Assessment of bacterial viability / activity in water samples

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Assessment of Bacterial Viability / Activity in Water Samples

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ABSTRACT

Rapid bacteria detection and viability / activity assessments have been greatly enhanced by recent advances in the use of fluorescent stains in flow cytometry. However, complex environmental matrices pose difficulties in assessing bacterial parameters using flow cytometry in terms of label specificity, fluorescent response, and method sensitivity.

In this study, the *BacLight*TM bacterial kit (based on membrane integrity) and carboxyfluorescein diacetate (CFDA) (based on enzyme activity) have been chosen for assessing bacterial viability / activity. However, CFDA could not discriminate between active and dead cells of *E. coli* culture. The *BacLight*TM kit was successful in discriminating active cells for both *E. coli* and *E. faecalis*. The matrix effect of different water types, i.e. deionized water, tap water, reservoir water, groundwater and seawater were investigated to better understand the response of bacteria to *BacLight*TM staining in real environmental samples. Furthermore, the effects of fixation using 1% glutaraldehyde and storage at -80°C for one week were also studied to optimize the *BacLight*TM analysis protocol. It was found that although glutaraldehyde fixation could increase the green fluorescence ratio of active cells to dead cells for both *E. coli* and *E. faecalis*, it could also significantly reduce the percentage of active *E. faecalis* cells in fresh water (deionized water, tap water, reservoir water, and groundwater). Fixation was necessary for *E. coli* in seawater, since active *E. coli* cells could not pick up the SYTO-9 dye (fluoresces green) without fixation of glutaraldehyde. For analysis of fresh water samples, no fixation with glutaraldehyde was needed; for analysis of seawater samples, fixation with glutaraldehyde was necessary to obtain the detection of active cells.

When applying the *BacLight*TM kit to study the bacteriological quality of Kranji Reservoir water and its catchment runoff, the traditional culture based method of

heterotrophic plate count (HPC) and a commercial *SimPlate* technique were also used for comparison with the *BacLight*TM kit analysis. The result showed that a good linear relationship could be found between HPC and *SimPlate*, but both methods reported significantly fewer viable bacteria compared to the number of active bacteria detected using *BacLight*TM kit, where this difference appeared to be 0.7 – 4 log orders of magnitude. However, it is noted that with increasing bacteria numbers in stormwater, the correlation between *BacLight*TM and HPC was improved, with the regression slope increasing from -0.0239 to 0.7027. For indicator bacteria, *E. coli* and enterococci, the numbers met the U.S.EPA standards for Kranji Reservoir water. For catchment runoff, the numbers of indicator bacteria was as high as 7, 701 MPN / 100 ml and 19, 863 MPN / 100ml for *E. coli* and enterococci respectively. For stormwater, the numbers of indicator bacteria was as high as 45, 690 MPN / 100 ml and 241, 960 MPN / 100ml for *E. coli* and enterococci respectively.

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CHAPTER 1 INTRODUCTION

1.1 Background

In Singapore, surface reservoirs are important sources of drinking water. Surface water bodies are presumed to be more vulnerable to contamination than groundwater reservoirs due to the absence of natural soil protection and filtration. It is also noted that in the case of heavy rainfall the microbial loads of running waters may suddenly increase substantially and reach reservoir bodies very quickly (Kistemann et al., 2002). For this reason, monitoring microbiological raw water quality is an essential component of the protection strategy for catchment areas of surface drinking water reservoirs. In Singapore, there is very little data on the temporal variation of bacteria in catchment waters during a storm runoff. This information is particularly important in tropical regions where short bursts of high-intensity rainfall can trigger the release of both chemical and biological contaminants (Kistemann et al., 2002). Both traditional culture methods and the rapid detection using flow cytometry are used to assess the microbiological water quality of the Kranji Reservoir.

One of the most basic questions that microbiologists address is whether a bacterial cell is viable or dead. Traditional culture methods to detect viable cells rely on the ability of cells to actively grow and form visible colonies on solid media. Under some circumstances, the number of viable organisms may be severely under-stated by culture method as sub-lethally damaged organisms (Lopez-Amoros et al., 1997; Boulos et al., 1999), fastidious uncultivable bacteria (Ward et al., 1990) and viable cells that have lost the ability to form colonies under the test conditions will not be detected (Hoefel et al., 2003b).

However, the determination of viability of microorganisms turns out to be much more complex. This is illustrated by the varied nomenclature which exists nowadays to describe the state of microorganisms, which includes terms as dead, moribund, starved,

dormant, resting, quiescent, viable but non-culturable (VBNC), active but non-culturable (ABNC), injured, sublethally damaged, inhibited, resuscitable, living, active, etc. Some discussions concerning the use of these terms were published recently (Kell et al., 1998; McDougald et al., 1998; Barer et al., 2000). However, this issue is still not clear and the debate is still carrying on.

To decide the viability or activity of bacteria, there are several elementary requirements: a) an intact cyto-plasmic (plasma) membrane which functions as a barrier between the cytoplasm and the extracellular environment; b) DNA transcription, and RNA translation; c) generation of energy for maintenance of cell metabolism, biosynthesis of proteins, nucleic acids, polysaccharides, and other cell components; and eventually, d) growth and multiplication (Breeuwer and Abee, 2000). Except for the last point 'growth and multiplication', the first three points are measurements of the cell's different physiological functions and cellular structures. For the purpose of this study, active cells can be generally defined as those cells which are capable of performing cell functions necessary for survival, such as having an intact membrane, having enzyme activity and so on.

During the last two decades, the development of alternative methods was concomitant with advances in fluorescence dye / probe technology offering measurement for cells' physiological functions and cellular structures. Advantages of fluorescence techniques are high sensitivity (i.e., the number of molecules needed for detection is low), short analysis time duration, and the potential to analyze individual cells in combination with flow cytometry (FCM) or image analysis. These methods also enable the detection of VBNC and active but non-culturable (ABNC) cells.

1.2 Purpose and Scope

Practical and accurate microbial assessment of environmental systems is dependent on the detection and quantification of various microbial parameters in complex matrices. Traditional culture-based methods, considered to be both slow and biased, are increasingly being replaced by optical detection methods such as flow cytometry. The unique technical properties of flow cytometry have allowed the discrimination of bacteria based on nucleic acid staining, microbial identification based on genomic and immunologic characteristics and determination of cell activity. In this thesis, we ask the following questions:

1. What fluorescence dyes can be used to access bacterial viability/activity?
2. How does the fluorescence dye respond to different water matrices (tap water, reservoir water, groundwater, and seawater) when detected by flow cytometry?
3. How do the results obtained by flow cytometry relate to culture-based methods?
4. What are the relative numbers of viable or active bacteria population in a tropical reservoir/catchment system in Singapore?
5. How does the bacteria population change during stormwater runoff?
6. Is there any faecal contamination in the reservoir/catchment system?

To address question 1, a literature review was conducted to find out appropriate fluorescence dyes to be used in this study. The fluorescence dye selected would be used to detect the viability or activity of pure bacterial cultures (*Escherichia coli* representative of Gram negative cells and *Enterococcus faecalis* representative of Gram positive cells) in different water matrices (tap water, reservoir water, groundwater, and seawater) using flow cytometry. After developing the rapid protocols using flow cytometry to analyze the activity of cultured bacteria in water samples, these methods were applied to study the natural bacteria population in a tropical reservoir system, Kranji Reservoir (Question 4 & 5). Comparisons were made between traditional culture methods and rapid detection method by flow cytometry (Question 3). In addition,

bacteria indicators (*Escherichia coli* and enterococci) were measured using the culture based methods for the same reservoir (Kranji Reservoir) for comparison.

CHAPTER 2 LITERATURE REVIEW

2.1 Assessment of Bacterial Viability & Activity Using Fluorescence Techniques

Fluorescence-based methods have remained very useful for a wide diversity of applications ranging from industrial to environmental microbiology. In this chapter, the different strategies used for the detection of cell fluorescence and the different cellular target sites and fluorescent dyes / probes which are actually used in viability/activity assays are described. Figure 2.1 below summarizes the different physiological target sites of these dyes or probes.

Figure 2.1 Different cellular target sites for physiological and taxonomic fluorescence probes dyes (Joux and Lebaron, 2000)

2.1.1 Membrane Integrity

It is well known that the loss of membrane integrity represents significant damage for cells due to multiple functions linked to the plasma membrane (permeability barrier, transport, respiratory activity, etc). Therefore, the maintenance of membrane integrity is commonly measured in eukaryotic cells as an indicator of cell activity (Shapiro, 2003). Assessment of membrane integrity of bacteria is complicated due to the complexity of membrane structures. Gram-positive bacteria consist of two layers: the rigid peptidoglycan layer and the inner membrane (plasma membrane). For Gram-negative bacteria, there is an additional layer in the cell wall structure, i.e., the outer membrane, which makes the Gram-negative bacteria more resistant to host defence factors, such as lysozyme, β -lysin, and various toxic leukocyte proteins (Nikaido and Vaara, 1985).

Most of the membrane integrity assays use nucleic acid stains, such as propidium iodide (PI), ethidium bromide (EB), PO-PRO-3 and SYTOX Green, because of the high concentrations of nucleic acids within the cells and the large fluorescence enhancement exhibited by nucleic acid stains upon binding. Among these dyes, PI is the most commonly applied. In order to detect total cells as well as dead cells with damaged membrane, Molecular Probes has developed the Live/Dead *BacLight*TM Bacterial Kit containing two nucleic acid stains: SYTO-9 (green fluorescence, maximum emission at approximately 530 nm) and PI (red fluorescence, maximum emission at approximately 630 nm). It allows the simultaneous detection of active and dead cells, based on the capacity of active (intact membrane) cells to exclude PI (Joux and Lebaron, 2000). Cells with intact membranes would be stained only by SYTO-9 and cells with damaged membranes would be stained by both SYTO-9 and PI. When applied to FCM analyses, dead and active populations may be separated in a bivariate cytogram of green vs red fluorescence, since dead cells would exhibit lower green fluorescence due to quenching of the SYTO-9 fluorescence by PI (Molecular Probes, 1995). According to the manufacturer, SYTO-9 should penetrate intact membranes of a large number of Gram-negative and Gram-positive bacteria. However, Langsrud and

Sundheim (1996) found that 30% of *Pseudomonas aeruginosa* strains tested did not accumulate SYTO-9. Barbesti et al. (2000) suggested that this may be due to the fact that SYTO-9 does not act exclusively as a nucleic acid stain. To overcome this problem, the other membrane-permeable stain SYBR Green I has been tested in combination with PI. Barbesti et al. (2000) found that these two fluorochromes (SYBR Green I and PI) could be ideally combined to detect active bacteria in different samples with a two-color fluorescence analysis.

2.1.2 Membrane Potential

In microorganisms, a membrane potential is generally generated by the extrusion of H^+ ions by the H^+ ATPase or the electron transfer chain. It results from the selective permeability of bacterial membranes to a variety of cations and anions leading to a difference of electric potential across the membrane. Inside, the cell is negatively charged compared with outside the cell, and membrane potential plays a central role in different cell life processes, such as ATP synthesis, active transport, mobility, and regulation of intracellular pH. This transmembrane electrical potential gradient is typically on the order of 100 mV (Nebe-von-Caron et al., 2000). Hence, voltage-sensitive dyes have been developed to measure membrane potential in bacteria, such as Rhodamine 123 (Rh123), 3,3'-dihexyloxacarbocyanine ($DiOC_6(3)$) and bis-(1,3-dibutylbarbituric acid) tri-methine oxonol ($DiBAC_4(3)$), which can be excited by the 488 nm line and emit green fluorescence. Depending on the charge of the dye, they are accumulated in polarized (cationic dyes) or depolarized (anionic dyes) cells. Cells which have a membrane potential (negative inside) accumulate the cationic Rh123 and $DiOC_6(3)$, whereas $DiBAC_4(3)$ is excluded.

Rhodamine 123 is a lipophilic, cationic dye commonly used to detect membrane potential (Lopez-Amoros et al., 1997; Jacobsen et al., 1997). However, staining with Rh123 often requires a pretreatment step of the cells, generally performed by adding EDTA to permeabilise the outer membrane of Gram-negative bacteria (Diaper et al.,

1992). An additional problem is that Rh123 staining requires several cell washing steps, which are time consuming and may result in cell losses.

DiOC₆(3) is a cationic carbocyanine dye whose advantages include the absence of permeabilisation and washing steps. However, non-specific binding of carbocyanine dyes to hydrophobic regions of the cell and quenching of the fluorescence of intracellular dye have been reported (Diaper et al., 1992).

Lastly, the negatively charged oxonol, DiBAC₄(3), accumulates only in cells in which the membrane potential is dissipated. The fluorescent dye accumulates inside the cell by binding to lipid containing intracellular components. Intact cells with polarized membranes exclude the dye and therefore remain non-fluorescent. DiBAC₄(3) was used to determine the plasma membrane potential in bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bifidobacterium lactis*, *Bifidobacterium adolescentis*, *Micrococcus luteus*, *E. coli* and *Salmonella typhimurium* (Jepras et al., 1995; Amor et al., 2002; Novo et al., 2000; Lopez-Amoros et al., 1997; Comas and Vives-Rego, 1998; Novo et al., 1999;). In some cases, a pretreatment with EDTA or EGTA of 1~5 mM to increase membrane permeability is required. Without permeabilisation, uptake of oxonols would be more related to membrane integrity rather than membrane depolarization (Nebe-von Caron et al., 1998).

2.1.3 Enzyme Activities

Measurements of enzyme activities such as dehydrogenases or esterases provide indications of bacteria metabolic activities. They show the capacity of a cell to have synthesized these enzymes in the past and its ability to having maintained them in active form.

