EFFECTS OF FREEZING ON UV INACTIVATION OF WATERBOURNE MICROORGANISMS

By

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Abstract

In northern communities where source water may be frozen for parts of the year or wastewater is treated using freezing technology, the efficiency of UV inactivation may be affected. This research was carried out to investigate the response of *Escherichia coli*, *Enterococcus faecalis* and spores of *Bacillus subtilis* to UV irradiation after freezing and possible photoreactivation after the bacteria were exposed to UV. The inactivation of *E. coli* (doses 1.5 – 9 mJ/cm^2), *E. faecalis* (6 – 25 mJ/cm^2) and *B. subtilis* spores (20 – 120 mJ/cm^2) by ultraviolet light was investigated using a collimated beam UV apparatus. UV inactivation efficiency of *E. coli* after freezing treatments showed a significant decrease at all temperatures and freeze/thaw cycles. The greatest inactivation decrease, 1 log unit, occurred at intermediate UV fluences (2.5 and 4.5 mJ/cm^2). *E. faecalis* and *B. subtilis* spores showed no significant changes in log reduction after all treatments except for *E. faecalis* at higher temperatures (-7 and -15°C) and at lower fluences (6 and 8 mJ/cm^2).

The results suggest that UV inactivation could be less effective on bacteria pre-exposed to freezing. Therefore, water and wastewater treatment plants operating in cold climates may require an increased UV dosage compared to more temperate treatment plants in order to meet the same regulations.

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Chapter 1: Introduction

Ultraviolet (UV) inactivation has been used as an alternative to chlorination for the disinfection of water and wastewater. No known disinfection by-products and the effectiveness to inactivate *Giardia* and *Cryptosporidium* have made this technology increasingly popular over the past few decades (Taghipour, 2004, Batch *et al.*, 2004). However, efficiency of UV disinfection processes is affected by many factors, such as, water quality and varied bacterial sensitivity to irradiation (Shaban *et al.*, 1997, Mamane-Gravetz and Linden, 2005). With the increasing popularity of UV treatment, all known factors must be studied to have a better understanding and ability to use this technology to its highest potential.

The impact of cold temperatures on water and wastewater treatment processes, especially, on disinfection processes, is rarely studied. Little is known whether waterborne pathogens become more resistant or more vulnerable to the disinfection agents after experiencing freezing; either way, the efficiency of disinfection processes could be affected, especially in cold climates. A study by Nwachuku *et al.* (2005) suggested that viral particles show a greater susceptibility to UV inactivation by a range of fluences, 30 to 120 mJ/cm^2, after the number of freeze-thaw cycles increased from one to four. Conversely, *Bacillus megaterium* spores demonstrated resistance to a UV inactivation at 60 and 80 mJ/cm^2 after being exposed to freezing at -15°C (Gao *et al.*, 2007). In order to provide safe drinking water, or to achieve desired treatment objectives, consideration of the cold temperature impact on the treatment processes is necessary and important, especially, for those treatment plants operating in cold climates.

Recent studies reviewed that microorganisms that were irradiated by UV, may repair the damage after being exposed to visible light, therefore reducing UV disinfection efficiency. For example, *Escherichia coli* showed a significant ability to photoreactivate

(Tosa and Hirata, 1999), especially after low-pressure UV lamp exposure (Zimmer and Slawson, 2002). A higher fluence is required in order for photoreactivation to fail such as 28 mJ/cm^2 for *E. coli* strain 11299 (Zimmer and Slawson, 2002). No studies have investigated the ability of UV irradiated pathogenic bacteria to photoreactivate when they have been previously frozen.

1.1 Objectives

The objectives of this study are:

1. To study the response of *Escherichia coli, Enterococcus faecalis* and *Bacillus subtilis* spores to UV after freezing.
2. To investigate the effect of freezing temperature (-7, -15, -30°C) and freeze/thaw cycles (0, 1, 3, 5) on UV inactivation efficiency.
3. To examine if photoreactivation occurs, the level of repair and the effect freezing has on the selected bacteria's ability to repair.

The experimental results obtained from this study indicated that UV inactivation could be less effective on water pre-exposed to freezing. The bacteria *E. coli* showed the greatest difference between frozen and non-frozen samples with those frozen at -7°C having the lowest average inactivation. The largest difference in the inactivation level occurred in the cells that were exposed to only one freeze-thaw cycle. *E. faecalis* also showed a significant reduction in inactivation at lower fluences (6 and 8 mJ/cm^2); however *B. subtilis* spores showed no difference between frozen and non-frozen even after five freeze-thaw cycles.

All three microorganisms showed varied photoreactivation ability after exposure to UV light. *E. coli* had over one log photoreactivation, with frozen samples having a greater ability than unfrozen ones. Therefore, freezing can make bacteria respond with

resistance to UV inactivation and aids in its ability to photoreactivate. Both factors would require an increased fluence for the inactivation of *E. coli* compared to those found in unfrozen water.

Therefore, water and wastewater treatment plants operating in cold climates may require an increased dosage compared to more temperate treatment plants in order to meet the same regulations.

Chapter 2: Microbe Selection

In this study, three bacteria, *Escherichia coli*, *Enterococcus faecalis* and *Bacillus subtilis*, were chosen to deliver a wide range of inactivation (Hassen *et al.*, 2000). *E.coli* has been found to be the most sensitive of the three with *B. subtilis* spores showing the most resistance to UV disinfection (Mamane *et al.*, 2007, Sommer *et al.*, 1998).

One of the main differences between the bacteria chosen is their cell wall. The Gram stain is the most commonly used method for staining bacteria to distinguish between the two types of bacterial cell walls (Nester *et al.*, 2004). The bacteria are separated into two major groups, Gram-positive and Gram-negative. Gram-positive cells are able to retain the primary stain; crystal violet is commonly used, whereas Gram-negative cells become colourless after a decolourizing agent is applied. The difference in stain retention shows the fundamental difference in the chemical structure of the cell walls.

The cell walls of the Gram-positive bacteria are relatively thick and contain a dense layer of peptidoglycan which traps the stain. Gram-negative bacteria have a much thinner cell wall. The wall is high in lipid content and low in peptidiglycan. Gram-negative cell walls have an additional outer membrane made up of lipopolysacharides rather than phospholipids. Figure 2-1 is an illustration demonstrating both Gram-positive and Gram-negative cell walls.

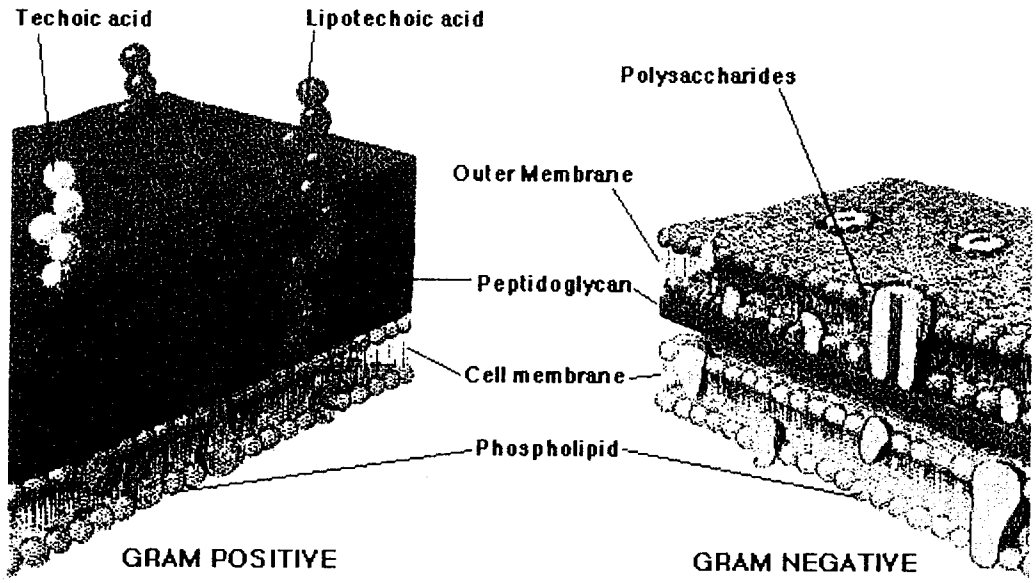

Figure 2-1 Cross-section of Gram positive and Gram negative cell walls (Betsy and Keogh, 2005)

2.1 *Escherichia coli*

Escherichia coli is a Gram-negative motile rod with most strains being non-pathogenic, however some, like *E. coli* O157:H7, can cause life-threatening illness (Salyers and Whitt, 2002). The non-pathogenic strains are part of the natural flora in the gastrointestinal tract and have a symbiotic relationship with their host. Fecal contamination is of great concern to the safety of drinking water and is therefore monitored (Nester *et al.*, 2004).

E. coli can survive outside of their hosts for long periods of time, which was shown by Watterworth *et al.* (2006) where strains survived greater than 56 days in well water. Therefore *E. coli* are useful for water monitoring and are the most frequently used fecal indicator organisms (Sommer *et al.*, 1998, Zimmer and Slawson, 2002). *E. coli* counts, along with heterotrophic plate counts and total coliforms counts are a means of quantifying UV efficiency at disinfection plants (Masschelein, 2002).

2.2 *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive cocci bacterium that also inhabits the gastrointestinal tracts of humans and other mammals. Enterococci may also be used as indicators of fecal contamination in a water supply (Nester *et al.*, 2004). It is part of the natural intestinal flora but can cause life-threatening infections when antibiotics diminish the other bacteria present, allowing the opportunistic bacterium to overrun the intestinal tract (Salyers and Whitt, 2002). *E. faecalis* can be considered a test organism that represents the group of enterobacteria (Masschelein, 2002).

2.3 *Bacillus subtilis*

Bacillus subtilis is a Gram-positive bacterium commonly found in soil. It has the ability to form a tough, protective endospore, which enables the bacterium to survive extreme environmental conditions. Spores are ubiquitous in natural waters, originating from the soil (Mamane-Gravetz and Linden, 2005).

Spores of *Bacillus* species are formed in sporulation, a process that is induced by low nutrient levels and they will be used in this study. Figure 2-2 illustrates the progression of a vegetative cell into an endospore. (A) The vegetative growth stops and DNA is duplicated through mitosis. (B) A septum forms that divides the cell into two asymmetric sections. (C & D) A forespore within a mother cell is formed when the larger compartment engulfs the smaller component. (E) Peptidoglycan-containing materials is laid down between the two membranes, forming the core wall and the cortex of the spore, meanwhile the mother cell produces proteins that create the spore coat and (F) the mother cell is degraded and the endospore released (Nester *et al.*, 2004).

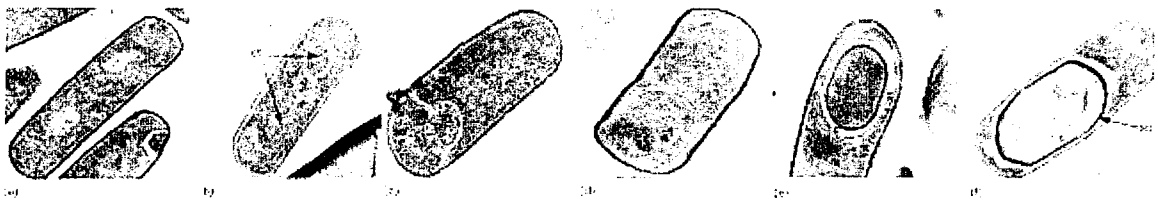

Figure 2-2 The sequential steps in the process of endospore formation in *Bacillus subtilis* (Todar, 2008)

B. subtilis spores are challenging to disinfect (Masschelein, 2002), which makes them ideal biodosimeters for prototype testing of commercial UV plants (Sommer *et al.*, 1998). They have also been studied in many UV studies (Qualls and Johnson, 1983). *B. subtilis* spores are one of the most commonly used microorganism for UV disinfection system validation, along with *MS2 coliphage* (Mamane-Gravetz *et al.*, 2005).

Chapter 3: UV Disinfection

3.1 History of UV Disinfection Technology

Ultraviolet (UV) disinfection is an accepted technology used for both water and wastewater (Masschelein, 2002). The germicidal properties of UV have been known since 1877 (USEPA, 2006). Even so, the prevalence of treatment facilities has not always been so abundant until recently. Disinfection by UV for water treatment was first used in Marseille, France in 1910 (USEPA, 2006). However, due to poor equipment reliability (Qualls *et al.*, 1985) and the low cost of chlorine, UV treatment did not become a widespread disinfection technology.

Then in the 1970s, chlorine was discovered to produce disinfection by-products (DBPs), such as trihalomethane which are harmful to aquatic life and possible carcinogens (NRC, 1980, Ward and DeGraeve, 1978). Accompanied by the increased effectiveness and reliability of the treatment, UV technology was again looked upon as a viable treatment (Severin, 1980, Qualls *et al.*, 1985). In addition, UV disinfection produced no known undesirable DBPs (Qualls and Johnson, 1983). Today, UV has continued to be a rapidly growing alternative to chlorination for wastewater and drinking water worldwide (USEPA, 2006).

3.2 Fundamentals of UV Inactivation of Microorganisms

3.2.1 UV Light

The wavelength of UV light ranges from 200 to 400 nanometers (nm) and can be separated into three sub-ranges: UVA (315-400 nm), UVB (280-315 nm), UVC (200-280 nm) (Masschelein, 2002). The ranges are also referred to as near, medium and far UV respectively. The UVC range is extremely dangerous since it is absorbed by proteins,

ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) and can lead to cell mutations, cancer, and even cell death (Jagger, 1967). The UVC range is also referred to as the germicidal range, since it is very efficient in inactivating bacteria and viruses.

3.2.2 UV Absorption

Most molecules have absorption bands in the UV region. When a molecule absorbs light, it is raised to an excited electronic state. In this excited state, it can react either by dissociation or by reacting with another molecule. The molecule can also return to the ground state either by releasing the excess energy as heat or by emitting a photon of light (fluorescence) (Jagger, 1967).

UV light is absorbed by proteins, RNA and DNA in a given microorganism. Absorption of UV by proteins in membranes at high fluences ultimately leads to the disruption of the cell membranes and hence death of a cell. The aromatic amino acids and peptide bonds are the important UV absorbers in proteins. All the bases in nucleic acids are aromatic and exhibit high absorption in the UVC range and are 10-20 times more likely to absorb than equal weights of protein (Jagger, 1967).

However, at much lower fluences, absorption of UV by DNA (or RNA in some viruses) can disrupt the ability of the microorganism to replicate. If a cell is unable to reproduce, it cannot cause disease and will die (Bolton, 2001).

DNA is a nucleic acid polymer in a double-stranded helix linked together by a sequence of four bases (adenine, cytosine, guanine and thymine), which make up the genetic code. These form base pairs, adenine with thymine and cytosine with guanine, and are held together by hydrogen bonds. The conjugated-ring structure of the bases allowed them to absorb UV wavelengths (Snyder and Champness, 1997). The energy resulting from the absorbed UV results in chemical bonds forming with nearby atoms.

Many different types of bonds are possible including abnormal linkages between bases in the DNA and other bases or between bases and the sugars of the nucleotides.

3.2.3 Dimer Formation

Absorption of UV light between 200 and 280 nm (UVC) can cause the production of pyrimidine dimers. The pyrimidine dimer is a common type of UV irradiation damage to both DNA and RNA. Dimers are formed primarily between thymines but they can also form in combinations of thymine-cytosine or cytosine-cytosine. A thymine dimer occurs when two thymine bases are located adjacent to each other and one of them adsorbs a UV photon and forms a chemical bond with the neighboring thymine. The action spectrum for this photochemical dimerization peaks at about 260 nm and follows closely the absorption spectrum of DNA (Bolton, 2001).

The photochemical dimerization of thymine pairs disrupts the structure of DNA, so that if enough thymine dimers are formed, the DNA cannot replicate in cell mitosis. This is the primary mechanism of UV disinfection (Bolton, 2001). The following schematic illustrates two photoproducts of thymine after UV light exposure.

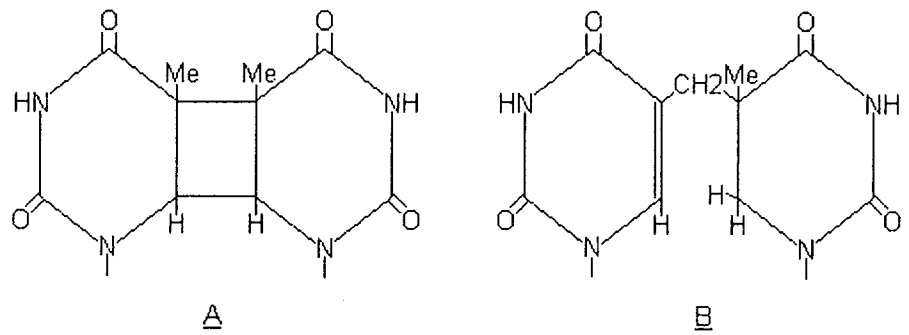

Figure 3-1 Structures of (A) cyclobutane-type thymine-thymine dimer and (B) spore photoproduct. Me - methyl group and N bonds at bottom are from the sugar residues in DNA (Adapted from Setlow, 2000)

For most organisms, the major photoproduct caused by 254 nm UV irradiation of growing cells is a *cis*, syn-cyclobutane thymine dimer shown on the left in Figure 3-1 (Setlow, 2000). On the right side, the major photoproduct formed when spores were irradiated is a thyminyl-thiymine adduct that is termed a spore photoproduct (Setlow, 2000).

3.3 Application in Drinking Water and Wastewater Industry

3.3.1 Current and Future of UV technology

UV has many commercial applications including UV disinfection of water and air, UV curing of inks and coatings, UV disinfection of foods, UV-based advanced oxidation destruction of pollutants in water and air (Hanes *et al.*, 2002).

3.3.1.1 Water and Wastewater Disinfection

Disinfection using UV irradiation is used in wastewater treatment and is increasing in usage in drinking water treatment. Many bottled water producers use UV to disinfect their water.

New York City has approved the construction of a 2 billion gallon per day UV drinking water disinfection facility. There are also several facilities under construction and several in operation that treat wastewater with several stages of filters, hydrogen peroxide and UV light to bring the water up to drinking water standards (USEPA, 2006).

Pairing UV technology with other disinfection methods has been tested and proved advantageous. The combined use of UV and ionizing radiation, when complete inactivation is desired, is advantageous because UV will be used to inactivate the freely suspended microorganisms and then the more expensive technology, ionizing radiation, will be used for inactivating particle-associated microorganisms (Taghipour, 2004).

3.3.1.2 Other Applications

Ultraviolet irradiation is also used in several food processes to kill unwanted microorganisms. UV light can be used to pasteurize fruit juices by flowing the juices over a high intensity ultraviolet light source. In a study by Hanes *et al.* (2002), UV technology was used to treat *Cryptosporidium parvum* oocysts, a protozoan parasite that has caused food related outbreaks. They were able to achieve a 5 log reduction without compromising the organoleptic qualities of fresh cider. As consumer demand for fresh and "fresh-like" food products increases, the demand for non thermal methods of food processing is likewise on the rise.

Ultraviolet reactive inks and coatings require a high intensity source of ultraviolet light to initiate a chemical reaction, curing the ink or the coating almost instantaneously. Certain inks, coatings and adhesives are formulated with photoinitiators and resins that when exposed to UV light, polymerize, causing the adhesives to harden or cure. Applications include glass and plastic bonding, optical fiber coatings, the coating of flooring, UV coating and paper finishes in offset printing and dental fillings.

3.3.2 Terminology

Before looking more in depth at its role in drinking water and wastewater applications, a background of terms and equipment must be established.

3.3.2.1 Fluence

Fluence is defined as the total radiant energy of all wavelengths passing from all directions through an infinitesimally small sphere of cross-sectional area dA divided by dA. If the fluence rate is constant, fluence is given as the fluence rate times the exposure

time in seconds. The fluence rate is defined as the total radiant power incident from all directions onto an infinitesimally small sphere of cross-sectional area d*A* divided by d*A*.

Irradiance, which is defined as the total radiant power incident from all upward directions on an infinitesimally element of surface of area d*S* containing the point under consideration divided by d*S*, is sometimes confused with fluence rate. Figure 3-2 shows the difference in the angles of absorbance. Irradiance and fluence rate become identical when using a collimated beam apparatus (Bolton, 2001). Nevertheless, the appropriate term for UV disinfection is fluence rate because a microorganism can receive UV power from any direction, especially when there is more than one lamp used in the process (Bolton, 2001).

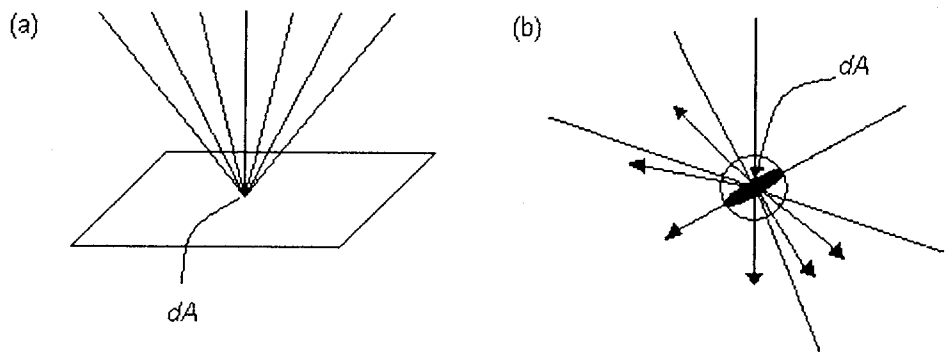

Figure 3-2 Illustration of the concept of irradiance and fluence rate: (a) irradiance onto a surface, (b) fluence rate through an infinitesimally small sphere of cross-sectional area (Adapted from Bolton, 2001)

Similarly, the term "UV dose" is often used in UV disinfection literature. It represents the UV exposure of a given organisms in the germicidal range (Bolton, 2001). The term dose is normally applied in situations where the radiation is totally absorbed (eg. UV in sunlight absorbed by the skin causing sun tanning or burning). However, since less than 1% of the UV incident on a microorganism is absorbed, the term dose is not appropriate for UV treatment applications.

Most scientists and engineers now use the unit milijoule per square centimetre (mJ/cm^2) or joule per square meter (J/m^2) for fluence. The units J/m^2 are used in most parts of the world except for North America where mJ/cm^2 are used (1 mJ/cm^2 = 10 J/m^2). The old term milliwatt seconds per square centimetre (mWs/cm^2) is equivalent to mJ/cm^2 since a watt-second is the same as a joule. (1000 microwatt = 1 milliwatt therefore 10 J/m^2 = 1 mJ/cm^2 = 1 mWs/cm^2).

The survival of microorganisms is a function of fluence:

$N_s/N_o = f$ (fluence) (Equation 3-1)

where N_o and N_s are the density of organisms before and after irradiation respectively.

3.3.2.2 Biodosimetry

Bacteria are commonly used to test UV efficiency. A wide range of bacteria are used in order to see how a varied group of these organisms would react. The cell wall of bacteria is a common difference among bacteria that would show varying responses to UV light. Bacteria with thicker cell walls have proven to be more resilient to the effects of UV damage (Sommer *et al.*, 1998).

Inactivation data of microorganisms causing waterborne diseases have to be studied in order to establish a UV fluence for safe drinking water disinfection (Sommer *et al.*, 1995). Biodosimetry is a reliable method used to measure UV fluence in a UV reactor (Bolton, 2001). This method involves seeding harmless microorganisms into the influent of a UV reactor and taking samples of the influent and effluent to calculate the inactivation. A fluence-response curve can also be calculated in the laboratory using a collimated beam apparatus. The effluent results to the fluence-response curve can be compared to estimate the fluence of the reactor (Bolton, 2001).

3.3.2.3 UV Fluence-Response

UV inactivation efficiency, meaning how effective UV is at inactivating cells, is measured by dose-response curves. Microbial response to UV varies for each microorganism. UV dose-response is determined by irradiating water samples containing the microorganism with various UV doses using a collimated beam apparatus and measuring the concentration of infectious microorganism before and after exposure.

$$\text{Log inactivation} = \log_{10} N_0/N \qquad \text{(Equation 3-2)}$$

where N_0 = Concentration of microorganisms before exposure to UV light

N = Concentration of microorganisms after exposure to UV light

UV dose-response can be expressed as either the proportion of microorganisms inactivated or the proportion of microorganisms remaining as a function of UV dose (fluence). The UV dose-response curves show a positive and negative slope respectively. Most literature refers to microbial response as log inactivation and is shown with log inactivation on a logarithmic scale and UV dose on a linear scale.

The curves usually show an initial steep decline in cell viability which can be attributed to free-swimming organisms (Gehr *et al.*, 2003). A second stage, with a much shallower slope (tailing) follows and can be due to shielding effects or clumping of the organisms.

An issue with published data and experiments run by researchers around the world are the inconsistencies in the findings and the varied equipment and different parameters used to study this treatment process (Sommer *et al.*, 1995). Efforts have been made to standardize testing (Sommer *et al.*, 1995, Bolton and Linden, 2003).

3.3.3 Types of UV Lamps

A wide choice of alternatives exists for lamp technologies (Masschelein, 2002). Conventional UV technology is based on continuous-wave mercury vapour lamps in either low-pressure (LP) (monochromatic at 253.7 nm) or medium-pressure (MP) (polychromatic in the UV and visible light range from 200 to 400 nm) formats (Bohrerova *et al.*, 2008). A mercury vapor germicidal lamp is constructed of quartz glass, argon gas, mercury and the filaments used to ignite the mixture (USEPA, 2006).

In wastewater treatment, most present and existing applications are based on low-pressure lamp technologies (Masschelein, 2002). UV light can be produced by either low-pressure (LP) mercury vapour lamps or low-pressure high-output (LPHO) mercury vapour lamps. LPHO can achieve up to 18 times greater germicidal UV output than LP, therein lays its advantage (USEPA, 2006). The higher the output, the exposure time necessary is lowered.

From investigations, MP high emission intensity systems can be more economical than the more conventional LP lamp systems, in both capital investment and lifetime costs (Masschelein, 2002). MP UV lamps have much higher germicidal UV power output than both LP and LPHO (Bolton and Linden, 2003). The number of plants making use of MP lamps is increasing rapidly (Masschelein, 2002). Bukhari *et al.* (1999) proved that MP lamps were as capable of inactivation of *Cryptosporidium parvum* oocysts as LP lamps. Full-scale drinking water applications generally use LP, LPHO or MP mercury vapour lamps (USEPA, 2006).

Some alternatives to mercury lamps are eximer and pulsed lamps, UV lasers and light emitting diodes (LEDs). Until now, the use of eximer lamps and pulsed Xenon lamps have been experimental but are looked at as a possible alternative lamp source for future UV technologies (Masschelein, 2002).

Pulsed wave UV (PUV) technology is relatively new and has been limited in the application to water disinfection (Bohrerova *et al.*, 2008). PUV lamps are mercury-free and do not require a warm-up period. A high power electrical pulse is discharged in microsecond bursts to produce intense light pulses. The discharge is in a rare gas (xenon or krypton) that is non-toxic. In a study by Bohrerova *et al.* (2008), PUV was able to inactivate *E. coli* at a faster rate than LP or MP UV. They explained that additional damage from the high intensity of light could damage other cell enzymes and cause the cell to reach its inactivation threshold faster, resulting in an increased inactivation when compared to LP or MP mercury lamps (Bohrerova *et al.*, 2008).

3.3.3.1 Correction factors for LP UV lamps

UV studies usually use a collimated beam apparatus to carry out research. Several correction factors are required to determine the average irradiance in the water sample in order to determine the average fluence rate to which each microorganism is exposed. From there, a delivered fluence to a sample can be calculated (Bolton and Linden, 2003). The four correction factors are as follows:

1. Reflection Factor (R*f*) – When a beam of light passes from one medium to the other, some of the light reflects off the interface between the media (Bolton and Linden, 2003). A constant Reflection Factor of 0.975 corrects for the 2.5% of incident UV irradiance that is reflected back from the surface of the sample (Bolton and Linden, 2003).

2. Petri Factor (P*f*) – The Petri Factor is necessary to account for the variance of irradiance over the surface of the sample. Measurements are taken out from the center of the Petri dish and divided by the center irradiance, then calculated to find an average of ratios known as the Petri Factor (Bolton and Linden, 2003).

