Freeze-Thaw Effect on Selected Fecal Indicator Bacteria: *Escherichia coli* and *Enterococcus faecalis*

Submitted in partial completion of degree requirements

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Abstract

The survival of two selected fecal indicator organisms, two strains of *Escherichia coli*, a Gram-negative bacterium, and two strains of *Enterococcus faecalis*, a Gram-positive bacterium, after freezing and thawing successively for five cycles was determined using a drop plating method. It was found that all bacterial strains, when an initial concentration of 1.0 x E^{+08} was used, showed significant decreases in their ability to be cultured ($p < 0.05$) when frozen and thawed at -15°C, compared to three other freezing temperatures, -7, -30 and -80°C, when all four were frozen for 24 hours. In addition, the number of culturable cells at -7, -30, or -80°C were not significantly different from each other ($p > 0.05$). The differences in cell inactivation between the two strains of each species of bacteria tested at all temperatures was not significantly different after five freeze-thaw cycles; while the difference between species was shown to be significant, depending on the temperature and condition tested ($p < 0.05$). When comparing small sample volume sizes (100µl) to larger sample volume sizes (100ml) the observed differences were that *Escherichia coli* strains showed a decrease in cell culturability at both -7°C and -15°C when cycled in the larger volume; whereas, *Enterococcus faecalis* strains showed a decrease in cell culturability at -7°C and an increase in cell culturability at -15°C when cycled at the larger volume.

Additional studies investigating culturability, cell wall integrity, and membrane damage of the bacterial strains were conducted using 100ml samples, cycled at -7, -15, and -30°C, and evaluated by three microbiological methods: drop plating, epi-fluorescent microscopy, and flow cytometry, respectively. In all instances, the plate counting method indicated that there was a decrease in cells that were culturable. Results from flow

cytometry indicated a smaller decrease in cell culturability, followed, lastly, by results using the epi-fluorescent microscope. Thus, these studies would suggest that the most damage that occurs to frozen and thawed cells, when cycled five times at -7, -15 or -30°C, would be due to damages occurring at the cellular level, rather than damages occurring on the cell envelope, since less cells were able to uptake nutrients from the culture plates.

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Table of Contents

List of Figures

Chapter 1: Literature Review

1.1 Introduction

From the beginning of the 20th Century it has been known that cultures of bacteria can be cooled to very low temperatures without destroying all of the cells present (Haines, 1938). Many bacteria often naturally encounter very cold temperatures, including the Arctic and Antarctic regions, where the temperature is extremely cold (Kawahara, 2002). Also, in many Northern rural communities, rivers and streams experience natural freezing and thawing, and it is these rivers and streams that are the source of drinking water, or the place where waste water is deposited. Bacteria that are within these aquatic environments can possibly be diluted through the freezing and thawing processes, whereby, rendering them dead or extremely injured without requiring expensive disinfectants (Parker *et al.*, 2000). Also in these regions, freezing is becoming a feasibly economic technique for sludge conditioning or waste water treatment (Gao *et al.*, 2007).

The stress of freezing and thawing of bacterial cells can result in many profound alterations within the cells, most likely affected by an altered structure of the outer membrane, both in the lipopolysaccharide and protein components (Calcott and Calcott, 1984). It is possible that damage to these components explain the variability in sensitivity of Gram-negative bacteria in stressful environments such as water bodies, frozen foods, and other locales where the organisms with injured walls may be detected (Calcott and Calcott, 1984).

1.2 Properties of the bacterial cell envelopes

The nature of the bacterial cell wall plays an important role in the bacterium's resistance to freezing and thawing (Calcott, 1978). Gram-positive bacterial cells have a complex cell wall that consists of a plasma membrane, lipoteichoic acids, many layers of a lattice structured peptidoglycan, and wall teichoic acids (Tortora *et al*, 2001). The wall teichoic acids are highly charged anionic polymers of polyol phosphate (D'Elia *et al.*, 2006), and they take on a role in cell growth, preventing extensive wall breakdown and possible cell lysis (Tortora *et al.*, 2001). The multiple layers of peptidoglycan also help to give the cell a rigid structure, which allows for increased resistance to both physical (e.g., freezing and thawing) and chemical (e.g., chlorine) stresses.

On the other hand, Gram-negative bacterial cells have a plasma membrane, a lipopolysaccharide layer, and a single, thin, layer of peptidoglycan (Tortora *et al.*, 2001). Both the plasma membrane and the lipopolysacharide layers are very weak, and as such they do not help hold the structure of the cell; also they are the first layers to be affected when the cell undergoes either physical or chemical stresses (Tortora *et al.*, 2001).

1.3 Fecal Indicator organisms

In the aquatic environment, bacteria are exposed to many physical and chemical stressors that sometimes create difficulties when enumeration is required during water inspection. Since it is difficult to test for all possible water-borne pathogens, indicator organisms are enumerated, such as fecal coliforms, and their presence is an estimation of the total amount of pathogenic organisms in the tested water. The occurrence of coliform bacteria in otherwise high-quality drinking water has been the nemesis of the water industry (LeChevalier, 1990). Sometimes the injury incurred upon the indicator

organisms is more than would be found on any of the pathogenic bacteria, and thus an inappropriate measure of the possible contaminants results (McFeters and Camper, 1983).

Accepted indicators of potential health hazards associated with fecal pollution are fecal coliforms and fecal streptococci (Pettibone *et al.*, 1987). These indicator bacteria are not considered to be part of the indigenous flora of soil and aquatic ecosystems, and as such they can be killed or injured by environmental stresses (Moss and Speck, 1966), which offers them the benefit of being identified as indicator organisms. Two examples of fecal indicator organisms are *Escherichia coli* and *Enterococcus faecalis* and both are often tested for during routine water safety checks in many water systems, however, it is more common to test for *Escherichia coli* than *Enterococcus faecalis*.

1.3.1 *Escherichia coli*

Escherichia coli is a Gram-negative bacterium, rod in shape, and usually found singly or in pairs. It can be found in the intestinal track of warm blooded animals and humans, and it usually does not cause disease. However, there are a few serotypes that are pathogenic, disease-causing, bacteria; for instance, the O157:H7 serotype, also known as enterohemorrhagic *Escherichia coli*, can cause epidemic and sporadic gastroenteritits, and can lead to hemolytic uremic syndrome (HUS), a devastating and sometimes fatal complication characterized by microangiopathic hemolytic anemia, thrombocytopenia, and renal failure (Besser *et al.*, 1999). *Escherichia coli* infections are most often acquired through consumption of contaminated food or water; also it is possible for person-to-person contact to cause spreading (Besser *et al.*, 1999).

1.3.2 *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive bacterium, round, or cocci, in shape, and generally found in pairs. It can be found as normal flora in humans and some warm-blooded animals (Geldreich and Kenner, 1969), and does not usually cause disease in healthy individuals. However, there are a few strains of *Enterococcus faecalis* that have grown antibiotic resistance to many drugs, most recently vancomycin, which causes a problem if infection were to occur, because antibiotic choice would be very limited (Huycke *et al.*, 1998).

1.4 Freezing and thawing of bacterial cells

In 1918, Hilliard and Davis concluded that cycling the bacteria through freezing and thawing can be considered a more effective germicidal action than continuous freezing.

1.4.1 Freezing of bacterial cells

Cells often become frozen once they past the supercooled stage (at the freezing point, but not yet frozen), thus resulting in freezing (Lund, 2000). For some microorganisms, this exposure to freezing can either physically disable or extremely injure them, such that, many microorganisms, upon enumeration, may not be cultured using common enumeration methods (Bissonnette *et al.*, 1975). During their study, Bissonnette *et al.* (1975) discovered that as the exposure time in the aquatic environment increased from 0 to 4 days, an increasing proportion of the cells surviving exhibited non-lethal injury. Thus they were able to be enumerated on the non-selective medium, but not on the selective medium employed by these investigators. It is a possibility that water treatment industries, while checking the quality of the water, would eliminate the non-

selective media, as they are only interested in selecting for fecal coliforms, fecal streptococci, and total coliforms. The use of a non-selective medium may result in too much background bacteria growing on the agar, with most not being recognized as acceptable indicator organisms. However, using a selective agar medium to enumerate indicator organisms that are injured may result in low detection of the injured or disabled pathogenic bacteria. Furthermore, the pathogenic bacterial cells that are not detected, may recover while in the water system, and cause a public outbreak of disease, such as hemolytic uremic syndrome that can occur with *Escherichia coli* O157:H7 serotype (Besser *et al.*, 1999).

1.4.1.1 Factors that affect freezing of bacterial cells

There are many factors that can affect bacteria cells during freezing, some of which include: 1) thickness of cell wall, dependent on the type of bacteria; 2) freezing rate; and 3) freezing temperature.

Generally, it is known that Gram-positive bacteria are more resistant than Gram-negative bacteria to all physical stressors including freezing and thawing (Lund, 2000). One possible difference between Gram-positive bacteria and Gram-negative bacteria is their permeability to water (Mazur, 1963). Thus, the permeability of the cell relies partly on the depth of cell membrane a bacterium has. For example, *Escherichia coli*, a Gram-negative bacterium, has a very thin cell membrane, which would help to make the cell very permeable to water; whereas, *Enterococcus faecalis*, a Gram-positive bacterium, has a thicker cell wall that would help keep water inside the cell, aiding this bacterium to maintain viability and avoid injury during freezing and thawing.

The amount of injury that is incurred is dependent on the type of microorganisms; however, generally, bacterial cells have an optimal freezing rate of between 6° and 11°C per minute (Parker and Martel, 2002). At freezing rates that are faster than this optimal (>10°C/min, Mazur, 1966), water is unable to move from the cell to the surrounding medium, resulting in intracellular ice nucleation. At freezing rates that are slower than the optimal (<10°C/min, Mazur, 1966), water is able to efflux from the cell to the surrounding medium, resulting in extracellular ice nucleation as well as dehydration to the bacterial cell (Dumont *et al.*, 2006). In their study, Dumont *et al.* (2004) determined that both lower cooling rates and very rapid cooling rates (>100°C/min, Mazur, 1966) (both rates are in relation to the optimal) are less detrimental for all cells tested (two yeasts cells and two bacterial strains, including one *Escherichia coli* - K12 strain).

Different holding temperatures below freezing can have differing effects on bacterial cells. At lower holding temperatures, it is possible for the cell to continuously lose water, initially dehydrating the cell, but eventually denaturing proteins and breaking down the cells membrane (Mazur, 1966). At higher holding temperatures, the ice that forms intracellularly remains; however, the damage can occur when the cell is thawed from this temperature, possibly resulting in increased injury or damage to the cell (discussed in section 1.4.1.2.2).

1.4.1.2 Mechanisms that affect freezing of bacterial cells

During the freezing and thawing process, whether singly or in a cycling method, there are a few mechanisms that might be expected to contribute to the damage of microbial cells. Two of which include extracellular ice formation and intracellular ice

formation, both of which are affected by the rate of both freezing and thawing (Lund, 2000; Gao, 2006).

1.4.1.2.1 Dehydration of frozen bacterial cells

When the cell is in a suspending medium and is subjected to warmer freezing temperatures, the water outside of the cell begins to freeze first. This occurs because the cytoplasm is more concentrated than the suspending medium, and the water in the suspending medium is able to freeze before the cells interior does, increasing the osmotic pressure of the medium (Dumont *et al.*, 2006). When the water that is in the extracellular solution freezes, turning to ice, the remaining solute becomes highly concentrated (Dumont *et al.*, 2006). The difference in solute concentration inside the cell as compared to outside the cell creates an osmotic gradient which results in an efflux of water from the cytoplasm to the extracellular fluid; with the rate of the efflux limited by the permeability of the plasma membrane to water (Mazur, 1963; Muldrew and McGann, 1990). Eventually the water that was lost by the cell also becomes crystallized, extracellularly, increasing the osmotic gradient and the cell continues to lose water. Consequently, the cell loses water, dehydrating the cell, causing it to shrink (Haines, 1938), and the solute inside of the cell to become quite concentrated. Interestingly, Dumont *et al.* (2004) proposed that cell death occurs from ice crystallization forming during the outflow of water from the cell, which involves lethal membrane damage.

1.4.1.2.2 Ice crystallization in cells

In 1918, Hilliard and Davis observed that ice crystallization plays a key role in the killing of bacterial cells, more so than the cold temperatures themselves. This they determined by comparing fluid sugar solutions to solidified water kept at the same

temperature, and noted that with all other factors held as constant as possible, the death-rate was observed to be much higher in the solidified water (Hilliard and Davis, 1918).

Most often, at slower freezing rates, ice crystals form on the outside of the bacterial cell, resulting in water osmotically flowing to the suspending medium, causing dehydration, as discussed previously. On the contrary, at faster freezing rates, internal ice can form, because water inside the cell does not have a sufficient amount of time to escape (Mazur, 1984), and as such the ice that forms can potentially affect the cellular organelles or the whole cytoplasm, both of which will cause injury to the cell (Dumont *et al.*, 2006).

Once ice is formed within the cell, osmotic equilibrium is maintained through increasing ice formation rather than by movement of water across the plasma membrane (Muldrew and McGann, 1990). As such, it is assumed that increased amounts of intracellular freezing decreases the amount of bacterial survival (Bank and Mazur, 1973).

1.4.2 Thawing of bacterial suspensions

In prior decades it was difficult to determine the damage that occurred during freezing separate from that which occurred during thawing. Fortunately, there has been an increase in technology that now allows scientists the ability to detect the injury that occurs from, first, freezing and then, secondly, thawing while observing the bacterial cell using a cryo-microscope (Muldrew and McGann, 1990), or other such device.

Thawing of bacterial cells may increase the physical damage that occurs during the freeze-thaw process, because as thawing occurs, after the cell is subjected to higher than optimal freezing rates, the small crystals that formed during freezing, dissolve, and the dissolved water then attaches to neighbouring ice crystals, causing larger ice crystals

to form (Bank and Mazur, 1973, Mazur and Schmidt, 1968). Also, according to Dumont *et al.* (2006) high freezing rates and temperatures below -70°C are necessary to promote recrystallization during warming; as well, it is suggested that the rate of recrystallization increases with increasing temperature (Mazur and Schmidt, 1968). However, Calcott (1978) indicated that the rate of thawing generally has little to no effect on the survival of bacterial cells that are frozen at cooling rates less than 100°C/min.

1.5 Objectives

Incidentally, in 2001 it was estimated that twenty percent of the world's population lacked access to safe drinking water, and that more than five million people died annually from illnesses associated with unsafe drinking water or inadequate sanitation (Hunter *et al.*). Thus, it is important to ensure that the enumeration by plate counting is a true representation of what would occur in natural rivers or lakes, which is addressed in the next chapter.

For this study, examining the two different fecal indicator organisms gives a representation of the effects that would occur to other Gram-negative as well as other Gram-positive bacteria, which is very important when trying to expand this *in vitro* study to what would actually be observed in the natural aquatic environment.

The objectives of this study are to (i) determine the effects of freezing and thawing on the survival of selected fecal indicator species (*Escherichia coli* and *Enterococcus faecalis*) by examining the influence of freezing temperature and freeze-thaw cycling, (ii) determine if and how individual strains of different bacteria differ in their ability to survive freezing and thawing, and (iii) compare different methods of injury determination, to understand how the freeze-thaw causes damage on cell structure.

All of these objectives are to help indicate if freezing and thawing is a good pre-treatment technique for waste water and/or sludge in Northern rural communities. It has already been noted that these communities should use the natural freeze-thaw cycles that occur to dilute pathogens in sludge and waste water; however, the effect of such cycling on selected fecal indicator organisms has not been tested, as well as the potential for different effects on pathogenic and non-pathogenic strains of the same indicator species. This study also takes a look at what types of injury occur in the bacterial cells, in which very little is known.

Chapter 2: Freeze-Thaw on Bacterial Viability

2.1 Introduction

Freezing and frozen storage has been investigated in the past to have a germicidal effect on bacteria (Hilliard and Davis, 1918). They discovered that bacteria were killed by low temperatures and they suggested that it was because the bacteria were either ruptured by internal pressure from freezing of the cell contents, or by external pressure during crystallization with the expansion of frozen medium, leading to slow death from starvation (Hilliard and Davis, 1918). They also suggest that there is a greater germicidal influence by intermittent freezing and thawing, than continuous freezing; however, they mentioned that their work is not extensive enough to render the statement final.

Freezing at warmer temperatures creates a dehydration effect on the bacteria cell (Dumont *et al.*, 2006). Freezing at colder temperatures causes ice crystals to form on the inside of the bacterial cell (Mazur, 1984). This interior freezing generally corresponds to increased amounts of physical damage, by either cell rupture or membrane damage, to the bacterium (Bank and Mazur, 1973).

