application of EMA-qPCR and PMA-qPCR in the quantification of viable *E.faecalis* in tropical water samples. Protocols for EMA-qPCR and PMA-qPCR were optimized and validated for the detection of the target bacteria, *E.faecalis*, in environmental waters.

4.2 Materials and Methods

4.2.1 Bacterial strains and cultivation

The bacteria strains used in this study are shown in Table 4.1. All enterococci bacteria were grown overnight in Brain Heart Infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA) at 37°C under constant shaking (120rpm). Other non-enterococci strains were cultivated overnight in Tryptic Soy broth (Becton Dickinson, Sparks, MD, USA) under similar incubation conditions (i.e. 37°C; 120 rpm constant shaking). All bacteria strains in Table 4.1 were tested for the specificity of primers and probe design. Both *E. faecalis* ATCC 29212 and *E. faecalis* 56R were used for EMA and PMA optimization. Meanwhile, *E.faecalis* 56R was used for the laboratory microcosm study. For long term storage, the

bacteria strains were kept at -80°C in the presence of glycerol as the cryoprotective agent.

Table 4.1 List of bacteria strains

Bacteria strain	Strain	Source
<i>Enterococcus faecalis</i>	ATCC 29212	American Type Culture Collection
<i>Enterococcus faecalis</i>	56R	Clinical isolate (Lleo et al., 1998)
<i>Enterococcus faecalis</i>	JH2-2	Clinical isolate (Signoretto et al., 2005)
<i>Enterococcus faecalis</i>	JH2-2R	Clinical isolate (Signoretto et al., 2005)
<i>Enterococcus faecalis</i>	MR-9a	Environmental isolate (Local reservoir catchment)
<i>Enterococcus faecium</i>	VR1	Clinical isolate (Ligozzi et al., 1996)
<i>Enterococcus faecium</i>	MR-9f	Environmental isolate (Local reservoir catchment)
<i>Enterococcus faecium</i>	MR-11a	Environmental isolate (Local reservoir catchment)
<i>Enterococcus duran</i>	MR-11i	Environmental isolate (Local reservoir catchment)
<i>Streptococcus bovis</i> I	MR-9b	Environmental isolate (Local reservoir catchment)
<i>Aerococcus viridans</i> 2	MR-11d	Environmental isolate (Local reservoir catchment)
<i>Encherichia coli</i>	K-12	American Type Culture Collection

4.2.2 Pre-concentration methods for environmental water samples

The pre-concentration of bacteria is important in improving its detection sensitivity. The main objective of pre-concentration is to reduce the sample volume size, and at the same time recover all the bacterial populations of interest. Thus, pre-concentration is often required to capture relative low densities of bacteria in natural environmental waters. Three different pre-concentration methods were examined for their recovery efficiency: (1) centrifugation at 11,000 x g for 15 minutes; (2) filtration through 0.45 µm pore size nylon membrane; and (3) filtration through

0.45 µm pore size nitrocellulose membrane followed by centrifugation at 11,000 x g for 15 minutes.

To test the recovery efficiency for each pre-concentration method, two experiments (in duplicate) were set up to measure the recovery concentration and control concentration respectively. The cultures were spiked into phosphate buffer saline (PBS) and environmental water matrices to measure the recovery rate. In measuring the recovery concentration, 1ml of overnight pure culture was spiked separately into 1L of PBS or an environmental water matrix. The seeded water samples were then concentrated according to the different concentration methods and resuspended with PBS to a final volume of 10ml. For the control concentration, 1ml of overnight pure culture was spiked directly into a 9ml of PBS without going through any concentration method. Enumeration of bacteria for both recovery concentration and control concentration were done by the membrane filter technique (APHA, 1998). The percentage of recovery efficiency was calculated by dividing the recovery concentration by the control concentration. Each measurement was performed in duplicate.

4.2.3 Dead cells for control experiments

The overnight grown culture was killed using two different treatments: (a) heat treatment at 100°C for 10 min and (b) ethanol treatment (final concentration 70%) for 10 min. The heat-killed cells were pelleted by centrifugation at 13,000 g for 10 min. The residual ethanol was removed by washing with ultrapure water followed by pelleting the bacterial cells through centrifugation at 10,000 g for 5 min. The cells were resuspended in ultrapure water before the optimum EMA or PMA concentration was added. The viability of cells were examined by spreading 100 µl of sample on to Tryptic soy agar (TSA) (Becton Dickinson, Sparks, MD, USA), followed by overnight incubation at 37°C.

4.2.4 Laboratory microcosms

Two laboratory microcosms were used for examining the decay kinetics of the stressed cells where the cells were stressed with starvation and nutrient depletion. The overnight grown culture was spiked into the two separate laboratory microcosms which contained (a) water from a local reservoir catchment and (b) ultrapure water which served as the control experiment. Both water matrices were filtered twice with 0.2- μm pore size membrane and autoclaved twice in order to eliminate the interference of indigenous bacteria in the study. The laboratory microcosms were maintained at room temperature under direct illumination of 36Wm^{-2} light intensity. All microcosms were set in duplicate.

4.2.5 Optimization of the ethidium monoazide treatment

EMA was purchased from Molecular Probe (Invitrogen, Carlsbad, CA). EMA powder was dissolved in ultrapure water to a stock concentration of 1 mg/ml. The stock solution was aliquoted and kept at -20°C in the dark. Optimization of the EMA treatment process is illustrated in the flow chart in Figure 4.1. Briefly, different EMA concentrations were examined by adding EMA into 500 μl of bacterial suspension (live or killed cells) to final concentrations of 7.5, 10, 50 and 100 $\mu\text{g/ml}$. The EMA treated cells were incubated in the dark, at room temperature for 5 min. Following dark incubation, the cells were exposed to a 500-W halogen light source (Philips) for 1, 3, 5 and 10 min at a distance of 20 cm between the light source and the samples. The cell suspensions were kept on ice when exposed to halogen light to minimize the elevation of temperature in the samples. All samples were prepared in duplicate for each optimization.

4.2.6 Optimization of the propidium monoazide treatment

PMA (Biotium, Hayward, CA) was dissolved with 20% of DMSO to a stock solution of 20 mM and aliquoted to a light-impermeable microcentrifuge tube for

storage at -20°C. Optimization experiments were carried out for different PMA concentrations (2, 10, 20, 80 and 100 µM) as well as different light exposure times (Figure 4.1). The protocol for PMA treatment is the same as for the EMA treatment. The optimized PMA treatment was tested on artificial turbid water by adding kaolin (Sigma-Aldrich Corporation, MO) in ultrapure water to a turbidity level of 20 NTU, 50 NTU and 100 NTU.

Figure 4.1 Flow chart illustrates the optimization process for EMA and PMA pretreatment

4.2.7 DNA extraction

Three different DNA extraction methods were tested: (1) the commercial DNA extraction kit, Wizard[®] Genomic DNA Purification Kit (Promega, Madison, WI, USA); (2) the conventional DNA extraction method using phenol-chloroform-isoamyl-alcohol (He and Jiang, 2005); and (3) the commercial DNA extraction kit, QIAamp[®] DNA Mini Kit (Qiagen, Hilden, Germany).

DNA extraction using the commercial kit, Wizard[®] Genomic DNA Purification Kit was performed in accordance to manufacturer's instructions. 1ml of overnight culture or concentrated sample was centrifuged at 14,000 rpm for two minutes to pellet the cells. The cell pellet was resuspended with 480µl of 50mM EDTA and 120µl of 10mg/ml lysozyme to weaken the cell walls and subsequently improve the efficiency of cell lysis. The sample was then incubated at 37°C for 60 minutes. After incubation, the sample was centrifuged at 14,000 rpm for 2 minutes and the pellet was resuspended with 600µl Nucleic Lysis Solution. The cells were further incubated at 80°C for 5 minutes to lyse the cells. Then, 3µl of RNase Solution was added to the cell lysate before an hour of incubation at 37°C. After that, 200µl of Protein Precipitation Solution was added to the RNase-treated cell lysate. Subsequently, the mixture of the solution and cell lysate was vortexed vigorously. The sample was then incubated on ice for 5 minutes. The protein was precipitated as a pellet after centrifugation at 14,000 rpm for 3 minutes. DNA remained in the supernatant was transferred into a new microcentrifuge tube containing 600µl of isopropanol. The sample was then centrifuged at 14,000 rpm for another 2 minutes and the DNA pellet was washed with 600µl of 70% ethanol. The sample was then centrifuged again. The ethanol was carefully aspirated and the pellet was allowed to air-dry. 100µl of DNA Rehydration Solution was added to the DNA pellet. The DNA was rehydrated by incubating at 65°C for 1 hour.

In conventional DNA extraction methodology, 1ml to 10ml of pure culture or environmental water was centrifuged at 6654 x g for 5 minutes. The pellet was

resuspended in 100µl of lysis buffer which consists of 10mM Tris-HCl (pH 8.3) 100mM NaCl, 1mM EDTA and 1% (vol/vol) Tween 20. Besides lysis buffer, 10µl of 10mg/ml of proteinase K was added. The sample was incubated at 45°C for 3 hours. After 3 hours incubation, the sample was centrifuged and the cell lysates were extracted with 100µl of phenol-chloroform-isoamyl-alcohol (24:25:1, pH 8). The mixture was centrifuged at top speed for 2 minutes to separate it into phases. After that, the supernatant was transferred to a new microcentrifuge tube, followed by the precipitation of DNA with isopropanol. The precipitated DNA was then washed with 70% ethanol. Finally, the DNA pellet was dissolved in 30µl of TE buffer.

Another commercial kit, QIAamp[®] DNA Mini Kit was evaluated for its performance. In brief, the bacterial suspension was centrifuged at 7500 rpm for 10 minutes to pellet the bacterial cells. The bacterial pellet was resuspended in 180µl of prepared enzyme solution. The enzyme solution consisted of 20 mg/ml of lysozyme, 20 mM of Tris-HCl, 2 mM of EDTA and 1.2% of Triton. The sample was then incubated at 37°C for at least 30 minutes. After incubation, 20 µl of proteinase K and 200 µl of Buffer AL were added into the sample followed by vortex mixing. Next, the sample was incubated at 56°C for 30 minutes and followed by a further incubation of 15 minutes at 95°C. 4 µl of 100 mg/ml RNase A and 200 µl of Buffer AL were added into the sample before being incubated at 70°C for 10 minutes. After the incubation, 200 µl of 99% ethanol was added and mixed. The mixture was transferred into a QIAamp Mini spin column and centrifuged at 8000 rpm to spin down the solution. The filtrate solution was discarded. The retentate was subsequently washed with 500 µl of Buffer AW1 and Buffer AW2 each. The purified DNA was finally eluted by the addition of 100 µl of Buffer AE.

The purity and concentration of the isolated DNA was examined by a NanoDrop[®] ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The concentration of bacterial genomic DNA was converted to a genome equivalent (GE) according to Equation 4.1.

$$\frac{\text{Genome copy number}}{\mu\text{l}} = \frac{\text{Avogadro's number} \times \text{DNA concentration}}{\text{genome molecular weight}} \quad (4.1)$$

where Avogadro's number = 6.022×10^{23} / mol

DNA concentration = A_{260} reading x dilution factor x 50 $\mu\text{g/ml}$

Genome molecular weight = Genome size (bp) x 660 g/mol.bp

4.2.8 Conventional PCR

Extracted DNA from pure culture and an environmental isolated strain were used to test the PCR amplification for the primers listed in Table 4.2. Detection of *E.faecalis* was carried out by the primers targeting the genes that encode the penicillin-binding proteins (*pbp5*), manganese-dependent superoxide dismutase (*sodA*), D-alanine:D-alanine ligase (*ddl*), collagen-binding protein (*ace*) and endocarditis antigen (*efaA*). Table 4.2 lists the nucleotide sequences for each primer set.

Table 4.2 Summary of the primers used in this study to detect *E.faecalis*

Target gene	Sequence 5' – 3'	Product size (bp)	Reference
<i>pbp5</i>	CATGCGCAATTAATCGG CATAGCCTGTCGCAAAAC	444	(Lleo et al., 2005)
<i>sodA</i>	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	(Jackson et al., 2004)
<i>ddl</i>	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	941	(Dutka-Malen et al., 1995)
<i>ace</i>	GGAATGACCGAGAACGATGGC GCTTGATGTTGGCCTGCTTCCG	616	(Creti et al., 2004)
<i>efaA</i>	GCCAATTGGGACAGACCCTC CGCCTTCTGTTTCTTCTTTGGC	688	(Creti et al., 2004)

Optimization was carried out for the concentration of primers and reagents, as well as the conditions for PCR reaction. PCR amplification was performed in an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). The optimized reagent mixes and the reaction profile for each primer set are described in Tables 4.3 and 4.4.

Table 4.3 PCR reagent mixes

Reagents \ Primer	<i>pbp5</i>	<i>sodA</i>	<i>ddl</i>	<i>ace</i>	<i>efaA</i>
PCR buffer	1x	1x	1x	1x	1x
MgCl ₂ (mM)	3	1.5	7	4	2
dNTP mix (mM)	0.2	0.2	0.5	0.2	0.2
Forward primer (μM)	1	1	0.5	1	0.4
Reverse primer (μM)	1	1	0.5	1	0.4
Taq DNA Polymerase (U)	2	2	2	1.5	0.25
DNA template (μl)	5	5	5	5	5

Table 4.4 PCR reaction profile

Conditions \ Primer	<i>pbp5</i>	<i>soda</i>	<i>ddl</i>	<i>ace</i>	<i>efaA</i>
Pre-incubation	95°C, 10min	95°C, 10min	94°C, 10min	95°C, 10min	95°C, 10min
Amplification					
- Denaturing	95°C, 1.5min	95°C, 30s	94°C, 1min	95°C, 1min	95°C, 1min
- Annealing	54°C, 1.5min	55°C, 1min	54°C, 1min	58°C, 1min	58°C, 1min
- Extension	72°C, 2min	72°C, 1min	72°C, 1min	72°C, 1min	72°C, 1min
- Number of cycle	30	30	30	30	30
Final extension	72°C, 5min	72°C, 7min	72°C, 10min	72°C, 10min	72°C, 10min

The PCR products were resolved by electrophoresis on a 1.5% agarose-Tris-acetate-EDTA agarose gel containing 0.8µg of ethidium bromide/ml. The DNA molecular weight marker (100-bp, Promega, Madison, WI, USA) was used as the standard. An external power supply was set at 75 Volts for an hour to allow amplicons to migrate towards the positive electrode. The PCR products were visualized with a UV transilluminator and photographed using a Kodak DC290 camera.