2.1.3.1 Dehydrogenase activity

Cell-specific assays to detect the respiratory activity of bacteria have been developed based on the use of different tetrazolium salts. Methods have been developed using 2-

(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT), and more recently, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), to indicate the fraction of respiring bacteria in the population. This is done through reduction of the soluble tetrazolium salts to their corresponding colored (INT), or fluorescent (CTC), insoluble, intracellular formazan crystals which can be viewed using epifluorescence microscopy (EFM) or flow cytometry (FCM) (Pyle et al., 1995; Gruden et al., 2003). Smith and McFeters (1997) study shows detailed mechanisms of INT and CTC reduction in *E. coli*.

However, this method remains controversial since some authors promote this technique while others pointed out several drawbacks of the method, such as the low fraction of positive CTC cells found in natural aquatic ecosystems, i.e. only 0~10% (Servais et al., 2001). Some authors also showed that some bacterial strains cannot be efficiently stained with CTC (Yamaguchi and Nasu, 1997), and some active bacteria cannot reduce CTC under certain conditions (Smith and McFeters, 1997).

2.1.3.2 Esterase activity

Detection of esterase activity is measured using lipophilic, uncharged and non-fluorescent fluorogenic substrates. Once within active cells, the substrate is cleaved by intracellular enzymes to produce a fluorescent product. There are two requirements for the cleaved substrate accumulated within the cell to fluoresce: a) the cell has intracellular esterase activity; and b) the cell has an intact cell membrane. As a result, it provides two indicators of bacterial activity (Joux and Lebaron, 2000; Veal et al., 2000)

Carboxyfluorescein diacetate (CFDA) is an esterified fluorogenic substrate that has been widely used for bacterial esterase activity assessment. Comparison made by Jepras et al. (1995) of different fluorogenic esters shows that CFDA is superior to FDA (fluorescein diacetate) and CFDA-AM (carboxyfluorescein diacetate acetoxymethyl ester) due to fluorophore retention and dye solubility problems. CFDA is a cell permeant and undergoes hydrolysis of the diacetate (DA) groups into fluorescent

carboxyfluorescein (CF) by intracellular non-specific esterases (Figure 2.2). CFDA has been used to detect a range of bacterial cultures including *Aeromonas hydrophila*, *Bacillus subtilis*, *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* (Hoefel et al., 2003a), and *Listeria monocytogenes* (Jacobsen et al., 1997), and for measuring the activity of bacteria in fresh water (Porter et al., 1995) using FCM. Double staining with CFDA and propidium iodide (PI) is also commonly used to distinguish between active and dead cells (Bunthof et al., 1999; Amor et al., 2002; Ananta et al., 2004)

Figure 2.2. Mechanism for cellular labeling by fluorogenic esterified substrate 5 (and 6)-CFDA (Hoefel et al., 2003a)

The main limitations encountered when using fluorogenic esterases are poor dye uptake for Gram-negative bacteria. Pretreatment using EDTA is required to increase the permeability of the cell membranes for the esterase substrates (Diaper and Edwards, 1994).

2.1.4 Pump Activities

Several observations have confirmed that different dyes can be loaded into bacteria and subsequently actively removed by energized cells. Active extrusion of these dyes, such

as Rh123 (Ueckert et al., 1997) and CFDA (Bunthof et al., 1999) can lead to biased results. To limit this interference, Nebe-von Caron et al. (1998) suggested the addition of sodium azide in the staining solution. When active dye extrusion is not inhibited, probe efflux can be used as a measurement of cell activity. However, pump activity assays are not popular for pure cultures and environmental samples.

2.1.5 Nucleic Acids

The presence of intact DNA sequences has been used as an indicator of cell activity with the assumption that DNA would be degraded in a dead cell more rapidly than other cellular components (Jamil et al., 1993). However, the precise correlation of cell activity with detection of DNA was shown to be poorly related, since DNA can persist in actively killed cells for significant periods of time (Masters et al., 1994). As a result, attention turned to the use of rRNA and mRNA as a marker of activity, as mRNA is a highly labile molecule with a very short half-life and, therefore, should provide a more closely correlated indication of activity status than DNA-based methods (Keer and Birch, 2003).

There are various analytical methods for detection of DNA or RNA in the cell. Most DNA detection is undertaken using PCR (Norton and Batt, 1999). The most commonly used amplification techniques for detecting mRNA are reverse transcriptase PCR (RT-PCR) and nucleic acid sequence-based amplification (NASBA) (Chan and Fox, 1999). Both these methods have been applied for the determination of bacterial activity, with variable success. For rRNA detection, fluorescence in situ hybridization (FISH) of oligonucleotides carrying a fluorochrome has been developed recently (Fuchs et al., 1998; Harmsen et al., 1999; Pernthaler and Amann, 2004). Moreover, in the case of complex communities, this assay could be developed to detect activity of specific bacteria using appropriate oligonucleotide probes. More details of specific detection using FISH will be discussed in later sections.

2.2 Environmental Matrix Interference

It is well known that environmental matrices pose difficulties when doing microbial analysis due to their inherent complexity (Gruden et al., 2004). This complexity is determined by the combination of physical and chemical characteristics of an environmental matrix. Environmental systems represent a broad range of biogeochemical conditions, such as ionic strength, pH, particulate matter, and dissolved matter. For analyzing environmental samples using flow cytometry, the limitations on fluorescent-based assays may be dependent on the following factors: a) the presence of background fluorescence; b) the heterogeneity of the sample; c) the fluorescence distribution; d) the signal-to-noise ratio and e) the efficiency of hybridization between the probe and the target biological molecule (Fuchs et al., 1998).

In addition to background fluorescence, the abundance of non-living bacterial sized particles in aqueous and subsurface microbial samples impart obvious challenges to the flow cytometry method (Proctor and Souza, 2001). The physical affinity of selected fluorochromes to abiotic particles will dominate their efficacy and use in certain environmental matrices. Particulate matters in natural waters include humic substances that are characterized by organic acids of biological origin. These humic substances typically interfere with nucleic acid-based microbial detection methods, such as PCR, because they are PCR inhibitors (Bischoff et al., 2005).

When applying the *BacLight*TM kit to actual environmental samples, considering the complex nature of the matrices as well as the heterogeneity of the microbial populations, it is anticipated that more than simply two distinct populations of 'active' and 'dead' may appear in the cytograms. For instance, in a study conducted by Hoefel (2003b), it was noted that the separation of active and dead populations was not as pronounced in raw waters compared to the populations in deionized water. The inherent complexities of environmental samples arise from a combination of physical and chemical characteristics, representing a broad range of biogeochemical conditions

(e.g., conductivity, ionic strength, pH) which may affect the response of cells to the nucleic acid stains.

Finally, differences in the biogeochemistry of fresh water and marine environments may substantially affect microbial diversity and activity, as well as method applicability due to interferences from ionic strength with probe hybridization and DNA/RNA recovery. In a study conducted by Lebaron et al. (1998), it was shown that among seven blue nucleic acid dyes from Molecular Probes (SYTO-9, SYTO-11, SYTO-13, SYTO-16, SYTO-BC, SYBR Green-I, and SYBR Green-II), SYTO-9 yields higher fluorescence signals in freshwater samples, whereas SYBR Green-II dye is most efficient in saline waters. However, to date, no studies have been published on the matrix effect when applying *BacLight*TM to different water samples and analyzed by flow cytometry.

2.3 Sample Preservation Effects

2.3.1 Fixation Effect

Fixation is a general procedure for preserving samples. There are several fixatives and even commercial kits available which can be used, e.g. formaldehyde, paraformaldehyde, glutaraldehyde, ethanol, methanol, FACSTM Perm, and PermeaFix (Koester and Bolton, 2000). Formaldehyde, paraformaldehyde and glutaraldehyde are considered as cross-linking agents. Ethanol and methanol are less efficient cross-linking agents, known as coagulant fixatives. These coagulant fixatives denature proteins, resulting in permeabilisation of cells, by extracting phospholipids from the cell membrane. Commercial kits (FACSTM Perm and PermeaFix) can also effectively permeabilize the cells (Koester and Bolton, 2000).

Formaldehyde, paraformaldehyde and glutaraldehyde do not have permeabilisation effects (Koester and Bolton, 2000). However, Bullock (1984), reviewing the effects of

different fixatives reported that formaldehyde and paraformaldehyde can increase the cell permeability. Gorman et al. (1980), reviewing the mechanism of antimicrobial activity of glutaraldehyde, showed that its main effect is to seal more or less the outer layers of the cell envelope depending on its chemical composition. Troussellier et al. (1995) also showed increasing fluorescence intensity using formaldehyde and paraformaldehyde fixation after 24 hours, but not unfixed or glutaraldehyde fixed cells.

Glutaraldehyde is very effective in stabilizing surface as well as intracellular structures for conventional scanning and transmission electron microscopy and high voltage electron microscopy. Hayat (1986) concluded that glutaraldehyde surpasses all other reagents in preserving the cell structure by introducing irreversible intra- and intermolecular cross-links into cellular proteins. Furthermore, it may preserve membrane structures by cross-linking amino lipids. However, increased concentrations of glutaraldehyde generally result in increased shrinkage of cells. The reason is that during the early stages of fixation, glutaraldehyde exerts osmotic pressure because it does not penetrate the plasma membrane as easily as does water. As a result, the water moves out of the cell, causing cell shrinkage. After the plasma membrane has been fixed partly or completely, it becomes freely permeable to ions and also to glutaraldehyde. At this stage of fixation, glutaraldehyde will not exert any osmotic pressure.

In using the *BacLight*TM Bacterial Kit, the manufacturer suggests the addition of glutaraldehyde as a preservative when storing samples. However, Boulos et al (1999) reported a loss of 63% activity after storage of *E. coli* cells for 24 hrs at 4°C, following glutaraldehyde fixation (at a final concentration of 5%). This may be due to the high concentration of glutaraldehyde used, since Madigan et al. (2003) suggested that 2% glutaraldehyde solution has already been used as high-level disinfectant or sterilant.

2.3.2 Storage Effect

The loss of cell activity with increasing length of sample storage is a well established fact (Vaulot et al., 1989; Troussellier et al., 1995). Boulos et al (1999) reported a loss of 63% activity after storage of *E. coli* cells for 24 hrs at 4°C, following glutaraldehyde fixation at 5%. Freeze preservation is a common method used to prolong the storage shelf-life of microbial samples prior to analysis. Troussellier et al. (1995) found that FCM bacterial cell counts showed no significant decrease of bacterial cell counts over time (4 months) when formaldehyde or paraformaldehyde (at a final concentration of 2%) was used to fix bacterial cells stored in liquid nitrogen (-196°C). However, on the other hand, bacterial cell counts obtained using refrigerator storage temperature (5°C) dropped by more than 50% over time (4 months).

Regarding the effect of freeze preservation on light scattering properties (forward scatter and side scatter) and fluorescence intensities detected by flow cytometry, Troussellier et al. (1995) reported that when fixed bacterial cells were stored in liquid nitrogen (-196°C), mean fluorescence of detected bacterial cells increased over storage time, while forward scatter values showed relatively large variations during the storage time.

2.4 Flow Cytometry

Each year microbiologists analyze millions of clinical, food, beverage, and water samples to assess the numbers and types of microorganisms present. Most of these analyses are conducted using culture based or direct microscopic techniques. Such techniques are often labor intensive, slow, tedious and may take several hours or even days to yield a result. Furthermore, culture based techniques cannot be used for the vast majority of microorganisms which cannot be cultured (Bernard et al., 2001). The traditional microbiological techniques also do not lend to rapid, on-line, microbial monitoring that is essential in many areas of industrial, environmental and clinical microbiology to enable correct and timely decisions to be made.

Increasingly, flow cytometry is being applied to bacteria as an automated microbial detection system. Such systems lend themselves to rapid, in-situ analysis of individual microorganisms. Flow cytometry applications have been most comprehensively reviewed by Shapiro (2003) and also by Porter *et al.* (1997), Nebe-von-Caron *et al.* (2000), Veal *et al.* (2000), Rieseberg *et al.* (2001) and Gruden *et al.* (2004). The aim of this section is to review flow cytometry as a technology for monitoring water microorganisms.

2.4.1 Basic Principles of Flow Cytometry

Flow cytometry is the measurement of physico-chemical characteristics of cells as they flow through an observation channel. It has been described as automated microscopy that has the advantages of automation, objectivity and speed (flow cytometers can analyze many thousands of cells per second). A single-file flow of microbes inside the observation channel (see Figure 2.3) is achieved by injection of the sample into a coaxial fluid stream where the mixture is focused using a sheath flow of aqueous solution. The basis of the flow is a jet of isotonic sheath fluid (approximately 100 μm in diameter) that travels at about 20 km h^{-1} into which samples are injected at a controlled rate, typically between 10 and 60 $\mu\text{l min}^{-1}$. A laser beam is directed at the observation point and the resulting light scatter and emitted fluorescence is detected with a photosensor (photodiode or photomultiplier tubes). Several parameters of detection are monitored: low angle or forward light scatter (FSC), right angle light scatter or side scatter (SSC) and several fluorescence detection channels with wavelength ranges defined by selected short and long pass filters.

Figure 2.3 Conceptual illustration of flow cytometry (Gruden et al., 2004)

While scatter information is related to particle size and cellular characteristics, specific fluorescent dyes / probes can also be added into the sample such that fluorescence only occurs when target microbes are present. As a result, flow cytometry can distinguish between ordinary particles and microbial cells. Furthermore, it is possible to differentiate some of the major microbial taxa based on their light scatter and auto-fluorescence characteristics alone. For example, yeasts can be distinguished from bacteria based on light scatter characteristics due to their size and shape. Fluorescence detection can be used further to distinguish photosynthetic from non-photosynthetic microorganisms using the auto-fluorescence emanating from photosynthetic pigments, such as *Prochlorococcus* which auto-fluoresces red when subjected to a 488 nm laser source.

