University of Strathclyde
Department of Electrical and Electronic Engineering

THE EFFECTS OF UV-RICH LIGHT PULSES ON PATHOGENIC MICROORGANISMS IN LIQUIDS

by

Yvonne Lamont, BSc (honours)

A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy

2005

DECLARATION OF AUTHOR'S RIGHTS

ABSTRACT

The work presented in this thesis is concerned with investigating the use of UV-rich light pulses for the inactivation of problematic microorganisms. UV radiation is an effective means of disinfecting surfaces and liquids and of reducing contamination in air. The germicidal effects are primarily due to the UV-C region of the electromagnetic spectrum, which interferes with the nuclear core of a microorganism, resulting in a loss of ability to replicate and initiate infection. When UV radiation is delivered as pulses of light however, the results are even more appealing, with higher levels of microbial inactivation achieved in much shorter timescales.

Drinking water and wastewater disinfection is normally provided by the use of chemicals such as chlorine. These are disadvantaged by the production of harmful chemical by-products and the resistance of certain types of microorganism to chemical treatment. The main aim of this investigation was therefore to look at the role of pulsed UV-rich light for inactivating a range of microorganisms suspended in liquid media and to determine how successful the treatment process would be as an alternative disinfection method. The results show that pulsed UV-rich light treatment is extremely effective against many types of bacteria, virus and *Cryptosporidium*. It was also demonstrated that the sensitivities of microorganisms to UV radiation can vary significantly depending on the cell-wall structure, growth phase, strains and microorganism species.

Studies were also undertaken to identify electrical and biological parameters that may influence the inactivation success. It was found that high operating voltages and low pulse repetition frequencies give desirable levels of inactivation. Other important factors investigated were sample depth, volume and reflection.

Finally, the possible limitations to pulsed UV-rich light treatment were investigated. It was found that the success of the treatment is primarily determined by the transmittance of UV pulses through a sample. As expected, transmittance depends

upon microorganism size and population. The major limitation of UV treatment is the ability of microorganisms to reactivate following exposure to visible light (photoreactivation). Studies showed photoreactivation to occur following pulsed UV-rich light treatment, only when the microorganisms do not receive sufficient UV treatment or if they are exposed to high intensities of visible light.

ABBREVIATIONS

The following list contains abbreviations, which are used regularly throughout this thesis:

BGLBB	Brilliant Green Lactose Bile Broth
CFUml-1	Colony Forming Units per ml
CNS	Central Nervous System
CPD	Cyclobutane Pyrimidine Dimers
CPE	Cytopathic Effect
CSL	Central Science Laboratory
DAPI	4'-6-diamidino-2-phenylindole
DIC	Differential Interferance Contrast
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EHEC	Enterohemorrhagic *E. coli*
FDA	Food and Drug Administration
GIT	Gastrointestinal Tract
HBSS	Hanks Balanced Salt Solution
HSV-1	Herpes Simplex Virus Type 1
LMG	Laboratory of Microbiology, University of Gent
MCL	Maximum Contaminant Level
MEM	Minimum Essential Medium
MMGM	Minerals Modified Glutamate Medium
MPN	Most Probable Number
NCTC	National Collection of Type Cultures
PEF	Pulsed Electric Fields
PCT	Pulsed Corona Treatment

PI	Propidium Iodide
PRF	Pulse Repetition Frequency
PUV	Pulsed Ultraviolet Light Treatment
6-4 PP	Pyrimidine-Pyrimidone (6-4) Photoproducts
RNA	Ribonucleic Acid
RO	Reverse Osmosis
SARS	Severe Acute Respiratory Syndrome
SMPS	Switch Mode Power Supply
$TCIU_{50}ml^{-1}$	Tissue Culture Infectious Units (able to destroy 50 % of tissue culture cells)
UV	Ultraviolet
WHO	World Health Organisation

CONTENTS

Chapter 1

INTRODUCTION

Despite the progress seen in recent times in medical care and food technology, food- and waterborne diseases are still (and increasingly) of major concern for human health, both in developing and developed countries [1]. Numerous types of bacterium, virus, yeast, fungus and protozoan are responsible for causing these diseases through contamination of food and water supplies. Any method that can therefore reduce or eliminate contamination of these sources will have a significant effect on the incidence of foodborne disease [2].

The most widely used method of killing pathogenic and spoilage microorganisms is by means of thermal processes, which include pasteurisation and sterilisation. A major problem that arises with thermal treatment is that not only does it kill contaminating microorganisms, but it also can affect taste, colour, flavour and the nutritional quality of foods [3]. Non-thermal methods such as the use of chemicals are extensively used for inactivating pathogens on surfaces such as those used for food preparation and on materials used in packaging of foods. One of the major applications of chemical treatment is the use of chlorine in the disinfection of water. However, there is increasing concern about the use of chemical disinfectants, as the formation of chemical by-products can occur, and these are potentially harmful to humans and the environment.

New approaches to sterilisation include the development of non-thermal pulsed power technologies in the form of PEF [4], PCT [5] and PUV [6]. These electrotechnologies can achieve successful microbial reduction without conventional heat sterilisation or chemical inactivation [7].

Inactivation of microorganisms using ultraviolet light (UV) has been used for many years. It is a physical process that disinfects by interacting with nucleic acids of the pathogen and induces damage, which interferes with nucleic acid replication [8]. Conventional UV systems are disadvantaged by the long exposure times required to achieve the desired levels of inactivation [9]. Pulsed UV light treatment however overcomes this problem and has proven to be very effective for disinfection and sterilisation purposes. Unlike conventional UV systems that emit UV at one wavelength (monochromatic), pulsed UV systems emit a broad range of wavelengths (polychromatic) with high intensity emissions in the germicidal UV-C region. Pulsed UV light is generated by storing electrical energy in a capacitor, and then releasing it through a gas as short, high intensity pulses, resulting in the generation of high peak-power levels [9]. The outcome is increased penetration, higher levels of inactivation and shorter treatment times [10]. Pulsed UV systems have many possible applications, which include the disinfection of water (potable, waste, ingredient, recreational), surfaces, packaging and medical instruments, and air sterilisation. Their most important application however would be in the disinfection of potable water, as UV can kill protozoa such as *Cryptosporidium* oocysts that are resistant to chlorination and it is more effective against viruses than chlorine.

The overall aim of this investigation was to study the effect of pulsed UV-rich light on microorganisms, and to evaluate how successful a pulsed-power system would be as an alternative or addition to sterilisation technology. The investigation involved microorganisms suspended in water or liquid media, the potential applications of the technology being in the treatment of liquids, primarily drinking water and possibly wastewater.

Prior to commencing the investigation several parameters had to be examined so that optimum levels of inactivation could be established. The pulsed light source system used throughout this study was developed by Samtech Ltd and could operate at between 400 and 1000 V and produce pulses in the frequency range of 0.1-10 pulses per second. The effects on the inactivation level by varying the voltage and pulse frequency were investigated along with various other electrical factors. Because the

investigation focussed on microorganisms suspended in liquid media, a small-scale study was also carried out on the significance of the volume and depth of the suspension, in order to determine a suitable test-cell geometry.

Once a suitable treatment protocol was determined, inactivation of a range of pathogenic bacteria was examined. As well as looking at the inactivation differences between various species and strains of bacteria, studies were carried out on the influence of growth phase. In addition to treatment of bacteria, the effects of UV light pulses on viruses and the problematic protozoan *Cryptosporidium* were investigated. While the majority of work concentrated on inactivating microorganisms suspended in liquid laboratory media, a study was also made with some private water samples in order to investigate the efficiency of UV-rich light pulses for disinfecting potable water.

Some inactivation experiments did not achieve complete inactivation. This was observed as a "tail" on an inactivation curve. A study was therefore made to determine the factor(s) responsible for inhibiting complete inactivation.

Finally, the phenomenon of photoreactivation was studied. A major disadvantage of UV disinfection is the ability of a microorganism treated by a sub-lethal UV dose to repair damage caused to its DNA [11]. One of these repair mechanisms is photoreactivation, whereby a microorganism exposed to certain levels of visible light, after treatment with UV, is able to repair its DNA and hence continue to survive. Whereas most of the published work on this topic concerns photoreactivation following continuous UV treatment, this study investigates photo repair following treatment with pulses of UV-rich light.

Chapter 2 provides a brief background of present sterilisation/disinfection technologies and the problems that can arise with these. Pulsed-power technologies are then introduced, with the remainder of the chapter concentrating on ultraviolet radiation and its use as an alternative sterilisation technology.

Chapter 3 provides information on all microorganisms, media, equipment and methods used in the investigation.

Chapter 4 discusses and shows results of various factors which can affect microbial inactivation levels. Taking these factors into account it also describes the experimental protocol chosen for the investigation.

Chapter 5 presents results on the use of pulsed UV light on a range of bacterial species and comparisons are made between type and strain of bacterium and the growth phase of the bacterium. In addition results are presented following the treatment of private water samples.

Chapter 6 presents results of *Cryptosporidium* and virus inactivation using pulsed UV light and compares the sensitivity of these microorganisms to that of bacteria.

Chapter 7 focuses on the problem of microorganisms possibly receiving protection from pulsed UV light treatment and how this can be prevented or kept to a minimum.

Chapter 8 concerns photoreactivation and results are presented from experiments undertaken to investigate whether repair of pulsed UV-light treated bacteria can occur following exposure to visible light.

Chapter 9 discusses all of the results obtained throughout this investigation and looks at the possible applications of this system as a future disinfection technology. Recommendations for further work in this area are also discussed.

Chapter 2

BACKGROUND AND LITERATURE REVIEW

2.1 An Introduction to Foodborne Infection

Despite the significant advances that have been made towards a better understanding of microbial transmission and pathogenicity, illness caused by the consumption of contaminated food (foodborne disease) and water (waterborne disease) still remains a major cause of human suffering, and a very significant cause of death throughout the world. Not only is the impact on human health huge, but so also is the annual economic burden [2, 12].

2.1.1 Foodborne Illness

There are more than 250 foodborne diseases known worldwide [13]. These are caused by viruses, bacteria, parasites, toxins, metals and prions, with the symptoms of illness ranging from mild gastroenteritis to more serious problems such as life-threatening neurologic hepatic and renal syndromes [14]. The actual incidence of foodborne infection is difficult to assess because cases are not always reported or recognised as foodborne. Recent estimates for the UK however, are around 9 million cases per year, and in the US, estimates are approximately 75 million cases with 5,000 deaths per year [15]. Gastroenteritis is an infection of the gastrointestinal tract, which causes inflammation of the lining of the stomach and the intestines. It is the most common cause of vomiting and diarrhoea and is caused by the consumption of contaminated foods and water. Every year there are more than 100 million cases of gastroenteritis amongst children under the age of five and up to 5 million deaths in underdeveloped countries. [16].

2.1.2 Contamination of Food

Food-borne transmission involves the presence of pathogens in and on foods that are poorly processed, undercooked, or poorly refrigerated [59]. There are many opportunities in which such pathogens may contaminate food/water, as they are produced and prepared, these include:

(i) Fruit and vegetables may become contaminated if they are washed with water that is contaminated with animal manure or human sewage.

(ii) Products such as milk, eggs, seafood, poultry, and meat from food-producing animals may become contaminated due to poor farming practices.

(iii) Foods may become contaminated during processing due to malfunctioning or improperly sterilized equipment, misuse of cleaning materials, improper storage and rodent and insect infestations.

(iv) Foods may become contaminated in supermarkets and in the home through the use of poor food handling practices [130, 20].

Cross contamination is another important cause of foodborne infection. *Salmonella*, for example, is found in raw poultry and other meats, and food prepared on surfaces that previously contained such meats, can in turn become contaminated with the bacteria. [13]. Foods in the refrigerator may also become contaminated with juices dripping from raw chicken. This is an important cause of *Campylobacter* infections [20]. Most foodborne diseases however are associated with poor hygiene practices. Whether by water or food transmission, the fecal-oral route is the vital link between food and the host [20].

2.1.3 Initiation of Infection and Immune Responses

Infection can occur when microorganisms present in unprocessed or uncooked foods multiply and produce toxins within the food. The microorganisms may be destroyed during food preparation but the toxin(s) remain viable, and when consumed can act within hours. If the microorganism itself survives food preparation, it can initiate infection in the gastrointestinal tract (GIT) [17]. For microorganisms to survive within a living host they must adopt a strategy for survival. The five functions which a microorganism must be able to carry out to make it a successful pathogen are: (i) gain entry into the host, (ii) find a suitable place within the host, (iii) evade the innate defence mechanisms of the host, (iv) multiply, and (v) exit the host in a way that it transmits to a new susceptible host [18]. The route of infection in the gastrointestinal tract is shown in Figure 2.1.3.1. The ability of a microorganism to initiate infection depends on (i) the types and numbers of food-poisoning microorganisms, (ii) the susceptibility of the host, and (iii) the immune response of the host.

Young children, pregnant women, old people, and immunodeficient individuals are particularly at risk from foodborne illness [12]. Over recent years increases in the numbers of immunodeficient/immunocompromised individuals have led to a rise in the number of food borne illnesses, as well as the emergence of new pathogens. Some of these so-called "new pathogens" do not affect healthy individuals but they can cause infection in these sub-populations [19].

The body produces both innate and adaptive immune responses in an effort to prevent the initiation of infection in the gastrointestinal tract. These include the highly acidic gastric juices in the stomach, pancreatic enzymes, bile and secretory IgA. Mucus on epithelial cells plays an important role, binding to microbial adhesions and thus blocking attachment to host cells. Motile organisms however can propel themselves through mucus layers allowing attachment to epithelial cells. When intestinal epithelium is

penetrated, final pathogenicity depends on toxin production, cell damage, multiplication, inflammation and the immune response [20, 17].

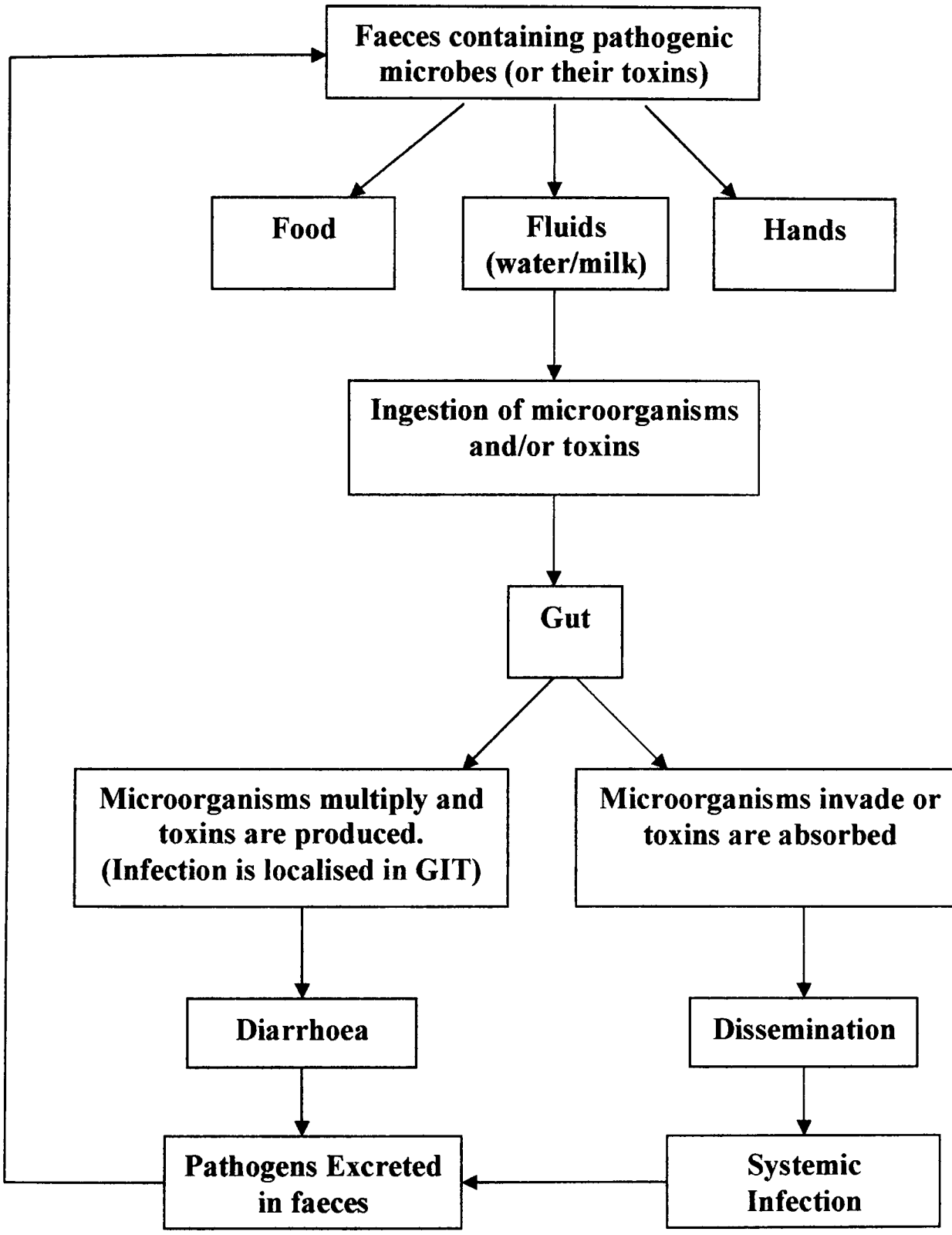


Figure 2.1.3.1 Route of microbial infection by microorganisms in the gastrointestinal tract (GIT). * Diagram from "Medical Microbiology" 2nd Edition, Mimms *et al* [17].

2.2 Traditional Food Preservation Methods

Due to the microbiological risks associated with contaminated foods, any method of either reducing or eliminating food contamination will have a significant effect on the incidence of foodborne disease [21]. Disinfection of objects or surfaces provides a means of killing, inhibiting or removing problematic microorganisms such as bacteria and viruses, whereas sterilisation technologies are employed to completely destroy microorganisms [20]. Traditional methods of sterilisation/disinfection include both thermal and non-thermal treatments.

2.2.1 Thermal Treatment

Thermal treatment (heating and cooking) of foods to destroy microorganisms has been practised for more than five thousand years [22]. Today, foods are thermally processed by subjecting them to temperatures between 60 °C and 100 °C for a few seconds to minutes, by a process known as pasteurisation. Pasteurisation kills pathogens in food and liquid and also reduces levels of non-pathogenic spoilage microorganisms to acceptable levels. This however can have several disadvantages, including changes in flavour, smell and appearance and the destruction of heat-sensitive compounds such as vitamins and proteins [23, 9]. Certain microorganisms are also capable of mounting adaptive stresses to the lethal temperatures involved in pasteurisation. Although such adaptation may only allow problematic bacteria to survive rather than grow, in the context of food preservation and safety, survival of microorganisms is a serious concern [24]. For some high-risk foods such as meats, fresh fruit and fresh vegetables, thermal treatment is not applicable.

2.2.2 Non-Thermal Treatment

Non-thermal processes hold the temperature of the food below the temperature used in thermal processing, so that the degradation of quality often experienced at high temperatures is minimal [9]. Some of the widely used non-thermal treatments are now described.

2.2.2.1 γ- Irradiation

After heat, the most cost-effective method of elimination of many vegetative pathogens from raw food, is by the use of γ-radiation. It is also used in the cold sterilisation of antibiotics, hormones and disposable plastics, for example, syringes. An advantage of γ-irradiation, is that it can be used on frozen foodstuffs. It is the only preservation method available for inactivating pathogenic microorganisms such as *Salmonella* in frozen foods [9]. Both the Food and Drug Administration (FDA) and the World Health Organisation (WHO) have approved food irradiation and declared it safe, although consumers are wary and furthermore γ-irradiation tends to be a relatively time-consuming process [20].

2.2.2.2 High Pressure

The use of high pressure in the food industry has been commercialised only over the past decade, even though its ability to inactivate microorganisms has been recognised for the past 100 years. The process involves applying a pressure of 4000 to 9000 atm for a specified period of time to packaged foods. The result is inactivation of enzymes and bacteria without the nutrients and flavour being affected, and the preservation of the food is uniform. High pressure inactivates microorganisms by denaturing proteins thus inhibiting the uptake of amino acids for cell growth. The process also increases the permeability of the cell membrane so that the contents of the cell leak out, disrupting the functioning of the cell. High-pressure technology can be applied to extend the shelf life

of foods and modify the texture and sensory properties of foods. The major limitation of high-pressure treatment is that it is difficult to produce high volume vessels that can withstand the very high pressures [9].

2.2.2.3 Chemicals

Chemicals have been used for years to preserve foods, with salt being one of the oldest known methods of food preservation [9]. Over the years various chemical agents have been recognised as safe and are closely regulated by the U.S. Food and Drug Administration. For example, sodium nitrite is used to preserve cured meats, and chlorine compounds are used to wash food and packaging materials [20]. The most widely used chemical is chlorine which has traditionally been used to disinfect drinking water; however a significant level of disinfection by-products (primarily trihalomethanes and haloaccetic acids) are formed as a result of the reaction of chlorine with dissolved organic matter. These by-products can be harmful to humans and alternative disinfection technologies are warranted [25].