4.2.9 Quantitative PCR

Quantitative PCR (qPCR) can be performed with two different detection options: (i) with non-specific DNA binding dyes; and (ii) sequence specific probes. Both have their own advantages and disadvantages as discussed in Chapter 2. These detection options were tested in this study and examined for their performances.

4.2.9.1 Quantitative PCR (non-specific DNA binding dye detection)

For non-specific DNA binding, SYBR Green I was selected for the qPCR analyses. The reaction was carried out in 20-µl reaction mixtures consisting of 1x reaction master mix (LightCycler® FastStart DNA Master^{PLUS} SYBR Green I, Roche Diagnostics), 0.5µM of forward and reverse primers and 5µl of template DNA. The primer sequences for *pbp5* gene are listed in Table 4.2. The samples were amplified, detected and quantified by LightCycler (Roche Diagnostics). The qPCR amplification conditions were optimized following the manufacturer's recommendations. The thermal cycling profile for *E.faecalis* targeting the *pbp5* gene was carried out at 95°C for 15min, followed by 45 cycles of 10s at 95°C, 5s at 55°C and 15s at 72°C. Determinations for cycle threshold (Ct) values were performed automatically by the LightCycler® Software 4.05.

Melting curve analysis is based on the temperature as the DNA strand separates or melts when heated. Basically, the melting temperature of DNA depends

on the sequence, length of strand, as well as the GC content of the strand. Thus, melting temperature profiles allow identification of the amplification products. In this study, the melting program was set at 65°C for 60s followed by the melting process at 95°C with a ramp rate of 0.1°C/s.

4.2.9.2 Quantitative PCR (Probe-based detection)

Dual-labeled fluorescent probes were selected for qPCR probe-based detection. The probe was labeled with FAM (6-carboxyfluorescein) at the 5' end as the reporter and was quenched at 3' end with TAMRA (6-carboxytetramethylrhodamine) or BHQ-1 (BlackHoleQuencher-1). Two different target gene regions were tested and presented in this study (Table 4.5). The existing primer and probe set from Santo Domingo et al. (2003) was used to detect and quantify the number of 16S rRNA gene in *E.faecalis*. To perform amplification of the 16S rRNA gene, the qPCR reaction mixture (20 µl) consisted of 10 µl of QuantiTect Probe master mix (Qiagen, Hilden, Germany), 1.8 µl each of 10 µM forward and reverse primers, 0.5 µl of 10 µM probe, 0.9 µl DNase free water and 5 µl DNA template were added into a glass capillary (Roche Diagnostics). The thermal conditions for 16S rRNA gene amplification were: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

A new primers and probe set which targeted *pbp5* gene in *E.faecalis* was designed in this study. The design of these primers and probe will be further discussed in section 4.2.9.2.1. The probe-based qPCR reactions were prepared with QuantiTect Probe PCR kit (Qiagen, Hilden, Germany). Each qPCR reaction for *pbp5* gene amplification contained 1x QuantiTect Probe PCR master mix (Qiagen, Hilden, Germany), 0.5µM forward primer, 0.5µM reverse primers, 0.1µM of fluorescent probe, and 5µl of DNA template in a 20-µl reaction volume. Amplifications were carried out in a LightCycler (Roche Diagnostics) with 15 min incubation at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Determinations for cycle threshold (Ct) values were performed automatically by the

LightCycler[®] Software 4.05. Each sample was analyzed in duplicate. A negative control (DNase free water) was incorporated in every set of qPCR experiments.

4.2.9.2.1 Design and synthesis of primers and probe

Primers targeting *pbp5* gene were modified from Lleo et.al. (2005) by using the Primer3 software (Steve Rosen and Helen Skaletsky, 2000; <http://primer3.sourceforge.net>). In addition, a new fluorescent probe was designed using the LightCycler Probe Design Software 2.0 (Roche Diagnostics). The primer and probe sets were designed based on published nucleic acid sequences in GenBank (GenBank: X78425.1). After the design analysis, the selected primers and probe sequences were verified by doing a comparison with other genome sequences using a BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>). The primer set was validated with SYBR Green I to test its specificity and its amplification efficiency. Optimization of primer concentrations was carried out to achieve one amplicon under high efficiency amplification and at the same time provide reproducible results. The probe was then validated and further optimized by using the TaqMan[®] assay. Figure 4.2 illustrates the work flow for the primers and probe design.

Figure 4.2 Work flow for primers and probe design

The sensitivity test and specificity test were carried out for the newly designed primer and probe set. The sensitivity of primer and probe sets were tested with triplicate dilutions of culture, whereas specificity was tested using the pure cultures from ATCC, the clinical and locally isolated bacteria strains (Table 4.1).

Table 4.5 Primers and fluorescence probes for the specific detection of *E.faecalis*

Target gene	Description	Sequence (5'-3')	Product length (bp)	Reference
16S rRNA	Forward primer	CGCTTCTTTCTCCCGAGT	143	(Santo Domingo et al., 2003)
	Reverse primer	GCCATGCGGCATAAACTG		
	Probe	6-FAM – CAATTGGAAAGAGGAGTGG CGGACG - TAMRA		
pbp5	Forward primer	AGGGACATTGAAGCCAGAT	125	This study
	Reverse primer	G AAGCGGTACGCAGATTGAC		
	Probe	T 6-FAM - TGTCGCAAATAGCCGCCC CAAGATT – BHQ-1		

4.2.9.3 Nucleic acid quantification analysis

A whole bacterial genomic DNA of *E.faecalis* was extracted to generate a standard curve for *E.faecalis*. The isolated DNA concentration was measured by utilizing a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The concentration of bacterial genomic DNA was converted to a genome equivalent (Eq.4.1) and a serial dilution was performed. The standard curve was derived from the serial dilutions of genomic DNA and plotted against the CT (Threshold cycle) values. Quantification can be performed by comparing the CT value from the blind sample to the CT value from the standard curve.

For quantification purposes and for easy comparison with colony forming unit (CFU), the gene copy number was converted to genome equivalent (GE) where one (1) GE equals a single cell. Since there are two different target regions (16S rRNA and *pbp5*) to be tested in this study, the conversion to GE are different for both target regions. Klappenbach et al. showed that *E.faecalis* species has an average of 4 copies of 16S rRNA gene in a chromosome ((Klappenbach et al.,

2001); <http://ribosome.mmg.msu.edu/rndb/index.php>,). Hence, we assumed 4 copies of 16S rRNA gene per chromosome for quantification purposes and reported it as a genome equivalent (GE). In the case of *pbp5*, we assumed that there is only a single *pbp5* gene in an *E.faecalis* chromosome.

4.2.10 Statistical Analyses

Statistical analyses were carried out using MS Excel software. The paired t-test was selected to test the mean difference between the readings for culture spiked into two different water matrices. Linear regression was used to examine the dispersion about the trend line for the two different water matrices. The significant level was set at the 95% confidence level.

4.3 Results

4.3.1 Comparison of recovery efficiencies for different pre-concentration methods

Three different pre-concentration methods were tested in PBS seeded samples and environmental water seeded samples. Among the three concentration methods, PBS seeded water samples concentrated by the combination of filtration and centrifugation achieved the highest recovery efficiency (76.3%) compared to filtration (49.5%) and centrifugation (28.1%) (Figure 4.3).

For environmental water matrices (i.e. Stamford Canal and Kallang River), membrane concentration methods were shown to have higher tendency of clogging the membranes, thus making the processing of large samples unfeasible. The recovery rate obtained from filtration method was 18.8% and 12.7% for Stamford Canal and Kallang River respectively. A great reduction in recovery rate was found between the PBS and environmental water matrices. This may be attributed to insufficient elution of bacteria from the filter membrane in environmental water

matrices where most of the bacterial attached suspended solids were clogged within the pores in the membrane.

Figure 4.3 Recovery efficiency of *E. faecalis* spiked in three different water matrices with three pre-concentration methods

The combination of filtration and centrifugation pre-concentration method was found to improve the recovery rate and achieved the highest recovery rate in PBS spiked samples. However, the combination of two pre-concentration methods gave the least recovery in spiked samples from the environmental water matrices. When the combination of filtration and centrifugation preconcentration methods was tested, low recovery rate of 11.5% and 10% were obtained for Stamford Canal spiked samples and Kallang River spiked samples, respectively.

Even though the centrifugation method gave the smallest recovery efficiency for the seeded experiment in PBS, this method was found to be most suitable for concentrating environmental waters. A higher recovery rate with an average of 64.4% was achieved for the centrifugation method for seeded environmental water samples. Results have shown that the centrifugation method highly depends on the type of water analyzed. Water samples with more settleable

silt gave better recovery efficiency than water samples with more suspended/floating dirt. For instance, water samples collected from Stamford Canal consist of a high percentage of suspended particles, thus giving a recovery efficiency of 52.7%. A high percentage of settleable silt was observed in water samples collected from Kallang River where the recovery efficiency was increased to 76.2%.

Mull and Hill (2009) reported that the centrifugation method gave higher recovery rates than membrane filtration when tested on *E.coli* O157:H7 in surface waters (Mull and Hill, 2009). In addition, the authors also showed that centrifugation can increase the sensitivity of the downstream IMS-qPCR detection although with bacterial background flora (Mull and Hill, 2009). Hence, the authors suggest centrifugation as a better alternative for pre-concentration compared to membrane filtration particularly for turbid environmental waters.

In conclusion, the pre-concentration method is highly dependent on the composition of environmental water matrix. Hence, the centrifugation method was chosen as the pre-concentration method to process environmental water samples for this study.

4.3.2 Evaluation of DNA extraction methods

The ability to efficiently extract DNA from environmental samples often constrains the utility of any molecular analyses. Therefore, DNA extraction is an extremely important procedure which releases DNA from the microorganism for downstream molecular analyses.

The DNA extraction methods were evaluated according to the purity of extracted DNA and the relative recovery efficiency of extraction.

4.3.2.1 Purity of DNA

The purity of DNA was analyzed by calculating the ratio of absorbance (OD) at wavelength 260 nm to 280 nm (A_{260}/A_{280}). The A_{260}/A_{280} ratio was measured by a NanoDrop® ND-1000 Spectrophotometer. A ratio ranging from 1.8 to 2 indicates a high purity of DNA being extracted. Among these DNA extraction methods, the commercial kits Wizard® Genomic DNA Purification Kit and QIAamp® DNA Mini Kit showed a higher purity of DNA product. The A_{260}/A_{280} ratio was within the range of 1.8 to 2 (Table 4.6). In contrast, the conventional DNA extraction method gave an A_{260}/A_{280} ratio of 1.54 to 1.56. This indicated that the isolated DNA could be contaminated with protein. Isolated DNA which was contaminated with proteins was shown to inhibit conventional PCR and quantitative real-time PCR (qPCR) reactions (Wilson, 1997).

Table 4.6 Comparison of three DNA extraction methods from PBS spiked with pure culture (*E.faecalis*)

	Extraction method	DNA purity ^a	Recovery efficiency (%)
Initial experiment	Wizard® Genomic DNA Purification Kit	1.89 ± 0.05	0.0004
	Conventional	1.54 ± 0.02	0.003
Second experiment	Wizard® Genomic DNA Purification Kit	2.01 ± 0.05	4.02
	Conventional	1.56 ± 0.06	12.19
	QIAamp® DNA Mini Kit	1.92 ± 0.03	12.08

Notes: ^a The values are averages ± standard errors based on two independent extractions

The isolated DNA is further amplified by PCR and qPCR to examine their purity and their possibility to inhibit PCR or qPCR reactions. For DNA template isolated from the Wizard® Genomic DNA Purification Kit, both PCR and qPCR showed no inhibition substances in the isolated DNA by giving clear amplification bands on the gel image (Figure 4.4A and Figure 4.4B). DNA isolated from the conventional DNA extraction method was shown to inhibit the PCR reaction

(Figure 4.5A, lane 1). Inhibitors can be reduced through dilution where further dilutions can give amplification bands on the gel image (Figure 4.5A, lane 2-8). Nevertheless, contamination inhibited the whole qPCR reaction as no band could be visualized in Figure 4.5B. There was no inhibition effect been observed with another commercial kit, QIAamp DNA Mini Kit (Figure 4.6). Details of PCR and qPCR will be discussed further in Sections 4.3.3 and 4.3.4.

Figure 4.4 Gel image from (A) PCR and (B) qPCR results using the DNA template isolated from the commercial kit, Wizard® Genomic DNA Purification kit. Lane M, 100-bp molecular marker; Lane 0, negative control; Lane 1, pure culture; Lane 2, 10-fold dilution of pure culture; Lane 3, 50-fold dilution of pure culture; Lane 4, 100-fold dilution of pure culture; Lane 5, 500-fold dilution of pure culture; Lane 6, 1000-fold dilution of pure culture

Figure 4.5 Gel image from (A) PCR and (B) qPCR using the DNA template isolated from conventional DNA extraction method. Lane M, 100-bp molecular marker; Lane 1, pure culture; Lane 2, 10-fold dilution of pure culture; Lane 3, 50-fold dilution of pure culture; Lane 4, 100-fold dilution of pure culture; Lane 5, 500-fold dilution of pure culture; Lane 6, 1000-fold dilution of pure culture; Lane 7, 5000-fold dilution of pure culture; Lane 8, 10,000-fold dilution of pure culture; Lane 9, negative control

Figure 4.6 Gel image from qPCR using the DNA template isolated from the commercial DNA kit, QIAamp® DNA Mini Kit. Lane M, 100-bp molecular marker; Lane 1, negative control; Lane 2 and 3, 10^6 -fold dilution of pure culture; Lane 4 and 5, 10^7 -fold dilution of pure culture; Lane 6 and 7, 10^8 -fold dilution of pure culture; Lane 8, 10^9 -fold dilution of pure culture; Lane 9 negative control.

4.3.2.2 Relative extraction efficiency

The relative efficiency of extraction was determined by comparing the number of gene copies recovered after DNA extraction with the known number of cells before DNA extraction. Equation 4.1 shows the formula to calculate the genome copy number.