2.4.2 Advantages and Disadvantages of Flow Cytometry

The advantages and disadvantages of flow cytometry are listed below.

2.4.2.1 Advantages_(Nebe-von-Caron et al., 2000; Veal et al., 2000; Vives-Rego et al., 2000)

- **Heterogeneity Analysis**

Analyses are performed on single cells which allows for heterogeneity within the sample to be detected and quantified. This is particularly important for specific detection of pathogens.

- **Speed**

Secondly, flow cytometry is a rapid technique. Most commercial instruments allow measurements at rates of up to thousands of cells per second. Thus flow cytometric data sets often represent 10,000 to 500,000 cells for a given population leading to statistically significant results.

- **Sensitivity**

Flow cytometry uses very sensitive electronic detectors called photomultiplier tubes to measure the intensity of scattered light or fluorescence at a given wavelength. These detectors are much better than the human eye at distinguishing different levels of fluorescence.

- **Multi-parameters**

Flow cytometric measurements of several different characteristics of each cell can be made simultaneously. Multiparametric measurements are useful because they allow one to correlate the different characteristics and thus define subpopulations and / or distinguish between different cell types.

- **Sorting**

Specialized flow cytometers allow physical separation of cells. This technique is an extension of flow cytometry and is often referred to as fluorescence-activated cell sorting. In simple terms, following analysis the sample stream is broken into a series of droplets. Most of those droplets will simply go to the waste collection vessel, but those

which contain a cell of a user-selected type will be electrostatically charged and deflected into a sorted fraction container.

2.4.2.2 Disadvantages

Flow cytometry offers accurate and precise data with simple manipulation for most water samples. However, for some water matrices, e.g. wastewater samples or turbid source waters, dilution of samples is needed due to blockage. Such samples require dilution and this increases the probability of error in calculating numbers, since some microbes may be diluted out and go undetected. Furthermore, the disadvantage of flow cytometry is the high capital cost and the need for highly skilled staff.

Table 2.1 listed below shows some applications of flow cytometry.

Table 2.1 Examples of flow cytometry applications	
Immunology	Phenotypic analysis, developmental studies All that antibodies can detect
Biochemistry	Total DNA content / ploidity Cell cycle analysis Apoptosis detection Enzyme activities
Functional studies	Live – dead analysis Phagocytosis pH inside the cell Ca ²⁺ flux detection Membrane potential Microviscosity of cell membranes
Cancer research	Residual analysis
Molecular biology	Chromosome analysis and sorting Rare event detection with PCR expanding Marker genes, green fluorescence protein

2.5 Microbial Distribution and Microbial Load during Storm Runoff and Rainfall for Reservoirs

Several studies showed that urban stormwater runoff correlated well with deterioration of water quality in reservoir/lake bodies or estuaries (Jin et al., 2000; Kistemann et al., 2002; Noble et al., 2004; Jeng et al., 2005). Surface water bodies are presumed to be more vulnerable to faecal contamination than groundwater reservoirs due to the absence of natural soil protection and filtration. It is argued that especially in the case of heavy rainfall the microbial loads of running waters may suddenly increase substantially and reach reservoir bodies very quickly (Kistemann et al., 2002). For this reason, monitoring microbiological raw water quality is an essential component of the protection strategy in catchment areas of surface drinking water reservoirs. The main criteria for assessing microbiological raw water quality are viable bacteria count and indicator bacteria. Although indicator bacteria do not necessarily cause illness, they are abundant in human waste where pathogenic organisms, such as pathogenic bacteria, viruses and parasites are also likely to exist. The bacterial groups that are most frequently used as indicators of faecal contamination are faecal coliforms (of which *E. coli* are a subset) and enterococci. However, the U.S. Environmental Protection Agency (EPA) has recommended *E. coli* and enterococci to replace faecal coliforms as indicators to monitor the water quality of freshwater and marine waters, respectively (U.S. EPA, 1986).

For viable bacteria count, the traditional method is pour plate method or spread plate method (heterotrophic plate count, HPC) approved by EPA. The commercially available kit, *SimPlate*, for HPC method is also used for the quantification of heterotrophic plate counts (HPC) in water. It is based on IDEXX's patented Multiple Enzyme Technology which detects viable bacteria in water by testing for the presence of key enzymes known to be present in these organisms. It uses multiple enzyme substrates that produce a blue fluorescence when metabolized by waterborne bacteria. The sample and media are added to a *SimPlate* plate, incubated, and then examined for

fluorescing wells. However, the culture based methods are time-consuming and take a minimum of two days to return a result. On the other hand, flow cytometry, coupled with advancements in fluorescent dye technology, can acquire and process water samples rapidly, usually within 30 minutes.

Stormwater is recognized as a major source of impairment of water quality in many receiving waters (Kistemann et al., 2002; Marsalek & Rochfort, 2004; Davies et al., 2004). While it is known that stormwater pollution can impact on receiving waters in many ways, the most difficult to control appears to be microbiological pollution. Stormwater pathways may eventually lead to water bodies that serve recreational purposes. As a result, typical levels of indicator bacteria in stormwater are in the range of 10^3 to 10^5 *E. coli* / 100 ml (Marsalek & Rochfort, 2004). The sources of bacteria in stormwater include domestic pet populations, urban wildlife (particularly birds), cross-connections between storm and sanitary sewers (human faecal pollution), lack of sanitation, deficient solid waste collection and disposal, accumulations of sediment in sewers, rodent habitation in sewers, land wash and growth of bacteria in nutrient-rich water standing in storm sewers between events (Olivieri et al., 1989)

CHAPTER 3 MATERIALS AND METHODOLOGIES

3.1 Culturing of Bacterial Cells

Cultures used in all control pure culture study were *Escherichia coli* (*E. coli*, ATCC NO. 700609) and *Enterococcus faecalis* (*E. faecalis*, ATCC No. 29212), which were obtained from the American Type Culture Collection.

Cultures of *E. coli*, as well as *E. faecalis* were cultured separately into lag, exponential and stationary phase by inoculating overnight cultures of the bacteria into fresh Luria-Bertani (LB, Difco) broth and Brain-Heart-Infusion (BHI, Oxoid) broth (2% inoculum) for periods of 1 hr, 4 hrs and 8hrs, respectively, at 37 °C with shaking at 150 rpm. Cells were then washed by centrifugation at 4,000 rpm for 15 min at 4 °C with re-suspension of the pellet in sterile phosphate buffered saline (PBS, 8 gL⁻¹ NaCl, 0.2 gL⁻¹ KCl, 1.44 gL⁻¹ Na₂HPO₄, 0.24 gL⁻¹ KH₂PO₄, pH 8.0). Dead cell suspensions were prepared by suspending cells in 70% v/v ethanol, and continuously mixed for one hour. Ethanol was removed by centrifugation at 4,000 rpm for 15 min at 4 °C and the pellet re-suspended in sterile PBS (pH 8.0).

3.2 BacLight™ Bacterial Kit

3.2.1 Re-suspension of Bacteria into Difference Water Matrices

Bacterial cells labelled “active” and “dead” were adjusted to 10⁸ cells ml⁻¹ in PBS followed by a 100-fold dilution in deionized water (DW, pH 6.09, conductivity 190 µS/cm), tap water (TW, pH 8.07, conductivity 301 µS/cm), reservoir water (RW, pH 8.23, conductivity 218.6 µS/cm), groundwater (GW, pH 8.61, conductivity 480 µS/cm) and seawater (SW, pH 8.49, conductivity 53.2 mS/cm). Reservoir water was obtained from Kranji Reservoir in north Singapore, groundwater was obtained from a sea sand constructed aquifer to the east of Singapore Island, and seawater was obtained from

costal waters off the eastern part of Singapore. All the water matrices were autoclaved and passed through 0.22 μm sterile filters prior to use.

3.2.2 Cell Fixation and Freeze Preservation

Each cell suspension was split into three equal fractions. One fraction was stained and analyzed without prior cell fixation (immediate analysis without fixation of GA); the second fraction was fixed with 1% glutaraldehyde (final concentration) in the dark for 10 min at room temperature prior to staining and FCM analysis (immediate analysis with fixation of GA); and the last fraction was fixed with 1% glutaraldehyde and stored at -80°C for one week prior to staining and FCM analysis (after one week storage at -80°C).

3.2.3 BacLight™ Staining

Staining was performed by combining equal volumes of SYTO-9 (3.34 mM) and PI (20 mM) dissolved in dimethyl sulfoxide (DMSO), diluted 1:10 in a NaCl solution (0.085%) prior to use and adding 30 μl of this mixture directly into 1.0 ml of sample. Incubation was performed for 15 to 20 min in the dark at room temperature before flow cytometry analysis (Boulos, 1999; Hoefel, 2003b).

3.2.4 Data Analysis

In order to compare the cellular responses to BacLight™ staining under varying conditions, several criteria were identified that could be used to determine the effectiveness of the assay for activity assessment. Based on the working principles of the BacLight™ kit, dead cell populations are distinguishable from active cell populations due to the quenching of SYTO-9 green fluorescence in dead cells by PI. This quenching effect is explained by the fluorescence resonance energy transfer (FRET) phenomenon that occurs between the two dyes when both are closely bound to

the nucleic acids (Grégori et al., 2003). The Figure 3.1 below is exacted from *BacLight*TM Product Information sheet, and shows the relative position of active cell population and dead cell population of *E. coli*. Hence one of the criteria we used to determine the efficacy of the *BacLight*TM assays was the extent of reduction in green fluorescence of the dead population relative to the active population. Results are calculated as the ratio of logarithm of mean green fluorescence of active to dead population (GFR, Equation 1). The logarithms of fluorescence values were calculated in order to reflect the visualization of the populations in the log-scale cytograms, which were used in all the analyses. Another discerning criterion for the assay was the coefficient of variation (CV) in the green fluorescence of the two populations, which is a measure of the spread, or dispersion of values around the mean of the population. The CV of the two populations is developed as shown in Equation 2. Generally, populations with low CV are considered better stained. Last but not least, the percentage of cells detected as active was also used as a judgement criterion (% active Equation 3).

Figure 3.1 Analysis of relative viability of *E. coli* suspensions by flow cytometry. The majority of the bacteria are represented by region I (dead cells) and region II (live cells). *E. coli* organisms appearing in region III are generally considered as uncharacterized in terms of viability. (Molecular Probes, 2003)

$$GFR = \frac{\log(\text{mean green fluorescence of active population})}{\log(\text{mean green fluorescence of dead population})} \quad (1)$$

$$CV = CV_{\text{active}} + CV_{\text{dead}} \quad (2)$$

$$\% \text{ active} = \frac{\text{concentration of active cells}}{\text{concentration of active cells} + \text{concentration of dead cells}} \times 100\% \quad (3)$$

3.3 CFDA (5-(and-6)-Carboxyfluorescein diacetate)

3.3.1 Re-suspension of Bacteria into Buffer

Bacterial cells labeled “active” and “dead” were adjusted to 10^8 cells ml^{-1} in PBS followed by a 100-fold dilution in PBS for *E. faecalis* cells, but in $1\times$ and $10\times$ Tris-EDTA buffer ($1\times$ TE buffer, 10 mM Tris, 1 mM EDTA, adjust to pH 8.0 with HCl) for *E. coli* cells. *E. coli* cells (Gram-negative bacteria) were re-suspended in sterile TE buffer to increase permeability of the cell membranes for the esterase substrates (Jepras et al., 1995; Porter et al., 1995; Hoefel et al., 2003a).

3.3.2 CFDA Staining & CFDA-PI Double Staining

Stock CFDA (Biotium) was prepared at a concentration of 10 mM in dimethyl sulfoxide (DMSO), and 10 μl aliquots were placed into 1.5-ml centrifuge tubes. Stock solutions were stored at -20°C in the dark.

Immediately prior to use, 10 μl CFDA dye was diluted 1: 10 by adding 90 μl ultra-pure water. Then 10 μl diluted CFDA dye was added to the 1 ml cell suspensions to give a final concentration of 10 μM . The cell suspensions were then incubated at 37°C in the dark for 30 min before flow cytometry analysis.

After running samples with CFDA staining, PI was added at a final concentration of 20 μM to distinguish enzyme active and membrane damaged populations. Samples were kept in an ice bath for 10 min to allow labeling of membrane damaged cells. No washing steps were performed with CFDA dyes (Ananta *et al*, 2004).

3.3.3 Data Analysis

Histogram plot analysis of both green fluorescence (CFDA) and red fluorescence (PI) was carried out to compare the fluorescence properties of the populations measured by flow cytometry. Based on these plots, the median fluorescence intensity and coefficient of variation (CV) can be obtained. The median refers to the middle of the population, the point at which there are 50% of the collected events on either side of a particular channel; and the CV measure of the spread, or dispersion of the population. In addition, dual-parameter dot plot analysis of red fluorescence (PI) vs. green fluorescence (CFDA) was also applied to detect active and dead populations simultaneously.

3.4 Kranji Reservoir Sampling

3.4.1 Study Site

The Kranji Reservoir is a freshwater storage reservoir located in the northwest of Singapore (1°25'N, 103°43'E). It was formed by damming of the estuary of the Kranji River in 1972. The reservoir has a surface area of about 450 ha and a maximum storage capacity of 15,850,000 m³. The mean depth of the reservoir is 3.5 m with a maximum depth of 17 m. It has three tributaries upward, namely Sungei KangKar (KK), Sungei Tengah (TG) and Sungei PengSiang (PS) (see Figure 3.2). Now it serves as a drinking water supply, non-body contact recreation, and freshwater aquatic habitat.