3. Water Quality Factor (W*f*) – A water quality correction is made for UV that is absorbed as it travels through the sample. This value is derived form integrating the Beer-Lambert Law over sample depth and takes into account the water absorption coefficient. Bolton and Linden (2003) define the Water Factor as:

$$\text{Water Factor} = \frac{1-10^{-al}}{al\ln(10)} \qquad \text{(Equation 3-3)}$$

where a = absorbance for a 1 cm path length

l = vertical path length (cm) of the water in the Petri dish

4. Divergence Factor (D*f*) – When using a collimated beam for testing, the beam is never completely collimated (Bolton and Linden, 2003). The UV irradiance diverges significantly and can be determined by the following equation

$$\text{Divergence Factor} = \frac{L}{(L+l)} \qquad \text{(Equation 3-4)}$$

where L = distance from the UV lamp to the surface of the cell suspension

l = vertical path length of the cell suspension in the Petri dish

Therefore the average germicidal fluence rate is given by

Ave. Fluence rate = Fluence rate X P*f* X R*f* X W*f* X D*f* (Equation 3-5)

3.4 Factors Influencing the Efficiency of UV Disinfection Processes

Equipment variables such as UV intensity, water quality, including total suspended solids (TSS) and turbidity, water temperature and pH are all factors that affect UV efficiency.

3.4.1 UV Intensity

Sommer *et al.* (1995) found that higher UV intensities would produce higher inactivation effects. Liu and Zhang (2006) also illustrated that higher intensities was

more efficient at inactivating microorganisms than at lower intensities. A lower fluence was required when using a higher UV intensity to achieve the same inactivation rate compared to when using a lower intensity (Liu and Zhang, 2006).

3.4.2 Water Quality

3.4.2.1 Total Suspended Solids (TSS)

Suspended particles can have a varied effect on UV efficiency including; scattering or absorbing light, shielding of microorganisms, and occlusion of microorganisms into the suspended particle (Qualls *et al.*, 1983). In all cases, the larger the particle, the more effect will be shown (Madge and Jensen, 2006).

Madge and Jensen (2006) separated wastewater into three categories and found that 55 to 65% were free-floating bacteria, 30 to 45% were bacteria associated with 20 µm particles and the remaining bacteria were associated with particles less than 20 µm.

A possible solution to TSS affecting UV efficiency is for a pre-filtering that would remove larger particles. A study by Jolis *et al.* (2001) showed that when particles greater than 7 µm were removed by sand filtration, a fluence reduction of 940 to 820 J/m^2 was observed to achieve a 4 log inactivation.

3.4.2.2 Turbidity

Another factor affecting UV efficiency is turbidity. This is especially evident when nephelometric turbidity units (NTU) are greater than 5 (Shaban *et al.*, 1997). Similar to TSS, turbidity can affect UV disinfection in two ways; first it might decrease the UV transmittance of the water and secondly it can shield microorganisms by shadowing or having them embedded into the surface of the particle. Unfiltered urban wastewater is typically 1.5 to 6 NTU (Masschelein, 2002).

Turbidity influenced the effect of UV inactivation of bacteria when it was greater than 4 NTU (Liu and Zhang, 2006). Therefore, water with low turbidity is more suitable for UV treatment, indicating that turbidity should be lowered using pre-treatments prior to UV disinfection. The same factors that may affect UV irradiations ability to inactivate microorganisms may also hinder their ability to photoreactivate (Tosa *et al.*, 2003).

3.4.2.3 Temperature and pH

The water temperature can affect the UV output of LP UV lamps but not as much for LPHO or MP UV lamps (USEPA, 2006). The optimum water temperature is about 22°C and the output drops to about 80% at 0°C. When the lamps are encased in a quartz sleeve with water on the other side, the effects are not so large. The effect of water temperature on the lethal dose is negligible in drinking water by either an increase or decrease of 10°C (Masschelein, 2002).

3.5 Photoreactivation (mechanisms and current findings)

One major disadvantage to UV disinfection is that following UV irradiation there is a possibility for repair. As a result of exposure to UV radiation from sunlight, many organisms have developed mechanisms to compensate for the damaging effects of UV radiation (Zimmer and Slawson, 2002). Microorganisms are able to repair by an enzyme-mediated microbial process where damaged strands of DNA are repaired. Energy for this process can be derived by light energy (photoreactivation) or chemical energy (dark repair).

3.5.1 Photoreactivation

The first observation of photoreactivation was by Albert Kelner in the 1940s when the bacterium *Streptomyces griseus* was more likely to survive UV irradiation in the light than in the dark (Snyder and Champness, 1997).

Photoreactivation begins when a photoreactivating enzyme called photolyase complexes with a pyrimidine dimer. Visible light ranging from 350 nm to 500 nm can then be absorbed by a reduced flavin adenine dinucleotide group contained by the enzyme (Snyder and Champness, 1997). The light energy then enables photolyase to separate the fused thymine bases.

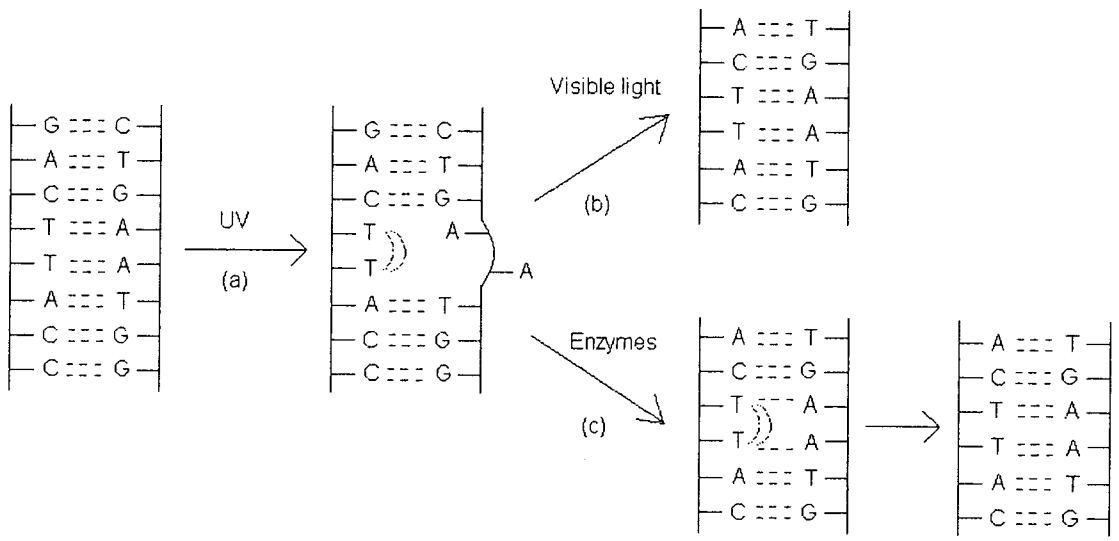

Figure 3-3 Schematic of (a) dimerization of the thymine base, (b) photoreactivation and (c) dark repair (Adapted from Masschelein, 2002)

Possible photoreactivation in wastewater treated by UV-C is usually calculated using a log-increase approximation (Masschelein, 2002):

$$\text{Log } (N_{pr}/N_o) - \text{log } (N/N_o) = \text{Log } [(N_{pr}/N_o)/ (N/N_o)] = \text{log } (N_{pr}/N) \qquad \text{(Equation 3-6)}$$

where: N= concentration of organisms surviving UV disinfection

N_o = concentration of organisms prior to UV disinfection

N_{pr} = concentration of organisms after photoreactivation

3.5.2 Dark Repair

Reactivation can also occur in the absence of light and is therefore termed dark repair. The main mechanism for dark repair, which can also occur in the presence of light, is called nucleotide excision repair and is the most common form of DNA repair in all organisms. This repair process involves the action of more than a dozen proteins that coordinate the removal of dimers in DNA or RNA produced by exposure to germicidal UV light (Shaban *et al.*, 1997, Bohrerova and Linden, 2007). DNA or RNA can then be resynthesized using the existing complementary strand of DNA (Kashimada *et al.*, 1996). Therefore repair by excision repair occurs only with double stranded DNA or RNA. The excised DNA fragments containing pyrimidine dimers and other types of damage by UV can be found in the media outside of the cell (Snyder and Champness, 1997).

The photoreactivating system may also help repair pyrimidine dimers even in the dark by making the sites more recognizable to the nucleotide excision repair system (Snyder and Champness, 1997).

This repair method (dark) is slower and less effective than photoreactivation (Salcedo *et al.*, 2007). In a study by Zimmer and Slawson (2002) *E. coli* strain 11229 had an average effective log repair of 0.4 whereas under photoreactivating light after the same fluence of 8 mJ/cm^2, there was an average effective log repair of 2.6. In other studies and at most fluences, maximum activation by dark repair was less than 1% (Salcedo *et al.*, 2007, Zimmer and Slawson, 2002).

3.5.3 Factors Affecting Repair

Repair can range from 1 to 3.4 log photoreactivation, which suggests that this is not a simple process (Masschelein, 2002). There are many factors that affect photoreactivation such as initial UV fluence, wastewater quality, exposure time to photoreactivating light, temperature and the species of the microorganism (Hassen *et al.*, 2000).

3.5.3.1 Temperature

An increase in temperature allows for a greater number of cells to photoreactivate. Also, the increase in temperature allows those that would repair to do so at a faster rate. Therefore, temperature is affecting photoreactivation in two ways; increased reaction rate and increased overall photoreactivation. After UV irradiation of 100 mJ/cm^2, Salcedo *et al.* (2007) exposed wastewater to photoreactivation light at temperatures ranging from 5 to 30°C and observed a maximum percent survival of 0.881 total coliforms at 30°C. Reported in the same study, dark repair also demonstrated maximum percent survival of 0.0198 at the highest temperature (Salcedo *et al.*, 2007).

As temperature increases, chemical reactions also increase and since reactivation by DNA repair is subject to enzymes, then it is likely to be affected by temperature. Therefore a way of inhibiting photoreactivation is to keep temperatures low, which is common in cool temperate climates.

3.5.3.2 Repairing light and exposure time

In most photoreactivation literature, the repair studies are reported on a time-basis, often ignoring the importance of lamp intensity (Tosa and Hirata, 1999, Zimmer *et al.*, 2003, Salcedo *et al.*, 2007). Photoreactivation fluence can be calculated and used

similarly to UV fluence by multiplying average light irradiance and time (Bohrerova and Linden, 2007). However many reports are still using time as a way of calculating photoreactivation, mainly due to the fact that photoreactivation is more dependant on the time of exposure to photoreactivating light than to the irradiance of the light (Oguma *et al.*, 2002).

It has also been noted that light intensity should be standardized because if not enough photoreactivating light is used in experiments false negatives could occur. Therefore, Zimmer and Slawson (2002) expressed the need for a positive photoreactivation control in order to avoid false negatives.

Photoreactivation may occur under sunlight however, inactivation also occurs with exposure (Tosa *et al.*, 2003). Light intensity could be at least 10 times higher with sunlight than under laboratory conditions (Bohrerova and Linden, 2007). Some reactivation curves reach a maximum at 15 minutes (Kim *et al.*, 2005), especially in the case of sunlight irradiation (Kashimada *et al.*, 1996, Bohrerova and Linden, 2007). *Mycobacterium terrae* reached its maximum photoreactivation after 30 minutes (Bohrerova and Linden, 2006).

3.5.3.3 UV Irradiation

The type of UV lamp and fluence can affect the level of repair after irradiation. Zimmer and Slawson (2002) showed that there is a substantial difference between photoreactivation following low- and medium–pressure UV irradiation with the latter showing limited or no photoreactivation. It has been suggested that the additional wavelengths involved with medium pressure mercury lamps may cause additional damage to cell structures in the organism (Zimmer and Slawson, 2002). Photochemical alteration of a tertiary structure in an amino acid, can cause the complete loss of enzyme activity (Jagger, 1967).

The rate of photoreactivation of fecal coliforms was influenced by light intensity (Kashimada *et al.*, 1996). Reactivation is also dependent on the amount of UV fluence initially used. If the disinfecting fluence is not high enough for complete inactivation, repair is possible (Masschelein, 2002). At UV fluences of 160 and 400 mJ/cm^2, *Giardia lamblia* cysts showed no signs of repair, however, at lower fluences, reactivation can occur (Linden *et al.*, 2002). Reactor design also played a part in the disinfection efficiency when considering photoreactivation. Photoreactivation occurred more extensively when quartz-sleeve reactors were used when compared to polytetrafluoroethylene tube design reactors (Harris *et al.*, 1987).

When effluent is released into water bodies, photoreactivation may be negatively affected by sunlight irradiation but also such factors as sedimentation, dilution and predation (Whitby and Palmateer, 1993, Tosa *et al.*, 2003). For these reasons Whitby and Palmateer (1993) concluded that photoreactivation of microorganisms may not be a major concern when wastewater is disinfected with UV light.

3.5.3.4 Type of Microorganism

Photoreactivation is a widespread phenomenon among many organisms, including viruses, but has exceptions in bacterial and mammalian groups, including humans (Jagger, 1967, Snyder and Champness, 1997). The level and rate of reactivation varies significantly within strains and species of microorganisms and moreover between different microorganisms (Jagger, 1967, Kashimada *et al.*, 1996, Bohrerova and Linden, 2006).

Fecal streptococci showed little evidence of photoreactivation whereas fecal coliforms showed a noticeable reduction of inactivation when photoreactivation was facilitated (Harris *et al.*, 1987). Zimmer *et al.* (2003) found that UV-irradiated *Cryptosporidium parvum* oocysts showed no signs of repair by either photoreactivation

or dark repair even though conditions favourable to repair were applied, including low UV fluences, various temperatures and longer detention times. *Giardia lamblia* also exhibited no ability to repair its DNA by either photoreactivation or dark repair when exposed to 16 or 40 mJ/cm^2 of low pressure UV (Linden *et al.*, 2002).

Tosa and Hirata (1999) found that in order to inactivate 90% of *E. coli* O157:H7 cells with photoreactivation, a maximum UV fluence was 2.2-fold higher than those without photoreactivation. A study by Tosa *et al.* (2003) found similar results where fluences required to inactivate *E. coli* (ATCC 11229) by 90% with and without photoreactivation were 7.0 and 2.5 mJ/cm^2, which is 2.8 fold greater. Zimmer *et al.* (2003) found that *E. coli* (ATCC 11229) was able to photoreactivate at various temperatures but was unable to repair without light.

3.5.4 Current Amendments

Photoreactivation of irradiated bacteria is especially problematic for wastewater systems where effluents are discharged into exposed receiving bodies such as lakes and rivers (Tosa and Hirata, 1999). Most studies do take potential reactivation as a serious issue to consider when determining a UV fluence (Salcedo *et al.*, 2007, Tosa *et al.*, 2003, Tosa and Hirata, 1999, Kashimada *et al.*, 1996, Harris *et al.*, 1987). Any repair, even 1% could cause detrimental health and environmental problems, making it a concern when designing a UV disinfection system. Therefore when assigning a disinfection fluence, repair should be a factor included in the equation.

Chapter 4: Responses to Freezing Stress

4.1 Freezing and Thawing Microbial Cells

Application of microbial freezing technology can be used for two conflicting means: cryopreservation, to preserve microbial cultures for medical and industrial purposes and disinfection, to prevent the spread of harmful pathogens in our food and water. In both instances, bacteria can be injured or die as a result of cold shock, freezing, storage at freezing temperatures and thawing (Parker and Martel, 2002).

4.1.1 Cold Shock

Before a solution can freeze it must drop in temperature, which can cause microbes to die. Cold shock, also referred to as thermal shock, occurs in some bacteria with loss of viability reaching as much as 10 000 times (Mazur, 1966). Cold shock can damage the cytoplasmic membrane and DNA of bacteria. The outer membrane of Gram-negative bacteria can be damaged; however Gram-positive bacteria are also affected by cold shock (Parker and Martel, 2002).

The effects of cold shock alone can be observed when a suspension is lowered to just above freezing or when it is supercooled, which is when solutions are cooled below their freezing point without nucleation (Baker, 1967). Once the supercooled liquid comes into contact with ice crystals, it will form ice until the water reaches the equilibrium at the freezing point.

Bacterial injury caused by chilling cells down to sub-zero temperature in a supercooled state was observed by Moussa *et al.* (2008). They suggested that the cell membrane was in a permeable state, allowing uncontrolled mass transfer to and from the cell, producing membrane damage and cell death.

Cold shock is mainly observed when cells are rapidly frozen in log growth phase

(Mazur, 1966). Other factors affect the sensitivity of bacteria to cold shock (Mazur, 1966). Cell suspension medium that contains divalent cations protect against the chilling effect and aid in cell recovery whereas some media can enhance injury. Also cell number, the loss of viability is greater the smaller the initial cell population. The growth media and storage temperature are both factors affecting cell loss.

Thermophiles and mesophiles were more susceptible to low temperature shock than psychrophiles (Davies and Obafemi, 1985). This is because cold shock depends more on the magnitude of any temperature drop, therefore psychrophiles have the least temperature difference from optimal growth.

4.1.2 Freezing

Once the freezing temperature is reached, external water freezes before the cells content. Freezing has adverse effects on cells because the removal of water during intracellular and extracellular freezing causes mechanical injury to the cell and changes the physical and chemical nature of the solutions within and around cells (Moussa *et al.*, 2008). Freezing rates are the primary factor of how injury occurs. At slow freezing rates extracellular ice causes solute concentrations to be the main factor whereas at fast freezing rates, intracellular ice causes damage (Meryman, 1974).

4.1.3 Frozen Storage

Ice crystal size and form can change in water that has been previously frozen during the cooling stages (Mazur, 1966). Given sufficient time and suitable temperature, ice crystals can configure into their most stable form, damaging cells as this occurs (Mazur, 1966).

During holding time in the frozen state, cells are also exposed to cold stress. Cells can remain suspended in the unfrozen fraction of the liquid (Moussa *et al.*, 2008).

As the cooling rate increases, so does the probability of intracellular ice formation. The ice formed this way will likely be less stable and will therefore change and grow as storage time increases (Mazur, 1966).

4.1.4 Thawing

Thawing of bacterial cells may increase the physical damage that occurs during the freeze-thaw process. Ice crystals that formed during rapid freezing dissolve during thawing and attach to neighboring ice crystals, causing larger crystals to form, however, the rate of thawing has little to no effect on the survival of bacterial cells that are frozen at cooling rates less than 100°C/min (Calcott *et al.*, 1975). However it is difficult to determine the damage that occurs during thawing because of the added damage incurred during cooling and freezing.

4.2 Mechanisms of Inactivation by Freezing

Freezing and thawing can alter the cell wall of Gram-negative bacteria more so than Gram-positive, making them more susceptible to other treatments such as antibiotics (Sage and Ingham, 1998).

4.2.1 Dehydration

During optimal osmotic conditions, microbes maintain a high cytoplasmic solute concentration relative to outside the cell. This indicates a lower water activity (a_w) inside the cell.

$$a_w = x / (x+c) \qquad \text{(Equation 4.1)}$$

where c = osmolarity of solute(s)

x = moles of water per liter (55.6)

When a solution freezes, ice is preferentially formed by pure water crystals. At

slow freezing rates, solutes are left in the remaining water and increase in concentration, meanwhile it decreases in freezing point and a_w. Cells found in solution are affected by the rise in osmotic pressure and become dehydrated (Chalmers, 1959).

Cells dehydrate either by plasmolysis or internal freezing (Mazur, 1966, Calcott and MacLeod, 1975b).

4.2.2 DNA Damage

Freeze-thaw can damage DNA which is important in determining cryosurvival (Calcott and Thomas, 1981). *E.coli* exposed to both slow and rapid freezing appeared to be dealt with by the same pathways and may not play a role in DNA damage (Calcott and Thomas, 1981). Calcott and Gargett (1981) conclude that DNA damage occurs after the external medium freezes and the cell dehydrates.

4.2.3 Ice Crystallization

Intracellular ice is also recognized as a mechanism for freeze damage. At slow freezing rates, ice crystals form on the outside of the cell, resulting in water osmotically flowing out of the cell and into the suspending medium, causing dehydration (Meryman, 1974). On the other hand, when more rapid freezing occurs, internal ice can form because the internal water does not have time to move out of the cell. Therefore the internal ice can damage the cellular organelles or the whole cytoplasm, causing cell damage. Also, if the water contains an impurity that is ionized, the positive and negative ions may be unequally rejected from the crystal, resulting in a separation of electric charges (Chalmers, 1959).

After freeze-thaw, many studies have reported leakage of material into the surrounding medium caused by permeability damage (Calcott and MacLeod, 1975a). Calcott and MacLeod (1975a) measured freeze-thaw damage to the membrane by the

amount of UV-absorbing material, enzymes and potassium released.

4.3 Microbial Response to Freeze-Thaw Damage

There are many factors that contribute to a microorganism's ability to grow under optimal conditions including available nutrients, growth temperature, pH, oxygen levels and solute concentrations (Hengge-Aronis, 2000). In reality, an environmental stress is always present which forces the bacteria to remain in a stressed state that must always respond to its ever changing surroundings. Bacteria react to a variety of stresses including osmotic and thermal stress.

Responses are generated from sigma factors that are activated under different environmental conditions. These specialized sigma factors bind to the promoters of genes appropriate to the environmental conditions, increasing the transcription of those genes.

4.3.1 Osmotic Stress

Osmotic stress is brought on during dehydration from slow freezing. The disruption of lysosomes and the release of lysosomal enzymes have been shown to be the primary factor in freeze injury (Meryman, 1974).

A low level of mutation results from osmotic shock caused by an increased level of solute concentration outside of the cell (Calcott and Gargett, 1981). Mutations occur when damaged DNA is repaired in an error-prone fashion (Calcott and Gargett, 1981).

4.3.2 Thermal Stress

Cells that have been frozen and thawed had damage to their cell envelope and their cell membranes (Calcott *et al.*, 1979).

Freeze-thaw can cause lateral gene transfer between bacteria. Bacteria transfer genes in order to adapt to various environments (Ishimoto *et al.*, 2008). Freeze-thaw treatment causes the cells to release cations and plasmid DNA from the damaged cells, allowing uptake by surviving cells. *E. coli* has shown to transmit non-conjugative, non-viral DNA under freezing conditions (Ishimoto *et al.*, 2008). A condensed amount of cells were studied, 4×10^9 CFU/ml, which increased the probability of transfer. This concentration of cells can be found in the environment as biofilms, feces, rotten food, and carcasses (Ishimoto *et al.*, 2008).

Freeze-thaw can be mutagenic (Calcott and Thomas, 1981). Mutational events are thought to occur during the freezing and thawing of the microbes and not during the time spent frozen (Calcott and Gargett, 1981). Calcott and Gargett (1981) showed that freeze-thaw cycles did increase the amount of mutagens, therefore supporting the theory that mutagenicity occurs during the freeze-thaw stages. Salinity also increases the number of mutations (Calcott and Gargett, 1981).

The two main pathways for DNA damage from freezing are *rec*-dependent and excision repair (Calcott and Thomas, 1981). Similarities between the response of bacteria to UV light and freeze-thaw are understandable once DNA was found to be a factor in the inactivation by both treatments (Calcott and Gargett, 1981).

4.3 Factors Influencing Freezing Inactivation Capacity

Knowledge of the factors affecting cryosurvival and the mechanisms of freeze damage of microbial cells will enable treatments to increase inactivation of targeted pathogens. Similar to cold shock, there are many factors that contribute to the inactivation of microbes by freezing.

4.3.1 Microbial factors

The types and strains of microorganisms are a major factor influencing freezing inactivation. Generally, most Gram-positive organisms are resistant to freezing, whereas those that are Gram-negative tend to be sensitive (Davies and Obafemi, 1985). Bacterial endospores are extremely resistant to freezing and to storage at sub-zero temperatures with survival exceeding 90% (Davies and Obafemi, 1985). Their possession of a large insulating capsule and a dehydrated spore protoplast, containing the majority of its water bound in an unfreezable state, might protect it from the frozen environment (Calcott and Macleod, 1974b).

The nutritional status, what nutrients were in the growth media, is a factor in freezing inactivation. Bacteria rich in carbohydrates are more resistant to freeze-thaw stress. Reasons for this are unknown. Calcott and MacLeod (1974b) speculate that carbohydrates associated with lipopolysaccharide may render the cell wall and membrane chemically and physically stronger. Nitrogen-limited cells that accumulated higher carbohydrate contents were found to be more freezing resistant. A theory behind this is that polyglucose and glycogen-like reserve material could be cryoprotective by strengthening the cell envelope or outer membrane (Davies and Obafemi, 1985).

The growth phase and the rate of growth play a role in freezing inactivation. The general rule of thumb is that exponential phase cells are more sensitive to freeze-thaw stress than stationary phase cells (Davies and Obafemi, 1985). However, there are conflicting studies where freezing rates and growth temperatures influenced results as to the sensitivity of the growth phase (Calcott and MacLeod,1974b).

4.3.2 Experimental factors

Once grown, the composition of the cooling and freezing media can alter the

outcome of bacterial survival during the freezing process (Calcott and MacLeod, 1974b). In a study by Calcott and Macleod (1974a), when compared with distilled water, saline proved to be a more lethal environment for *E. coli* cells. Freezing cells in saline was found to cause permeability damage to both the cell membrane and wall (Calcott and Macleod, 1975a, Calcott and Macleod, 1975b). However, not all microorganisms react similarly. A Gram-negative cocci showed no difference in survival when frozen in both saline and water (Calcott *et al.* 1975).

The rate of cooling, as mentioned above, can alter the mechanism of freezing damage. Cooling rates have been defined as slow (about 10°C/min), rapid (about 400°C/min) and ultrarapid (6000°C/min) by Calcott and Macleod (1975a). In their study, peak survival of *E. coli* was observed at a cooling of 8°C/min (Calcott and Macleod 1975a). There is an optimal cooling rate for cells and when that rate is increased or decreased, survival is reduced. Cooling rate is not the same as freezing rate. Cell freezing rate is very difficult to determine; most studies only investigated the effect of cooling rates.

At slow cooling rates the cell membrane is an effective barrier to ice because the membrane pore size is smaller than the critical radius of curvature of ice (Meryman, 1974). Therefore solute concentration is what leads to cell death. When slow to moderate cooling rates are observed, about 90% of cells do not remain supercooled at -20°C or below (Mazur, 1966). The remaining 10% is supercooled or bound (Mazur, 1966).

As the rate of cooling increases, the degree of supercooling also increases (Calcott and MacLeod, 1974a). Solutions that are cooled below their freezing point without nucleation show metastability and can become subject to flash freezing (Baker, 1967). Once the supercooled liquid comes into contact with ice crystals, it will form ice until the water reaches the equilibrium at the freezing point. Therefore, it is more difficult

to start the formation of ice than it is for ice to continue once it has started (Chalmers, 1959).

Storage duration and temperature account for some of the loss in viability of cells. After freezing, death caused by storage parameters, gradually slows until viable numbers remain constant (Davies and Obafemi, 1985). Generally, much like freezing, Gram-negative microbes tend to be sensitive to frozen storage, whereas most Gram-positive organisms are resistant (Davies and Obafemi, 1985). Storage death has been attributed to a variety of stresses including high solute concentrations, pH changes and recrystallisation (Davies and Obafemi, 1985). The survival rate of *E.coli* decreases with increasing storage temperature from -79°C (Mazur, 1966). Viability of cells will decline at higher temperatures than -135°C (ATCC, 2008).

4.4 Application of Freezing Technology in Various Industries

Cooling to very low temperatures is commonly applied to preserve living cells and tissues while maintaining their biotechnological properties. In the medical field, freezing has preserved blood for transfusions, organs for transplants and eggs for artificial insemination. Research in many fields relies on frozen biobanks, repositories of biological material where frozen samples are stored and preserved for later use, which is offered because of this technology. Freezing has become an important technology that has many applications.

Freezing has been looked at as a means to inactivate pathogenic microorganisms in various industries such as food preservation and disinfection. Freeze technology is used to preserve food and to extend its shelf life while limiting microbial growth. Poultry meat is highly perishable and is a great medium for microbial growth (Patsias *et al.*, 2008). Freezing technology is being looked at as an effective means of

preserving meat. Samples frozen at -40°C provided a lower amount of microbial growth compared to those chilled at 4°C.

Effective antimicrobial treatments for unpasteurized juices have included UV light, high pressure, pulsed electric fields and frozen storage (Yamamoto and Harris, 2001). Many small processors do not have their own pasteurization equipment and would prefer to limit the amount of chemicals necessary (Sage and Ingham, 1998). Companies are already freezing their juice for ease of transportation and to extend product shelf-life, therefore to determine the possible disinfection capabilities of freezing and its optimization would be beneficial. Yamamoto and Harris (2001) have studied the effects of freezing on the survival and injury of *E. coli* O157:H7 in apple juice and found that freeze-thaw conditioning could be a method of disinfection when coupled with another treatment.

Another use for freezing technology is for microbial preservation. Freezing is the principal process of cell preservation (Moussa *et al.*, 2008). Freezing microbial cultures and storing them at low temperatures enable medical and research personal access to bacteria for study at any time. The bacteria used in this study came from the American Type Culture Collection (ATCC) which freezes some of their bacteria at liquid nitrogen temperatures. They have some cultures that date back to the 1960's and those cultures have not had a significant loss in viability (ATCC, 2008).