The initial recovery efficiencies obtained for Wizard® Genomic DNA Purification Kit and conventional DNA extraction method were extremely low, i.e. 0.0004% and 0.003% respectively (Table 4.6). Some modifications were done for both methods to improve the recovery efficiency. For Wizard® Genomic DNA Purification Kit, the initial lysozyme concentration of 5mg/ml was increased to 10mg/ml. In addition, the lysozyme volume was increased from 35µl to 120µl. The first washing step in the conventional method was modified by replacing ethanol with isopropanol. After these modifications, the recovery efficiency improved significantly for both methods. Among both methods, the conventional method scored higher recovery efficiency (12.2%) than the Wizard® Genomic DNA Purification Kit (4.0%). QIAamp® DNA Mini kit achieved a high recovery efficiency of 12.1%. Although the recovery efficiency for QIAamp® DNA Mini kit is slightly lower than the conventional method, it is still preferred as this method can achieve higher DNA yield while maintaining a high purity of DNA.

4.3.2.3 Summary on recovery of DNA

In summary, the results of the study suggest that QIAamp® DNA Mini kit is the most sensitive DNA extraction method compared to the Wizard® Genomic DNA Purification Kit and conventional method. The purification step in QIAamp® DNA Mini Kit which involved the silicon membrane cartridge is believed to be the best way to remove most of the humic acids and other PCR inhibitors (Boddinghaus et al., 2001). Although both QIAamp® DNA Mini Kit and Wizard® Genomic DNA

Purification Kit gave high purity in isolated DNA, QIAamp[®] DNA Mini Kit has the advantage of a better recovery rate.

4.3.3 Detection sensitivity of conventional PCR

Conventional PCR analyses have been tested on five different primers set to detect five different target genes in *E.faecalis* (Table 4.2). The sensitivity of PCR reaction was examined for each primer set with 10-fold dilutions of the isolated *E.faecalis* DNA. Among these primer sets, the primer set targeting the *pbp5* gene was shown to give the highest sensitivity. Figure 4.7 shows that the lowest detection limit achieved by PCR reaction with *pbp5* gene is 987 gene copy/ μ l.

Figure 4.7 Gel image for PCR targeting the *pbp5* gene. Lane M, 100-bp molecular marker; Lane 1 to 9, 10-fold dilutions (from 9.78×10^7 gene copy/ μ l to 0.978 gene copy/ μ l) of DNA extracted from *E.feacalis*; Lane 10, negative control.

4.3.4 Detection sensitivity and specificity of quantitative PCR (SYBR Green I)

4.3.4.1 Standard curve

For quantification and to assess the sensitivity of the qPCR assay, a standard curve of fluorescence versus concentration was developed. The isolated DNA was serially diluted to a range from 0.866 genomic copies/ μl to 8.66×10^6 genomic copies/ μl and used to generate the standard curve. The CT value showed a linear correlation with the isolated DNA concentration over a seven log dynamic range (Figure 4.8). This indicated that the sensitivity of qPCR for *E.faecalis* assay was about 1 genomic copies/ μl at a CT value of 35.

Figure 4.8 Standard curve and sensitivity of SYBR Green I qPCR for detection of *E.faecalis*. The resulting CT values for each dilution of DNA template were plotted as a function of log initial *pbp5* gene copy number. All samples were measured in duplicate.

The efficiency of the standard curve was 1.871, as indicated by the slope of the curve together with the calculation in Equation 4.2. A perfect amplification reaction will give an efficiency reading of 2 for the standard curve. This means that the amount of target DNA will double with each amplification cycle.

$$E = 10^{(-1/\text{slope})} \quad (4.2)$$

4.3.4.2 Melting curve analysis

Melting peaks generated from the melting curve analysis allows identification of the PCR products. Figure 4.9 shows the melting peaks for SYBR Green I qPCR with primers set that targets the *pbp5* gene. The dilutions of *E.faecalis* DNA showed a sharp and single peak at around 82°C. However, the negative control gave another peak at around 83.3°C. This phenomenon could be attributed to the contamination in the negative control sample, possibly mispriming, or primer-dimer artifacts. These are the common problems in SYBR Green based qPCR as SYBR Green will bind to any double-stranded DNA. Often, the amplification of target DNA and non-target DNA can be differentiated through the different melting peaks generated.

Figure 4.9 Specificity of SYBR Green I qPCR determined from melting peaks for *pbp5* gene. Dilution of *E.faecalis* showed the consistence peaks at around 82°C. Negative control of non-template control gave a melting peak at 83.3°C.

4.3.5 Quantitative PCR (Dual-labeled probe)

4.3.5.1 Primers and probe design

Good primers and probe design are the prerequisites for a successful qPCR reaction. A good primer and a good probe design should have the following criteria:

(1) hybridize only the target gene without cross-hybridization; (2) minimal secondary structure which will affect the primer binding in the amplicons; and (3) minimal formation of primer dimers which can reduce the sensitivity of the qPCR reaction. In addition to the above, other attributes in the primers and probes will also contribute to the success of the qPCR reaction. These attributes in the design parameters include the primers and probes length, GC content, melting temperature (T_m), and the sequence of the primers and probes. These criteria are important in primer and probe design to achieve sensitive, specific and reproducible results.

In this study, the primer and probe set was designed based on the published gene sequence (GenBank: X78425.1). The main reason that the region of *pbp5* was selected for the quantification is the continuous expression of *pbp5* gene during the stress state in Enterococcal cells (Lleo et al., 2000). Table 4.5 shows the primers and probe sequences designed in this study.

4.3.5.2 Detection sensitivity and specificity of qPCR (dual-labeled probe)

The newly designed primer and probe set was tested for sensitivity with the qPCR assay and also tested for its specificity as well. A standard curve was plotted with serial dilution of the DNA template. The standard curve for *pbp5* gene by dual-labeled probe qPCR assay gave an amplification efficiency of 1.920 with a lowest detection limit of 0.9 gene copy/ μ l (Figure 4.10).

Figure 4.10 Standard curve of dual-labeled probe qPCR assay for detection of the *pbp5* region in *E.faecalis*. The resulting CT values for each dilution of the DNA template were plotted as a function of log initial *pbp5* gene copy number. All samples were measured in duplicate.

The specificity of primer and probe set was tested against different strains of *E.faecalis* as well as environmental isolates *E.faecalis*. The specificity test was also tested on non-*E.faecalis* species which have similar biochemical properties as *Enterococcus*, e.g. the colonies that gave false positive results on selective mE agar and esculin iron agar (Difco, Detroit, MI). Figure 4.11A shows the amplification curves generated by dual-labeled probe qPCR for different bacterial species. The corresponding CT values for each bacterial species are reported in Figure 4.11B. The lower CT value in qPCR indicates a higher amount of initial DNA template in the sample. In this specificity test, all bacterial DNA were adjusted to the same initial cell concentration (10^8 gene copy/ml). The CT value shows the high specificity of the newly designed primer and probe set which targets the *pbp5* gene of *E.faecalis* species (Note that the CT values for *E.faecalis* range from 18.62 to 20.23). In contrast, other non-*E.faecalis* pure cultures and environmental isolates, including *E.faecium* and *E.duran*, had CT values which were higher than 38.23 although the initial cell concentrations were the same for all bacteria species. Higher CT values have been shown to increase the error in standard deviation (Karlen et al., 2007). Thus, results with high CT values should be interpreted with care.

Figure 4.11 Specificity test for dual-labeled probe qPCR (target *pbp5* region): (A) Amplification curves generated from different species of bacteria with a constant initial cell concentration of 10^8 gene copy/ml, (B) list of the bacteria species tested in the specificity test.

4.3.6 EMA optimization

EMA optimization was carried out under different operating environmental conditions, i.e. light intensity for inactivation, distance between the light source and samples, duration of light irradiation and EMA concentration. A light intensity of 500W at a distance of 20 cm away from the samples gave the best results in terms of highest inhibition in dead cells and minimal inhibition in viable cells. This is also in agreement with other studies (Rueckert et al., 2005; Pisz et al., 2007).

The EMA optimization process was carried out with heat-killed cells, ethanol treated cells and live cells. Before the heat and ethanol treatment, all samples were diluted to a constant cell concentration of 10^8 CFU/ml. Five different EMA concentrations (0 μ g/ml, 7.5 μ g/ml, 10 μ g/ml, 50 μ g/ml and 100 μ g/ml) together with a control study (direct qPCR without EMA pre-treatment) have been tested for their performance. Figure 4.12 shows the number of cells at different EMA concentrations for three different types of cells (A. Heat-killed cells; B. Ethanol treated cells; C. Live cells).

Figure 4.12 The relationship between EMA concentration and enumeration of *E. faecalis* using qPCR for the 16S rRNA gene and the standard culture method on TSA media for A) heat-killed cells; B) ethanol treated cells; and C) live cells. The initial cell concentration was approximately 10^8 CFU/ml. The detection limit for qPCR is 3×10^2 GE/ml in natural water without pre-concentration.

From the graphs (Figure 4.12), all enumeration readings obtained from 0 µg/ml EMA pre-treatment were very close to the readings from direct qPCR without going through the pre-treatment process. The maximum difference between the reading from 0 µg/ml EMA-qPCR and direct qPCR was 0.19 log unit. These results indicate that there are almost negligible losses during EMA pre-treatment. The data show that EMA pre-treatment is very important as the heat-killed cells and ethanol treated cells remain giving extremely high counts in both the 0 µg/ml EMA

pre-treatment and direct qPCR assay, whereas the colony counts have reduced significantly.

The detection of heat-killed cells reduces significantly to 4.5 log units as the EMA concentration increases to 10µg/ml. However, the degree of inhibition does not increase with the increase in EMA concentration after exceeding 10µg/ml in heat-killed cells. The amplification of heat-killed cells increased gradually for EMA concentrations of 50µg/ml and 100µg/ml. This phenomenon clearly shows that the excess of EMA dyes may not necessarily improve the elimination of dead cells, but may, however, complicate the amplification steps.

In the case of ethanol treated cells, the greatest inhibition was achieved at 10µg/ml and 50µg/ml EMA pre-treatment. Similar to heat-killed cells, an excess of EMA dye had the reverse impact for the pre-treatment. This is shown in Figure 4.12B where a slight increase in detection of ethanol treated cells was observed for the 100µg/ml EMA pre-treatment.

In conclusion, the optimum EMA concentration was 10µg/ml. At this concentration, the greatest inhibition for dead cells (≈ 4.5 logs) and ethanol treated cells (≈ 4 logs) was achieved (Figure 4.12A and 4.12B). Although EMA pre-treatment is shown to reduce the false positive signals in dead cells (both heat-killed and ethanol treated cells) significantly, the influence of EMA on live cells hampers its application in pre-treatment. Approximately 1.5 log units of live cells were inhibited from downstream amplification at an EMA concentration of 10µg/ml (Figure 4.12C).

4.3.7 PMA optimization

PMA optimization was initially performed with *E.faecalis* at a cell concentration of 10^8 CFU/ml. Similar to EMA pre-treatment, there is very minimal difference between the qPCR results with 0µM PMA pre-treatment and direct qPCR

without PMA pre-treatment as the control (Figure 4.13). The difference between 0 μ M PMA-qPCR and direct qPCR for heat-killed cells, ethanol treated cells and live cells were 0.22 log unit, 0.1 log unit and 0.22 log unit respectively. This indicates that the losses attributed from PMA pre-treatment is not statistically significant and can be neglected.

Figure 4.13A and 4.13B show that the heat-killed cells and ethanol treated cells that were not treated with PMA (i.e. 0 μ M PMA pre-treatment and direct qPCR) can be amplified and subsequently gave false-positive detection. This phenomenon clearly shows that the DNA from dead cells can persist in the water for long periods, and thus qPCR cannot differentiate live and dead cells. Hence, an optimized PMA pre-treatment is an important step to ensure the accuracy of the downstream molecular detection method.

In the initial experiment with a cell concentration of 10⁸ CFU/ml, the amplification for heat-killed cells decreases with an increase in the PMA concentration (Figure 4.13A). The greatest inhibition of approximately 4.7 log units was achieved in heat-killed cells at 100 μ M of PMA pre-treatment. Ethanol treated cells achieved 3 log units reduction at 80 μ M PMA pre-treatment (Figure 4.13B). 80 μ M of PMA dye could be the saturated concentration for the pre-treatment, as an increase in PMA dye to 100 μ M caused a reduction in inhibition for ethanol treated cells.

The most important advantage of PMA pretreatment is the minimal impact on live cells. An average difference of 0.22 log unit was found between the readings from PMA-qPCR and colony count (Figure 4.13C).

Figure 4.13 The relationship between PMA concentration and enumeration of *E.faecalis* using qPCR for the 16S rRNA gene and standard culture method on TSA media for A) heat-killed cells; B) ethanol treated cells; and C) live cells. The initial

cell concentration was approximately 10^8 CFU/ml. The detection limit for qPCR is 3×10^2 GE/ml in natural water without pre-concentration.

Interestingly, when cell concentration was reduced to 10^6 CFU/ml, the degree of inhibition was not proportional to the PMA concentration. In fact, the degree of inhibition for heat-killed cells was the same after 20 μ M of PMA pretreatment (Figure 4.14A). This phenomenon was observed in qPCR with two different target genes, namely 16S rRNA and *pbp5*.

Figure 4.14 The relationship between PMA concentration and enumeration of (A) heat-killed *E. faecalis* cells and (B) live *E. faecalis* cells, using qPCR for 16S rRNA gene and *pbp5* gene, and standard culture method on TSA media. The initial cell concentration was diluted to approximately 10^6 CFU/ml. The detection limit for qPCR is 3×10^2 GE/ml in natural water without pre-concentration.

For easy reference, the CT values obtained for different PMA concentrations with heat-killed cells are plotted in Figure 4.15. The influence of PMA on viable cells increased as the PMA concentration increased beyond 20 μM . However, the heat-killed cells achieved equilibrium once the PMA concentration reached 20 μM and further increases in PMA concentration did not cause further inhibition for the heat-killed cells.

Figure 4.15 The CT values from qPCR for different concentrations of PMA. The initial cell concentration was diluted to approximately 10^6 CFU/ml.