3.4.2 Background Sampling

Background samples were collected from six sites in the catchment and six sites in the Kranji Reservoir. The six catchment sites are CP1 to CP6, while the six sites in the reservoir are Sungei KangKar (KK), Sungei Tengah (TG), Sungei PengSiang (PS), Junction (JTN), Station 3, and Station 4 respectively (see Figure 3.2). Catchment samples and Kranji Reservoir samples were sampled randomly from September 2005 – December 2005. Samples were taken from different depth only for Station 3 (0, 4, 8, and 14m) and Station 4 (0, 4, and 8m), other samples were only take from the surface.

Surface samples were collected by hand using autoclaved 1-L bottles and screw caps from approximately 0 – 5 cm below the water surface. Samples below the surface were collected using a 5-L Niskin bottle. After the Niskin bottle was retrieved, it was shaken several times and 1-L samples were transferred into autoclaved 1-L bottles. All water samples were chilled on ice after collection and analyzed within 24 hours of collection.

Figure 3.2 Kranji Reservoir sampling site

3.4.3 Stormwater Sampling

An automatic sampler (Model #900 max, American Sigma, Loveland, CO) collected a flow-weighted stormwater sample from an open channel drain (CP1). The storm drain collects rainfall water from a total of 556 ha area which consists of 181 ha high-density residential area and 375 ha unknown and undeveloped area (Figure 3.3) in Choa Chu Kang, Singapore. The automatic sampling began when the flowing water depth in the storm drain reached 0.4 m, and eight samples were collected at 15 minutes interval. A

calibrated peristaltic pump transferred the sample to a pre-cleaned, 1 L polypropylene container. The sample was collected during two rain events on November 23, 2005 and January 18, 2006. The sample was recovered, placed in a cooler with ice and transported to the laboratory for processing. All samples are analyzed within 24 hours of auto-sampler collection.

Figure 3.3 Subcatchment Area for CP1

3.4.4 Water Microbiological Quality Analysis

3.4.4.1 Total bacteria

Three methods for total viable / active bacterial detection were used: traditional culture method, commercially available kit *SimPlate*, and rapid detection using *BacLight*TM kit with flow cytometry.

- Heterotrophic Plate Count (HPC)

HPC were performed in accordance to standard guidelines (APHA, 1998) using the spread plate technique on Tryptic Soy Agar (TSA) with incubation for 48 hours at $35 \pm 0.5^\circ\text{C}$. The bacterial plate counts were performed in duplicate from 10-fold series dilutions in PBS buffer.

- *SimPlate*

SimPlate for HPC (IDEXX Laboratories, Westbrook, ME) was purchased in multi-dose form. Samples were processed in accordance with the manufacturer's specifications. Briefly, the *SimPlate* reagent was hydrated by filling the reagent vessel to the 100 ml mark with deionized water, and shaken to dissolve. 1 ml sample was added onto the center of the plate base. Dilutions of 10^{-1} for Kranji Reservoir water, 10^{-2} and/or 10^{-3} for catchment tributaries water, and 10^{-3} and 10^{-4} for stormwater runoff were required. After that, another 9 ml of the hydrated reagent was added on to the center of the plate base and the plate was covered. To distribute the sample into the wells, the plate was swirled gently several times. Excess sample was drained by tipping the plate $90^\circ - 120^\circ$ into the absorbent pad. Finally, plates were inverted and incubated for 48 hours at $35 \pm 0.5^\circ\text{C}$. After 48 hours, the number of positive wells was determined by fluorescence emitted upon illumination with long wavelength UV light (365 nm). The most probable number (MPN) was obtained using the MPN table provided and multiplied by 10 or 100 if the 1 ml sample was diluted by $10\times$ or $100\times$.

- *BacLight*TM Bacterial Kit

*BacLight*TM kit staining on raw water was performed on 1 ml samples immediately after sampling without any treatment (fixation with GA or freeze preservation) accordance to section 3.2.3. In this study, there is no intention to remove phototrophic bacteria. As a result, it was included in the total bacterial count.

3.4.4.2 Indicator bacteria – *Escherichia coli* and enterococci

The IDEXX kits, *Colilert-18* and *Enterolert* were used for detection of *Escherichia coli* and enterococci respectively. The tests were performed according to manufacturer's instructions. 100 mL samples were used in each test and placed in 100-ml sterile Wirlpak bags (Nasco, Fort Atkinson, WI) prior to use. If a high number of the indicator organisms was anticipated, serial 10-fold dilutions were then made at 10^{-1} to 10^{-2} to obtain readable results. The defined substrate medium (*Colilert-18* or *Enterolert* reagent) was added into the 100 mL sample, and shaken to dissolve. The mixture was poured aseptically into a Quanti-TrayTM 2000, followed by sealing and incubation at $35 \pm 0.5^{\circ}\text{C}$ for 18 hours for *E. coli* and $41 \pm 0.5^{\circ}\text{C}$ for 24 hours for enterococci respectively. Fluorescence was interpreted under UV light at 365 nm.

3.4.4.3 Quality assurance and quality control (QA/QC)

QA/QC procedures were followed throughout the course of the study. For the laboratory microbial analysis, samples were analyzed in duplicate for each method. Additional QA/QC procedures for the IDEXX kits (*SimPlate*, *Colilert-18*, and *Enterolert*) were conducted following the methodology indicated in the product manual.

3.5 Flow Cytometry Analysis

3.5.1 Flow Cytometry

All flow cytometric analyses were performed using a FACSCaliburTM flow cytometer (Becton Dickinson, San Jose, CA, USA), equipped with an air-cooled argon ion laser emitting 15 mW of blue light at 488 nm. There are six parameters of detection: forward scatter (FSC), side scatter (SSC), and four fluorescence intensity detectors (FL1, FL2, FL3, FL4). Among six parameters, except FSC detector which was a photodiode, other detectors were photomultiplier tubes (PMT). Figure 3.4 below shows a detailed optical layout of the FACSCaliburTM flow cytometer. The specifications of the fluorescence detector channels were as follows: FL1 (yellow/green: $\lambda = 530 \pm 30$ nm); FL2 (orange: $\lambda = 585 \pm 42$ nm); FL3 (dark red: $\lambda = 670$ nm LP); and FL4 (red: $\lambda = 661 \pm 16$ nm). Ultra-pure water was used as the sheath fluid.

3.5.2 Calibration and Instrument Settings

Daily calibration was completed by analyzing unlabeled beads (to simulate unstained microorganisms) and fluorescein isothiocyanate (FITC: yellow-green), phycoerythrin (PE: red-orange), and peridinin chlorophyll protein (PerCP: red) beads (to simulate fluorochrome labeled microorganisms) (CaliBRITETM 3 beads [Cat. No. 340486], Becton Dickinson). The beads have expected values for FSC, SSC, and fluorescence, and these values are used to adjust instrument settings to set fluorescence compensation, and to check instrument sensitivity (BD FACSCompTM software) before any sample was processed. The unlabeled beads were used to discriminate actual events from background debris using the FSC detector. The photomultiplier tube voltages were adjusted so that the mean channel values of the beads corresponded to the programmed target values. The threshold was adjusted to minimize background noise.

Figure 3.4 FACSCalibur™ Optical Layout

3.5.3 Data Acquisition and Analysis

For acquiring data, all bacterial samples were analyzed with the 'LOW' flow rate setting. Before running each sample, cleaning with 70% v/v ethanol for a minute was carried out to avoid dye carry-over problem. Quantification was carried out based on the flow rate measured. A 12×75-mm plastic tube containing 1-ml ultra-pure water was weighed before and after 10 minutes running at 'LOW' speed on the flow cytometer. The difference in weight was converted to volume analyzed (density of water is 10^3 g L^{-1}). And then the flow rate was calculated based on volume per minute (flow rate was stable at around $12 \mu\text{l min}^{-1}$). This method was shown to be better than the bead ratio method, which was consistent with the observation made by Hoefel et al (2003b). For fluorescence intensity reference, 2- μm -diameter green-yellow fluorescent beads (YG Fluoresbrite beads, Polysciences) were added into samples.

All parameters were collected as logarithmic signals, and recorded as listmode files. The data were analyzed and converted into histogram or dot plots by using the Windows Multiple Document Interface computer program (WinMDI; Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA; available at <http://facs.scripps.edu/software.html>).

CHAPTER 4 RESULTS AND DISCUSSIONS

4.1 BacLight™ Bacterial Kit

The BacLight™ bacterial kit developed by Molecular Probes (Eugene, Oreg., USA), is a simple-to-use kit for the determination of cell activity based on membrane integrity. It consists of two nucleic acid stains, SYTO-9 (green fluorescence, maximum emission at approximately 530 nm) and Propidium iodide (PI) (red fluorescence, maximum emission at approximately 630 nm). Cells with intact membranes would be stained only by SYTO-9 and cells with damaged membranes would be stained by both SYTO-9 and PI. When applied to FCM analyses, damaged and intact cell populations may be separated in a bivariate cytogram of green vs red fluorescence, since damaged cells would exhibit lower green fluorescence due to quenching of the SYTO-9 fluorescence by PI (Molecular Probes, 1995). Figures 4.1, 4.2 and 4.3 below summarize values for log mean green fluorescence ratio of active to dead population (GFR), coefficient of variation in green fluorescence of active and dead population (CV), number of total cells, and percentage of cells detected as active (% active) obtained from this study. Further discussions are carried out in subsequent sections.

4.1.1 Matrix Effect

A comparison of BacLight™-stained populations of *E. coli* cultures (stationary phase, prepared with equal proportions of active to dead cells) in deionized water, tap water, reservoir water, groundwater and seawater is presented in Figure 4.4. Theoretically, active cells, being absent of PI, should stain SYTO-9 positive and fluoresce green. However, it is noted that simultaneous staining of active cells by SYTO-9 and PI can occur, which leads to similar red fluorescence intensity for both active and dead populations (Hoefel et al., 2003b; Swarts, et al., 1998). Stocks (2004) explained this simultaneous staining using cell-free DNA by suggesting that the relative intensities

Figure 4.1 Green fluorescence ratio (GFR) of active/dead population for *E. coli* and *E. faecalis* (stationary phase, prepared with equal proportions of active to dead cells) in different water matrix. DW is deionized water, TW is tap water, RW is reservoir water, GW is groundwater, and SW is seawater

Figure 4.2 Coefficient of variation in green fluorescence of active and dead population (CV) for *E. coli* and *E. faecalis* (stationary phase, prepared with equal proportions of active to dead cells) in different water matrix. DW is deionized water, TW is tap water, RW is reservoir water, GW is groundwater, and SW is seawater

Figure 4.3 Percentage of cells to be detected as active (% active) for *E. coli* and *E. faecalis* (stationary phase, prepared with equal proportions of active to dead cells) in different water matrix. DW is deionized water, TW is tap water, RW is reservoir water, GW is groundwater, and SW is seawater.

Figure 4.4 Comparison of *BacLight*TM stained populations of *E. coli* cultures (stationary phase, prepared with equal proportions of active to dead cells) in deionized water (A&B), tap water (C&D), reservoir water (E&F), groundwater (G&H), and seawater (I&J). (A)(C)(E)(G) and (I) shows immediate analysis without fixation of GA, while (B)(D)(F)(H) and (J) shows immediate analysis with fixation of GA. R1 defines the dead cells, R2 defines the active cells, and R3 defines the 2.0 μm beads.

Figure 4.5 Comparison of *BacLight*TM stained populations of *E. faecalis* cultures (stationary phase, prepared with equal proportions of active to dead cells) in deionized water (A&B), tap water (C&D), reservoir water (E&F), groundwater (G&H), and seawater (I&J). (A)(C)(E)(G) and (I) shows immediate analysis without fixation of GA, while (B)(D)(F)(H) and (J) shows immediate analysis with fixation of GA. R1 defines the dead cells, R2 defines the active cells, and R3 defines the 2.0 μ m beads.

of the stains or the concentration of PI relative to nucleic acid were not properly designed. As a result, it is possible for DNA bound SYTO-9 to have a component in the red region equal to that of DNA bound PI, which means potentially confusing emissions would occur when PI was not in sufficient excess to saturate the nucleic acid. Furthermore, the live-dead protocol developed by Barbesti et al. (2000) for cultures of *E. coli* using dual staining of SYBR Green I and PI shows three population: green, green plus orange-red, and orange-red *E. coli* cells which were identified as live, damaged, and dead cells, respectively. Cells with a partially damaged membrane will enable various amounts of PI to bind some nucleic acids that will result in a corresponding decrease of the green fluorescence and an increase of the red fluorescence. However, for whole cells in this experiment, there is no possibility for the two stains to be simultaneously intercalated into active cells' DNA. As a result, the possible reason is due to the broad emission spectrum of SYTO-9, which overlaps part of the emission spectrum of PI. Therefore, regardless of red fluorescence, membrane-intact populations showing higher green fluorescence intensities should appear on the right of membrane-compromised populations which exhibit relatively lower green fluorescence intensities.