2.2.2.4 Ozone Treatment

Ozone is an unstable allotrope of oxygen. Its generation can be induced by electrically creating a corona discharge [23]. Reactions of ozone with various chemical compounds in aqueous systems occur in two different and coexisting modes. These are direct reactions of molecular ozone and free-radical mediated destruction by superoxide anions (O_2^-) and hydroxyl radicals (OH·). These mechanisms are also thought to be involved in the destruction of bacteria. The major advantage of ozone is its spontaneous decomposition to a non-toxic product [26], making it a suitable method of surface disinfection. Applications of ozone in the food industry are primarily in the decontamination of surfaces, drinking water and wastewater.

2.2.2.5 Continuous Ultraviolet Light

Continuous ultraviolet irradiation is another non-thermal alternative that is successful in killing a range of microorganisms (bacteria, viruses, fungi, protozoa). The treatment process has minimum health risks and produces no residuals that can react with organics in the products being treated. [27]. Its main application is in the disinfection of drinking water although others include surface treatment, air sterilisation and sterilisation of medical instruments. A disadvantage is that long exposure times are required before desired inactivation levels are achieved [9].

2.3 Pulsed Power Technologies

A new approach to non-thermal sterilisation is that of advanced pulsed power. Existing pulsed power technologies are capable of inactivating microorganisms through the use of Pulsed Electric Field treatment (PEF), Pulsed Corona Treatment (PCT) and Pulsed Ultra Violet light treatment (PUV). The principle of these pulsed power systems is the ability to store large amounts of electrical energy over a long period of time (typically millisecond to seconds), to allow high peak powers to be produced (up to hundreds of megawatts), and then release this energy in a very short time [28]. All of these treatments have potent anti-microbial properties.

2.3.1 Pulsed Electric Fields (PEF)

PEF pasteurisation involves the application of a short pulse of high voltage to liquid foodstuff placed between two electrodes. The resultant inactivation of microorganisms is caused by irreversible structural changes in the cellular plasma membrane, which leads to pore formation, membrane lysis and eventual cell death. The process is known as electroporation [15]. The treatment is conducted at relatively low temperatures for less than 1 second and detrimental changes to the sensory and physical properties of food are

minimal [22]. Wall *et al* have [4] suggested that PEF may also cause internal membrane damage by compromising the nuclear membrane, which also leads to cell death. To date PEF has been mainly applied to preserve the quality of foods and increase the shelf life of products such as fruit juices and milk [22]. One of the major uses of PEF may be in the beer and wine industries as a means of inactivating spoilage microorganisms. [29]. A disadvantage of PEF treatment is that it can only be applied to liquids and cannot be used, for example in surface sterilisation. PEF treatment is also unable to efficiently inactivate all forms of microorganism, including viruses, spores and oocysts [30].

2.3.2 Pulsed-corona

Pulsed-corona treatment is an advanced oxidation technology that uses non-thermal plasma to produce free radicals, ozone (if oxygen is present) and UV photons all of which react with microorganisms [5]. Applying a series of high voltage pulses across an ionised gaseous mixture, in a non-uniform electrode gap, creates an electrical discharge, which in turn creates free radicals and other reactive plasma species. The treatment kills microorganisms by irreversible oxidative damage [5] and it has the potential to be used for water treatment and sterilisation of food and contact surfaces.

2.3.3 Pulsed Ultraviolet Light

Pulsed ultraviolet light treatment involves the use of intense and short-duration pulses of light, emitted from an ultraviolet-rich light source, to kill microbial populations on surfaces, in the air and in liquids [2, 31]. Pulsed UV treatment holds many advantages over continuous UV treatment including shorter treatment times, higher levels of inactivation and increased effective depth of penetration [10].

This thesis focuses on the use of pulsed ultraviolet light for the inactivation of microorganisms and the remainder of this chapter concentrates on the nature and source of ultraviolet radiation and its application to disinfection technology.

2.4 Ultraviolet Radiation

Ultraviolet radiation is a form of electromagnetic radiation, which is emitted as a result of energy changes in the orbital electrons of atoms [32]. This section describes how different types of electromagnetic radiation, mainly UV, are produced; the effect UV radiation has on human health and the various sources of ultraviolet light.

2.4.1 Electromagnetic Radiation

Electromagnetic radiation results whenever electrons oscillate, decelerate or change energy levels in an atom [32]. Figure 2.4.1 shows what happens when an electron in an atom moves down an energy level. An electron moves down to a lower energy level when it receives energy and becomes "excited". The atom gives up the excess energy as a photon of electromagnetic radiation. Photons are massless particles that travel in a wave-like pattern, and move at the speed of light.

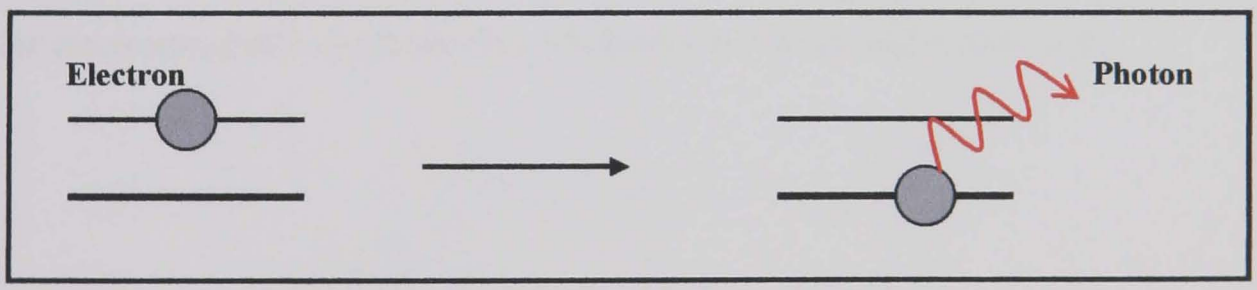


Figure 2.4.1 Production of a photon from an "excited" electron

The amount of energy carried by each photon is proportional to its frequency: the higher the frequency, the greater the energy. The frequency is the number of oscillations the light travels in 1 second [32, 33]. There are different types of electromagnetic radiation, which all travel at the same constant velocity ($c = 3 \times 10^{10}$ cm.s $^{-1}$), but have different

wavelengths (λ) and different frequencies (f). The three are related by the following equation [34]:

$$\mathbf{c = f \lambda}$$

The energy carried by a photon is known as a quantum of energy and is given by the frequency of the light (f) multiplied by Planck's constant ($h = 6.62 \times 10^{-27}$ Js) [35]:

$$\mathbf{E = h f} \qquad \textbf{or} \qquad \mathbf{E = h c / \lambda}$$

As the wavelength (λ) of the radiation increases the energy of the photons decrease therefore short-wave UV-C photons carry more energy than medium-wave UV-B photons.

The Sun gives the Earth energy in the form of electromagnetic radiation. The light we can see is made up of this radiation, but there are also other types of electromagnetic radiation produced by the sun which we cannot see. The electromagnetic spectrum covers an extremely broad range, from radio waves with wavelengths of 1 m or more down to gamma rays with wavelengths of less than 10 nm [36]. The different types of electromagnetic radiation are shown in Figure 2.4.1.1. Ultraviolet radiation is the portion of the electromagnetic spectrum that lies between X-rays and visible light.

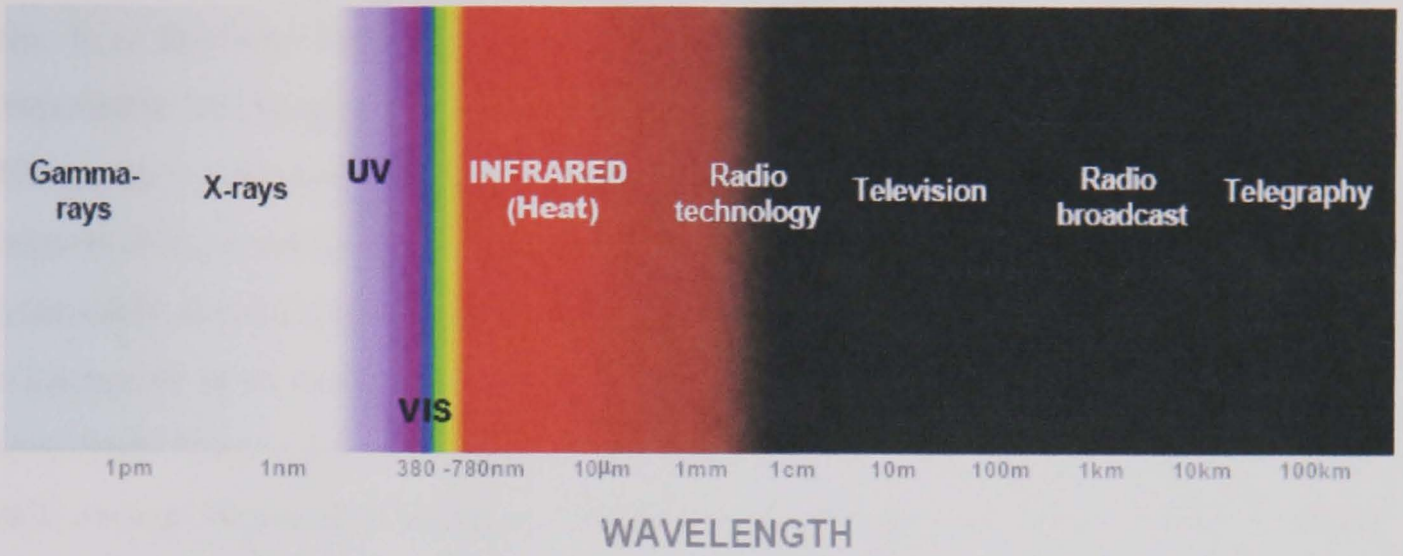


Figure 2.4.1.1 Distribution of electromagnetic radiation produced by the sun according to wavelength. *diagram obtained from Noblelight website [37].

2.4.2 Ultraviolet Region of the Electromagnetic Spectrum

Ultraviolet light occupies a band of wavelengths in the non-ionising region of the electromagnetic spectrum between X-rays and visible light (100-400 nm) [31]. The UV spectrum can then be further sub-divided into four regions, shown in Figure 2.4.2.1.

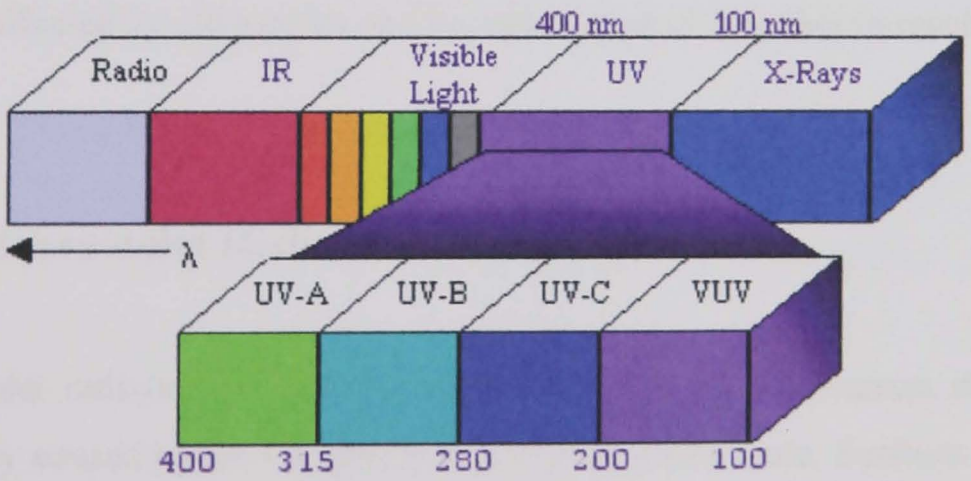


Figure 2.4.2.1 Ultraviolet Region of the Electromagnetic Spectrum, obtained from the IUVA website [38].

The region with the longest wavelength is UV-A, which extends from 315 nm to 400 nm. It is the least harmful because it has the lowest energy. It is also the region responsible for changes in human skin that lead to tanning [36, 22]. UV-B extends from 280 nm to 315 nm and is the most destructive form of UV light which reaches the earth; because it has enough energy to damage biological tissues, yet not quite enough to be completely absorbed by the atmosphere. UV-B is the region that causes skin burning and is known to cause skin cancer [36]. UV-C extends from 200 nm to 280 nm, is almost completely absorbed in air within a few hundred metres. When UV-C photons collide with oxygen atoms, the energy exchange causes formation of ozone. UV-C is almost never observed in nature, since it is absorbed so quickly. UV-C lamps are often used to kill bacteria and viruses since the radiation is absorbed by DNA/RNA, thus preventing replication [36, 31]. Vacuum UV extends from 100 nm to 200 nm and is absorbed by almost all materials thus can only be transmitted in a vacuum. [39].

Ultraviolet radiation is produced naturally by the sun (solar), but the intensity that reaches the earth depends on the attenuation by the atmosphere through absorption and scattering. UV-C has the shortest wavelength therefore very little reaches the earth's surface. UV-B has a slightly longer wavelength therefore some is able to reach the earth's surface, although it is also affected by attenuation. UV-A on the other hand is hardly affected by attenuation and therefore most of it is able to reach the earth's surface [31].

2.4.3 Ultraviolet Radiation and Human Health

Ultraviolet radiation is harmful to humans. The most common damage is sunburn, primarily caused by UV-B, although UV-A can contribute. Sunburn is characterised by blistering and swelling of the skin, which is a result of the body's immune response to UV. The greatest danger that UV poses on human health is skin cancer [40]. Damage to the eyes can also occur, with chronic UV exposure causing changes in the structure of the lens, leading eventually to cataracts, a loss of transparency [34].

Not all UV effects on human health are negative however. UV can be used to treat Vitamin D deficiency in skin cells. Vitamin D is essential for the growth and development of healthy bones [32] and small doses of UV-A can also be used to treat certain skin complaints such as psoriasis [40]. A significant positive attribute of UV radiation is its germicidal properties, and these are used in industrial and medical applications as discussed later in the chapter.

2.5 Artificial Sources of UV

Because the earth's ozone layer prevents UV-C (germicidal wavelengths) from reaching the earth's surface, practical application of UV disinfection therefore depends on the use of artificial UV sources [41]. There are several laboratory sources of UV radiation available, the most common being the electric arc and the mercury lamp, which are continuous sources of ultraviolet light. One of the most effective germicidal sources however, is the xenon flash lamp, which emits high intensity pulses of ultraviolet radiation [10].

2.5.1 Continuous UV Lamp Systems

The traditional laboratory source of UV is the mercury vapour lamp that can be adapted to give emissions that are either primarily in the UV-A, B or C region [31]. Most of these lamps contain a small amount of elemental mercury and an inert gas, which are contained in a tube made from a UV transmitting material such as quartz, with electrodes at either end. Figure 2.4.4.1 shows a diagram of the construction of a typical lamp. The mechanism of the lamp is the passage of electrons through the gas resulting in an electrical discharge causing the emission of radiation from excited mercury atoms. The wavelength of radiation that is emitted depends on the nature of the gas, pressure and the electrical conditions in the gas discharge. [34, 35].

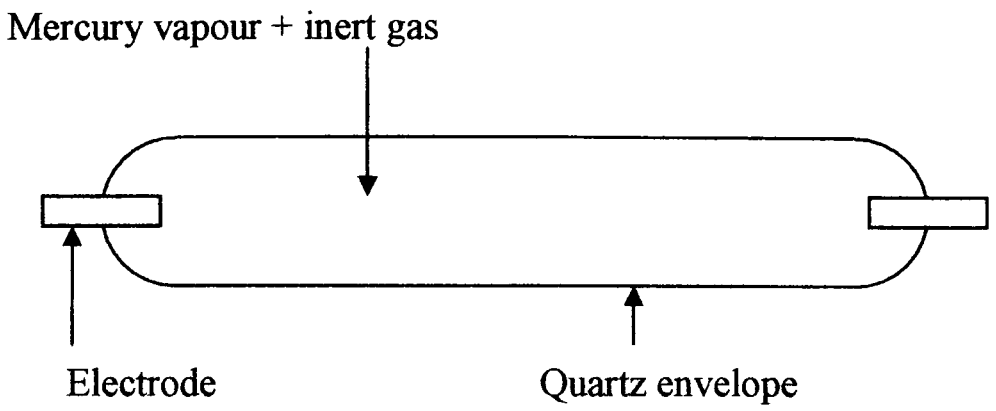


Figure 2.4.4.1 Ultraviolet lamp construction.

The emission lines of a mercury lamp are only sharp (monochromatic) when the pressure of the gas is low i.e. a pressure of less than 10 torr can result in wavelengths in the region of 250-260 nm being emitted. When the pressure is increased, the lamp intensity increases, but the emission lines broaden (polychromatic/broadband) resulting in the emission of wavelengths in the region of 250-500 nm [42]. Lamp types are therefore separated by gas pressure into two principal types: low pressure and medium pressure. Medium-pressure lamps are designed to operate at pressures above 100kPa, and the high plasma temperatures within medium-pressure lamps cause the vaporised mercury to exist in a number of excited states. Transition of the excited states to lower energy levels results in the emission of light at several wavelengths. Low-pressure lamps are operated at a lower power and are therefore more electrically efficient than medium-pressure lamps but medium-pressure lamps produce a greater UV content [41]. A comparison of the emissions of low- and medium-pressure lamps in the ultraviolet region is shown in Figure 2.4.4.2.

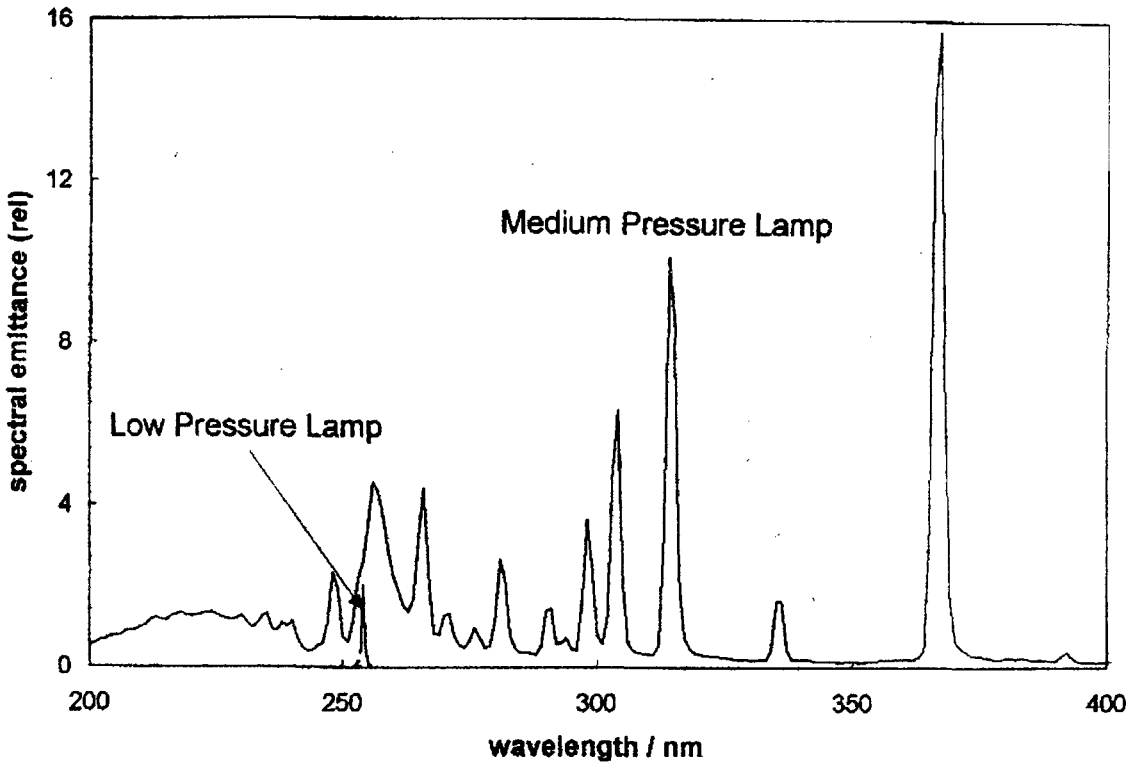


Figure 2.4.4.2 Relative spectral emittance from low-pressure and medium-pressure lamps. *Diagram obtained from "Ultraviolet Applications Handbook" by Bolton Photosciences Inc [42].

2.5.2 Pulsed UV Lamp Systems

Pulsed gas-filled flash-lamps are often used to produce pulsed UV light for sterilisation purposes [9]. Various inert gases can be used to fill the lamp, but xenon is the most widely used. A primary characteristic of the xenon flash-lamp is its capability of generating broadband radiation with high intensity emissions in the UV-C region, which has the most potential for directly damaging DNA [31, 10]. Pulsed UV light is created by storing electrical energy in a capacitor, and then releasing this as a short, high-intensity pulse, with a duration of between 1 μs and 0.1 s [2]. A modest energy input of a few joules can result in high peak-power dissipation of about 10^7-10^8 W [15]. The electrical pulse is applied to a xenon flash-lamp (normally a quartz or sapphire envelope containing the gas), in which the pulse ionises the gas to create plasma that expands to fill the lamp. During this process, outer-shell electrons are stripped away and intense

pulses of UV light are emitted [35]. It has been suggested that the shorter the pulse duration, the more effective the treatment process is [10]. The efficacy of the pulsed light system is attributed to the unique effects of the high peak power and broad-spectrum UV content as well as the ability to regulate both the pulse duration and the frequency of the output of the flash lamp [43, 10]. Changing the current of the system allows predetermined changes in the flash-lamp spectrum. Table 2.5.2.1 summarises the characteristics of the three types of UV lamp.