Apart from PMA concentration, turbidity is another parameter which can influence the PMA pretreatment method. The previous optimized PMA pretreatment (i.e. PMA concentration of 20 μM) was shown to give the same reading for both the control (0 NTU) and turbid water samples (20 NTU) for heat-killed cells (Figure 4.16), although a slight difference was observed for samples with live cells (less than 1 log unit). However, for turbid water samples of 50 NTU and 100 NTU, greater inhibition was found for both live cells and heat-killed cells. This could be due to the incomplete photoactivation of PMA in the turbid water samples, as sediment particles attenuate the light.

Figure 4.16 The effect of turbidity in the optimized PMA pretreatment.

4.3.8 Validation of optimized PMA treatment with environmental samples

The optimized PMA pretreatment was further validated with environmental water matrices collected from local catchment waters. The water matrices were autoclaved twice to kill all the indigenous microorganisms in the water. The overnight grown cultures were spiked into the environmental water matrices, as well as ultrapure water which was used as control. The optimized PMA-qPCR was used to enumerate the spiked bacteria culture. The percentage difference between the spiked environmental water matrices and control study (i.e. spiked into ultrapure water) ranged from 0.19% to 10.66%, as measured with PMA-qPCR. Statistical analysis using the paired t-test showed that there is no significant difference between samples spiked into the environmental matrices and ultrapure water ($R^2=0.9833$) (Figure 4.17). These results indicate that there is no significant interference of local catchment water matrices on the performance of PMA-qPCR.

Figure 4.17 Regression plot to show the relationship between the cell number of spiked culture into the (i) environmental water matrices and (ii) ultrapure water (as the control study).

4.3.9 Validation of optimized PMA treatment with stressed cells

Two laboratory microcosms were set up to provide stressed growth conditions for the cells (i.e. ultrapure water and an environmental freshwater matrix). In the first set of experiments, the enumeration of cells was done by conventional plate count and qPCR. Figure 4.18A and 4.19A show a rapid decay in plate count enumeration but consistent results for qPCR throughout the experiments (i.e. 50 days).

With EMA pretreatment in the second set of experiments, a gradual decrease in cell number was observed in the EMA-qPCR results (Figure 4.18B and 4.19B). However, the initial GE obtained from EMA-qPCR was much lower than the CFU obtained from the plate count. This further confirms that the EMA dye tends to penetrate into intact cells, as reported in other studies (Rueckert et al., 2005; Kobayashi et al., 2009).

The third set of experiments was carried out using PMA-qPCR. Interestingly, the initial reading from PMA-qPCR was higher than the plate count method (Figure 4.18C and 4.19C). This could be due to injured cells or the sudden shock of environmental changes triggering survival mechanisms in the cells such as ceasing replication in the cells.

Figure 4.18 Decay curve plotted with cell number against the stress exposure duration in the ultrapure water microcosm. Cell numbers were examined with (i) culture-based plate count; (ii) molecular techniques which targeted the 16S rRNA gene and (iii) molecular techniques which targeted the *pbp5* gene. Molecular techniques included (A) qPCR without pretreatment; (B) qPCR with EMA pretreatment; and (C) qPCR with PMA pretreatment.

Figure 4.19 Decay curve plotted with cell number against the stress exposure duration in the environmental water microcosm. Cell numbers were examined with (i) culture-based plate count; (ii) molecular techniques which targeted the 16S rRNA gene and (iii) molecular techniques which targeted the *pbp5* gene. Molecular techniques included (A) qPCR without pretreatment; (B) qPCR with EMA pretreatment; and (C) qPCR with PMA pretreatment.

4.4 Discussion

4.4.1 Comparison between SYBR Green I qPCR and Dual-labeled probe qPCR

qPCR has been widely used for microbial detection and quantification. SYBR Green and dual-labeled probe are the most common chemistries applied in qPCR reaction. qPCR based on SYBR Green I and dual-labeled probe chemistries were examined in this study. In term of sensitivity, both SYBR Green I qPCR and dual-labeled probe qPCR gave very sensitive detection, which corresponds to around 1 gene copy/ μ l. In addition, both methods gave high amplification efficiency of 87.1% and 92% for SYBR Green I qPCR and dual-labeled probe qPCR respectively. Unfortunately, multiple melting peaks have often been observed in SYBR Green I qPCR for environmental samples (Figure 4.20A and 4.20B). This could be due to the non-specific binding in the qPCR reaction as the SYBR Green I binds to any double-stranded DNA. Theoretically, different PCR products should

have different melting temperatures. Thus, different PCR products can be differentiated by carefully examining the melting curves generated by each amplification product. However, melting curve analysis often leads to a difficulty in interpretation especially for PCR products with very close melting temperatures. In addition, the formation of primer dimer and non-specific amplification products have shown to reduce the sensitivity in the qPCR reaction. SYBR Green I qPCR also faced the difficulty of inaccurate quantification for samples with low initial target DNA concentrations (Hein et al., 2001). In contrast, dual-labeled probe chemistry employs a third oligonucleotide which serves as a fluorescence probe and complements the target sequence of the primers set. The additional dual-labeled probe improved the specificity of the qPCR reaction in this study, particularly for detecting *E.faecalis* in environmental water samples.

A

B

Samples				Melting Peaks	
Include	Color	Pos	Name	Tm1	Tm2
☒	Blue	1	-ve	73.68	
☒	Green	2	+ve	82.43	
☒	Red	3	mc2 a	89.10	
☒	Black	4	mc2 a	83.18	88.81
☒	Pink	5	mc2 b	88.73	
☒	Green	6	mc2 b		88.65
☒	Blue	7	mc4 a		
☒	Grey	8	mc4 a		
☒	Orange	9	mc4 b		
☒	Purple	10	mc4 b		
☒	Yellow	11	mc9 a	80.74	90.16
☒	Teal	12	mc9 a	81.51	89.81
☒	Red	13	mc9 b	90.71	
☒	Grey	14	mc9 b	90.82	
☒	Pink	15	mc11 a	84.46	89.23
☒	Green	16	mc11 a	82.78	
☒	Purple	17	mc11 b	82.25	
☒	Red	18	-ve	73.94	

Figure 4.20 Melting curve analysis for environmental water samples from SYBR Green I qPCR assay: (A) Melting peaks generated from environmental water samples and *E.faecalis* pure culture as the positive control, (B) the corresponding melting peak temperatures.

4.4.2 Selection of pbp5 region for the primer and probe design

Enteric bacteria often have to face various stresses when released into the natural environment, including osmotic stress, radiation stress, starvation and nutrient depletion. Dormancy, spore-formation or changes in cell structure are some of the strategies that are being utilized by bacteria for survival in harsh environments. In enterococcal cells, the VBNC state has been reported as another physiological state when cells are facing stressed environments (Lleo et al., 1998; Lleo et al., 2005; Oliver, 2010). During this stage, cells still maintain their metabolic activity although they have lost their cultivation ability on nutrient agar.

E.faecalis has been shown to undergo specific cell wall modification when entering the VBNC state (Lleo et al., 2000). The enzymes that are involved in peptidoglycan metabolism, i.e. penicillin binding proteins (PBPs), were shown to have different expression patterns in different physiological states of the cells. Among the PBPs, PBP5 shows continuous expression in the VBNC state, even at

higher expression rates (Signoretto et al., 2000). Hence, the gene encoding PBP5 was employed for the detection of VBNC cells in this study.

In our study, a new primer and probe set was designed to detect and quantify the *pbp5* gene in *E.faecalis*. The existing primer set for PCR from Lleo et al. (1998) was modified for the current qPCR study, i.e. shorter amplicon (125-bp) was modified from the existing 444-bp amplicon primer set. Our results show that shorter amplicons gave higher amplification efficiency in qPCR when compared to longer amplicons. In addition, primer sets with shorter amplicons (close to 100-bp) are recommended for qPCR. The newly designed primer and probe set was shown to achieve high sensitivity in detection (detection limit of 1 gene copy/ μ l) and highly specific detection.

4.4.3 Precautions during the PCR analysis

Speed and accuracy are two of the most significant advantages of PCR analysis in comparison to the culture-based method. However, there are several factors that can affect the PCR analysis. Inhibition from inhibiting substances is the main factor that greatly affects the performance of PCR analysis. In environmental water samples, the inhibitors comprise of a wide range of humic acids, proteinases, polysaccharides, etc. Due to the tremendous variable nature of inhibitors, there is no single ideal method to remove the inhibitors.

In order to ensure the effectiveness of PCR analysis, the inhibitors need to be removed from the samples before processing the samples. Various attempts have been made to reduce the PCR inhibition in environmental water samples. The most common and straight forward method to remove inhibitors is through the DNA extraction steps. At the present time, there are numerous commercial DNA extraction kits which can extract the DNA and at the same time eliminate the inhibitors. In this study, three extraction methods (i.e. the conventional DNA extraction method using phenol-chloroform-isoamyl-alcohol (He and Jiang, 2005), the Wizard[®] Genomic DNA Purification Kit and the QIAamp[®] DNA Mini Kit were

studied and compared. Among these methods, QIAamp[®] DNA Mini Kit gave the most satisfactory results in term of DNA purity, DNA yield and recovery rate. The silica membrane provided in QIAamp[®] DNA Mini Kit played the main role in removing the inhibitors. The principle of silica membrane is based on the adsorption of nucleic acids on the silica membrane in the presence of chaotropic salts. Under these conditions, the major inhibitors (such as polysaccharides and proteins) are not able to adsorb onto the membrane. In other words, the membrane will filter and remove these inhibitor substances (Qiagen).

Another attempt to reduce inhibitors is to isolate the target organisms from the samples through magnetic beads. The magnetic beads can be coated with antibody or probe (i.e. labeled biotinylated oligonucleotide). This method has shown to remove the inhibitors in the shortest time (Arnal et al., 1999). The drawback of this method is the additional cost involved and the losses encountered during the isolation step. In addition, poor recovery was reported for the magnetic beads method for complex samples or highly contaminated samples (Arnal et al., 1999). The inhibitors can also be reduced through dilution of the samples. This is only workable for samples with high concentrations of target analytes.

Bovine serum albumin (BSA) is another option to overcome the residual inhibitors that are not eliminated during the DNA extraction steps (Kreader, 1996; Wang et al., 2007). However, BSA should be added with caution as the quality for BSA may vary between the supply source and excessive amount can also cause the reverse impact.

In addition, there are several standard practices and precautions which should be applied when conducting the molecular techniques analysis. These include a separate working environment and equipments for molecular technique experiments. A thoroughly sterilization should be carried out before and after the experiments (detail discussion in Chapter 2). As a precautionary step, positive and negative controls should always be added to the experiments.

4.4.4 PMA optimization

Numerous studies have been done on EMA or PMA pretreatment prior to the downstream molecular analysis. However, there is still no universal protocol for its application to different bacteria, as well as different sample matrices (e.g. food samples, wastewater samples, plant samples). This could be due to the different cell wall structures existing in different bacteria, whereas the fundamental theory for EMA and PMA pretreatment assumes penetration of the dye through membrane-compromised cells. The sample matrix is also another important factor which needs to be examined before application of EMA or PMA.

In this study, the potential application of EMA and PMA to differentiate between viable and dead cells was evaluated. The combination of qPCR and EMA or PMA allows the selective quantification of viable cells in a mixed population of bacteria. This study demonstrates that both EMA and PMA could be used to eliminate dead cells. However, there is a higher tendency for EMA to penetrate into live cells, thus giving false negative results for live cells (Nocker et al., 2006; Kobayashi et al., 2009).

PMA is an alternative DNA-intercalating dye that has been introduced to overcome the penetration of EMA through intact cell membranes (Nocker et al., 2006). PMA was shown to work better when compared with EMA due to its higher molecular charge (two positive charges in PMA compared to one positive charge in EMA) (Nocker et al., 2006). The higher molecular charge provides greater impermeability through intact cell membranes, thus achieving better selective staining of dead cells.

The PMA pretreatment was optimized to analyze the target bacteria *E.faecalis* in natural environmental waters. In the optimization process, we found that cell concentration is the main factor in determining the optimum PMA concentration. The PMA concentration of 100 μ M has been reported to differentiate viable and dead *Enterococcus* cells (Varma et al., 2009; Bae and Wuertz, 2009b). In

our study, a PMA concentration of 100 μM gave the optimum treatment for *E.faecalis* at a cell concentration of 10^8 CFU/ml. However, a cell concentration of 10^8 CFU/ml is too high when dealing with environmental surface waters. At a lower cell concentration (i.e. 10^6 CFU/ml), the optimum PMA concentration can be reduced to 20 μM . In other words, the optimized PMA concentration can lead to a significant cost savings of about 80%.

Another interesting finding was obtained during the course of PMA optimization. The enumeration of viable cells consistently showed higher counts in PMA-qPCR when compared to the conventional culture-based method for a PMA concentration of 20 μM . This could be due to the detection of the stressed cells, persistent cells, or the viable but nonculturable cells. The difference between the PMA-qPCR and culture-based method is more obvious when the initial cell concentration was diluted 100 times with phosphate buffer. The sudden change in media may have caused stress or injury to the cells, which subsequently affected their growth on nutrient rich agar (i.e. TSA or BHIA). In contrast, the cell count for PMA-qPCR was lower than the plate count with the 100 μM PMA pretreatment. This clearly shows that a higher concentration of PMA may not be suitable for lower cell concentrations. This may be attributed to residual levels of PMA, which were not successfully inactivated by the photo-inactivation process and subsequently bound to lysed DNA during the DNA extraction step.

Although the optimized PMA pretreatment works well in both control and environmental water samples in our study, the main limitation for this pretreatment is the turbidity level in water samples. High turbidity levels can reduce light penetration into the water samples, thus reducing the photo-inactivation efficiency. Hence, continuous stirring or shaking of water samples when exposing to strong light may help to allow greater light penetration. Future studies can also explore different triggering factors (besides light inactivation) to generate the DNA cross-linking events with PMA.