A clear distinction between active (membrane-intact) and dead (membrane-compromised) populations of *BacLight*TM-stained *E. coli* cells (stationary phase) in all five tested matrices could be obtained, i.e., they appeared as separate clusters in the cytograms (Figure 4.4 (A), (C), (E), (G), and (I)). Nevertheless, by comparing the mean green fluorescence intensities of active and dead *E. coli* populations in the different water matrices, it was apparent that the cellular response to *BacLight*TM staining varied when cells were suspended in different waters. Since the cells were essentially in the same state of activity prior to re-suspension in the different media, the variation in green fluorescence intensity is indicative of the presence of matrix interference, which resulted in the apparent difference in cellular interaction with the *BacLight*TM stains. A clearer expression of this variation is shown by comparing the ratio of log mean green fluorescence of active/dead (GFR) populations of *E. coli*, which is indicative of the

extent of PI quenching of green fluorescence in dead cells, i.e., the GFR was 3.14 ± 0.030 in deionized water, 3.88 ± 0.047 in tap water, 1.73 ± 0.021 in reservoir water, 2.61 ± 0.003 in ground water and 0.44 ± 0.003 in seawater (Figure 4.1, *E. coli*, immediate analysis without fixation of GA). The GFR should be greater than 1.00 in order to meet the quenching effect of SYTO-9 by PI. However, the low GFR value of 0.44 obtained in the seawater analysis falls short of this, as seen from the cytograms where the active *E. coli* population in seawater has a lower green fluorescence intensity than the dead population (Figure 4.4(I)). This apparent low green fluorescence is attributed to seawater matrix interference, although the exact nature of interference is unclear. The study conducted by Lebaron et al. (1998) also shows the formaldehyde fixed bacterial cells stained with SYTO-9 have lower mean relative green fluorescence intensity for the seawater. The fluorescence noise, from the presence of fluorescent organic particles naturally occurring in seawater, interfered with the cytometric signals of stained bacteria. These interferences resulted in biased estimations of bacterial counts (generally an overestimation). A possible explanation is that passive diffusion of SYTO-9 into *E. coli* active cells was reduced due to the high osmotic pressure of seawater, resulting in low green fluorescence.

4.1.2 Microbial Heterogeneity

In order to assess the applicability of the *BacLight*TM assay for analyses involving mixed bacterial populations, the results from the assay using *E. faecalis* (gram-positive cocci-shaped bacteria) was compared with the results from the assay using *E. coli* (gram-negative rod shaped bacteria). The cytograms of *BacLight*TM stained pure cultures of *E. faecalis* (stationary phase) in different matrices i.e. deionized water, tap water, reservoir water, groundwater, and seawater are shown in Figure 4.5 ((A), (C), (E), (G), and (I)). When comparisons of these cytograms are made with the corresponding cytograms for *E. coli* (Figure 4.4 (A), (C), (E), (G), and (I)), a significant difference in the positions of the active as well as dead populations relative to the 2 μm reference beads was observed. This implies that a high coefficient of

variation would be expected for both the active and dead populations of a mixed culture analysis, since different fluorescence signals would be generated from different cell types, regardless of activity state. This observed high coefficient of variation (CV) of green and red fluorescence intensities for the active and dead populations in turn translates to poor separation of the populations.

By comparing the GFR values (Figure 4.1, immediate analysis without fixation of GA), it can be seen that the separation between active and dead cells for *E. faecalis* in all water matrices were better than for *E. coli*, since the GFR for the *E. faecalis* samples, which were 5.81 ± 0.235 for deionized water, 5.47 ± 0.130 for tap water, 3.94 ± 0.016 for reservoir water, 5.35 ± 0.084 for groundwater and 1.50 ± 0.004 for seawater, were generally higher. However, the higher CVs (populations were more dispersed) for the *E. faecalis* populations more than offset the effect of the higher GFR (Figure 4.2, immediate analysis without fixation of GA). For example, in the groundwater analyses, $\text{GFR} = 2.61 \pm 0.003$, $\text{CV} = 31.95 \pm 0.78$ for *E. coli* and $\text{GFR} = 5.35 \pm 0.084$ and $\text{CV} = 56.17 \pm 1.19$ for *E. faecalis*. Therefore, although the majority of the active and dead *E. faecalis* cells were relatively far apart, the two populations were less well separated due to the greater variation in the green fluorescence intensities exhibited by the cells within each population (Figure 4.4 & Figure 4.5, (A), (C), (E), (G), and (I)).

4.1.3 Difference in Bacterial Growth Phase

*BacLight*TM staining was performed on *E. coli* in different growth phases: A) lag phase, B) exponential phase and C) stationary phase. The dot plot of red fluorescence intensity (y-axis) versus green fluorescence intensity (x-axis) in Figure 4.6 shows the comparison between different growth phases in deionized water. A clear separation of active population (high green fluorescence intensity) and dead population (low green fluorescence intensity) was observed in all the cytograms. Between different growth phases, there was no apparent difference in the mean green fluorescence intensities of the 'active' and 'dead' populations. However, an obvious difference in mean red

fluorescence intensities of the active populations was observed. The 'active' populations in lag and exponential phases had relatively higher red mean fluorescence compared to the 'dead' populations. 'Active' cell populations in stationary phase appeared to exhibit red mean fluorescence intensities comparable to the 'dead' cell populations. Another interesting observation was that a portion of cells in exponential phase appeared to exhibit a status of transition between 'active' and 'dead' (observed as a trail on the left of the 'active' population). In another study done by Barbesti et al. (2000), there was no fluorescence intensity difference for active exponential phase *E. coli* cells and stationary phase *E. coli* cells. The differences in this study are likely to be damaged cells which are metabolically active but have incipient membrane damage so that the incomplete exclusion of PI results in incomplete energy transfer from SYTO 9 to PI. Hence, these cells emit both green and red fluorescence (Barbesti et al., 2000; Ziglio et al., 2002).

Figure 4.6 Comparison of *BacLight*TM stained populations of *E. coli* cultures A) lag phase, B) exponential phase, C) stationary phase, (prepared with equal proportions of active to dead cells, fixed with glutaraldehyde) in deionized water. R1 defines the dead cells, R2 defines the active cells, and R3 defines the 2.0 μm beads.

4.1.4 Effect of Fixation with Glutaraldehyde

A major advantage of membrane integrity based assays over other activity assays is that they allow the flexibility of cell fixation and preservation prior to analysis. In environmental analyses, fixation and preservation is frequently necessary prior to sample analysis, since large quantities of samples are often collected simultaneously and it may not be feasible to analyze all the samples before cells start to die or multiply in the absence of preservation.

Fixation is a general procedure for preserving samples. It is very effective in stabilizing surface as well as intracellular structures for conventional scanning and transmission electron microscopy and high voltage electron microscopy (Hayat, 1986). In testing the effect of cell fixation, the impact of the fixative on membrane integrity and hence, cell activity is the main concern for this experiment. Thus the proportion of active cells is an important criterion in the assessment. Our results suggest that glutaraldehyde fixation did not have a significant impact on the membrane integrity of *E. coli* cells. However, the percentage of active (% active) *E. faecalis* cells suspended in fresh waters (TW, RW and GW) were very much reduced following fixation (Figure 4.3 immediate analysis without or with fixation of GA).

Figure 4.4 shows the comparisons between unfixed and fixed cells of *E. coli* (stationary phase) in different water matrices. Glutaraldehyde fixation resulted in an obvious increase in the green fluorescence ratios of active to dead, i.e., GFR for fixed samples were increased from 3.14 to 6.86 for deionized water, 3.88 to 5.59 for tap water, 1.73 to 3.75 for reservoir water, 2.61 to 5.29 for groundwater, and 0.44 to 1.23 for seawater, respectively (Figure 4.1). More cohesive populations were also obtained after fixation for *E. coli* in seawater, i.e., CV = 42.51 for unfixed cells and 15.42 for fixed cells (Figure 4.2). However, there was not much change in the CV of the populations in the other samples except for deionized water in which the CV increased from 24.79 to 43.14 (Figure 4.2). The relatively low mean green fluorescence value of the unfixed

membrane-intact *E. coli* population in seawater (value was even lower than that for the corresponding dead population) suggests inefficient SYTO-9 staining. Glutaraldehyde fixation probably improved the permeability of cell membranes resulting in better dye penetration and staining efficiency. This is consistent with the observation made by Troussellier et al. (1995), in that glutaraldehyde fixed cells emitted higher fluorescence compared to unfixed cells due to increased cell membrane permeability.

The GFR value of 0.44 in the unfixed *E. coli* seawater analysis indicates that the mean green fluorescence of the 'dead' cells was actually higher than the 'active' cells. This appears to be contradictory to the 'FRET' phenomenon. By comparing Figures 4.4 (I) and (J), it can be seen that glutaraldehyde fixation resulted in an increase in the mean green fluorescence of the 'active' cells, but had no effect on the fluorescence of the 'dead' cells. We believe that the preparation of the 'dead' cells by ethanol treatment probably resulted in a good degree of cell wall permeabilization (effectively much greater than that obtained through glutaraldehyde fixation) so that further permeabilization with glutaraldehyde fixation was negligible. However, SYTO 9 penetration into ethanol-killed cells was probably much more efficient than that of PI (due to seawater matrix interference as explained earlier), which resulted in incomplete quenching of the SYTO 9 green fluorescence by PI.

The corresponding results for unfixed and fixed cells of *E. faecalis* (stationary phase) in different water matrices are shown in Figure 4.5. Following glutaraldehyde fixation of *E. faecalis* cells, higher GFR values were obtained for most of the analyses except for the seawater samples (Figure 4.1). However, despite the generally higher GFR obtained after glutaraldehyde fixation, we observed poorer separations of membrane-intact and membrane-compromised populations of *E. faecalis*. Except for the seawater analyses, all other analyses on *E. faecalis* produced much higher CVs upon glutaraldehyde fixation. A group of cells in the stage of transition between active and dead apparently contributed to the higher CVs and hence poorer separation of the populations.

By comparing the total cell numbers (data not shown) and % active (Figure 4.3), we found that although the total cell numbers remained largely unchanged, there was a drastic reduction in the numbers of % active *E. faecalis* cells following glutaraldehyde fixation for all the samples, except seawater. It appears that the glutaraldehyde treatment had a severe damaging effect on the cell walls of *E. faecalis* in the freshwater samples. In view of this, we do not recommend glutaraldehyde fixation prior to analysis of Gram-positive bacterial populations in freshwater samples.

4.1.5 Effect of Freeze Preservation

The loss of cell viability / activity with increasing length of sample storage is a well established fact (Vaulot et al., 1989; Troussellier et al., 1995). Boulos et al. (1999) reported a loss of 63 % activity after storage of *E. coli* cells for 24 hrs at 4°C, following glutaraldehyde fixation. Freeze preservation is a common method used to prolong the storage shelf-life of microbial samples prior to analysis. This portion of study aims to provide information on the recovery of active cell as reflected by the *BacLight*TM assay, following fixation of GA and storage for one week at -80°C.

A significant decline in total cell numbers for both *E. coli* and *E. faecalis* (stationary phase) after one-week of storage at -80°C (data not shown) were observed. The percentage reduction of total cell numbers ranged from between 3% to 22% for *E. coli* and 15% to 54% for *E. faecalis* (data not shown). Nevertheless, the percentage of 'active' cells remained mostly unchanged in the case of *E. coli*, but fell to below detection limit for *E. faecalis* in fresh water, i.e. deionized water, tap water, reservoir water, and groundwater (Figure 4.7).

Following freeze preservation, the quality of the *BacLight*TM assays also deteriorated for both cell types in almost all the matrices, except seawater. The results show that there was a reduction in the mean green fluorescence of the 'active' populations, thus

resulting in reductions of the GFR values (Figure 4.1). In addition, following freeze preservation, an increase in background noise level (probably due to lysed cell debris) was detected in the green fluorescence channel, which interfered with the discrimination of the dead cell population. These interferences may prove to be of substantial hindrance in the analyses, especially of environmental samples.

Figure 4.7 Comparison of BacLight™ stained populations of *E. coli* cultures (A&B) & *E. faecalis* cultures (C&D) at stationary phase in ground water matrix, prepared with equal proportions of active to dead cells, and fixed with glutaraldehyde. (A) and (C) show results of cells with immediate analysis with fixation of GA, while (B) and (D) show results of cells after one week storage at -80°C. R1 defines the dead cells, R2 defines the active cells, and R3 defines the 2.0 μm beads..

4.2 CFDA (5-(and-6)-Carboxyfluorescein diacetate)

Carboxyfluorescein diacetate (CFDA) is an esterified fluorogenic substrate that has been widely used for assessing esterase activity in bacteria. It is a cell permeant, and can be cleaved by intracellular enzymes to produce a fluorescent product. Provided that a cell has intracellular esterase activity and an intact cell membrane, CFDA will be cleaved and the fluorescent product can accumulate within the cell. Table 4.1 below summaries values of green fluorescence intensity for both *E. coli* (Gram-negative) and *E. faecalis* (Gram-positive) bacteria cultures after CFDA staining. Further discussions on the effect of TE buffer treatment and the possibility of double staining by CFDA-PI are carried out in subsequent sections.

Table 4.1 Flow cytometric fluorescent intensities of CFDA for both active and ethanol-killed bacterial cultures ^a

		Green Fluorescence Intensity			
		Active		Ethanol-killed	
		Median	CV ^b	Median	CV
<i>E. coli</i>	PBS	1.79	59.36	8.90	14.37
	1× TE	3.08	50.65	4.07	26.72
	10× TE	16.85	35.40	34.91	9.45
<i>E. faecalis</i>	PBS	562.34	6.28	4.14	51.69

^a Median fluorescent intensities & CVs are the average of duplicate samples

^b CV, percentage coefficient of variation

4.2.1 Performance of intracellular esterase for *E. coli* and *E. faecalis*

For Gram-negative bacteria, an additional permeabilisation step is required since the Gram-negative bacteria contains an additional wall layer called the lipopolysaccharide layer (LPS) that is impermeable to macromolecules and hydrophobic substances (Vaara, 1992; Nikaido, 1996). To increase the permeability of CFDA into Gram-negative bacteria, EDTA is usually added to the sample (Diaper and Edwards, 1994; Hoefel et al., 2003a). For this experiment, bacteria were resuspended in PBS buffer, 1× TE buffer which contains 1 mM EDTA, or 10× TE buffer which contains 10 mM EDTA.