In this study, the effects of different freezing temperatures are considered. It is thought that the warm freezing temperatures tested indicate a slow freezing rate, whereas, the low freezing temperatures indicate a fast freezing rate. Freezing rate is considered to be the difference between the initial temperature and the final temperature divided by the time it took to freeze, and is measured in °C/h (Lund, 2000). Dumont *et al.* (2006) froze yeast cells to -196°C by using four different freezing rates to determine specific causes of cell mortality (25°C/min, 115°C/min, 180°C/min, and 1800°C/min). They observed that a common decrease in cell viability occurred at temperatures between 0°C and -5°C, and

that a second cell mortality relationship occurred within the two higher freezing rates, at approximately -70°C. They determined that the first increase in cell mortality was due to both crystallization outside of the cell, in the medium, and to the freezing rate, while the second increase in cell mortality was related to the influence of the warming rate in which recrystallization was able to occur.

Two *Escherichia coli* strains (an O157:H7 and a non-diarrheagenic strain) and two *Enterococcus faecalis* strains (a vancomycin resistant and a vancomycin sensitive strain) were used in this study. Unfortunately, most drinking water outbreaks are caused by the use of contaminated, untreated water, or due to inadequacies in treatment; the majority tend to occur in small water treatment systems (Craun, 1986). Thus, the objectives of this study were to determine: (1) the effect of different freezing temperatures on the survival of the four selected fecal indicator bacteria strains, (2) the effect of freeze-thaw cycles on these bacteria, and (3) the effect of the volume of the cell suspension on the results of the freezing treatments to the studied bacterial cells. The long-term goal of this study was to examine the effects of freezing and thawing on two common fecal indicator organisms so that knowledge can be acquired as to the effects on these bacteria of the natural freeze-thaw cycles of surface water, or sludge, in small, rural waste water treatment industries.

2.2 Materials and Methods

2.2.1 Bacterial strains

Four different strains of bacteria: *Escherichia coli* American Type Culture Collection (ATCC) (Manassa, VA, USA) strain 25922, *Escherichia coli* O157:H7 strain 961019 (also known as *Escherichia coli* H22) (Watterworth, *et al.*, 2006), *Enterococcus*

faecalis ATCC strain 29212, and *Enterococcus faecalis* ATCC strain 51299 were used in this study. Each of these strains were chosen for their difference in pathogenicity or antibiotic capability; *Escherichia coli* ATCC strain 25922, *Enterococcus faecalis* ATCC strain 29212, and *Enterococcus faecalis* ATCC strain 51299 (with a vancomycin resistance phenotype) are considered to be opportunistic pathogenic strains, whereas *Escherichia coli* strain 961019 is the pathogenic strain. The four bacterial strains were maintained in 25% glycerol, stored at -80°C, and re-grown in BD Bacto™ Tryptic Soy Broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA) at 37°C when needed.

2.2.2 Preparation of bacterial suspensions

To prepare the bacterial samples for testing at 100µl volumes, the four fecal bacterial strains were inoculated into four separate Erlenmeyer flasks (Kimble Glass Incorporated, Vineland, New Jersey, USA) that contained 25ml of sterile TSB which were placed in an Innova™ 4430 large stackable refrigerated incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA), set at 37°C and rotating at 150 rpm, overnight. Vancomycin (Sigma-Aldrich Inc., St. Louis, MD, USA) was added to the growth medium of *Enterococcus faecalis* ATCC 51299 to a final concentration of 4µg/ml because this strain is known to have a resistance to vancomycin, to ensure that the resistance did not diminish.

The optical density, set at 600nm (OD_{600nm}), of the overnight cultures were determined using a Biochrom Novaspec II visible spectrophotometer, RS232C (Biochrom Ltd., Cambridge, England) before inoculating each strain into 25ml of fresh TSB, all without antibiotic, to an OD_{600nm} of approximately 0.1. The four new flasks were then placed back into the large stackable refrigerated shaking incubator set at 37°C

and 150 rpm for approximately 1.5 hours or until the OD_{600nm} was approximately 0.6, or mid-log phase.

Individual cell cultures were poured into 50ml sterile, disposable centrifuge tubes (Fisher Scientific Ltd., Whitby, ON, Canada) and centrifuged using a table-top centrifuge (Thermo IER Centra CL3R) at 3000 x g for 10 minutes. The supernatant was discarded and the cells were resuspended in 25ml of sterile double distilled water and spun for another 10 minutes at 3000 x g to wash the cells. This wash step was repeated three times, and 50ml of sterile double distilled water was used to suspend the final pellet of the centrifuged cells, to give an OD_{600nm} of approximately 0.3, which is equal to a cell density of approximately $1x10^8$ CFU/ml. The cell suspensions were used immediately for freezing treatments.

The above procedure was also used to prepare 100ml volumes of bacterial suspension except that the overnight cultures and the re-inoculation of the cells were dispensed into 150ml sterile TSB, and once they had grown to the mid-log phase, the cells were poured into sterilized 250ml centrifuge bottles and centrifuged using a Sorvall centrifuge (Ivan Sorvall Inc., Newton, Connecticut, USA). After pouring off the supernatant, 100ml of sterile double distilled water was used to wash the cells twice, and then the cells were resuspended in 250ml sterile double distilled water to give an OD_{600nm} of approximately 0.3, again corresponding to a cell density of approximately $1x10^8$ CFU/ml.

2.2.3 Dilutions and drop plates

The samples that were kept out for further testing were diluted in a 10x serial dilution, using disposable borosilicate glass (16 x 150 mm) culture tubes (Fisher

Scientific Ltd., Whitby, ON, Canada) containing 4.5ml sterile double distilled water. All small volumetric measurements were made using Gibson Pipettes with sterile disposable pipette tips. Six 5µl drops were dropped onto selected plates separated into 4 quadrants, so that 4 different dilutions could be dropped onto the same plate. The media that was selected to be used to select for *Escherichia coli* was BD Difco™ membrane Fecal Coliform (mFC) agar (Becton, Dickinson and Company, Sparks, MD, USA) and to select for *Enterococcus faecalis* was BD Difco™ membrane *Enterococcus* (mE) agar (Becton, Dickinson and Company, Sparks, MD, USA). Both of these mediums were used because they are commonly used in detection of indicator organisms during water quality assessment, which would allow this study to show the possible outcomes that would arise if identical testing were performed in water treatment industries. The mFC agar plates, once the drops had dried, were incubated upside-down in a large stackable refrigerated shaking incubator, without shaking, set at 44.5°C incubator for 24 hours. The mE agar plates, once the drops had dried, were incubated upside-down in a Fisher Econotemp incubator model 30D (Fisher Scientific Inc., Whitby, ON, Canada), set at 37°C, for 48 hours.

Once the plates were taken out of their respective incubators, at the proper time intervals, the plates were counted. The dilution that had between 30 and 250 visible colonies in the quadrant was counted, and from that count, and through the use of the following equation the cell concentration was obtained:

Cell concentration (in CFU/ml)

$= [(\text{colony number in a quadrant}/30)/1\text{x}10^{-3}\text{ml}] \text{ x dilution.}$

The cell concentration was then converted into survival fraction using the equation:

Survival fraction = N/N_o,

where N_o is the initial cell concentration and N is the cell concentration at any given time, *t*.

2.2.4 Determination of the length of freezing time

Time intervals of 0.5-, 2.5-, 24-, 48- and 168-hours were tested, at -80°C, using the non-pathogenic strain of *Escherichia coli* in 100µl volumes, to determine the optimal length of time to keep the bacterial suspensions frozen, such that the greatest reduction in cell density could occur. For freezing at -80°C, a Thermo Electron Corporation 700 Series Forma ULT Freezer (Marietta, Ohio, USA) was used. For freezing at -30°C, a Thermo Forma dual chamber digitally regulated freezer, (Thermo Electron Corporation, Marietta, Ohio, USA) was used, and for freezing at both -7°C and -15°C a temperature and humidity controlled walk-in room (Climatic Testing Systems Incorporated, Warminster, PA, USA) was used.

For thawing, an Isotemp 228 water bath (Fisher Scientific Ltd., Whitby, ON, Canada), set at 22°C, was used. The length of time it took to completely thaw the frozen samples was rounded to the nearest half hour, and thus the 100µl samples were thawed for half an hour and the 100ml samples were thawed for one hour.

2.2.5 Determining the number of freeze-thaw cycles

Once the length of time to keep the samples frozen between cycles was determined, the number of freeze-thaw cycles needed to be determined. One hundred microlitres test samples of the non-pathogenic *Escherichia coli* were frozen at -80°C for 24 hours, and then thawed for 30 min. Three 0.2ml flat cap PCR tubes with attached cap (RNase/DNase/Pyrogen Free) (Fisher Scientific Ltd., Whitby, ON, Canada) were kept out

for further testing while the rest were placed back into freezing at -80°C. This cycling was done for five cycles and after each cycle, dilutions and drop plates were performed on the tubes that were kept out for further testing. The 0.2ml flat cap PCR tubes were used because the tubes have a thinner wall than most other tubes of a similar size, aiding in more uniform freezing of the bacterial suspensions within the tubes.

2.2.6 Experimental treatments

2.2.6.1 100µl portion samples (0.2ml PCR tubes)

One hundred microlitres of each of the prepared cell suspensions (from section 2.2.2) were dispensed into sterile 0.2ml flat cap PCR tubes and then frozen at -7, -15, -30 or -80°C for 24 hours. The 0.2ml flat cap PCR tubes were then taken out of the appropriate freezer to thaw for half an hour. Three tubes, equivalent to three replications, of each bacterial suspension were kept out for further testing, while the rest of the tubes were placed back into the appropriate freezer for another 24-hour interval. This cycling was repeated for five cycles and the experiment was repeated at least three times.

For samples tested at -7°C, freezing was very inconsistent, therefore 0.01g of washed and sterilized sand (Nu-Gro Corporation, Woodstock, Ontario, Canada) was added to the 0.2ml flat cap PCR tubes before freezing as an ice nucleator to ensure freezing. To wash the sand, 50g of unwashed sand was placed into a 250ml beaker. 50ml of double distilled water was used to rinse the sand three times. The beaker was then autoclaved for one hour twice to sterilize against spore-forming organisms, and then placed into a drying oven overnight. 0.01g of dry sand was then placed into 0.2ml flat cap PCR tubes and autoclaved again for 20 minutes, then left for a couple of hours to overnight for drying purposes.

Unfortunately, freezing was still not completely consistent even with the ice nucleator added, thus the tubes were checked for freezing sampled. Only the frozen cell suspension samples were collected for the drop-plating assay and continued onto the thawing stage of the cycling.

2.2.6.2 100ml portion samples (500ml Nalgene® bottles)

Fifty millilitres of each of the cell suspensions (as described in section 2.2.2) were dispensed into four separate sterile 500ml wide-mouth polypropylene Nalgene® bottles (Nalge Nunc International, Rochester, NY, USA) (i.e. 4 replications) containing 50ml of sterile double distilled water. One millilitre from each bottle was dispensed into sterile 1.5ml flat top microcentrifuge tubes (Fisher Scientific Ltd., Whitby, ON, Canada) for further testing at Day 0. The remaining contents of the bottles were then frozen at -7 and -15°C for 24 hours. The 100ml samples frozen at -7°C were supercooled and agitation was required to create nucleation for freezing.

After 24 hours, the bottles were taken out of the appropriate freezer and thawed in a water bath set at 22°C for one hour. One millilitre from each bottle was again dispensed into sterile 1.5ml flat top microcentrifuge tubes for further testing. These freezing and thawing cycles were continued until five cycles had been completed and the experiment was repeated at least three times.

2.2.7 Statistical analysis

One-way ANOVA tests were performed on all replicates used to create all the graphs using the Sigma Stat program. Letters were added to all graphs to indicate statistical significant difference at the 95% confidence interval, where the letters in each graph are independent of the next graph.

2.3 Results

2.3.1 Determination of the length of freezing time

Figure 2.1 shows the non-pathogenic *Escherichia coli* frozen at -80°C for 0.5, 2.5, 24, 48, and 168 hours, in 100μl bacterial suspension, to determine the amount of time the cell suspensions should be kept in the freezer before thawing began. From the figure, it is shown that the log reduction (3.41) of the culturable cells is plateau at the 24-hour time interval, whereas at the two earlier time points log reductions of 2.67 and 2.36 are observed, for the 0.5-hour time interval and the 2.5-hour time interval, respectively. At the 48-hour time interval a log reduction of 3.38 is observed, and at the 168-hour time interval a log reduction of 3.15 is observed. Statistically, the 24-hour interval is significantly different from 0.5-hour interval, with a $p < 0.01$ and the 2.5-hour interval, with a $p < 0.001$. This trial was repeated 3 times of which similar results were obtained. For future reference, the 24-hour freezing interval was chosen as it could be assumed that an appropriate amount of cells were non-culturable, and any length of freezing beyond this chosen interval would have little to no effect on inactivating the remainder of the population.

2.3.2 Determining the number of freeze-thaw cycles

Figure 2.2 shows the non-pathogenic strain of *Escherichia coli* cycled at -80°C, in 100μl bacterial suspensions; two other trials were performed and similar results were obtained for all three trials. A log reduction of 0.86 is observed after the first cycle; a log reduction of 1.26 is observed after the second cycle; a log reduction of 1.54 is observed after the third cycle; a log reduction of 2.27 is observed after the fourth cycle; and a log reduction of 2.20 is observed after the fifth cycle. For future analysis, five freeze-thaw

cycles were chosen for all experimental treatments so that the full effects of freezing and thawing, during the cycling process, could be examined.

2.3.3 Effects of sand as an ice-nucleator and on cell culturability

Each 100µl bacterial suspension was initially cycled at -7°C without sand, but because of inconsistencies, sand was added as an ice nucleator. Samples were tested with both sand and no sand at least three times, with the average results of the trials shown in Figure 2.3. Figure 2.3(a), pathogenic strain of *Escherichia coli*, shows that a significant difference between the sand and no sand is only observed at cycle three, with a $p < 0.05$; whereas, there is no significant difference, at the 95% confidence interval, at any other point between cycling the pathogenic *Escherichia coli* with 0.01g sterile sand or no sand. A log reduction of 1.73 is observed, after five freeze-thaw cycles, for the average with sand and a log reduction of 2.05 is observed after the same five freeze-thaw cycles for the average without sand.

The non-pathogenic strain of *Escherichia coli* shows statistical significant difference at cycles 1, 2, and 5 ($p < 0.05$, 0.01, and 0.01, respectively). After five freeze-thaw cycles, the average amount of culturable cells in the tubes with sand showed a log reduction of 1.61, while the average amount of culturable cells in the tubes without sand showed a log reduction of 2.42.

The vancomycin resistant strain of *Enterococcus faecalis* shows no statistical significant difference at any of the five cycles. After five freeze-thaw cycles, the amount of cell reduction that were culturable in the tubes with sand was found to be 1.08 log, while the amount of cell reduction without sand was found to be 0.90 log.

The vancomycin sensitive strain of *Enterococcus faecalis* also shows a significant difference at cycles 3 and 4 ($p < 0.05$ and 0.01, respectively). After five freeze-thaw cycles, the average amount of culturable cell reduction in the tubes with sand was 1.11 log, while the average cell inactivation in the tubes without sand 1.03 log.

Although there was no statistical significant difference observed after five freeze-thaw cycles, the tubes with sand froze more consistently, therefore, 0.01g of sterile sand was used with all 0.2ml flat capped PCR tubes containing 100µl bacterial suspension when future testing at -7°C was necessary.

2.3.4 Comparison of the effect of freezing temperature on the four bacterial strains

Figure 2.4 represents the effect of the four different freezing temperatures on all four bacterial strains frozen and thawed, in 100µl bacterial suspensions, for five cycles. All four strains showed a similar trend in the amount of non-culturable cells, in that, at -15°C the greatest amount of non-culturable cells was observed, when comparing each strain to the amount of non-culturable cells that occurred at -7, -30 or -80°C.

When looking at the first graph in Figure 2.4, the pathogenic strain of *Escherichia coli*, reductions of 1.27-, 4.67-, 3.39-, and 2.60-log were observed for -7, -15, -30 and -80°C, respectively, after five freeze-thaw cycles. At the end of the five cycles, there is no statistical significant difference between the bacterial suspension cycled at -80°C or -30°C, on a 95% confidence interval. However, those cycled at -7°C were statistically different from all other temperatures ($p < 0.001$ for -15, -30 and -80°C). Also, those cells that were cycled at -15°C showed a statistical significant difference to all other temperatures ($p < 0.001$).