4.5 Application of Freezing in Water and Wastewater Treatment

Undesirables can be removed from water by freeze concentration, a technology that uses freezing for separation. Salt and most other solutes are rejected from forming the ice crystal. The solute accumulates in front of the advancing ice surface. Therefore freezing may be a practical way to desalt sea water because it takes less energy to freeze water than to vaporize it (Chalmers, 1959). The freeze-thaw process has also

been looked at for dewatering sludge from water treatment facilities (Parker *et al.*, 2000) and separate dissolved chemicals and impurities from wastewater (Beier *et al.*, 2007, Gay *et al.*, 2003). Freeze concentration is being used to concentrate waste prior to incineration for a practical and cost-effective wastewater treatment system (Holt, 1999). The ice produced in these treatments can be used for cold heat storage (Wakisaka *et al.*, 2001).

Freeze-thaw is looked at as a viable treatment process in cold climates, especially where electricity costs are low and waste disposal is high (Parker and Collins, 2000). Freeze-thaw technology can also be used for inactivation of pathogens in water and wastewater.

Sanin *et al.* (1994) found that while decreasing sludge volume, another advantage to freeze concentration was the inactivation of pathogens and therefore their ability to meet regulations with less disinfection needed. Sanin *et al.* (1994) reported that the lowest freezing temperature tested, -25°C, gave the greatest log reduction of fecal coliforms with an overall average of 1.10. Storage time did play an important role, with 7 days maximizing the log reduction however, the storage temperature was not a factor.

Natural freezing of wastewater has also been examined to inactivate pathogenic bacteria (Gao *et al.*, 2006 and Gao *et al.*, 2007). Gao *et al.* (2006) observed that *E. coli* frozen at -5°C was more sensitive to frozen storage and freeze-thaw cycles than those frozen at colder temperatures (-15 and -35°C). The cells used in the 2006 study by Gao *et al.* were not naturally occurring in the environment and were grown in the lab which may have caused the discrepancy between optimal freezing temperature for inactivation observed by Sanin *et al.* (1994). Also the cells tested at -35°C were very close to the inactivations observed at -5°C, making them a close second, with -15°C being the most optimal for cell survival.

There have been studies calculating the cost effectiveness of implementing freezing as a treatment (Parker *et al.*, 2000, Beier *et al.*, 2007). However, further studies are needed to properly estimate the amount of inactivation that is consistently achieved by freezing in order to reduce and save on chemical and electricity costs necessary to reduce the number of remaining pathogens to concentrations below regulations.

Chapter 5: Materials and Methods

5.1 Test Organisms

Experiments were performed using *Escherichia coli* strain 25922 (American Type Culture Collection, Manassas, Va.), *Enterococcus faecalis* strain 29212 (American Type Culture Collection, Manassas, Va.) and endospores of *Bacillus subtilis* strain 6633 (American Type Culture Collection, Manassas, Va.).

5.2 Microbial Preparation

Original cultures were kept in frozen storage at -80°C in a Thermo Electron Corporation 700 Series Forma ULT Freezer (Mariette, Ohio, USA). Frozen cultures were prepared by transferring 0.5 ml of stationary phase culture in Trypticase soy broth (TSB) to 0.5 ml of 50% glycerol solution. Cultures were also stored at 5°C in the refrigerator and plated every two weeks to keep cells viable.

5.2.1 Storing Bacterial Cultures

A variety of media were used in order to grow and store the bacterial cultures throughout the experiment. Modified BD Difco™ membrane Fecal Coliform (mFC) agar (Becton, Dickson and Company, Sparks, MD, USA) was used to store pure cultures of *E. coli*. *E. faecalis* cultures were stored on BD Difco™ membrane *Enterococcus* (mENT) agar (Becton, Dickson and Company, Sparks, MD, USA). BD BBL Trypticase Soy Broth™ (TSB) (Becton, Dickson and Company, Sparks, MD, USA) was mixed with granulated BD Difco Agar (Becton, Dickson and Company, Sparks, MD, USA) in order to store *B. subtilis* cultures.

5.2.2 *E. coli* and *E. faecalis* Growth and Cell Suspension

E. coli and *E. faecalis* were cultured from fridge cultures in individual TSB flasks and grown overnight in an Innova™ 4430 large stackable incubator shaker (New Brunswick Scientific Co., Inc., Edison,NJ, USA), set at 37°C and 150 rpm. The following day, fresh sterile TSB was inoculated from the overnight culture to an optical density of 0.1 using a Biochrom Novaspec II visible spectrophotometer, RS232C (Biochem Ltd., Cambridge, England), when set at 600nm (OD_{600nm}). The freshly inoculated TSB was then placed in an incubator shaker until an OD_{600nm} of 0.6 was reached which took approximately 2 hours. An OD_{600nm} of 0.6 for both *E. coli* and *E. faecalis* was comparable to a cell suspension of 1×10^9 colony forming units (CFU)/ml.

Once log growth was observed (OD_{600nm} of 0.6), the cells were poured into sterilized 250 ml centrifuge bottles and centrifuged at 4000 rpm (or 3600 x g) for 10 minutes at a temperature of 4°C using a Sorvall superspeed RC2-B automatic refrigerated centrifuge (Ivan Sorvall Inc., Newton, Connecticut, USA). The supernatant was then discarded and the pellet (bacteria) was re-suspended in sterile double distilled (DD) H_2O. The bacteria were then centrifuged and re-suspended in DD H_2O twice more in order to remove as much media as possible. The cells were brought to an OD_{600nm} of 0.3 and placed in 500 ml bottles at a volume of 300 ml. The final suspension of an OD_{600nm} of 0.3 was between 8×10^7 and 2×10^8 CFU/ml.

5.2.3 *Bacillus subtilis* Spore Production and Cell Suspension

Refrigerator cultures of *B. subtilis* were inoculated into a nutrient poor media to stimulate sporulation. Schaeffer media (Appendix B) was used to produce *B. subtilis* spores. Schaeffer *et al.* (1965) reported that sporulation is determined by the intracellular concentration of at least one nitrogen-containing catabolite repressing directly or

indirectly the expression of all the sporulation genes. They concluded that the nature of both the carbon and nitrogen source in the media are important in order to optimize sporulation (Schaeffer *et al.*, 1965).

Spores were harvested after 3 days of incubation in an innova™ 4430 large stackable incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA), set at 37°C and 150 rpm. The spore suspension was poured into 50 ml centrifuge tubes and spun down at 4000 rpm by a benchtop BioMax refrigerated centrifuge (Thermo IER Centra CL3R) for 10 minutes at 4°C. The supernatant was removed and the pellet was re-suspended in sterile DD H_2O.

After the cells were washed three times with sterile DD H_2O, they were re-suspended in sterile DD H_2O to 10 ml and treated with lysozyme (0.25 mg lysozyme/ml) to break down the vegetative cell wall. The mixture was placed in the incubator shaker at 37°C for 60 minutes. After the allotted time, the mixture of *B. subtilis* vegetative cells and endospores were placed in a digital Isotemp 228 water bath (Fisher Scientific Ltd., Whitby, ON, Canada) at 80°C for 10 minutes to destroy the remaining vegetative cells and then centrifuged. The supernatant was removed and the pellet was re-suspended in sterile DD H_2O. This method of inactivating the vegetative *B. subtilis* by heat is a reliable method of spore cultivation that ensures that spores alone are able to survive for further experimentation (USEPA, 2006).

An OD_{600nm} of 1.0 was achieved in the tubes and from that 3 ml of suspension were resuspended in 500 ml bottles containing 300 ml of DD H_2O. This concentration of *B. subtilis* spores is comparable to a cell suspension of 1×10^5 CFU/ml.

5.4 Freezing

Cell suspensions of 200 ml at room temperature were frozen at -7, -15 or -30°C for either 24 or 48 hours, depending on the cycle number. Cycles 1 and 3 had a 24 hour

freezing period whereas cycle 5 had a 48 hour freezing period. Samples frozen at -7 and -15°C were frozen in a temperature and humidity controlled walk-in freezer (Climate Testing Systems Incorporated, Warminster, PA, USA). Those frozen at -30°C were frozen in a Thermo Forma dual chamber digitally regulated freezer (Thermo Electron Corporation, Marietta, Ohio, USA). Samples frozen at -7°C were shaken after an hour to promote freezing because supercooling was occurring at this temperature. After the appropriate time, samples were removed and thawed for approximately 2 hours in a digital Isotemp 228 water bath (Fisher Scientific Ltd., Whitby, ON, Canada) set at 20°C.

As mentioned, there are many factors that influence a microbes ability to survive freezing. For that reason a variety of factors will be set as controls in order to observe the effects of selected treatments.

1. Freezing temperature: samples were frozen at either -7, -15 or -30°C.
2. Freeze-thaw cycles: 0, 1, 3 and 5 freeze-thaw cycles were chosen
3. UV dose: low, low-moderate, moderate-high, high

For each bacterial sample chosen, every treatment will be conducted with all four UV doses at least three times each. For example *E. coli* suspensions were tested at each freezing temperature, at every freeze-thaw cycle and every sample and corresponding control were irradiated at four UV fluences. An example of one trial of *E. coli* is shown in a schedule, Table B-1, found in Appendix B.

5.5 UV Inactivation

5.5.1 UV Bench-scale apparatus

The bench-scale apparatus was set up at the Atlantic Avenue Water Pollution Control Plant (WPCP) in the City of Thunder Bay. The apparatus was designed and built

by Trojan Technologies (Collimated Tube Assembly, 120 Volt, Part No. 910057) and was on loan from the City of Winnipeg.

The apparatus consisted of a manual plastic shutter used to regulate the calculated time of exposure, a 120 volt AC power supply, a collimating tube that provided a uniform irradiation field on the water surface, a stirrer to ensure equal mixing throughout the suspension and between samples, a low pressure mercury vapor lamp (monochromatic at 253.7 nm)

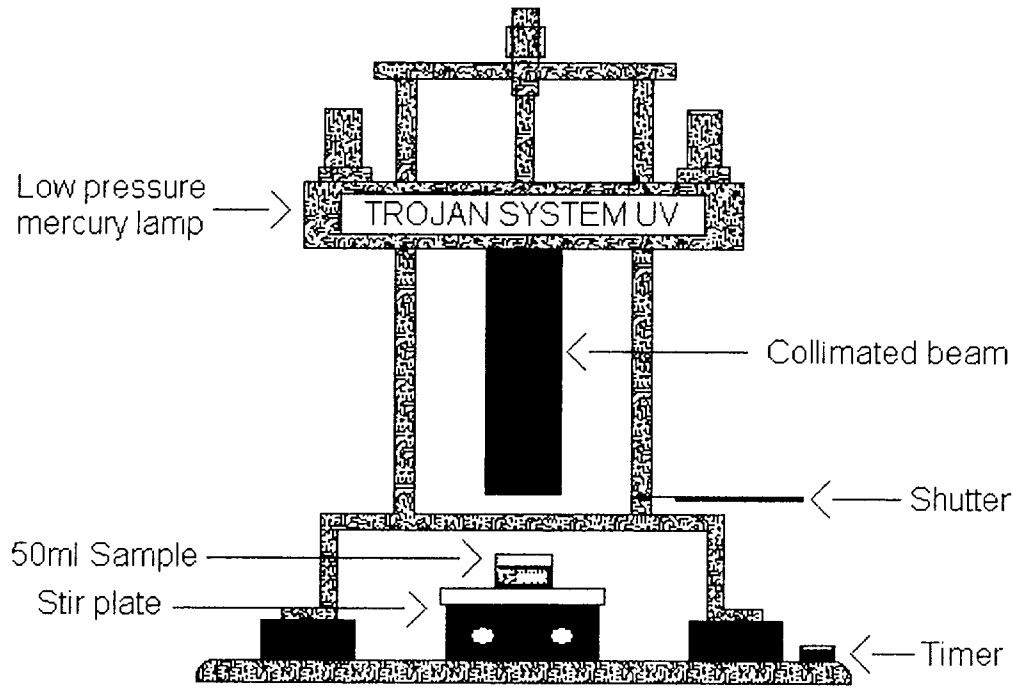

Figure 5-1 Collimated beam apparatus

5.5.3 Irradiation

The lamp was on prior to all experiments to ensure a stable UV output. The exposure times were calculated using a spreadsheets provided by Trojan Technology. The collimated beam correction factors required to determine the exposure times were UV irradiance values, Petri dish specifications, sample volume, distance from UV lamp to sample surface, UV transmittance as a percentage. Only UV transmittance and

irradiation values were recorded for each sample because the remaining factors were constants.

The UV irradiance was measured with a calibrated radiometer (IL1400A, International Light, S/N 6976) equipped with a SEL240 narrow band germicidal detector (S/N 5871) and a NS254 bandpass filter (S/N 25514) for 249-259 nm UV radiation. The detector was placed at the same level as where the sample surface would be in the irradiation Petri dish (Fisher). Irradiance was measured every 0.5 cm in the x and y directions over 3 cm.

Prior to each test, the percent UV transmittance was measured using a UV spectrophotometer (P254C UV Photometer, Trojan Technologies Inc.).

Samples, 50 ml in volume, were poured into sterile Petri dishes which contained a magnetic stir bar. The sample was placed under the collimated beam with the shutter closed. The stir plate was on prior and during each sample to achieve a completely mixed sample throughout testing. The timer was started and the shutter was manually opened simultaneously. After the allotted time, the shutter was closed immediately and the sample was poured into a sterile 250 ml bottle. The bottle was then placed inside a cooler to prevent as much visible light from reaching the irradiated sample as possible (<20 s).

5.5.2 Fluence Determination

The samples were initially exposed to fluences previously observed to deliver a 1, 2, 3 and 4 $\log_{10}$ inactivation. After preliminary testing, a range of doses were selected for each bacterial species. The fluences used for *Escherichia coli* strain 25922 were 1.5, 2.5, 4.5 and 9 mJ/cm^2. *Enterococcus faecalis* strain 29212 was exposed to 6, 8, 20 and 25 mJ/cm^2. Lastly, the endospores of *Bacillus subtilis* strain 6633 were irradiated at 20, 40, 80 and 120 mJ/cm^2.

The average fluence rate is a function of several factors mentioned previously, which takes into account the incident fluence rate, the spectral UV absorbance, path of light travelling through the water, reflection factor, Petri factor and divergence factor. The UV fluence was obtained by multiplication of the average microbial irradiance by exposure time. A computer program set up by Trojan Technologies was used for all calculations and fluences were given after the input of factors, % transmittance of the sample and irradiance measurements for the day.

For drinking water, 40 mJ/cm^2 is commonly used design fluence for treatment on residential systems prior to release into the distribution system (Linden *et al.*, 2002). For wastewater, there is a wider range of fluences used due to the varying types of effluent and requirements of the receiving water bodies (Masschelein, 2002). Solids content plays an important role in significantly altering the fluence needed to meet regulations. Therefore there is no definitive required fluence for UV treatment.

5.6 Photoreactivation

The objective of this study is to determine what response the selected bacteria have to exposure to photoreactivating light following UV treatment. Also if they are able to photoreactivate, does prior freezing have any effect on its photoreactivation?

Samples tested were cultivated and suspended as above and were either frozen at -15°C or were not frozen. Two fluences were investigated for each bacterial species. The fluences used for *Escherichia coli* strain 25922 were 2.5 and 9 mJ/cm^2, *Enterococcus faecalis* strain 29212 were exposed to 6 and 20 mJ/cm^2 and *Bacillus subtilis* strain 6633 spores were irradiated at 40 and 120 mJ/cm^2.

An initial aliquot was removed from each sample to determine the initial UV inactivation. The irradiated sample was then divided into three separate sterile plastic Petri dishes at a volume of 15 ml/plate. Three dishes were used to allow one to be

removed at each time point, 30, 90 and 180 minutes for sampling and enumeration. A sample that had not been irradiated was separated into three Petri dishes to observe the effect fluorescent light may have on the cells and act as a control.

Visible light irradiation was carried out using four cool white fluorescent light tubes (F40CW, 40 watts, General Electric Company, Fairfield, CT). The air temperature was measured and maintained between 25 and 27°C. The intensity of the lights at the sample surface was measured to be approximately 3,200 to 5,600 lux using a light meter (Fisher Scientific Traceable Dual-Range Light Meter) in order to ensure consistency. The samples were placed approximately 30 cm from four over-hanging lamps.

5.3 Enumeration

Cell concentrations were measured by drop plating. Serial dilutions of samples were made in sterile DD H_2O in order to reduce the cell density to a countable number. Because there were no background microorganisms present, drop plating on TSA was used for enumeration of all bacterial species. From selected dilutions, 5 µl of sample were dropped 5 times for each chosen dilution. Inverted plates were incubated for approximately 15 and 30 hours, with *E. faecalis* requiring the extended growth period for more visible colonies. Colonies in each drop were counted and $\log_{10}$ CFU/ml values were calculated using the following equation

$$\text{Cell concentration (CFU/ml)} = \frac{N \times D}{(25 \times 10^{-3} ml)} \qquad \text{(Equation 5.1)}$$

where N = Total colony number

D = Dilution number

5.9 Data Analysis

All experiments were replicated at least three times and data are reported as averages with standard deviations. Graphs were made using Sigmaplot 2000 (SPSS Inc., Chicago, Ill) where 95% confidence error bars were applied. Log inactivation of the test microorganisms was calculated as

$$\text{Log inactivation} = \log_{10}\left(\frac{N_0}{N}\right) \qquad \text{(Equation 5.2)}$$

where N_0 = the concentration (in CFU/ml) of the non-irradiated sample

N = the concentration of microorganisms able to reproduce after irradiation

Graphs illustrating the photoreactivation levels of the bacterium tested were calculated by using the following equation

$$\text{Ratio of photoreactivation} = \left(\frac{N}{N_0}\right) \qquad \text{(Equation 5.3)}$$

One-way and multiple factors ANOVA tests were used to analyze the data using SigmaStat 2.03 (SPSS Inc., Chicago, Ill). Statistical results are found in the Appendix C.

Chapter 6: Results and Discussion

6.1 Freezing Inactivation

Firstly, the effect of freezing on the selected microbes was investigated in order to record what changes may have occurred from this process alone. Conclusions on the effects of UV on samples pre-exposed to freezing can then be made on the cumulative effects of freezing and UV treatment.

6.1.1 Effect of Freezing Temperature

Freezing temperature was an important factor in bacterial inactivation for both *Escherichia coli* and *Enterococcus faecalis*. The endospores of *Bacillus subtilis* were not affected by freezing temperature, nor by freezing itself, which was shown by no statistical difference between frozen and non-frozen samples ($\alpha = 0.05$). Freezing inactivation of *E. coli*, *E. faecalis* and *B. subtilis* spores at various freezing temperatures are shown in Figure 6-1.

After allowing for effects of bacteria, there was a statistically significant difference in the mean values between -7°C and -15°C. *E. coli* had significantly different log inactivations than both *B. subtilis* spores and *E. faecalis* at -7°C. However *B. subtilis* spores and *E. faecalis* inactivation were not statistically different at -7°C. Similar statistical analysis was computed for comparisons between *E. coli* and both *B. subtilis* spores and *E. faecalis* at -15 and -30°C.

There was a significant effect between bacterial species and freezing temperature (p=0.008). The effect of freezing on log inactivation depends on which bacterial species is tested and what freezing temperature is used.

Freezing temperature had the greatest effect on the inactivation of *E. coli* samples of the three opportunistic pathogens tested. Results of analysis of variance

(ANOVA) indicated that freezing temperature had a significant effect ($p<0.001$) on the inactivation of *E. coli*. As shown in Figure 6-1, *E. coli* was observed to have a maximum average reduction of 1.3 log when cells were frozen at the warmest freezing temperature (-7°C). After freezing at -15 and -30°C, *E. coli* had an average of 0.694 and 0.897 log inactivation respectively.

There was significantly higher inactivation at -7°C than freezing at -15 and -30°C, where similar results have been reported in previous studies. Gao *et al.* (2006) found that *E. coli* strain 15597 had the greatest log inactivation when frozen at -5°C, with -35°C having greater reductions than -15°C. It was suggested that -15°C might be close to the optimal cooling rate of *E. coli*, causing the least amount of damage from freezing (Mazur, 1966). The *E. coli* in the Gao *et al.* (2006) study were a different strain, grown to stationary phase, suspended in buffer, frozen at 50 ml volumes and had cell concentrations of 10^6 to 10^7 CFU/ml. There are key differences between this study and that done by Gao *et al.* (2006), however the general trend of the effect of freezing temperatures are similar, implying a general observation for *E. coli* inactivation. When frozen, *E. coli* inactivation is optimized at lower and higher freezing temperatures with an intermediate temperature of -15°C providing minimal cell inactivation.

There have also been studies that have observed opposing results. Sanin *et al.* (1994) found that fecal coliforms were not reduced after freezing at -7°C and that over 1 log reduction was observed at freezing temperatures of -25°C. In the coliform study actual effluent was used, therefore the cells were in stationary phase, compared to log phase cells used in this study. Other discrepancies include a lower initial cell concentration in the 1994 study, an increased resistant to the effects of freezing because of their physiological state (Calcott and MacLeod, 1974b) and also the differences in strains. Also *E. coli* are only part of the make up of fecal coliforms tested and there are many interactions between bacteria taking place.

In a related study where *E. coli* strain 15597 was frozen at -15°C, Gao *et al.* (2007) observed an average 0.67 log inactivation when using selective media for plating and 0.35 log inactivation for non-selective media. The results of the selective media are more comparable to reductions found for the strain used in this study. Moreover the plating media, TSA, was found to be the least selective when comparing with non-selective and selective m-FC agar. This observation implies that *E. coli* strain 15597 was more resistant to freezing than strain 25922 used in this study.

Freezing temperature was not a factor for either *E. faecalis* or *B. subtilis* spores. No significant difference was found between unfrozen samples and samples frozen at all temperatures (-7, -15 and -30°C). Results were similar to other studies where spore-formers were more resistant to freeze-thaw damage than Gram-negative bacteria (Gao *et al.*, 2007, 2006, Walker *et al.*, 2006).

The varying levels of bacterial inactivation could be explained by the difference in cell envelope and how Gram-negative are more sensitive to freezing than Gram-positive bacteria. The warmest freezing temperature resulting in the greatest reductions may be caused by the slower freezing rate. The external freezing of the outside liquid would cause the cell to dehydrate and consequently damage the cell membrane leading to cell death (Mazur, 1966). Therefore it is likely that at higher freezing temperatures the cell reduction will be optimal when comparing the relatively slow cooling rates of -7, -15 and -30°C.

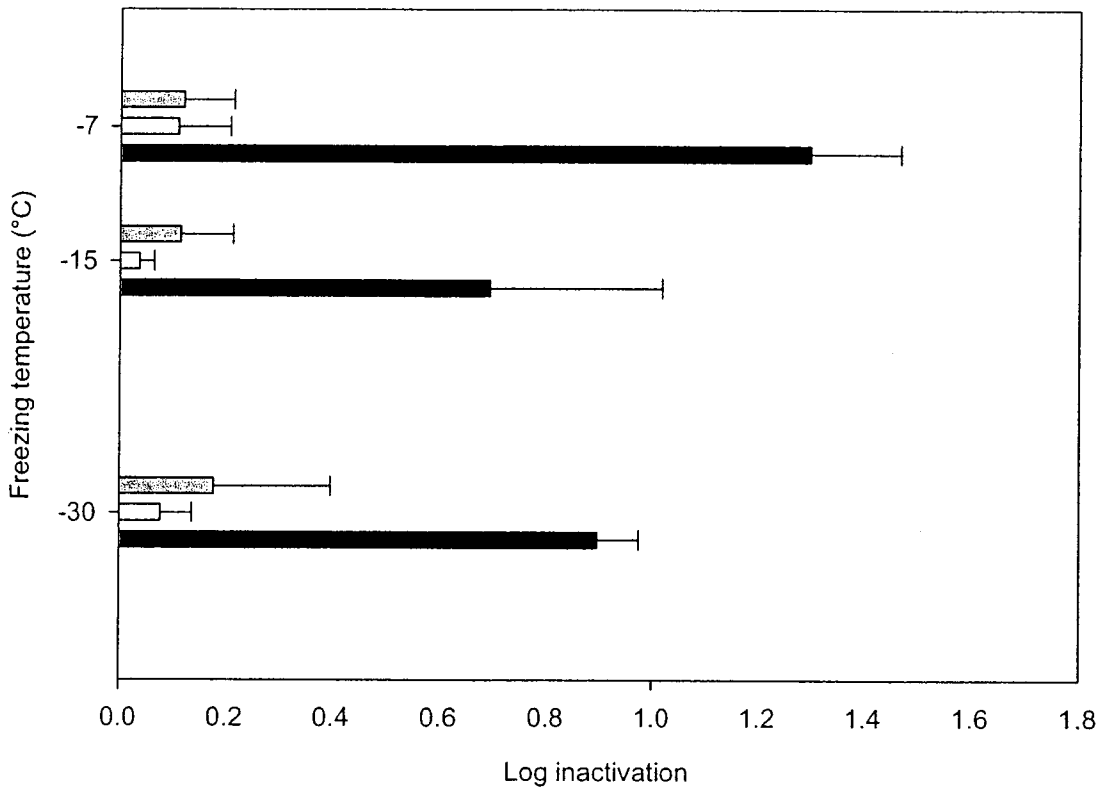

Figure 6-1 Bacterial log inactivation after freezing at -7, -15 and -30°C
B. subtilis spores, *E. faecalis*, *E. coli*

6.1.2 Effect of Freeze-Thaw Cycles

Freeze-thaw cycles did have a significant effect on the survival of *Escherichia coli* and *Enterococcus faecalis*. Again, *Bacillus subtilis* spores were unaffected by freeze-thaw cycle. In order to illustrate the effects of freeze-thaw cycles, Figure 6-2 shows the average log inactivation of *E. coli*, *E. faecalis* and *B. subtilis* spores after 1, 3 and 5 freeze-thaw cycles at combined freezing temperatures.

There was a statistically significant interaction between freezing temperature and freeze-thaw cycles for *E. coli* and *E. faecalis* cells. For *E. coli*, the inactivation increased with the increase of freeze-thaw cycles. The average inactivation increased from 0.95 log after one freeze-thaw cycle to 4.36 log after five freeze-thaw cycles.

Freezing at -7°C was observed to be the most lethal during the cycling process. *E. coli* had the greatest log inactivation caused from the increase in freeze-thaw cycles. A study by Gao *et al.* (2006) found similar results when *E. coli* inactivation increased with additional freeze-thaw cycles. Freezing temperature was an important factor in the reduction of cells and inactivation capacity at -5°C > -35°C > -15°C.

The log reductions are greater in this study than those reported by Gao *et al.* (2006) which could be attributed to the differences in cell culturing. Those used in the 2006 study were grown to stationary phase which could provide greater resistance to freezing inactivation.

E. faecalis also demonstrated an increased inactivation with the increase of freeze-thaw cycles. The maximum log inactivation was 0.78 after five freeze-thaw cycles for the combined temperature results (Figure 6-2). However the maximum average after five freeze-thaw cycles at -7°C was 1.49 log inactivation, the most lethal for *E. faecalis* cells. Similar results were found with a study using *Enterococcus flavens* (Walker *et al.*, 2006), showing that species may not play a role in the susceptibility of damage caused be freeze-thaw cycles to *Enterococcus* bacteria.

After statistical comparison, as shown in Table 6-1, *E. coli* and *E. faecalis* were more sensitive to freezing temperature and freeze-thaw cycles than *B. subtilis* spores. The results were similar to other studies where *E. coli* was also more sensitive to freeze-thaw cycles than *Enterococcus* strains and *B. subtilis* endospores (Gao *et al.*, 2007, 2006, Walker *et al.*, 2006). Walker *et al.* (2006) reported greater than 3 log inactivation of *E. coli* after 3 freeze-thaw cycles, whereas there was less than a 1 log inactivation of an unknown *Enterococcus* strain.

The increase in log inactivation of *E. coli* and *E. faecalis* cells as freezing temperature increased supports the theory that slow freezing is more damaging than fast freezing. At warmer freezing temperatures the crystals form outside of the cell,

concentrating the solutes, causing the cell to dehydrate as water flows out of the cell which causes membrane and protein denaturation (Walker *et al.*, 2006). Fast freezing and thawing are less damaging to microorganisms and result in intracellular ice formation or perhaps even a glassy state where no ice is formed (Walker *et al.*, 2006).