FCM analysis of the *E. coli* cells (Figure 4.8) shows that CFDA could not discriminate between metabolically active and inactive *E. coli* cells due to the low green fluorescence of the active cells. Although TE buffer was used to permeabilize the cell membrane, the increase in median green fluorescence intensity for active cells was not significant, from 1.79 to 16.85 after 10× TE buffer permeabilisation. This may be due to the fact that *E. coli* cells after permeabilisation, did not have the same capacity for intracellular retention of the fluorescent product carboxy-fluorescein (CF), whereby the product may passively leak out of the cells or the presence of active extrusion pumps may be involved in pumping out the product (Nebe-von-Caron et al., 2000). For the dead cells of *E. coli*, the weak green fluorescence was probably caused by non-enzymatic hydrolysis of CFDA (Hoefel et al., 2003a).

For *E. faecalis* cells, FCM analysis shows that CFDA could successfully discriminate between active and dead cells (Figure 4.9), which gives a median green fluorescence of 562.34 for active cells and 4.14 for dead cells respectively (Table 4.2).

Figure 4.8 Flow cytometric fluorescence histograms of *E. coli* bacteria stained with CFDA in PBS Buffer (A & B), 1× TE Buffer (C & D) and 10× TE Buffer (E & F). (A) (C) and (E) show the active *E. coli*; (B) (D) and (F) show ethanol-killed *E. coli*.

Figure 4.9 Flow cytometric fluorescence histograms of *E. faecalis* bacteria stained with CFDA in PBS Buffer. Filled histograms represent ethanol-killed cells, and non-filled histograms represent active cells

4.2.2 CFDA-PI Double Staining for *E. faecalis*

A mixture of active (with esterase activity) and ethanol-killed (without esterase activity) *E. faecalis* in logarithmic phase was stained with CFDA and PI, and analyzed by flow cytometry to examine the possibility of simultaneously detecting active and dead *E. faecalis* (Yamaguchi and Nasu, 1997; Ananta et al., 2004). CFDA is used primarily for the evaluation of cellular enzymatic activity. It is a lipophilic, non-fluorescent precursor that readily diffuses across the cell membranes. Once it is inside the cell, it undergoes hydrolysis of diacetate groups by unspecific esterases into a membrane-impermeant green fluorescent compound carboxy-fluorescein (CF). The cell can only remain fluorescent green if the cell membrane is intact and CFs are unable to diffuse out. In contrast, PI is a membrane-impermeant, nucleotide-binding probe which cannot penetrate cells with intact membranes. Following loss of membrane integrity, the PI diffuses into and stains the cells, fluorescing red under blue light excitation. Thus, after CFDA-PI double staining, *E. faecalis* cells with esterase activity should show only green fluorescent (CFDA) while dead cells show only red fluorescence (PI) with blue light irradiation (Yamaguchi and Nasu, 1997; Ananta et al., 2004).

From Figure 4.10, a clear distinction between ‘esterase active’ and ‘dead’ populations of CFDA-PI double stained *E. faecalis* cells can be obtained with a high background noise level at the left hand bottom corner. This background noise is due to the unspecific binding of CFDA and PI dyes to cell debris or small particles in the samples. The active population has a higher mean green fluorescence compared to the dead population, which are 463.20 ± 4.38 and 1.90 ± 0.01 respectively. For the mean red fluorescence, the respective values are 49.40 ± 0.57 and 187.00 ± 1.20 for active and dead populations. The dead cells have a higher red fluorescence because of PI penetration into the cell, but the active cells also have a significant red fluorescence. This observation of significant red fluorescence for active cells is not observed in other studies (Yamaguchi and Nasu, 1997; Tanaka et al., 2000). This unexpected red fluorescence of active cells is probably due to the interference of PI since for only

CFDA stained active cells, the red fluorescence of the active population is at the noise level, whereas the same population is lifted up from the baseline after PI addition (Figure 4.11). Based on the principle of PI staining, PI can only penetrate cells with non-intact membranes. Therefore, it is not possible for an intact cell to fluorescence red since there is no PI present in the cell. The same phenomenon has also been observed when using the *BacLight*TM kit (Figure 4.4 & Figure 4.5), i.e., the active populations of both *E. coli* and *E. faecalis* have significant red fluorescence. The explanation from Molecular Probes is that “the green color SYTO-9 dye has a broad emission spectrum and hence, if the concentration of the SYTO-9 is high, it could bleed into the Propidium Iodide spectrum around 550-580 nm”. It is true that only SYTO-9 stained cells also have red fluorescence (data not shown), but only CFDA stained cells do not have significant red fluorescence (Figure 4.11). As a result, a possible explanation is the PI interference because of binding to the membrane cell wall of *E. faecalis* (Gram-positive) cells.

Figure 4.10 Flow cytometric analysis of the mixture of active and dead *E. faecalis* (logarithmic phase) prepared with equal proportions, stained with CFDA and PI. R1 defines the dead cells, R2 defines the active cells, and R3 defines the 2.0 μ m beads.

Figure 4.11 Comparison for red fluorescence intensity of active population of *E. faecalis* cultures (logarithmic phase) stained with CFDA only (A) and CFDA+PI (B). R3 defines the 2.0 μ m beads.

4.3 Microbial Indicators in the Kranji Reservoir / Catchment System

During the five-month sampling period from September 2005 to January 2006, 3 sets of background samples for Kranji Reservoir and its three main tributaries (KK, TG, PS, JTN, Station 3, and Station 4), 4 sets of catchment samples for catchment tributaries (CP1 to CP6), and 2 sets of stormwater samples (at CP1) were collected for evaluation. All samples were analyzed by three different methods for total bacteria viability/activity: traditional HPC spread plate method, commercial available kit *SimPlate* method, and rapid detection using *BacLight*TM kit by flow cytometry. Furthermore, indicator bacteria *E. coli* and Enterococcus were assessed by commercial available kits *Colilert-18* and *Enterolert* from IDEXX.

4.3.1 Total Bacterial Counts

4.3.1.1 Detection of active bacteria using *BacLight*TM kit

The determination of active and dead bacterial cells using the *BacLight*TM kit for Kranji water samples was carried out successfully. In the cytograms of red versus green fluorescence, active populations were clearly distinguished from dead populations (Figure 4.12). The dye SYTO-9 is freely permeant to most cells and its affinity for nucleic acids is moderate. Therefore, it can be displaced by higher-affinity nucleic acid stain PI. Because the membrane of intact cells offers a barrier to the entry of PI which is a larger molecule, cells with damaged membranes would be stained by PI only if PI is in excess of DNA content. In most cases, both SYTO-9 and PI are present in the cells with damaged membrane, but the emission spectrum of SYTO-9 overlies the excitation spectrum of PI, and hence, SYTO-9's green fluorescence is quenched due to fluorescence resonance energy transfer (FRET) (Grégori et al., 2003; Stocks, 2004).

Green Fluorescence

Figure 4.12 Flow cytometry analysis of Kranji Reservoir water (JTN, 29 September 2005) using the *BacLight*TM kit to detect active and dead cells. (A) shows the density plot; (B) shows the contour plot.

However, the separation between active and dead bacteria in raw water (Kranji Reservoir water) was not as good as for pure culture, as expected. This is due to low DNA content in raw water samples compared to lab culture samples. As a result, the green fluorescence intensity of the active population of Kranji Reservoir water is lower than the corresponding active *E. coli* culture in reservoir water. In addition, the dye PI yielded significantly greater non-specific binding to the matrix (Biggerstaff et al. 2006) e.g. organic and inorganic matter, can consume PI, which can cause insufficient PI to DNA content in dead cells, leading to a lower quenching effect. However, using dual staining by SYTO-9 and PI, the background noise is much reduced compared to single staining by SYTO-9. The background noise in single staining by SYTO-9 is due to the non-specific binding of SYTO-9 to non-biological material. As a result, the level of background noise in the green fluorescence channel is quite high.

4.3.1.2 Numbers of viable bacteria using spread plate method

The number of viable bacteria count by traditional spread plate method for the 6 sampling locations inside Kranji Reservoir, 6 sampling locations in the catchment tributaries and 2 sets of stormwater are summarized in Figures 4.13, 4.14 and 4.15 respectively. The number ranged from $2.50\text{E}+02$ to $3.40\text{E}+03$ cfu/ml for Kranji Reservoir water (Figure 4.13), from $1.40\text{E}+03$ to $3.41\text{E}+05$ cfu/ml for catchment runoff (Figure 4.14) and from $1.30\text{E}+05$ to $1.22\text{E}+06$ cfu/ml for stormwater (Figure 4.15). It is note that the viable bacteria number in catchment tributaries runoff is about 1 to 2 log value higher than the number in main body. The number for the catchment tributaries varies significantly in different locations and different dates. For example, at CP4, the number reaches $3.41\text{E}+05$ cfu/ml on 23 November 2005, which is around 40 times larger than $8.00\text{E}+03$ cfu/ml on the other two sampling dates. However, the number varies little inside the reservoir itself with the largest difference occurring at PS, $3.30\text{E}+03$ cfu/ml on 29 September 2005 and $2.50\text{E}+02$ cfu/ml on 12 October 2005 (around 10 times difference). The numbers for the stormwater are typically 1 to 2 log

scale higher than the normal catchment tributaries runoff, and 2 to 4 log scale higher than the Kranji Reservoir main body water.

Generally speaking, the differences between the water in the reservoir main body (JTN, Station 3 and Station 4) are not obvious (typically within 1 log difference). However, the viable bacteria number shows a modest increasing trend with depth for Station 3 (0m, 4m, 8m, and 14m) and Station 4 (0m, 4m, and 8m). The average number increases from $7.67\text{E}+02$ cfu/ml (0m) to $2.00\text{E}+03$ cfu/ml (14m) for Station 3, from $9.83\text{E}+02$ cfu/ml (0m) to 2.25 cfu/ml (8m) for Station 4. It represents a more than 100% increase in the absolute number from surface to bottom. Higher bacteria numbers at the bottom have been attributed to association with settling particulates, increased availability of nutrients, and protection from sunlight inactivation (Davis et al., 2005).

Figure 4.15 below shows the fluctuation in numbers of the viable bacteria during 2 hours of sampling during a storm. The number for viable bacteria is usually in the range of 10^5 cfu/ml (from $1.30\text{E}+05$ to $5.05\text{E}+05$ cfu/ml) for stormwater, with one exception on November 23, 2005 where the number increased rapidly to $1.22\text{E}+06$ cfu/ml. Comparing the results from the two sets of stormwater, it can be seen that the viable bacteria number remains roughly constant regardless of the water flow rate or the rainfall intensity. However, the number for stormwater is still 1 log order of magnitude higher than the normal catchment water (dry weather) flow.

Figure 4.13 Number of viable bacteria (heterotrophic plate count) by spread plate method in Kranji Reservoir and its three main tributaries

Figure 4.14 Number of viable bacteria (heterotrophic plate count) by spread plate method in the Kranji Reservoir catchment tributaries

Figure 4.15 Number of viable bacteria (heterotrophic plate count) by spread plate method for stormwater at different flow rate Q

4.3.1.3 Comparison of active bacteria counts obtained with spread plate method, *SimPlate*, and *BacLight*TM

Three different approaches, i.e. traditional culture method (HPC spread plate method), *SimPlate*, and *BacLight*TM, were taken to assess the viability/activity of bacteria from Kranji Reservoir, its catchment points and the stormwater. The number of viable/active bacteria of the samples for *SimPlate* (MPN/ml) and *BacLight*TM (cells/ml) were compared to the CFU/ml from the spread plate method. Since bacteria levels in waters are generally log-normally distributed, numbers are presented as $\log_{10}$ numbers for analysis. This simplifies the graphical presentation of data, since a linear scale with non-log transformed data would obscure trends present at low bacterial levels (Davis, et al., 2005).

Linear regression analysis (correlation coefficient [r], y intercept, and slope) was used to compare the three methods. Table 4.2 indicates the number of samples tested for Kranji Reservoir water, catchment water, and stormwater along with the linear regression analysis comparing results of the three methods. Figure 4.16 below represents the combined linear regression graph for all samples analyzed. A total of 74 samples consisting of 34 Kranji Reservoir waters (46.0%), 24 catchment waters (32.4%), and 16 stormwater (21.6%) were evaluated. The number ranges (in $\log_{10}$ scale) using the spread plate method are 2.40 to 3.53 for Kranji Reservoir waters, 3.15 to 5.53 for catchment water, and 5.11 to 6.08 for stormwater. A strong positive correlation was found between the *SimPlate* and spread plate method, with a slope of 1.02 and a y intercept of -0.09 for all samples analyzed. On the other hand, between the *BacLight*TM and spread plate method, there is a nonlinearity with a slope of -0.01 and a y intercept of 6.38. However, it is noted that with increasing bacteria number, the correlation between *BacLight*TM and spread plate method improved, where the slope increases from -0.02 to 0.70, and the y intercept decreases from 6.55 to 2.58.

Table 4.2 *SimPlate* / *BacLight*TM versus spread plate method. Field data for Kranji reservoir, catchment and stormwater were pooled together for the analysis

	n	<i>SimPlate</i>			<i>BacLight</i> TM		
		R ²	Slope	y intercept	R ²	Slope	y intercept
Kranji Reservoir	34	0.73	1.15	−0.53	0.003	−0.02	6.55
Catchment	24	0.93	0.89	0.53	0.26	0.32	4.68
Stormwater	16	0.97	0.99	0.02	0.65	0.70	2.58
Combined	74	0.97	1.02	−0.09	0.002	−0.01	6.38

The traditional spread plate method measures the culturability of bacteria, which requires a single cell to form visible colonies on a solid medium under specified test conditions (e.g. medium nutrients, incubation time, incubation temperature etc.). The *SimPlate* developed by IDEXX Laboratories contains substrates that are hydrolyzed by microbial enzymes to release 4-methylumbelliferone, which fluoresces blue under a long-wavelength (365 nm) 6 W UV light after incubation for 48 hours at 35 °C. The bacteria are then detected as fluorescent wells on the plate, and the number of fluorescent wells was converted into most probable number (MPN) using the MPN table provided. The *SimPlate* method is also considered a culture based method, since it is accessed under specified test conditions which are the same as the spread plate method. The positive correlation between these two methods suggests that *SimPlate* is a suitable replacement for the spread plate method in raw waters (Hoefel et al., 2003b; Jackson et al., 2000). It has advantages of no plate preparation and ease of counting fluorescent wells compared to colony counting. Although it may be a suitable replacement, it has similar problems to the spread plate method in the requirement of time consuming incubation time which is usually 48 hours (Hoefel et al., 2003b).