Characteristic	**Low-Pressure**	**Medium-Pressure**	**Pulsed-UV**
Wavelength	Monochromatic (85-90 % at 254 nm)	Polychromatic (185-1400 nm)	Polychromatic (185 to 800 nm)
Emission	Continuous-wave	Continuous-wave	Up to 30 pulses per second
Mercury Vapour Pressure	10^{-3} to 10^{-2} torr	10^{2} to 10^{4} torr	Typical Xe pressure is 450 torr.
Operating Temperature	40 to 60 °C	500 to 800 °C	15, 000 °C
Arc Length	40 to 75 cm	5 to 40 cm	15 cm
Lifetime	8,000 to 10,000 h	2,000 to 5,000 h	> 9,000 h at 30 pulses/sec
Relative Light Intensity	Low	Medium	High

Table 2.5.2.1 Characteristics of typical low-pressure, medium-pressure and pulsed-UV lamps. * Table adapted from "Pulsed-Ultraviolet Light for Drinking Water Disinfection", found at www.epri.com [44].

2.5.3 Advantages of Pulsed Over Continuous UV

It is well documented that ultraviolet light is effective in killing those microorganisms that contaminate drinking water, food and contact areas. The way in which the UV is applied (continuous or pulsed) can have a significant effect on the inactivation results, with pulsed UV being considered to be the most successful. The main advantage of pulsed lamps over continuous lamps is that they have a high peak-power dissipation which allows for more rapid inactivation. A continuous 10 W lamp needs to be operated for 10 seconds to achieve the same result (supply the same energy) as a pulsed lamp of typically 1 MW operated for 100 μs. Higher energies provide higher levels of penetration into the medium containing the microorganism [10].

When using continuous UV radiation, a phenomenon known as *tailing* is observed to occur after 3 to 5 logs of inactivation. Tailing is what can be observed on inactivation curves whereby an initial population decrease occurs, followed by very little or no further inactivation, which is characterised as a tail on the inactivation curve. This causes the process to become inefficient and large increases in exposure time are required to produce further improvement in the level of inactivation. It has been demonstrated by Otaki *et al*, 2003 that tailing occurs when using continuous UV to treat high turbidity solutions, whereas none occurs when a pulsed xenon lamp is used [45].

Most continuous UV lamps contain mercury, which is toxic and can present a hazard should the lamp envelope become damaged. Pulsed lamps however generally contain xenon, which is not considered environmentally unfriendly.

Finally, conventional continuous UV exposure primarily affects DNA by mechanisms that are reversible under certain conditions (Photoreactivation). However if using pulsed UV, adjusting the required light intensity, pulse duration and number of pulses can lead to ultimate genetic destruction of various microorganisms [10].

2.6 Effects of UV on Microorganisms

The germicidal effects of radiant energy from the sun were first reported in 1878 [41]. In the spectrum from the sun, ultraviolet radiation has the shortest wavelength and thus is the highest energy solar radiation reaching the earth. It is the high energy of UV that makes it potentially damaging to living organisms. Practical application of UV led to the development of the mercury vapour lamp as a laboratory UV source (1901) and the recognition of quartz as the ideal material for the lamp envelope (1905) [41].

UV light has been recognised as an effective antimicrobial agent since the end of the 19th Century, with UV-C radiation in the range of 250 nm to 260 nm being the most lethal to most microorganisms (bacteria, viruses, protozoa, mycelial fungi, yeasts and algae). Ultraviolet light photons of different energies have various effects on DNA, with many reporting that the maximum effect of UV-C can be observed at 254nm [23, 31, 10]. It can be seen from Figure 2.6.1 that the maximum absorbance of DNA occurs at around 260 nm. This suggests that light of this wavelength may produce maximum inactivation of microorganisms.

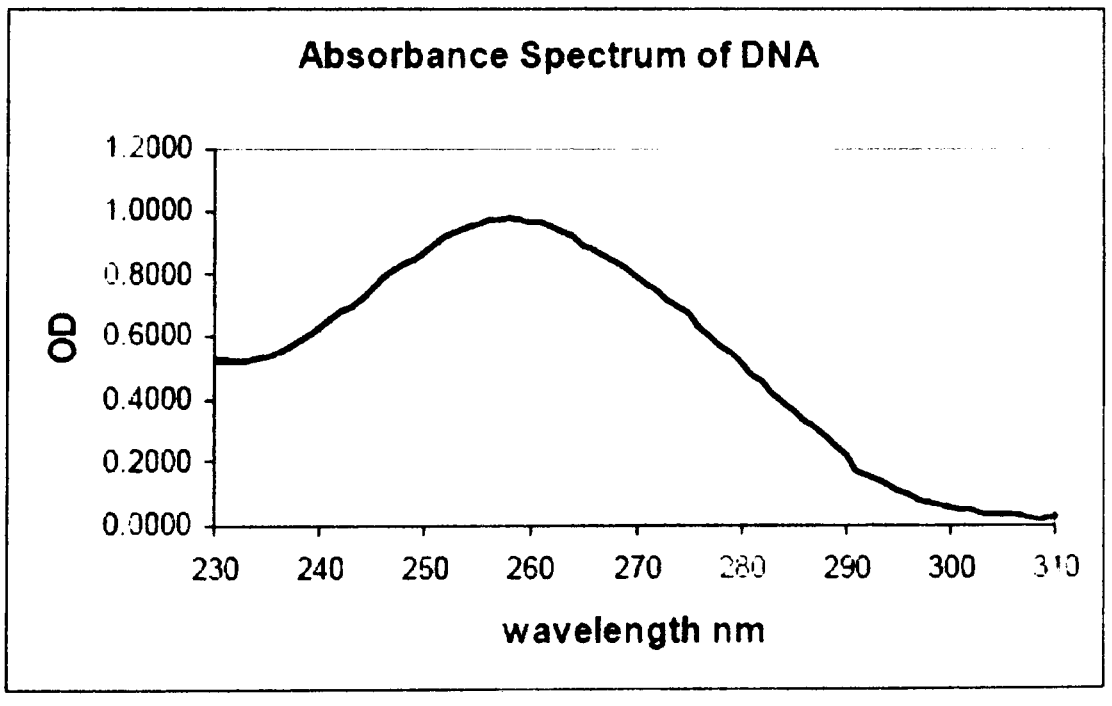


Figure 2.6.1 Absorbance Spectrum of DNA. *diagram obtained from http://www.tecan.com [46].

2.6.1 Microbial Damage

The antimicrobial effects of UV wavelengths are primarily mediated through the absorption of a UV photon, by highly conjugated carbon-to-carbon double-bond systems in proteins and nucleic acids of microorganisms [9]. The part of a cell most vulnerable to UV damage is the DNA and RNA. This is due to their unique function as the depository of the cell genetic code and also because of their highly complex structure and size [47]. In DNA molecules the backbone of ribose sugars and phosphates does not absorb UV significantly above 220 nm. The nucleotide/nitrogenous bases do absorb UV radiation above this wavelength and are where most of the damage occurs [35, 47]. The structure of a DNA molecule is shown in Figure 2.6.1.2

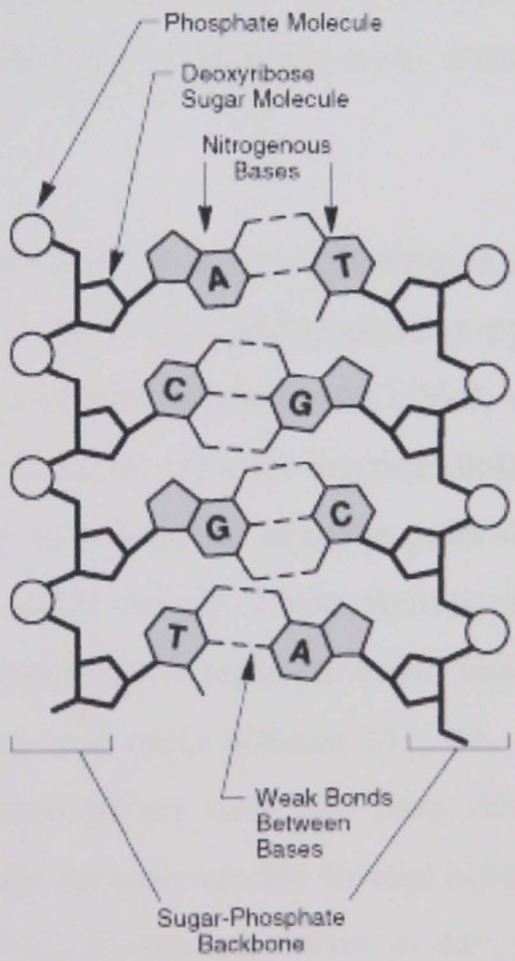


Figure 2.6.1.2 DNA Structure. *Image credit: U.S. Department of Energy Human Genome Program: http://www.ornl.gov/hgmis [48].

The four nucleotide bases of DNA (shown in Figure 2.6.1.2) are arranged along the sugar-phosphate backbone. They consist of:

1. Cytosine (C) } Pyrimidines
2. Thymine (T) (Uracil in RNA) }
3. Adenine (A) } Purines
4. Guanine (G) }

Cytosines form a strong hydrogen bond with Guanine and are therefore known as complementary bases. Adenine and Thymine are also complementary bases. The purine bases (adenine and guanine) are about 10 times more resistant to the effects of UV than the pyrimidines (cytosine and thymine) and, the major effects of UV radiation on biological systems are attributed to photochemical transformations of the pyrimidine bases [35].

The two most common forms of UV-induced damage in DNA involve the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PPs) [35]. CPDs are produced by UV-C and UV-B, which act directly on DNA by producing covalent bonds between adjacent thymine molecules to give thymine dimers, that is, instead of pairing with adenine, thymine pairs with another thymine base (in RNA, uracil pairs with another uracil). These thymine dimers form a four-membered cyclobutyl ring, which inhibits DNA replication and function, resulting in the bacteria being unable to reproduce and cause disease [31, 35, 39]. Cytosine-thymine mixed dimers and cytosine-cytosine dimers have also been identified but are less frequently found [35]. Thymine dimers are more readily formed because thymine absorbs UV more effectively than cytosine [49]. Cytosine however, is the pyrimidine most often involved in the formation of 6-4 PP's, either as cytosine dimers or mixed dimers [35].

UV radiation also causes photochemical reactions in proteins, including enzymes and other molecules within the cell. Absorption by proteins peaks at around 280 nm and there is some absorption by the peptide bond within proteins at wavelengths below 240 nm. Proteins are more complex and variable in their responses compared with the much more sensitive nucleic acids. Proteins with high tryptophan, tyrosine, phenylalanine and cysteine content are particularly prone to damage [35]. Other biological molecules with unsaturated bonds, for example, coenzymes, hormones and electron carriers, may also be susceptible to destruction by UV. This is important in larger microorganisms such as fungi and protozoa. Although UV radiation may be unable to penetrate these microorganisms as far as the DNA, it could still have a lethal effect by damaging other molecules [47].

Other direct effects produced by UV-C and UV-B include formation of DNA-protein cross-links, rare base adducts and inter- and intra- strand cross-links [39]. UV-A is also known to damage microorganisms mainly by exciting photosensitive molecules inside the cell to produce active oxygen species. These damage the genome and other intracellular molecules causing lethal and sublethal effects such as mutations and growth delay [50]. In bacterial spores treated with UV radiation, the formation of 5-thyminyl-5, 6-dihydrothymine, has been shown to cause lethal effects by preventing replication [51].

2.6.2 Reactivation of Microorganisms

Because many microorganisms are exposed to potentially high levels of UV irradiation on a daily basis, the ability to repair damaged DNA is essential to an organism's survival and consequently many organisms are known to have DNA repair mechanisms that act to reduce or eliminate UV-induced damage. The process by which they do this is known as *reactivation*, which is a natural defence mechanism that has evolved as a result of exposure to UV radiation from sunlight [52, 47]. Reactivation can take place under both light (photoreactivation) and dark (dark repair) conditions [47].

Photoreactivation was first identified in the late 1940s and early 1950s, when it was observed that the detrimental effects of UV light could be reversed by illumination with longer wavelengths [53]. Photoreactivation involves specific recognition and binding of a small enzyme called a photolyase to a cyclobutane dimer. Photolyases are monomeric proteins of 50-60 kDa with stoichiometric amounts of two noncovalent cofactors. One of these cofactors is $FADH^-$, and the second is methenyltetrahydrofolate (MTHF) [131]. MTHF is a light harvesting molecule that absorbs mostly in the UV-A and visible wavelengths (300-500 nm). On absorption of a photon within these wavelengths MTHF transfers its excitation energy to $FADH^-$ which in turn transfers an electron to the pyrimidine dimer causing it to split [131, 132, 54]. The original structure is then restored, allowing replication to take place [39]. Figure 2.6.2.1 shows the reaction that takes place between two thymine dimers when UV-C is present, and the reversal of the reaction when photolyase is activated by UV-A/visible light.

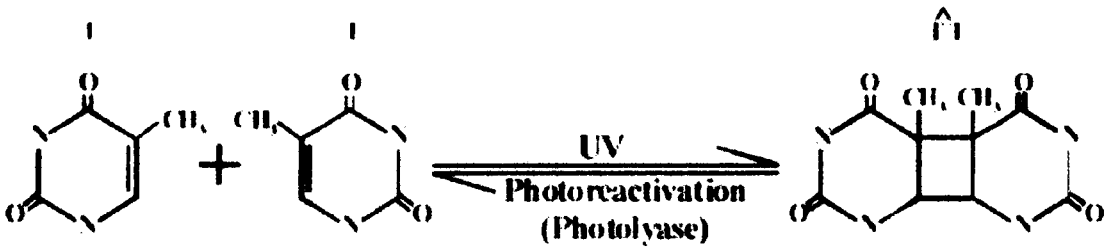


Figure 2.6.2.1 Formation of a thymine dimer and its reversal when photolyase is activated.

UV-A radiation is essential for photoreactivation, although it also has lethal and sublethal effects on microorganisms. This phenomenon is called concomitant photoreactivation because the inactivating light itself has the potential to repair the dimers [50]. Once photolyase has bound to a CPD, the efficiency of reactivation is extremely high: approximately one dimer split for every photon absorbed [55]. Several classes of photo-repair enzyme have been characterised according to their photoreceptors and whether or not they act on cyclobutane-pyrimidine dimers or pyrimidine-pyrimidone photoproducts [53]. The photolyase of *E.*coli for example, is

specific for repair of pyrimidine dimers, while some organisms have been found to have a photoreactivating enzyme specific for (6-4) photoproducts [50].

The ability of a microorganism to reactivate varies significantly depending on the type of UV damage and the level of biological organisation of the microorganism. The repair mechanism is universal and there are no clearly defined characteristics determining which species can or cannot repair themselves [47]. Other factors affecting photoreactivation include UV dose, water quality, exposure time to photoreactivating light and the type of microorganism [11]. Viruses have no repair mechanism that can reverse the damage created by UV light [41] and Belosevic *et al* have demonstrated that *C. parvum* oocysts do not undergo reactivation [56].

UV-induced transformations in the nucleic acids other than thymine dimers, for example, cytosine dimers, can only be repaired by dark-repair mechanisms [41]. These systems are inducible suggesting that cells will likely use them to respond to damage to DNA arising from increasing UV exposure [39]. There are 3 distinct DNA dark repair mechanisms; (i) Nucleotide-excision repair, (ii) SOS-error prone repair and (iii) Post-replication recombinational repair [39]. The nucleotide excision repair process involves the action of more than a dozen proteins that coordinate the removal of DNA damage [52]. The energy source that activates the dark repair enzymes is nutrients within the cell [47]. The activated enzymes recognise and bind to the helical distortion created at the damaged site, and thus initiate nucleotide-excision repair. A repair complex is assembled and cleaves the DNA at positions a few bases to either side of the lesion, leaving a gap. This gap is filled by a DNA polymerase and the strand is ligated to restore the DNA duplex to its original state [54]. Nucleotide-excision repair removes a wide range of DNA distortion lesions including CPDs and 6-4PPs. The SOS-error prone and post-replication recombinational repair mechanisms only extend the life of the cell by repairing damaged DNA strands. They do not remove abnormal bases from the UV-damaged site therefore the actual damage must eventually be removed by the excision repair system [39].

2.6.3 Sensitivity of Microorganisms to Ultraviolet Radiation

There are many factors that determine the sensitivity of microorganisms to UV exposure:

Suspending Medium: Water turbidity, concentration of suspended solids and fluid thickness all affect the inactivation efficiency of UV irradiation [11]. In the laboratory for example, high-intensity UV radiation is required to sterilise microbes suspended in liquids such as broths, because turbid materials attenuate and scatter UV radiation more than transparent materials such as water or buffer [35, 22].

Cell Density: Cell density during treatment is an important factor. For example if *clumps* are present some of the microorganisms may be shielded from the UV radiation. It has been suggested that the aggregation of bacteria can be viewed as a natural defence mechanism for protection from UV [57].

Growth Phase: There are four distinct phases of growth for a microorganism and the sensitivity to UV radiation differs with phase [58]. Figure 2.6.3 shows the typical growth cycle of a bacterial population.

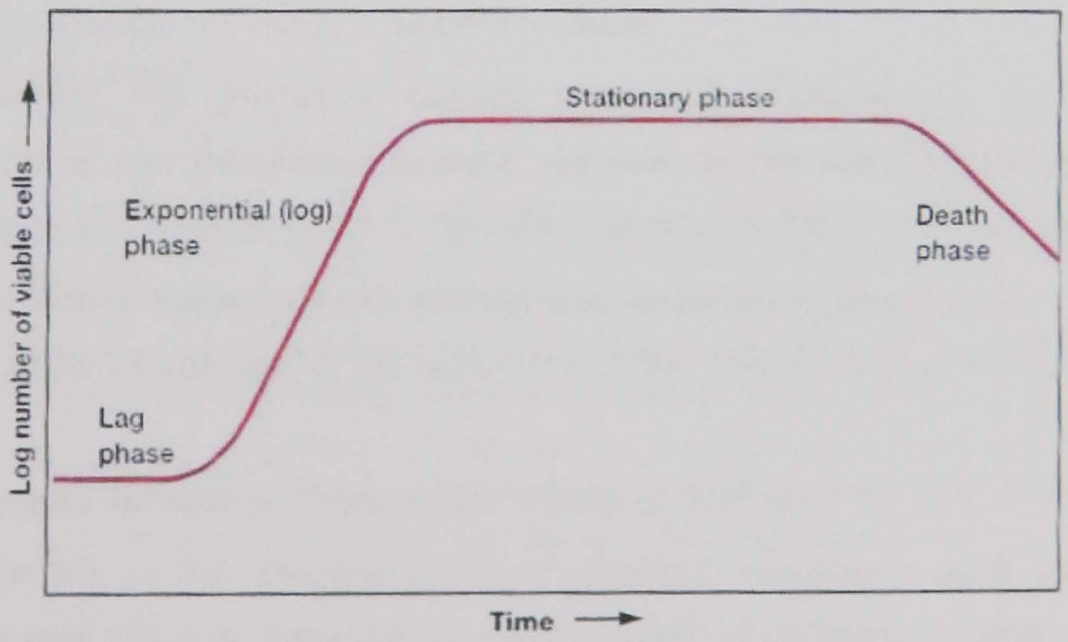


Figure 2.6.3 Bacterial Growth Cycle

The four stages of growth are:

1. Lag Phase: There is no immediate increase in number as cells adjust to their new environment and synthesise new components such as enzymes, in order to utilise new nutrients in the medium.
2. Exponential Phase: The organisms are growing and dividing at the maximum possible rate. The population increases exponentially, and the reproductive rate reaches a constant as DNA and protein synthesis are maximised.
3. Stationary Phase: This exists when the rates of production and loss of cells are equal. This balance develops as a result of nutrient limitations and accumulation of waste products.
4. Death: The death phase occurs when no nutrients are available and wastes are not removed. The cells then die at a faster rate than they are produced.

[20, 59].

Microorganisms are most sensitive to UV when in the exponential phase [35]. This may be due to the fact that when the majority of microorganisms enter stationary phase, they undergo structural and physiological changes that results in increased resistance to heat shock, acid stress etc [60]. These changes may also account for the decreased sensitivity of microorganisms to UV when in stationary phase.

UV Exposure: The amount of damage created by UV radiation, and hence the effectiveness of the disinfection process, depends on the intensity of light and the exposure time to that intensity [61]. Specific energies needed to achieve total or partial destruction of microorganisms vary according to the physio-chemical composition of the substance to be treated and to the sensitivity of the microorganism to UV irradiation [11].