The second graph in Figure 2.4 shows the cell culturability of the non-pathogenic strain of *Escherichia coli* with final reductions of 1.32-, 5.38-, 4.06-, and 1.46-log observed for -7, -15, -30 and -80°C, respectively, after five freeze-thaw cycles. When comparing five freeze-thaw cycles at -30°C and -80°C, there is no significant difference, at the 95% confidence interval. However, when comparing the differences of non-culturable cells on bacterial suspensions cycled at either -7°C or -15°C, there are significant differences, in that at -7°C there is a greater amount of culturability, while at -15°C, the least amount of culturability is present ($p < 0.001$ in comparison to all temperatures for both -7°C and -15°C).

In the third graph of Figure 2.4 the effects of cell culturability during five freeze-thaw cycles are shown for the vancomycin resistant strain of *Enterococcus faecalis*, with reductions of 0.94-, 2.21-, 0.50-, and 0.61-log being observed for -7, -15, -30 and -80°C, respectively. After the five freeze-thaw cycles, there is statistical significant difference when -15°C is compared to the other freezing temperatures ($p < 0.001$), in that there is a greater amount of cells that are not culturable at -15°C.

The fourth graph in Figure 2.4 shows the cell culturabilitiy of the vancomycin sensitive strain of *Enterococcus faecalis* cycled at all four freezing temperatures. After five freeze-thaw cycles reductions of 0.77-, 1.98-, 0.35-, and 0.53-log for -7, -15, -30 and -80°C, respectively, were observed. At the end of the five freeze-thaw cycles, -30°C and -80°C show no significant difference between each other, at the 95% confidence interval; whereas, -7°C and -15°C are significantly different from the other three temperatures tested, in comparison, with $p < 0.001$, 0.001, and 0.01 for -30, -15 and -80°C, respectively for -7°C, and $p < 0.001$ for all temperatures compared to -15°C. Thus for

both *Enterococcus faecalis* strains the least amount of non-culturable cells, after five freeze-thaw cycles, was observed at -30°C, closely followed by -80°C, and finally by -7°C, with -15°C consistently showing the greatest amount of non-culturable cells.

The difference between species was shown to be significant, depending on the temperature and condition tested ($p < 0.05$). In Figure 2.4, there is a statistical difference between the pathogenic strain of *Escherichia coli* and the non-pathogenic strain of *Escherichia coli* at -80°C only, where the non-pathogenic strain showed a greater amount of inactivated cells ($p < 0.001$); at all other temperatures there is no significant difference between the pathogenic and non-pathogenic strains of *Escherichia coli*, at a 95% confidence interval. Also, for the vancomycin resistant strain of *Enterococcus faecalis* compared to the vancomycin sensitive strain of *Enterococcus faecalis* there is a statistical difference at -15°C, $p < 0.05$; while at all other temperatures there is no significant difference at a 95% confidence interval between the vancomycin resistant and the vancomycin sensitive strains of *Enterococcus faecalis*.

2.3.5 Comparison of volume effect between 100µl and 100ml samples

Figure 2.5 compares all four bacterial strains cycled at -7°C and -15°C in two different suspension volumes, 100µl and 100ml. For all four bacterial strains the smallest decrease in non-culturable cells occurred when the 100µl suspensions were cycled at -7°C, while the trend for the larger volume cycled at -7°C and both volumes cycled at -15°C varied between each bacterial strain tested.

In the first graph of Figure 2.5, cell culturability of the pathogenic strain of *Escherichia coli* is presented. After five freeze-thaw cycles, log reductions of 3.28 and 3.75 are observed for the 100ml suspensions cycled at -7°C and -15°C, respectively,

while log reductions of 1.27 and 4.67 are observed for the 100µl suspensions cycled at -7°C and -15°C, respectively. There is a significant difference between the volumes tested at both -7°C and -15°C, where the 100ml bacterial suspension shows significantly more non-culturable cells at -7°C ($p < 0.001$), while at -15°C, the 100µl bacterial suspension shows significantly more cell inactivation ($p < 0.001$).

In the second graph of Figure 2.5, cell culturability of the non-pathogenic strain of *Escherichia coli* is presented, and after five freeze-thaw cycles log reductions of 3.96 and 4.99 are observed for the 100ml suspensions cycled at -7°C and -15°C, respectively, while log reductions of 1.32 and 5.38 are observed for the 100µl suspensions cycled at -7°C and -15°C, respectively. There is a significant difference between the volumes tested at both -7°C and -15°C, where the 100ml bacterial suspension shows significantly more cells that are non-culturable at -7°C ($p < 0.001$). In contrast, at -15°C, the 100µl bacterial suspensions showed significantly more non-culturable cells ($p < 0.001$).

From these two graphs it can be seen that the non-pathogenic strain of *Escherichia coli* shows a greater log reduction for all four experimental freezing temperatures. However, only when cycling 100ml at -15°C is there a significant difference between the pathogenic and non-pathogenic strains of *Escherichia coli*, with the non-pathogenic strain showing a greater amount of inactivated cells ($p < 0.05$).

From the third graph in Figure 2.5 it is observed that the vancomycin resistant strain of *Enterococcus faecalis* shows log reductions of 1.66 and 2.74 for cycling of the 100ml suspensions at -7°C and -15°C, respectively, while log reductions of 0.94 and 2.21 are shown for the 100µl suspensions cycled at -7°C and -15°C, respectively. There is a significant difference between the volumes tested at both -7°C and -15°C, where the

100ml bacterial suspension has significantly more non-culturable cells at both temperatures ($p < 0.01$ and 0.001, respectively).

The fourth graph of Figure 2.5 shows the vancomycin sensitive strain of *Enterococcus faecalis* cycled at both -7°C and -15°C, at either a 100µl or 100ml bacterial suspension. Log reductions of 2.09 and 2.82 were observed for the 100ml suspensions cycled at -7°C and -15°C, respectively, while log reductions of 0.77 and 1.98 were observed for the 100µl suspensions cycled at -7°C and -15°C, respectively. There is only statistical difference between the bacterial suspensions being cycled at -7°C at a volume of 100µl versus 100ml ($p < 0.001$).

From these two graphs it can be seen that after five freeze-thaw cycles, the vancomycin sensitive strain of *Enterococcus faecalis* shows a greater amount of non-culturable cells than the vancomycin resistant strain for both of the 100ml suspensions, whereas, for the 100µl suspensions the vancomycin resistant strain shows a greater log reduction than the vancomycin sensitive strain, however, a significant difference is only observed at -15°C when using 100µl of bacterial suspension, with the vancomycin sensitive strain showing a greater amount of inactivated cells ($p < 0.05$), after five freeze-thaw cycles.

2.4 Discussion

In determining the optimal number of freeze-thaw cycles, our finding agrees with others that 5 cycles of freeze-thaw give an optimum kill of the *E. coli* and *Enterococcus faecalis* (Calcott and Calcott, 1984; Yamamoto and Harris, 2001; Hilliard and Davis, 1918). In Hilliard and Davis' (1918) experimental results, it was found that after five freeze-thaw cycles, *B. typhosus* showed no visible growth of colonies.

The number of cells that are able to grow visible colonies on the selective media used in this study continues to decrease, at least until the fifth cycle, which corresponds with findings by Calcott and Calcott (1984). When Calcott and Calcott (1984) subjected their *Escherichia coli* strains to cycles of rapid freeze-thaw in water and saline, the viability of the population decreased with increasing number of cycles of stress. These observations were also observed when repeated with a slow freeze-thaw protocol, where the loss of viability may be proceeding by a different mechanism (Calcott and Calcott, 1984).

The data in this current study can also be compared to Yamamoto and Harris (2001), who froze and thawed various bacteria in apple juice and found that each cycle resulted in further death and injury, with *Escherichia coli* O157:H7 showing a 4.0- to 6.4-$\log_{10}$ reduction in colony forming units per milliliter (CFU/mL). However, in the current study, after the fifth freeze-thaw cycle, the final amount of cells that are inactivated is less than the final amount that is observed for the fourth cycle, which would indicate that either some cells recovered while being frozen or incubated, or that something interrupted the cells from growing in the fourth cycle, producing a false number of cells that were actually inactivated. It is quite possible that more cells from the fourth cycle, compared to the fifth cycle, were in a viable but non-culturable state, where the bacterial cells are unable to grow on selective agar media, having the ability to only grow on enriched agar media. In order to determine if this was happening, one trial of each bacterial strain was drop plated onto Tryptic Soy Agar (TSA) plates, an enriched agar medium. It was found that there was no significant difference between the amount of

visible colonies that grew on the selective media (mFC agar or mE agar) compared to the growth of visible colonies on TSA (data not shown).

Referring to Figure 2.3, where 100µl of the bacterial suspensions were frozen and thawed with either 0.01g of sterile sand or 0g of sand, it was shown that after five freeze-thaw cycles, a statistical difference was only observed for the non-pathogenic strain of *Escherichia coli*, $p < 0.01$, while the other three strains showed no significant difference between cycling with 0g of sand or 0.01g of sand, at the 95% confidence interval. However, in all instances, the tubes with sand froze more consistently than the tubes without sand, and although a larger number of cells were non-culturable in the tubes without sand, due to cooling rather than freezing, the tubes without sand did not freeze. Since this study was interested in the different effects of freezing at different temperatures, the 0.2ml flat cap PCR tubes that were used for all future testing at -7°C had 0.01g of washed, sterilized sand in them.

The difference in freezing rate determines the amount of damage that the bacterial cell undergoes because the freezing rate determines the size of the ice crystals that occur during freezing (Dumont *et al.*, 2006). For example, at a low freezing rate, all of the water inside the cell can osmotically flow out of the cell, resulting in no intracellular ice forming; at medium freezing rates, the water in the cell is reduced to a point where irreversible damage to the cell occurs; and at very high freezing rates the water in the cell does not have time to flow out of the cell, which may allow for higher amounts of viable cells (Dumont *et al.*, 2003). However, the amount of cells that become inactivated during freeze-thaw cycling, also depends on the thawing rate, such that, the thawing rate should match the freezing rate, otherwise more damage can occur to the cells. For example,

cells that are frozen quickly, show the most survival if thawed quickly as well (Calcott, 1978).

Also, as stated in section 2.1, thawing of the frozen samples is equally important as the freezing process because the slower the thawing process, after a fast freezing process, the more damage that could possibly occur, from larger ice crystals forming, in the cells (Dumont *et al.*, 2006). Thus, the amount of time for thawing in this study was kept as constant as possible by utilizing the same temperature for both the 100µl samples and the 100ml samples, in a water bath set at 22°C, or room temperature, so that we were really only observing the effects of the freezing temperatures and the freeze-thaw cycling at such temperatures.

From the above information it can be speculated that more cells had compromised membranes at -15°C, if it is considered that this temperature is a medium freezing rate, which would indicate that the damage that occurred to those cells is irreversible damage. If this is compared to the bacterial suspensions frozen at -7°C, considering this temperature to be a slow freezing rate, then it can be stated that water leaked out of the cell, leading to dehydration, and possibly causing only minor damage to the cells. Interestingly, more cells may endure damage, resulting in less cells being culturable, if they are held at -7°C for more than 24-hours because increasing amounts of water will continue to leak out of the cell (Gao *et al.*, 2006). This is because freezing at slow freezing rates, such as -7°C here (hypothetically), allows the bacterial cell to potentially lose all water within the cell, which would then denature the protein and possibly cause damage to the membrane (Mazur, 1966).

The difference noted at -80°C, in Figure 2.4 for the pathogenic and non-pathogenic strains of *Escherichia coli*, may not be as publicly significant as one might initially imagine, as most rivers and streams would not have a freezing temperature below -80°C; which means that any pathogenic bacteria that are surviving freezing and thawing at this low freezing temperature would not normally be a concern for industrial waste water treatment plants. However, it is important to keep in mind that all indicator organisms used by water treatment facilities need to be able to detect most, if not all, pathogenic organisms, by portraying similar characteristics of their pathogenic counterparts. If an indicator organism fails to imitate the pathogenic organisms in question, then some pathogenic organisms may be overlooked. Unfortunately, it has been noted that the non-pathogenic indicator organisms are less resistant to the natural stresses of the aquatic environment, compared to pathogenic organisms as determined by McFeters and Stuart (1972). McFeters and Stuart (1972) determined that fecal coliform bacteria are among the first to die in the aquatic environment, while such things as viruses and pathogenic bacteria are capable of survival, maintaining the health hazard of contaminated water.

Figure 2.4 shows that at -80°C the *Enterococcus faecalis* strains are less susceptible to freezing damage than the *Escherichia coli* strains. This could be due to the difference in cell wall structures as described in section 1.2, where it is stated that *Enterococcus faecalis* is a Gram-positive bacterium that has a multi-layer lattice structure peptidoglycan cell wall, while *Escherichia coli* is a Gram-negative bacterium that only has a single, thin layer of peptidoglycan in the cell wall. This structural difference, among others, makes the Gram-positive bacterium more resistant to the freeze-thaw

pressures, allowing it to survive more often in such aquatic environments. Ray and Speck (1972) determined, through their study of injury repair on *Escherichia coli*, that freezing and thawing produces some kind of damage to the cell envelope of *Escherichia coli* and renders them sensitive to deoxycolate, a bile acid; however, for some bacteria this injury is reversible.

In this study, the most damage was observed at the intermediate freezing temperature of -15°C, while at -7, -30 and -80°C the damage to each strain was less after five freeze-thaw cycles. In comparison, Dumont *et al.* (2004) found that lower cooling rates and very rapid cooling rates were less damaging to the cells. Furthermore, Lund (2000) commented that the rate of injury accumulates with the increasing temperature to reach a maximum near, but not at, the freezing point.

At higher freezing temperatures, the cells are more likely to become dehydrated due to freezing of the extracellular solution first, possibly causing cell death (Gao *et al.*, 2006). Gao *et al.* (2006) determined that the amount of injury, or the number of cells that are still viable after being frozen and thawed at warmer freezing temperatures, declined as the amount of time kept in the freezer increased. This could be a significant factor in many water treatment plants where natural freezing occurs in the surface water source that the treatment plant uses. It is possible that after only two natural freeze-thaw cycles, and a long freezing time before the third cycling, that a large proportion of the *Escherichia coli* cells that may have been viable, will more than likely not be viable anymore.

In examining Figure 2.4, the amount of non-culturable cells of the vancomycin resistant *Enterococcus faecalis* strain at -15°C continued to be similar to the other three

freezing temperatures tested, -7, -30 and -80°C, until after the third freeze-thaw cycle. It is possible that the bacteria were slowly becoming more sensitive to the freeze-thaw stresses, but were able to recover when enumerated, then finally succumbed. Or it could be that the cells that were injured in the first three cycles were no longer able to repair themselves on the selective media used in this study. Also, for all temperatures, when the vancomycin resistant strain of *Enterococcus faecalis* was drop plated after two or more successive freeze-thaw cycles, some of the colonies on mE agar, supplemented with 4ppm vancomycin, were noticeably smaller. Similarly, Pettibone *et al.* (1987) noticed that the Gram-positive cocci produced smaller colonies when they were plated on antibiotic agar and with increased exposure to the estuarine water.

By increasing the volumes in the samples tested at -7°C and -15°C from 100µl to 100ml, the corresponding freezing rates decreased, with the results shown in Figure 2.5. It was observed that at the warmer freezing temperature (-7°C) when the volume of bacterial suspension was increased there was a greater amount of cells that could not be cultured on the selective media used in this study (approximately from a 1-log reduction to a 3-log reduction). Using the information that was obtained by Dumont *et al.* (2006), as explained in section 2.1, it is possible that this decrease in freezing rate caused more cells to be damaged through increased dehydration because of increased ice crystal formation outside of the cells, during the thawing process. On the other hand, both observations of increasing and decreasing cell numbers were obtained at -15°C, when increasing the volume size from 100µl to 100ml, thus it is difficult to conclude how the cells were actually affected by the decrease in the freezing rates. However, it is possible to say that the difference in behaviour between the two bacterial strains could possibly be

due to their difference in cell permeability (Mazur, 1963). For instance, both of the *Escherichia coli* strains showed a decrease in the amount of cells that were inactivated (approximately 1.0- and 0.5-log difference for the pathogenic and non-pathogenic strain, respectively), whereas both of the *Enterococcus faecalis* strains showed an increase in the amounts of non-culturable cells (approximately 0.5- and 1.0-log difference for the vancomycin resistant and vancomycin sensitive strain, respectively).

All results were reproducible through a minimum of three trials; therefore, it can be assumed that these are consistent results, and the implications towards bacteria in the natural environment can be inferred from this study.