4.4.5 Comparison between the 16S rRNA gene and *pbp5* gene in stressed cells

The primer and probe sets for both 16S rRNA and *pbp5* gene were found to be exclusive to *E.faecalis* detection. Besides species specific detection, the *pbp5* gene played another important role in monitoring the VBNC enterococcal cells (Leo et al., 2000). In stressed microcosms, the quantification of viable *E.faecalis* was carried out by targeting these two regions. In the initial stage, all three experiments (i.e. without pretreatment, EMA pretreatment and PMA pretreatment) gave almost the same results for both the 16S rRNA gene and *pbp5* gene (Figure 4.18A-4.18C and Figure 4.19A-4.19C). However, when the cells continued to face stresses, the GE numbers reported by the *pbp5* gene were shown to be consistently higher than the 16S rRNA. This difference was compounded further in the PMA-qPCR laboratory microcosm with the environmental matrix (Figure 4.19C). It is hypothesized that the lower nutrient level in environmental water compared to nutrient broth may have helped to induce the cells into the VBNC state. To our knowledge, this is the first study in comparing the qPCR, EMA-qPCR and PMA-qPCR in enterococcal cells in freshwater under stressed conditions.

4.5 Conclusion

The method development involved both upstream sample preparation and the downstream molecular analysis. Currently, many advanced molecular analyses have been developed to identify, detect and quantify microorganisms in various sample matrices. Unfortunately, the developments in upstream sample preparation have often been neglected. Sample preparation is the most important step to ensure that the downstream advance molecular analysis can achieve the best performance.

This study was carried out carefully by examining the current available sample preparations methods and subsequently selecting the most suitable method for the downstream analyses. Centrifugation was found to be the most suitable pre-concentration method for this study, particularly for local catchment water samples.

The application of silica membranes in DNA extraction was also proven to enhance the DNA recovery with minimum interference of inhibitors.

The newly designed primer and probe set for qPCR achieved very sensitive detection and has greatly improved the specificity detection of *E.faecalis*. The developed methods were incorporated with EMA or PMA to detect all viable *E.faecalis* cells, including the VBNC *E.faecalis* cells.

In conclusion, both EMA-qPCR and PMA-qPCR were shown to reduce the detection of heat-killed *E.faecalis* cells efficiently. The major draw-back for EMA-qPCR is the penetration of EMA into intact cells which can inhibit the amplification of live cells. This draw-back can be overcome by using PMA-qPCR. PMA-qPCR signifies an alternative technique that offers three main advantages: (i) minimizes false positive results in the conventional qPCR method; (ii) provides rapid results when compared to the time-consuming yet non-species-specific culture-based method; and (iii) detection of all viable cells including the VBNC cells. However, the cost involved for PMA pretreatment is relatively high. The optimized protocol can save PMA usage by up to 80%, which directly lowers the cost for PMA pretreatment for routine water quality monitoring. Although PMA-qPCR can significantly suppress the detection of dead cells, this method is still unable to totally discriminate the dead cells from qPCR detection. This is currently the main obstacle for PMA-qPCR method. Studies have suggested that the presence of high numbers of membrane compromised cells which have exceeded the dye's capacity, may cause insufficient modification to the DNA in the membrane compromised cells (Yáñez et al., 2011). Other studies have suggested that the short amplicon size in PCR or qPCR cannot be suppressed completely by PMA pretreatment (Luo et al., 2010). In the latter case, Luo et al. (2010) overcame this problem through a combination of two-step nested PCR. Nevertheless, PMA-qPCR is currently the most straight forward method to exclude the false positive detection from membrane compromised cells.

The EMA-qPCR and PMA-qPCR methods which target the *pbp5* gene gave higher cell counts than 16S rRNA during the course of the experiments. The reason behind this could be due to the occurrence of the VBNC state in stressed *E.faecalis* cells. Future studies should incorporate more viability tests such as direct viable count (Kogure et al., 1979), membrane potential (Lopez-Amoros et al., 1995) and membrane integrity (Sachidanandham et al., 2005) to confirm the physiological state of *E.faecalis*.

CHAPTER 5 KINETICS AND OCCURRENCE OF VIABLE BUT NON-CULTURABLE (VBNC) BACTERIA UNDER VARIOUS ENVIRONMENTAL STRESSES

5.1 Introduction

Bacteria indicators such as fecal coliforms and fecal streptococci, have been used to monitor microbial water quality for many decades. In recent years, enterococci have emerged as a suitable bacterial indicator for measuring microbial water quality as recommended by the U.S. Environmental Protection Agency and the World Health Organization (USEPA, 2004; WHO, 2006). Water quality standards currently dictate the monitoring of these organisms using culture based methods. For primary contact recreational water quality, this standard is set at 33 cfu/100 mL and 35 cfu/100 mL for freshwaters and marine waters respectively (USEPA, 2004). However, studies have shown that under stressed environmental conditions, bacteria are able to enter a physiological state where the cells remain VBNC (Oliver, 2005; Oliver, 2010). Upon release of the stressed conditions, these cells can be resuscitated and regain normal cell activity, including pathogenicity (Baffone et al., 2003; Chaveerach et al., 2003). This means that there is potential danger of not detecting the actual microbial populations in environmental water samples if the cells have entered the VBNC state. It was shown that several enterococci strains, such as *E.faecalis* and *E.faecium*, are able to enter the VBNC state when confronted with unfavourable conditions (Lleo et al., 1998; Oliver, 2010). The reliability of the routine microbial water quality monitoring that employs the culture-based method is therefore doubtful and uncertain.

Studies have shown that starvation (Byrd et al., 1991; Lleo et al., 2001), temperature, salinity and direct illumination of light (Lleo et al., 2005) are among the factors that induce *E.faecalis* to enter the VBNC state. In this study, sunlight and salinity are the two main environmental factors to be studied. As tropical

environmental waters receive approximately 12 hours of sunlight everyday with different light irradiation intensities throughout the day, the impact of sunlight in the formation of VBNC cells is an important factor to investigate for tropical waters. Although salinity has been well studied as a factor which induces the VBNC state in *E.faecalis*, the combination of both salinity and sunlight has not been studied. Thus, here we investigate these factors simultaneously to better understand how multiple stresses contribute to the formation of VBNC cells in tropical water.

Current microbial models for aquatic environments are typically incorporated into large scale hydrodynamic models, dispersion models, coupled to water quality, sediment processes and ecological models. The most common microbial decay model is a simple first order decay model, also known as Chick's law (Chick, 1908), which is used to predict the number of microorganisms over time. Although there are numerous complex models derived from Chick's laws, the decay rates of microorganisms are still based on measurements taken from the conventional culture-based method. However, the conventional culture-based method has been reported to underestimate the number of viable cells, particularly when cells are confronted with stresses from the environment (Oliver, 2005). The molecular technique, propidium monoazide quantitative PCR (PMA-qPCR) is currently an alternative method to enumerate the viable cells, including the VBNC cells. The subpopulation of VBNC cells can be estimated from the difference in counts between the PMA-qPCR and the culture-based methods.

There have been many attempts to prove the entry of bacteria, including enterococci, into the VBNC state. However, to our knowledge, there is no mathematical model available currently, to predict the occurrence of the enterococcal VBNC cells in the natural water environments. Modelling the subpopulation dynamics of enterococcal VBNC cells is important to give a clearer picture of the survival of this bacteria indicator under different stressed environments. The mathematical models which draw the relationship between the environmental variables and the occurrence of enterococcal VBNC cells provide information which is needed for a valuable risk assessment. This model can be

further incorporated into the existing water quality models which predict bacteria/pathogen contamination. The new model will be able to enhance the current models by (i) providing an accurate picture of the actual microbial contamination level, and (ii) monitoring the fate of the entire bacteria community and not just the culturable cells. Ultimately, this will provide early warning to the public of microbial contamination.

The objective of this study is to examine the decay kinetics of *E.faecalis* under different environmental stresses by using both the conventional culture-based method and the molecular techniques. The data from the laboratory microcosm study is then used to generate models to predict the occurrence of enterococcal VBNC cells. This will enable a better prediction of the actual microbial risk in recreational waters.

5.2 Fundamentals of the model

The possible changes in the physiological state of microorganisms, particularly when the enteric bacteria are being released from the animals' gut to the natural environment, are illustrated in the schematic diagram below (Figure 5.1). In the natural environment, the enteric bacteria could face the stresses of starvation, nutrient depletion, sunlight irradiation, osmotic pressure, temperature, as well as pH. We hypothesize that cells entering stressed environments could enter one of three possible physiological states, i.e. Culturable, VBNC and Dead.

The culturable state is the physiological state where cells are able to maintain their cultivation ability. This phenomenon is usually observed in the first few hours to days after the release of microorganisms into the natural environment (Flint, 1987; Na et al., 2006; Avery et al., 2008). It is believed that cells may contain a fraction of cellular biomass which will be consumed by endogenous metabolism (Martin et al., 1998). The duration of the cell's survival, either in the culturable state or VBNC state, is highly dependent on the cell's ability to regulate its maintenance energy demand (Martin et al., 1998).

In response to natural environmental stresses, the culturable cells will either enter the VBNC state (k_{C-N}) or death phase (k_{C-D}). If the cells are confronted with mild stress, the cells will gradually enter the VBNC state. On the other hand, when the cells are facing multiple or higher level of stress, the cells will lose their culturability much faster and subsequently enter the VBNC state in a shorter period (Nocker et al., 2011) (more discussion in section 5.4.4). These VBNC cells may regain their cultivation ability once the environmental stresses are removed (Oliver, 2010). This ‘resuscitation’ pathway is illustrated as k_{N-C} in Figure 5.1.

The high level of stresses, multiple stresses and continuous stresses could eventually lead to the death phase of the cells. The pathway to the death phase of culturable cells could be immediate when cells are confronted with adverse environmental stresses (denoted as k_{C-D}). The culturable cells may also follow the pathway k_{N-D} by entering the VBNC state before entering the death phase.

Figure 5.1 The multi-reaction kinetic model illustrates the physiological changes in microorganism in the natural environment. The schematic diagram outlines the kinetic models for each physiological state. C_C , C_N and C_D represent the concentration of culturable cells, VBNC cells and dead cells, respectively. Transformation rate constants are depicted by a k value with subscripted alphabets.

5.3 Materials and Methods

5.3.1 Bacterial strain and cultivation

The bacterial strain used in this study was *E.faecalis* 56R (University of Verona, Italy). The bacteria was cultivated overnight in Brain Heart Infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA) at 37°C under constant shaking (120rpm). For long term storage, the bacteria strain was kept at -80°C in the presence of glycerol as the cryoprotective agent.

5.3.2 Laboratory microcosms

For consistency of experiments, all water matrices were prepared in sterilized form in the laboratory. Water matrices with four different salinities were tested in this study. Ultrapure water was used to represent the water matrix with 0 ppt. Artificial seawater was prepared according to Lleo et al. (2005). The recipe for a litre of artificial seawater consists of 24.7g NaCl, 0.67g KCl, 1.36g CaCl₂.H₂O, 4.66g MgCl₂.6H₂O, 6.29g MgSO₄.7H₂O and 0.18g NaHCO₃ (Lleo et al., 2005). The artificial seawater was diluted with ultrapure water to a final salinity of 10 ppt, 20 ppt and 30 ppt.

The laboratory microcosm setup involved light irradiation from a sunlight simulator (Suntest CPS+, Atlas). Different voltages of light irradiation (36Wm⁻², 250Wm⁻², 350Wm⁻², 450Wm⁻², 550Wm⁻² and 650Wm⁻²) were tested against the water matrices. Meanwhile, two parallel white fluorescent lamps (Philips, TL-D 18W/54 Daylight) were used for the lowest light irradiation (36Wm⁻²). Figure 5.2 and 5.3 show the spectral power distributions from sunlight simulator (Suntest CPS+, Atlas) and white fluorescent lamp (Philips, TL-D 18W/54 Daylight), respectively. All laboratory microcosms were set in duplicate and maintained at around 26 to 27°C by a circulating water bath.

Figure 5.2 Spectral power distributions of Suntest lamps from sunlight simulator (Suntest CPS+, Atlas)

Figure 5.3 Spectral power distributions from white fluorescent lamp (Philips, TL-D 18W/54 Daylight)

5.3.3 Plate counting

The culture-based method was performed by plating the water sample onto the Tryptic Soy Agar in triplicate. 100- μ l of sample was spread onto Tryptic soy agar (TSA) (Becton Dickinson, Sparks, MD, USA), followed by overnight incubation at 37°C. The water sample was concentrated by membrane-filtration once the colony forming unit (CFU) on the agar plate was less than 10, according to Standard Methods (APHA, 1998).

5.3.4 Real-time quantitative PCR

The molecular technique was carried out by combining the quantitative PCR and propidium monoazide pretreatment (PMA-qPCR). PMA is a DNA-intercalating dye which can eliminate free DNA prior to the downstream molecular analysis. PMA (Biotium, Hayward, CA) was dissolved with 20% of DMSO to a stock solution of 20 mM. The PMA was added to water samples to an optimized final concentration of 20 μ M (discussed in Chapter 4). The PMA treated samples were incubated in the dark at room temperature for 5 min. After dark incubation, the samples were exposed to 500-W halogen light source (Philips) for 5 min. Following the PMA pretreatment, the water samples were ready for DNA extraction. The bacterial DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers and probe set were designed to target the penicillin binding protein 5 gene (*pbp5*) in qPCR (discussed in Chapter 4). The qPCR reaction mixture and thermal conditions are described previously (discussed in Chapter 4).

5.3.5 Staining of cells with LIVE/DEAD BacLight™ viability kit

LIVE/DEAD BacLight™ utilizes two nucleic acid stains, SYTO 9 and propidium iodide (PI) to differentiate the living and dead cells based on the integrity of cell membranes. Briefly, the SYTO 9 which gives green fluorescence will

penetrate the intact cell membrane due to its low molecular weight, while PI which gives red fluorescence will only pass through damaged cell membrane due to its high molecular weight. Thus, the intact cell emits green fluorescence while the damaged cell emits significantly less green fluorescence but relatively strong red fluorescence. The classification of the cells can be done according to the fluorescence signal emitted from the cells.

To perform the viability test, 1ml of each water sample (drawn from the laboratory microcosms in duplicate) was mixed with 1 μ l of SYTO9 (3.34mM) and 6 μ l of PI (20mM). The mixture was vortexed and incubated in the dark at 30°C for 30min. After the staining step, the excess dyes were washed away with PBS twice before being resuspended in PBS for flow cytometry analysis (Rezaeinejad and Ivanov, 2011).