Environmental Influences: Temperature variations between 5 °C and 37 °C have little if any influence on the microbial action of radiation. Moisture is quite an important factor because, when bacteria are suspended in air, an increase in relative humidity results in a greatly reduced death rate [31]. pH has been proven to have little impact on the rate of microbe inactivation [41].

Other Factors: The cleanliness of the surface being treated is important because if dirt is present it may absorb UV radiation [31]. When treating water the presence of suspended contaminating particles can result in absorption and scattering of UV radiation. Some bacteria have a unique UV-absorbing protective exterior, for example, bacterial spores, which require relatively high doses of UV radiation to produce inactivation [41].

2.7 Applications of Ultraviolet Irradiation

Decontamination/sterilisation using UV radiation is an effective method of either eliminating or reducing the bacterial load in liquids, in the air or on surfaces. It does not affect taste, smell, colour or nutrient content of the products(s) being treated, and the efficiency can be further improved when used along with other methods such as heat, chemicals and ozone. The following sub-section describes some of the major uses of ultraviolet radiation (pulsed and continuous) for disinfection.

2.7.1 Contact Surfaces and Foodstuffs

Present disinfection and sterilisation methods of surfaces generally involve the use of biocidal chemicals, such as hydrogen peroxide. Residues of these chemicals however are highly undesirable, as they pose a potential occupational hazard and lead to large waste streams that must be treated to avoid adverse environmental impacts. UV light can however be used to reduce or eliminate the need for chemical disinfectants and preservatives [30, 9]. When disinfecting surfaces the nature of the material on which the microorganism is deposited is important. Substances such as aluminium and glass can be successfully sterilised whereas porous materials such as wood, rubber and paper cannot [35]. Work surfaces in operating theatres, surgical and dental instruments and surfaces involved in food processing can be decontaminated using UV light. [62, 35]. When irradiating three-dimensional objects, such as a dental drill, it is necessary to ensure that

all surfaces receive adequate exposure to the UV [63]. *Pseudomonas* and *Streptococcus* and fungi such as *Candida* and *Aspergillus* have been inactivated on contact lens surfaces after 20 minutes of treatment with UV irradiation [2].

Surfaces of foods often become contaminated with pathogens. The use of UV however has proven to be an effective technology for reducing the level of these pathogens and thus prolong shelf lives [64]. Bacterial loads on fresh meats, for example, chicken, can be effectively reduced by UV irradiation without adversely affecting poultry carcass colour or increasing meat rancidity [16]. Foods that have been successfully treated with UV include beef, poultry, sausages, fish, eggs and chocolate [63]. Pulsed light has been used to provide dramatic shelf-life extension and preservation of a variety of foods including baked goods, seafood, fruit and vegetables [65]. Bread is prone to contamination with mould spores but when treated with UV, as it emerges from the oven, its shelf life can be usefully extended [31]. Prepared and processed meat products such as sausages and ground meat pâté can also be successfully treated with UV light pulses to increase their shelf life under refrigeration without the necessity for freezing [9].

UV sterilisation has also been successfully used for aseptic yoghurt filling, and the associated packaging materials. It was found that when stored at 5-7 ^{o}C, the shelf life of the yoghurt was extended by about 2 weeks [31]. UV light treatments that achieve high inactivation rates on media, packaging or relatively simple surfaces generally only give a 1-3 log reduction on complex surfaces, such as meats. This is due to the presence of small surface recesses, fissures and folds that allow some microorganisms to avoid exposure. UV irradiation of meats in general produces only a reduction in the bacterial load, but this does provide a useful increase in shelf life [65].

2.7.2 Air

Ultraviolet light has been demonstrated to effectively inactivate airborne bacteria, viruses and mould species, and its application is now widespread throughout the process industries [66]. Air purification by UV radiation is used by health care providers and those with an interest in indoor air quality [66]. Recent concern about *sick building* syndrome, the anthrax scare and the SARS crisis have led to an increased interest in UV air treatment for disinfection and removal of contaminants [64].

Hospitals in the United States have used ultraviolet radiation for air sterilisation for over 50 years, where it has been shown to reduce 60 % of airborne contamination in operating theatres. An additional advantage is that if a wound infection occurs in a patient, the ultraviolet radiation can exert a bactericidal effect. A drawback however is that both staff and patients must be protected from the radiation. The protective clothing necessary for this is inconvenient to use and uncomfortable which is the main reason ultraviolet radiation has not gained acceptance by surgeons in the UK [67]. The use of UV lamps in quarantine and other infectious-disease control rooms has been shown to achieve 30-100 % reduction in the levels of *Micrococcus luteus*, *Bacillus subtilis* and *E. coli* [68].

In areas where large numbers of people are brought together in close proximity, there is the potential for the build up of substantial airborne contamination and therefore cross-infection. Maclean *et al*, have demonstrated that pulses of UV-rich light are effective in reducing levels of airborne bacteria in university lecture theatres, where airborne contamination was found to rise significantly over the period of a lecture [69].

Ultraviolet radiation has been incorporated into fan-powered filtration and heating, ventilating and air-conditioning duct systems. In combination with filtration this technology has been shown to achieve inactivation efficiencies in the high 90 % range [68]. When a UV system was installed in the recirculating air duct at a call centre in the

UK, a 40 % reduction in absence due to sickness was reported [66]. When UV is combined with an air sterilisation unit the microbiological quality of air in cold stores can also be improved [31]. The use of UV radiation along with titanium dioxide as a photocatalyst has been reported to kill many air contaminants including *Serratia marcescens* and *Escherichia coli* [70].

2.7.3 Liquids

The most widely used germicidal application of UV radiation is in the treatment of liquids, especially water. It is considered as a credible alternative to chemical disinfection, because of the absence of toxic by-products which are usually generated and identified during chemical disinfection [11]. When treating natural water supplies however, suspended solids must be filtered out prior to treatment to prevent unwanted absorption of the UV [31].

UV water purification has been applied to water wells, cisterns and swimming pools to avoid heavy chlorination. It is also used where biologically pure water is required, for example, in the production of beer, soft drinks, pharmaceuticals and cosmetics. [35]. UV radiation is used for treating seawater used in the shellfish industry, because chlorine has an adverse effect on the feeding activities of oysters [35].

A disadvantage of UV treatment of liquids is that there can be a lack of penetration. In distilled water, UV radiation at 254 nm suffers a 30 % intensity reduction over a distance of 40 cm. In seawater the same reduction occurs over 10 cm and in a solution of sucrose (10 %), or natural spring water containing high levels of iron, the same loss occurs within 5 cm. UV radiation can however, be combined with ozone purification to provide a powerful oxidizing action that reduces the organic content of water to extremely low levels [31].

The treatment of opaque and coloured liquids such as milk presents more of a problem [31], although there has been some success with the treatment of fruit juices. Fruit juices are normally pasteurised, but this can alter the taste. UV on the other hand, despite the fact that it is strongly absorbed by fruit juices, has been shown to successfully disinfect them without changing flavour. The process requires a dedicated UV reactor that enables the UV radiation to interact efficiently within the volume of liquid [25]. Other areas of research have found that absorptive or coloured materials, which are not treatable at filled-container depths, can be successfully treated in the form of millimetre-thick layers [35].

There is a worldwide problem of blood-supply contamination by various microorganisms, which has led to the need for effective methods of inactivating these pathogens [10]. Human plasma has been successfully sterilised by treating it with a combination of beta-propiolactone (BPL) and UV irradiation. Patients receiving this plasma (581 patients) did not develop transfusion hepatitis [35]. Because the method of inactivation is by cross-linking of DNA/RNA, the blood components used for transfusions (platelets plasma and red blood cells) retains their biological activity after exposure, since they do not contain DNA or RNA [10].

2.8 Drinking Water and Wastewater Treatment

Water is implicated as a medium for the transmission of many diseases. Estimates by the World Health Organisation indicate that about 500 million people are affected yearly by waterborne or water-associated diseases, and of these about 10 million die [71]. Most of these diseases are caused by contamination of water by human and animal excreta [72]. Chlorination is the established disinfectant for both drinking water and waste water in the UK and US respectively, but increasing concern over the environmental impact of chlorine has led to the development of alternatives [73]. UV-light disinfection is being increasingly used in the treatment of both wastewater and potable drinking water since

such treatment does not produce disinfectant by-products and it is effective against protozoans that are resistant to chlorination [74].

2.8.1 Drinking Water

The transmission of pathogens in drinking water is a widespread problem, affecting not only the countries with low hygienic standards but also industrialised countries [76]. Since 1990, the number of people without access to safe water sources has remained constant at around 1.1 billion, of whom approximately 2.2 million die of waterborne disease each year [77]. The most common source of pathogenic diarrhoea-causing microorganisms is contaminated drinking water which is either untreated, inadequately treated, or becomes contaminated during collection, handling, storage or use [78, 72].

The primary source of drinking water is rain. When it rains, water flows into rivers, streams, lochs and reservoirs, and this surface-water must be filtered and disinfected to protect against the threat of microbiological contaminants. Alternatively, water can seep through the ground until it reaches rocks that it cannot pass through. It then forms water pools and this is known as ground-water. Ground-water needs either no treatment or only disinfection before use as drinking water, because soil and rock act as a filter to remove pathogenic microorganisms [79, 80]. In Scotland, most customers receive their tap water from surface-water sources [79]. The principal objective of water treatment should be the reduction of pathogens in the water to levels that will not cause disease. The standards for drinking water quality as recommended by the World Health Organisation are 10 Total Coliforms per 100 ml and 0 Faecal Coliforms per 100 ml [81]. Contamination of public drinking water can occur by several means with the main risk areas being [82]:

(a) Abnormal contamination of the raw water source

(b) Water treatment breakdown

(c) Water treatment operating above design capacity or under stress

(d) Non-availability of electricity, treatment chemicals or essential materials

(e) Water mains burst and repairs

(f) Mains renovation and renewal

(g) Structural faults in service reservoirs

(h) Vandalism

Figure 2.8.1.1 shows a flow chart of the typical treatment processes involved in drinking water disinfection [79, 20]. Filtration removes up to 99 % of the bacteria before the water is treated with a disinfectant, usually involving chlorination [20]. A concern however, is the by products which are produced when chlorine reacts with organic matter; these include haloacetic acids (HAA's), total trihalomethanes and chlorite. Their potential health effects include anaemia, liver, kidney and central nervous system problems as well as an increased risk of cancer [78]. If chlorines and chloramines are present at levels above the maximum contaminant level (MCL), set by the National Primary Drinking Water Standards, they can cause eye/nose irritation and stomach discomfort [78]. The potential harmful effects of using chlorine, as well as the resistance of some microorganisms to chemical disinfectants, has created the need for alternative disinfection processes. The alternative is ozone treatment, while treatment with ultraviolet radiation is becoming increasingly attractive. UV technology was first used in water treatment in Ft. Benton, USA in the early 1970's. There are now over 2000 installations in Europe and over 1000 installations in the United States using UV radiation to disinfect drinking water [41]. Chlorine and UV disinfection are the principal drinking-water disinfectants used in Norway [83].

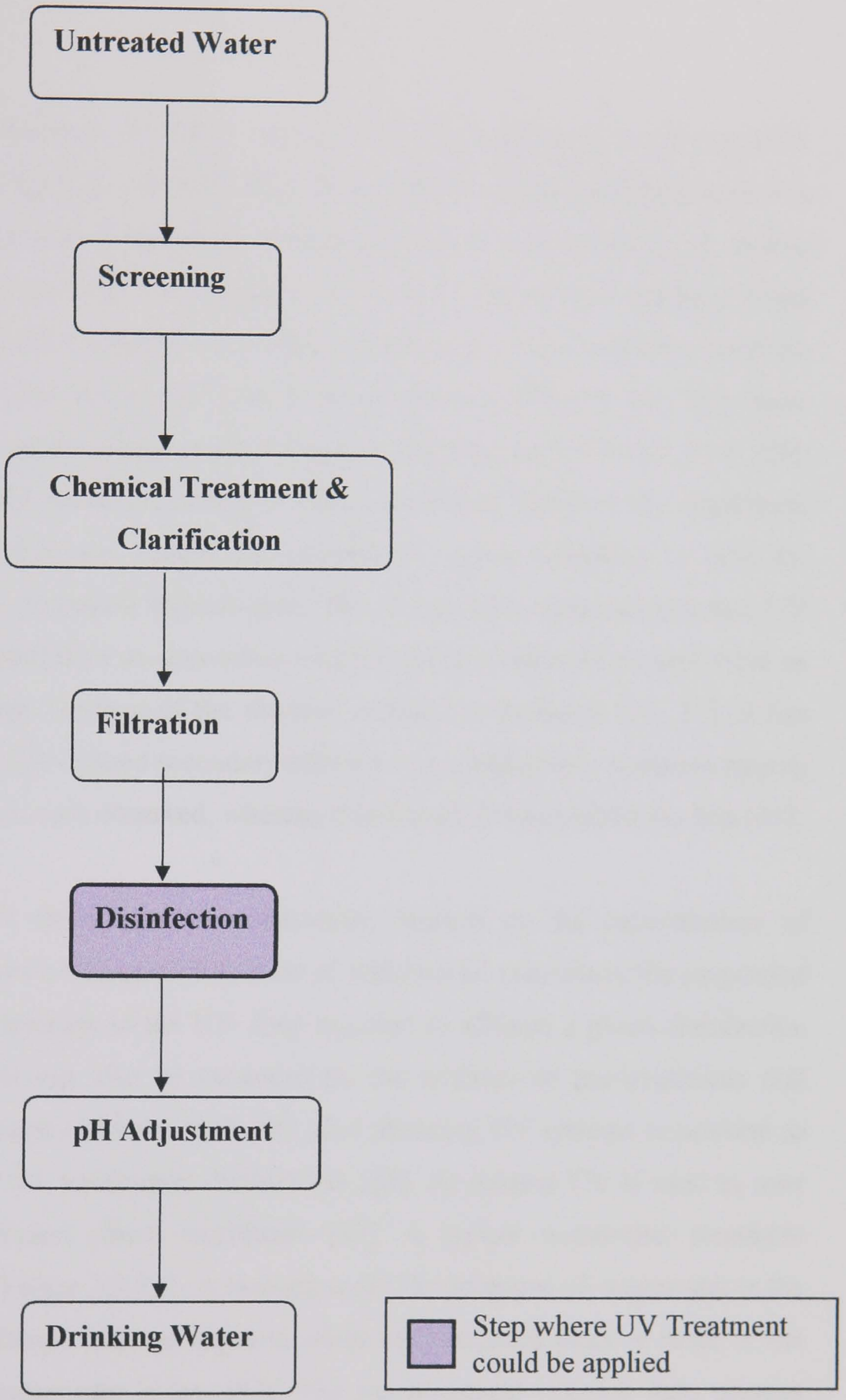


Figure 2.8.1.1 Typical Drinking Water Treatment Process.

2.8.2 Wastewater

Wastewater is a combination of human faeces, urine and graywater (water used for washing, bathing and meal preparation) [80]. Wastewater is treated and disinfected to reduce contamination to environmentally acceptable levels before returning the treated water to rivers and the sea [84]. The European Community Directive for Bathing Water has set standards for both total and faecal coliform indicators. Total coliforms must not exceed 10,000/100 ml and faecal coliforms must not exceed 2,000/100 ml. They have also set a standard for enteroviruses which is 0 plaque forming units (PFU) in 10 L [85]. Chlorine is traditionally used to disinfect treated wastewater; however the significant level of by-products that are formed has provided a strong incentive to look for alternative wastewater treatment technologies [86]. It has been demonstrated that UV irradiation may be regarded as an alternative method to chlorination of sewage from an activated sludge process because of the absence of toxic by-products [35, 11]. It has been shown that when UV-treated secondary effluent was released into water containing trout, no adverse effects were observed, whereas chlorinated effluent killed the fish [35].

The efficiency of UV disinfection of wastewater depends on the concentration of suspended solids and their diameters. Filtration of wastewater, can reduce the suspended solids and provide a decrease in the UV dose required to achieve a given disinfection target. UV disinfection can also be enhanced by the addition of pre-treatments that increase UV transmittance and lower turbidity, thus allowing UV systems to perform to the standards required for wastewater disinfection [86]. At present UV is used in over 1500 wastewater treatment plants worldwide [87]. A typical wastewater treatment process is depicted in Figure 2.8.2 .1. A limitation of UV treatment of wastewater is the large number of UV lamps that are required when the treatment plant is large or the transmittance of the wastewater is low [87]. The use of pulsed UV-rich light sources such as the xenon flash-lamp can help overcome this limitation because fewer lamps are needed as a result of the high intensity emission of the pulsed source and its broadband spectral output.

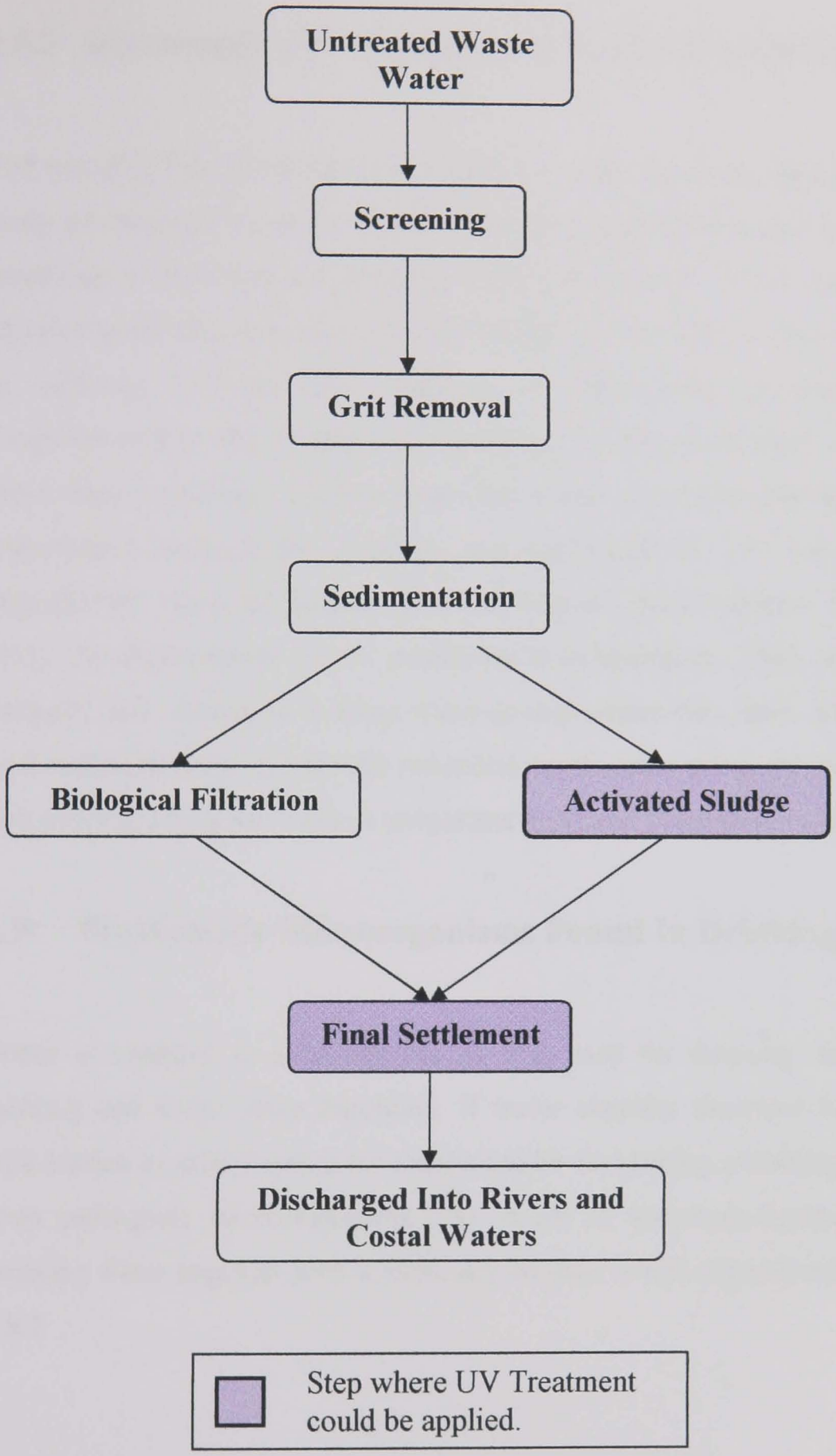


Figure 2.8.2.1 Typical Wastewater Treatment Process.

2.8.3 Advantages of UV Disinfection Over Chemical Disinfection

The use of UV as a disinfection method for water treatment (potable and waste) holds many advantages over using standard chemical disinfectants such as chlorine. The major advantage is that there are minimal health risks because no carcinogenic or mutagenic chloro-organic by-products are formed by the UV radiation as occurs with chlorine [41]. In addition, UV is very effective at inactivating protozoan cysts such as *Cryptosporidium* and *Giardia*. Chlorination, on the other hand, is ineffective against these microorganisms. Another advantage is that many microbial molecules such as the sugar-based extracellular polymers are unaffected by UV light, whereas they are degraded by chemical disinfectants and become good nutrients for microbial growth [41]. The effectiveness of UV irradiation is independent of pH, temperature and ionic strength, and variations in these water-quality parameters have minimal impact on the disinfection process. Successful microbial inactivation using chlorine on the other hand is dependent on factors such as temperature, pH and intracellular diffusion [8].