Unfortunately, our model is a small representation of a large-scale freeze-thaw technique and there are many challenges that arose, making this type of *in vitro* testing a bit difficult. For example, there was an issue of adding an ice nucleator to the 100µl samples at -7°C so that the bacterial suspensions could freeze. An expansion to this study is required to determine if both the pathogenic bacteria and the non-pathogenic bacteria are capable of re-growth in natural river water or lake water, after all points of the freeze-thaw cycling, because the bacteria are not as stressed in laboratory studies as they would be in the natural aquatic environment (McFeters, 1989). As stated in a coliform regrowth review written by Le Chevallier (1990), coliform regrowth in potable water does occur, and the presence of these coliform bacteria may mask the presence of the indicator bacteria. It is mentioned that this causes a breakdown of treatment barriers, and such possible outcomes of water treatment should be addressed with all new, and old, ideas. Also, it was stated by Kitrell and Furfari (1963) that greater numbers of fecal indicator bacteria survive in water for a longer time during the winter months than during

the summer months, indicating that the natural cold water, with all the aquatic environmental elements, are possibly not as detrimental to the bacteria as the temperatures used in this study.

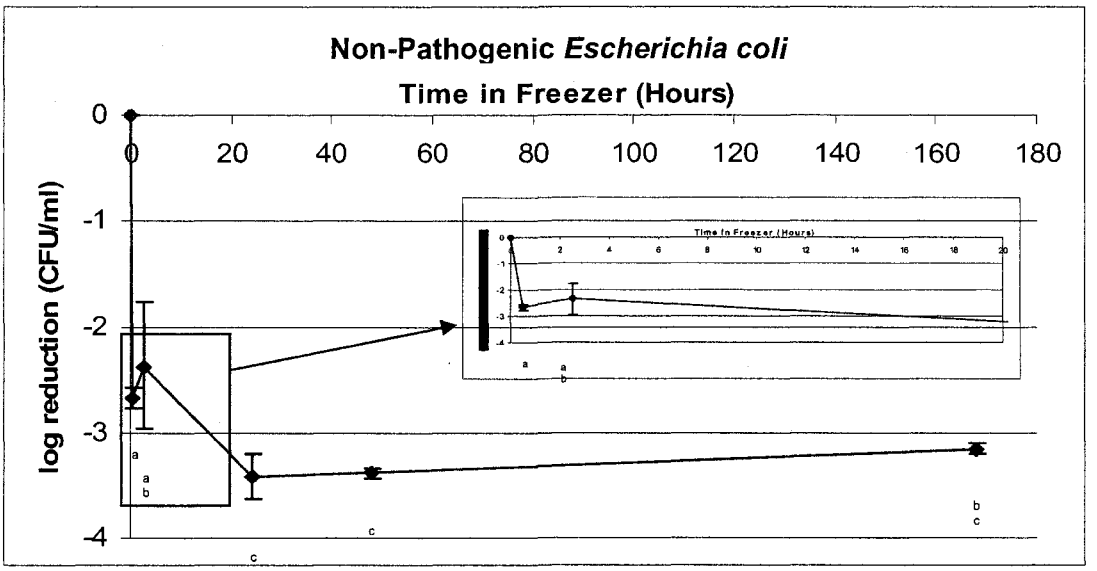


Figure 2.1 –Non-pathogenic *Escherichia coli* frozen at -80°C for varying lengths of time: 0.5-, 2.5-, 24-, 48-, and 168-hours, for freezing time determination. Each individual letter represents a significant difference at a 95% confidence interval.

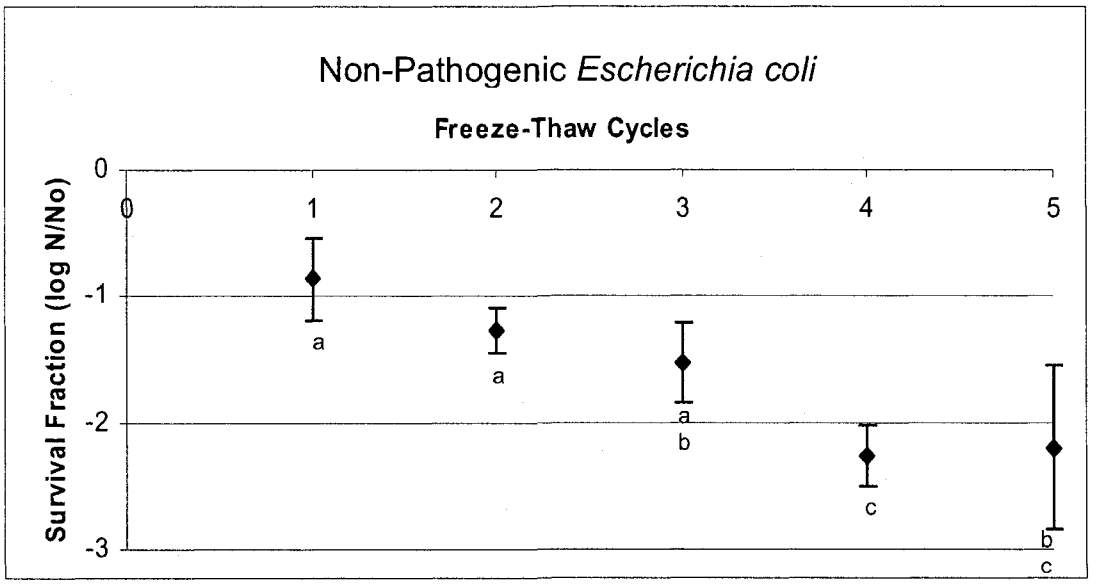
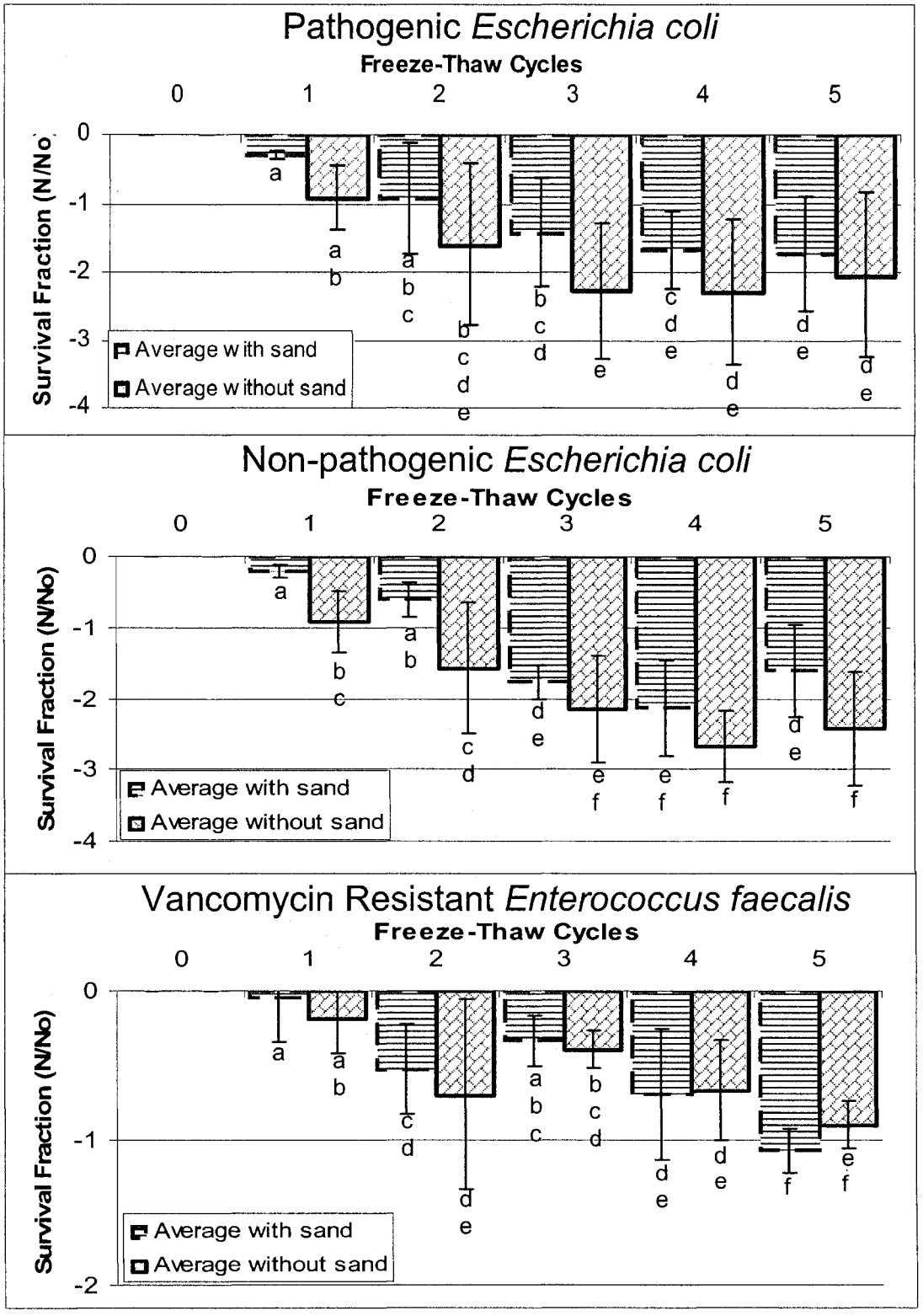


Figure 2.2 – Non-pathogenic strain of *Escherichia coli* cycled at -80°C for five freeze-thaw cycles. Each letter indicates a significant difference at a 95% confidence interval.


Pathogenic *Escherichia coli*
Freeze-Thaw Cycles
0 1 2 3 4 5
Survival Fraction (N/No)
0 -1 -2 -3 -4
Average with sand
Average without sand
Non-pathogenic *Escherichia coli*
Freeze-Thaw Cycles
0 1 2 3 4 5
Survival Fraction (N/No)
0 -1 -2 -3 -4
Average with sand
Average without sand
Vancomycin Resistant *Enterococcus faecalis*
Freeze-Thaw Cycles
0 1 2 3 4 5
Survival Fraction (N/No)
0 -1 -2
Average with sand
Average without sand

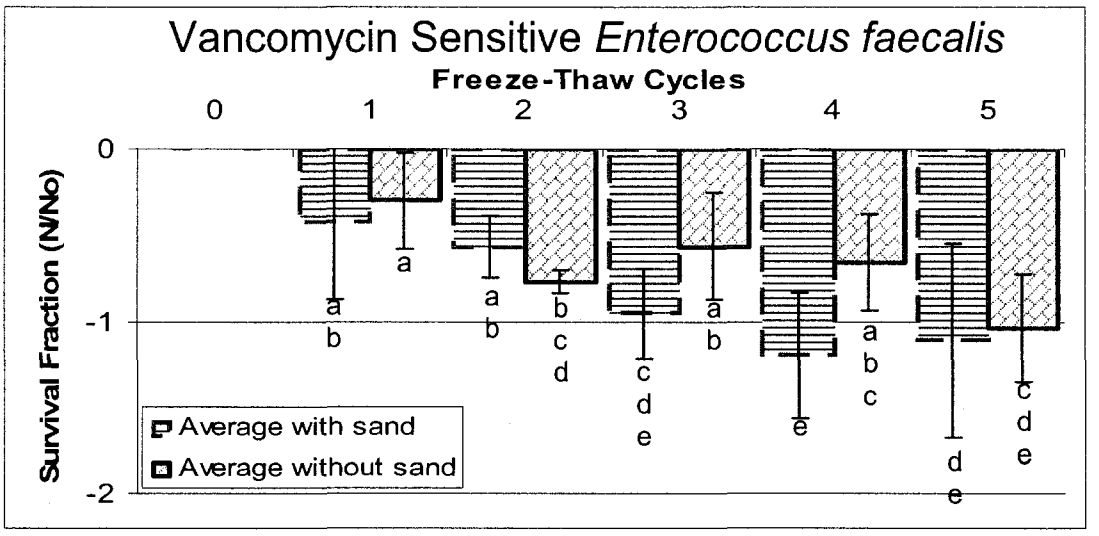
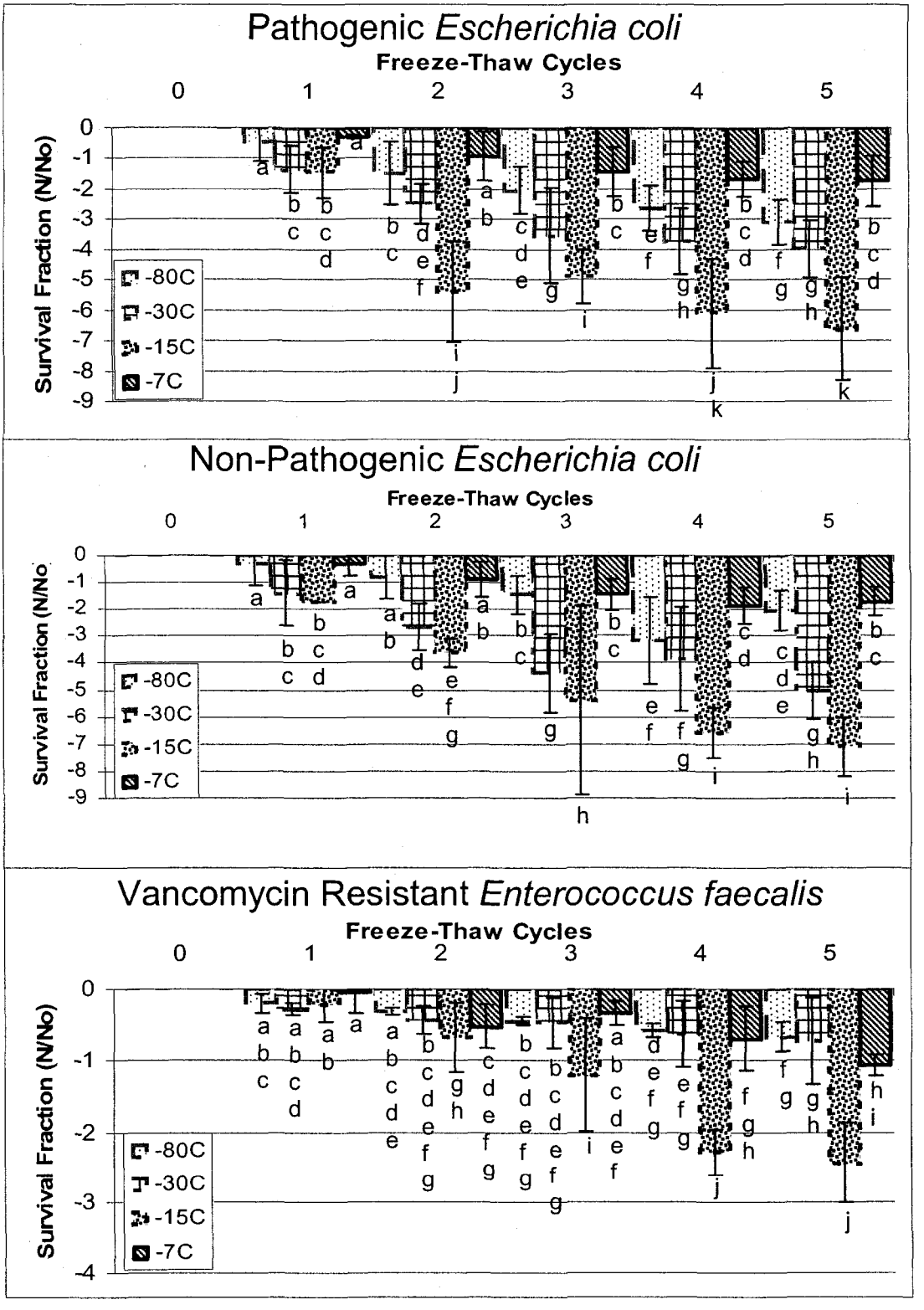


Figure 2.3 – Comparison of 100µl bacterial suspensions frozen and thawed in 0.2ml flat cap PCR tubes with either 0.01g of sand (maroon stripes) or with 0g of sand (purple bricks) at -7°C, for five cycles. Each unique letter, in each graph, indicates significant difference at a 95% confidence interval.