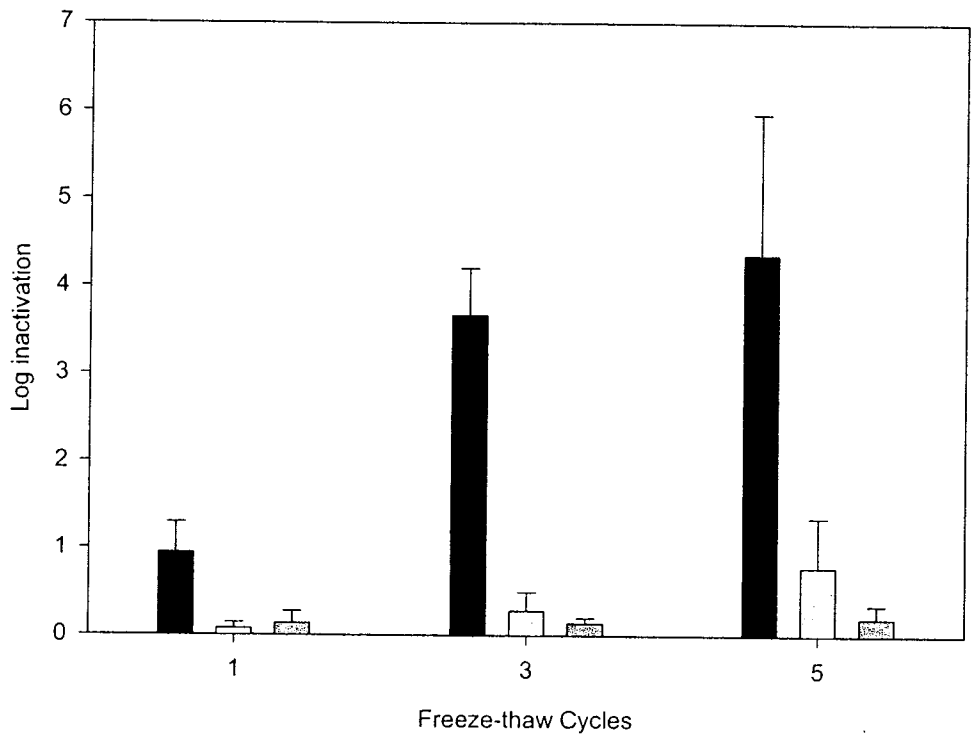

Figure 6-2 Effect of freeze-thaw cycles on tested bacteria using the combined freezing temperatures

E. coli, *E. faecalis*, *B. subtilis* spores

Table 6-1 Two Way ANOVA comparing Freezing Temperature and Freeze-thaw Cycle (*p*-values)

Source of Variation	*E. coli*	*E. faecalis*	*B. subtilis* spores
Freezing Temperature	**<0.001**	**<0.001**	0.287
Freeze-thaw Cycles	**<0.001**	**<0.001**	0.4
Freezing Temperature x Freeze-thaw cycles	**<0.001**	**<0.001**	0.422

6.2 UV Inactivation

UV inactivation data for bacteria tested are revealed in Figure 6-3. *Bacillus subtilis* strain 6633 spores required a greater fluence in order to achieve inactivation.

Escherichia coli strain 25922 was inactivated at the lowest fluences and *Enterococcus faecalis* strain 29212 was the intermediate bacterium tested. An average of 4.4 log inactivation of *E. coli* was achieved at 9 mJ/cm^2. *E. faecalis* had similar inactivation results after a fluence of 20 mJ/cm^2 was used, whereas *B. subtilis* spores did not reach inactivation levels greater than 3.78 log which was achieved by a fluence of 120 mJ/cm^2. The greatest average log inactivation of *E. faecalis* was 5.14 after irradiation of fluences 25 mJ/cm^2. Therefore, endospores were observed to be the most UV resistant of the three bacteria studied. These results were similar to those for UV inactivation in previous studies (Tosa *et al.*, 2003, Mamane *et al.*, 2007, Chang *et al.* 1985).

Chang *et al.* (1985) demonstrated that *B. subtilis* spores were 9 times more UV-resistant than vegetative cells including *E. coli.* The resistance of endospores to UV inactivation can be attributed to the extra protection of the spore coat, the unique photoproduct formed as a result of UV irradiation and its unique repair pathway involving SP lyase produced during sporulation (Setlow, 2000). Sporulation on a solid medium produces *B. subtilis* spores less resistant to UV compared to those grown in liquid media (Mamane-Gravetz *et al.*, 2005).

The inactivation of spores depends not only on the disinfection used but also on the nature of spores (indigenous versus cultured), the spore strain and the culturing method (Mamane-Gravetz and Linden, 2005). Mamane-Gravetz and Linden (2004) found that indigenous spores were more resistant to UV disinfection compared to ATCC strain spores. The inactivation of spiked spores was much more rapid than the indigenous spores, reaching 3.5 log inactivation at an UV fluence of 60 mJ/cm^2 compared to less than 1 log inactivation for the indigenous spores at this fluence. A greater than 1 log inactivation was shown for the spores grown in this study at the same fluence.

Bacillus subtilis spores exhibited a log-linear fluence-response inactivation (Figure 6-3). The spread of the standard deviation grew as fluence increased. The fluctuation could be attributed to a tailing phenomenon known to occur with *B. subtilis* spores because of the possibility of enumeration sensitivity, spore repair systems or spore-spore/spore-particle aggregation (Mamane-Gravetz and Linden, 2004).

In this study, *E. faecalis* strain 29212 required a higher UV fluence to achieve similar log inactivations as *E. coli* strain 25922. The opposite was true for a study by Koivunen and Heinonen-Tanski (2005) where *E. coli* showed a log reduction of 0.55 at fluence 10 mJ/cm^2 whereas at the same fluence, *E. faecalis* demonstrated a 1.20 log reduction. The strains used in this study were *E. coli* 15597 and *E. faecalis* 19433, which implies that UV inactivation can vary among strains, species or genus and must be further researched.

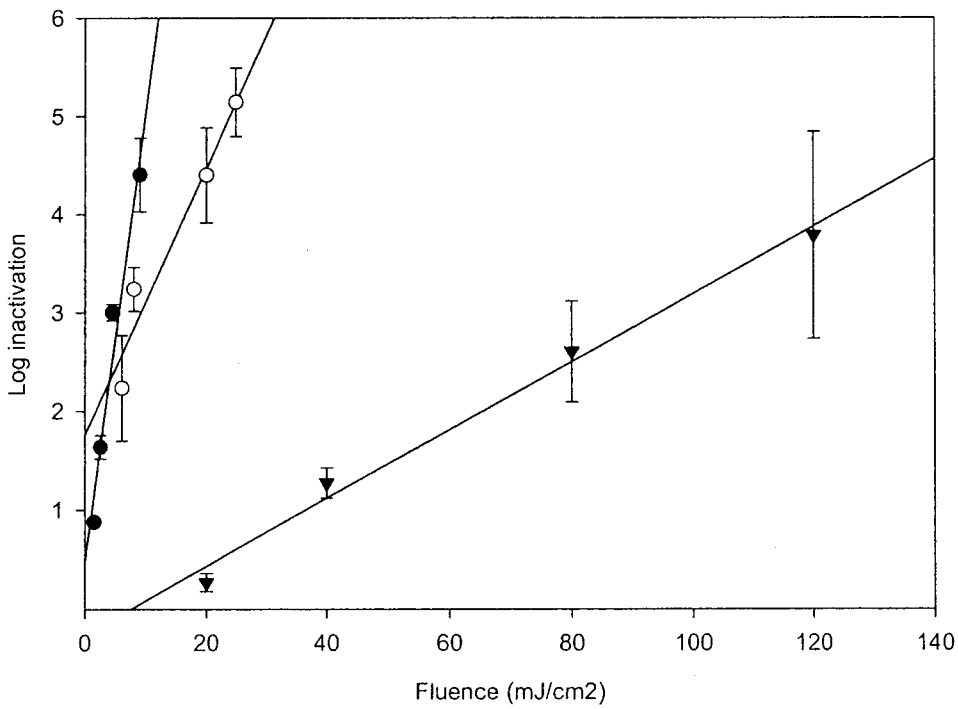

Figure 6-3 UV inactivation of tested bacteria
E. coli —●—, *E. faecalis* —○—, *B. subtilis* spores —▼—

6.3 Effect of Freezing on UV Inactivation

The effect of freezing on UV inactivation of *E. coli*, *E. faecalis* and *B. subtilis* spores was observed after samples were first frozen at -7, -15 and -30°C, thawed and then irradiated at the bacteria's respective fluences. A room temperature control was also tested for comparison. Bacteria could have a variety of responses to the effect of freezing prior to UV inactivation. Apart from no effect at all, there could be an increased susceptibility comparable to a Hurdle effect in food disinfection. This is when a series of treatments are used where each treatment alone would not effectively disinfect the product, but when combined each step has an additive effect that equates to an overall acceptable inactivation of the target organism. Lastly, there is the possibility of resistance where freezing would make UV treatment less effective than if the samples were not frozen. Referring to the only other study to have examined the effects of freezing on UV inactivation could lead to a hypothesis being made that frozen bacteria could respond differently to UV treatment (Gao *et al.*, 2006).

Experimental data indicated that freezing prior to UV irradiation showed a significant effect on the ability of UV to inactivate *E. coli*. The average inactivation results for *E. coli* at all fluences after one freeze cycle are presented in Figure 6-4. Log inactivation at -7°C showed the lowest average of inactivation at all fluences except for at fluence 1.5 mJ/cm^2 where samples frozen at -30°C had a 0.227 log inactivation.

The greatest difference in log inactivation between frozen and unfrozen samples was at 4.5 mJ/cm^2 when samples were frozen at -7°C. The inactivation level of unfrozen samples was statistically significant at 1.0 log unit higher than that which was frozen (p=0.01). At 2.5 and 9 mJ/cm^2 there were differences of between average log inactivation's of 0.71 and 0.83 at -7°C respectively (p=0.003, p=0.07). The fluence 1.5 mJ/cm^2 had the greatest difference of 0.597 log inactivation at -30°C (p<0.001).

Statistical analysis indicated that cells frozen at -7 and -30°C were more effective at resisting UV inactivation than those frozen at -15°C, especially at the fluences 1.5 and 9 mJ/cm^2. These results may be skewed by the increased inactivation capacity of freezing prior to UV at -7 and -30°C. It was mentioned that at these temperatures the maximum amount of inactivation is attained therefore the cells that survive freezing may be selected for resistance. As for cells frozen at -15°C, freezing may not have inactivated cells but may have weakened them and made them more likely to be killed when exposed to UV irradiation.

A hurdle-effect was not shown when freezing and UV irradiation were used in concert; conversely a resistance was shown by *E. coli* cells. Cells damaged by freeze-thaw, release UV-absorbing material (Calcott and MacLeod, 1975a), which might lead to a lower response to UV. Calcott and MacLeod (1975a) observed that as cooling rates were increased from about 6-7°C/min, *E. coli* released an increased amount of UV-absorbing material. Therefore, perhaps the cells that were damaged and died during freezing protected those that survived by absorbing the UV light when the sample was irradiated. This could explain why the samples frozen at -7°C did not show more resistance overall to inactivation by UV than those frozen at -15 and -30°C where more damage occurred from freezing.

The stress of freezing initiates a response that causes changes in the cytoplasmic membrane, DNA, mRNA and ribosomes that may enable the cell to withstand UV inactivation to a greater degree (Phadtare *et al.*, 2000). Therefore, when *E. coli* cells undergo UV irradiated directly coming from thawing after freezing, there may be an increased amount of DNA repair enzymes available or UV damage preventing structure within the cell. This could account for the decrease in inactivation after UV for those cells that have been previously frozen. This would be especially true for the dark repair pathway because the same pathway is used in freezing repair (Calcott and

Gargett, 1981). Conversely, Koivunen and Heinonen-Tanski (2005) suggest that a combination of disinfection methods could overload the repair mechanisms of the bacteria and lead to their inability to repair, followed by cell death.

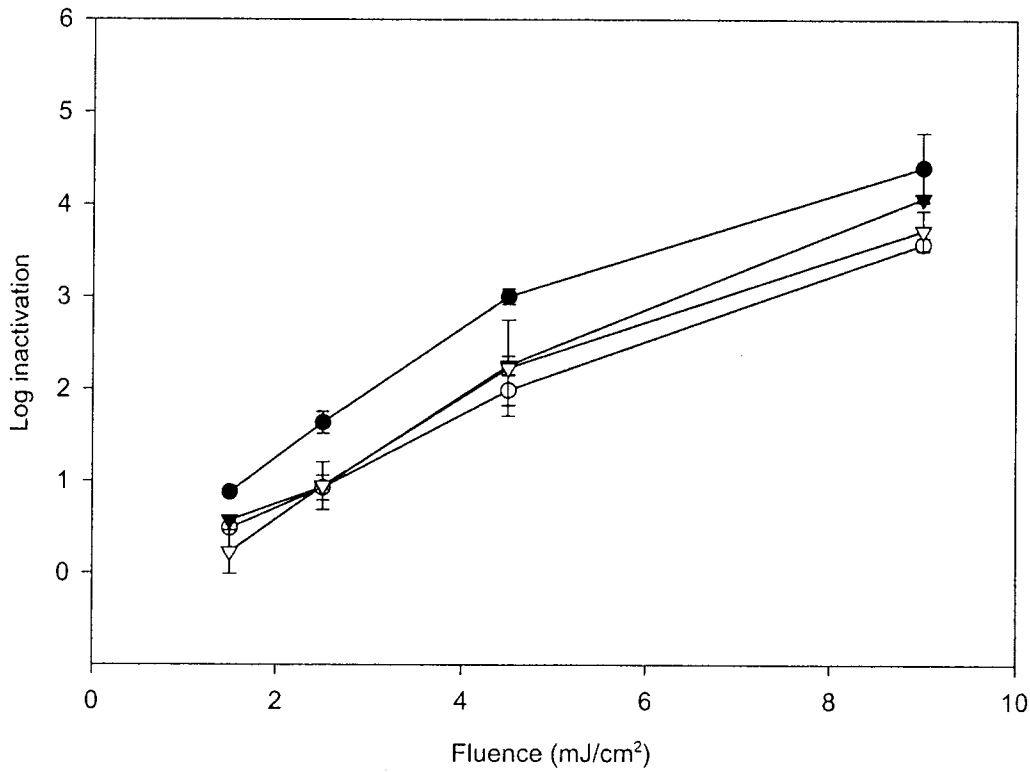

Figure 6-4 Effect of freezing on UV inactivation of *E. coli*
Control —●—, -7°C —○—, -15°C —▼—, -30°C —▽—

Freezing at various temperatures prior to UV treatment showed a significant effect on the ability of UV to inactivate *E. faecalis* at a low fluence. The average inactivation results for all fluences after one freezing cycle are shown in Figure 6-5.

Results of statistical analysis listed in Table 6-2, indicated that frozen *E. faecalis* cells have a significant resistance to inactivation when compared to unfrozen ones. The greatest difference between frozen and unfrozen samples was 0.479 log inactivation at 8 mJ/cm^2 when samples were frozen at -7°C (p=0.023). There was a statistically

significant difference between the control and the samples that were frozen at all temperatures when the fluence was 8 mJ/cm^2. The *p* values for temperatures -7, -15, -30°C were 0.023, 0.004 and 0.007 respectively. At all other fluences there was no statistical difference between UV inactivation of frozen and unfrozen samples. Samples frozen once at -7, -15, -30°C demonstrated no difference from the unfrozen samples when inactivated at all other fluences (6, 20 and 25 mJ/cm^2).

There was a greater difference at fluence 8 mJ/cm^2 than other fluences which suggests that UV fluence plays a greater role on the ability to inactivate cells than the temperature at which *E. faecalis* cells were frozen. At higher UV fluencies, such as 20 and 25 mJ/cm^2, *E. faecalis* cells were killed no matter if they were frozen or not.

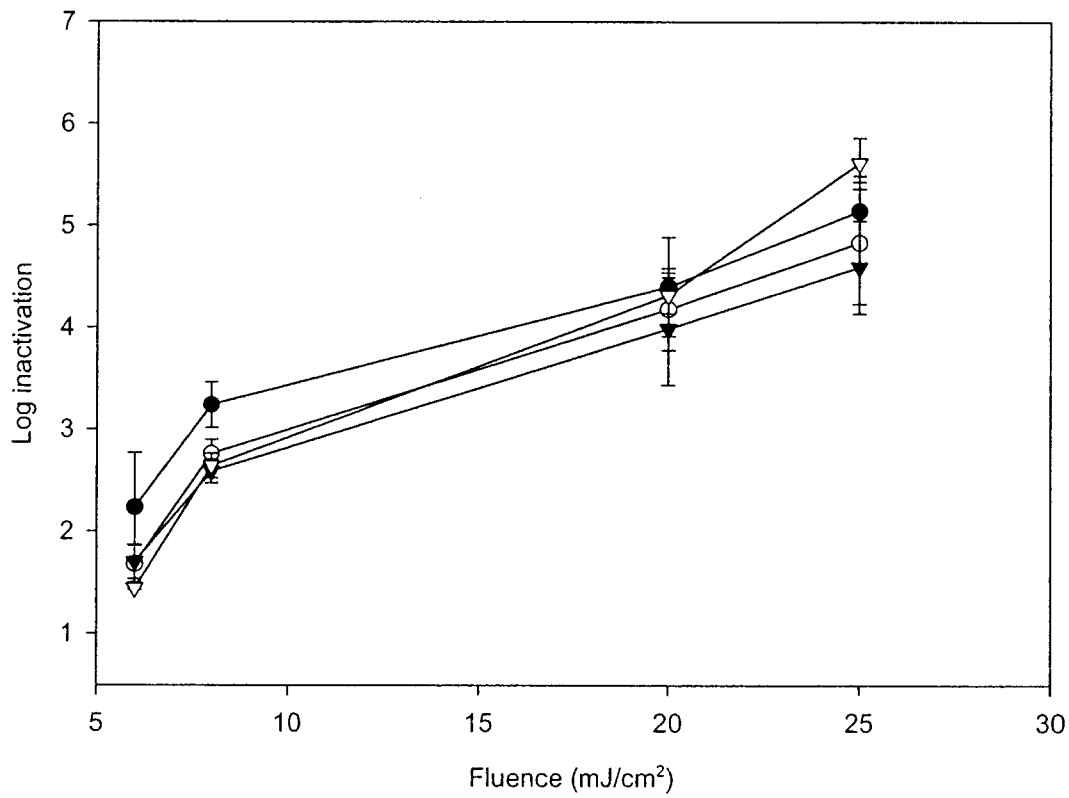

Figure 6-5 Effect of freezing on UV inactivation of *E. faecalis*
Control —●—, -7°C —○—, -15°C —▼—, -30°C —▽—

Statistical analysis, in Table 6-2, indicated that freezing samples had a significant effect on the UV inactivation of the spores of *Bacillus subtilis* ($p<0.001$). As shown in Figure 6-6, freezing temperature does affect the average inactivation results at fluences 80 mJ/cm^2 and above.

Table 6-2 Two Way ANOVA comparing UV inactivation fluence and freezing temperature

Source of Variation	*E. coli*	*E. faecalis*	*B. subtilis* spores
Freezing Temperature	**<0.001**	**0.005**	**0.026**
UV Fluences	**<0.001**	**<0.001**	**<0.001**
Freezing Temperature x Fluences	0.171	0.106	**0.009**

Freezing had no effect on spore inactivation however, when paired with UV irradiation, *B. subtilis* spores proved to be affected by the added stress of freezing by showing resistance to UV treatments at higher fluences. The results found in this study are similar to those published by Gao *et al.* (2006), where the freezing process made *Bacillus megaterium* spores more resistant to UV inactivation at higher fluences (60 to 80 mJ/cm^2).

A tailing effect is shown in Figure 6-6 which was also seen in the study by Gao *et al.* (2006). They cited a variety of explanations including clumping of spores in order to explain their observations of tailing. Also mentioned was a possible resistant subpopulation to UV. The resistant spores would provide similar results found for *E. coli* strain 25922 and *E. faecalis* strain 29212. Initial irradiation would result in the expected inactivation levels, however as exposure increases, only resistant spores remain. Therefore, fewer spores are inactivated as exposure continues, compared to non frozen spores.

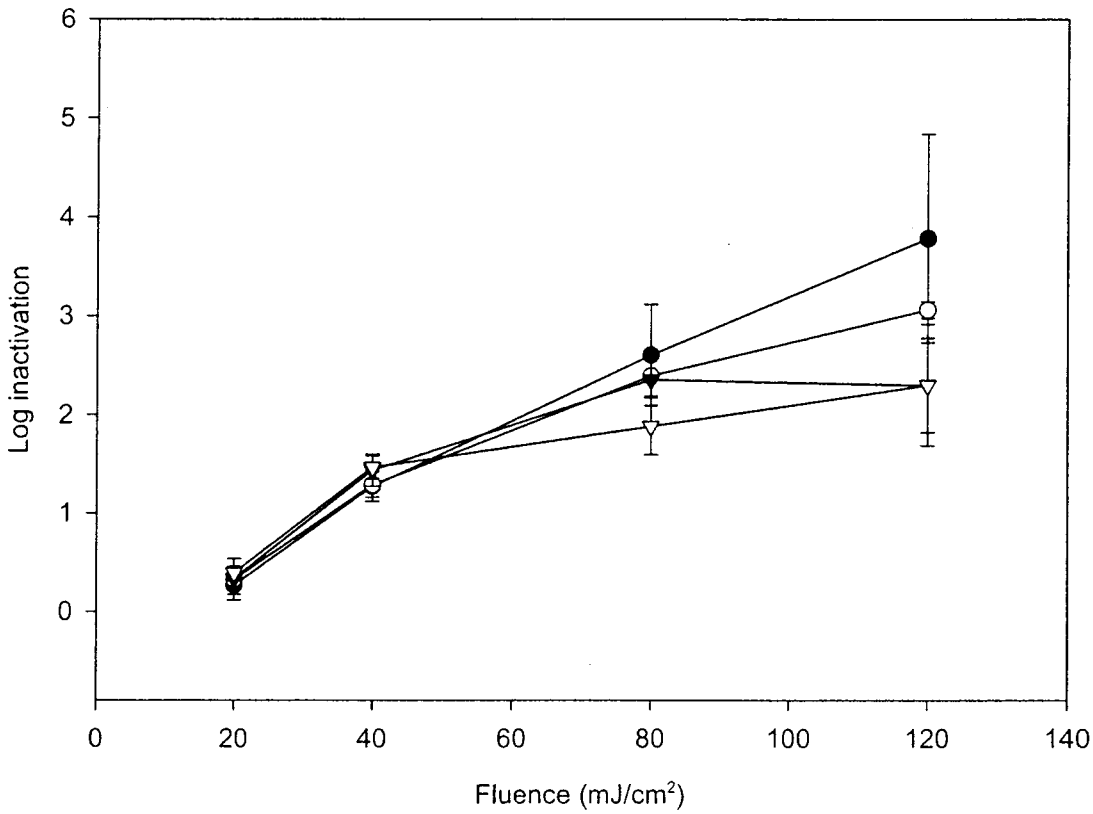

Figure 6-6 Effect of freezing on UV inactivation of *B. subtilis* spores
Control —●—, -7°C —○—, -15°C —▼—, -30°C —▽—

When all three bacteria were compared by a two-way ANOVA, a statistically significant difference was found between the bacteria however, the difference in bacteria does not depend on the freezing temperature.

6.4 Effect of Freeze-Thaw Cycles on UV Inactivation

The effect of freezing and thawing on UV inactivation of *E. coli*, *E. faecalis* and *B. subtilis* spores was observed over 1, 3 and 5 cycles at the following temperatures: -7, -15, -30°C. A room temperature control was also noted for comparison.

The number of freeze-thaw cycles significantly affected the UV inactivation of *E. coli* strain 29522 and *E. faecalis* strain 29212 (p=0.026 and p<0.001). Comparatively, *B. subtilis* strain 6633 spores were found to be unaffected by freeze-thaw cycles (p=0.148).

For *E. coli*, the average inactivation results for all freeze-thaw cycles at -7, -15 and -30°C are shown in Figure 6-7. The greatest average difference in loss of viability occurred at fluence 4.5 mJ/cm^2 where cells were able to survive greater than 1.0 log inactivation difference at both cycles 1 and 3. Experiments were carried out for cycle 5 but were unable to be recorded because the concentration of cells reached the detection limit for the method of calculating concentrations for this study. Cycle 3 at fluence 9 mJ/cm^2 also was beyond the detection limit for 4 trials.

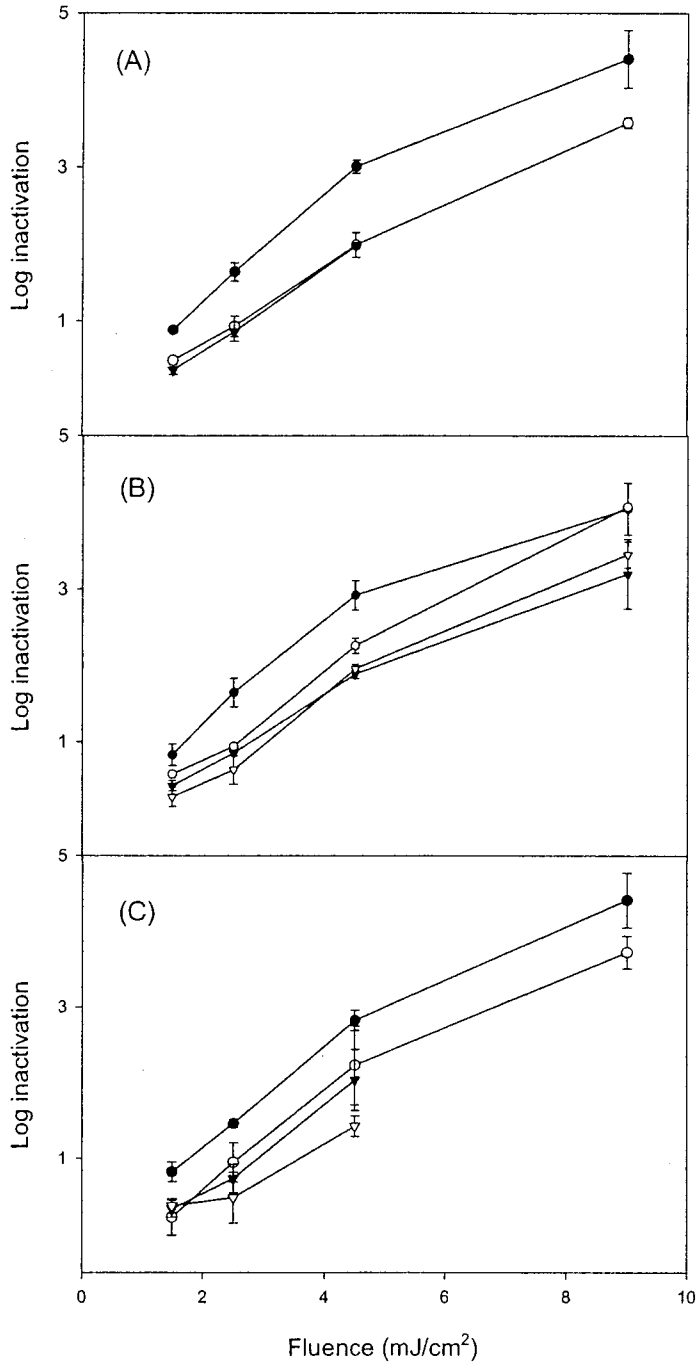

Figure 6-7 Effect of freeze-thaw cycles on UV inactivation of *E. coli* at (A) -7°C, (B) -15°C, (C) -30°C
cycle 0 —●—, cycle 1 —○—, cycle 3 —▼—, cycle 5 —▽—

Statistical analysis found that freeze-thaw cycles had a significant effect on the ability of UV to inactivate *E. coli*. There was a significant difference between the control and freeze-thaw cycles 1 and 3 at fluences 1.5, 2.5 and 4.5 mJ/cm^2, however no significant difference was found at fluence 9 mJ/cm^2. There was no statistical difference between the freeze-thaw cycles or freezing temperature. Therefore, it is expected that the microbial cells will be killed after exposure to high strength UV light whether or not they were frozen.

Table 6-3 Three Way Analysis of Variance comparing freezing temperature, freeze-thaw cycles and fluences (*E. coli* with 1 and 3 F/T cycles and an additional 5 for the other two bacteria)

Source of Variation	*E. coli*	*E. faecalis*	*B. subtilis* spores
Freezing Temperature	0.552	**<0.001**	**<0.001**
Freeze-thaw cycles	**0.026**	**<0.001**	0.148
UV Fluences	**<0.001**	**<0.001**	**<0.001**
Freezing Temperature x Freeze-thaw	0.561	0.897	0.662
Freezing Temperature x UV Fluences	0.347	**<0.001**	**<0.001**
Freeze-thaw cycles x UV Fluences	0.585	0.327	0.937
Freezing temp x Freeze-thaw x Fluences	0.498	0.836	0.995

A further statistical comparison was carried out in order to compare the effect of freeze-thaw cycles and fluences on cell viability by adding the data from the fifth cycle. A significant relationship between *E. coli* inactivation and freeze-thaw cycles when the fifth cycle was added to the computed data set.