2.9 Problematic Microorganisms Found In Drinking Water

Water is essential to everyday life as it is used for drinking, recreational purposes, farming and many other functions. If water supplies therefore become contaminated with human or animal waste the results can be devastating, resulting in illness and deaths from pathogenic microorganisms [71]. A list of waterborne pathogens transmitted in drinking water together with a summary of their health significance, is shown in Table 2.9.1.

Pathogen	Health Significance	Persistence In Water Supplies	Resistance To Chlorine	Relative Infectious Dose	Important Animal Reservoir
Bacteria					
Campylobacter jejuni, C. coli	High	Moderate	Low	Moderate	Yes
Pathogenic *Escherichia coli*	High	Moderate	Low	High	Yes
Salmonella typhi	High	Moderate	Low	High	
Other *Salmonellae*	High	Long	Low	High	Yes
Shigella spp	High	Short	Low	Moderate	No
Vibrio Cholerae	High	Short	Low	High	No
Yersina enterocolitica	High	Long	Low	High (?)	No
Pseudomonas aeruginosa	Moderate	May Multiply	Moderate	High (?)	No
Aeromonas spp	Moderate	May Multiply	Low	High (?)	No
Viruses					
Adenoviruses	High	?	Moderate	Low	
Enteroviruses	High	Long	Moderate	Low	No
Hepatitis A	High	?	Moderate	Low	No
Enterically transmitted non-A, non-B hepatitis viruses, hepatitis E	High	?	?	Low	No
Norwalk virus	High	?	?	Low	No
Rotavirus	High	?	?	Moderate	No (?)
Small round viruses	Moderate	?	?	Low (?)	No
Protozoa					
Entamoeba histolytica	High	Moderate	High	Low	No
Giardia intestinalis	High	Moderate	High	Low	Yes
Cryptosporidium parvum	High	Long	High	Low	Yes
Helminths					
Dracunculus medinensis	High	Moderate	Moderate	Low	Yes

Table 2.9.1 Orally transmitted waterborne pathogens and their significance in water supplies. * Table obtained from World Health Organisation website[81].

From table 2.9.1 it can be observed that there is a variety of different microorganisms responsible for transmitting disease through contaminated water. A list of microorganisms and the number of outbreaks of illness associated with UK water supplies, for the period 1991-2000, is shown in Table 2.9.2.

Pathogen	**Public Supplies**	**Private Supplies**
Cryptosporidium	24	4
Giardia		1
Campylobacter	4	16
E. coli 0157	2	4
Salmonella		1
Unknown		2

Table 2.9.2 Outbreaks of illness associated with public and private drinking-water supplies in the UK (1991-2000). * Data obtained from the Environment Agency Report 2002 [82].

It can be observed from Table 2.9.2 that *Cryptosporidium* is responsible for a large number of outbreaks in public drinking-water supplies, whereas in private supplies *Campylobacter* spp has produced the largest number of outbreaks.

Most drinking-water supplies are generally disinfected by chemical means or ozone exposure. Numerous water sources (mainly private water supplies) do not receive this treatment and therefore pose an unacceptable risk to human health. Pulsed UV treatment could be used for such water supplies or as an additional treatment where levels of particular microorganisms (for example, *Cryptosporidium*) are high. Information on the waterborne pathogens that present a serious risk to humans is presented next.

2.9.1 Coliform Bacteria

Coliform bacteria are considered suitable microbial indicators of drinking water quality. These are rod-shaped bacteria, capable of growth in the presence of bile salts and able to ferment lactose [20]. Generally these bacteria originate as microorganisms in soil or vegetation and in the intestinal tract of warm-blooded animals and humans [88]. Many types of coliform bacteria are harmless, but their presence in drinking water indicates that disease-causing microorganisms may be present [89, 90]. Within the coliform group, a sub-group exist known as faecal coliform bacteria which appear in high numbers in sewage and polluted-water sources. Their presence in drinking water indicates potential faecal pollution and a failure of treatment and disinfection procedures. The faecal coliform group includes *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* [20]. If *E.coli* is detected in drinking water this almost always indicates recent faecal contamination [72].

Harmless strains of *E. coli* can be found widely in nature, within the intestinal tracts of humans and animals. Harmful strains are a frequent cause of both intestinal and urinary genital tract infections [13]. A particularly hazardous form is Enterohemorrhagic *E. coli* (EHEC), which can cause mild non-bloody diarrhoea, haemorrhagic colitis and/or haemolytic uraemic syndrome [WHO]. EHEC strains have been responsible for several outbreaks of food poisoning in Japan due to drinking water contaminated by waste-water [91]. One of the most problematic strains is O157:H7, which has a low infectious dose (less than 100 cells) and was responsible for an outbreak of infection from a private water supply in Scotland in 1999 [92]. O157:H7 attacks the intestinal lining and may cause severe damage. Other types of *E. coli* include Enterotoxigenic *E. coli* (ETEC), which is prevalent in food and water in developing countries, and Enteropathogenic *E. coli* (EPEC) normally transmitted by contaminated water. Both types cause diarrhoeal disease [13].

2.9.2 Other Bacteria

Campylobacter species are the major cause of bacterial gastroenteritis in the developed world, with the bacterium being responsible for between 5 and 14 % of all diarrhoeal illness worldwide [60, 13]. In the United States alone, *Campylobacter* is thought to cause more than 2 million cases of diarrhoea annually [93]. The natural habitats of most *Campylobacter* species are the intestines of birds and other warm-blooded animals. *Campylobacter* cells may enter the environment (water supplies) by contamination from sewage effluent, agricultural run-off, grazing animals and wild birds [94]. *Campylobacter* spp are transmitted to humans by way of contaminated food and water [81] and as little as 500 cells can cause human illness [93]. Although symptoms can be severe, the associated illness, is usually self-limiting, consisting of diarrhoea, fever, abdominal pain and vomiting [13]. Serious outcomes include neuromuscular paralysis, due to Guillain-Barre syndrome, and Miller-Fisher syndrome which effects one in 1000 patients [60]. A possible explanation for the prevalence of *C. jejuni* as a human pathogen is that contamination levels in food and water can be high, and although the death rate of the organism is high, infectious doses can still remain [95].

Salmonella is another pathogen which can cause three types of infection; gastroenteritis, bacteraemia/septicaemia and enteric fever. The infective dose of *Salmonella* is considered to be about 100 CFU in healthy individuals, and infection is most commonly caused by the ingestion of food, water or milk contaminated by human or animal excreta [72]. Waterborne outbreaks are associated with contaminated groundwater, surface water and drinking water that has not been sufficiently disinfected. The species of clinical importance are *S. typhi, S. typhimurium* and *S. enteritidis.*

Pseudomonas aeruginosa is an environmental bacterium that can grow in water distribution systems and is also found in faeces, soil, and sewage. It is one of the most important opportunistic pathogens causing nosocomial infections in immunocompromised people and patients with underlying diseases [19]. Although it is

not responsible for enteric infections following ingestion, its presence in drinking water is often associated with complaints about taste, odour and turbidity. Water containing *Pseudomonas aeruginosa* may contaminate food and pharmaceutical products, causing their deterioration [81].

2.9.3 Protozoa

Protozoa are unicellular eukaryotic cells measuring 1-150 μm [140]. They have a relatively complex internal structure and can carry out complex metabolic activities [96]. Protozoa that infect humans and animals have two morphological forms: (i) a feeding and reproductive form called a trophozoite, which lives within the host, and (ii) a dormant cyst form, which can survive in the environment and infect new hosts. Figure 2.9.3.1 shows how these two forms are produced.

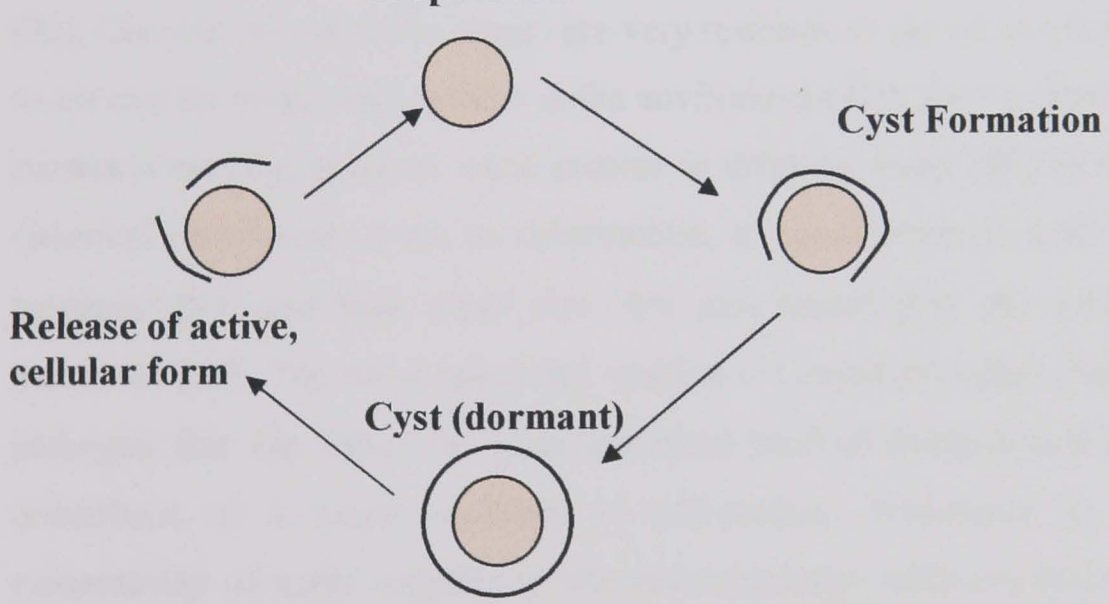


Figure 2.9.3.1 Production of trophozoite and cyst stages in protozoa.

Waterborne protozoa that present major problems are *Giardia lamblia* cysts, *Cryptosporidium* oocysts and *Cyclospora*, as these are not often removed during water purification.

Giardia lamblia was first observed in 1681 and it is the most common waterborne pathogen in the United States [20]. It causes Giardiasis (also known as traveller's diarrhoea) and is transmitted primarily through untreated stream water. The cysts are hardy and can survive in cold water for months. Infection can occur by the ingestion of cysts in contaminated water, food or by the faecal oral route [97]. Symptoms of giardiasis include diarrhoea and abdominal cramps. A major problem is that *Giardia* cysts survive standard concentrations of chlorine used for water purification systems [81] and its small size (~ few μm) allows evasion of some water purification stages. Consistent removal is achieved with slow sand filters [20].

In the last few years *Cryptosporidium* has become of even greater concern than *Giardia* [20]. Oocysts (the infective stage) are very resistant to environmental stress and are able to survive for weeks and months in the environment [19]. Due to their robust nature they present a massive problem when present in drinking water [98] as they are resistant to chemical disinfection such as chlorination, at concentrations used for drinking water treatment [99] and their small size (4-6 μm) means they can escape some filtration processes [19]. The most important species is *Cryptosporidium parvum*, a waterborne pathogen that can infect the gastrointestinal tract of humans and livestock [19]. The occurrence of a major outbreak in Milwaukee, Wisconsin in 1993 showed the vulnerability of water supplies to this microorganism with an estimated 400, 000 cases of *Cryptosporidiosis* occurring [20]. Infection can arise from as little as 30 oocysts [100] and normally occurs from faecal-oral spread between humans and animals or ingestion of contaminated water [52]. Activities associated with cattle farming, particularly muck spreading, slurry spraying and run off from contaminated grazing land, have been proposed as causes of many outbreaks [101]. The life cycle for *Cryptosporidium* is complex and is depicted in Figure 2.9.3.2.

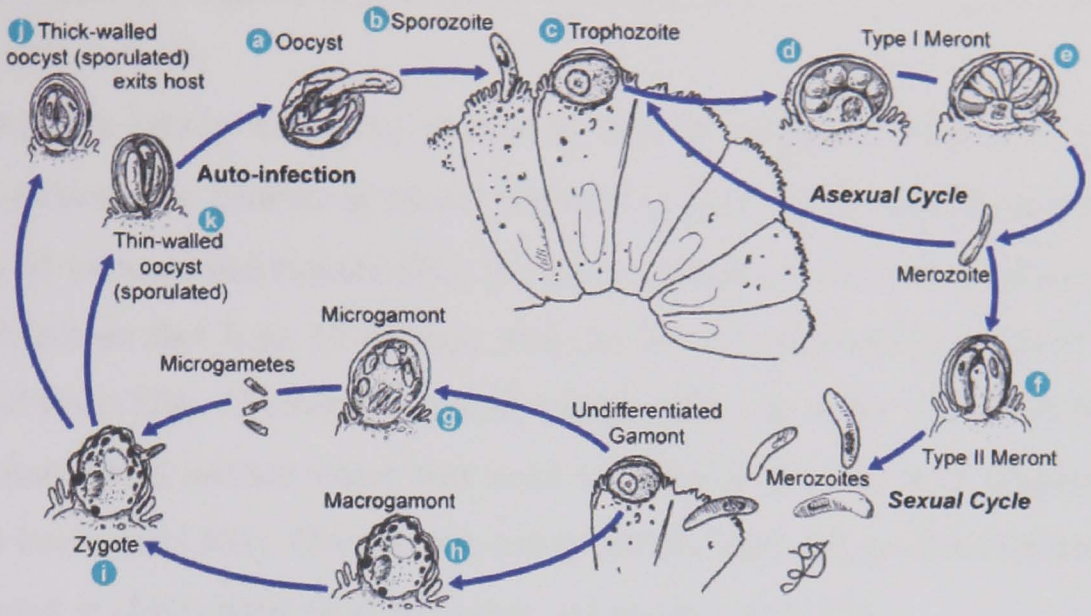


Figure 2.9.3.2 Life cycle of *Cryptosporidium parvum* [102].

When an oocyst is ingested (a) 4 sporozoites (individual parasites) are released which parasitise epithelial cells of the gastrointestinal tract. In these cells the parasites undergo asexual multiplication (d, e, f) followed by sexual multiplication producing microgamonts and macrogamonts (g, h). Upon fertilisation (i) of the macrogamonts by the microgametes an oocyst develops that sporulates in the infected host. Thick-walled oocysts (j), which are infective, are excreted from the host and thin-walled oocysts (k) are involved in autoinfection [97]. When a host is infected, symptoms are normally mild and self-limiting causing gastroenteritis in healthy individuals, but in the immunocompromised patients, illness tends to be more chronic and can be life-threatening [103].

In 1999 regulations, which amended the existing Water Supply (Water Quality) Regulations, made it a criminal offence to supply water from a treatment works that contains more than one *Cryptosporidium* oocyst in 10 Litres of water. Because of the difficulty in removing *Cryptosporidium* by sand filters and its extreme resistance to disinfectants, control in drinking waters is a subject of intensive research [20]. Studies

have shown that UV light is effective for the inactivation of *Cryptosporidium* oocysts [56, 104, 105].

Cyclospora is a newly emerging protozoan human pathogen, which is larger than *Cryptosporidium*. The parasite produces resistant oocysts (8-10 μm) that are excreted in the faeces of infected individuals [81]. It causes cyclosporiasis, which results in a self-limiting diarrhoea that lasts 19-43 days and can be accompanied by nausea, vomiting, cramps and fever [20]. *Cyclospora* caused a large epidemic in the United States in 1996 where contaminated surface water was used to spray raspberries with fungicide before they were harvested [106]. Oocysts are exceptionally resistant to disinfection and have been detected in chlorinated drinking water and waste water [72].

2.9.4 Viruses

A virus is a very small, acellular, infectious agent that contains either DNA or RNA. Almost all viral DNA is double stranded, and it can have either a circular or linear arrangement. Almost all viral RNA is single stranded; it is usually linear, and it may be segmented or non-segmented. Individual viruses (virions) have a protective coat, which is also known as the capsid. The capsid surrounds the nucleic core and is comprised of proteinaceous subunits called capsomeres. The virus capsid can be either helical or icosahedral [107]. Some viruses also have a phospholipid membrane surrounding the capsid, which is known as an envelope. The outer layer (whether it is the capsid or envelope) provides protection and recognition sites for virus attachment to a host cell. When a virus enters a host cell, the capsid is removed and it exists as a nucleic acid. Unlike bacteria, viruses are not capable of their own metabolic activity and have to invade a host cell where they take control of the metabolic machinery of the host cell to produce more molecules of viral nucleic acid and proteins. These then assemble into new viruses [59]. Viruses are capable of causing many types of disease in animals, plants and even bacteria. Enteric viruses are responsible for many waterborne outbreaks associated with drinking water. These include hepatitis and viral gastroenteritis.

Although viral gastroenteritis is caused by a number of viruses, it is estimated that Norovirus is responsible for about one third of annual cases. Gastroenteritis caused by norovirus is transmitted by the faecal-oral route through contaminated water and food. The disease is self-limiting, mild, and characterised by nausea, vomiting, diarrhoea, and abdominal pain. The infectious dose is unknown but is presumed to be quite low [108]. Although the Caliciviridae family of viruses is the leading cause of viral gastroenteritis, a number of other viruses, such as the adenovirus have been implicated in outbreaks. In addition to gastroenteritis, adenovirus can cause infections of the eyes and respiratory tract, as well as various sub-clinical infections. The adenovirus occurs in large numbers in water environments and is exceptionally resistant to purification and disinfection [81]. Adenoviruses are responsible for 5-20 % of gastroenteritis cases in young children [108].

Hepatitis is another viral disease that is spread through water. Hepatitis A can be transmitted through faecal contamination of food or water, with shellfish and salads being the most frequent sources. The disease is usually mild and is characterised by a sudden onset of fever, nausea and abdominal discomfort, followed several days later by jaundice. The infectious dose is unknown but is presumed to be 10-100 virus particles [133]. Each year, approximately 30 – 50, 000 cases occur in the United States, with an estimated 100 deaths as a result of acute liver failure caused by Hepatitis A [109]. Hepatitis E was responsible for one of the largest viral waterborne outbreaks ever documented. The outbreak occurred in 1955 in New Delhi, India and it affected 35,000 young adults with 75 deaths [80]. Outbreaks of Hepatitis E virus are usually associated with faecally contaminated drinking water [81], symptoms of the virus consisting of malaise, anorexia, abdominal pain and fever. The disease is more often seen in young to middle-aged adults (15-40 years old). Pregnant women appear to be particularly susceptible to severe disease, and excessive mortality (20 %) has been reported in this group [133].

In water purification, coagulation and filtration reduces virus levels by 90-99 %. The small size (25-80 nm) of enteric viruses however allows their passage through conventional filtration processes [20, 73] and further inactivation by chemical oxidants, high pH and photo-oxidation, does not provide complete protection [20]. Ultraviolet inactivation of viruses in water shows potential [73, 74].

Chapter 3

MATERIALS AND METHODS

3.1 General

In this chapter, the microorganisms, media and essential equipment used to conduct the research are listed. The chapter also includes descriptions of the experimental techniques used in the investigation.

3.2 Microorganisms

This section lists all of the microorganisms used in the experimental work, where they were obtained, and how they were cultured and maintained. The majority of the experiments used bacteria, but some work was also carried out with several viruses and a protozoan.

3.2.1 Bacteria

All bacterial strains used were obtained from the Microstock Bead System (used for long-term storage of bacteria) where they were stored at –70 °C. Strains were cultured by removing a bead of the desired microorganism, which was then streaked onto the appropriate type of agar plate and incubated for the required period of time (see Table 3.2.1.1). After the incubation period, a single, well-isolated colony was removed and streaked onto an agar slope for further incubation. After the suitable incubation period, the slope could then be refrigerated at 4 °C and kept as a source of inoculum. The cultures were maintained by routinely sub-culturing them onto fresh agar, approximately

every 4 weeks, and microscopic examination was performed, which included Gram staining.

All bacterial strains were obtained from the National Collection of Type Cultures (NCTC), Colindale, London, UK, with the exception of the *Pseudomonas* species, which was obtained from the LMG Bacteria Collection, Laboratory of Microbiology, University of Gent (LMG), Gent, Belgium. The table below shows all of the bacterial strains used, their preferred growth media and their incubation conditions.

Bacterium	**Growth Media**	**Incubation Conditions**
Escherichia coli NCTC 9001	Nutrient Broth/Agar	37 °C for 18 hours
Salmonella enteritidis NCTC 132344	Nutrient Broth/Agar	37 °C for 18 hours
Bacillus megaterium NCTC B17/97	Nutrient Broth/Agar	37 °C for 18 hours
Pseudomonas aeruginosa LMG 9009	Nutrient Broth/Agar	37 °C for 18 hours
Listeria monocytogenes NCTC 11994	Tryptone Soya Yeast Extract Broth/Agar	37 °C for 18 hours
Campylobacter jejuni NCTC 11352	Brucella Broth (+ Supplement)/Agar	30 °C for 48 hours
Campylobacter jejuni NCTC 11322	Brucella Broth (+ Supplement)/Agar	30 °C for 48 hours
Campylobacter coli NCTC 11366	Brucella Broth (+ Supplement)/Agar	30 °C for 48 hours

Table 3.2.1.1 The bacterial strains, their growth media and their incubation conditions.