Pathogenic Escherichia coli
Freeze-Thaw Cycles
0
1
2
3
4
5
Survival Fraction (N/No)
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-80C
-30C
-15C
-7C
Non-Pathogenic Escherichia coli
Freeze-Thaw Cycles
Survival Fraction (N/No
Vancomycin Resistant Enterococcus faecalis
Freeze-Thaw Cycles
Survival Fraction (N/No)

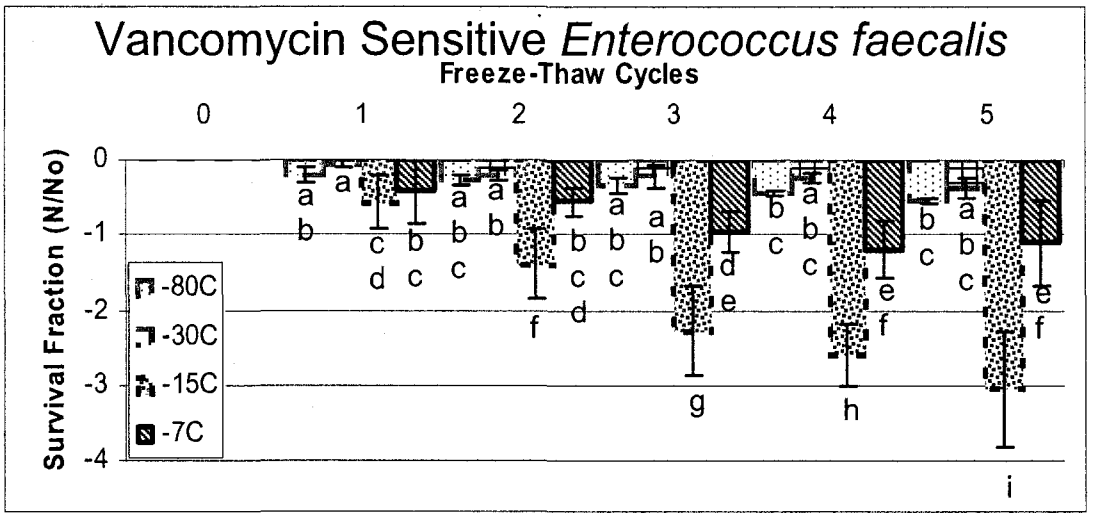
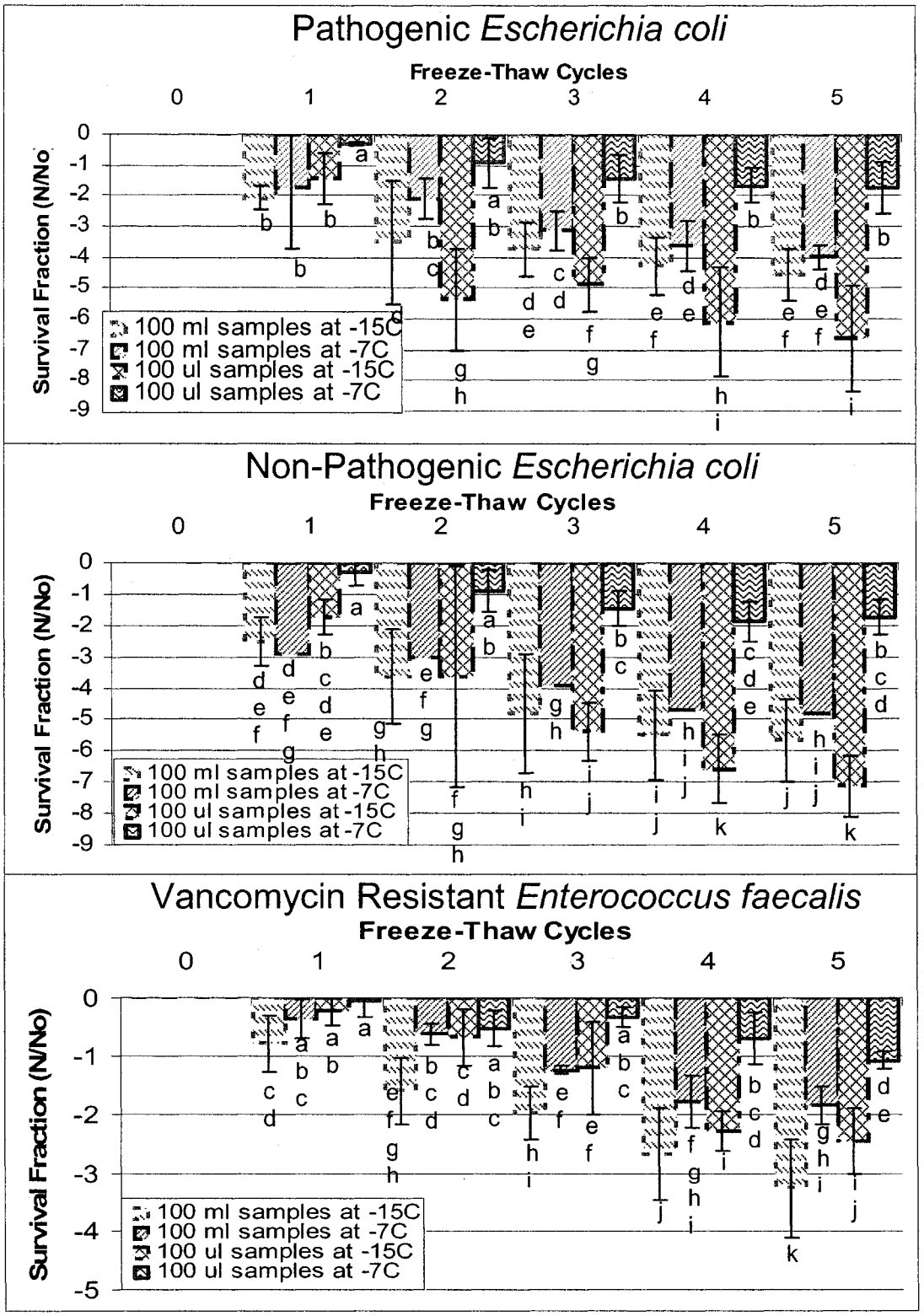


Figure 2.4 – Comparison of 100µl of bacterial suspension frozen and thawed in 0.2ml flat cap PCR tubes at -80°C (pink dots), -30°C (blue grid), -15°C (green confetti), or -7°C (purple diagonal lines), for five cycles. Each individual letter in each graph represents a significant difference at a 95% confidence interval.


Pathogenic Escherichia coli
Freeze-Thaw Cycles
0
1
2
3
4
5
Survival Fraction (N/No
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
100 ml samples at -15C
100 ml samples at -7C
100 ul samples at -15C
100 ul samples at -7C
Non-Pathogenic Escherichia coli
Freeze-Thaw Cycles
Survival Fraction (N/No)
100 ml samples at -15C
100 ml samples at -7C
100 ul samples at -15C
100 ul samples at -7C
Vancomycin Resistant Enterococcus faecalis
Freeze-Thaw Cycles
Survival Fraction (N/No)
100 ml samples at -15C
100 ml samples at -7C
100 ul samples at -15C
100 ul samples at -7C

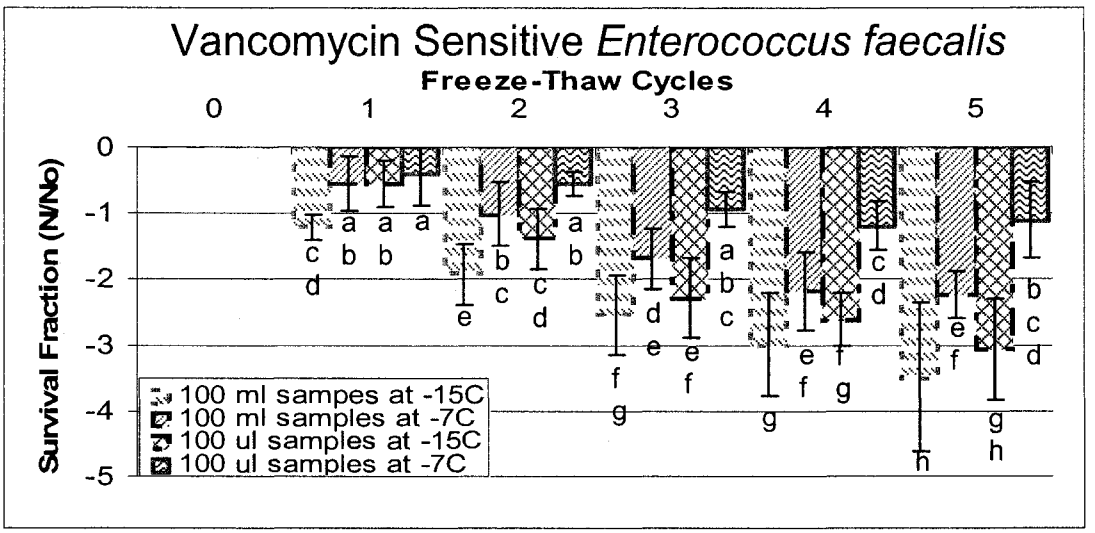


Figure 2.5 – 100µl of bacterial suspension frozen and thawed in 0.2ml flat cap PCR tubes at -15°C (pink dashed diagonal) or -7°C (orange diagonal) compared to 100ml of bacterial suspension frozen and thawed in 500ml Nalgene® bottles at -15°C (blue diamond) or -7°C (purple zig zag), for five cycles. Each unique letter in each graph represents a significant difference at a 95% confidence interval.

Chapter 3: Effect of Freeze-Thaw on Cell Injury

3.1 Introduction

As stated in Chapter 1, damage to bacterial cells can occur through freezing and/or thawing by two mechanisms, dehydration and ice crystallization. These damaged cells can either be dead or injured. The injured cells could be considered to be in a latent state, where they need to recover before they can grow on selective media. For example, Davenport *et al.* (1976) researched optimizing the recovery of viable but non-culturable cells, which according to McFeters (1989) are theoretically the same as injured cells, and found that cells can recover from injury if they are first grown on non-selective medium at 35°C for four hours. However, viable but non-culturable bacteria are likely to be detected only after extended nutrient limitations, whereas the first observable change is the progressive increase in sensitivity to certain media (e.g., injury) (McFeters, 1989). The injured cells that are not detected through regular means of enumeration can be a danger to communities, especially if they are left undetected in consumable items such as food or water (Lund, 2000) and they are of the pathogenic origin.

Many water treatment industries employ the heterotrophic plate count (HPC) technique that assesses the number of bacteria in the water samples that are able to form visible colonies on a solid medium, under specified test conditions (e.g., medium nutrients, incubation time, incubation temperature, etc.) (Hoefel *et al.*, 2003). However, this only detects those cells that are culturable, and does not detect those cells that may be non-culturable. Although detection of injured cells through plate counting is possible, with the extra recovery step, it is time consuming. It is also possible that the routine plating method could underestimate the real bacterial presence, which could be a public

health concern, especially if the viable but non-culturable cells maintain their virulence (Pianetti *et al.*, 2006).

Fortunately, the membrane damaged cells are able to take up fluorescent stains, two examples are propidium iodide and SYTO® 9, that can be detected using an instrument that can detect fluorescence, for example a flow cytometer or an epi-fluorescence microscope, to name a few. Propidium iodide is a larger particle that is too big to enter the cell through the cell membrane, and therefore is unable to stain the double stranded nucleic acids unless the cell membrane has been compromised in some way (López-Amorós *et al.*, 1995). Additionally, propidium iodide is an impermeant dye, which helps to measure the membrane integrity by the amount of propidium iodide that the cells can exclude. Propidium iodide, and other similar stains are insoluble in the hydrophobic membrane phase of the cell (Ben-Amor *et al.*, 2005), which is another reason why propidium iodide is unable to enter the cell if the membrane is intact. On the contrary, SYTO® 9 is able to stain all cells because it is small enough to enter through the pores of the cell membrane and stain the double stranded nucleic acids (López-Amorós *et al.*, 1995). The cells that stain with both propidium iodide and SYTO 9® will fluoresce varying shades of orange because when the cell is double stained, and the fluorescent emission of SYTO 9® is absorbed by propidium iodide (the energy transfer phenomena) SYTO 9® is no longer as visible, but the cell still emits both green and red fluorescence because the energy transfer is not complete when the cells are only injured (Ben-Amor *et al.*, 2005). Interestingly, Gram-positive bacteria lack a cell membrane and this enables SYTO 9® to enter the cells more readily; also, it is known that staining properties are dependent on the physiological state of the bacteria (Berney *et al.*, 2007).

Flow cytometry, coupled with advancements in fluorescent dye technology has become a valuable tool for the detection of bacteria in aquatic environments (Hoefel *et al.*, 2003). Flow cytometry allows for fast enumeration of non-homogeneous populations (Allman *et al.*, 1993) and is able to detect cells on the single-cell level (Berney *et al.*, 2007), which allows for corrective action in water treatment plants to be implemented a lot earlier than conventional culture-based techniques (Hoefel *et al.*, 2003), which generally require more than one day before definitive results are available (Yamaguchi *et al.*, 2003). Yamaguchi *et al.* (2003) noticed that it takes more than 90 minutes for a trained technician to detect 10,000 bacterial cells by fluorescent microscopy, while, detecting the same number of bacterial cells by flow cytometry takes only a few minutes, as long as the bacterial number in the sample is high enough (counting greater than 300 cells per second). However, it is quite common to use both an epi-fluorescent microscope and flow cytometry to detect fluorescence of bacterial cells; however, the epi-fluorescent microscope is usually only used to ensure that the cells are showing expected fluorescence. Thus, the total procedure for testing samples using a flow cytometer ranges from two to three hours, making flow cytometry faster than many other methods of detection (Yamaguchi *et al.*, 2003).

The viability of pathogenic organisms is of great concern to all water treatment facilities, especially after treatment has been administered. If any pathogenic organisms are capable of recovering from the disinfection treatment, and then multiplying in the municipal waters after all treatments have been given, then a large public disease outbreak is likely to occur. Therefore, it is important to understand the amount if injury

that is encountered by each indicator organism, so that proper secondary treatment of waste water sludge can be implemented.

The objectives of this study were to determine the type of injury that occurs to the bacterial cell as it undergoes freezing and thawing, through five cycles, by common conventional techniques that will indicate growth ability (drop plating), cell wall breakage (epi-fluorescence microscopy), or membrane damage (flow cytometry).

3.2 Materials and Methods

3.2.1 Bacterial strains

The bacterial strains for this portion of the project were the same as those used in the previous chapter and are described in section 2.2.1.

3.2.2 Preparation of bacterial suspensions

Preparations for the bacterial suspensions used in this part of the study were the same as those used in the previous chapter and are described in section 2.2.2.

3.2.3 Experimental treatments

The experimental treatments employed during this part of the study were similar to those used in the previous chapter, and are described in section 2.2.6.2. In addition to the procedures described, a few alterations were made: 1) 50ml of each bacterial suspension were dispensed into three sterile 500ml wide mouth polypropylene Nalgene® bottles (Nalge Nunc International, Rochester, New York, USA), instead of four, with 50ml filter and autoclaved sterilized double distilled water; giving three replicates of each bacterial suspension; 2) filter and autoclave sterilized double distilled water was used in place of autoclave sterilized double distilled water; 3) instead of one millilitre of sample

being dispensed from each bottle, two millilitres were pipetted into two separate sterile 1.5ml flat top microcentrifuge tubes for further testing.

3.2.4 Preparation of samples for determination of injury

3.2.4.1 Drop plating technique

The drop plating technique was used in the prior chapter, and details of how it was performed are described in section 2.2.3.

3.2.4.2 Flow cytometry

3.2.4.2.1 Preparation of positive control curve samples

Each bacterial strain was prepared as described in section 2.2.2. After the cell suspensions were prepared, 4ml of each bacterial suspension were equally separated into four sterile 1.5ml flat top microcentrifuge tubes. The tubes were sealed and placed into a 70°C water bath (Isotemp 205, Fisher Scientific Ltd., Whitby, ON, Canada) for 40 minutes to heat-kill the bacteria, an average of other known heat-kill methods. The rest of the bacterial suspensions were placed in the refrigerator to be used later with the heat killed bacteria.

After the 40 minute heat-kill, the tubes containing the bacterial suspensions were taken out of the water bath, allowed to cool for 10 minutes, and then mixed in ratios with the bacterial suspensions from the refrigerator. The live, from the refrigerator, to dead, heat-killed bacterial suspensions, ratios used were: 0:10, 1:9, 3:7, 5:5, 7:3, 9:1, and 10:0. These were made in duplicate and repeated daily for the five freeze-thaw cycles, or six days. The cells in ratio were then stained with 2mM propidium iodide, from the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit and the volume used was from suggestions made by the manufacturer of the staining kit (3µl/ml), for 15 minutes in the

dark, at room temperature. An extra tube of each live bacterial suspension was left unstained as a negative control for the positive control curve samples. The tubes were then kept on ice until they were run on the BD FacsCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada), using a 15mW air-cooled argon ion laser, emitting at a fixed wavelength of 488nm.

3.2.4.2.2 Preparation of the frozen and thawed samples

Once the bacterial suspensions were thawed, and diluted, 0.5ml of each of the three 10^1 dilution replicates were dispensed equally into two sterile 1.5ml flat top microcentrifuge tubes, giving a total of 12 samples of 2 tubes each. Twelve of these tubes were then stained the same way as the positive control curve samples, in that 3µl/ml propidium iodide, from the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit was added to one tube of each sample, left to stain for 15 minutes in the dark at room temperature. The other twelve tubes were left unstained and used as negative controls. All tubes were kept on ice until they were run on the flow cytometer.