Freeze-thaw cycles affected the UV inactivation of *E. coli*. An increase in the number of freeze-thaw cycles showed a decreased inactivation after UV treatment. This could be an important factor to consider when UV facilities in cold climates irradiate water that has been previously frozen and has continual freezing and thawing during the fall and spring months.

Cycles 3 and 5 were unable to be recorded due to detection limitations at -15 and -30°C. Cycle 5 was not tested at -15°C because of results found in previous experiments. By following the trend at lower fluences it could be suggested that a similar

trend, of decreased inactivation, would follow at higher fluences. The greatest average difference in loss of viability occurred at fluence 4.5 mJ/cm^2 where cells were able to survive greater than 1 log inactivation difference at both cycles 1 and 3.

Mutations could have occurred during the freezing and those that survive could have passed the resistance genes on to other cells also enabling them to survive. After repeated freeze-thaw cycles, Calcott and Gargett (1981) observed an increase in the frequency of mutation suggesting that freezing and/or thawing was a mutagenic process. Ishimoto *et al.* (2008) found that *E. coli* can horizontally transmit non-conjugative, non-viral DNA between bacterial cells.

Experimental data obtained from this study indicates that freeze-thaw cycles had a significant effect on the ability of UV to inactivate *E. faecalis* at lower fluences. The average inactivation results for all freeze-thaw cycles are shown in Figure 6-8.

The greatest average inactivation difference between the frozen and unfrozen samples occurred at fluence 6 mJ/cm^2 where the difference was 0.897 log inactivation at cycle 5. As the fluence increased for samples frozen at 5 freeze-thaw cycles, the difference between it and the control decreased.

Experimental data obtained from this study indicates that UV inactivation of *B. subtilis* spores was not affected by freeze-thaw cycles. The average inactivation results for all freeze-thaw cycles are shown in Figure 6-8 and the statistical analysis is shown in Table 6-3.

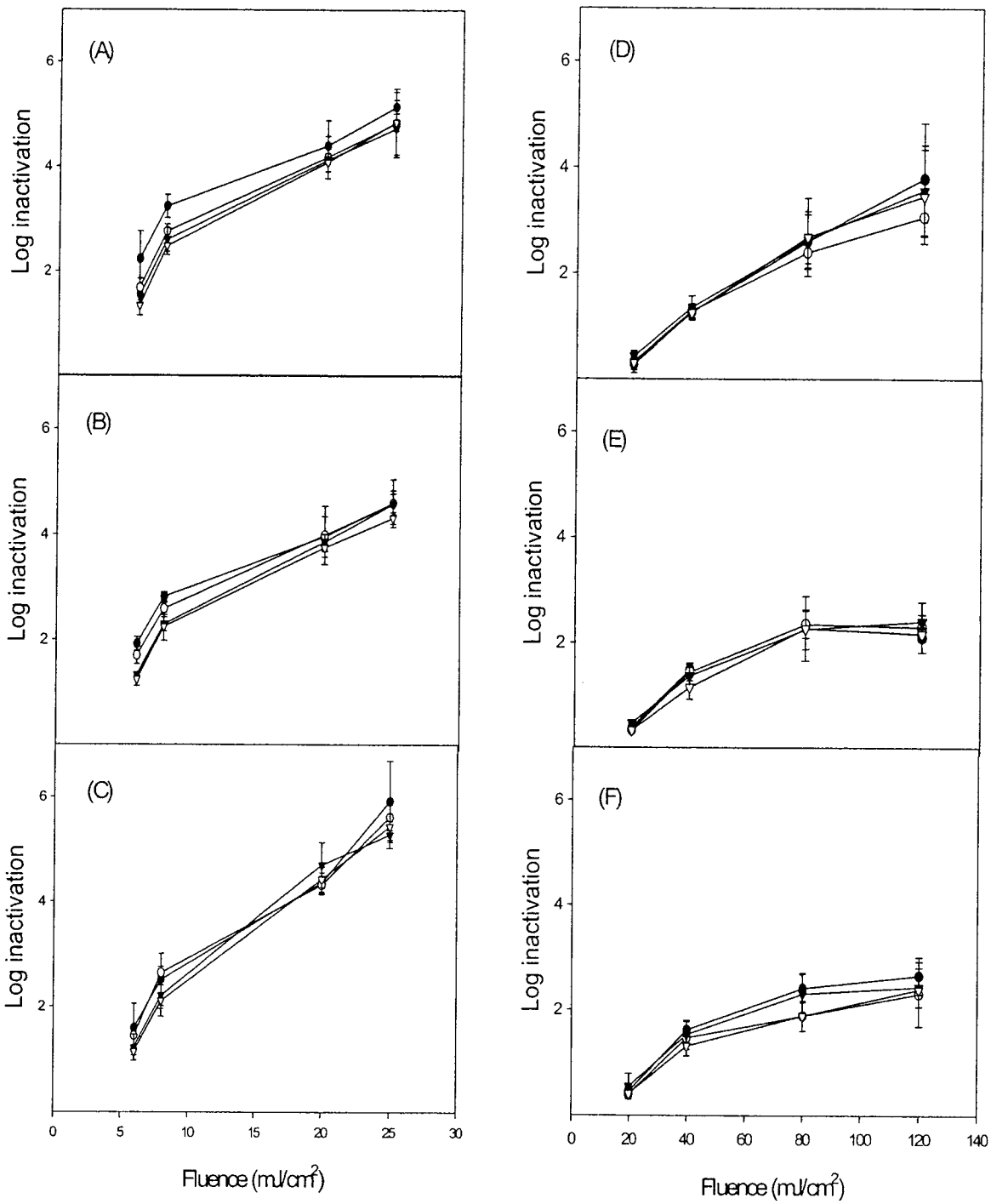

Figure 6-8 Effect of freeze-thaw cycles on UV inactivation of *E. faecalis* at (A) -7°C (B) -15°C (C) -30°C and of *B. subtilis* spores at (D) -7°C (E) -15°C (F) -30°C cycle 0 —●—, cycle 1 —○—, cycle 3 —▼—, cycle 5 —▽—

6.5 Photoreactivation

In this experiment, bacteria were exposed to photoreactivating light in order to determine if the bacterial strains used were able to photoreactivate. Of the three bacteria tested, *Escherichia coli* strain 29522 and *Enterococcus faecalis* strain 29212 were able to photoreactivate after exposure to photoreactivating light.

Experimental results shown in Figure 6-9 indicated that *E. coli* 29522 photoreactivated after UV inactivation fluences of 2.5 and 9 mJ/cm^2. It took 30 minutes of light exposure when previously irradiated at 9 mJ/cm^2 and 90 minutes when irradiated at 2.5 mJ/cm^2.

Statistical analysis suggests that cells irradiated at 2.5 and 9 mJ/cm^2 were able to significantly photoreactivate after 90 (p<0.001) and 180 minutes (p<0.001) of exposure to visible light. After 30 minutes of exposure time, only the samples irradiated at 9 mJ/cm^2 were able to photoreactivate significantly (p=0.025). There was an obvious difference in the level of photoreactivation between the two fluences used in this study after 30 minutes (p<0.033). The maximum average increase in viable cells was 0.953 log photoreactivation observed 180 minutes after UV irradiation at 9 mJ/cm^2.

The results from this study support previous research indicating photoreactivation of *E. coli* following irradiation from low-pressure UV lamps. Photoreactivation of *E. coli* strain 11229 has been well documented in the literature (Zimmer and Slawson, 2002, Bohrerova and Linden, 2007, Tosa *et al.*, 2003, Shaban *et al.*, 1997, Hassen *et al.*, 2000, Zimmer *et al.*, 2003), to the extent that Zimmer *et al.* (2003) used this strain of bacteria as a positive control known to photoreactivate. However, there is little evidence regarding the *E. coli* strain 29522 used in this study.

E. coli strain 11229 was observed to photoreactivate after fluences of 5, 8 and 10 mJ/cm^2. A maximum repair was noted after 120 minutes of light exposure with 2.8 log

reactivation after 10 mJ/cm^2 was recorded. Photoreactivation after 5 and 8 mJ/cm^2 were 2.6 and 0.7 log repair with the latter being more comparable to the degree of photoreactivation observed in this study.

Samples of *E. coli* strain 29522 were shown to photoreactivate after UV inactivation fluences of 2.5 and 9 mJ/cm^2. The maximum average increase in viable cells of 0.95 log photoreactivation was lower than photoreactivation values reported in previous studies. Bohrerova and Linden (2007), Zimmer and Slawson (2002) and Sommer *et al.* (2000) reported photoreactivation as high as 2.7, 2.6 and 3.6 log respectively after 8 mJ/cm^2 by *E. coli* strain 11229.

A lag time was detected at higher fluences, as the time needed for repair increased with increase in UV fluence (Zimmer and Slawson, 2002). This was shown in this study when, at 90 minutes, the lower fluence, 4.5 mJ/cm^2 had greater recovery than samples that were irradiated at 9 mJ/cm^2. Zimmer and Slawson (2002) attributed the lag time to the increase in DNA damage by the higher fluence, suggesting that it would take longer to repair DNA damage because there are only approximately 20 photolyase enzymes in each *E. coli* organism and each enzyme can repair only approximately 5 dimers per minute (Zimmer and Slawson, 2002).

Although photoreactivation was observed, the level of repair never reached the initial concentration of *E. coli* prior to UV exposure. Therefore, complete repair did not occur, which indicates that irreversible damage occurred to cells (Zimmer and Slawson, 2002).

Enterococcus faecalis 29212 was able to photoreactivate after UV inactivation fluences of 20 mJ/cm^2, but not after 6 mJ/cm^2. The difference in photoreactivation between the two fluences are contradictory to published studies where photoreactivation decreases when irradiation fluence increases (Bohrerova and Linden, 2006, Oguma *et al.*, 2002, Tosa and Hirata, 1999, Zimmer and Slawson, 2002). In this study the large

experimental error bars resulting from experimental error could be the cause of this inconsistency.

E. faecalis that was irradiated at 20 mJ/cm^2 demonstrated less photoreactivation than *E.coli* after exposure to the fluorescent light. The average photoreactivation results for *E. faecalis* are shown in Figure 6-10.

Statistical analysis, shown in Table 6-4, suggests that cells irradiated 20 mJ/cm^2 were not significantly different after 30, 90 and 180 minutes of exposure to visible light. There was, however, a significant difference between the average log values of the two fluences used in this study. Samples irradiated at fluence 6 mJ/cm^2 were significantly lower in concentration than those irradiated at 20 mJ/cm^2 at all time points (30, 90 and 180 minutes). *E. faecalis* photoreactivation has been observed in a few studies (Tosa *et al.*, 2003, Hassen *et al.*, 2000), however the strain 29212 used in this study *E. faecalis* has little recorded accounts of its ability to photoreactivate.

B. subtilis spores are not able to photoreactivate after UV inactivation fluence of either 40 or 120 mJ/cm^2. The average photoreactivation results for *B. subtilis* spores are shown in Figure 6-11 and the statistical analysis overview is in Table 6-4. Vegetative cells of *B. subtilis* strain 6633 have been shown to photoreactivate (Hassen *et al.*, 2000), however their spores have not yet shown an ability to photoreactivate.

Table 6-4 ANOVA results for bacteria comparing photoreactivation exposure time and fluences

Source of Variation	*E. coli*	*E. faecalis*	*B. subtilis* spores
UV Fluences	**<0.001**	**<0.001**	0.076
Exposure Time	**<0.001**	0.092	0.213
Fluences x Exposure Time	**<0.001**	0.448	0.054

6.6 Effect of Freezing on Photoreactivation after UV Inactivation

The effect of freezing on photoreactivation after UV inactivation of *E. coli*, *E. faecalis* and *B. subtilis* spores was observed after one freeze-thaw cycle at -15°C. A

room temperature control was also noted for comparison. Freezing had a negative effect on the ability of photoreactivating bacteria to repair once irradiated. Bacteria were able to photoreactivate, however to a lesser extent than samples that had not been frozen.

When *E. coli* cells are previously frozen at -15°C and inactivated at 2.5 and 9 mJ/cm^2, photoreactivation can occur after 180 minutes of light exposure. However, the maximum photoreactivation of samples previously frozen were still well below the photoreactivation capabilities of cells irradiated without freezing. The average photoreactivation results for *E. coli* inactivated at 2.5 and 9 mJ/cm^2 are shown in Figure 6-9.

Freezing had a negative effect on the ability of *E. coli* to photoreactivate. Only slight photoreactivation was observed after 180 minutes, which was still less than the initial concentration of cells after UV inactivation. That means that the negative effect of visible light to frozen cells was greater than the bacteria's ability to photoreactivate. Table 6-6 illustrates the three-way comparison of factors for *E. coli* photoreactivation after exposure to UV irradiation.

There was photoreactivation of *E. faecalis* cells when placed under visible light after prior freezing at -15°C when inactivated at 6 mJ/cm^2. The opposite was observed for samples irradiated at 20 mJ/cm^2. The average log results and ANOVA results for *E. faecalis* are listed in Table 6-5 and Table 6-6.

Freezing and fluence, two factor interactions, had a significant impact on the viability of *E. coli* and *E. faecalis* while the combined effect was not obvious on *B. subtilis* spores ($p<0.001$). Frozen samples irradiated at 6 mJ/cm^2 showed more photoreactivation than non frozen samples. The opposite was true for frozen samples irradiated at 20 mJ/cm^2 because they showed less ability of photoreactivation. Therefore, as fluence increases the effect of freezing has more of a negative effect on photoreactivation.

Samples of *B. subtilis* spores frozen at -15°C prior to UV irradiation were not able to photoreactivate when placed under visible light when inactivated at 40 and 120 mJ/cm^2. There was no statistical difference between frozen and non-frozen samples, both were unable to photoreactivate, shown in Table 6-5 and Figure 6-11. The average log values did show a difference between 90 and 180 minutes, however, this was not due to photoreactivation because the average at 90 minutes was indicating that this amount of exposure time was killing the cells. This is more likely due to experimental error than the effect of visible light since, at 180 minutes, there was an increase in cell counts but photoreactivation has not been shown to be an ability that *B. subtilis* spores posses.

Table 6-5 Average log photoreactivation after exposure to photoreactivating light

		E. faecalis (N/N_0) (avg ± SD)		*B. subtilis*(N/N_0) (avg ± SD)	
Fluence (mJ/cm^2)		6	20	40	120
	Time(min)				
Cycle 0	30	-0.007 ± 0.021	0.217 ± 0.050	0.000 ± 0.069	0.100 ± 0.173
	90	-0.253 ± 0.146	0.150 ± 0.020	-0.013 ± 0.006	-0.260 ± 0.262
	180	-0.100 ± 0.225	0.297 ± 0.110	0.103 ± 0.100	-0.173 ± 0.148
Cycle 1	30	0.0567 ± 0.031	0.0067 ± 0.040	-0.133 ± 0.146	-0.120 ± 0.06
	90	-0.0533 ± 0.117	0.0533 ± 0.040	-0.033 ± 0.015	-0.200 ± 0.09
	180	-0.0167 ± 0.049	-0.0267 ± 0.130	-0.013 ± 0.058	0.090 ± 0.29

Table 6-6 Three Way ANOVA comparing photoreactivation exposure time, freezing and fluences

Source of Variation	*E. coli*	*E. faecalis*	*B. subtilis* spores
Freeze	**<0.001**	0.281	0.724
UV Fluences	**<0.001**	**<0.001**	0.061
Exposure Time	**<0.001**	0.118	**0.047**
Freeze x UV Fluences	**0.018**	**<0.001**	0.285
Freeze x Exposure Time	**<0.001**	0.072	0.136
UV Fluences x Exposure Time	**<0.001**	0.265	0.153
Freeze x Fluences x Exposure Time	0.193	0.664	0.061

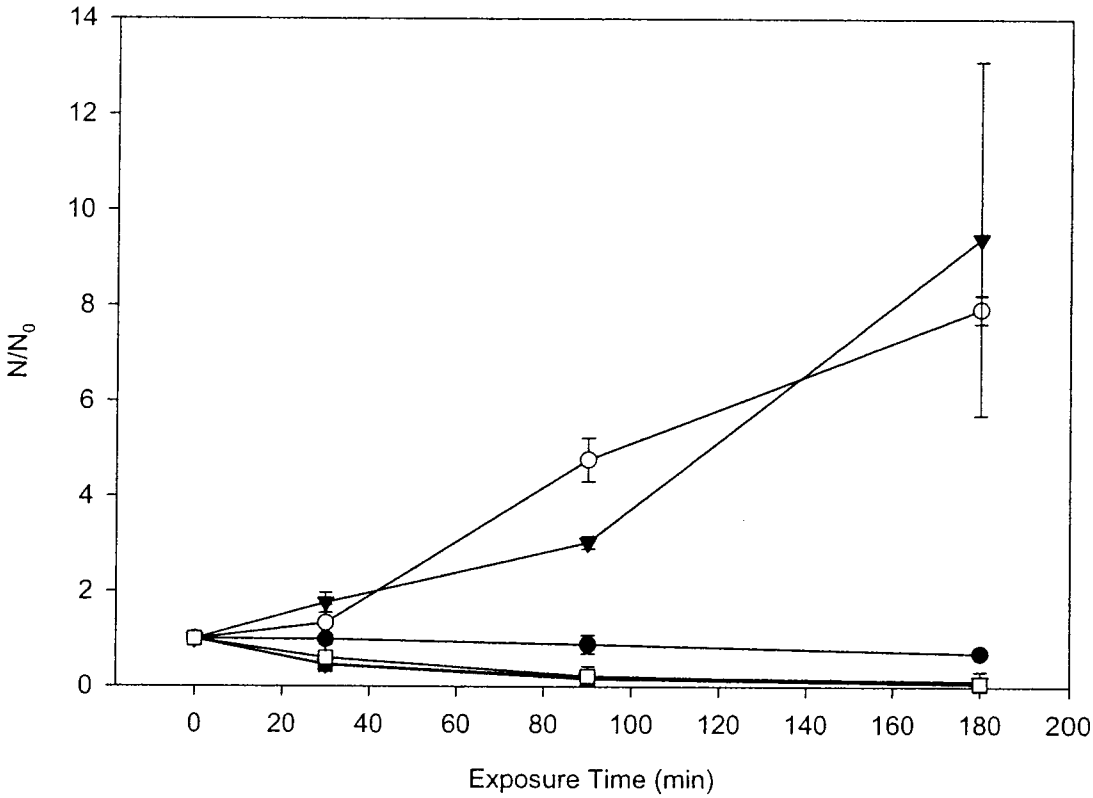

Figure 6-9 Ratio of *E. coli* photoreactivation after frozen and unfrozen cells were exposed to photoreactivating light
Control —●—, Control 2.5 mJ/cm^2 —○—, Control 9.0 mJ/cm^2 —▼—,
Frozen Control —▽—, Frozen 2.5 mJ/cm^2 —■—, Frozen 9.0 mJ/cm^2 —□—

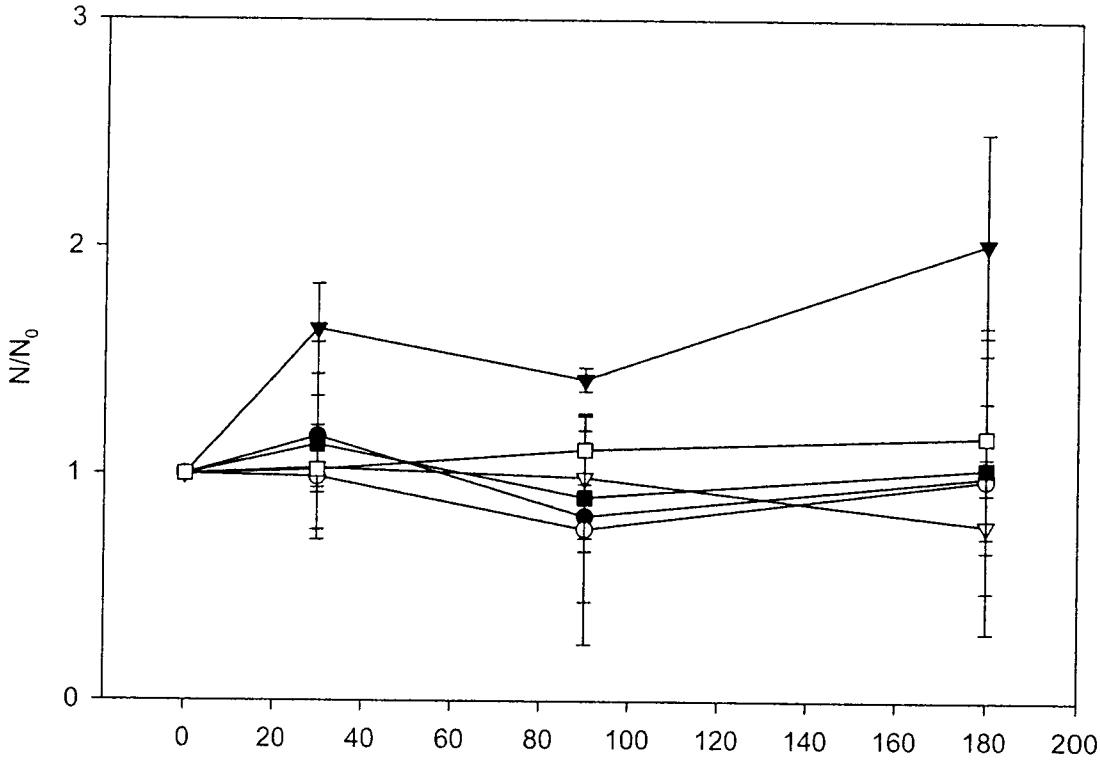

Figure 6-10 Ratio of *E. faecalis* photoreactivation after frozen and unfrozen cells were exposed to photoreactivating light

Control —●—, Control 6.0 mJ/cm^2 —○—, Control 20 mJ/cm^2 —▼—,
Frozen Control —▽—, Frozen 6.0 mJ/cm^2 —■—, Frozen 20 mJ/cm^2 —□—

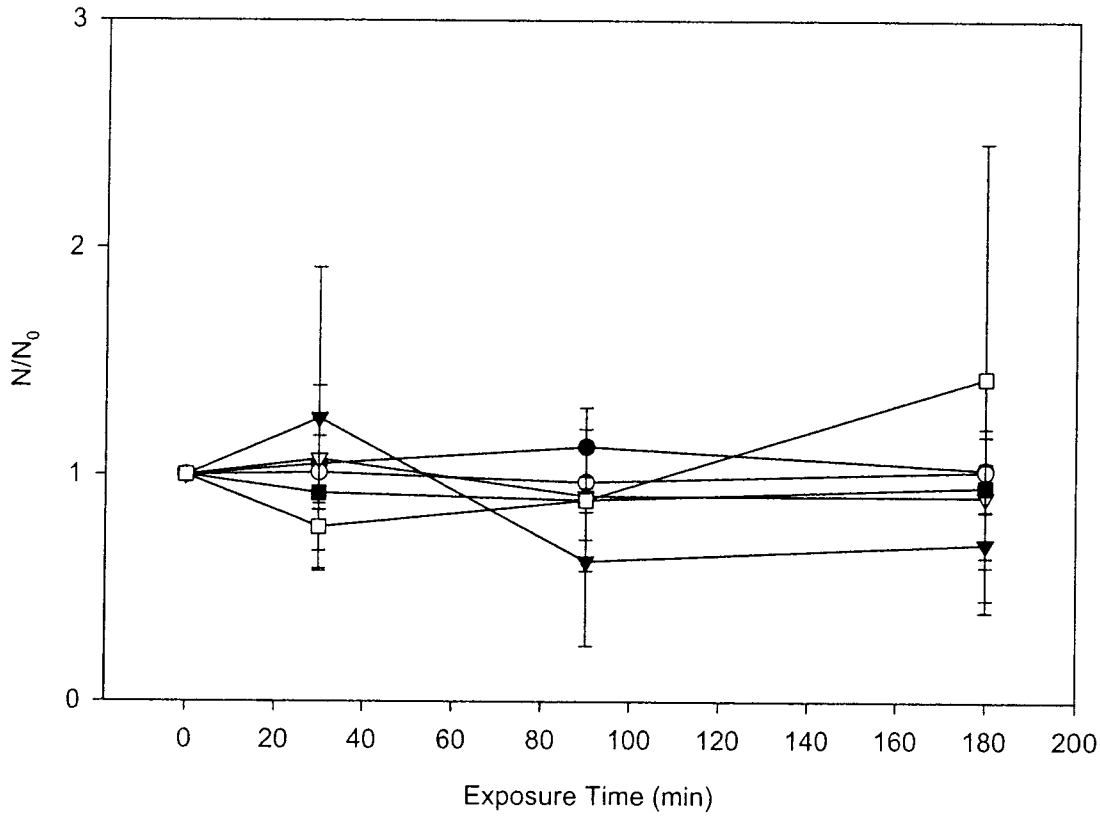

Figure 6-11 Ratio of *B. subtilis* spores photoreactivation after frozen and unfrozen cells were exposed to photoreactivating light

Control —●—, Control 40 mJ/cm^2 —○—, Control 120 mJ/cm^2 —▼—,
Frozen Control —▽—, Frozen 40 mJ/cm^2 —■—, Frozen 120 mJ/cm^2 —□—

Chapter 7: Conclusions and Recommendations

7.1 Conclusions

The main purpose of this study was to examine the effects of freezing on UV inactivation of *Escherichia coli*, *Enterococcus faecalis* and *Bacillus subtilis*. The following conclusions have been made:

The effect of freezing on UV efficiency is species dependant. When there was a discernible difference by the frozen samples to the control, a resistance was observed.

- *E. coli* had the greatest log difference of 1 log inactivation, suggesting a resistance to UV affect freezing.
- *E. faecalis* had a negative response to UV after freezing at low fluences.
- Spores of *B. subtilis* were only affected at high fluences at low freezing temperatures.

Freezing temperature proved to be a factor as well as freeze-thaw cycles which can make waterborne microorganisms more tolerant to UV treatment.

- Inactivation of *Escherichia coli* strain 29522 and *Enterococcus faecalis* strain 29212 were both affected by freezing temperature and freeze-thaw cycles.
- Conversely, samples of *Bacillus subtilis* strain 6633 endospores were not affected by either freezing temperature or by freeze-thaw cycles.

Therefore, in cold climates, freezing may render UV inactivation less efficient and require greater UV fluences to compensate.

Photoreactivation was observed by two of the bacteria tested and freezing had a negative effect on photoreactivation

- *Escherichia coli* 29522 have shown to be able to photoreactivate after the fluences tested. The repair observed was the greatest of the three microorganisms tested.
- *Enterococcus faecalis* 29212 showed slight photoreactivation after the higher fluence was tested.
- *Bacillus subtilis* 6633 spores showed no photoreactivation after all fluences and exposure times tested.

Freezing had a negative effect on the bacteria that were able to photoreactivate. Freezing had a negative effect on the ability of the bacteria to survive after irradiation when exposed to visible light. The ability of either *E. coli* or *E. faecalis* to photoreactivate was overcome by the negative effect of freezing and they were unable to photoreactivate to levels equal to that of the concentration immediately after inactivation and definitely not to concentrations comparable to irradiated non-frozen samples. For *B. subtilis* spores, freezing had no effect on the ability of the bacteria to photoreactivate. They were unable to repair when exposed to visible light and that still remained the case when frozen.

Therefore, some microorganisms have the ability to require a 1 log inactivation increase in fluence to compensate for the possibility of repair in order to meet effluent requirements. Furthermore, the effects of freezing are not magnified by some bacteria's ability to photoreactivate.

In conclusion, freezing may affect the efficiency of UV treatment when target pathogens are irradiated by UV. This could occur in Northern communities in Canada and in parts of USA. Also with the increased interest in alternative disinfection technologies, freezing technology could be used in concert with UV treatment which may also encounter the effects of freezing on UV irradiation.

7.2 Recommendations

1. Inactivation data of microorganisms causing waterborne diseases have to be investigated to establish the required UV dose for safe water disinfection, including further studies of microorganisms under environmental stresses that could affect UV inactivation.

2. Further studies on how freeze-thaw affects UV inactivation of other waterborne pathogens, focusing on possible variety of strain, species and genus of waterborne pathogens.

3. Investigate cells grown to stationary phase and because of the diversity of microorganisms varies, testing actual effluent from communities where freezing and thawing can occur and applying UV inactivation to waste that has been treated to freeze-thaw.

4. Photoreactivation studies of other possible waterborne pathogens in the presence of turbidity, which may react differently since the turbidity would hinder the microorganisms' ability to photoreactivate

5. Further study is needed to determine the cause of tailing found when *Bacillus subtilis* spores are UV inactivated. Investigate clumping as a source of tailing by sonicating spore samples or passing samples through a filter prior to irradiation.