E. coli, *S. enteritidis*, *Ps. Aeruginosa* and *B. megaterium* were all routinely maintained on Nutrient Agar slopes, and *L. monocytogenes* was kept on a Tryptone Soya Agar slope. The *Campylobacter* species however, were slightly more difficult to maintain so they were kept in an incubator at 37 °C in ~20ml of Brucella Broth. An inoculation loop was used to inoculate the culture into fresh broth every 48 hours to ensure good

maintenance of the microorganism. Details about the preparation of the media are given in section 3.3.

3.2.2 Protozoa

Cryptosporidium parvum was the only protozoan used. The oocysts were purchased from the University of Arizona, where they had been collected from the faeces of deer calves, which had previously been infected with the protozoan. The experiments involving *Cryptosporidium* oocysts were carried out at Stobhill Hospital, Glasgow and more information about their growth is provided in Chapter 6.

3.2.3 Viruses

All the work involving the use of viruses was carried out at the Central Science Laboratory, Sand Hutton, York. The viruses used are listed below.

Adenovirus (Group D)

Poliovirus (1a)

Herpes Simplex type 1

The adenovirus had previously been isolated from a clinical (faecal) sample and both the polio and herpes virus were obtained from the European Collection of Cell Cultures (EACC), Health Protection Agency, Porton Down, UK. More information about the viruses is provided in Chapter 6.

3.3 Media

Culture media was prepared by dissolving the appropriate weight in distilled water and then autoclaving at 121 °C for 15 mins, to ensure complete sterilisation. All media was produced by OXOID LTD, Basingstoke, UK with the exception of Brucella Broth, which is produced by Becton Dickinson & Co, Cowley, Oxford, UK.

3.3.1 Agar

Brucella Agar: 28 g/ L + 10 g Bacteriological Agar/ L.

Nutrient Agar: 28 g/ L.

Tryptone Soya Yeast Extract Agar: 40 g Tryptone Soya Agar/ L + 6 g Yeast Extract/ L.

After autoclaving, all agar was placed in a water bath maintained at 55 °C, to allow it to cool but not solidify. Once cool, the agar was used to either pour into Petri dishes to make agar plates or to pour into Universals to make agar slopes.

3.3.2 Broths

Brucella Broth: 28 g/ L + 2 vials of *Campylobacter* Growth Supplement (Code SR084E)/L.

Nutrient Broth: 13 g/ L.

Tryptone Soya Yeast Extract Broth: 30 g Tryptone Soya Broth/ L + 6 g Yeast Extract/ L.

3.3.3 Diluents and Reagents

Quarter Strength Ringer: 2 tablets/ L.

Brilliant Green Bile Broth: 40 g/ L

Minerals Modified Glutamate Medium

Gram Reagents (Lugols Iodine, Ethanol, Crystal Violet and Safronin)

CampyGen sachets (Oxoid Ltd)

3.4 Equipment

The main items of equipment and their application are now described. The pulsed UV source and its associated components are described in a separate section (3.5).

3.4.1 Centrifuge

The centrifuge was used to spin down cultures in order to remove the bacterial cells from their growth media. The type of centrifuge used in the laboratory was a Heraeus Labofuge 400R (Labcare, Buckinghamshire, UK). Cells were spun down by dispensing equal volumes of bacterial cultures into centrifuge tubes and placing them inside the centrifuge, ensuring that it was well balanced. The machine was set to 4000 g and the samples were spun for 10 minutes at 25 °C. After centrifuging, the supernatant was discarded and the remaining pellets were re-suspended in the appropriate diluent, usually quarter-strength ringer solution.

3.4.2 Spiral Plater and Colony Counter

The spiral plater (Figure 3.4.2.1) is an automatic machine, which is used for enumerating bacterial samples. The spiral plater works by dispensing a liquid sample of culture onto the surface of a rotating agar plate. It deposits decreasing amounts of sample as it moves from near the centre of the plate to the outside (an Archimedes spiral). After incubation, the plates are counted by centering them over a counting grid (each marked area on the grid corresponds to a known, constant volume of sample deposited on the plate). The colonies on the appropriate areas are counted by hand, either on a manual colony counter or on a PC using the ACOLYTE software package. Figure 3.4.2.2 displays photographs of the equipment used for counting the colonies. The spiral plater was a WASP 2, and it and the counters were supplied by Don Whitley Scientific, Shipley, UK.

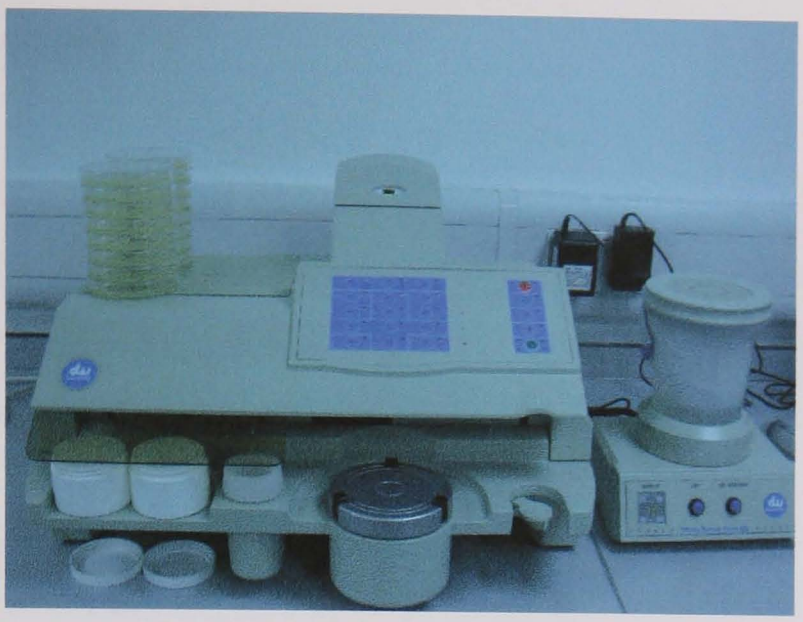


Figure 3.4.2.1 Spiral Plater

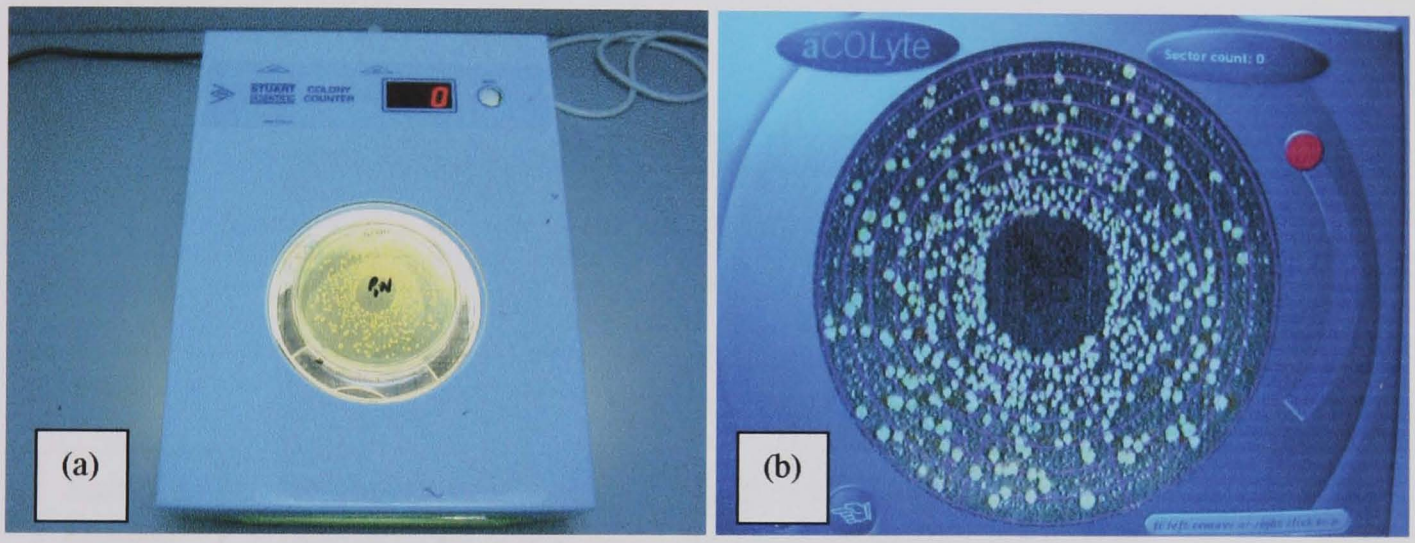


Figure 3.4.2.2 Photographs of (a) manual plate counter, and (b) image of plate that appears on the computer screen when using the Acolyte package.

The colony counter shown in Figure 3.4.2.2 (a) has an illuminated circular centre where a plate is placed, to make the colonies more visible for counting. The colonies are then marked with a marker pen and the pressure on the illuminated circle registers as one colony on the digital counter. The image shown in Figure 3.4.2.2 (b) is what appears on the computer screen when a plate is placed under a specially adapted web camera. Colonies are counted by clicking the mouse pointer on them. Once counted, they change colour and the PC then calculates the number of colonies on the whole plate.

3.4.3 Microscope

A Nikon E400 microscope (Surrey, UK) was used to observe all bacterial cultures before and after pulsed UV treatment and to check the purity of cultures. To obtain images of bacterial cells, a Nikon Coolpix 4500 digital still camera was mounted onto the microscope. The digital picture files could thereafter be transferred to a PC.

3.4.4 Spectrophotometer

A BioMate 5 Spectrophotometer, Thermo Spectronic (Witchford, UK), (Figure 3.4.4.1), was used to scan bacterial samples and measure the absorbance and percentage transmittance at particular wavelengths.

Figure 3.4.4.1 Spectrophotometer

Most scans were over the wavelength range 200-500 nm, with the mode set to absorbance or transmittance. Before a sample was scanned, a baseline scan was performed using distilled water or quarter-strength ringer to "zero" the instrument. A 3 ml sample was then placed in a quartz cuvette, which was placed inside the

spectrophotometer, in the light path. Measurements were made in 1 nm steps. Absorbance is a measurement of the absorption of light energy per unit depth (1 cm) whereas Transmittance is the percentage of original light that is not absorbed, reflected or scattered by the properties of a 1 cm^3 sample. Percentage transmittance is a parameter commonly used to determine the suitability of UV radiation for disinfection.

3.4.5 Photoreactivation Light Cabinet

The light cabinet used in the photoreactivation experiments was a Fi-totron growth chamber, model 600H (Fisons Environmental Equipment). The cabinet was equipped with 12 x Phillips 40 W white fluorescent lamps, which provided an intensity of around 17, 000 lux at the surface of a sample. The light intensity was measured using a digital Lux meter. The emission spectrum of the light inside the cabinet is shown in Figure 3.4.5.1. It consists of a continuum emitted by the fluorescent phosphor of the lamps together with three mercury lines from the mercury discharge used to excite the fluorescence.

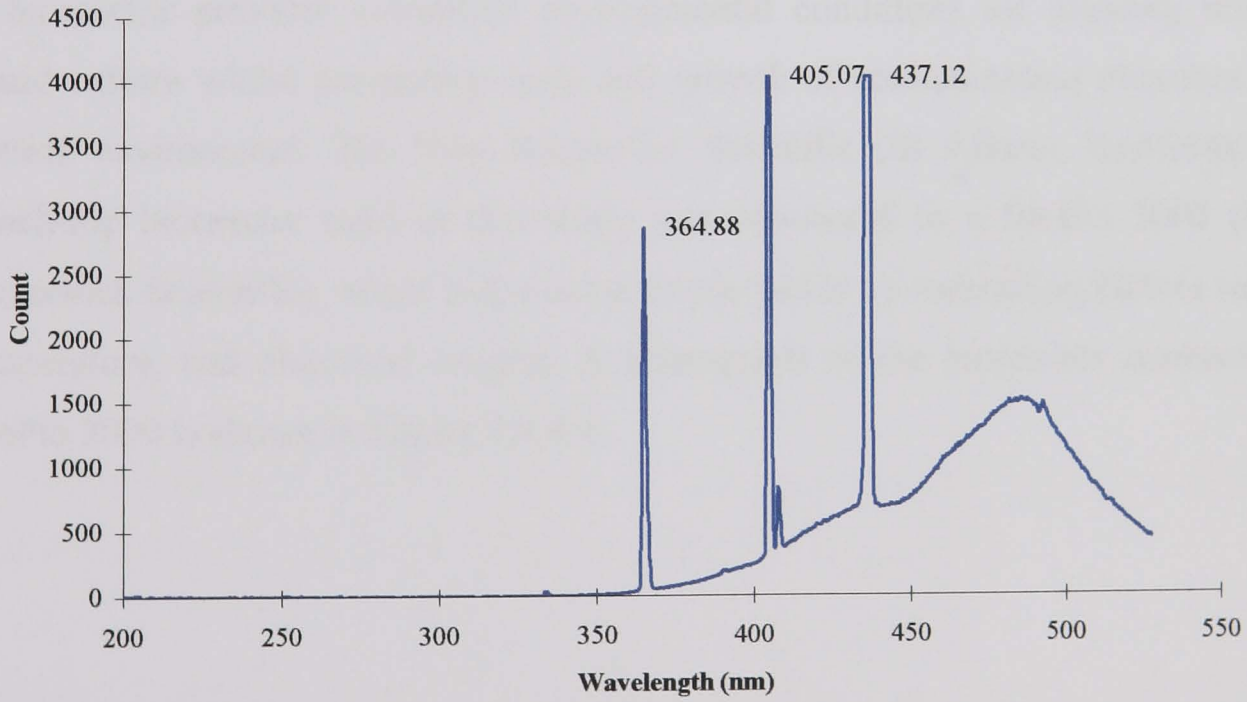


Figure 3.4.5.1 Spectrum of the light inside the photoreactivation light cabinet.

It can be observed from Figure 3.4.5.1 that there is no emission of light in the UV-C (200-280 nm) or UV-B (280-315 nm) regions; the wavelengths responsible for microbial inactivation. There is however substantial emissions in the UV-A/visible range (315-400 nm), the wavelengths commonly associated with photoreactivation. The temperature of the cabinet was thermostatically maintained at 37 °C, throughout.

3.4.6 Autoclaves

Culture media was sterilised using a Dixons (ST 2228) Portable Autoclave, Essex, UK. When the temperature reached 100 °C the bleed valve was closed, and the temperature and pressure were monitored until they reached 121 °C and 15 psig respectively. The temperature was maintained at 121 °C for 15 mins, before switching off and allowing the pressure to fall to ambient. An automatic KESTREL autoclave was used to sterilise waste and discards.

3.4.7 Bioreactor

A bioreactor provides controlled environmental conditions for growing microbes in liquid culture whilst preventing entry and growth of contaminating microbes from the outside environment. The New Brunswick Scientific (St Albans, Hertfordshire, UK) bench-top bioreactor used in this study was connected to a BioFlo 3000 (also New Brunswick Scientific), which helps to maximise yields by controlling factors such as pH, temperature, and dissolved oxygen. A photograph of the bioreactor connected to the BioFlo 3000 is shown in Figure 3.4.8.1.

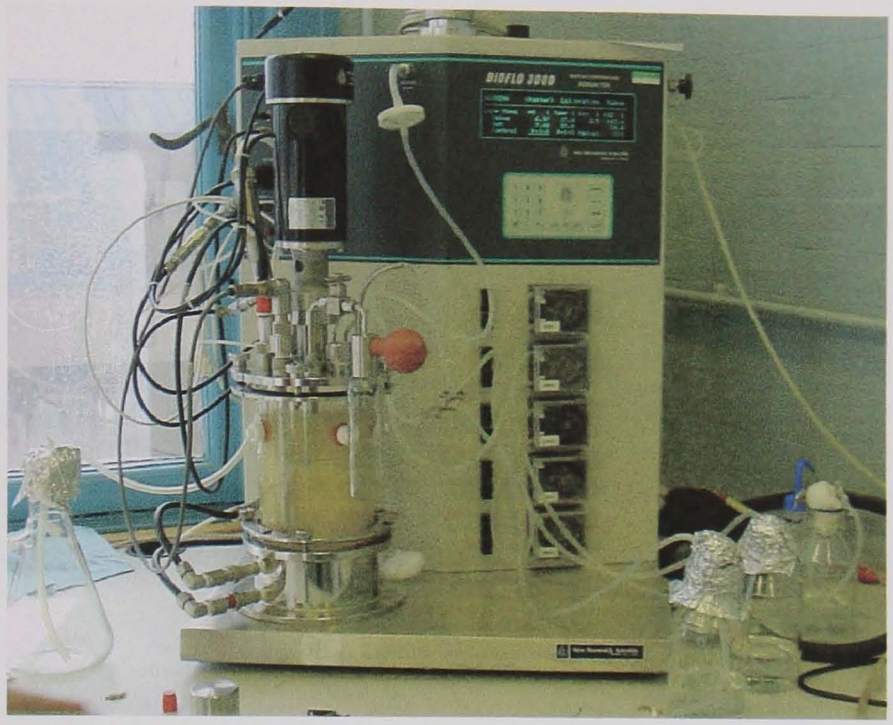


Figure 3.4.7.1 The bioreactor system while culturing *Campylobacter jejuni*.

3.4.8 Other Essential Equipment

Other items of equipment used in the project were:

Fridges (Lec Medical, Benfleet, Essex, UK), maintained at 4 °C and used to store all bacterial cultures.

2 x IP250 Incubators (Scientific Laboratory Supplies, Coatbridge, Lanarkshire, UK), used to culture bacteria. These were maintained at 30 and 37 °C.

A C25KC Incubator Shaker (New Brunswick Scientific, St Albans, Hertfordshire, UK) was used for aerobically culturing bacteria in liquid media. Flasks were placed onto the shaker, which was maintained at 125 oscillations per minute, to help introduce aeration

into the liquid; therefore producing ideal growth conditions. The temperature could be adjusted to whatever was suitable for the microorganism being cultivated (normally 30 or 37 °C).

A Grant Waterbath (Scientific Laboratory Supplies, Coatbridge, Lanarkshire, UK) was maintained at 55 °C and was used to cool agar to a temperature suitable for pouring.

A Whirlimixer (Fisher Scientific, Loughborough, Leicestershire, UK) was used to ensure adequate mixing of bacteria when carrying out serial dilutions. It was also used to re-suspend bacterial pellets after a culture had been centrifuged.

A Merit W4000 Distil (Barloworld Scientific, Staffordshire, UK) was used to distil tap water, to ensure that any chemical residues did not lower bacterial populations.

Gilson Pipettes, Luton, Bedfordshire, UK (10, 000 µl, 1000 µl & 200 µl) were used with sterile tips to accurately measure out volumes of bacteria. Measuring cylinders were used for measuring out volumes of liquid when preparing culture media.

A Brand dipensette II Dispenser was used when making up bottles of Ringer solution. A large volume of Ringer could be prepared, then the dispenser was attached to the bottle, and the desired volume (9 ml) could be dispensed into small glass bottles.

CampyGen sachets (Oxoid Ltd, Basingstoke, UK) were placed inside AG25AnaeroJar's (Oxoid Ltd, Basingstoke, UK) and plates inoculated with microaerophillic bacteria (*Campylobacter* species) were placed inside before being put into the incubator. The sachet provides reduced oxygen levels to help achieve optimum bacterial growth.

OHAUS Navigator, digital scales (Benfleet, Essex, UK) were used to measure out desired weights of media, during their preparation. A Yellowline MSH basic magnetic stirrer was then used to dissolve the media in solution.

A Lutron LX-101 digital Lux meter was used to take measurements of the light intensity inside the photoreactivation light cabinet.

100-QG LP 10 mm Quartz Cuvettes were filled with sample and placed inside the spectrophotometer for absorbance and transmittance readings.

A Thanda TG 105 Pulse Generator was connected to the external trigger input at the back of the pulsed UV system to produce higher pulse frequencies.

An OCEAN OPTICS 2000 spectrometer (Florida, 34698, USA) was used to obtain emission spectra from the UV flashlamp.

90 mm Single Vent Petri-Dishes

Universal Containers (Fisher Scientific, Loughborough, Leicestershire, UK)

3.5 Pulsed UV System

The pulsed UV system used to carry out the microbial inactivation experiments consists of two main components: the flashlamp chamber and the driver circuit. A photograph and schematic diagram of the pulsed-light source, developed by Samtech Ltd, are shown in Figures 3.5.1 and 3.5.2, respectively.