3.2.4.2.3 Running the flow cytometer

All positive control curve tubes were diluted with filter sterilized and autoclave sterilized double distilled water, into 5ml Falcon® tubes (BD Biosciences Discovery Labware, Two Oak Park, Bedford, MA, USA), such that approximately 2000 events per second were read on the low setting of the BD FacsCalibur flow cytometer (uptake of 12µl/sec). All frozen and thawed samples were left undiluted, as they had already been diluted ten-fold prior to staining; however, the contents of the 1.5ml flat top microcentrifuge tubes were transferred into unused 5ml Falcon tubes. The frozen and thawed samples were read at approximately 500 events per second.

Each sample was run until 50000 events were counted, and the amount of time it took and the percentage of cells showing red fluorescence was recorded. For the positive control curves, the percentages at each ratio were graphed with the amount of red fluorescence on the x-axis and the percentage of cells with compromised membranes on the y-axis. A trend-line was added to the graph, in which both the equation and the r^2 value were displayed, and from this equation, the proper amount of red fluorescence, indicating the amount of cells with compromised membranes, for the frozen and thawed samples was calculated (data not shown). The average percentage of cells with compromised membranes from the three freeze-thaw sample replicates were calculated and multiplied by the percentage of intact cells obtained by the epi-fluorescent microscopy direct count; this was determined for each cycle. This additional calculation was required to compensate for the presence of lysed cells in the bacterial suspensions in which the flow cytometer could not detect. These new values were then used to determine the survival fraction by dividing the daily value with the value determined at Day 0, N/N_o, where N_o is the initial count and N is the daily count, which were obtained to create the flow cytometry lines in the figures at the end of this chapter.

3.2.4.3 Epi-fluorescent microscopy direct count

One 1.5ml flat top microcentrifuge tube from each frozen and thawed bottle was set aside to stain it with the LIVE/DEAD® *Bac*Light™ Bacterial Viablility Kit. SYTO® 9 was diluted to a 100 μM concentration and propidium iodide was diluted to a concentration of 20 μM. They were then used in a 1:1 staining ratio of 3μl stain per millilitre of bacterial suspension, as suggested by the manufacturer. The bacterial suspensions were incubated at room temperature for 15 minutes in the dark. 2μl of each

suspension was then dropped onto a clean microscope slide (75 mm x 25 mm, Corning Glass Works Scientific Glassware Dept., Corning, NY, USA), and immediately covered by a 22 mm x 22 mm coverslip (Fisher Scientific, Whitby, ON, Canada) and sealed using nail polish. The slides were kept in the dark until the nail polish dried and were then viewed using an epi-fluorescent microscope under the 100x oil immersion objective. Pictures were taken and the total cell counts were determined using Image Pro, a computer program.

3.2.4.3.1 Counting cells using pictures from epi-fluorescence microscopy

For counting of cells with the epi-fluorescent microscope, all cells were stained with both a green and a red fluorescent dye, contained in the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit from Molecular Probes, and counted as either being live (green), dead (red), or injured (orange); however, the colour indication was not considered when compiling the data, as the direct count was what was of interest. The number of total cells counted in each picture was averaged, and then to make the microscopy line in the figures at the end of this chapter, the count that was determined each day was divided by the count that was determined on Day 0, N/N_o, where N_o is the initial count and N is the daily count. All figures are an average of ten individual pictures taken each day between two trials of freeze-thaw cycling, at temperatures of -7, -15 and -30°C.

3.2.5 Statistical analysis

One-way ANOVA tests were performed on all replicates used to create all the graphs using the Sigma Stat program. Letters were added to all graphs to indicate statistical significant difference at the 95% confidence interval, where the letters in each graph are independent of the next graph.

3.3 Results

3.3.1 Examining cells using flow cytometry

Figure 3.1 represents the four bacterial strains stained with propidium iodide and counted using the BD FacsCalibur flow cytometer. For each histogram, the y-axis represents the number of cells that are counted by the BD FacsCalibur flow cytometer, while the x-axis represents the accumulation of red fluorescence, in log, that is emitted from each counted cell. After only one freeze-thaw cycle, each bacterial strain shows a population shift towards increasing amounts of cells with compromised membranes, indicated by an increase in red fluorescence. The populations continue to shift to increasing amounts of cells with compromised membranes through to Day 5, in which a large proportion of the cells, counted by the flow cytometer, show red fluorescence, indicated by the large peak at approximately 2-log, representing compromised membranes.

3.3.2 Examining cells using epi-fluorescence microscopy

Pictures that were taken using the epi-fluorescence microscope, with samples corresponding to the histograms in Figure 3.1, are presented in Figure 3.2. The green cells were counted as live and the red cells were counted as dead, with the cells that were orange, or in between bright red and bright green, were counted as injured. All of the cells that were counted were combined to create the direct count, which often decreased through the five freeze-thaw cycles, indicating that some cells were lysed, resulting in some cells not being counted.

3.3.3 Comparison of the three injury identification techniques (drop plating, microscopy, and flow cytometry)

In Figure 3.3 the three injury identification techniques are examined at three different freezing temperatures, -7, -15, and -30°C, through five freeze-thaw cycles. For all of the graphs in each figure the drop plate technique shows the amount of cells that remains culturable; the BD FacsCalibur flow cytometer shows the amount of cells with compromised membranes; and the epi-fluorescence microscopy shows the amount of cells that are not lysed. In all instances, the drop plating technique showed the most amount of cell inactivation, followed by flow cytometry, and then epi-fluorescence microscopy, as the flow cytometer was unable to detect lysed cells, as discussed in section 3.2.4.2.3.

In Figure 3.3i when the pathogenic strain of *Escherichia coli* is cycled at -7°C, non-culturable injury, as determined through drop plating, after five freeze-thaw cycles, results in a log reduction of 5.72, while detection of lysed cells through microscopy yields a log reduction of 0.58 and detection of cells with compromised membranes through flow cytometry detects a log reduction of 1.46. After five freeze-thaw cycles, the drop plate technique statistically shows a greater amount of cells that were non-culturable through the freeze-thaw cycling, when compared to the other two techniques, $p < 0.001$ for both, while microscopy, lysed cells, and flow cytometry, cells with compromised membranes, showed no significant difference (95% confidence interval).

When the same strain is cycled at -15°C, detecting non-culturable injury through drop plating results in a log reduction of 6.73, after five freeze-thaw cycles, while for microscopy and flow cytometry log reductions of 0.61 and 0.74, respectively, were observed. At the end of five freeze-thaw cycles, drop plating has statistically more cells that were non-culturable than microscopy, lysed cells ($p < 0.001$) or flow cytometry, cells

with compromised membranes ($p < 0.001$), while microscopy and flow cytometry only differed from each other at the second cycle, with the flow cytometer showing a greater amount of cells with compromised membranes ($p < 0.05$).

When the pathogenic strain of *Escherichia coli* is cycled at -30°C, detection of injury, after five freeze-thaw cycles, through drop plating results in a cell inactivation of 4.94-log, while detecting injury through microscopy and flow cytometry yields log reductions of 0.66 and 1.77, respectively. At the end of five freeze-thaw cycles, all three identification techniques are significantly different from each other. Counting culturable cells through drop plating demonstrates a significant difference in the increased amount of cells that are inactivated when compared to microscopy, lysed cells ($p < 0.001$), and flow cytometry, cells with compromised membranes ($p < 0.001$), while microscopy compared to flow cytometry results in a p 0.004, with flow cytometry showing more inactivated cells, at a 95% confidence interval.

In Figure 3.3ii, when the non-pathogenic strain of *Escherichia coli* is cycled at -7°C, the injury detected through drop plating, after five freeze-thaw cycles, results in a log reduction of 4.68, whereas for injury detected through microscopy and flow cytometry, log reductions of 0.42 and 0.89, respectively, were produced. After five freeze-thaw cycles, the drop plate technique statistically shows a greater amount of cells that were non-culturable, compared to the other two techniques, $p < 0.001$ for both, while microscopy, lysed cells, and flow cytometry, cells with compromised membranes, showed no significant difference (95% confidence interval).

When the same strain is cycled at -15°C, detection of injury through drop plating, after five freeze-thaw cycles, results in a log reduction of 6.33, while for microscopy and

flow cytometry, log reductions of 0.53 and 0.99 are obtained. At the end of five freeze-thaw cycles, all three techniques show a significant difference from each other, at the 95% confidence interval. Counting cells through drop plating demonstrates a significant difference in the increased amount of cells that are inactivated when compared to microscopy ($p < 0.001$) and flow cytometry ($p < 0.001$), as well when microscopy is compared to flow cytometry a $p < 0.001$ results.

When the non-pathogenic strain of *Escherichia coli* is cycled at -30°C, injury detected, after five freeze-thaw cycles, through drop plating results in a log reduction of 4.75, while detection through microscopy and flow cytometry produces log reductions of 0.44 and 0.94, respectively. After five freeze-thaw cycles, all three identification techniques differ significantly, with the drop plating showing more cells that are inactivated, compared to microscopy ($p < 0.001$) and flow cytometry ($p < 0.001$), while flow cytometry shows a greater amount of cell inactivation when compared to microscopy ($p < 0.005$).

In comparing the pathogenic strain to the non-pathogenic strain of *Escherichia coli*, significance is detected at -7°C and -15°C when determining culturable cells by drop plating, and at -15°C when counting the number of cells left unlysed, through microscopy. At -7°C, the pathogenic strain shows a greater amount of cells that were non-culturable through drop plating ($p < 0.001$), as well at -15°C, the pathogenic strain shows a greater amount of cells that were non-culturable through drop plating ($p < 0.005$). Also at -15°C, the pathogenic strain of *Escherichia coli* shows more cells were lysed, as detected through microscopy, than the non-pathogenic strain ($p < 0.001$).

In Figure 3.3iii, the vancomycin resistant strain of *Enterococcus faecalis* is cycled at -7°C, the injury detected through drop plating, after five freeze-thaw cycles, results in a log reduction of 3.59, whereas for injury detected through microscopy and flow cytometry, log reductions of 0.75 and 1.77, respectively, were observed. When the same strain is cycled at -15°C, detection of injury through drop plating resulted in a log reduction of 3.30, after five freeze-thaw cycles, while for microscopy and flow cytometry, log reductions of 0.41 and 1.32 were observed. And finally, when the vancomycin resistant strain of *Enterococcus faecalis* is cycled at -30°C, injury detected through drop plating results in a log reduction of 2.35, after five freeze-thaw cycles, while detection through microscopy and flow cytometry show log reductions of 0.48 and 1.58, respectively. At the end of the five freeze-thaw cycles, all three detection techniques differ statistically, in all three graphs, in the amount of cell inactivation. Counting through drop plating results in the greatest amount of cell inactivation, when compared to microscopy ($p < 0.001$) or flow cytometry ($p < 0.001$), also, flow cytometry shows a greater amount of cell inactivation when compared to microscopy ($p < 0.001$).

In Figure 3.3iv, when the vancomycin sensitive strain of *Enterococcus faecalis* is cycled at -7°C, the injury detected through plate counting, after five freeze-thaw cycles, results in a log reduction of 3.23, whereas for injury detected through microscopy and flow cytometry, log reductions of 0.82 and 1.53, respectively, are observed. When the same strain is cycled at -15°C, detection of injury through plate counting, after five freeze-thaw cycles, results in a log reduction of 3.51, while for microscopy and flow cytometry, log reductions of 0.62 and 1.45 are obtained. And finally, when the vancomycin sensitive strain of *Enterococcus faecalis* is cycled at -30°C, injury detected

through plate counting results in a log reduction of 2.58, while detection through microscopy and flow cytometry show log reductions of 0.61 and 1.50, respectively. At the end of the five freeze-thaw cycles, all three identification techniques differ statistically in all three graphs, in the amount of cell that were inactivated. Counting through drop plating results in the greatest amount of cell inactivation, when compared to microscopy ($p < 0.001$) or flow cytometry ($p < 0.001$), also, flow cytometry shows a greater amount of cell inactivation when compared to microscopy ($p < 0.001$).

In comparing the vancomycin resistant strain to the vancomycin sensitive strain of *Enterococcus faecalis*, significant difference is only observed at -7°C for the drop plate technique, in which the vancomycin resistant strain has a greater amount of cells that were inactivated by the five freeze-thaw cycles ($p < 0.05$). At the other two temperatures and for all techniques used, there is no statistical significance between the vancomycin resistant and the vancomycin sensitive on a 95% confidence interval.

3.4 Discussion

Analyzing cells on the flow cytometer can result in two population peaks, as seen in some of the histograms shown in Figure 3.1, where some of the cells are detected to have fluorescence, the peak on the right side of 10^1 on the x-axis, while some do not show fluorescence, as seen by the peaks that are on the left side of 10^1 on the x-axis. Contrary to this, Berney *et al.* (2007) also observed two peaks in population fluorescence after subjecting their cells, one strain of *Escherichia coli* (ATCC 700926) and one strain of *Enterococcus faecalis* (ATCC 29212), to freezing and thawing and then ultra-violet irradiation. They saw the double peaks in the double stained samples, but especially in the single stained samples, when the single stain was SYTO® 9; the epi-fluorescence

microscope revealed that these cells were in an unfinished state of division (Berney *et al.*, 2007). In this study, the cells were also examined using an epi-fluorescence microscope, however, it was difficult to determine if the cells were in a state of division, or simply in a state of distress (see Figure 3.2).

To coincide with the increasing amounts of red fluorescence as detected by flow cytometry, which indicated increasing amounts of cells with compromised membranes, increasing amounts of cells were unable to grow on the selective media used in this study. This was similarly observed in chapter 2, as the freeze-thaw cycles progressed from Day 0 to Day 5. Unfortunately, when indicator organisms are injured, they are often not capable of growing on typical selective media used for enumeration. Therefore, a non-selective media is needed, initially, so that the bacteria can repair themselves before they are subjected to the selective media for the remainder of their incubation period (McFeters and Camper, 1983). A few trials of freeze-thaw cycling were drop plated on BD Bacto™ Tryptic Soy Agar (Becton, Dickinson and Company, Sparks, MD, USA) and compared to the selective medium with no significant difference observed between the two mediums (data not shown).

The bacterial strains tested showed more cells that were unable to grow when cycled at -15°C than at either -30°C or -7°C, except for the vancomycin resistant strain of *Enterococcus faecalis*, which showed greatest reduction of cell growth at -7°C, followed by -15°C, then by -30°C, as shown in Figures 3.3. Hackney *et al.* (1979) noted that injured cells can lose their ability to recover in the presence of selective agents. Similarly noted, Ray and Speck (1972) observed that injury in *Escherichia coli* after freezing can be explained by the response of cells to a restricted supply of nutrients or to the presence

of compounds in media that infringe additional stresses on the cells. Both results from these investigators suggest that the decreasing amounts of cells that are culturable may result in the cell being unable to use the nutrients that are available to it on a selective medium, and as such, require nutrient rich media to be culturable. However, as stated previously, one trial was performed in which drop plates were plated in duplicate (one on selective media and one on non-selective media), and no significant difference was observed (data not shown).

However, using a fluorescent dye and the flow cytometer, the same observations of similar reduction in inactivated cells was not detected. Thus, some of the cells possibly had become injured to the point that they were too 'weak' to recover on the selective media, and the enriched media used did not contain the proper supplements to promote visible cultures. For water testing purposes, it is possible to recover the cells so that they can be enumerated through drop plating, by first giving them the opportunity to grow on nonselective medium at room temperature, and then exposing them to the specific selective environment to promote the selective growth and colony formation of the fecal coliforms, as Hackney *et al.* (1979) have done in their study on repair of fecal coliforms and *Enterococci.* In agreement to Hackney *et al.*, Ray and Speck (1972) also observed that the injury that is induced by freezing and thawing of *Escherichia coli* was repairable. It is important for water quality testing to be able to recover all injured indicator organisms; however, it is not feasible to only drop plate on enriched medium, as too much background microorganisms could result, such as moulds, which would swarm the plate, making detection of bacteria nearly impossible.

Unfortunately, when running bacteria on a flow cytometer, it is nearly impossible to set the forward- and side-scatter thresholds at levels that the whole bacterial population could be detected but no debris would be counted. This is because bacteria are as small as some debris, which may have resulted in some of the bacteria, in this study, to be not counted, as the boundaries set by the operator were quite strict. Also, as the samples were increasingly cycled at each temperature, the flow cytometer took longer to detect the 50,000 cells that were requested before stopping each run. For example, when cycling the pathogenic strain of *Escherichia coli* at -15°C, on Day 0 it took approximately 25 seconds, whereas on Day 5 it took approximately 50 seconds, and this is consistent for the four bacterial strains tested at each temperature. Slower detection could indicate lysing of bacterial cells causing less cells per milliliter on Day 5 than on Day 0, and as such a longer amount of time was needed to detect the same number of cells. This idea of increasing amounts of cells lysing was confirmed from the results obtained from counting intact cells using an epi-fluorescence microscope.