Glossary of Terms

The following definitions were derived from existing UV literature and textbooks. Some concepts have more than one acceptable term or definition, but for consistency, only one term is used.

Absorption – the transformation of UV light to other forms of energy as it passes through a substance.

Biodosimetry – a procedure used to determine the reduction equivalent dose (RED) of a UV reactor. Biodosimetry involves measuring the inactivation of a challenge microorganism after exposure to UV light in a UV reactor and comparing the results to the known UV dose-response curve of the challenge microorganism (determined via bench-scale collimated beam testing).

Cell envelop – made up of the cell membrane, cell wall and outer membrane if present

Cell membrane (cytoplasmic membrane) – composed of a phospholipid bilayer and acts as a barrier to hold nutrients, proteins and other essential components of the cytoplasm within the cell.

Cell wall – made of peptidoglycan in bacteria, provides structural support to protect the cell from turgor pressure.

Collimated Beam Test – a controlled bench-scale test that is used to determine the UV fluence-response of a challenge microorganism. Both time and UV light intensity are directly measured; the UV dose is calculated using the intensity of the incident UV light, UV absorbance of the water, and exposure time.

Dark Repair – an enzyme-mediated microbial process that removes and regenerates a damaged section of deoxyribonucleic acid (DNA), using an existing complimentary strand of DNA. Dark repair refers to all microbial repair processes not requiring reactivating light.

Endospores – resting spores produced by Gram-positive bacteria to survive harsh conditions that are surrounded by a cortex layer and protected by an impermeable and rigid coat.

Fluence (UV dose) – the total radiant energy of all wavelengths passing from all directions through an infinitesimally small sphere of cross-sectional area *dA* divided by *dA* (J/m^2) (mWs/cm^2).

Fluence Rate (dose rate) – the total radiant power incident from all directions onto an infinitesimally small sphere of cross-sectional area *dA* containing the point under consideration divided by *dA* (W/m^2).

Germicidal Range – the range of UV wavelengths responsible for microbial inactivation in water (200 to 300 nm).

Gram-Negative Bacteria – have a thin layer of peptidoglycan surrounded by an outer lipid membrane containing lipopolysaccharides and phospholipids. Does not retain crystal violet dye because of their outer membrane and needs to be stained with a couterstain (safranin) during the Gram stain process.

Gram-Positive Bacteria – have a cell wall which consists of a very thick layer of peptidoglycan, which stains dark blue/violet when crystal violet is used during the Gram stain process.

Gram Stain – used to distinguish between the two types of cell wall in bacteria called Gram-positive and Gram-negative.

Hurdle Effect – each inhibitory factor contributes to a food's overall stability and safety.

Inactivation – in the context of UV disinfection, a process by which a microorganism is rendered unable to reproduce, thereby rendering it unable to infect a host.

Irradiance – the total radiant power incident from all upward directions on an infinitesimal element of surface of area dS containing the point under consideration divided by dS (W/m^2).

Low-pressure (LP) Lamp – a mercury-vapour lamp that operates at an internal pressure of 0.13 to 1.3 Pa (2 x 10-5 to 2 x 10-4 psi) and electrical input of 0.5 watts per centimetre (W/cm). This results in essentially monochromatic light output at 254 nm.

Low-pressure high-output (LPHO) Lamp – a low-pressure mercury-vapour lamp that operates under increased electrical input (1.5 to 10 W/cm), resulting in a higher UV intensity than low-pressure lamps. It also has essentially monochromatic light output at 254 nm.

Medium-pressure (MP) Lamp – a mercury vapour lamp that operates at an internal pressure of 1.3 and 13,000 Pa (2 to 200 psi) and electrical input of 50 to 150 W/cm. This results in a polychromatic (or broad spectrum) output of UV and visible light at multiple wavelengths, including wavelengths in the germicidal range.

Monochromatic – light output at only one wavelength, such as UV light generated by LP and LPHO lamps.

Peptidoglycan – made from polysaccharide chains cross-linked by peptides containing D-amino acids.

Photoreactivation (Photorepair) – a microbial repair process where enzymes are activated by light in the near UV and visible range, thereby repairing UV induced damage.

Polychromatic – light energy output at several wavelengths such as with MP lamps.

Radiometer – an instrument used to measure UV irradiance.

Turbidity – the presence of suspended material such as clay, silt, finely divided organic material, plankton, and other particulate matter (nephlometric turbidity unit, NTU).

UV Fluence Distribution – the probability distribution of delivered UV doses that microorganisms receive in a flow-through UV reactor; typically shown as a histogram.

UV Fluence-Response – the relationship indicating the level of inactivation of a microorganism as a function of UV dose.

UV Intensity – the power passing through a unit area perpendicular to the direction of propagation. UV intensity is used in this guidance manual to describe the magnitude of UV light measured by UV sensors in a reactor and with a radiometer in bench-scale UV experiments.

UV Light – light emitted with wavelengths from 200 to 400 nm.

UV Transmittance – the fraction of UV intensity transmitted through 1cm path length of the sample (measured in %).

Visible Light – Wavelengths of light in the visible range (380 – 720 nm).

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Appendix A: Effect of Turbidity

A.1 Introduction

A preliminary study was done to observe the effects of turbidity on UV inactivation of *E. faecalis*. As mentioned in the literature review, turbidity is a factor that affects UV performance. The particles that contribute to turbidity may decrease the UV transmittance of the water and affect dose delivery or protect target microorganisms from irradiation, either by shielding them or by having the target microbes embedded into the particle (Liu and Zhang, 2006).

There have been several studies on this topic and it was found that turbidity levels under 5 NTU have very little effect on inactivation. Many studies have spiked the effluent as to measure the efficiency thereby, making the results dependant on a wide variety of factors regarding the strain and physiology of the tested microorganism.

A.2 Materials and Methods

Enterococcus faecalis (ATCC 29212) were grown following protocol outlined in 5.2.2 of Materials and Methods in the main body of the thesis. The control samples were brought to an OD_{600nm} of 0.3 by adding 1 ml of a concentrated cell solution at a time until the appropriate final suspension of an OD_{600nm} of 0.3 was reached. The number of 1 ml additions was noted and the concentrated solution was brought with the controls to the WWTP along with empty sterile 500 ml plastic bottles.

Once at the WWTP, grab samples were collected after the biological aerated filter (BAF) process. Samples were then measured to 300 ml in 500 ml sterile bottles. The noted amount of concentrated cell suspension was then used to spike the BAF effluent and a concentration of approximately between 8×10^7 and 2×10^8 CFU/ml was

achieved. Two types of samples were compared, spiked BAF effluent and *E. faecalis* in DDH_2O.

UV protocol was as noted in 4.5 and the fluences used in inactivation *E. faecalis* were 6, 8, 20 and 25 mJ/cm^2. Turbidity was measured using a turbidimeter (Hach 2100P portable Turbidimeter, Loveland, Colorado).

Enumeration was the same as Section 4.3, with the exception that mENT Agar was used as a selecting media to count colonies. This was so that the background microorganisms did not alter the counts given by the plate counting method used.

Graphs were made using Sigmaplot 2000 where 95% confidence error bars were applied. One-way ANOVA tests were used to analyze the data using SigmaStat 2.03.

A.3 Results and Discussion

A spiked sample was found to have lower inactivation at the highest fluence, shown in Figure A-1. Table A-1 reports all average log inactivation results for the seeded BAF and control samples. The maximum reductions of *E. faecalis* strain 29212 at 25 mJ/cm^2 to be 4.23 log, whereas the seeded effluent had a >0.7 log lower average maximum after irradiation at the same fluence. There was a statistical difference at low dosages when *E. faecalis* was irradiated with UV light. Preliminary trials of *E. coli* samples showed that turbidity significantly decreased the inactivation capability of UV at all fluences tested (6, 8, 20 and 25 mJ/cm^2).

Tailing was visible for the cells inactivated in the seeded sample. After 20 mJ/cm2 or perhaps before there is a significant drop in inactivation compared with the control samples. Similar results were observed by Mamane-Gravetz and Linden (2005) when indigenous *B. subtilis* spores were seeded into treated drinking water.

Table A-1 Effect of turbidity on average UV log inactivation of *E. faecalis*

Fluence	Control 87.625 NTU		Seeded Effluent 88.825 NTU	
(mJ/cm2)	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
6	1.1633	0.1332	0.8667	0.0666
8	2.4300	0.0361	1.9467	0.0404
20	3.6867	0.1721	3.5200	0.2944
25	4.2367	0.2301	3.5100	0.2138

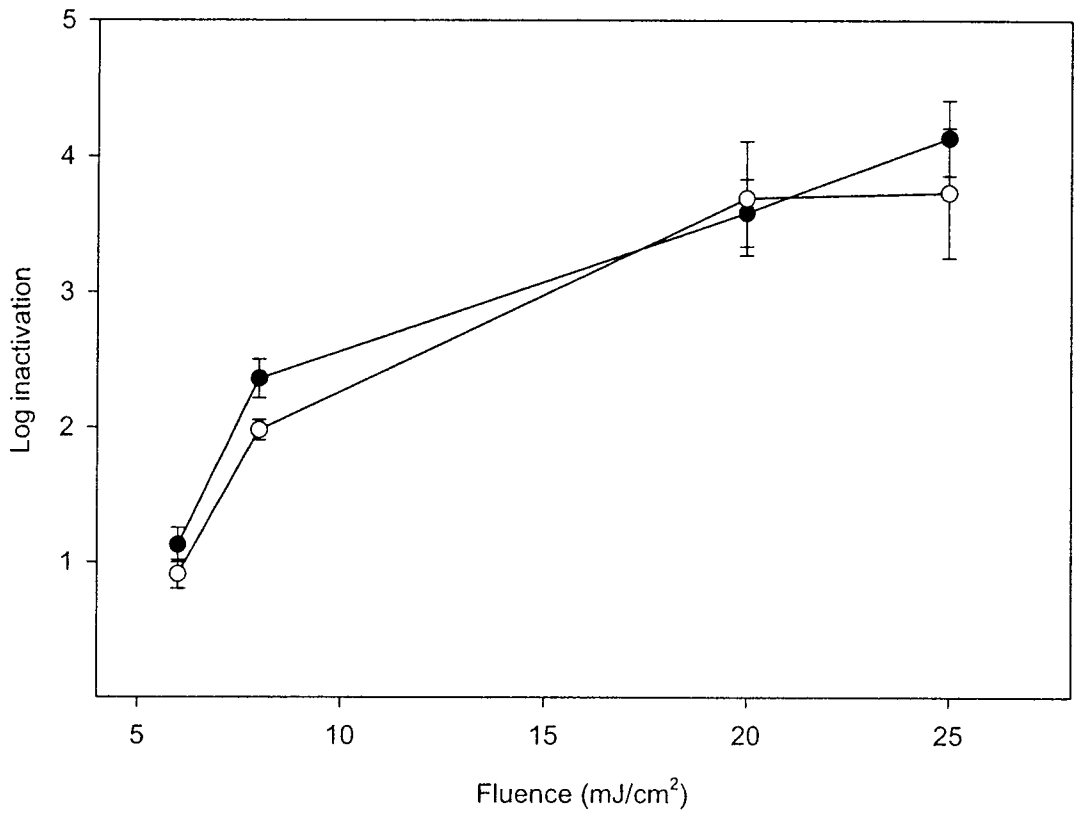

Figure A-1 Effect of turbidity on UV inactivation of *E. faecalis*
Control —●—, Seeded Effluent —○—

There was no statistical difference between the turbidity of the control sample and the spiked effluent. From table A-2 it can be noted that at fluence 25 mJ/cm^2 there was a significant decrease in log-inactivation in spiked effluent compared to the control.

Table A-2 One Way ANOVA comparing the control and the spiked effluent by fluences

Normality Test:	Passed	(P > 0.200)			
Equal Variance Test:	Passed	(P = 0.265)			
Source of Variation	DF	SS	MS	F	P
Between Groups	7	3.31E+01	4.72E+00	1.57E+02	**<0.001**
Residual	16	4.80E-01	3.00E-02		
Total	23	3.35E+01			
All Pairwise Multiple Comparison Procedures (Tukey Test):					
Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor:					
Control vs. Spiked at 25	7.27E-01	8	7.27E+00	0.002	**Yes**
Control vs. Spiked at 20	1.67E-01	8	1.67E+00	0.927	Do Not Test
Control vs. Spiked at 8	4.83E-01	8	4.83E+00	0.054	No
Control vs. Spiked at 6	2.97E-01	8	2.97E+00	0.455	No

Shielding of microorganisms would be caused by other bacteria rather than the particles in the effluent. Other studies have found that effluent under 5 NTU did not show to cause any inhibition of UV inactivation. The effluent particles could even lessen the effects of photoreactivation where applicable.

A.4 Conclusions and Recommendations

At high fluences, the turbidity of the BAF effluent did decrease the efficiency of UV inactivation. However, the results are not comparable with those in the literature because the initial turbidity of the control sample was well beyond those found in other studies.

The high turbidity was a result of the high concentration of the *E. faecalis*. Using a lower concentration of cells would bring the turbidity levels to a more realistic level as well as a level to which an *E. faecalis* outbreak would occur.

The concentration of cells was necessary in this study due to the unreliability of cell counts at lower cell densities. A solution could be to test natural waters with high fecal coliform levels in order to observe actual effects of turbidity.

Appendix B: Additional Data

B.1 Materials and Methods

Table B-1 Schedual for one trial at a temperature for *E. coli* and *E. faecalis*

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Grow bacteria overnight	Make cell suspensions **UV** control **Freeze** samples for cycles 1, 3 & 5	**Thaw** samples for cycles 1 & 3 **UV** cycle 1 **Freeze** sample for cycle 3	**Thaw** samples for cycles 3 & 5 **Freeze** samples for 3 & 5	**Thaw** samples for cycle 3 **UV** cycle 3	**Thaw** sample for cycle 5 **Freeze** sample for cycle 5
Thaw sample for cycle 5 **Freeze** sample for cycle 5		**Thaw** sample for cycle 5 **Freeze** sample for cycle 5		**Thaw** sample for cycle 5 **UV** cycle 5	

Bacillus subtilis endospore growth media

Schaeffer media (Chang *et al.*, 1985) (Shaeffer *et al.* 1965)

Media:

Nutrient broth (Difco)	8 g/L	
$MgSO_4 \cdot 7H_2O$	0.25 g/L	246.48 g/mol
KCl	1 g/L	74.56 g/mol
$MnCl_2 \cdot 4H_2O$	0.002 g/L	197.91 g/mol
Distilled water	1 litre	

Adjust pH to 7.0 and autoclave. Then sterile additions are made of:

$CaCl_2$ ($CaCl_2 \cdot 2H_2O$)	0.5 mM	147.02 g/mol	0.07351g/L
$FeSO_4$ ($FeSO_4 \cdot 7H_2O$)	1 µM	278.02 g/mol	0.00027802g/L

The corresponding solid media contains 17g/L agar (Difco) which is used for plating.

B.2 Freezing Inactivation

Table B-1 Log inactivation after freezing

	Frozen at -7°C					
	Cycle 1		Cycle 3		Cycle 5	
Species	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
E. coli	1.3000	0.1700	4.1900	0.4945	6.7800	0.0212
E. faecalis	0.1100	0.0983	0.4850	0.1330	1.4900	0.1486
B. subtilis spores	0.1200	0.0949	0.1300	0.0294	0.1850	0.0656
	Frozen at -15°C					
	Cycle 1		Cycle 3		Cycle 5	
	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
E. coli	0.6940	0.3255	3.5300	0.3075	3.2900	1.1918
E. faecalis	0.0375	0.0275	0.2180	0.2065	0.5400	0.1734
B. subtilis spores	0.1150	0.0988	0.1650	0.0719	0.1030	0.0772
	Frozen at -30°C					
	Cycle 1		Cycle 3		Cycle 5	
	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
E. coli	0.8970	0.0777	3.2200	0.3353	4.5200	0.2524
E. faecalis	0.0775	0.0591	0.1450	0.1370	0.3230	0.3251
B. subtilis spores	0.1780	0.2198	0.1280	0.0793	0.3080	0.2089

B.3 UV Inactivation

Table B-2 Log UV inactivation of *E. coli*

Fluence	Frozen at -7°C							
	Cycle 0		Cycle 1		Cycle 3		Cycle 5	
(mJ/cm2)	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
1.5	0.8800	0.0200	0.4867	0.0462	0.3600	0.0624		
2.5	1.6367	0.1193	0.9267	0.1343	0.8567	0.1242		
4.5	3.0033	0.0850	1.9867	0.1620	1.9733	0.0462		
9	4.4033	0.3753	3.5733	0.0702				
	Frozen at -15°C							
1.5	0.8267	0.1405	0.5733	0.0252	0.4200	0.0693	0.2733	0.1242
2.5	1.6433	0.1872	0.9367	0.0321	0.8500	0.0400	0.6300	0.1905
4.5	2.9167	0.1922	2.2567	0.1012	1.8833	0.0513	1.9567	0.0569
9	4.0467	0.3383	4.0733	0.0321	3.1967	0.4539	3.4500	0.1709
	Frozen at -30°C							
1.5	0.8233	0.1305	0.2267	0.2386	0.3367	0.1115	0.3767	0.0945
2.5	1.4600	0.0557	0.9467	0.2597	0.7367	0.1877	0.4833	0.3350
4.5	2.8233	0.1343	2.2300	0.5212	2.0367	0.4028	1.4250	0.1344
9	4.4133	0.3620	3.7233	0.2155				

Table B-3 Log UV inactivation of *E. faecalis*

Fluence	Frozen at -7°C							
	Cycle 0		Cycle 1		Cycle 3		Cycle 5	
(mJ/cm2)	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
6	2.2333	0.5348	1.6800	0.1803	1.5200	0.0794	1.3367	0.1861
8	3.2393	0.2238	2.7600	0.1389	2.6000	0.2307	2.4800	0.1744
20	4.4000	0.4854	4.1800	0.4034	4.1267	0.0751	4.0833	0.1793
25	5.1433	0.3485	4.8333	0.5992	4.7333	0.5450	4.8533	0.1662
	Frozen at -15°C							
6	1.9200	0.1311	1.7000	0.1670	1.3300	0.0200	1.2400	0.1217
8	2.8167	0.0907	2.5867	0.1150	2.2933	0.1343	2.2467	0.2650
20	3.9600	0.3831	3.9867	0.5518	3.8567	0.1484	3.7500	0.0700
25	4.5967	0.2386	4.5933	0.4554	4.5800	0.1970	4.3133	0.1097
	Frozen at -30°C							
6	1.5933	0.4605	1.4433	0.0153	1.2233	0.2479	1.1433	0.0709
8	2.5100	0.4952	2.6400	0.1212	2.2067	0.2499	2.1067	0.2984
20	4.3567	0.1966	4.3167	0.1779	4.7000	0.4267	4.4267	0.2354
25	5.9167	0.7726	5.6133	0.2479	5.2800	0.2571	5.4333	0.2554

Table B-4 Log UV inactivation of *B. subtilis* spores

Fluence	Frozen at -7°C							
	Cycle 0		Cycle 1		Cycle 3		Cycle 5	
(mJ/cm2)	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
20	0.2700	0.0917	0.3300	0.2095	0.4300	0.0800	0.3100	0.1418
40	1.2733	0.1537	1.2900	0.1277	1.3567	0.2183	1.2600	0.1277
80	2.6033	0.5125	2.3933	0.2003	2.6400	0.5311	2.6867	0.7353
120	3.7867	1.0538	3.0633	0.0850	3.5667	0.8584	3.4567	0.8896
	Frozen at -15°C							
20	0.3800	0.0173	0.3367	0.0208	0.4667	0.0611	0.3300	0.0265
40	1.4833	0.1332	1.4367	0.1620	1.3700	0.2261	1.1500	0.2265
80			2.3533	0.2572	2.2567	0.3790	2.2733	0.6150
120	2.1100		2.3000	0.4776	2.4100	0.1353	2.1700	0.1375
	Frozen at -30°C							
20	0.4533	0.0808	0.3967	0.0666	0.5467	0.2444	0.4067	0.1026
40	1.6167	0.1617	1.4667	0.1185	1.5267	0.2774	1.3100	0.1819
80	2.4100	0.2687	1.8833	0.2892	2.3033	0.4020	1.8867	0.0404
120	2.6500	0.3536	2.3033	0.6144	2.4333	0.3711	2.3867	0.0902

B.4 Photoreactivation

Table B-5 Photoreactivation of *E. coli* after UV inactivation and freezing at -15°C

Time	Fluence 2.5mJ/cm2			
	Cycle 0		Cycle 1	
(min)	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
30	0.1100	0.1229	-0.3500	0.0781
90	0.6767	0.0404	-0.8200	0.2163
180	0.9000	0.0200	-1.2533	0.2532
	Fluence 9mJ/cm2			
30	0.2433	0.0493	-0.2533	0.1877
90	0.4800	0.0173	-0.7200	0.2498
180	0.9533	0.1617	-1.1933	0.2845

Table B-6 Photoreactivation of *E. faecalis* after UV inactivation and freezing at -15°C

Time	Fluence 6mJ/cm2			
	Cycle 0		Cycle 1	
(min)	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
30	-0.0067	0.0208	0.0567	0.0306
90	-0.2533	0.1457	-0.0533	0.1168
180	-0.1000	0.2254	-0.0167	0.0493
	Fluence 20mJ/cm2			
30	0.2167	0.0503	0.0067	0.0404
90	0.1500	0.0200	0.0533	0.0404
180	0.2967	0.1102	-0.0267	0.1301

Table B-7 Photoreactivation of *B. subtilis* spores after UV inactivation and freezing at -15°C

Time	Fluence 40mJ/cm2			
	Cycle 0		Cycle 1	
(min)	Ave.(log N/No)	Std.dev.	Ave.(log N/No)	Std.dev.
30	0.0000	0.0693	-0.1333	0.1457
90	-0.0133	0.0058	-0.0333	0.0153
180	0.1033	0.1002	-0.0133	0.0577
	Fluence 120mJ/cm2			
30	0.1000	0.1732	-0.1200	0.0600
90	-0.2600	0.2623	-0.2000	0.0917
180	-0.1733	0.1484	0.0900	0.2858

Appendix C: Data Analysis

C.1 Freezing Inactivation

Table C-1 Two Way ANOVA comparing bacteria and freezing temperature

Normality Test:	Failed	(P = 0.008)			
Equal Variance Test:	Passed	(P = 0.247)			
Source of Variation	DF	SS	MS	F	P
Bacteria	2	5.72E+00	2.86E+00	1.01E+02	<0.001
Freezing temp	2	3.20E-01	1.60E-01	5.65E+00	0.009
Bacteria x Freezing temp	4	4.84E-01	1.21E-01	4.27E+00	0.008
Residual	27	7.65E-01	2.84E-02		
Total	35	7.27E+00	2.08E-01		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.05
Comparisons for factor: Bacteria					
EC vs. EF	8.88E-01	3	1.81E+01	<0.001	Yes
EC vs. BSS	8.25E-01	3	1.68E+01	<0.001	Yes
BSS vs. EF	6.25E-02	3	1.29E+00	0.639	No
Comparisons for factor: Freezing Temper					
-7 vs. -15	2.27E-01	3	4.75E+00	0.007	Yes
-7 vs. -3	1.25E-01	3	2.51E+00	0.197	No
-30 vs. -15	1.02E-01	3	2.07E+00	0.324	No
Comparisons for factor: Freezing Temper within E.coli					
-7 vs. -15	6.04E-01	3	7.56E+00	<0.001	Yes
-7vs. -30	4.01E-01	3	4.41E+00	0.012	Yes
-30 vs. -15	2.03E-01	3	2.33E+00	0.243	No
Comparisons for factor: Freezing Temper within E.faecalis					
-7 vs. -15	7.25E-02	3	8.61E-01	0.817	No
-7 vs. -30	3.25E-02	3	3.86E-01	0.96	Do Not Test
-30vs. -15	4.00E-02	3	4.75E-01	0.94	Do Not Test
Comparisons for factor: Freezing Temper within B.subtilis					
-30 vs. -15	6.25E-02	3	7.42E-01	0.86	No
-30 vs. -7	5.75E-02	3	6.83E-01	0.88	Do Not Test
-7 vs. -15	5.00E-03	3	5.94E-02	0.999	Do Not Test
Comparisons for factor: Bacteria within -7					
EC vs. EF	1.19E+00	3	1.41E+01	<0.001	Yes
EC vs. BSS	1.18E+00	3	1.40E+01	<0.001	Yes
BSS vs. EF	1.00E-02	3	1.19E-01	0.996	No
Comparisons for factor: Bacteria within -15					
EC vs. EF	6.57E-01	3	8.22E+00	<0.001	Yes
EC vs. BSS	5.79E-01	3	7.25E+00	<0.001	Yes
BSS vs. EF	7.75E-02	3	9.21E-01	0.794	No
Comparisons for factor: Bacteria within -30					
EC vs. EF	8.19E-01	3	9.01E+00	<0.001	Yes
EC vs. BSS	7.19E-01	3	7.91E+00	<0.001	Yes
BSS vs. EF	1.00E-01	3	1.19E+00	0.682	No

C.1.1 Effect of Freeze-Thaw Cycles on *E. coli*

Table C-2 Two Way ANOVA comparing *E. coli* freezing temperature and freeze-thaw cycles

Normality Test:	Failed	(P = 0.002)			
Equal Variance Test:	Passed	(P = 0.795)			
Source of Variation	DF	SS	MS	F	P
Freezing temp	2	1.44E+01	7.20E+00	2.35E+01	<0.001
F/T Cycle	2	8.27E+01	4.13E+01	1.35E+02	<0.001
Freezing temp x F/T Cycle	4	9.28E+00	2.32E+00	7.56E+00	<0.001
Residual	25	7.67E+00	3.07E-01		
Total	33	1.02E+02	3.09E+00		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Freezing temp					
-7 vs. -15	1.58E+00	3	9.58E+00	<0.001	Yes
-7 vs. -30	1.21E+00	3	6.55E+00	<0.001	Yes
-30 vs. -15	3.73E-01	3	2.26E+00	0.265	No
Comparisons for factor: F/T Cycle					
5 vs. 1	3.90E+00	3	2.22E+01	<0.001	Yes
5vs. 3	1.22E+00	3	6.93E+00	<0.001	Yes
3 vs. 1	2.68E+00	3	1.64E+01	<0.001	Yes
Comparisons for factor: F/T Cycle within -7					
5 vs. 1	5.48E+00	3	1.62E+01	<0.001	Yes
5 vs. 3	2.59E+00	3	7.63E+00	<0.001	Yes
3 vs. 1	2.89E+00	3	1.04E+01	<0.001	Yes
Comparisons for factor: F/T Cycle within -15					
3 vs. 1	2.83E+00	3	1.14E+01	<0.001	Yes
3 vs. 5	2.32E-01	3	9.37E-01	0.787	No
5 vs. 1	2.60E+00	3	1.05E+01	<0.001	Yes
Comparisons for factor: F/T Cycle within -30					
5 vs. 1	3.62E+00	3	1.13E+01	<0.001	Yes
5 vs. 3	1.30E+00	3	4.08E+00	0.021	Yes
3 vs. 1	2.32E+00	3	7.26E+00	<0.001	Yes
Comparisons for factor: Freezing temp within 1					
-7 vs. -15	6.04E-01	3	2.30E+00	0.254	No
-7 vs. -30	4.01E-01	3	1.34E+00	0.616	Do Not Test
-30 vs. -15	2.03E-01	3	7.09E-01	0.872	Do Not Test
Comparisons for factor: Freezing temp within 3					
-7 vs. -30	9.71E-01	3	3.25E+00	0.075	No
-7 vs. -15	6.62E-01	3	2.52E+00	0.197	Do Not Test
-15 vs. -30	3.09E-01	3	1.08E+00	0.728	Do Not Test

C.1.2 Effect of Freeze-Thaw Cycles on *E. faecalis*

Table C-3 Two Way ANOVA comparing *E. faecalis* freezing temperature and freeze-thaw cycles