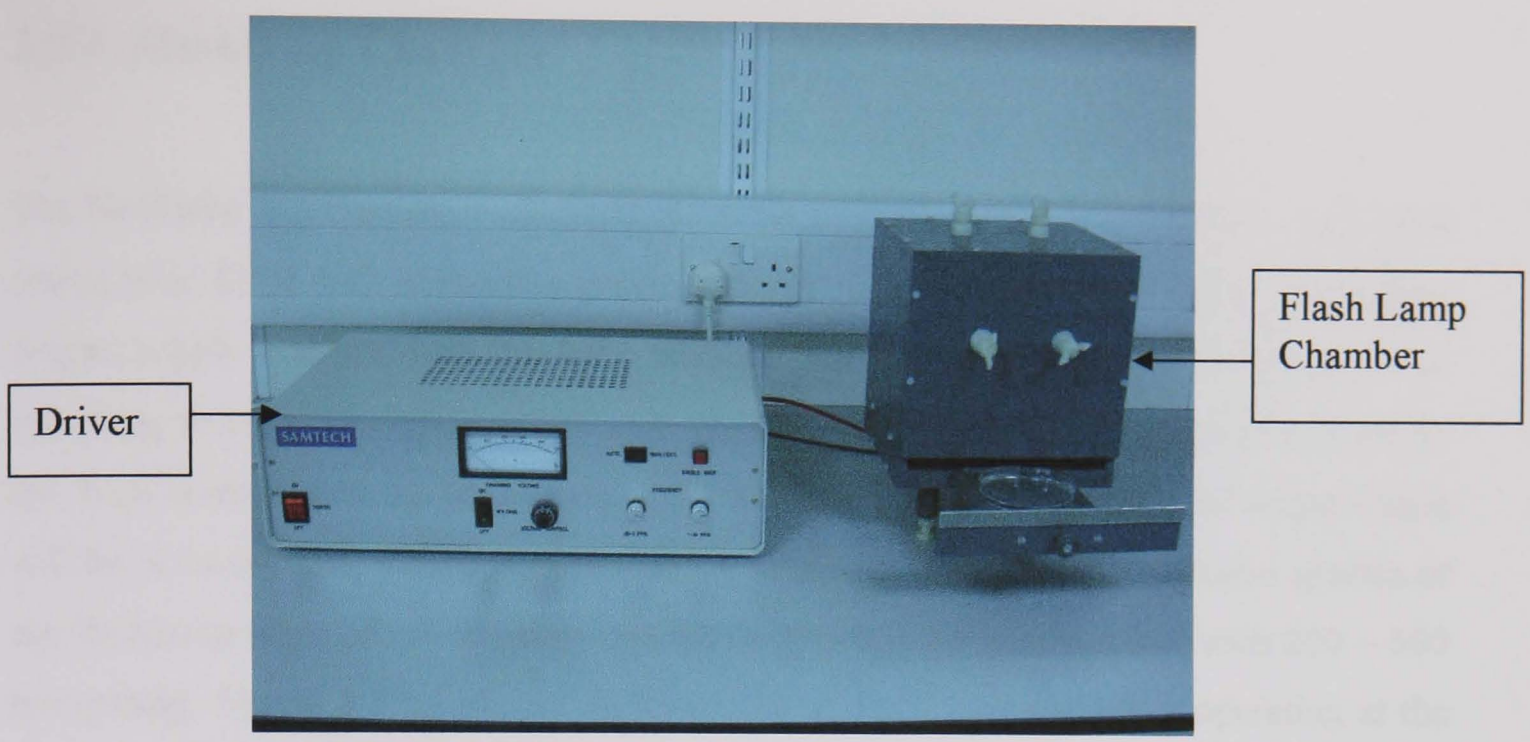


Figure 3.5.1 Pulsed UV-Rich Light System

The driver allows the supply voltage and pulse frequency to be controlled. The flashlamp chamber comprises the xenon flash lamp and a drawer for holding the sample.

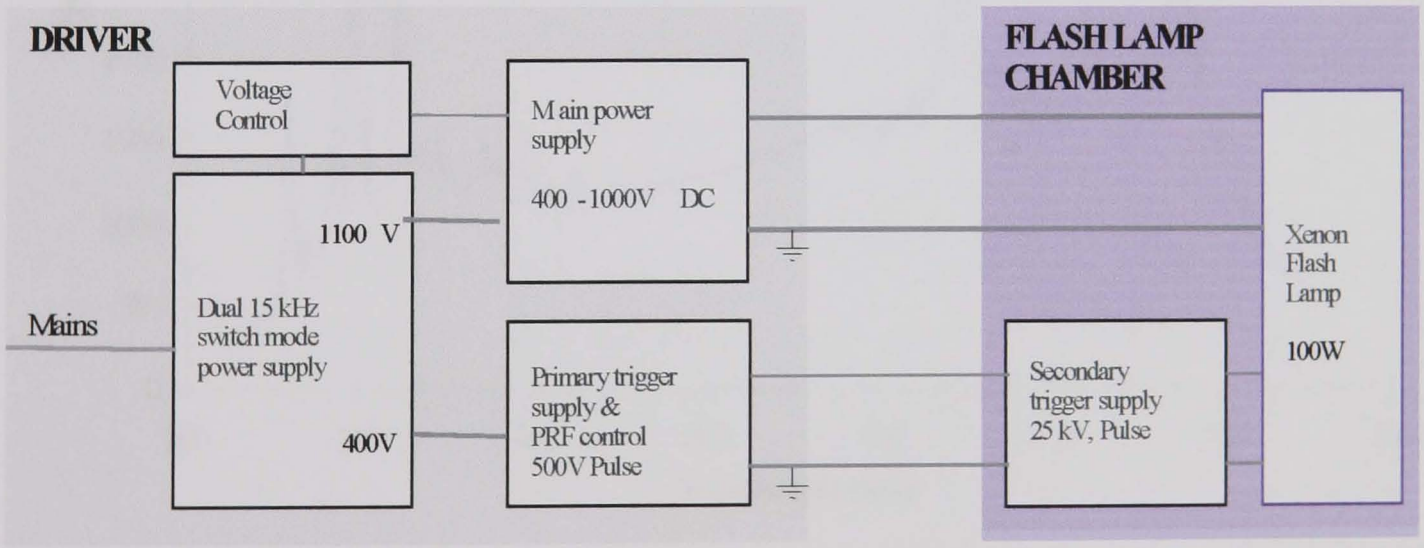


Figure 3.5.2 Schematic diagram of the main components of the pulsed UV light source.

3.5.1 Flashlamp Chamber

The flashlamp is a Heraeus Noblelight XAP Series that is constructed from a clear fused quartz tube, filled with xenon to a pressure of 450 torr. The chamber houses a secondary trigger supply that provides the lamp with 25 kV pulse to trigger the flash-lamp. The operating voltage, gas type and pressure determine the wavelength spectrum emitted by the flash lamp. When set to the maximum voltage, the UV component of emitted light will be at its highest, which is desirable for microbial inactivation. Emission spectra of the flashlamp were obtained using an Ocean Optics 2000 spectrometer with 200 – 500 nm grating. Figure 3.5.1.1 shows the spectrum of the flashlamp, when operating at the maximum charging voltage (1 kV).

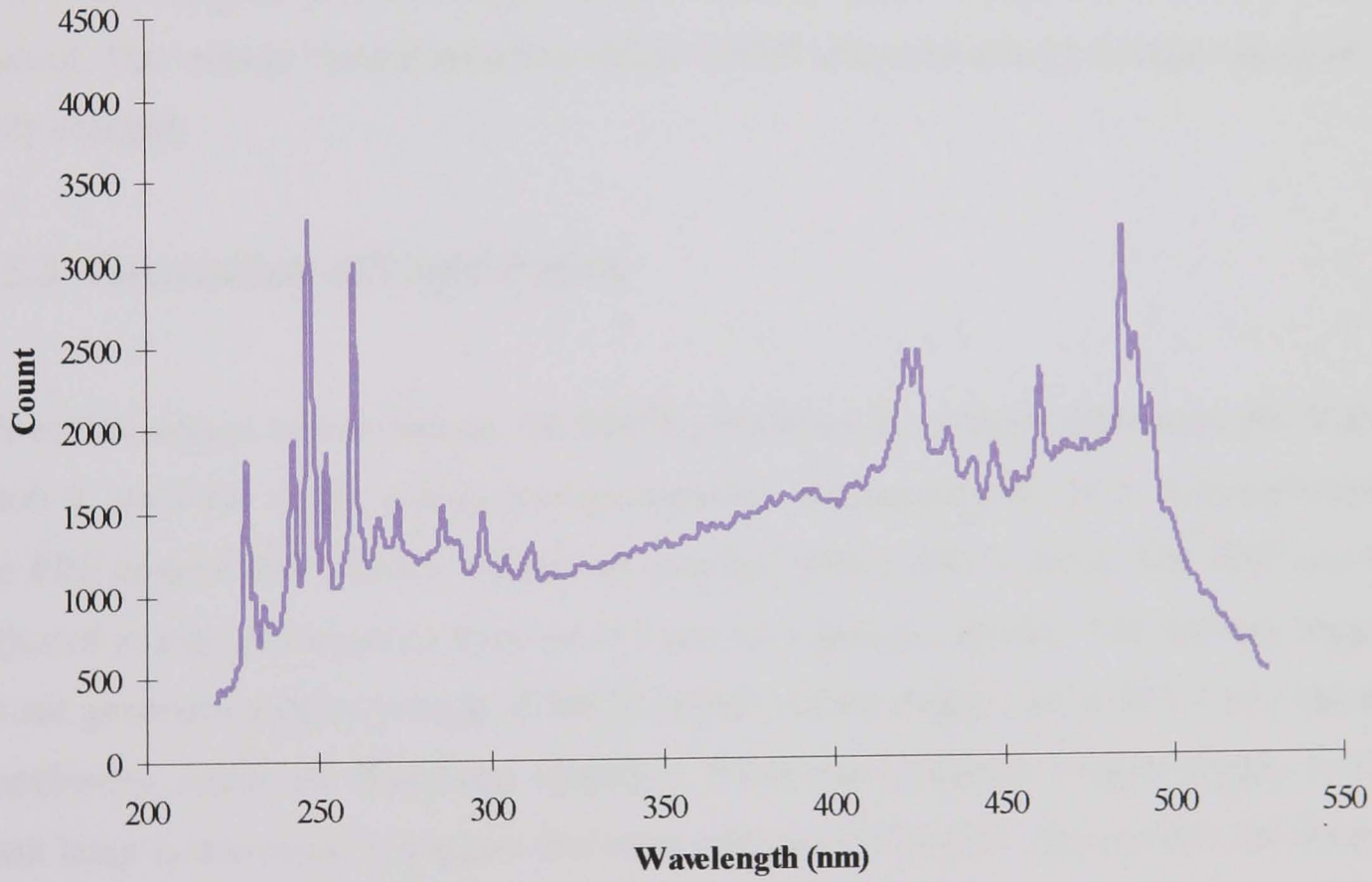


Figure 3.5.1.1 Emission spectrum of the flashlamp as measured using the Ocean Optics spectrometer.

In Figure 3.5.1.1, the UV-rich section can be observed between 200 – 280 nm. There are three distinct peaks at 260 nm, 248 nm and 230 nm, which are desirable wavelengths for UV disinfection. Measurements were undertaken and it was found that for microbial samples to receive the maximum light intensity then the flashlamp should be placed 80 mm above the sample surface.

3.5.2 Driver Circuit

As illustrated in Figure 3.5.2, the 15 kHz switch mode power supply (SMPS) provides output voltages to two major components inside the driver. It supplies 1100 V to charge an energy-storage capacitor. The voltage in the main power supply can be set to between 400 V and 1000 V, in order to vary the energy of each pulse. The SMPS also provides 400 V to charge a primary trigger circuit and the pulse repetition frequency (PRF) control. The voltage control switches off the SMPS when the energy storage capacitor is fully charged.

3.5.3 Generation of Light Pulses

When the voltage is switched on, the SMPS provides a dc voltage of between 400 V and 1000 V. At 1000 V, the energy-storage capacitor is charged with 20 J. Simultaneously the PRF control and primary trigger are supplied with a 400 V pulse. The PRF can be adjusted to a pulse frequency between 0.1 and 10 pulses per second. The primary trigger circuit generates a pulse voltage of 500 V, which is then stepped up to 25 kV by a pulsed transformer inside the flashlamp chamber. When the secondary trigger supply in the flash lamp is activated, it triggers the lamp with a 25 kV pulse. This causes the energy storage capacitor to discharge and transfer the stored energy of 20 J to the lamp resulting in a pulse of UV-rich light which lasts for approximately 40 μs. The average power per pulse is 0.1 MW and the instantaneous peak power reaches 1 MW. The efficiency of pulsed light for inactivation studies is attributed to the high peak power, the broad UV

spectral output and the methods used to regulate both the pulse duration and frequency output of the flashlamp [10]. When generating UV-rich light pulses, precautions should always be taken to shield the eyes, not only from direct radiation but from exposure to radiation reflected from room surfaces, clothing and furnishings [35].

3.6 Bacterial Enumeration

This study focuses on how effective UV-rich light pulses are at inactivating different bacterial species; therefore it is essential that the number of bacterial cells before and after treatment is accurately calculated. To do this plating techniques were employed, which are methods of counting viable microbial cells. These techniques involve spreading a microorganism onto the surface of an agar plate then incubating them appropriately. After incubation, a single microorganism or a group of microorganisms will develop to a distinct colony. Because it is not known whether the colony has developed from an individual cell, the result is expressed as colony forming units (CFU) rather than the number of bacteria. Original numbers of viable bacteria in a sample can then be calculated from the colonies which have formed using the dilution factor. In this study, serial dilutions of samples were carried out and then the appropriate plating techniques were employed.

3.6.1 Serial Dilutions

Serial dilutions were carried out by adding 1 ml of sample to 9 ml of quarter-strength Ringer to give a 10^{-1} dilution. This was then mixed on the Whirlimixer for approximately 10 seconds (or until a vortex was achieved) to ensure thorough mixing of the sample. From this dilution 1 ml was then added to a subsequent 9 ml bottle of ¼ Strength Ringer to give a 10^{-2} dilution. This procedure was normally carried out down to about a 10^{-6} dilution, as diluting any more than this would result in too few colonies forming and statistically inaccurate results.

3.6.2 Plating Techniques

Once all the appropriate dilutions were carried out, samples from each required dilution were plated onto agar by one of three methods:

(a) **Spiral Plate:** Using the spiral plater described in section **3.4.2**, 50 µl of sample was dispersed onto an agar plate. After incubation the colonies were counted using the counter also described in section **3.4.2**. Results are given in colony-forming units per ml (CFU ml^{-1}).

(b) **Pour Plate:** If a sample was expected to contain less than 250 CFU ml^{-1}, pour plates were carried out. This involved pipetting 1 ml of normally undiluted bacterial suspension onto the centre of an empty sterile Petri dish. 20 ml of molten agar, cooled to approximately 50 °C, was then poured over the sample and the plate was gently rotated clockwise, and then anti-clockwise; to ensure the bacteria and agar mixed. The plate was then left for the agar to solidify before incubating at the appropriate temperature. After incubation the number of colonies on the whole plate was counted to give the CFUml^{-1}.

(c) **Spread Plates:** These were produced by pipetting 100 µl of sample onto the surface of a pre-poured agar plate. The sample was then spread out as evenly as possible over the entire plate using a sterile L-shaped spreader. After incubation, the number of colonies on the whole plate was counted, and then multiplied by ten to obtain the number of CFU present per ml.

3.7 Pulsing and Culturing Routine

For the majority of experiments pulsing and culturing followed a fixed routine. This routine is now described while the media used and any variations in the routine are described as they apply.

3.7.1 Bacterial Preparation

An inoculation loop was used to remove bacteria from the culture stored in the fridge. This was inoculated into a flask containing 100 ml of broth which was subsequently placed onto an incubator shaker and incubated for 18 hours at 37 °C. After incubation, the contents of the flask were poured into two 50 ml centrifuge tubes, placed in the centrifuge and spun down (described in Section 3.4.1). After centrifuging, the pellets were re-suspended in 100 ml of quarter-strength Ringer, and the suspension was either used as it was, or further diluted to obtain a lower concentration of bacterial cells.

3.7.2 Pulsing of Samples

An aliquot of 20 ml was pipetted into a standard sized Petri dish, which resulted in a sample depth of 3.28 mm. The dish was then placed in the sample-holder drawer, and the drawer closed. The sample was directly under the lamp and the distance from the sample surface to the lamp was 80 mm. This ensured the sample received the maximum UV dose. The pulsed light system was then switched on and the voltage adjusted to the desired voltage, normally 1000 V. This provided a pulse energy of 20 J. The pulse repetition frequency was then set (normally to 1 pulse per second), and the automatic trigger switch activated. The number of pulses was counted until the sample had received a chosen number of pulses of UV rich light, when the trigger was switched off. Immediately after pulsing, the sample was pipetted into a Universal tube and wrapped in aluminium foil to prevent photoreactivation. The procedure was repeated for different numbers of pulses.

3.7.3 Microbial Enumeration

After pulsing, the samples were serially diluted and enumerated by one of the methods described in section 3.6. As well as the pulsed samples, an untreated (control) sample was enumerated to determine the population of microorganisms present before pulsing.

3.7.4 Statistical Analysis

Experiments were carried out in triplicate and the data presented for most of the experimental work is the average recovery of microorganism from treated samples, expressed as Log_{10}. The data was then used to produce inactivation curves for most sets of experiments. Standard deviations were calculated using Microsoft Excel ® 2002.

3.8 Additional Microbiological Methods

This section describes other essential microbiological techniques employed throughout the experimental work. These were used for the identification of bacteria under the microscope and to determine the purity of water samples.

3.8.1 Gram Stains

Gram stains were routinely carried out to check the purity of bacterial cultures and to look at the appearance of different microorganisms under the microscope. To prepare a slide for staining, a colony of the desired microorganism (no older than 24 hours) was removed from an agar plate and mixed with a drop of water on a microscope slide. The sample was then left to air dry before fixing by passing through a blue Bunsen flame 3-4 times. The first step of the staining procedure is to cover the slide with crystal violet for about 30 seconds. This is then poured off and the slide is covered with Lugols iodine. After approximately 1 minute the iodine is poured off and the slide is rinsed with

absolute alcohol until no more violet colour comes away. The alcohol is then rinsed off with tap water and the slide covered with safronin. After approximately 30 seconds this is washed off with tap water and the slide is blotted dry. The slide can then be observed under the oil immersion lens of the microscope.

Gram-positive bacteria have cell walls that are made up of several layers of peptidoglycan. When they are washed with the alcohol they become dehydrated causing the pores in the walls to close. This prevents the insoluble crystal violet from escaping and the cells remain purple. In gram-negative bacteria, the alcohol readily penetrates the lipid-rich outer layer and the thin peptidoglycan layer does not prevent solvent passage, so that the crystal violet is easily removed. When the safronin is then added, the cells stain pink.

3.8.2 Multiple-Tube Method

The multiple-tube method is used to count coliforms in water samples. This involves adding measured volumes of a sample to a series of tubes containing a liquid differential medium. After incubation, each tube that has received one or more microorganisms will show growth, and the most probable number of microorganisms in 100 ml of the sample is estimated from the number and distribution of tubes showing positive reactions. The numbers are calculated using a table of Most Probable Numbers (See Appendix A).

Minerals Modified Glutamate Medium (MMGM) was used for the multiple-tube method and the volumes chosen were 1 x 50 ml and 5 x 10 ml (these are the preferred choice for waters which are expected to be of good quality). The 50 ml bottle was inoculated with 50 ml of the water sample and each of the 10 ml bottles was inoculated with 10 ml of sample (all bottles contained a Durham tube to detect gas production). After inoculation the bottles were incubated at 37 °C for 48 hours.

After incubation, the MMGM bottles were observed for positive results. This is indicated by a change in the colour of the medium from purple to yellow (production of acid from lactose) with or without gas production. All bottles showing positive results were then sub-cultured into two tubes of Brilliant Green Lactose Bile Broth (BGLBB) containing Durham tubes. One bottle was incubated at 37 °C for 48 hours and the other at 44 °C for 24 hours. After incubation, the tubes were examined for positive results. These are indicated by turbidity and gas production. Positive growth at 37 °C indicates the presence of coliforms and growth at 44 °C indicates the presence of thermotolerant microorganisms.

Chapter 4

INVESTIGATION OF FACTORS EFFECTING INACTIVATION

4.1 General

Many different parameters can influence how pulsed light treatment inactivates microorganisms. This chapter describes an investigation of these factors that are important for achieving optimum levels of inactivation. Once these key factors have been identified, they can be used to establish a standard procedure that can be applied throughout the investigation. For comparative purposes, the same microorganism, namely *Escherichia coli* (NCTC 9001), was used throughout the investigation of control parameters. Choice of suspension medium is also important because a limitation of UV-light treatment is the lack of penetration of opaque liquids such as milk and fruit juice, and the absorbance of UV in liquids that are slightly coloured, such as broths (growth media) and wine. The chosen media was Quarter-strength Ringer solution, which provides a clear, isotonic suspension that shows low absorbance in the UV.

Using samples of *E. coli* in Quarter-strength Ringer solution, the parameters influencing pulsed-UV inactivation were then examined: these parameters are depth and volume of sample, optical alignment, electrical pulse energy, pulse frequency and the wavelength of the UV-light.