When running the positive control curve samples, generally, the 10:0 ratio of live to dead cells (100% live) showed some cells that expressed red fluorescence. This was similarly seen in an experiment performed by Hoefel *et al.* (2003), in which they mention that although the supposed 100% physiologically active suspension showed permeability to propidium iodide, the correlation was not significantly affected. Thus, the equations determined in this study, through the positive control curves, was thought to not be affected either. Additionally, Hoefel *et al.* (2003) had difficulties separating active and inactive bacteria because there was a range of cells that had an intermediate capacity for propidium iodide uptake, which was also observed in this study in which some of the

middle ratios tested, of the live to dead cells, was not represented as expected on the flow cytometer. Furthermore, staining bacterial cells with SYTO® 9 and propidium iodide does not always produce distinct "live" and "dead" populations, as determined by (Berney *et al.*, 2007), instead intermediate states are often observed. These intermediate states that are observed could cause a potential risk when decisions towards the effectiveness of disinfection methods have to be made (Berney *et al.*, 2007).

Even though there were difficulties in the Hoefel *et al.* (2003) experiment, they believe that the technological advances in both flow cytometry and fluorescent dyes offer a realistic approach for direct bacterial activity assessment in waters, returning bacteriological data rapidly and with high accuracy. Interestingly, Sachidanandham *et al.* (2004) subjected a two-day old seawater microcosm of *Escherichia coli* to three cycles of cold-shock and analyzed it by both flow cytometry and plate counting on mFC agar. They determined that a sharp decline in cell counts in the first cycle was detected by both methods, and subsequent cold-shock still showed viability through flow cytometry, but not by plate counting. They repeated their experiment with a seven-day old seawater microcosm and found similar results. The results determined in the current study are similar in that, generally, the most cell decline is seen in the first cycle of the freeze-thaw protocol, for all three detection techniques.

Calcott and Calcott (1984) confirmed that the proportion of survivors exhibiting wall damage, as detected through microscopy in the current study, was higher than those exhibiting membrane damage, as detected through flow cytometry in the current study. The difference between the cell concentration enumerated through drop plating, in the current study, and that determined by flow cytometry represents an injury that is not

explained by either wall damage or membrane damage. Thus, cells that undergo freezing and thawing in cycles incur damage to the cellular components within the cell that does not allow them to be enumerated through drop plating. This was similarly determined by Moss and Speck (1963) who froze *Streptococcus lactis* bacteria. They observed that there was a decrease in the number of colonies enumerated by plate counting on selective and non-selective agar media used. Thus they suggested that frozen and thawed cells were injured in such a way that their nutritional requirements were altered, and, as a result, the cells no longer grew on the selective agar media.

Notably, it has been suggested by Pettibone *et al.* (1987) that the use of antibiotic-resistant mutants to follow the fate of bacteria in the environment is inappropriate without adequate preliminary studies to ensure that resistant and wild-type strains react similarly to environmental stressors. The preliminary data collected here suggests that the antibiotic resistant strain of *Enterococcus faecalis* shows similar injury results to the antibiotic sensitive strain of *Enterococcus faecalis*, and as such is a good representation of what may actually be observed in the environment.

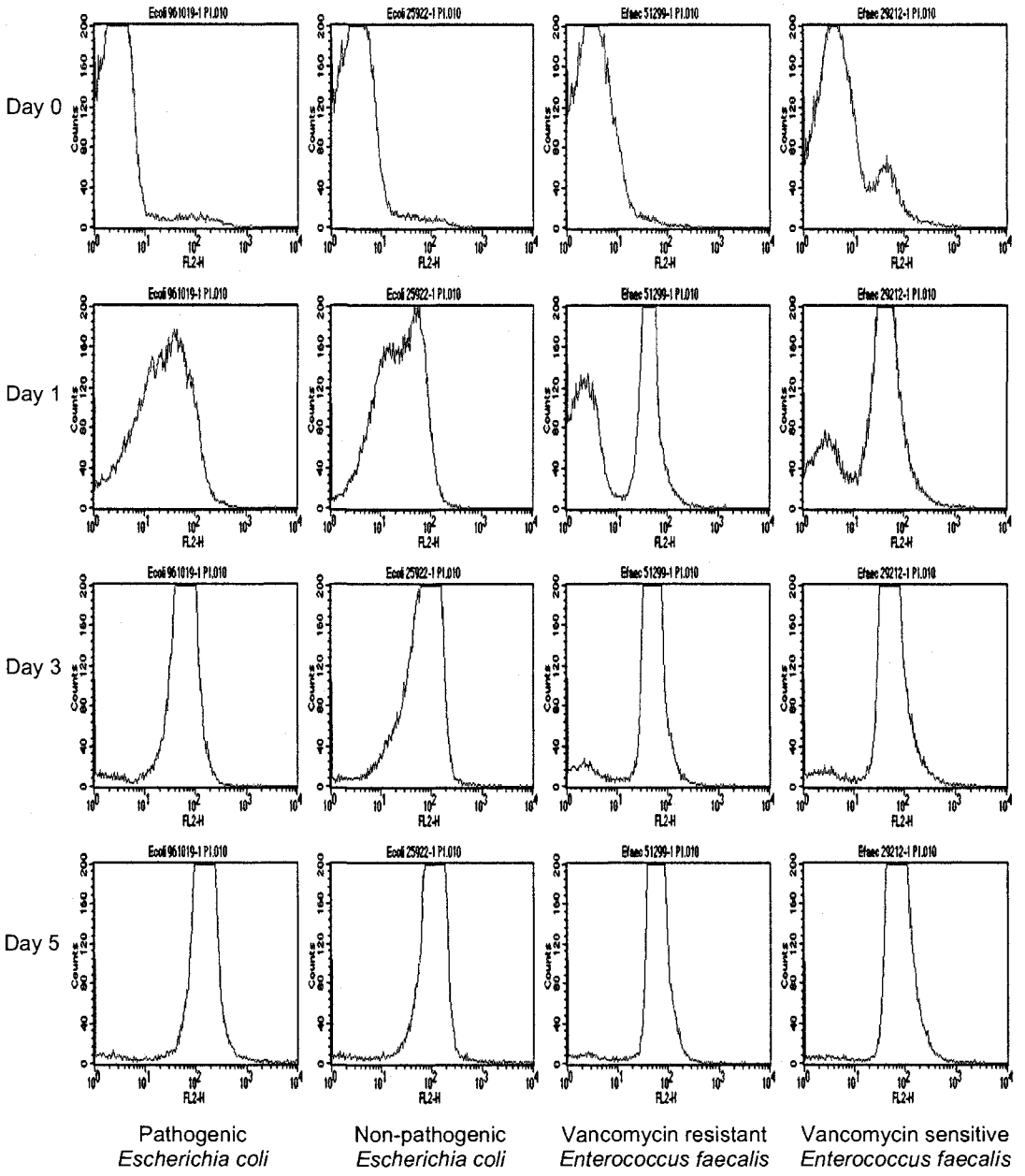


Figure 3.1 – Histograms from one trial of all bacterial suspensions cycled at -15°C. Data was collected daily for the five cycles, with data from Day 0, Day 1, Day 3, and Day 5 shown here. The y-axis represents the number of cells that were counted by the flow cytometer, and the x-axis represents the accumulation of red fluorescence, in log, that is emitted from each counted cell.

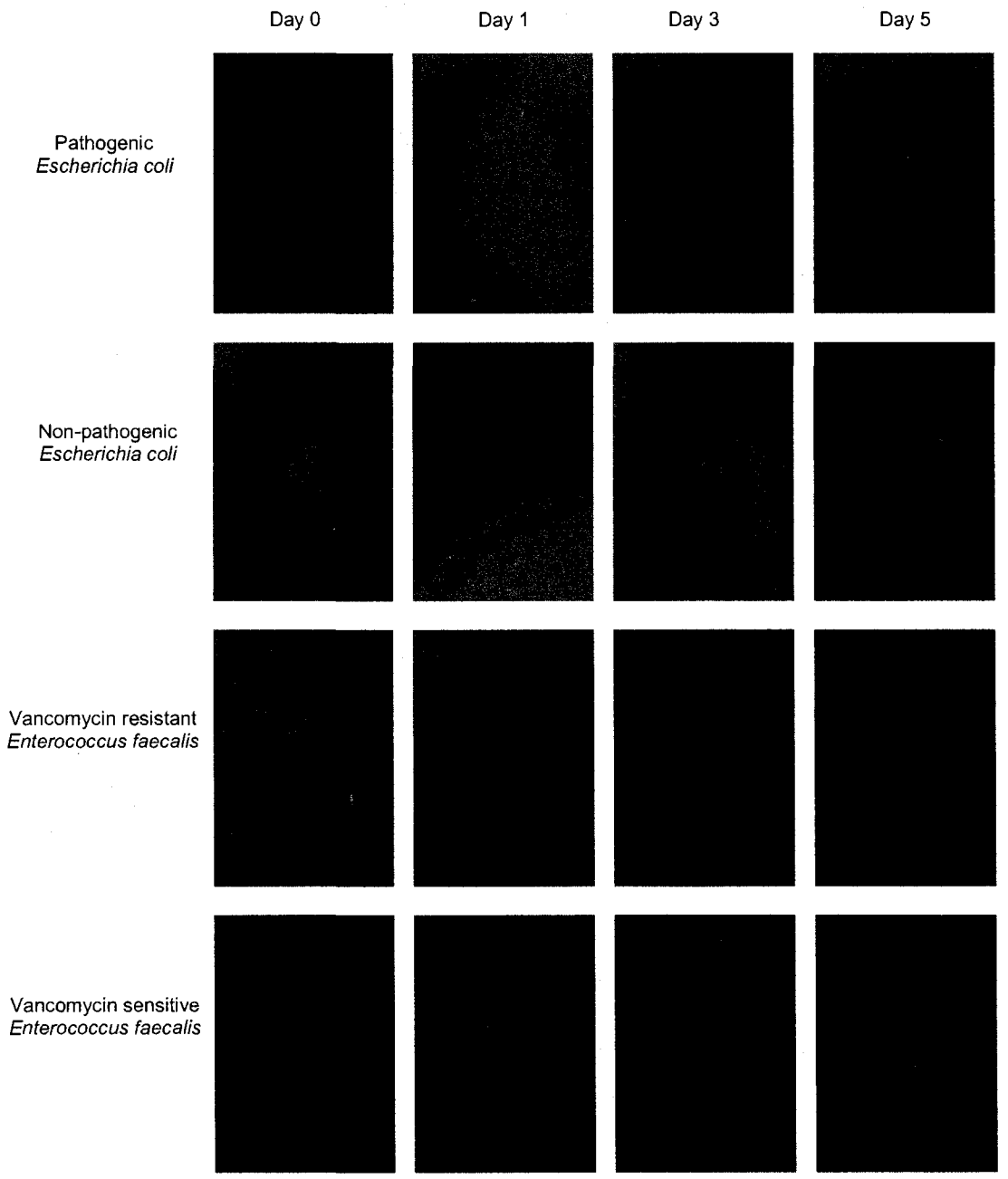
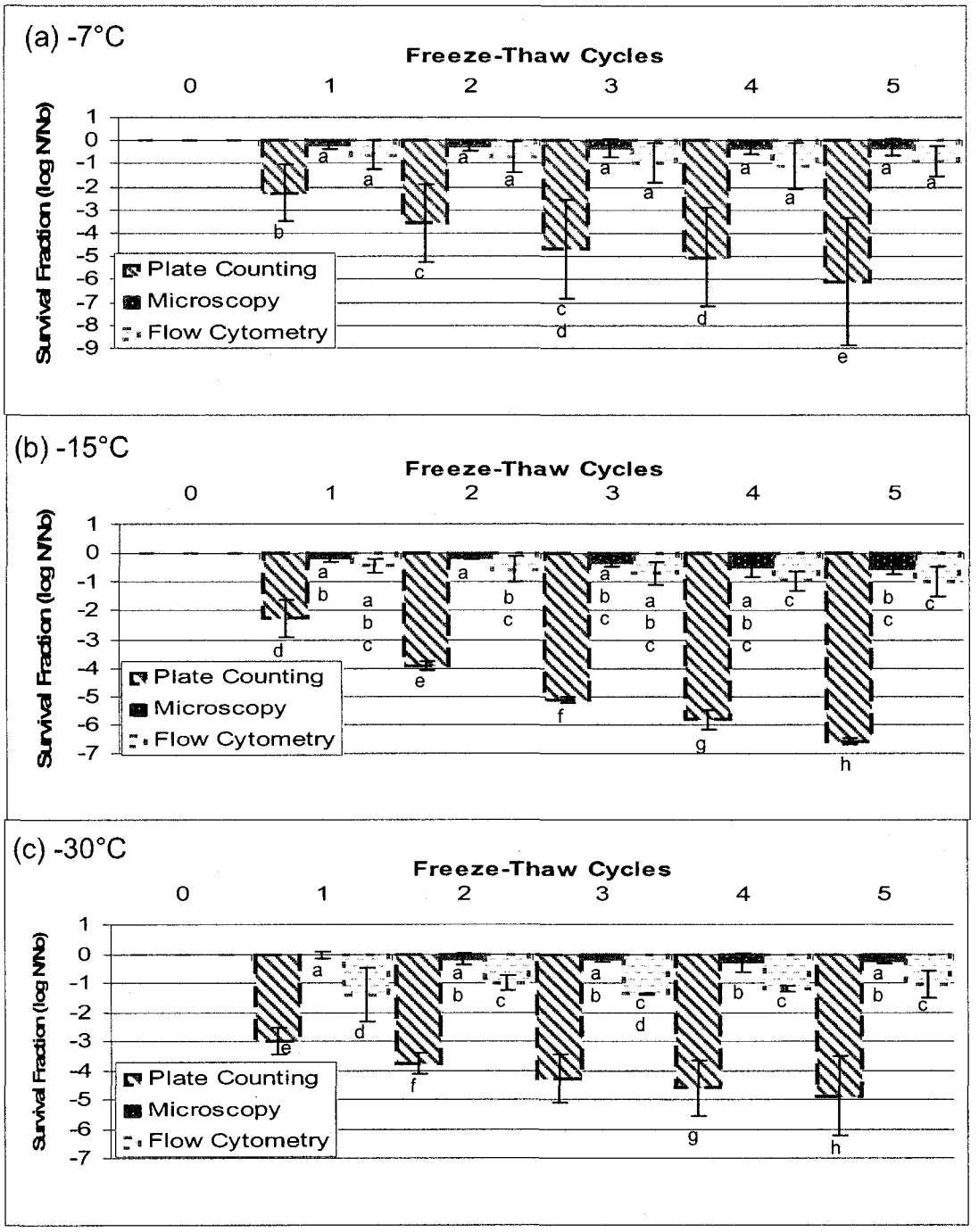
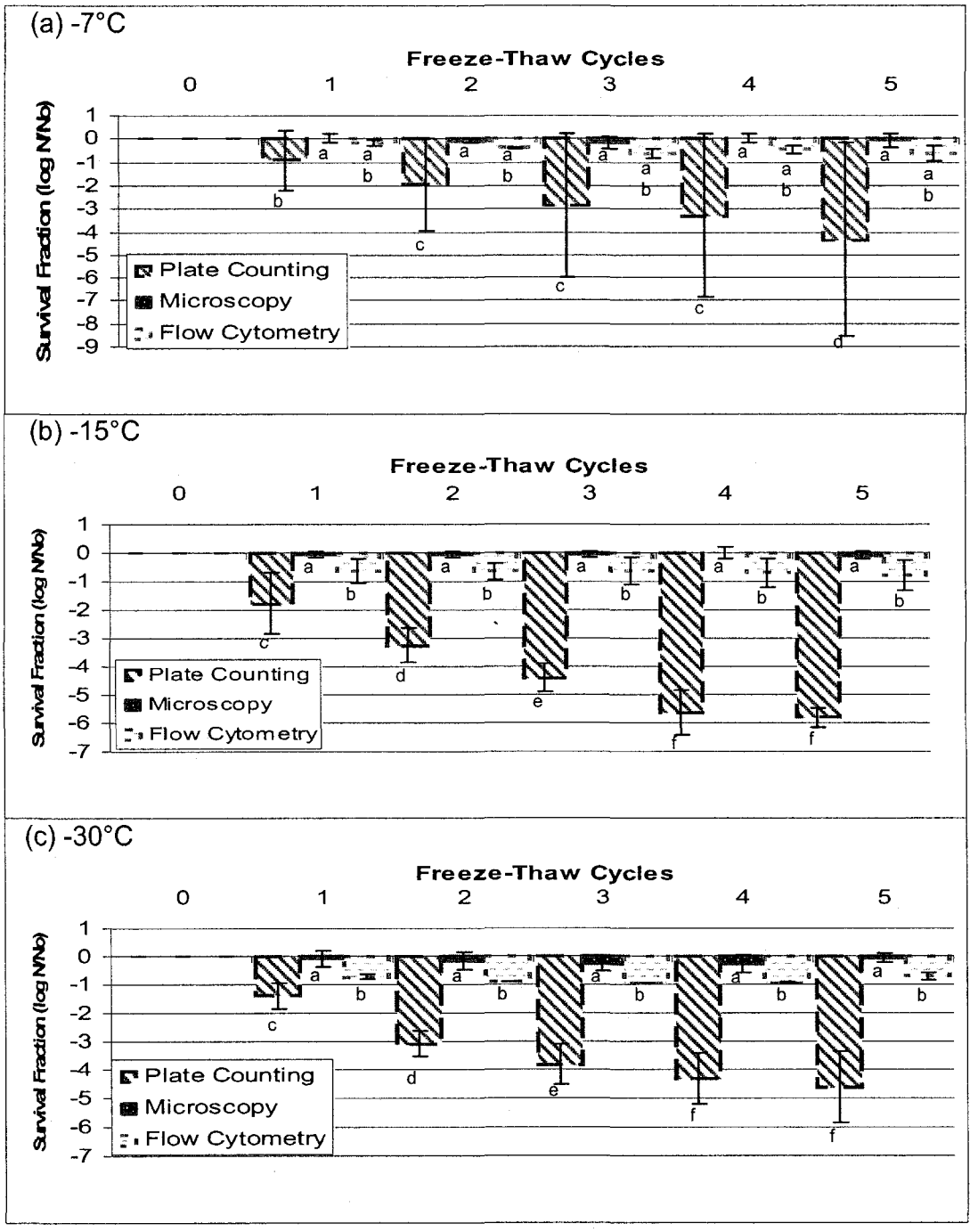


Figure 3.2 – Epi-fluorescence microscopy images from Days 0, 1, 3, and 5. Cells were stained with LIVE/DEAD® *Bac*Light™ Bacterial Viability kit from Molecular Probes; however all intact cells were counted to determine the direct viable count.