On the other hand, the rapid detection method using *BacLight*TM dyes can give faster results, typically within 30 minutes. The principle of *BacLight*TM dyes is based on membrane integrity rather than culturability. Although it does not directly measure the viability/culturability of bacteria, it measures one of the physiological activities – membrane integrity, which is the key criteria to be satisfied before the conclusion of viability (Breeuwer and Abee, 2000). From this study, it is found that the number of active bacteria reported by using the *BacLight*TM was 2.44 – 4.06 log fold higher than the spread method for Kranji Reservoir water (number between 2.50E+02 cfu/ml and 3.40E+03 cfu/ml measured by spread plate method), 0.69 – 2.85 log fold higher for catchment water (number between 1.40E+03 cfu/ml and 3.41E+05 measured by spread plate method), and 0.70 – 1.21 log fold higher for stormwater (number between 1.30E+05 cfu/ml and 1.22E+06 cfu/ml). These results are similar to other studies which concluded the level of difference to be 2 – 4 log fold higher when using bacteria physiological activity assessment compared to culturability assessment (Hoefel et al., 2003b; Porter et al., 1995; Yamaguchi and Nasu, 1997). Hoefel et al. (2003b) also found the difference between numbers of physiologically active and culturable bacteria to be nonlinear. Although the traditional spread plate method was consistent in reporting fewer culturable bacteria compared to the rapid assays, such as *BacLight*TM, a linear correlation did not exist. As a result, a culturable number could not correlate to the number of physiologically active bacteria by estimation via a standard curve against the rapid assay results. The reason for this nonlinear relationship may include differences in the percentage of heterotrophic versus non-heterotrophic bacteria between samples, in addition to different proportions of sublethally injured or ABNC bacteria (Hoefel et al., 2003b).

Stormwater contains higher levels of nutrient which favor the maintenance and growth of bacteria. Bacteria in stormwater, which is considered as polluted water contains more nutrient, are highly active and their physiological conditions may be similar to laboratory strains (Yamaguchi and Nasu, 1997). On the other hand, the water inside the Kranji Reservoir has a lower nutrient level compared to stormwater, where the bacteria

cells could be subjected to stress and become debilitated until cell death (McDougald et al., 1998). At this stage, bacteria cells may maintain some metabolic activity but are not able to be cultured. These cells are considered to be in active but non culturable (ABNC) state (Barer et al., 2000; Hoefel et al., 2003b; Kell et al., 1998; McDougald et al., 1998). Hence, they would not be detected by culturable (plate) method, but would be detected by the *BacLight*TM method.

Figure 4.16 *SimPlate* / *BacLight*™ combined field test data results compared with spread plate method

4.3.2 Indicator Bacteria *E. coli* and Enterococci

Health officials have traditionally used indicator organisms, such as total coliforms and faecal coliforms, to monitor safety of water quality. Nowadays, the U.S. EPA has recommended using *Escherichia coli* (*E. coli*) and enterococci as indicators for freshwater and brackish water, respectively (U.S. EPA, 1986). The *E. coli* and enterococci counts for the Kranji Reservoir water, its catchment tributaries, and stormwater are summarized in Figures 4.17, 4.18 and 4.19.

Generally, the *E. coli* and enterococci numbers met the U.S. EPA standards for recreational waters inside the Kranji Reservoir and its three main tributaries, which is 30 MPN/100 ml for *E. coli* and 33 MPN/100 ml for enterococci respectively: *E. coli* counts ranged from 0 to 16.1 MPN/100 ml and enterococci counts ranged from 0 to 37.3 MPN/100 ml. However, there is an exception on 29 September 2005 for the tributary Sungei PengSiang (PS) where very high *E. coli* and enterococci numbers were measured, i.e. 261 and 770 MPN/100 ml respectively. This is an indication of pollution probably due to high urban runoff loading or sewage leakage. For station 3 and station 4 with sampling at different depths (Figure 4.17), there is no trend of increasing indicator bacteria number with increasing depth as was previously found for total viable bacteria (Figure 4.13). The vertical trends for Kranji Reservoir, which shows total viable bacteria varies with depth but not for indicator bacteria, is contrary to some other studies (Davis et al., 2005).

For the catchment tributaries, the *E. coli* and enterococci numbers vary significantly for different catchment tributaries and on different dates. The *E. coli* number ranged from 110 to 7,701 MPN/100 ml and the enterococci number ranged from 10 to 19,863 MPN/100 ml. It is observed that on 23 November 2005, the number for both indicator bacteria are quite high at most catchment tributaries. This is probably due to the rain that occurred in the early morning (4am). Relationships between meteorological

parameters and microbial loads have been well described. The numbers of *E. coli*, coliform bacteria, and enterococci increased after rainfall (Ferguson et al., 1996).

Figure 4.19 below shows the fluctuation in numbers of the indicator organisms during 2 hours of stormwater sampling. For the first rainfall event (November 23, 2005), the highest numbers of *E. coli* and enterococci were 45,690 and 241,960 MPN/100 ml, respectively. This followed a 4.04 cm rainfall event with highest water flow quantity of 4.7 m³/s of stormwater. For another rainfall sampling event on January 18, 2006, the highest numbers of indicator organisms observed for *E. coli* and enterococci were 7,890 and 7,880 MPN/100 ml, respectively. For this 0.96 cm rain event, the highest water flow was 1.2 m³/s. Comparing two sets of stormwater data, it was observed that increased numbers of indicator organisms were associated with higher flow (Q), which in turn was affected by rainfall intensity. This trend for indicator bacteria contradicts the trend for total viable bacteria (heterotrophic plate count in Figure 4.15), which shows stable numbers regardless of flow rate or rainfall intensity. However, for each storm, the numbers of indicator organisms were not affected by the flow quantity (Q), and the highest enterococci number occurred suddenly whilst the *E. coli* number remained stable. It is interesting that elevated enterococci numbers were higher than those for *E. coli* (Figure 4.19). The literature notes that enterococci found in the environment can have sources other than sewage or faeces, such as plant material, reptiles and insects (Grant et al., 2001). Higher enterococci number with lower *E. coli* number may indicate other sources of microbial contribution other than sewage leakage (Jeng et al., 2005).

Figure 4.17 Indicator bacteria *E. coli* and enterococci count by *Colilert*-18 and *Enterolert* in Kranji Reservoir and its three main tributaries

Figure 4.18 Indicator bacteria *E. coli* and enterococci count by Colilert-18 and Enterolert for catchment tributaries

Figure 4.19 Indicator bacteria *E. coli* and enterococci count by Colilert-18 and Enterolert for stormwater at different flow rates Q

CHAPTER 5 CONCLUSION AND RECOMMENDATION

The study demonstrated the successful application of *BacLight*TM bacterial kit in conjunction with flow cytometry to effectively discriminate between active (membrane-intact) and dead (membrane-compromised) populations of *E. coli* (representative of Gram negative bacterial cells) and *E. faecalis* (representative of Gram positive bacterial cells) in diverse environmental water matrices. It was shown that different cell types generated different fluorescence signals regardless of activity state, resulting in a large coefficient of variation for the populations in analyses of samples containing a heterogeneous mix of bacterial populations. The results showed the presence of a population of cells with damaged cell membranes that existed in a state of transition between membrane-intact and membrane-compromised, in the analyses using stationary phase cells. It was also observed that differences in the biogeochemistry of fresh water and seawater environments substantially affect the performance of *BacLight*TM staining. This may be due to interferences from ionic strength with probe hybridization and DNA/RNA recovery. As a result, fluorescent dyes or probes used must be carefully chosen to best suit different water matrices. Furthermore, it is also necessary to carry out matrix-specific calibrations prior to actual analyses in order to determine the exact positions of target population in different water matrices.

In the case of esterified fluorogenic substrate, CFDA, it was found that the chemical could discriminate between metabolically active and inactive cells only for *E. faecalis*, but not for *E. coli*. The inability of CFDA to discriminate between active and inactive *E. coli* was due to the presence of active extrusion pumps involved in pumping out the fluorescent product carboxy-fluorescein (CF). As a result, CFDA is not suitable as an indicator of bacterial activity when analyzing environmental samples involving both Gram positive and Gram negative bacteria.

The investigation on the effects of glutaraldehyde fixation on the efficiency of the assays suggests that Gram negative cells such as *E. coli* respond positively to the treatment, with better staining efficiency, specially for *E. coli* in seawater. On the other hand, treatment with the fixative caused significant damage to the cell membranes of Gram positive cells such as *E. faecalis* in fresh water, resulting in an apparent loss in cell activity. Similarly, freeze preservation following glutaraldehyde fixation appeared to be harsher for *E. faecalis* cells than for *E. coli* cells in fresh water. Due to the detrimental effects of glutaraldehyde fixation and freeze preservation on *E. faecalis* cell activity in fresh water, it is recommended that analyses of fresh water involving Gram positive bacterial cells should be carried out without undergoing such treatments. The result also showed that for seawater analysis, glutaraldehyde treatment should be applied to avoid misleading results.

In accessing the water quality of Kranji Reservoir, its catchment runoff and stormwater, the rapid detection method *BacLight*TM kit with flow cytometry, together with the traditional culture based methods of HPC and *SimPlate* method were used to access the viable / active bacteria in water samples. HPC method reported good linear relationship with the *SimPlate* method. However, both HPC and *SimPlate* methods give significantly fewer viable counts when compared to the number of active cells detected using the *BacLight*TM kit with flow cytometry. Although there was no consistent proportionality between numbers of active and culturable bacteria, it was noted that the more nutrient enriched the water, e. g. stormwater, the better the linearity obtained between HPC and *BacLight*TM result. It is also because the cells in stormwater are “fresh”, i.e. they have only recently left their original source and therefore show increased levels of viability. Comparing these three methods, *BacLight*TM kit with flow cytometry can be processed within one hour, and it yields both quantitative (cell count) and qualitative (for membrane-damaged and membrane-compromised cells) information for each analyzed sample. This quick and more accurate approach is very useful for routine sampling and analyses.

In addition to the viable / active bacteria, the indicator bacteria *E. coli* and enterococci were also analyzed using the commercial kits, *Colilert-18* and *Enterolert*, from IDEXX. The number of indicator bacteria inside the Kranji Reservoir met U.S.EPA standards for recreational water. However, for the catchment runoff, the number of indicator bacteria varied significantly for different catchment points, which maximum values of 7,701 MPN / 100 ml and 19,863 MPN / 100ml for *E. coli* and enterococci respectively. For stormwater, the number of indicator bacteria was even higher for large rainfall events compared to small rainfall events. However, within the same stormwater event, there was no apparent relationship between the number of indicator bacteria and the stormwater flow Q .

It was clear that the bacterial quality of surface water runoff was poor, yielding high bacterial loads and pathogens to the reservoir. Natural conditions, as well as human activities, in catchment areas of surface reservoirs significantly affect the quality of surface runoff. In addition to routine analytical monitoring of microbial parameters of water samples, it is important to evaluate systematically the environmental condition of the catchment areas and their roles in microbial contamination of surface water. Further studies on nutrient loading, source of pathogens, and survival of pathogenic bacteria can be carried out to enhance the understanding of pathogens and their indicators in tropical surface waters.

REFERENCES

- Amor, K.B., Breeuwer, P., Verbaarschot, P., Rombouts, F.M., Akkermans, D.L., Vos, M.D., Abee, T., 2002. Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead bifidobacterium cells during bile salt stress. *Applied and Environmental Microbiology* 68, 5209-5216.
- Ananta, E., Heinz, V., Knorr, D., 2004. Assessment of high pressure induced damage on *Lactobacillus rhamnosus* GC by flow cytometry. *Food Microbiology* 21, 567-577.
- Barbesti, S., Citterio, S., Labra, M., Baroni, M.D., Neri, M.G., Sgorbati, S., 2000. Two and three-color fluorescence flow cytometric analysis of immunoidentified viable bacteria. *Cytometry* 40, 214-218.
- Barer, M.R., Smith, R.J., Cooney, R.P., Kimmitt, P.T., 2000. Relationships between culturability, activity and virulence in pathogenic bacteria. *Journal of Infection and Chemotherapy* 6, 108-111.
- Bernard, L., Courties, C., Duperray, C., Schafer, H., Muyzer, G., Lebaron, P., 2001. A new approach to determine the genetic diversity of viable and active bacteria in aquatic ecosystems. *Cytometry* 43, 314-321.
- Biggerstaff, J.P., Puil M.Le., Weidow, B.L., Prater, J., Glass, K., Radosevich, M., and White, D.C., 2006. New methodology for viability testing in environmental samples. *Molecular and Cellular Probes* 20, 141-146.
- Bischoff, C., Lüthy, J., Altwegg, M., and Baggi, F., 2005. Rapid detection of diarrheagenic *E. coli* by real-time PCR. *Journal of Microbiological Methods* 61, 335-341.

Boulos, L., Prevost, M., Barbeau, B., Coallier, J., Desjardins, R., 1999. Live/Dead BacLight™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal of Microbiological Methods* 37, 77-86.

Breeuwer, P., Abee, T., 2000. Assessment of viability of microorganisms employing fluorescence techniques. *International Journal of Food Microbiology* 55, 193-200.