5.3.6 Flow cytometry data acquisition and analysis

The fluorescence of SYTO 9 and PI were measured using a FACSCaliburTM flow cytometer (Becton Dickinson, Mountain View, CA, USA). A 15mW air cooled blue argon-ion laser with 488nm excitation was used for the experiment. Fluorescence data for cells stained with SYTO 9 and PI were collected in channel FL1 and channel FL3 by using a band pass filter $530 \pm 15\text{nm}$ and $620 \pm 10 \text{ nm}$ respectively. Data acquisition was performed by CELLQuestTM software. A total of 10,000 cells were counted in each experiment to ensure the efficiency and significance of the test. In addition, the sheath flow rate was adjusted to 10ml min^{-1} in order to control the sample analysis rate below $1000 \text{ event s}^{-1}$. Data analysis was carried out using FlowJo V.7.5.5 software (www.flowjo.com).

5.3.7 Statistical analysis and model development

Statistical analyses were carried out using SPSS software (SPSS. Inc.). One-way analysis of variance (ANOVA) was also employed to test the difference

between the decay coefficient (k value) and the environmental variables. Multiple linear regressions were used to examine whether the dynamic changes in subpopulation could be explained by the environmental variables such as sunlight irradiation and salinity. The coefficient of determination (r^2) was used to measure the goodness of fit of the model produced by the regression equation. Mathematical modelling was performed using MATLAB (The MathWorks, Inc.). The simple first-order reaction, also known as Chick's Law (Chick, 1908) was used to predict the decay coefficient.

$$\frac{N_t}{N_o} = e^{-kt} \quad (5.1)$$

where N_t and N_o represent the microbial count at time t and initial microbial count, respectively; k is the decay rate or decay coefficient.

The decay coefficient for the culture-based method (k_C) and PMA-qPCR (k_P) were determined from each experiment by using curve fitting analysis. Regression models were developed to predict the k_C and k_P value as a function of environmental variables.

5.3.8 Validation of the models

The models developed in this study were validated with data collected from independent microcosms. The performance of the predictive models was assessed using the accuracy factor (A_f) and the bias factor (B_f) (Ross, 1996). The A_f and B_f are defined by Equation 5.2 and 5.3 respectively.

$$A_f = 10^{\left(\frac{\sum \left| \log\left(\frac{X_p}{X_o}\right) \right|}{n}\right)} \quad (5.2)$$

$$B_f = 10^{\left(\frac{\sum \log\left(\frac{X_p}{X_o}\right)}{n}\right)} \quad (5.3)$$

where X_p is the predicted value (log); X_o is the observed value (log); and n is the total number of observations.

The A_f measures the degree of closeness of the predicted values to the observed values. An A_f of 1 indicates a perfect agreement between the predicted and observed values. The larger the A_f value, the less accurate the prediction. B_f indicates how far the average observed values lie from the predicted values. A $B_f < 1$ means the predictive model underestimates the percentage of the VBNC subpopulation and potentially leads to ‘fail-dangerous’ prediction. Overestimation of the VBNC subpopulation in the model will give a $B_f > 1$.

The overall performance of the models was determined by the percentage of residuals in the acceptable prediction zone (APZ) as described in Oscar (2009). Residuals for individual prediction cases, also known as prediction error (PE) were calculated based on Equation 5.4.

$$PE = X_o - X_p \quad (5.4)$$

where X_o is the observed value (log) and X_p is the predicted value (log). An acceptable model should have a PE within APZ, where APZ is defined in Equation 5.5.

$$-1.0 \log < \text{acceptable } PE < 0.5 \log \quad (5.5)$$

The overall performance of the models was measured based on the percentage of the prediction cases with PE in the APZ (Equation 5.6).

$$\% APZ = \left(\frac{n_{inAPZ}}{n_{total}} \right) \times 100 \quad (5.6)$$

where n_{inAPZ} is the number of prediction cases with PE in the APZ; n_{total} is the total number of prediction cases. A model with %APZ greater than 70% indicates that the model is able to provide valid predictions with acceptable bias and accuracy (Oscar, 2005).

5.4 Results and Discussion

5.4.1 Viability of cells

In this study, four different methods have been utilized to examine the viability of cells. The culture-based method is the typical growth dependent assay to

examine the viability of cells. The individual cell which is able to form a colony is scored as viable, or most commonly known as culturable. In contrast, the PMA-qPCR method is a growth independent assay to determine the cells' viability by examining the persistence of DNA in a cell, as well as the membrane integrity of the cell. The PMA-qPCR method has been shown to detect viable cells effectively in wastewaters and seawaters (Nocker et al., 2006; Bae and Wuertz, 2009a; Bae and Wuertz, 2009b).

In addition, the study confirmed the cells' viability by employing the commercial viability test kit, i.e. the LIVE/DEAD BacLight viability kit. This viability kit consisted of two different nucleic acid stains to dye the live and dead cells and give different fluorescent colours. The fluorescent colours were visualized under the epifluorescence microscope. Figure 5.4 shows the images from epifluorescence microscope for the microcosms study conducted over a period of 90 days. At day 0, the spiked-in live cells in both freshwater and seawater were stained mainly by SYTO9 and gave green fluorescence under blue light (488nm) excitation. The viability of cells was monitored as the cells were continuously placed under placed under the environmental stresses of starvation and light irradiation (36W/m^2). After 15 days under the stressed conditions, the culture-based method showed that no colony was detected. However, some of the cells in freshwater remained alive in term of membrane integrity. It is interesting to note that, there were always higher percentages of live cells found in seawater when compared to live cells found in freshwater under similar stress conditions. We hypothesized that the *E.faecalis* could survive better in saline water than freshwater and salinity could be one of the factors to induce cells to enter into the VBNC state. A small population of cells in seawater can even remain in the viable state for up to 90 days by maintaining the integrity of its cells (Figure 5.4).

From the microscope images, an interesting phenomenon was observed. In the seawater microcosm, *E.faecalis* culture tended to organize itself in long chains. However, paired or short chains of *E.faecalis* were observed in freshwaters. The main mechanism for chaining in *E.faecalis* is still unknown to date. In addition, the

degree of chaining in *E.faecalis* seems to be highly variable among the strains (Arias et al., 2007). In this laboratory microcosm study, different chaining degrees were found although a single *E.faecalis* strain was tested. The stress from salinity seems to increase the length of the cell chain. Studies have shown a correlation between the gelatinase content (GelE) and *E.faecalis* cellular chain length (Waters et al., 2003; Singh et al., 2005), where a disruption of GelE production leads to an increase in cell chain length (Waters et al., 2003). In addition, GelE production has been shown to be the main factor for biofilm formation (Hancock and Perego, 2004; Kristich et al., 2004) and also responsible for the pathogenesis of periapical inflammation (Steck et al., 2011) and translocation across the epithelial barrier (Zeng et al., 2005). Nevertheless, the exact mechanism for cellular chaining, as well as virulence of GelE in *E.faecalis* under stressed conditions still remain to be explained.

Figure 5.4 Bacterial viability was visualized by staining with the Live/Dead BacLight Kit. Images from epifluorescence microscopy of Live/Dead BacLight stained cells in freshwater and seawater microcosms at day 0, day 15, day 35 and day 90.

In addition to microscope visualization, the cells stained with LIVE/DEAD BacLight were also quantified using flow cytometry (FCM). Figure 5.5 shows the flow cytometry dot plots of *E.faecalis* from the freshwater microcosm which was monitored from day 0 to day 90. At day 0, the majority of cells (71.44%) were shown to be live cells, showing a high intensity of green fluorescence and accumulation in the Q3 region. As the cells faced the stresses of starvation, nutrient depletion and light irradiation, a subpopulation (27.08%) of cells were formed at the Q2 region during the 18th day of sampling, while the majority of cells shifted to the Q4 region. The cells in the Q4 region have relatively high red fluorescence compared to green fluorescence, and thus can be identified as dead cells. The distinct subpopulation in Q2 gradually shifted to Q4 when the cells continued to face stressed conditions. At day 96, most of the cells (82.49%) were identified as dead cells. The shifting of the dot plot pattern can be linked to changes in the cells' physiological state.

Figure 5.5 Flow cytometry analysis of *E. faecalis* viability in a freshwater microcosm using the LIVE/DEAD BacLight Viability kit.

In this study, results from PMA-qPCR method were compared with results from FCM coupled to the LIVE/DEAD BacLight Viability kit. Statistical analysis showed a significant correlation ($r = 0.889$) between the results from PMA-qPCR and FCM ($P < 0.01$, $n = 40$) (Figure 5.6). The paired-t test showed that there was no significant difference in means between PMA-qPCR and FCM data ($P = 0.948$).

Figure 5.6 The scattergram shows the percentage of live cells measured from PMA-qPCR and FCM. A strong correlation was found between PMA-qPCR and FCM data.

5.4.2 Kinetics of *E.faecalis* decay

In this study, laboratory microcosms were set up to examine the decay kinetics of *E.faecalis* under different stress conditions by using two methods, i.e. the culture-based method and PMA-qPCR. Bacterial decay when facing environmental stresses was assumed to follow first-order decay kinetics. The data collected from both culture-based method and PMA-qPCR were analysed using curve fitting analysis. Figure 5.7A and 5.7B show the decay kinetics of *E.faecalis* at a low intensity of light irradiation (36Wm^{-2}) in freshwater and seawater microcosms. Decay kinetics of *E.faecalis* at a high intensity of light irradiation (650Wm^{-2}) in freshwater and seawater microcosms are presented in Figure 5.7C and 5.7D. Decay coefficients (k value) and the corresponding r^2 obtained from curve-fitting analysis for all experiments are summarized in Table 5.1.

Figure 5.7 Decay kinetics at low light irradiation (36Wm⁻²) and high light irradiation (650Wm⁻²) for freshwater microcosm (salinity = 0 ppt) and seawater microcosm (salinity = 30 ppt). Note that the decay coefficient, k values are reported in hr⁻¹.

Table 5.1 k values obtained from both culture-based method (k_C) and PMA-qPCR (k_P) for each experiment.

Experiment	Salinity (ppt)	Light (Wm^{-2})	Culture-based method		PMA-qPCR	
			k_C (hr^{-1})	Adjusted r^2	k_P (hr^{-1})	Adjusted r^2
1	0	36	0.0122	0.9870	0.0021	0.8968
2	0	250	0.3458	0.9672	0.0883	0.9633
3	0	350	0.3490	0.6780	0.0740	0.8636
4	0	450	0.6617	0.8833	0.2310	0.8995
5	0	550	0.8427	0.7650	0.2228	0.9036
6	0	650	0.9745	0.8742	0.2373	0.9324
7	10	36	0.0088	0.7447	0.0019	0.8910
8	10	250	0.4554	0.9008	0.0355	0.8735
9	10	350	0.6230	0.9200	0.1536	0.9067
10	10	450	0.6407	0.9597	0.0980	0.8029
11	10	550	0.7286	0.9233	0.1182	0.9359
12	10	650	0.9600	0.9493	0.1304	0.9560
13	20	36	0.0117	0.8686	0.0019	0.9231
14	20	250	0.3387	0.9815	0.0463	0.8587
15	20	350	0.4463	0.8786	0.0710	0.8547
16	20	450	0.6520	0.9414	0.0966	0.8513
17	20	550	0.6782	0.8182	0.0599	0.9170
18	20	650	0.9155	0.9317	0.1108	0.9073
19	30	36	0.0122	0.8925	0.0019	0.9203
20	30	250	0.5130	0.9658	0.0431	0.7488
21	30	350	0.5177	0.9483	0.0632	0.8880
22	30	450	0.6627	0.9476	0.0663	0.8232
23	30	550	0.7236	0.9052	0.0760	0.8434
24	30	650	0.9655	0.9375	0.1003	0.9051

In all microcosms, the k_C value (decay coefficient from culture-based method) is higher than the k_P value (decay coefficient from PMA-qPCR method). The k_C values obtained in this study were within the range of 0.0088 hr^{-1} to 0.9745 hr^{-1} . This translates to a T_{90} of 262 hours (11 days) and 2.4 hours (0.1 day) respectively. The k_C values show an increasing trend with an increase in light irradiation.

On the other hand, the slow decay rates of k_P imply that cells are able to retain their viability for a much longer period than what is shown by the culture-based method. In the freshwater microcosm (i.e. 0 ppt), the k_P values were 2.9 to 6 times lower than the k_C values for different light irradiation conditions. This is in agreement with the study conducted by Guillaud et al. who found that the time required to decrease *E.coli* counts by one decimal logarithm using the direct viable count method were generally 3 to 5 times more when compared with the plate count method (Guillaud et al., 1997). In the case of our seawater microcosms, the difference showed an increase in k_C values of up to 12 times that of k_P . These results suggest that *E.faecalis* can adapt well to seawater and still maintain their viability although they have lost their culturability. Similar to k_C values, k_P values also show an increasing trend with light irradiation but within a narrow margin. However, there is no significant correlation between salinity and k_C or k_P (further discussion in section 5.4.3). From the curve fitting analysis, the r^2 obtained from all experiments generally gave high correlations ($r^2 \geq 0.65$) between the measured data and predicted first-order model (Table 5.1). Hence, decay coefficients (k_C and k_P) from the first-order decay model were used to generate the regression models to examine the influence of environmental stresses (e.g. sunlight and salinity) on the formation of VBNC cells.

The predictive models to estimate the subpopulation of culturable, VBNC and dead cells are developed and described in Equations 5.7 to 5.9. Basically, the culturable cells are estimated based on first-order decay kinetics using the culture-based method. As the PMA-qPCR method detects all viable cells including the culturable and VBNC cells, the difference in cell counts from the culture-based method and PMA-qPCR method is defined as the VBNC cells. The subpopulation of dead cells can be predicted by comparing the difference between the initial PMA-qPCR counts with the decay kinetics from PMA-qPCR. Although the PMA-qPCR method is still not a perfect method to discriminate all the dead cells (see discussion at section 4.5), this study has shown significant suppression of dead cell detection in the stressed microcosms (see section 4.3.9). Hence, an assumption that

PMA-qPCR only detects intact cells, i.e. viable cells, is made for the development of mathematical models in this study.

$$\text{Culturable cell} \quad : \quad C_{C(t)} = C_{Co} e^{(-k_C t)} \quad (5.7)$$

$$\text{VBNC cell} \quad : \quad C_{V(t)} = C_{Vo} e^{(-k_P t)} - C_{Co} e^{(-k_C t)} \quad (5.8)$$

$$\text{Dead cell} \quad : \quad C_{D(t)} = C_{Vo} - C_{Vo} e^{(-k_P t)} \quad (5.9)$$

where C_{Co} and C_{Vo} represent the initial count from culture-based method and PMA-qPCR method respectively. k_C and k_P represent the decay coefficient from the culture-based method and PMA-qPCR method respectively.