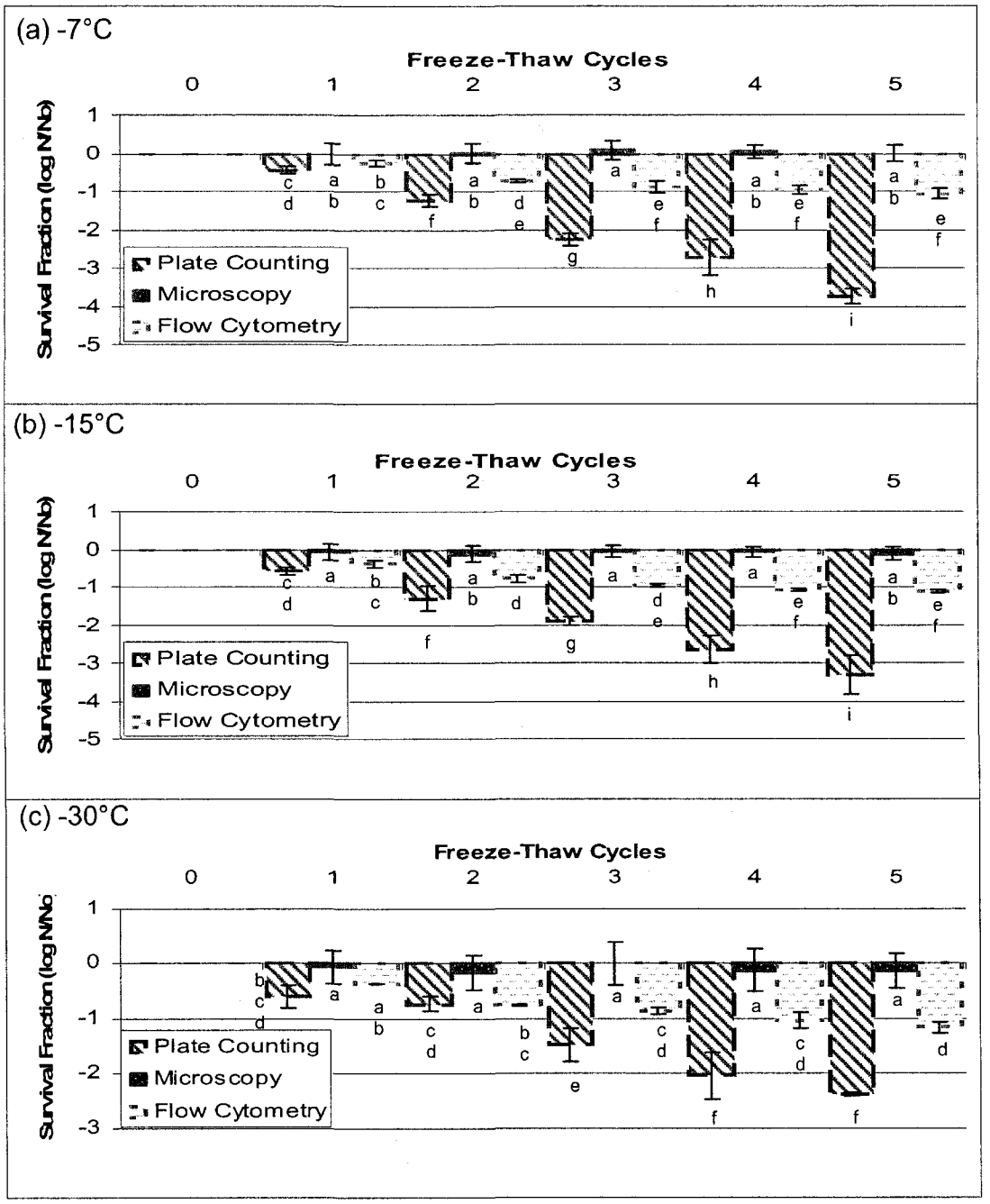
Pathogenic *Escherichia coli*

Figure 3.3i – Comparison of the pathogenic strain of *Escherichia coli* using the three different identification techniques to assess damage at the three different temperatures tested.



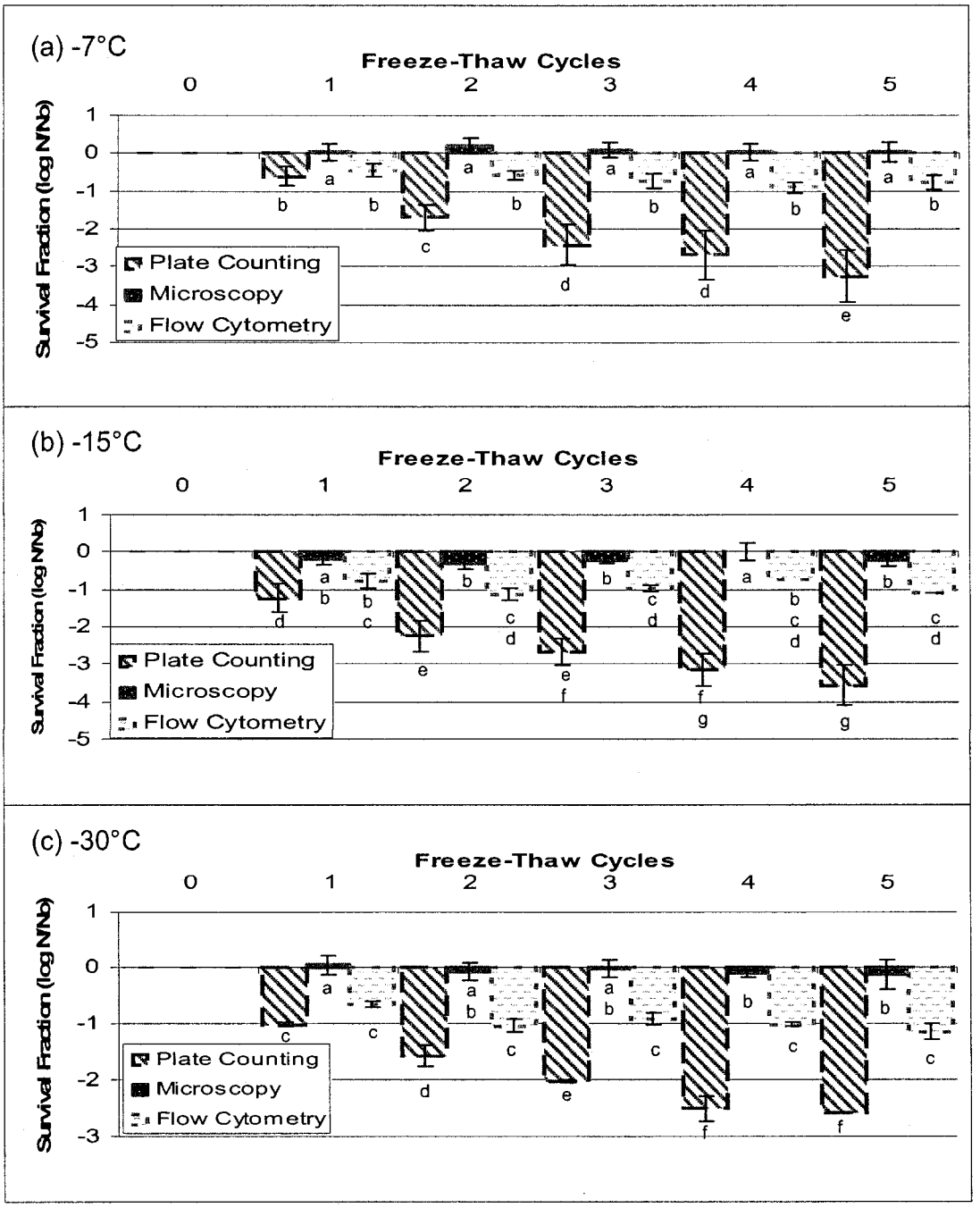
Non-pathogenic *Escherichia coli*

Figure 3.3ii – Comparison of the non-pathogenic strain of *Escherichia coli* using the three different identification techniques to assess damage at the three different temperatures tested.



Vancomycin resistant *Enterococcus faecalis*

Figure 3.3iii – Comparison of the vancomycin resistant strain of *Enterococcus faecalis* using the three different identification techniques to assess damage at the three different temperatures tested.



Vancomycin sensitive *Enterococcus faecalis*

Figure 3.3iv – Comparison of the vancomycin sensitive strain of *Enterococcus faecalis* using the three different identification techniques to assess damage at the three different temperatures tested.

Figure 3.3 – Comparison of three different identification techniques, on injury, for all four bacterial strains. The survival fraction of cells counted through drop plating, as indicated by the blue diagonal lines, shows the amount of cells that remained to be culturable after freeze-thaw cycling. The survival fraction of cells counted through microscopy, as indicated by the maroon dots, represents the amount of cells that were not lost through lysing; while the survival fraction of cells counted through flow cytometry, as indicated by the green dashed lines, depicts the amount of cells that still had intact membranes. Each letter in each graph represents statistical significance at a 95% confidence interval, with each graph being independent of the others.

Chapter 4: Conclusions

Four freezing temperatures were examined in this study, and it was determined that cell inactivation, as determined initially through drop plating, then by counting with an epi-fluorescence microscope and a flow cytometer, occurred at all four freezing temperatures; however, a greater amount of cells were non-culturable when the cells were frozen at -15°C.

This study mainly considered the effects of the freezing temperatures after five freeze-thaw cycles, and all temperatures showed an increase in the amount of inactivated cells at the end of the five cycles.

For the pathogenic *Escherichia coli*, at -30°C and -80°C there was no significant difference in culturability. While -7°C and -15°C both showed a significant difference between each temperature, with -15°C having a greater amount of non-culturable cells. Both -7°C and -15°C showed greater amounts of non-culturable cells compared to the other two freezing temperatures ($p < 0.001$). Also, the non-pathogenic *Escherichia coli* strain showed no significant difference in the amount of culturable cells at -30°C and -80°C. However, at -7°C and -15°C there was a significant difference between each temperature, with -15°C observed to have a greater number of non-culturable cells. Interestingly, -7°C showed fewer amounts of non-culturable cells, while -15°C showed greater amounts of non-culturable cells, compared to the other two freezing temperatures (-30°C and -80°C) ($p < 0.001$). Between the two strains, there was a greater amount of non-pathogenic *Escherichia coli* cells that were non-culturable than that of the pathogenic strain at -80°C, $p < 0.001$.

For the vancomycin resistant *Enterococcus faecalis*, at -7, -30, and -80°C there was no significant difference in the amount of culturable cells ($p > 0.05$). While at -15°C, a greater amount of non-culturable cells were observed when compared to the three other freezing temperatures ($p < 0.001$). The vancomycin sensitive *Enterococcus faecalis* strain at -30°C and -80°C showed no significant difference in the amount of culturable cells, while -15°C showed a greater amount of cells that were not culturable ($p < 0.001$) and at -7°C, a greater amount of non-culturable cells were observed ($p < 0.001$ for -30°C; $p < 0.005$ for -80°C). Between the two strains, there were significantly more cells that were non-culturable in the vancomycin sensitive strain than the vancomycin resistant strain, $p < 0.05$.

A part of this study also took a look at volume difference, 100µl and 100ml, at -7°C and -15°C. For both the pathogenic and non-pathogenic strains of *Escherichia coli* the 100ml volumes at -7°C showed a greater amount of cells that were non-culturable ($p < 0.001$), and at 100µl at -15°C showed a greater amount of non-culturable cells ($p < 0.001$). Between these two strains, there was a greater amount of cells that were non-culturable at -15°C in the 100ml volumes for the non-pathogenic strain, compared to the pathogenic strain ($p < 0.05$).

For the vancomycin resistant strain of *Enterococcus faecalis* the least amount of cell culturability was observed using the 100ml volumes at both -7°C and -15°C ($p < 0.005$ and < 0.001, respectively). The vancomycin sensitive strain of *Enterococcus faecalis* showed the least amount of cell culturability when using the 100µl at -7°C ($p < 0.001$), whereas at -15°C, there was no significant difference between the two volumes. Between the two *Enterococcus faecalis* strains, there was a greater amount of

cells that were non-culturable at -15°C in the 100µl volumes for the vancomycin sensitive strain, compared to the vancomycin resistant strain ($p < 0.05$).

During the last part of this study, the culturabilty, cell wall integrity, and membrane damage were examined using drop plating, epi-fluorescence microscopy, and flow cytometry, respectively. For the four strains, the amount of cells that remained culturable was significantly less than the amount of cells that were unlysed or had intact membranes ($p < 0.001$).

When examining the pathogenic strain of *Escherichia coli*, there were significantly more cells that were shown to be membrane damaged than cell wall breakage at -15°C and -30°C ($p < 0.05$ and 0.005, respectively). As well, the non-pathogenic strain of *Escherichia coli* showed similar results, in that, more cells were shown to be membrane damaged than cell wall breakage at -15°C and -30°C ($p < 0.001$ and 0.005, respectively). Between the two strains, the pathogenic strain shows significantly more cells to be non-culturable by drop plating at -7°C and -15°C, and to have cell wall breakage by epi-fluorescence microscopy at -15°C, than the non-pathogenic strain ($p < 0.001$, 0.005, and 0.001, respectively).

When examining both strains of *Enterococcus faecalis* there was a greater amount of cells that were not culturable, compared to those cells that had damaged cell walls or membranes at all three temperatures (-7, -15 and -30°C; $p < 0.001$). Also, the amount of cells that were observed to have damaged membranes was greater than the amount of cells that had broken cell walls, at all three temperatures ($p < 0.001$). Between the two strains, vancomycin resistant and vancomycin sensitive, the vancomycin resistant strain showed a greater amount of cells that were not culturable ($p < 0.05$), only.

Therefore, this study has shown that freezing and thawing affects bacterial cells that have a Gram-negative cell wall the most; although, some evidence is seen for injury to Gram-positive bacteria as well. Also, most of the injury is observed to be biochemical in nature; the cell has an inability to take up nutrients that are offered in selective media, and thus they are unable to grow on conventional selective media agar plates during drop plate enumeration. Interestingly, most of the injury that is incurred to the bacterial cells is not cell rupture, as initially hypothesized by many scientists. Only a small amount of cells that are unable to be detected through conventional enumeration methods are ruptured, and furthermore, only relatively few of them show a loss in membrane integrity.

4.1 Future Research

It would be interesting to complete this study even further by subjecting the frozen and thawed cells to conventional ultraviolet irradiation, as this technique of water treatment has become very popular, and has experimentally shown itself to be a healthier disinfectant than chlorine. As well, it would be interesting to see if freeze-thaw cycling has similar effects on other potential water-borne pathogens, such as *Cryptosporidium* or *Giardia lamblia*, as these have be noted to be resistant to normal amounts of chlorine disinfection.

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