Bullock, G.R., 1984. The current status of fixation for electron microscopy: a review. *Journal of Microscopy* 133, 1-15.

Bunthof, C.J., Braak, S.V., Breeuwer, P., Rombouts, F.M., Abee, T., 1999. Rapid fluorescence assessment of the viability of stressed *Lactococcus lactis*. *Applied and Environmental Microbiology* 65, 3681-3689.

Chan, A.B., Fox, J.D., 1999. NASBA and other transcription-based amplification methods for research and diagnostic microbiology. *Review in Medical Microbiology* 10, 185-196.

Comas, J., Vives-Rego, J., 1998. Enumeration, viability and heterogeneity in *Staphylococcus aureus* cultures by flow cytometry. *Journal of Microbiological Methods* 32, 45-53.

Davies, C.M., Ferguson, C.M., Kaucner, C., Krogh, M., Altavilla, N., Deere, D.A. and Ashbolt, N.J., 2004. Dispersion and transport of *Cryptosporidium* oocysts from fecal pats under simulated rainfall events. *Applied and Environmental Microbiology* 70, 1151-1159.

Davis, K., Anderson, M.A., Yates, M.V., 2005. Distribution of indicator bacteria in Canyon Lake, California. *Water Research* 39, 1277-1288.

Diaper J.P., Tither K., Edwards C., 1992. Rapid assessment of bacterial viability by flow cytometry. *Applied Microbiology and Biotechnology* 38, 268-272.

Diaper, J.P., Edwards, C., 1994. The use of fluorogenic esters to detect viable bacteria by flow cytometry. *Journal of Applied Bacteriology* 77, 221-228.

Ferguson, C.M., Coote, B.G., Ashbolt, N.J., and Stevenson, I.M., 1996. Relationships between indicators, pathogens and water quality in an estuarine system. *Water Research* 30, 2045-2054

Fuchs, B.M., Wallner, G., Beisker, W., Schwiippl, I., Ludwig, W., Amann, R., 1998. Flow cytometry analysis of the in situ accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. *Applied and Environmental Microbiology* 64, 4973-4982.

Gorman, S.P., Scott, E.M., and Russell, A.D., 1980. Antimicrobial activity, uses and mechanism of action of glutaraldehyde. *Journal of Applied Bacteriology* 48, 161-190.

Grant, S.B., Sanders, B.F., Boehm, A.B., Redman, J.A., Kim, J.H., 2001. Generation of enterococci bacteria in a coastal saltwater marsh and its impact on surface zone water quality. *Environmental Science Technology* 35, 2407-2416.

Grégori, G., Denis, M., Lefèvre, D., Romano, J.D., 2003. Viability of heterotrophic bacteria in the Bay of Marseilles. *Comptes Rendus Biologies* 326, 739-750.

Gruden, C.L., Khijniak, A., Adriaens, P., 2003. Activity assessment of microorganisms eluted from sediments using 5-cyano-2,3-ditolyl tetrazolium chloride: a quantitative comparison of flow cytometry to epifluorescent microscopy. *Journal of Microbiological Methods* 554, 865-874.

Gruden, C., Skerlos, S., Adriaens, P., 2004. Flow cytometry for microbial sensing in environmental sustainability applications: current status and future prospects. *FEMS Microbiology Ecology* 49, 37-49.

Harmsen, H.J.M., Gibson, G.R., Elfferich, P., Raangs, G.C., Wildeboer-Veloo, A.C.M., Argai, A., Roberfroid, M.B., Welling, G.W., 1999. Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human fecal bacteria. *FEMS Microbiology Letters* 183, 125-129.

Hayat, M.A., 1986. Glutaraldehyde: role in electron microscopy. *Micron and Microscopica Acta* 17, 115-135.

Hoefel, D., Grooby, W.L., Monis, P.T., Andrews, S., Saint, C.P., 2003a. A comparative study of carboxyfluorescein diacetate and carboxyfluorescein diacetate succinimidyl ester as indicators of bacterial activity. *Journal of Microbiological Methods* 52, 379-388.

Hoefel, D., Grooby, W.L., Monis, P.T., Andrews, S., Saint, C.P., 2003b. Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. *Journal of Microbiological Methods* 55, 585-597.

Jackson, R.W., Osborne, K., Barnes, G., Jolliff, C., Zamani, D., Roll, B., Stillings, A., Herzog, D., Cannon, S., Loveland, S., 2000. Multiregional evaluation of the *SimPlate* heterotrophic plate count method compared to the standard plate count agar pour plate method in water. *Applied and Environmental Microbiology* 66, 453-454.

Jamil, S., Keer, J.T., Lucas, S.B., Dockrell, H.M., Chiang, T.J., Hussain, R., Stoker, N.G., 1993. Use of polymerase chain reaction to assess efficacy of leprosy chemotherapy. *The Lancet* 342, 264-268.

Jacobsen, C.N., Rasmussen, J., Jakobsen, M., 1997. Viability staining and flow cytometric detection of *Listeria monocytogenes*. *Journal of Microbiological Methods* 28, 35-43.

Jeng, H.A.C., Engle, A.J., Baker, R.M., and Bradford, H.B., 2005. Impact of urban stormwater runoff on estuarine environmental quality. *Estuarine, Coastal and Shelf Science* 63, 513-526.

Jepras, R.I., Carter, J., Pearson, S.C., Paul, F.E., Wilkinson, M.J., 1995. Development of a robust flow cytometric assay for determining numbers of viable bacteria. *Applied and Environmental Microbiology* 61, 2696-2701.

Jin, G., Engle, A.J., Bradford, H., Jeng, H.C., 2000. Indicator organisms of water quality in lake Pontchartrain. *Proceeding of WEFEC' 00*, June 10-13, 2000, New Orleans.

Joux, F., Lebaron, P., 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes and Infection* 2, 1523-1535.

Keer, J.T., Birch, L., 2003. Molecular methods for the assessment of bacterial viability. *Journal of Microbiological Methods* 53, 175-183.

Kell, D.B., Kaprelyants, A.S., Weichert, D.H., Harwood, C.R., Barer, M.R., 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie van Leeuwenhoek* 73, 169-187.

Kistemann, T., Claben, T., Koch, C., Dangendorf, F., Fischeder, R., Gebel, J., Vacata, V., and Exner, M., 2002. Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. *Applied and Environmental Microbiology* 68, 2188-2197.

Koester, S.K., Bolton, W.E., 2000. Intracellular markers. *Journal of Immunological Methods* 243, 99-106.

Langsrud S., Sundheim G., 1996. Flow cytometry for rapid assessment of viability after exposure to a quaternary ammonium compound. *Journal of Applied Bacteriology* 81, 411-418.

Lebaron, P., Parthuisot, N., and Catala, P., 1998. Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. *Applied and Environmental Microbiology* 64, 1725-1730.

López-Amorós, R., Castel, S., Comas-Riu, J., Vives-Rego, J., 1997. Assessment of *E. coli* and *Salmonella* viability and starvation by confocal laser microscopy and flow cytometry using Rhodamine 123, DiBAC4(3), Propidium Iodide, and CTC. *Cytometry* 29, 298-305.

Madigan, M.T., Martinko, J.M., and Parker J., 2003. *Brock Biology of Microorganisms*, 10th edition, Prentice Hall.

Marsalek, J., Rochfort, Q., 2004. Urban wet-weather flows: sources of fecal contamination impacting on recreational waters and threatening drinking-water sources. *Journal of Toxicology and Environmental Health* 67A, 1765-1777.

Masters, C.I., Shallcross, J.A., Mackey, B.M., 1994. Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. *Journal of Applied Bacteriology* 77, 73-79.

McDougald, D., Rice, S.A., Weichart, D., Kjelleberg, S., 1998. Nonculturability: adaptation or debilitation? *FEMS Microbiology Ecology* 25, 1-9.

Molecular Probes, 1995. Live/Dead *BacLight*TM bacteria viability kit technical sheet. Molecular Probes Inc.

Molecular Probes, 2003. Live/Dead *BacLight*TM bacteria viability kit product information sheet. Molecular Probes Inc.

Nebe-von-Caron, G., Stephens, P., Badley, R.A., 1998. Assessment of bacterial viability status by flow cytometry and single cell sorting. *Journal of Applied Microbiology* 84, 988-998.

Nebe-von-Caron, G., Stephens, P.J., Hewitt, C.J., Powell, J.R., Badley, R.A., 2000. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting 42, 97-114.

Nikaido, H., Vaara, M., 1985. Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews* 49, 1-32.

Nikaido, H., 1996. Outer membrane. In: *Escherichia coli* and *Salmonella*. Washington D.C: American Society for Microbiology 29-47.

Noble, R.T., Leecaster, M.K., McGee, C.D., Weisberg, S.B., and Ritter K., 2004. Comparison of bacterial indicator analysis methods in stormwater-affected coastal waters. *Water Research* 38, 1183-1188.

Norton, D.M., Batt, C.A., 1999. Detection of viable *Listeria monocytogenes* with a 5' nuclease PCR assay. *Applied and Environmental Microbiology* 65, 2122-2127.

Novo, D., Perlmutter, N.G., Hunt, R.H., Shapiro, H.M., 1999. Accurate flow cytometric membrane potential measurement in bacteria using Diethyloxycarbocyanine and a ratiometric technique. *Cytometry* 35, 55-63.

Novo, D.J., Perlmutter, N.G., Hunt, R.H., Shapiro, H.M., 2000. Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. *Antimicrobial Agents and Chemotherapy* 44, 817-834.

Olivieri, V.P., Kawata, K., and Lim, S.H., 1989. Microbiological impacts of storm sewer overflows: some aspects of the implication of microbiological indicators for receiving waters. In *Urban discharges and receiving water quality impacts*, ed. J. B. Ellis, 47-54. Oxford: Pergamon Press.

Pernthaler, A., Amann, R., 2004. Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria. *Applied and Environmental Microbiology* 70, 5426-5433.

Porter J., Diaper, J., Edwards, C., Pickup R., 1995. Direct measurements of natural planktonic bacterial community viability by flow cytometry. *Applied and Environmental Microbiology* 61, 2783-2786.

Porter, J., Deere, D., Hardman, M., Edwards, C., Pickup, R., 1997. Go with the flow – use of flow cytometry in environmental microbiology. *FEMS Microbiology Ecology* 24, 93-101.

Proctor, L.M. and Souza, A.C., 2001. Method for enumeration of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC)- active cells and cell-specific CTC activity of benthic bacteria in riverine estuarine and coastal sediments. *Journal of Microbiological Methods* 43, 213-222.

Pyle, B.H., Broadaway, S.C., McFeters G.A., 1995. Factors affecting the determination of respiratory activity on the basis of cyanoditolyl tetrazolium chloride reduction with membrane filtration. *Applied and Environmental Microbiology* 61, 4304-4309.

Rieseberg, M., Kasper, C., Reardon, K.F., Scheper, T., 2001. Flow cytometry in biotechnology. *Applied Microbiology and Bitotechnology* 56, 350-360.

Servais, P., Agogu, H., Courties, C., Joux, F., Lebaron, P., 2001. Are the actively respiring cells (CTC+) those responsible for bacterial production in aquatic environments? *FEMS Microbiology Ecology* 35, 171-179.

Shapiro, H.M., 2003. *Practical flow cytometry*, 4th edition, Wiley Liss, New York

Smith, J.J., McFeters G.A., 1997. Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in *Escherichia coli* K-12. *Journal of Microbiological Methods* 29, 161-175.

Stocks, S.M., 2004. Mechanism and use of the commercially available viability stain, *BacLight*. *Cytometry* 61A, 189-195.

Swarts A.J., Hastings J.W., Roberts R.F., won-Holy A., 1998. Flow cytometry demonstrates bacteriocin induced injury to *Listeria monocytogenes*. *Current Microbiology* 36, 266-270.

Tanaka, Y., Yamaguchi, N., and Nasu, M., 2000. Viability of *Escherichia coli* O157:H7 in natural river water determined by the use of flow cytometry. *Journal of Applied Microbiology* 88, 228-236.

Troussellier, M., Courties C., Zettelmaier, S., 1995. Flow cytometric analysis of coastal lagoon bacterioplankton and picophytoplankton: fixation and storage effects. *Estuarine, Coastal and Shelf Science* 40, 621-633.

Ueckert, J.E., Nebe-von-Caron, G., Bos, A.P., ter-Steeg P.F., 1997. Flow cytometric analysis of *Lactobacillus plantarum* to monitor lag times, cell division and injury. *Letters in Applied Microbiology* 25, 295-299.

U.S. EPA, 1986. Ambient water quality criteria for bacteria. EPA 440/5-84-002. U.S. Environmental Protection Agency, Washington, D.C.

Vaara, M., 1992. Agents that increase the permeability of the outer membrane. *Microbiology Reviews* 56, 395-411.

Vaulot, D., Courties, C., Partensky, F., 1989. A simple method to preserve oceanic phytoplankton for flow cytometric analyses. *Cytometry* 10, 629-635.

Veal, D.A., Deere, D., Ferrari, B., Piper, J., Attfield, P.V., 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. *Journal of Immunological Methods* 243, 191-210.

Vives-Rego, J., Lebaron, P., Nebe-von-Caron, G., 2000. Current and future applications of flow cytometry in aquatic microbiology. *FEMS Microbiology Reviews* 24, 429-448.

Ward, D.M., Weller, R., Bateson, M.M., 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* 345, 63-65.

Yamaguchi, N., Nasu, M., 1997. Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. *Journal of Applied Microbiology* 83, 43-52.

Ziglio, G., Andreottola, G., Barbesti, S., Boschetti, G., Bruni, L., Foladori, P., Villa, R., 2002. Assessment of activated sludge viability with flow cytometry. *Water Research* 36, 460-468.