5.4.3 Regression model

Multiple linear regression analysis was carried out to examine the regression of decay coefficients as a function of salinity and light irradiation. Before performing multiple linear regression analysis, all environmental variables were tested for the difference in decay coefficient (k value) using ANOVA. As ANOVA is a parametric test, the assumption of a normally distributed population and homogeneity of variance was tested prior to the application of ANOVA analysis.

Pearson's r analysis was used to examine the correlation between the decay coefficients and the environmental variables. Table 5.2 shows the results from Pearson's r analysis. Light irradiation gave a strong positive correlation with both k_P and k_C . However, salinity gave a weak correlation for both decay coefficients, particularly k_C . In addition, salinity was found to be inversely related to k_P but positively related to k_C . The negative correlation between salinity and k_P can be explained by *E.faecalis* triggering its survival mechanism by entering the VBNC state in high osmotic stress conditions. This in turn causes the cells to lose their culturability. This result is consistent with the result obtained by Kay et al. (2005) where they reported enterococci being more resistant to deactivation with decreases in salinity.

Table 5.2 Pearson's r analysis for variables correlated with k_P and k_C

	k_P	k_C
Light	0.589 (0.002)	0.943 (<0.001)
Salinity	- 0.532 (0.007)	0.025 (0.907)

Note: significant levels for 2-tailed are given in parentheses.

In multiple regression analysis, the Backwards method was selected to remove the non-significant predictors. From multiple regression analysis, salinity was the variable which was omitted from the analysis. The k_P and k_C values for different light irradiation were plotted against the salinity level (Figure 5.8A and 5.8B respectively). The graphs show that both k_P and k_C values were greatly dependent on the light irradiation intensity as compared to salinity level. A low intensity of light irradiation (i.e. 36Wm^{-2}) gave lower k values for both the PMA-qPCR and culture-based method. In contrast, higher k values were achieved at higher intensities of light irradiation (i.e. 650Wm^{-2}). For salinity, there was no significant difference in k_C at each light irradiation condition over the salinity range from 0 ppt to 30 ppt (Figure 5.8B). However, a large difference in k_P was found between 0 ppt to 10 ppt, particularly for microcosms at higher intensities of light irradiation (Figure 5.8A): An increase in salinity from 0 ppt to 10 ppt clearly shows the dramatic decrease in k_P . The significant reduction in k_P from salinity 0 ppt to 10 ppt can be explained by the osmotic pressure faced by cells in freshwater. Although it is well-known that a Gram positive bacteria, such as enterococcus, has a thicker membrane layer which may better adapt to osmotic pressure, however, this study showed that these cells may have difficulty in maintaining their membrane integrity over a longer period under multiple environmental stresses.

It is interesting to note that no significant difference in k_P values was observed in the microcosms with salinity ranging from 10 ppt to 30 ppt (Figure 5.8A), although the decay rates did decrease for higher saline conditions. This is consistent with salinity being a factor that triggers VBNC formation (Lleo et al., 2005). This is further supported by Flahaut et al. (1996) who found the over expression of two proteins (DnaK and GroEL) which may contribute to the

maintenance of bacterial viability under stressed conditions induced by a salt medium.

Figure 5.8 The k values obtained from (A) PMA-qPCR (k_p), and (B) culture-based method (k_c) plotted against the salinity level. The solid black lines represent the lower limit of light irradiation (36Wm^{-2}) in the microcosms; the solid grey lines represent the upper limit of light irradiation (650Wm^{-2}) in the microcosms. Dashed lines indicate the data within the range of 250Wm^{-2} to 450Wm^{-2} and 450Wm^{-2} to 650Wm^{-2} respectively.

Strong correlations were found between k_C and light irradiation ($r^2 = 0.9498$), as well as k_P and light irradiation ($r^2 = 0.5001$). The regression models for k_P and k_C as a function of light irradiation are illustrated in Figure 5.9. In microcosms with low intensity of light, both k_P and k_C values were relatively low and found to be close to each other. As the light irradiation intensity increased, k_C values increased dramatically whereas only marginal increases were found in k_P . This suggests the entry of cells into the VBNC state as cells are no longer culturable, but can still be detected by PMA-qPCR. The regression models for k_C and k_P as a function of light irradiation are given in Equations 5.10 and 5.11.

$$k_C = 1.4333L \quad (5.10)$$

$$k_P = 0.2334L \quad (5.11)$$

where L is the variable for light in kW.m^{-2} ; k_C and k_P are the dependent variable in h^{-1}

Figure 5.9 The observed k_P (•) and k_C values (◦) at different light irradiation. The regression lines for k_P and k_C are represented by the solid line and the dashed line respectively.

5.4.4 Dynamic changes in bacterial populations

Dynamic changes in culturable, VBNC and dead subpopulations under different light irradiation intensities can be estimated from the predictive models generated in this study by fitting Equation 5.10 and 5.11 into Equation 5.7, 5.8 and 5.9. Assuming the initial spiked-in cells are all culturable cells, Figure 5.10 shows the dynamic changes of these three subpopulations at light irradiation of 36Wm^{-2} , 250Wm^{-2} and 650Wm^{-2} . From these graphs, the culturable subpopulation decreases slowly at lower light irradiation intensity and sharply decreases under high light intensity. The VBNC subpopulation increases gradually and is maintained in the VBNC state for a longer period in the microcosm with low light intensity. At higher light intensity, the VBNC subpopulation achieved its maximum concentration in a very short period and decreased soon after the peak. The dead subpopulation generally increases with higher light irradiation intensity.

Figure 5.10 Dynamic changes in culturable, VBNC and dead subpopulations at light irradiation intensities of (A) 36Wm^{-2} ; (B) 250Wm^{-2} ; and (C) 650Wm^{-2} , assuming the initial spiked-in cells are all culturable cells.

The decay coefficients as a function of light irradiation obtained from Equations 5.10 and 5.11 were fitted into Equation 5.8. The percentage of VBNC cells over time and under different light conditions can be estimated from this simple model. A three-dimensional model was generated from MATLAB to show the dynamic changes in the VBNC subpopulation for different light irradiation conditions (Figure 5.11).

Figure 5.11 3-D plot to show the effect of light irradiation and exposure time on the dynamic changes in the VBNC subpopulation.

From Figure 5.11, the dynamics of the VBNC subpopulation is greatly affected by the light irradiation intensity, as well as the duration of light exposure. Higher light intensity leads to a rapid decrease in the VBNC subpopulation. However, for environments with low light irradiation, the VBNC subpopulation is shown to increase gradually and remain in this physiological state for a longer period. Based on our data, *E.faecalis* kept in dark or low light conditions can still consist of a high percentage of VBNC cells even after 30 days. For example, Lleo et al. (2000) demonstrated that *E.faecalis* cells were able to remain in the VBNC state even after three months in a 4°C, nutrient-poor microcosm (Lleo et al., 2000).

5.4.5 Validation of models

The predictive models were validated with independent microcosms. The subpopulation of VBNC cells were measured from 16 independent sets of microcosms with four major light irradiation intensities (36Wm^{-2} , 250Wm^{-2} ,

450Wm⁻² and 650Wm⁻²) at four salinity levels (0 ppt, 10 ppt, 20 ppt and 30 ppt). The predicted subpopulation of VBNC cells were calculated according to the mathematical models generated in this study. The validation performance was evaluated by accuracy factor (A_f), bias factor (B_f) and percentage of prediction cases in the acceptable prediction zone (%APZ). Table 5.3 summarizes the validation performance results. The overall performance of the predictive models in these 16 sets of data was in the acceptable level where 73.33% of the prediction errors were in the APZ. The overall A_f and B_f scores were 1.103 and 1.014, respectively.

The individual evaluation of predictive models for each light irradiation condition showed that the predicted values at 36Wm⁻² and 450Wm⁻² do not fall inside the APZ. As the B_f for the 36Wm⁻² light irradiation condition scored less than 1, this indicates that the model under-predicts the subpopulation of VBNC cells. Hence, this model is potentially leading to “fail-dangerous” predictions when the light irradiation is low (i.e. 36Wm⁻²). The under-prediction of VBNC cells could be attributed to the influence of salinity upon entry into the VBNC state. In the freshwater microcosm (0 ppt; 36Wm⁻²), the B_f obtained was greater than 1, which means the prediction is still considered acceptable. However, the bias in B_f increases with salinity. This shows that salinity could be the main predictor variable in determining the subpopulation of VBNC cells for low light irradiation conditions (i.e. 36Wm⁻²).

In microcosms with 450Wm⁻² light irradiation, only 67% of the prediction cases fell within the APZ. Nevertheless, the B_f scored in these microcosms was greater than 1, which indicates that the model tends to over-predict the subpopulation of VBNC cells. As a result, the model is categorized to produce “fail-safe” predictions. The rest of the microcosms have achieved the minimum of 70% of prediction cases in the APZ.

As a conclusion, the predictive model developed in this study has been validated and shown to provide valid prediction with acceptable bias and accuracy.

However, extra precaution should be taken when analysing the data for low light irradiation at higher salinity. A possible bias towards under-prediction was found in the model for microcosms with low light irradiation level, particularly in saline water. Hence, this analysis suggests that other environmental factors (i.e. salinity) could be the main factor that triggers the VBNC state when the light irradiation intensity is low.

Table 5.3 Validation of models for predicting VBNC cells in stressed microcosms

Microcosm	Salinity (ppt)	Light (Wm^{-2})	A_f	B_f	%APZ
1	0	36	1.055	1.051	100
2	10	36	1.124	0.896	60
3	20	36	1.184	0.845	40
4	30	36	1.197	0.835	20
Subtotal			1.139	0.903	55
5	0	250	1.034	1.034	100
6	10	250	1.019	1.017	100
7	20	250	1.024	0.977	100
8	30	250	1.028	1.020	100
Subtotal			1.026	1.012	100
9	0	450	1.428	1.428	33
10	10	450	1.066	1.066	67
11	20	450	1.069	1.069	67
12	30	450	1.025	1.019	100
Subtotal			1.136	1.135	67
13	0	650	1.262	1.262	50
14	10	650	1.056	1.056	75
15	20	650	1.040	1.018	100
16	30	650	1.029	1.004	100
Subtotal			1.093	1.080	81
Overall			1.103	1.014	73.33

5.4.6 Application of model

Numerous studies have reported the impact of sunlight irradiation on bacterial survival (Fujioka et al., 1981; Alkan et al., 1995; Sinton et al., 1999; Sinton et al., 2002; Sinton et al., 2007; Schultz-Fademrecht et al., 2008). Most of

the studies examine the die-off rate of bacteria based on their growth on culture medium. There are few studies which have reported the higher survival rate of bacteria upon sunlight irradiation observed using culture-independent methods as compared to the culture-based method (Davies and Evison, 1991; Muela et al., 2000). The entry of bacteria into the VBNC state is one of the common explanations for this phenomenon (Pommepuy et al., 1996a). Conceptual models have been suggested to address the subpopulation of VBNC cells (Martin et al., 1998; Quiros et al., 2009). However, to our knowledge, there is no mathematical model to predict the subpopulation of VBNC cells under environmental stresses, especially for indicator bacteria such as, *E.faecalis*.

Singapore is geographically situated in the equatorial region and receives an average of 1,634 kWh/(m²year) of solar radiation (www.seris.sg). Figure 5.12 shows the average annual solar irradiation profile in Singapore (Liping and Hien, 2007). The primary application of this model is to predict the percentage of VBNC subpopulation of *E.faecalis* in recreational waters at different intensities of sunlight irradiation. The model is generated based on data collected at different irradiance levels at a temperature of around 26 to 27°C, which is the average water temperature in the Marina Reservoir catchment.

Figure 5.12 Average annual solar radiation profile in Singapore (Liping and Hien, 2007)

The light irradiation model can be modified to incorporate the Beer-Lambert law which measures the light intensity at different depths. According to the Beer-

Lambert law, light intensity decreases exponentially with depth in the water column (Eq. 5.12).

$$I(z) = I_o e^{-k_e z} \quad (5.12)$$

where $I(z)$ is the light energy (ly hr^{-1}); I_o , the surface light energy (ly hr^{-1}); k_e represents an extinction coefficient (m^{-1}); and z is the water depth (m). The extinction coefficient, k_e , is a function of the quantity of particulate matter and color of the water. k_e can be measured either using the Secchi-disk depth (Chapra, 1997) or suspended solids parameters (Takayama and Kjelleberg, 2000), as described in Equation 5.13 and 5.14.

$$k_e = \frac{1.8}{SD} \quad (5.13)$$

$$k_e = 0.55m \quad (5.14)$$

where SD is the Secchi-disk depth (m); m the suspended solids (mgL^{-1}).

By integrating Equation 5.12 over the depth, an average light intensity can be estimated and the bacterial decay due to light effect is presented in Equation 5.15 (Chapra, 1997).

$$k = \frac{\alpha I_o}{k_e H} (1 - e^{-k_e H}) \quad (5.15)$$

where k is the light decay rate (hr^{-1}); and α the proportionality constant. From our experimental data, the proportionality constant, α , for PMA-qPCR and culture-based method are 0.0027 ly^{-1} and 0.0167 ly^{-1} , respectively. Considering the transmissibility of light as the function of water depth and particulate matter, the decay coefficient for culture-based and PMA-qPCR method can be estimated from Equation 5.15 and illustrated in Figure 5.13. At low water level (i.e. 0.1 m), light is allowed to penetrate into the water column and thus higher inactivation of bacteria was achieved. As the water depth increases, a lower decay rate was observed due to less light is expected to penetrate into the water column. In addition, the decrease of decay coefficient was found with the increase of suspended solids content.

By incorporating the Beer-Lambert law into our mathematical model, the relationship between environmental parameters (i.e. light intensity, water depth,

water clarity, and suspended solids content) and the occurrence of enterococcal VBNC cells can be drawn. This is very useful to give accurate picture of actual microbial contamination level and subsequently provide information for valuable risk assessment.