Normality Test:	Passed	(P > 0.200)			
Equal Variance Test:	Failed	(P = 0.001)			
Source of Variation	DF	SS	MS	F	P
Freezing temp	2	1.82E+00	9.08E-01	3.26E+01	<0.001
F/T cycle	2	3.18E+00	1.59E+00	5.71E+01	<0.001
Freezing temp x F/T cycle	4	1.52E+00	3.80E-01	1.37E+01	<0.001
Residual	27	7.53E-01	2.79E-02		
Total	35	7.27E+00	2.08E-01		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Temp1					
-7 vs. -30	5.13E-01	3	1.06E+01	<0.001	Yes
-7 vs. -15	4.29E-01	3	8.91E+00	<0.001	Yes
-15 vs. -30	8.33E-02	3	1.73E+00	0.451	No
Comparisons for factor: F/T2					
5 vs. 1	7.08E-01	3	1.47E+01	<0.001	Yes
5 vs. 3	5.01E-01	3	1.04E+01	<0.001	Yes
3 vs. 1	2.08E-01	3	4.31E+00	0.014	Yes
Comparisons for factor: F/T2 within -7					
5 vs. 1	1.38E+00	3	1.65E+01	<0.001	Yes
5 vs. 3	1.00E+00	3	1.20E+01	<0.001	Yes
3 vs. 1	3.75E-01	3	4.49E+00	0.01	Yes
Comparisons for factor: F/T2 within -15					
5 vs. 1	5.03E-01	3	6.02E+00	<0.001	Yes
5 vs. 3	3.23E-01	3	3.86E+00	0.029	Yes
3 vs. 1	1.80E-01	3	2.16E+00	0.296	No
Comparisons for factor: F/T2 within -30					
5 vs. 1	2.45E-01	3	2.94E+00	0.114	No
5 vs. 3	1.78E-01	3	2.13E+00	0.305	Do Not Test
3 vs. 1	6.75E-02	3	8.09E-01	0.836	Do Not Test
Comparisons for factor: Temp1 within 1					
-7 vs. -15	7.25E-02	3	8.69E-01	0.814	No
-7 vs. -30	3.25E-02	3	3.89E-01	0.959	Do Not Test
-30 vs. -15	4.00E-02	3	4.79E-01	0.939	Do Not Test
Comparisons for factor: Temp1 within 3					
-7 vs. -30	3.40E-01	3	4.07E+00	0.02	Yes
-7 vs. -15	2.68E-01	3	3.21E+00	0.078	No
-15 vs. -30	7.25E-02	3	8.69E-01	0.814	No
Comparisons for factor: Temp1 within 5Comparison					
-7 vs. -3	1.17E+00	3	1.40E+01	<0.001	Yes
-7 vs. -15	9.48E-01	3	1.14E+01	<0.001	Yes
-15 vs. -30	2.18E-01	3	2.61E+00	0.175	No

C.1.3 Effect of Freeze-Thaw Cycles on *B. subtilis* Spores

Table C-4 Two Way ANOVA comparing *B. subtilis* spores freezing temperature and F/T cycles

Normality Test:	Passed	(P = 0.100)			
Equal Variance Test:	Passed	(P = 0.246)			
Source of Variation	DF	SS	MS	F	P
Freezing temp	2	3.87E-02	1.94E-02	1.31E+00	0.287
F/T cycle	2	2.81E-02	1.40E-02	9.48E-01	0.4
Freezing temp x F/T cycle	4	5.95E-02	1.49E-02	1.01E+00	0.422
Residual	27	4.00E-01	1.48E-02		
Total	35	5.26E-01	1.50E-02		

C.1.4 Comparing the Effect of Freeze-Thaw Cycles on All Three Bacteria

Table C-5 Three Way ANOVA comparing bacteria freezing temperature and freeze-thaw cycles

Normality Test:	Failed	(P = <0.001)			
Equal Variance Test:	Failed	(P = 0.006)			
Source of Variation	DF	SS	MS	F	P
Bacteria	2	5.64E+01	2.82E+01	6.30E+02	<0.001
Freezing temp	2	1.24E+00	6.19E-01	1.38E+01	<0.001
F/T cycle	1	1.22E+01	1.22E+01	2.73E+02	<0.001
Bacteria x Freezing temp	4	1.28E+00	3.20E-01	7.14E+00	<0.001
Bacteria x F/T	2	2.17E+01	1.08E+01	2.42E+02	<0.001
Freezing temp x F/T	2	3.33E-01	1.67E-01	3.72E+00	0.034
Bacteria x Freezing temp x F/T cycle	4	1.72E-01	4.30E-02	9.60E-01	0.441
Residual	36	1.61E+00	4.47E-02		
Total	53	9.49E+01	1.79E+00		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor:					
Bacteria					
EC vs. BSS	2.17E+00	3	4.36E+01	<0.001	Yes
EC vs. EF	2.16E+00	3	4.34E+01	<0.001	Yes
BSS vs. EF	1.22E-02	3	2.45E-01	0.984	No
Comparisons for factor: Freezing temp					
-7vs. -30	3.22E-01	3	6.45E+00	<0.001	Yes
-7 vs. -15	3.21E-01	3	6.43E+00	<0.001	Yes
-15 vs. -30	1.11E-03	3	2.23E-02	1	No
Comparisons for factor: F/T					
3 vs. 1	9.52E-01	2	2.34E+01	<0.001	Yes
Comparisons for factor: Freezing temp within *E. coli*					
-7 vs. -30	2.69E+00	3	3.12E+01	<0.001	Yes
-7 vs. -15	2.53E+00	3	2.93E+01	<0.001	Yes
-15 vs. -30	1.62E-01	3	1.87E+00	0.392	No
Comparisons for factor: Freezing temp within *E. faecalis*					
-7 vs. -15	1.97E+00	3	2.29E+01	<0.001	Yes
-7 vs. -30	1.95E+00	3	2.25E+01	<0.001	Yes
-30 vs. -15	2.67E-02	3	3.09E-01	0.974	No
Comparisons for factor: Freezing temp within *B subtilis* spores					

-7 vs. -15	1.98E+00	3	2.30E+01	<0.001	Yes
-7 vs. -30	1.89E+00	3	2.18E+01	<0.001	Yes
-30 vs. -15	9.83E-02	3	1.14E+00	0.702	No
Comparisons for factor: Bacteria within -7					
EC vs. BSS	2.69E+00	3	3.12E+01	<0.001	Yes
EC vs. EF	2.53E+00	3	2.93E+01	<0.001	Yes
BSS vs. EF	1.62E-01	3	1.87E+00	0.392	No
Comparisons for factor: Bacteria within -15					
EC vs. EF	1.97E+00	3	2.29E+01	<0.001	Yes
EC vs. BSS	1.95E+00	3	2.25E+01	<0.001	Yes
BSS vs. EF	2.67E-02	3	3.09E-01	0.974	No
Comparisons for factor: Bacteria within -30					
EC vs. EF	1.98E+00	3	2.30E+01	<0.001	Yes
EC vs. BSS	1.89E+00	3	2.18E+01	<0.001	Yes
BSS vs. EF	9.83E-02	3	1.14E+00	0.702	No
Comparisons for factor: F/T within *E. coli*					
1 vs. 3	8.68E-01	2	1.23E+01	<0.001	Yes
Comparisons for factor: F/T within *E. faecalis*					
1vs. 3	3.46E+00	2	4.90E+01	<0.001	Yes
Comparisons for factor: Bacteria within 1					
EC vs. EF	8.68E-01	3	1.23E+01	<0.001	Yes
EC vs. BSS	7.86E-01	3	1.11E+01	<0.001	Yes
BSS vs. EF	8.22E-02	3	1.17E+00	0.69	No
Comparisons for factor: Bacteria within 3					
EC vs. EF	3.53E+00	3	5.00E+01	<0.001	Yes
BSS vs. EF	3.46E+00	3	4.90E+01	<0.001	Yes
EC vs. BSS	7.00E-02	3	9.93E-01	0.764	No
Comparisons for factor: F/T within -7					
1 vs. 3	2.81E-01	2	3.99E+00	0.008	Yes
Comparisons for factor: F/T within -15					
1 vs. 3	3.60E-01	2	5.11E+00	0.001	Yes

C.2 UV Inactivation

C.2.1 Effect of Freezing on UV inactivation of *E. coli*

Table C-6 Two Way ANOVA comparing *E.coli* UV inactivation fluence and freezing temperature

Normality Test:	Failed	(P = 0.003)			
Equal Variance Test:	Passed	(P = 0.399)			
Source of Variation	DF	SS	MS	F	P
Freezing temp	3	4.15E+00	1.38E+00	3.31E+01	<0.001
Fluences	3	8.19E+01	2.73E+01	6.53E+02	<0.001
Freezing temp x Fluences	9	5.87E-01	6.52E-02	1.56E+00	0.171
Residual	32	1.34E+00	4.18E-02		
Total	47	8.80E+01	1.87E+00		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Freezing temp					
Control vs. -7	7.38E-01	4	1.25E+01	<0.001	Yes
Control vs. -30	6.99E-01	4	1.18E+01	<0.001	Yes
Control vs. -15	5.21E-01	4	8.82E+00	<0.001	Yes
-15 vs. -7	2.17E-01	4	3.67E+00	0.065	No
-15 vs. -30	1.78E-01	4	3.02E+00	0.164	Do Not Test
-30 vs. -7	3.83E-02	4	6.49E-01	0.967	Do Not Test
Comparisons for factor: Fluence					
9 vs. 1.5	3.40E+00	4	5.76E+01	<0.001	Yes
9 vs. 2.5	2.83E+00	4	4.80E+01	<0.001	Yes
9 vs. 4.5	1.57E+00	4	2.67E+01	<0.001	Yes
4.5 vs. 1.5	1.83E+00	4	3.10E+01	<0.001	Yes
4.5 vs. 2.5	1.26E+00	4	2.13E+01	<0.001	Yes
2.5 vs. 1.5	5.70E-01	4	9.65E+00	<0.001	Yes

C.2.2 Effect of Freezing on UV inactivation of *E. faecalis*

Table C-7 Two Way ANOVA comparing *E.faecalis* UV inactivation fluence and freezing temperature

Normality Test:	Passed	(P > 0.200)			
Equal Variance Test:	Passed	(P = 0.606)			
Source of Variation	DF	SS	MS	F	P
Freezing temp	3	1.88E+00	6.28E-01	5.21E+00	0.005
Fluence	3	7.68E+01	2.56E+01	2.12E+02	<0.001
Freezing temp x Fluence	9	1.96E+00	2.17E-01	1.80E+00	0.106
Residual	32	3.86E+00	1.21E-01		
Total	47	8.45E+01	1.80E+00		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Freezing temp 6					
Control vs. -15	5.37E-01	4	5.36E+00	0.003	Yes
Control vs. -7	3.91E-01	4	3.90E+00	0.045	Yes
Control vs. -30	2.51E-01	4	2.50E+00	0.307	No
-30 vs. -15	2.87E-01	4	2.86E+00	0.201	No
-30 vs. -7	1.40E-01	4	1.40E+00	0.757	Do Not Test
-7 vs. -15	1.47E-01	4	1.46E+00	0.73	Do Not Test
Comparisons for factor: Fluence 64					
25 vs. 6	3.28E+00	4	3.28E+01	<0.001	Yes
25 vs. 8	2.24E+00	4	2.24E+01	<0.001	Yes
25 vs. 2	8.25E-01	4	8.23E+00	<0.001	Yes
20 vs. 6	2.46E+00	4	2.45E+01	<0.001	Yes
20 vs. 8	1.41E+00	4	1.41E+01	<0.001	Yes
8 vs. 6	1.04E+00	4	1.04E+01	<0.001	Yes

C.2.3 Effect of Freezing on UV inactivation of *B. subtilis* Spores

Table C-8 Two Way ANOVA comparing *B. subtilis* spores UV inactivation fluence and freezing temperature

Normality Test:	Failed	(P = 0.002)			
Equal Variance Test:	Passed	(P = 0.669)			
Source of Variation	DF	SS	MS	F	P
Freeze temp	3	1.53E+00	5.11E-01	3.53E+00	0.026
Fluence	3	4.44E+01	1.48E+01	1.02E+02	<0.001
Freeze temp x Fluence	9	3.98E+00	4.42E-01	3.06E+00	0.009
Residual	32	4.63E+00	1.45E-01		
Total	47	5.46E+01	1.16E+00		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Freezing temp					
Control vs. -30.000	4.71E-01	4	4.29E+00	0.024	Yes
Control vs. -15.000	3.77E-01	4	3.43E+00	0.092	No
Control vs. -7.000	2.14E-01	4	1.95E+00	0.521	Do Not Test
-7.000 vs. -30.000	2.57E-01	4	2.34E+00	0.364	No
-7.000 vs. -15.000	1.63E-01	4	1.48E+00	0.724	Do Not Test
-15.000 vs. -30.000	9.42E-02	4	8.58E-01	0.929	Do Not Test
Comparisons for factor: Fluences					
120.000 vs. 20.000	2.53E+00	4	2.31E+01	<0.001	Yes
120.000 vs. 40.000	1.50E+00	4	1.36E+01	<0.001	Yes
120.000 vs. 80.000	5.55E-01	4	5.06E+00	0.006	Yes
80.000 vs. 20.000	1.98E+00	4	1.80E+01	<0.001	Yes
80.000 vs. 40.000	9.42E-01	4	8.58E+00	<0.001	Yes
40.000 vs. 20.000	1.03E+00	4	9.41E+00	<0.001	Yes
Comparisons for factor: Fluences within Control					
120.000 vs. 20.000	3.52E+00	4	1.60E+01	<0.001	Yes
120.000 vs. 40.000	2.51E+00	4	1.15E+01	<0.001	Yes
120.000 vs. 80.000	1.18E+00	4	5.39E+00	0.003	Yes
80.000 vs. 20.000	2.33E+00	4	1.06E+01	<0.001	Yes
80.000 vs. 40.000	1.33E+00	4	6.06E+00	<0.001	Yes
40.000 vs. 20.000	1.00E+00	4	4.57E+00	0.014	Yes
Comparisons for factor: Fluences within -7					
120.000 vs. 20.000	2.73E+00	4	1.25E+01	<0.001	Yes
120.000 vs. 40.000	1.77E+00	4	8.08E+00	<0.001	Yes
120.000 vs. 80.000	6.70E-01	4	3.05E+00	0.157	No
80.000 vs. 20.000	2.06E+00	4	9.40E+00	<0.001	Yes
80.000 vs. 40.000	1.10E+00	4	5.03E+00	0.006	Yes
40.000 vs. 20.000	9.60E-01	4	4.37E+00	0.02	Yes
Comparisons for factor: Fluences within -15					
80.000 vs. 20.000	2.02E+00	4	9.19E+00	<0.001	Yes
80.000 vs. 40.000	9.17E-01	4	4.18E+00	0.029	Yes
80.000 vs. 120.000	5.33E-02	4	2.43E-01	0.998	No
120.000 vs. 20.000	1.96E+00	4	8.94E+00	<0.001	Yes
120.000 vs. 40.000	8.63E-01	4	3.93E+00	0.043	Yes
40.000 vs. 20.000	1.10E+00	4	5.01E+00	0.007	Yes

Comparisons for factor: Fluences within -30					
120.000 vs. 20.000	1.91E+00	4	8.69E+00	<0.001	Yes
120.000 vs. 40.000	8.37E-01	4	3.81E+00	0.052	No
120.000 vs. 80.000	4.20E-01	4	1.91E+00	0.537	Do Not Test
80.000 vs. 20.000	1.49E+00	4	6.77E+00	<0.001	Yes
80.000 vs. 40.000	4.17E-01	4	1.90E+00	0.544	Do Not Test
40.000 vs. 20.000	1.07E+00	4	4.87E+00	0.008	Yes
Comparisons for factor: Freezing temp within 20					
-30.000 vs. Control	1.27E-01	4	5.77E-01	0.977	No
-30.000 vs. -7.000	6.67E-02	4	3.04E-01	0.997	Do Not Test
-30.000 vs. -15.000	6.00E-02	4	2.73E-01	0.997	Do Not Test
-15.000 vs. Control	6.67E-02	4	3.04E-01	0.997	Do Not Test
-15.000 vs. -7.000	6.67E-03	4	3.04E-02	1	Do Not Test
-7.000 vs. Control	6.00E-02	4	2.73E-01	0.997	Do Not Test
Comparisons for factor: Freezing temp within 40					
-30.000 vs. Control	1.93E-01	4	8.81E-01	0.924	No
-30.000 vs. -7.000	1.77E-01	4	8.05E-01	0.941	Do Not Test
-30.000 vs. -15.000	3.00E-02	4	1.37E-01	1	Do Not Test
-15.000 vs. Control	1.63E-01	4	7.44E-01	0.952	Do Not Test
-15.000 vs. -7.000	1.47E-01	4	6.68E-01	0.965	Do Not Test
-7.000 vs. Control	1.67E-02	4	7.59E-02	1	Do Not Test
Comparisons for factor: Freezing temp within 80					
Control vs. -30.000	7.20E-01	4	3.28E+00	0.115	No
Control vs. -15.000	2.50E-01	4	1.14E+00	0.852	Do Not Test
Control vs. -7.000	2.10E-01	4	9.57E-01	0.905	Do Not Test
-7.000 vs. -30.000	5.10E-01	4	2.32E+00	0.37	Do Not Test
-7.000 vs. -15.000	4.00E-02	4	1.82E-01	0.999	Do Not Test
-15.000 vs. -30.000	4.70E-01	4	2.14E+00	0.441	Do Not Test
Comparisons for factor: Freezing temp within 120					
Control vs. -15.000	1.49E+00	4	6.77E+00	<0.001	Yes
Control vs. -30.000	1.48E+00	4	6.76E+00	<0.001	Yes
Control vs. -7.000	7.23E-01	4	3.30E+00	0.112	No
-7.000 vs. -15.000	7.63E-01	4	3.48E+00	0.086	No
-7.000 vs. -30.000	7.60E-01	4	3.46E+00	0.088	Do Not Test
-30.000 vs. -15.000	3.33E-03	4	1.52E-02	1	Do Not Test

C.2.4 Comparing the Effect of Freezing on UV inactivation of All Three Bacteria

Table C-9 Two Way ANOVA comparing bacteria vs. freezing temperature and its effect on UV inactivation at approx. 3-log

Normality Test:	Passed	(P = 0.010)			
Equal Variance Test:	Passed	(P = 0.685)			
Source of Variation	DF	SS	MS	F	P
Bacteria	2	1.77E+00	8.86E-01	1.23E+01	<0.001
Freezing temp	3	2.59E+00	8.63E-01	1.20E+01	<0.001
Bacteria x Freezing temp	6	7.79E-01	1.30E-01	1.80E+00	0.141
Residual	24	1.73E+00	7.21E-02		
Total	35	6.87E+00	1.96E-01		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Bacteria					
EF vs. BSS	4.98E-01	3	6.43E+00	<0.001	Yes
EF vs. EC	4.37E-01	3	5.64E+00	0.002	Yes
EC vs. BSS	6.08E-02	3	7.85E-01	0.845	No
Comparisons for factor: Freezing Temp					
Control vs. -30.000	6.98E-01	4	7.79E+00	<0.001	Yes
Control vs. -7.000	5.69E-01	4	6.35E+00	<0.001	Yes
Control vs. -15.000	5.50E-01	4	6.14E+00	0.001	Yes
-15.000 vs. -30.000	1.48E-01	4	1.65E+00	0.653	No
-15.000 vs. -7.000	1.89E-02	4	2.11E-01	0.999	Do Not Test
-7.000 vs. -30.000	1.29E-01	4	1.44E+00	0.741	Do Not Test

C.2.5 Effect of Freeze-Thaw Cycles on UV inactivation of *E. coli*

Table C-10 Three Way ANOVA comparing *E. coli* freezing temperature and F/T and fluence

Normality Test:	Failed	(P = <0.001)			
Equal Variance Test:	Passed	(P = 0.674)			
Source of Variation	DF	SS	MS	F	P
Freezing temp	2	4.67E-02	2.34E-02	6.05E-01	0.552
F/T cycle	1	2.08E-01	2.08E-01	5.39E+00	0.026
Fluences	2	2.63E+01	1.32E+01	3.41E+02	<0.001
Freezing temp x F/T	2	4.53E-02	2.27E-02	5.87E-01	0.561
Freezing temp x Fluences	4	1.78E-01	4.46E-02	1.16E+00	0.347
F/T x Fluences	2	4.21E-02	2.10E-02	5.45E-01	0.585
Freezing temp x F/T x Fluence	4	1.32E-01	3.31E-02	8.58E-01	0.498
Residual	36	1.39E+00	3.86E-02		
Total	53	2.84E+01	5.35E-01		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: F/T					
1.000 vs. 3.000	1.24E-01	2	3.28E+00	0.026	Yes
Comparisons for factor: Fluence					
4.500 vs. 1.500	1.66E+00	3	3.59E+01	<0.001	Yes
4.500 vs. 2.500	1.19E+00	3	2.56E+01	<0.001	Yes
2.500 vs. 1.500	4.75E-01	3	1.03E+01	<0.001	Yes

Table C-11 Two Way ANOVA comparing *E. coli* F/T and fluence

Normality Test:	Passed	(P > 0.200)			
Equal Variance Test:	Passed	(P = 0.065)			
Source of Variation	DF	SS	MS	F	P
F/T cylce	2	1.48E+00	7.39E-01	1.55E+01	<0.001
Fluences	3	9.00E+01	3.00E+01	6.31E+02	<0.001
F/T cycle x Fluences	6	6.55E-01	1.09E-01	2.30E+00	0.044
Residual	74	3.52E+00	4.76E-02		
Total	85	1.19E+02	1.40E+00		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: F/T cycle					
1.000 vs. 5.000	3.09E-01	3	7.01E+00	<0.001	Yes
1.000 vs. 3.000	2.41E-01	3	5.94E+00	<0.001	Yes
3.000 vs. 5.000	6.80E-02	3	1.43E+00	0.575	No
Comparisons for factor: Fluences					
9.000 vs. 1.500	3.10E+00	4	5.59E+01	<0.001	Yes
9.000 vs. 2.500	2.71E+00	4	4.88E+01	<0.001	Yes
9.000 vs. 4.500	1.52E+00	4	2.71E+01	<0.001	Yes
4.500 vs. 1.500	1.58E+00	4	3.41E+01	<0.001	Yes
4.500 vs. 2.500	1.19E+00	4	2.56E+01	<0.001	Yes
2.500 vs. 1.500	3.94E-01	4	8.69E+00	<0.001	Yes
Comparisons for factor: Fluences within 1					
9.000 vs. 1.500	3.36E+00	4	4.62E+01	<0.001	Yes
9.000 vs. 2.500	2.85E+00	4	3.93E+01	<0.001	Yes
9.000 vs. 4.500	1.63E+00	4	2.25E+01	<0.001	Yes
4.500 vs. 1.500	1.73E+00	4	2.38E+01	<0.001	Yes
4.500 vs. 2.500	1.22E+00	4	1.68E+01	<0.001	Yes
2.500 vs. 1.500	5.08E-01	4	6.98E+00	<0.001	Yes
Comparisons for factor: Fluences within 3					
9.000 vs. 1.500	2.82E+00	4	2.75E+01	<0.001	Yes
9.000 vs. 2.500	2.38E+00	4	2.32E+01	<0.001	Yes
9.000 vs. 4.500	1.23E+00	4	1.20E+01	<0.001	Yes
4.500 vs. 1.500	1.59E+00	4	2.19E+01	<0.001	Yes
4.500 vs. 2.500	1.15E+00	4	1.58E+01	<0.001	Yes
2.500 vs. 1.500	4.42E-01	4	6.08E+00	<0.001	Yes
Comparisons for factor: Fluences within 5					
9.000 vs. 1.500	3.13E+00	4	2.87E+01	<0.001	Yes
9.000 vs. 2.500	2.89E+00	4	2.65E+01	<0.001	Yes
9.000 vs. 4.500	1.71E+00	4	1.52E+01	<0.001	Yes
4.500 vs. 1.500	1.42E+00	4	1.52E+01	<0.001	Yes
4.500 vs. 2.500	1.19E+00	4	1.27E+01	<0.001	Yes
2.500 vs. 1.500	2.32E-01	4	2.60E+00	0.263	No
Comparisons for factor: F/T cycle within 1.5					
1.000 vs. 5.000	1.04E-01	3	1.28E+00	0.64	No
1.000 vs. 3.000	5.67E-02	3	7.79E-01	0.846	Do Not Test
3.000 vs. 5.000	4.72E-02	3	5.81E-01	0.911	Do Not Test
Comparisons for factor: F/T cycle within 2.5					
1.000 vs. 5.000	3.80E-01	3	4.68E+00	0.004	Yes
1.000 vs. 3.000	1.22E-01	3	1.68E+00	0.464	No
3.000 vs. 5.000	2.58E-01	3	3.17E+00	0.071	No
Comparisons for factor: F/T cycle within 4.5					
1.000 vs. 5.000	4.14E-01	3	4.81E+00	0.003	Yes
1.000 vs. 3.000	1.93E-01	3	2.66E+00	0.152	No
3.000 vs. 5.000	2.20E-01	3	2.56E+00	0.173	No
Comparisons for factor: F/T cycle within 9					
1.000 vs. 3.000	5.93E-01	3	5.77E+00	<0.001	Yes
1.000 vs. 5.000	3.40E-01	3	3.31E+00	0.057	No
5.000 vs. 3.000	2.53E-01	3	2.01E+00	0.335	No

C.2.6 Effect of Freeze-Thaw Cycles on UV inactivation of *E. faecalis*

Table C-12 Three Way ANOVA for *E. faecalis* freezing temperature and F/T and fluence

Normality Test:	Failed	(P = <0.001)			
Equal Variance Test:	Passed	(P = 0.746)			
Source of Variation	DF	SS	MS	F	P
Temp	2	2.14E+00	1.07E+00	1.54E+01	<0.001
F/T cycle	2	1.10E+00	5.48E-01	7.92E+00	<0.001
Fluence	3	2.07E+02	6.91E+01	9.99E+02	<0.001
Temp x F/T cycle	4	7.43E-02	1.86E-02	2.69E-01	0.897
Temp x Fluence	6	4.49E+00	7.48E-01	1.08E+01	<0.001
F/T cycle x Fluence	6	4.89E-01	8.15E-02	1.18E+00	0.327
Temp x F/T cycle x Fluence	12	4.98E-01	4.15E-02	6.00E-01	0.836
Residual	72	4.98E+00	6.92E-02		
Total	107	2.21E+02	2.07E+00		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Freezing Temper					
-30.000 vs. -15.000	3.38E-01	3	7.71E+00	<0.001	Yes
-30.000 vs. -7.000	1.12E-01	3	2.56E+00	0.173	No
-7.000 vs. -15.000	2.26E-01	3	5.15E+00	0.002	Yes
Comparisons for factor: F/T cycle					
1.000 vs. 5.000	2.43E-01	3	5.55E+00	<0.001	Yes
1.000 vs. 3.000	1.57E-01	3	3.58E+00	0.036	Yes
3.000 vs. 5.000	8.64E-02	3	1.97E+00	0.35	No
Comparisons for factor: Fluences					
25.000 vs. 6.000	3.51E+00	4	6.94E+01	<0.001	Yes
25.000 vs. 8.000	2.48E+00	4	4.90E+01	<0.001	Yes
25.000 vs. 20.000	7.56E-01	4	1.49E+01	<0.001	Yes
20.000 vs. 6.000	2.76E+00	4	5.45E+01	<0.001	Yes
20.000 vs. 8.000	1.72E+00	4	3.40E+01	<0.001	Yes
8.000 vs. 6.000	1.03E+00	4	2.04E+01	<0.001	Yes
Comparisons for factor: F/T cycle within -7					
5.000 vs. 3.000	2.87E-01	3	3.78E+00	0.025	Yes
5.000 vs. 1.000	1.40E-01	3	1.84E+00	0.398	No
1.000 vs. 3.000	1.47E-01	3	1.93E+00	0.364	No
Comparisons for factor: F/T cycle within -15					
5.000 vs. 3.000	3.38E-01	3	4.45E+00	0.007	Yes
5.000 vs. 1.000	1.08E-01	3	1.42E+00	0.579	No
1.000 vs. 3.000	2.30E-01	3	3.03E+00	0.089	No
Comparisons for factor: F/T cycle within -30					
5.000 vs. 3.000	3.90E-01	3	5.14E+00	0.002	Yes
5.000 vs. 1.000	8.92E-02	3	1.18E+00	0.685	No
1.000 vs. 3.000	3.01E-01	3	3.96E+00	0.018	Yes
Comparisons for factor: Freezing temp within 1					
-30.000 vs. -15.000	2.87E-01	3	3.78E+00	0.025	Yes
-30.000 vs. -7.000	1.40E-01	3	1.84E+00	0.398	No
-7.000 vs. -15.000	1.47E-01	3	1.93E+00	0.364	No
Comparisons for factor: Freezing temp within 3					

-30.000 vs. -15.000	3.38E-01	3	4.45E+00	0.007	Yes
-30.000 vs. -7.000	1.08E-01	3	1.42E+00	0.579	No
-7.000 vs. -15.000	2.30E-01	3	3.03E+00	0.089	No
Comparisons for factor: Freezing temp within 5					
-30.000 vs. -15.000	3.90E-01	3	5.14E+00	0.002	Yes
-30.000 vs. -7.000	8.92E-02	3	1.18E+00	0.685	No
-7.000 vs. -15.000	3.01E-01	3	3.96E+00	0.018	Yes
Comparisons for factor: Fluences within -7					
25.000 vs. 20.000	1.34E+00	4	1.53E+01	<0.001	Yes
25.000 vs. 8.000	1.19E+00	4	1.36E+01	<0.001	Yes
25.000 vs. 6.000	1.10E+00	4	1.26E+01	<0.001	Yes
6.000 vs. 20.000	2.42E-01	4	2.76E+00	0.215	No
6.000 vs. 8.000	8.89E-02	4	1.01E+00	0.89	Do Not Test
8.000 vs. 20.000	1.53E-01	4	1.75E+00	0.606	Do Not Test
Comparisons for factor: Fluences within -15					
25.000 vs. 20.000	1.81E+00	4	2.07E+01	<0.001	Yes
25.000 vs. 8.000	1.75E+00	4	2.00E+01	<0.001	Yes
25.000 vs. 6.000	1.52E+00	4	1.73E+01	<0.001	Yes
6.000 vs. 20.000	2.96E-01	4	3.37E+00	0.089	No
6.000 vs. 8.000	2.38E-01	4	2.71E+00	0.23	Do Not Test
8.000 vs. 20.000	5.78E-02	4	6.59E-01	0.966	Do Not Test
Comparisons for factor: Fluences within -30					
25.000 vs. 8.000	9.42E-01	4	1.08E+01	<0.001	Yes
25.000 vs. 6.000	6.77E-01	4	7.72E+00	<0.001	Yes
25.000 vs. 20.000	3.26E-01	4	3.71E+00	0.051	No
20.000 vs. 8.000	6.17E-01	4	7.04E+00	<0.001	Yes
20.000 vs. 6.000	3.51E-01	4	4.01E+00	0.03	Yes
6.000 vs. 8.000	2.66E-01	4	3.03E+00	0.15	No
Comparisons for factor: Freezing temp within 8					
-30.000 vs. -15.000	1.81E+00	3	2.07E+01	<0.001	Yes
-30.000 vs. -7.000	1.75E+00	3	2.00E+01	<0.001	Yes
-7.000 vs. -15.000	5.78E-02	3	6.59E-01	0.887	No
Comparisons for factor: Freezing temp within 20					
-15.000 vs. -7.000	3.26E-01	3	3.71E+00	0.028	Yes
-15.000 vs. -30.000	3.11E-01	3	3.55E+00	0.038	Yes
-30.000 vs. -7.000	1.44E-02	3	1.65E-01	0.993	No
Comparisons for factor: Freezing temp within 25					
-7.000 vs. -30.000	3.68E-01	3	4.20E+00	0.011	Yes
-7.000 vs. -15.000	2.50E-01	3	2.85E+00	0.116	No
-15.000 vs. -30.000	1.18E-01	3	1.34E+00	0.611	No
Comparisons for factor: Fluences within 1					
25.000 vs. 20.000	1.42E+00	4	1.62E+01	<0.001	Yes
25.000 vs. 8.000	1.30E+00	4	1.49E+01	<0.001	Yes
25.000 vs. 6.000	1.05E+00	4	1.20E+01	<0.001	Yes
6.000 vs. 20.000	3.68E-01	4	4.20E+00	0.021	Yes
6.000 vs. 8.000	2.50E-01	4	2.85E+00	0.192	No
8.000 vs. 20.000	1.18E-01	4	1.34E+00	0.778	No
Comparisons for factor: Fluences within 3					
25.000 vs. 20.000	1.88E+00	4	2.15E+01	<0.001	Yes
25.000 vs. 8.000	1.79E+00	4	2.05E+01	<0.001	Yes

25.000 vs. 6.000	1.50E+00	4	1.71E+01	<0.001	Yes
6.000 vs. 20.000	3.84E-01	4	4.39E+00	0.014	Yes
6.000 vs. 8.000	2.96E-01	4	3.37E+00	0.089	No
8.000 vs. 20.000	8.89E-02	4	1.01E+00	0.89	No
Comparisons for factor: Fluences within 5					
25.000 vs. 20.000	9.27E-01	4	1.06E+01	<0.001	Yes
25.000 vs. 6.000	8.52E-01	4	9.72E+00	<0.001	Yes
25.000 vs. 8.000	7.86E-01	4	8.96E+00	<0.001	Yes
8.000 vs. 20.000	1.41E-01	4	1.61E+00	0.667	No
8.000 vs. 6.000	6.67E-02	4	7.61E-01	0.95	Do Not Test
6.000 vs. 20.000	7.44E-02	4	8.49E-01	0.932	Do Not Test

C.2.7 Effect of Freeze-Thaw Cycles on UV inactivation of *B. subtilis* Spores

Table C-13 Three Way ANOVA for *B. subtilis* spores freezing temperature and F/T and fluence

Normality Test:	Failed	(P = <0.001)		
Equal Variance Test:	Passed	(P = 0.286)		
Source of Variation	DF	SS	MS	F	P
Freezing temp	2	2.58E+00	1.29E+00	1.03E+01	<0.001
F/T cycle	2	4.92E-01	2.46E-01	1.97E+00	0.148
Fluences	3	8.46E+01	2.82E+01	2.25E+02	<0.001
Freezing temp x F/T cycle	4	3.02E-01	7.54E-02	6.02E-01	0.662
Freezing temp x Fluences	6	5.29E+00	8.82E-01	7.05E+00	<0.001
F/T cycle x Fluences	6	2.22E-01	3.70E-02	2.95E-01	0.937
Freezing temp x F/T cycle x Fluences	12	3.60E-01	3.00E-02	2.40E-01	0.995
Residual	72	9.01E+00	1.25E-01		
Total	107	1.03E+02	9.61E-01		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Freezing temp					
-7.000 vs. -30.000	3.28E-01	3	5.56E+00	<0.001	Yes
-7.000 vs. -15.000	3.28E-01	3	5.56E+00	<0.001	Yes
-15.000 vs. -30.000	2.78E-04	3	4.71E-03	1	No
Comparisons for factor: Fluences					
120.000 vs. 20.000	2.28E+00	4	3.35E+01	<0.001	Yes
120.000 vs. 40.000	1.33E+00	4	1.95E+01	<0.001	Yes
120.000 vs. 80.000	3.79E-01	4	5.57E+00	0.001	Yes
80.000 vs. 20.000	1.90E+00	4	2.80E+01	<0.001	Yes
80.000 vs. 40.000	9.46E-01	4	1.39E+01	<0.001	Yes
40.000 vs. 20.000	9.57E-01	4	1.41E+01	<0.001	Yes
Comparisons for factor: F/T cycle within -7					
1.000 vs. 5.000	2.57E-01	3	2.51E+00	0.185	No
1.000 vs. 3.000	1.63E-01	3	1.59E+00	0.502	Do Not Test
3.000 vs. 5.000	9.42E-02	3	9.22E-01	0.792	Do Not Test
Comparisons for factor: F/T cycle within -15					
1.000 vs. 3.000	3.73E-01	3	3.65E+00	0.032	Yes
1.000 vs. 5.000	2.96E-01	3	2.90E+00	0.108	No
5.000 vs. 3.000	7.67E-02	3	7.51E-01	0.857	No
Comparisons for factor: F/T cycle within -30					

1.000 vs. 3.000	4.48E-01	3	4.38E+00	0.008	Yes
1.000 vs. 5.000	4.31E-01	3	4.22E+00	0.011	Yes
5.000 vs. 3.000	1.67E-02	3	1.63E-01	0.993	No
Comparisons for factor: Freezing temp within 1					
-7.000 vs. -30.000	2.57E-01	3	2.51E+00	0.185	No
-7.000 vs. -15.000	1.63E-01	3	1.59E+00	0.502	Do Not Test
-15.000 vs. -30.000	9.42E-02	3	9.22E-01	0.792	Do Not Test
Comparisons for factor: Freezing temp within 3					
-7.000 vs. -15.000	3.73E-01	3	3.65E+00	0.032	Yes
-7.000 vs. -30.000	2.96E-01	3	2.90E+00	0.108	No
-30.000 vs. -15.000	7.67E-02	3	7.51E-01	0.857	No
Comparisons for factor: Freezing temp within 5					
-7.000 vs. -15.000	4.48E-01	3	4.38E+00	0.008	Yes
-7.000 vs. -30.000	4.31E-01	3	4.22E+00	0.011	Yes
-30.000 vs. -15.000	1.67E-02	3	1.63E-01	0.993	No
Comparisons for factor: Fluences within -7					
120.000 vs. 20.000	9.46E-01	4	8.02E+00	<0.001	Yes
120.000 vs. 40.000	9.24E-01	4	7.84E+00	<0.001	Yes
120.000 vs. 80.000	8.52E-01	4	7.23E+00	<0.001	Yes
80.000 vs. 20.000	9.33E-02	4	7.92E-01	0.944	No
80.000 vs. 40.000	7.22E-02	4	6.13E-01	0.973	Do Not Test
40.000 vs. 20.000	2.11E-02	4	1.79E-01	0.999	Do Not Test
Comparisons for factor: Fluences within -15					
120.000 vs. 20.000	1.27E+00	4	1.08E+01	<0.001	Yes
120.000 vs. 40.000	1.25E+00	4	1.06E+01	<0.001	Yes
120.000 vs. 80.000	1.14E+00	4	9.66E+00	<0.001	Yes
80.000 vs. 20.000	1.32E-01	4	1.12E+00	0.858	No
80.000 vs. 40.000	1.16E-01	4	9.80E-01	0.9	Do Not Test
40.000 vs. 20.000	1.67E-02	4	1.41E-01	1	Do Not Test
Comparisons for factor: Fluences within -30					
120.000 vs. 80.000	1.34E+00	4	1.14E+01	<0.001	Yes
120.000 vs. 40.000	1.07E+00	4	9.06E+00	<0.001	Yes
120.000 vs. 20.000	7.89E-01	4	6.69E+00	<0.001	Yes
20.000 vs. 80.000	5.49E-01	4	4.66E+00	0.008	Yes
20.000 vs. 40.000	2.79E-01	4	2.37E+00	0.346	No
40.000 vs. 80.000	2.70E-01	4	2.29E+00	0.375	No
Comparisons for factor: Freeze Temp within 40					
-30.000 vs. -7.000	1.25E+00	3	1.06E+01	<0.001	Yes
-30.000 vs. -15.000	1.14E+00	3	9.66E+00	<0.001	Yes
-15.000 vs. -7.000	1.16E-01	3	9.80E-01	0.768	No
Comparisons for factor: Freezing temp within 80					
-15.000 vs. -7.000	1.34E+00	3	1.14E+01	<0.001	Yes
-15.000 vs. -30.000	1.07E+00	3	9.07E+00	<0.001	Yes
-30.000 vs. -7.000	2.69E-01	3	2.28E+00	0.247	No
Comparisons for factor: Fluences within 1					
120.000 vs. 80.000	1.05E+00	4	8.90E+00	<0.001	Yes
120.000 vs. 20.000	1.04E+00	4	8.85E+00	<0.001	Yes
120.000 vs. 40.000	9.17E-01	4	7.77E+00	<0.001	Yes
40.000 vs. 80.000	1.32E-01	4	1.12E+00	0.858	No
40.000 vs. 20.000	1.27E-01	4	1.07E+00	0.872	Do Not Test

20.000 vs. 80.000	5.56E-03	4	4.71E-02	1	Do Not Test
Comparisons for factor: Fluences within 3					
120.000 vs. 80.000	9.70E-01	4	8.23E+00	<0.001	Yes
120.000 vs. 20.000	8.12E-01	4	6.89E+00	<0.001	Yes
120.000 vs. 40.000	7.92E-01	4	6.72E+00	<0.001	Yes
40.000 vs. 80.000	1.78E-01	4	1.51E+00	0.711	No
40.000 vs. 20.000	2.00E-02	4	1.70E-01	0.999	Do Not Test
20.000 vs. 80.000	1.58E-01	4	1.34E+00	0.78	Do Not Test

C.2.8 Effect of Freeze-Thaw Cycles on UV inactivation of All Bacteria

Table C-14 Three Way ANOVA comparing bacteria at approximately 3-log inactivation

Normality Test:	Failed		(P = 0.002)		
Equal Variance Test:	Passed		(P = 0.643)		
Source of Variation	DF	SS	MS	F	P
Bacteria	2	1.85E+00	9.27E-01	1.16E+01	<0.001
Freezing temp	2	2.90E-01	1.45E-01	1.82E+00	0.176
F/T cycle	1	1.34E-01	1.34E-01	1.68E+00	0.203
Bacteria x Freezing temp	4	5.66E-01	1.42E-01	1.78E+00	0.155
Bacteria x F/T cycle	2	5.90E-01	2.95E-01	3.70E+00	0.034
Freezing temp x F/T cycle	2	1.81E-01	9.07E-02	1.14E+00	0.331
Bacteria x Temp x F/T cycle	4	1.79E-01	4.48E-02	5.63E-01	0.691
Residual	36	2.87E+00	7.96E-02		
Total	53	6.66E+00	1.26E-01		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Bacteria					
EF vs. EC	4.53E-01	3	6.82E+00	<0.001	Yes
EF vs. BSS	2.09E-01	3	3.15E+00	0.08	No
BSS vs. EC	2.44E-01	3	3.67E+00	0.036	Yes
Comparisons for factor: Freezing temp within *E. coli*					
-15.000 vs. -7.000	7.00E-01	3	6.08E+00	<0.001	Yes
-15.000 vs. -30.000	1.63E-01	3	1.42E+00	0.58	No
-30.000 vs. -7.000	5.37E-01	3	4.66E+00	0.006	Yes
Comparisons for factor: Freeze Temp within E. faecalis					
-15.000 vs. -7.000	3.70E-01	3	3.21E+00	0.073	No
-15.000 vs. -30.000	1.35E-01	3	1.17E+00	0.688	Do Not Test
-30.000 vs. -7.000	2.35E-01	3	2.04E+00	0.33	Do Not Test
Comparisons for factor: Freeze Temp within *B. subtilis* spores					
-15.000 vs. -30.000	3.30E-01	3	2.87E+00	0.121	No
-15.000 vs. -7.000	2.90E-01	3	2.52E+00	0.191	Do Not Test
-7.000 vs. -30.000	4.00E-02	3	3.47E-01	0.967	Do Not Test
Comparisons for factor: Bacteria within -7					
EF vs. EC	7.00E-01	3	6.08E+00	<0.001	Yes
EF vs. BSS	1.63E-01	3	1.42E+00	0.58	No
BSS vs. EC	5.37E-01	3	4.66E+00	0.006	Yes
Comparisons for factor: Bacteria within -15					
EF vs. EC	3.70E-01	3	3.21E+00	0.073	No
EF vs. BSS	1.35E-01	3	1.17E+00	0.688	Do Not Test

BSS vs. EC	2.35E-01	3	2.04E+00	0.33	Do Not Test
Comparisons for factor: Bacteria within -30					
EF vs. BSS	3.30E-01	3	2.87E+00	0.121	No
EF vs. EC	2.90E-01	3	2.52E+00	0.191	Do Not Test
ECvs. BSS	4.00E-02	3	3.47E-01	0.967	Do Not Test
Comparisons for factor: F/T cycle within *E. coli*					
3.000 vs. 1.000	5.04E-01	2	5.36E+00	<0.001	Yes
Comparisons for factor: F/T cycle within *E. faecalis*					
3.000 vs. 1.000	4.02E-01	2	4.28E+00	0.005	Yes
Comparisons for factor: Bacteria within 1					
EF vs. EC	5.04E-01	3	5.36E+00	0.002	Yes
EF vs. BSS	4.52E-01	3	4.81E+00	0.005	Yes
BSS vs. EC	5.22E-02	3	5.55E-01	0.919	No
Comparisons for factor: Bacteria within 3					
BSS vs. EF	4.02E-01	3	4.28E+00	0.013	Yes
BSS vs. EC	1.57E-01	3	1.67E+00	0.474	No
EC vs. EF	2.46E-01	3	2.61E+00	0.169	No

C.3 Photoreactivation

C.3.1 Photoreactivation of *E. coli*

Table C-15 Two Way ANOVA comparing *E. coli* photoreactivation exposure time and fluences

Normality Test:	Passed	(P = 0.081)			
Equal Variance Test:	Passed	(P = 0.316)			
Source of Variation	DF	SS	MS	F	P
Fluences	2	2.28E+00	1.14E+00	1.49E+02	<0.001
Time	2	8.39E-01	4.19E-01	5.48E+01	<0.001
Fluences x Time	4	1.01E+00	2.52E-01	3.30E+01	<0.001
Residual	18	1.38E-01	7.65E-03		
Total	26	4.27E+00	1.64E-01		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Fluences					
2.500 vs. 0.000	6.19E-01	3	2.12E+01	<0.001	Yes
2.500 vs. 9.000	3.33E-03	3	1.14E-01	0.996	No
9.000 vs. 0.000	6.15E-01	3	2.11E+01	<0.001	Yes
Comparisons for factor: Time					
180.000 vs. 30.000	4.30E-01	3	1.48E+01	<0.001	Yes
180.000 vs. 90.000	1.85E-01	3	6.36E+00	<0.001	Yes
90.000 vs. 30.000	2.45E-01	3	8.40E+00	<0.001	Yes
Comparisons for factor: Time within 0					
30.000 vs. 180.000	2.09E-01	3	4.14E+00	0.023	Yes
30.000 vs. 90.000	6.84E-02	3	1.35E+00	0.612	No
90.000 vs. 180.000	1.41E-01	3	2.79E+00	0.148	No
Comparisons for factor: Time within 2.5					
180.000 vs. 30.000	7.90E-01	3	1.57E+01	<0.001	Yes

180.000 vs. 90.000	2.23E-01	3	4.42E+00	0.015	Yes
90.000 vs. 30.000	5.67E-01	3	1.12E+01	<0.001	Yes
Comparisons for factor: Time within 9					
180.000 vs. 30.000	7.10E-01	3	1.41E+01	<0.001	Yes
180.000 vs. 90.000	4.73E-01	3	9.38E+00	<0.001	Yes
90.000 vs. 30.000	2.37E-01	3	4.69E+00	0.01	Yes
Comparisons for factor: Fluences within 30					
9.000 vs. 0.000	2.07E-01	3	4.11E+00	0.025	Yes
9.000 vs. 2.500	1.33E-01	3	2.64E+00	0.177	No
2.500 vs. 0.000	7.39E-02	3	1.46E+00	0.565	No
Comparisons for factor: Fluences within 90					
2.500 vs. 0.000	7.09E-01	3	1.40E+01	<0.001	Yes
2.500 vs. 9.000	1.97E-01	3	3.90E+00	0.033	Yes
9.000 vs. 0.000	5.12E-01	3	1:02E+01	<0.001	Yes
Comparisons for factor: Fluences within 180					
9.000 vs. 0.000	1.13E+00	3	2.23E+01	<0.001	Yes
9.000 vs. 2.500	5.33E-02	3	1.06E+00	0.739	No
2.500 vs. 0.000	1.07E+00	3	2.13E+01	<0.001	Yes

C.3.2 Photoreactivation of *E. faecalis*

Table C-16 Two Way ANOVA comparing *E. faecalis* photoreactivation exposure time and fluences

Normality Test:	Passed	(P > 0.200)			
Equal Variance Test:	Passed	(P = 0.655)			
Source of Variation	DF	SS	MS	F	P
Fluences	2	5.33E-01	2.67E-01	1.88E+01	<0.001
Time	2	7.73E-02	3.86E-02	2.73E+00	0.092
Fluence x Time	4	5.50E-02	1.38E-02	9.70E-01	0.448
Residual	18	2.55E-01	1.42E-02		
Total	26	9.20E-01	3.54E-02		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Fluences					
20.000 vs. 6.000	3.41E-01	3	8.60E+00	<0.001	Yes
20.000 vs. 0.000	2.10E-01	3	5.29E+00	0.004	Yes
0.000 vs. 6.000	1.31E-01	3	3.30E+00	0.076	No

C.3.3 Photoreactivation of *B. subtilis* Spores

Table C-17 Two Way ANOVA comparing *B. subtilis* spores exposure time and fluences

Normality Test:	Passed	(P = 0.142)			
Equal Variance Test:	Passed	(P = 0.118)			
Source of Variation	DF	SS	MS	F	P
Fluences	2	9.69E-02	4.84E-02	2.99E+00	0.076
Time	2	5.46E-02	2.73E-02	1.69E+00	0.213
Fluences x Time	4	1.85E-01	4.63E-02	2.86E+00	0.054
Residual	18	2.92E-01	1.62E-02		
Total	26	6.28E-01	2.42E-02		

C.3.4 Effect of Freezing on *E. coli* Photoreactivation after UV inactivation

Table C-18 Three Way ANOVA comparing *E. coli* exposure time, freezing and fluences

Normality Test:	Failed	(P = <0.001)			
Equal Variance Test:	Passed	(P = 0.531)			
Source of Variation	DF	SS	MS	F	P
Freezing	1	2.07E+01	2.07E+01	8.54E+02	<0.001
Fluences	2	2.87E+00	1.43E+00	5.90E+01	<0.001
Time	2	9.72E-01	4.86E-01	2.00E+01	<0.001
Freezing x Fluences	2	2.19E-01	1.10E-01	4.51E+00	0.018
Freezing x Time	2	5.19E+00	2.59E+00	1.07E+02	<0.001
Fluencesx Time	4	1.10E+00	2.75E-01	1.13E+01	<0.001
Freezing x Fluences x Time	4	1.56E-01	3.91E-02	1.61E+00	0.193
Residual	36	8.74E-01	2.43E-02		
Total	53	3.21E+01	6.06E-01		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Freezing					
0.000 vs. 1.000	1.24E+00	2	4.13E+01	<0.001	Yes
Comparisons for factor: Fluences					
9.000 vs. 0.000	5.08E-01	3	1.38E+01	<0.001	Yes
9.000 vs. 2.500	4.11E-02	3	1.12E+00	0.711	No
2.500 vs. 0.000	4.67E-01	3	1.27E+01	<0.001	Yes
Comparisons for factor: Time 16					
30.000 vs. 180.000	3.29E-01	3	8.94E+00	<0.001	Yes
30.000 vs. 90.000	1.54E-01	3	4.20E+00	0.014	Yes
90.000 vs. 180.000	1.74E-01	3	4.74E+00	0.005	Yes
Comparisons for factor: Fluences within 0					
9.000 vs. 2.500	1.69E+00	3	3.24E+01	<0.001	Yes
9.000 vs. 0.000	6.19E-01	3	1.19E+01	<0.001	Yes
0.000 vs. 2.500	1.07E+00	3	2.05E+01	<0.001	Yes
Comparisons for factor: Fluences within 1					
0.000 vs. 2.500	1.37E+00	3	2.64E+01	<0.001	Yes
0.000 vs. 9.000	3.33E-03	3	6.42E-02	0.999	No
9.000 vs. 2.500	1.37E+00	3	2.63E+01	<0.001	Yes
Comparisons for factor: Freezing within 0					
0.000 vs. 1.000	1.07E+00	2	2.05E+01	<0.001	Yes
Comparisons for factor: Freezingwithin 2.5					
1.000 vs. 0.000	1.37E+00	2	2.63E+01	<0.001	Yes
Comparisons for factor: Freezing within 9					
0.000 vs. 1.000	2.69E+20	2	5.18E+21	<0.001	Yes
Comparisons for factor: Time within 0					
180.000 vs. 90.000	7.12E-01	3	1.37E+01	<0.001	Yes
180.000 vs. 30.000	2.45E-01	3	4.72E+00	0.006	Yes
30.000 vs. 90.000	4.67E-01	3	8.99E+00	<0.001	Yes
Comparisons for factor: Timewithin 1					
180.000 vs. 90.000	1.45E+00	3	2.79E+01	<0.001	Yes
180.000 vs. 30.000	1.85E-01	3	3.57E+00	0.042	Yes
30.000 vs. 90.000	1.27E+00	3	2.44E+01	<0.001	Yes

Comparisons for factor: Freezing within 30					
0.000 vs. 1.000	4.67E-01	2	8.99E+00	<0.001	Yes
Comparisons for factor: Freezing within 90					
1.000 vs. 0.000	1.45E+00	2	2.79E+01	<0.001	Yes
Comparisons for factor: Time within 2.5					
90.000 vs. 30.000	5.11E-01	3	8.03E+00	<0.001	Yes
90.000 vs. 180.000	4.83E-02	3	7.60E-01	0.854	No
180.000 vs. 30.000	4.63E-01	3	7.27E+00	<0.001	Yes
Comparisons for factor: Time within 9					
180.000 vs. 30.000	8.80E-01	3	1.38E+01	<0.001	Yes
180.000 vs. 90.000	5.67E-02	3	8.91E-01	0.805	No
90.000 vs. 30.000	8.24E-01	3	1.29E+01	<0.001	Yes

C.3.5 Effect of Freezing on *E. faecalis* Photoreactivation after UV inactivation

Table C-19 Three Way ANOVA of *E. faecalis* comparing comparing *E. coli* exposure time, freezing and fluences

Normality Test:	Failed	(P = <0.001)			
Equal Variance Test:	Passed	(P = 0.599)			
Source of Variation	DF	SS	MS	F	P
Freezing	1	1.25E-02	1.25E-02	1.20E+00	0.281
Fluences	2	2.89E-01	1.44E-01	1.39E+01	<0.001
Time	2	4.71E-02	2.35E-02	2.27E+00	0.118
Freezing x Fluences	2	2.46E-01	1.23E-01	1.19E+01	<0.001
Freezing x Time	2	5.90E-02	2.95E-02	2.84E+00	0.072
Fluences x Time	4	5.67E-02	1.42E-02	1.37E+00	0.265
Freezing x Fluences x Time	4	2.50E-02	6.25E-03	6.02E-01	0.664
Residual	36	3.74E-01	1.04E-02		
Total	53	1.11E+00	2.09E-02		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Fluences					
20.000 vs. 6.000	1.78E-01	3	7.43E+00	<0.001	Yes
20.000 vs. 0.000	1.03E-01	3	4.30E+00	0.012	Yes
0.000 vs. 6.000	7.50E-02	3	3.12E+00	0.083	No
Comparisons for factor: Fluences within 0					
6.000 vs. 20.000	1.34E-01	3	3.96E+00	0.022	Yes
6.000 vs. 0.000	3.33E-03	3	9.82E-02	0.997	No
0.000 vs. 20.000	1.31E-01	3	3.86E+00	0.026	Yes
Comparisons for factor: Fluences within 1					
20.000 vs. 0.000	3.41E-01	3	1.00E+01	<0.001	Yes
20.000 vs. 6.000	2.26E-01	3	6.64E+00	<0.001	Yes
6.000 vs. 0.000	1.16E-01	3	3.40E+00	0.055	No
Comparisons for factor: Freezing within 0					
1.000 vs. 0.000	3.33E-03	2	9.82E-02	0.945	No
Comparisons for factor: Freezing within 6					
1.000 vs. 0.000	2.26E-01	2	6.64E+00	<0.001	Yes
Comparisons for factor: Freezing within 20					
1.000 vs. 0.000	7.85E-308	2	0.00E+00	1	No

Comparisons for factor: Time within 6					
180.000 vs. 90.000	2.55E-01	3	6.13E+00	<0.001	Yes
180.000 vs. 30.000	9.50E-02	3	2.28E+00	0.253	No
30.000 vs. 90.000	1.60E-01	3	3.85E+00	0.026	Yes
Comparisons for factor: Time within 20					
180.000 vs. 90.000	1.93E-01	3	4.65E+00	0.006	Yes
180.000 vs. 30.000	1.38E-01	3	3.33E+00	0.061	No
30.000 vs. 90.000	5.50E-02	3	1.32E+00	0.622	No

C.3.6 Effect of Freezing on *B. subtilis* Spores Photoreactivation after UV inactivation

Table C-20 Three Way ANOVA comparing *B. subtilis* spores exposure time, freezing and fluences

Normality Test:	Passed	(P > 0.200)			
Equal Variance Test:	Passed	(P = 0.547)			
Source of Variation	DF	SS	MS	F	P
Freezing	1	2.02E-03	2.02E-03	1.27E-01	0.724
Fluences	2	9.65E-02	4.83E-02	3.03E+00	0.061
Time	2	1.06E-01	5.32E-02	3.34E+00	0.047
Freezing x Fluences	2	4.14E-02	2.07E-02	1.30E+00	0.285
Freezing x Time	2	6.72E-02	3.36E-02	2.11E+00	0.136
Fluences x Time	4	1.14E-01	2.84E-02	1.79E+00	0.153
Freezing x Fluences x Time	4	1.58E-01	3.95E-02	2.48E+00	0.061
Residual	36	5.73E-01	1.59E-02		
Total	53	1.16E+00	2.19E-02		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
Comparisons for factor: Time					
180.000 vs. 90.000	1.06E-01	3	3.55E+00	0.043	Yes
180.000 vs. 30.000	3.01E-02	3	1.01E+00	0.756	No
30.000 vs. 90.000	7.55E-02	3	2.54E+00	0.186	No