Figure 5.13 3D graphs showing the responses for (A) k_C ; and (B) k_P at different light intensity and suspended solids content. The upper surface and lower surface show the decay coefficient at average water depth of 0.1m and 2m, respectively.

5.5 Conclusions

Since the introduction of the terminology of VBNC in early 1980s, there have been more than sixty species of bacteria that have demonstrated the VBNC state (Oliver, 2005). However, debates in the use of the VBNC terminology have been raging over the decades. For example, there are differences of opinion as to whether the VBNC state is a programmed genetic response in a cell to overcome the adverse conditions in the environment or whether it is just a process before the end of life (McDougald et al., 1998; Trainor et al., 1999). Some researchers argue that the VBNC bacteria should not be confused with injured bacteria (Steck, 2006). Another issue frequently debated is the use of the term “viable” and “active” in the non-culturable bacteria (Weichart, 1999). The author suggested that the term “viable” should only refer to recoverable cells but the term “active but nonculturable” for non-recoverable (non-viable) cells such as lethally injured and senescent cells (Weichart, 1999).

One of the reasons that created the great debate is the lack of knowledge on the transition between culturable and nonculturable states in bacteria. In fact, the natural survival response of bacteria is so different among species and this is further complicated by the past “survival experience”, also known as pre-adaptation in the bacteria. This study is not intended to either clarify this terminology, or demonstrate the fundamentals of the VBNC process. On the contrary, this study would like to draw attention to the limitations of the culture-based method and highlight the existence of non-culturable bacteria which fail to form colonies on agar plates, but show the living characteristics such as the maintenance of cell membrane integrity and the presence of nucleic acids. The conventional culture-based method which employs nutrient rich agar as the growing medium contains environment conditions which are very different from the natural aquatic environment. For example, the solid surface of an agar may cause greater inhibition for bacteria growth when compared to liquid medium (Wai et al., 2000). In addition, studies have suggested that the high nutrient content in agar may be toxic to injured cells, as well as the VBNC cells (Whitesides and Oliver, 1997; Bloomfield et al., 1998). With this in

mind, the culture-based method appears to be suitable for the growth of healthy cells but poses a limitation for injured and VBNC cells.

The occurrence of VBNC enterococcal cells in environmental waters may have implications from a public health and water quality perspective. Standard water quality monitoring that employs culture-based methods is not able to detect the cells which are in this physiological state. Consequently, results using the standard culture-based methods could possibly misinterpret the health risk to the public, especially to swimmers in recreational surface waters.

Models developed in this study are the first step in predicting the occurrence of VBNC enterococcal cells in the environment. In this study, the impact of sunlight and salinity on the survival of *E.faecalis* and entry into the VBNC state was evaluated. Mathematical models generated in this study can be used in risk assessment procedures, which are currently a major development in recreational water management systems. The survival patterns of *E.faecalis* obtained from both the culture-based and PMA-qPCR methods for different irradiation conditions provide new data on how these factors trigger entry into the VBNC state. Although the salinity factor has been included in this study, no clear trend was observed between salinity and k_P or k_C . In addition, other factors capable of triggering cells to enter the VBNC state need to be examined including turbidity, pH, temperature, suspended solids, total dissolved solids and nutrient levels. These environmental variables should be systematically investigated for their impact on the occurrence of the VBNC subpopulation, and subsequently incorporated into this predictive model.

The models in this study were developed according to a constant sunlight irradiation (i.e. 36Wm^{-2} , 250Wm^{-2} , 450Wm^{-2} and 650Wm^{-2}). These models demonstrated the dynamic changes of the VBNC subpopulation under different sunlight irradiation intensities and duration of light exposure. However, future studies could investigate the dynamic changes of the VBNC subpopulation under the daily fluctuations in light irradiation intensity, instead of a constant sunlight irradiation. This could better represent actual solar irradiation conditions in tropical

environments. Subsequently, the models need to be validated with actual daily changes in sunlight irradiation. All these would be useful in the future water quality modeling development for an accurate quantification of microorganisms in various environmental samples.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Bacteria indicators have been widely used to indicate the occurrence of pathogenic microorganism in environmental water quality monitoring. In this study, the occurrence of *Enterococcus*, was quantified using the conventional culture-based method (MF) and enzyme-based method (Enterolert™). Related environmental factors were analyzed in parallel to draw a correlation with the bacteria indicator. MF was found to correlate with turbidity ($R = 0.526$) and salinity ($R = 0.416$), while Enterolert™ was correlated only with turbidity ($R = 0.633$). Biochemical tests were carried out to further identify the species of bacterial indicators. The major species in Marina Reservoir catchment is *E.faecalis* (38%), followed by *E.faecium* (29%). Approximately 21% false-positive detection was reported in this study with *Streptococcus bovis* as the major non-enterococcus species.

The study aims to develop a molecular detection method to detect enterococcal viable cells in tropical surface waters. From the field samples tested, *E. faecalis* was found to be one of the dominant species in Marina Reservoir catchments. Hence, a detection method was developed to target this species. Penicillin binding protein 5 gene (*pbp5*) was selected for the molecular study. Studies have reported the continuous expression of this gene in all physiological states, including the viable but nonculturable (VBNC) state (Lleo et al., 2000). Previous studies applied conventional PCR and RT-PCR method to detect this gene (Lleo et al., 1999; Lleo et al., 2000). However, these methods do not allow quantification. Hence, a new primer and probe set which is suitable for qPCR reaction was designed in this study to detect the *E.faecalis pbp5* gene. This primer and probe set has been validated for sensitivity and specificity using qPCR. The

results showed that the newly developed qPCR assay can detect as low as 9 gene copy/ μ l, and at the same time is highly specific to the *E.faecalis* species.

Pure cultures of *E.faecalis* were induced into the VBNC state under various stress conditions (e.g. nutrient depletion, light radiation). Different stress conditions showed different decay rates using the colony count method. However, molecular counts for all environmental stress conditions remained constant throughout the experiment. There are two possible reasons to explain this phenomenon. One of the reasons is the formation of VBNC cells. Another reason is the detection of naked DNA (free DNA from dead cells). To examine the possibility of the detection of naked DNA in qPCR, heat-killed cells and ethanol-treated cells were tested. The result showed the persistence of free DNA after cell death had lead to the overestimation of viable cells. Other studies also revealed that the inability of DNA-based molecular methods to differentiate between viable and dead cells had hampered their wide application (Young et al., 2007; Walters et al., 2009). Hence, it was critical to overcome this major drawback before further molecular analysis could be carried out.

DNA-intercalating dyes such as ethidium monoazide (EMA) or propidium monoazide (PMA) were then applied to prevent the detection of free DNA in the downstream molecular analysis. Eventually, an optimized PMA pre-treatment was obtained to efficiently reduce the amplification of DNA from dead cells with an optimum PMA concentration (20 μ M).

The optimized PMA-qPCR method was used in parallel with the culture-based method to examine the decay of *E.faecalis* under different environmental stresses (salinity, starvation and sunlight) under controlled conditions in the laboratory. The cell counts for the culture-based method were found to be generally lower than the cell counts from PMA-qPCR method over time in the stressed microcosms. This phenomenon suggests the entry of the cells into another physiological state, the VBNC state. The difference between the two enumeration methods was taken as the VBNC cell count, and was modeled as a function of

environmental variables. The decay coefficients (k values) showed strong correlation with light irradiation ($r = 0.943$ and $r = 0.589$ for culture-based method and PMA-qPCR method, respectively). In addition, this study demonstrated the ability of *E.faecalis* to adapt to a wide range of salinity. Mathematical models were developed to predict the occurrence of VBNC cells under stressed conditions from light irradiation. In addition, this study also incorporated the well-known Beer-Lambert law into the mathematical model to allow better prediction of occurrence of VBNC cells at different water depth and water clarity. Mathematical models developed in this study can enhance current water quality models by providing a more accurate picture of the microbial community's survival characteristics in surface waters.

6.1.1 Scientific contributions

This thesis aims at determining the survival of *E.faecalis* in tropical environmental waters. The survival of *E.faecalis* was examined not only by the conventional culture-based method, but also examined with a molecular-based method, flow cytometry and microscopy. The information obtained on the survival of *E.faecalis* examined by the different methods contribute to a better understanding of the actual viability of *E.faecalis* and thus provide useful information for assessing the reliability of current microbial water quality monitoring, as well as the possibility of health risk brought by *E.faecalis* VBNC cells in tropical environmental waters.

The molecular methods developed in this study included (i) the design of a novel primer and probe set to detect *E.faecalis pbp5* gene in qPCR, and (ii) the optimization of PMA pre-treatment in qPCR according to local natural aquatic environments, which can reduce the false positive detection in qPCR significantly. This study also provides decay coefficient data from both culture-based and molecular-based method for *E.faecalis* under a wide range of light intensities and salinity levels. These data are useful for the subsequent mathematical model development to predict the occurrence of *E.faecalis* VBNC cells under the

environmental stresses of light irradiation and salinity. To our knowledge, no mathematical model has been developed to predict the occurrence of VBNC cells in natural aquatic environments.

6.2 Recommendations

In this study, the VBNC cells were differentiated through the Live/Dead BacLight kit, as well as qPCR with EMA or PMA pretreatment. Technically, a living cell is capable of maintaining the integrity of its membrane together with protecting the nucleic acid inside the cell membrane (Vives-Rego et al., 2000). Nevertheless, the infectivity of bacteria in the VBNC state is still unclear. Dispute on the infectivity of VBNC cells has been widely debated. Future studies should incorporate a resuscitation experiment to determine the extent of cells reentering the normal state (from VBNC) with potential pathogenicity intact. The resuscitation experiment is proposed to be carried out in microcosms where the colony count is less than 0.1 CFU/ml. The VBNC cells are subsequently transferred to amended liquid culture media for the resuscitation experiment. Two main approaches are suggested to be used in the amended liquid culture media, (i) dilute the carbon source of liquid culture media, and (ii) addition of oxygen scavengers such as catalase enzyme or sodium pyruvate. Through the dilution of carbon source, it can reduce the sudden nutrient shock to the VBNC cells. A study reported that the stressed bacteria achieve better recovery when plated on minimal nutrient media in comparison with rich media (Rowan, 1999). This is mainly due to the oxidative-burst when the growing bacterial cells produce an abundance of intracellular free radicals. The production of intracellular free radicals has been reported to cause death in bacteria. Meanwhile, the second approach is to break down the hydrogen peroxide through the addition of oxygen scavengers (Bogosian et al., 2000; Kong et al., 2004; Tandon et al., 2007). A statistical counting method, MPN, is suggested to quantify the possibility of resuscitation. The resuscitation effect can be incorporated into proposed conceptual model in this study, and can give an overall picture of the fate of bacteria from the entry into VBNC state to resuscitation upon removal of stresses.

Preliminary data in this study showed that approximately 65% of the water samples gave higher enterococcus cell counts in qPCR (genome equivalent) than the standard culture-based method (CFU), while 78% of water samples reported higher counts in qPCR (genome equivalent) than the defined substrate based method (MPN). This phenomenon shows the possibility that VBNC cells can appear in natural aquatic environments where these VBNC cells escape detection by culture-based and defined substrate-based detection. Although this study had demonstrated the occurrence of the VBNC state in *E.faecalis* under light irradiation and salinity stresses in laboratory microcosms, a future study is needed to validate the model with field data. To carry out the field experiment, *E.faecalis* cells are proposed to be inoculated into a membrane diffusion chamber and submerged into the natural aquatic environment. These cells are contained inside the chamber and protected from grazing effects, while exposed to various environmental stresses such as elevated sunlight irradiation, changes in nutrient level, pH, dissolved oxygen, temperature and salinity. Further investigations to relate environmental parameters with the occurrence of VBNC enterococcal cells in the field are important to demonstrate the significance of this physiological state in bacteria when encountering different environmental stresses.

From this study, we gain a better insight into bacterial responses to environmental variables. The occurrence of the VBNC enterococcal cells can have a negative impact on the reliability of these bacteria as fecal indicator bacteria. Future studies should bridge the gap between the knowledge in VBNC and advanced water quality modeling tools. Current water quality models (i.e. HSPF and WASP) only allow single first-order decay rate in estimating the die-off of bacteria. Most studies used only the culture-based method to determine a single decay rate for modeling purposes. This is not sufficient to capture all bacteria, especially those bacteria (i.e. *E.coli*, enterococci) which could enter the VBNC state in harsh environments. The combination of microbiological knowledge in bacteria survival and bioinformatics in water quality models can provide significant improvements in water quality monitoring and management.

A deep insight into the survival responses of *E.faecalis*, the bacteria indicator species, in environmental waters was carried out in this study. A similar approach can be used to examine the survival responses, particularly the VBNC state of pathogenic bacteria and viruses. Little is known on the survival responses of these pathogenic microorganisms. With a better understanding of survival responses of pathogens, this would help to improve the estimation of pathogen risk for public health. The information on pathogen survival could be very useful to incorporate into the water quality model to give direct surveillance of the potential health risk due to pathogens, instead of relying on the current bacteria indicators.

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APPENDIX

mE agar

Basal medium ingredients:

Peptone	10 g
Sodium chloride	15 g
Yeast extract	30 g
Esculin	1 g
Actidione (cycloheximide)	0.05 g
Sodium azide	0.15 g
Agar	15 g
Reagent-grade distilled water	1 L

Esculin iron agar (EIA)

Ingredients:

Esculin	1 g
Ferric citrate	0.5 g
Agar	15 g
Reagent-grade distilled water	1 L

Brain heart infusion broth (BHIB)

Ingredients:

Calf brain infusion	200 g
Beef heart infusion	250 g
Proteose peptone	10 g
Sodium chloride	5 g
Disodium phosphate	2.5 g
Dextrose	2 g
Reagent-grade distilled water	1 L

Brain heart infusion agar (BHIA)

Ingredients:

Addition of 15 g agar per liter of BHIB

Brain heart infusion broth (BHIB) with 6.5% NaCl

Ingredients:

Addition of 60 g NaCl per liter of BHIB

Bile esculin agar (BEA)

Ingredients:

Bacto beef extract	3 g
Bacto peptone	5 g
Bacto oxgall	40 g
Bacto esculin	1 g
Ferric citrate	0.5 g
Bacto agar	15 g
Reagent-grade distilled water	1 L