4.2 Effect of Depth

The first set of parameters explored was the volume and depth of samples. It has been reported that in distilled water UV light at 254 nm loses 30 % of its intensity 40 cm below the surface [31]. In this study, such large depths are not intended to be used; but it is useful nevertheless to investigate the effect of depth on inactivation so as to determine a suitable sample depth. A 20.7 mm diameter, PVC container of length 90 mm was used for the investigation. Because the material that the tube was constructed from could not be sterilised by autoclaving, it was disinfected, prior to use, with 70 % ethanol and then rinsed with sterile distilled water several times. For this study it was decided to try treating samples at depths of 5, 10, 20, 30, 40 and 50 mm. Therefore the volumes of sample required to obtain these depths in the tube were calculated and are shown in Table 4.2.1.

Sample Depth (mm)	Sample Volume (ml)
5	1.7
10	3.4
20	6.7
30	10
40	13.5
50	16.8

Table 4.2.1 Volume of sample required to achieve depths of 5, 10, 20, 30, 40 and 50 mm.

For each test, the correct volume of sample was pipetted into the container using a sterile tip to give the desired depth. The height was adjusted for each test so that the distance (138 mm) between the flash lamp and the sample surface was kept constant throughout each experimental test. See Figure 4.2.1 for illustration.

It was decided that for this experiment a starting population of 10^6 CFUml^{-1} of *E. coli* should be used as this population is large enough to show adequate levels of inactivation and observe any differences between the sample depths. The *E. coli* was prepared as described in section 3.7.1 and each sample was then treated with 15 UV-rich light pulses as described in section 3.7.2 of the materials and methods chapter. The results obtained are displayed in Figure 4.2.2.

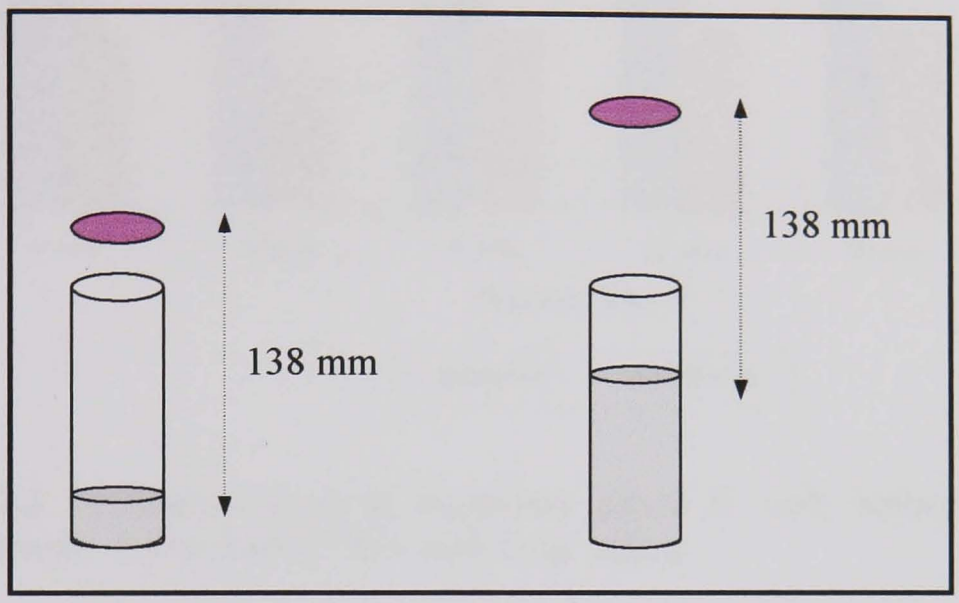


Figure 4.2.1 Illustration showing that as the sample depth increases the lamp is adjusted to a higher position, so that the distance between sample surface and the lamp is kept constant.

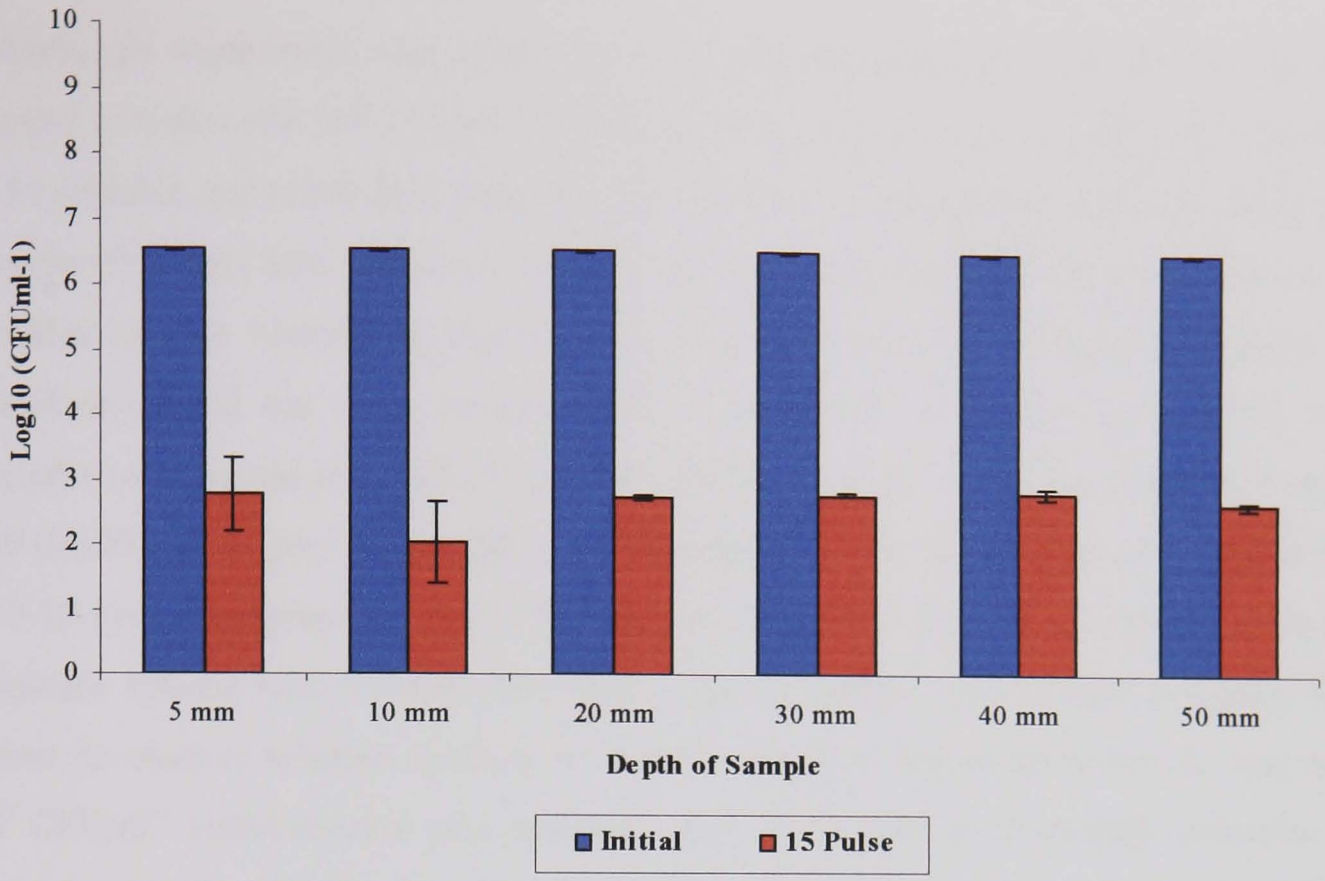


Figure 4.2.2 Population sizes of stationary phase *E. coli*, before and after treating different sample depths with 15 UV-rich light pulses.

The results in Figure 4.2.2 show an approximate 3.5-4 Log_{10} reduction in bacterial population for each of the different sample depths. The sample depth of 10 mm is shown to have a greater number of microorganisms inactivated. This is most likely due to uncertainty within the measurements rather than samples of this depth having an increased susceptibility to the treatment. There does however appears to be no significant decrease in bacterial inactivation as the depth of sample increases suggesting that inactivation rates appear to be little affected by sample depths up to 50 mm.

4.3 Effect of Volume

The volume of the sample being treated is considered an important parameter when using ultraviolet light for disinfection purposes, therefore experiments were carried out to determine how important this factor is when using pulsed UV-rich light.

Initially, an experiment was carried out in a Petri dish using 10, 20, 40, 50 and 60 ml sample volumes of a 10^9 CFUml^{-1} population of *E. coli*. The results obtained were found to be variable and unreliable, because as the volume of sample increases the depth of the sample obviously also increases. Because of this a conclusion could not be drawn as to whether sample volume or depth is the important factor. A second experiment was therefore carried out using samples with a volume of 10 ml and 20 ml. The 10 ml samples were pulsed in a small Petri dish, which resulted in a sample depth of 4.06 mm, and the 20 ml samples were pulsed in a standard size Petri dish that gave a sample depth of 3.28 mm. Changing the size of the treatment chamber allowed a comparison between a sample volume with a small depth and a smaller sample volume with a slightly larger depth, to observe whether volume or depth is the most important factor. *E. coli* with a 10^9 CFUml^{-1} concentration was prepared and 10 ml and 20 ml sample volumes were treated with 5, 20, 30, 50 and 100 light pulses. The results can be observed in Figure 4.3.1.

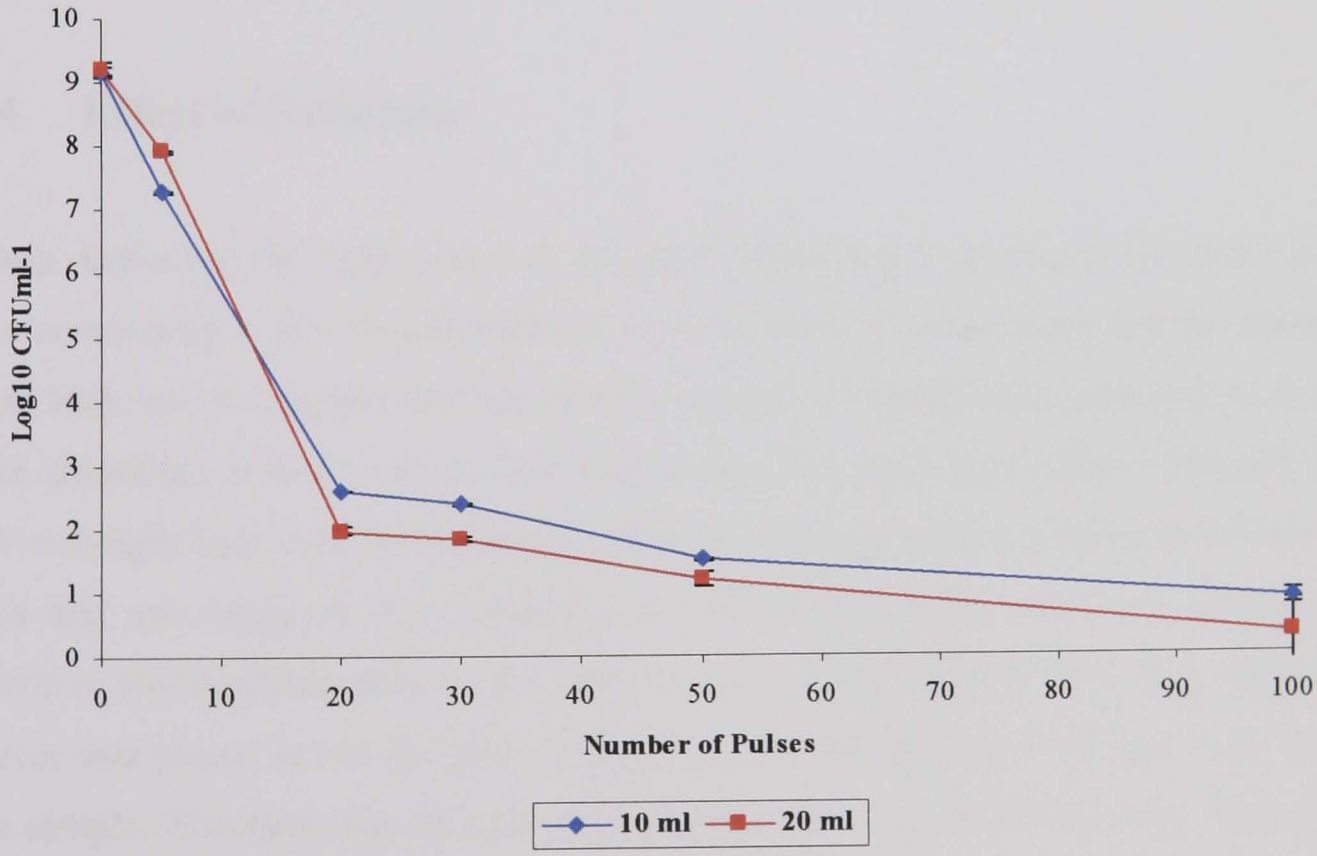


Figure 4.3.1 Differences in levels of inactivation of stationary phase *E. coli*, when pulsing 10 and 20 ml sample volumes with a range of UV-rich light pulses.

It can be observed from the results in Figure 4.3.1 that the inactivation levels for the two different sized sample volumes are very similar. After treatment of 100 light pulses, there is an 8.75 $\log_{10}$ reduction in bacterial population for the 20 ml samples and an 8.34 $\log_{10}$ reduction for the 10 ml samples. The results from this experiment therefore show that a large sample volume at a small depth, is inactivated to a similar degree as a small sample volume at a larger depth.

The above results indicate that large sample volumes are not problematic in pulsed UV-rich light inactivation as long as they are presented for treatment with small depths. If practical applications of pulsed UV-rich light require the treatment of large volumes, a reactor vessel should be constructed which would allow liquid samples to flow through at the lowest depth possible. Other ways of enhancing the level of inactivation would be to increase the pulse number which in turn would increase the amount of inactivation or if treating extremely large volumes, the number of lamps in operation could also be increased.

4.4 Effect of Reflection

When delivering the light pulses to a sample, reflection is an important factor to take into consideration. If reflected surfaces, such as mirrors, are present they can reflect the light back into the sample and enhance the amount of inactivation achieved. In order to take advantage of this a sample dish was constructed that would allow reflection of the UV-rich light back into the sample and thereby increase the inactivation efficiency. The dish that was designed had a quartz glass bottom and nylon sides. UV absorption in quartz is much smaller than in the material used in the standard Petri dish; therefore a mirror was placed below the dish, which would reflect the pulses of light back through the sample. The new dish had the same dimensions as a standard plastic Petri dish to allow a comparison between the two types of dish. A disadvantage of the new dish is that it could not be autoclaved because of the heat sensitive materials it was constructed

from. Cleaning with 70 % ethanol and rinsing with water several times before use therefore ensured the sterility of the dish.

In order to examine the effect of reflection, 20 ml samples of 10^9 CFUml^{-1} *E. coli* were treated with 5, 10, 20 and 30 UV-rich light pulses. The test was carried out in triplicate in both the new sample dish and in a standard Petri dish. The average recovery for both types of dish was calculated and the results are presented in Figure 4.4.1.

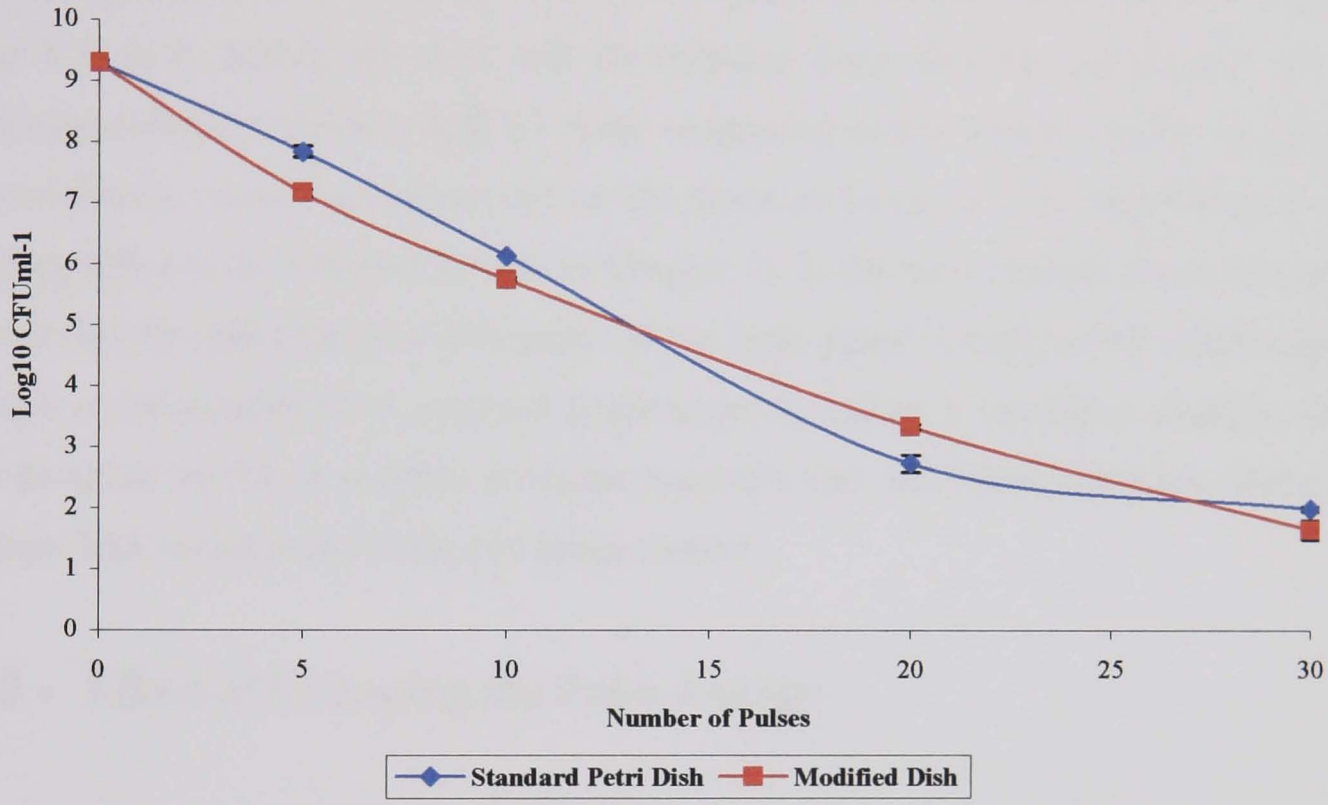


Figure 4.4.2 Inactivation levels achieved when stationary phase *E. coli* samples are treated in a standard Petri Dish and a modified dish that allows reflection of the light pulses.

On observation of the two sets of results for the different dishes, it can be seen that the inactivation curve, for samples treated in the new modified dish, shows a constant decrease in population size. The curve for samples treated in the standard Petri dish also shows an exponential decrease, but after 20 light pulses inactivation of the bacterial population starts to tail off. Overall however, the level of inactivation after 30 pulses is

very similar, with a 7.7 Log reduction achieved with the modified dish and a 7.3 Log reduction for the standard Petri dish.

The results demonstrate that using a sample dish that allows reflection of pulses back through the sample has no significant effect on the inactivation level achieved. It is however shown that using the modified dish did not exhibit tailing as observed with the standard Petri dish. An explanation for this is that the bacteria suspended at the bottom of the sample may not receive a sufficient dose of pulses due to shielding from other microorganisms. When a reflective mirror is place at the bottom of the dish however, any UV light exiting the dish will be reflected back into the sample and the first microorganisms it reaches will be those suspended at the bottom of the liquid. As a consequence, no tailing is observed on the inactivation curve. (The phenomenon of the tailing effect is investigated further in Chapter 7). It can be concluded from this area of work that the collection and redirection of the light pulses is essential for achieving high levels of inactivation. For practical applications therefore, a treatment chamber should be designed so that it contains adequate materials that will allow reflection of the light pulses back to the sample/material being treated.

4.5 Effect of Changing the Pulse Energy

The magnitude of the applied charging voltage and thereby the electrical energy, used for each light pulse is extremely important for achieving high levels of microbial inactivation. When the pulsed light system is operating at its maximum voltage, the energy of each pulse is 20 J. As this voltage is decreased the energy of each pulse also decreases. The two are linked by the following equation, where C is the size of the energy storage capacitor (40 μF) inside the pulsed light system and v is the operating voltage:

$$E = \tfrac{1}{2} Cv^2$$

Examining the differences in inactivation results when different charging voltages are used and observing what effect changing the delivery of the energy input has on levels of inactivation were the two types of experiment undertaken.

4.5.1 Effect of Varying the Applied Charging Voltage

As mentioned previously when the applied charging voltage is decreased, the energy of each pulse that is delivered decreases. In addition to this, the UV component of the emitted light also decreases. This section therefore looks at what effect increasing the voltage has on the UV component of emitted light and the associated inactivation levels that are achieved.

The three charging voltages studied were 600 V, 800 V and 1000 V which results in an electrical energy of 7.2 J, 12.8 J and 20 J respectively. To observe the emission spectra from the light source when operating at each of these voltages, an OCEAN OPTICS 2000 spectrometer was used. The emission spectra for each charging voltage are shown in Figure 4.5.1.1, over the range 200 to 500 nm.

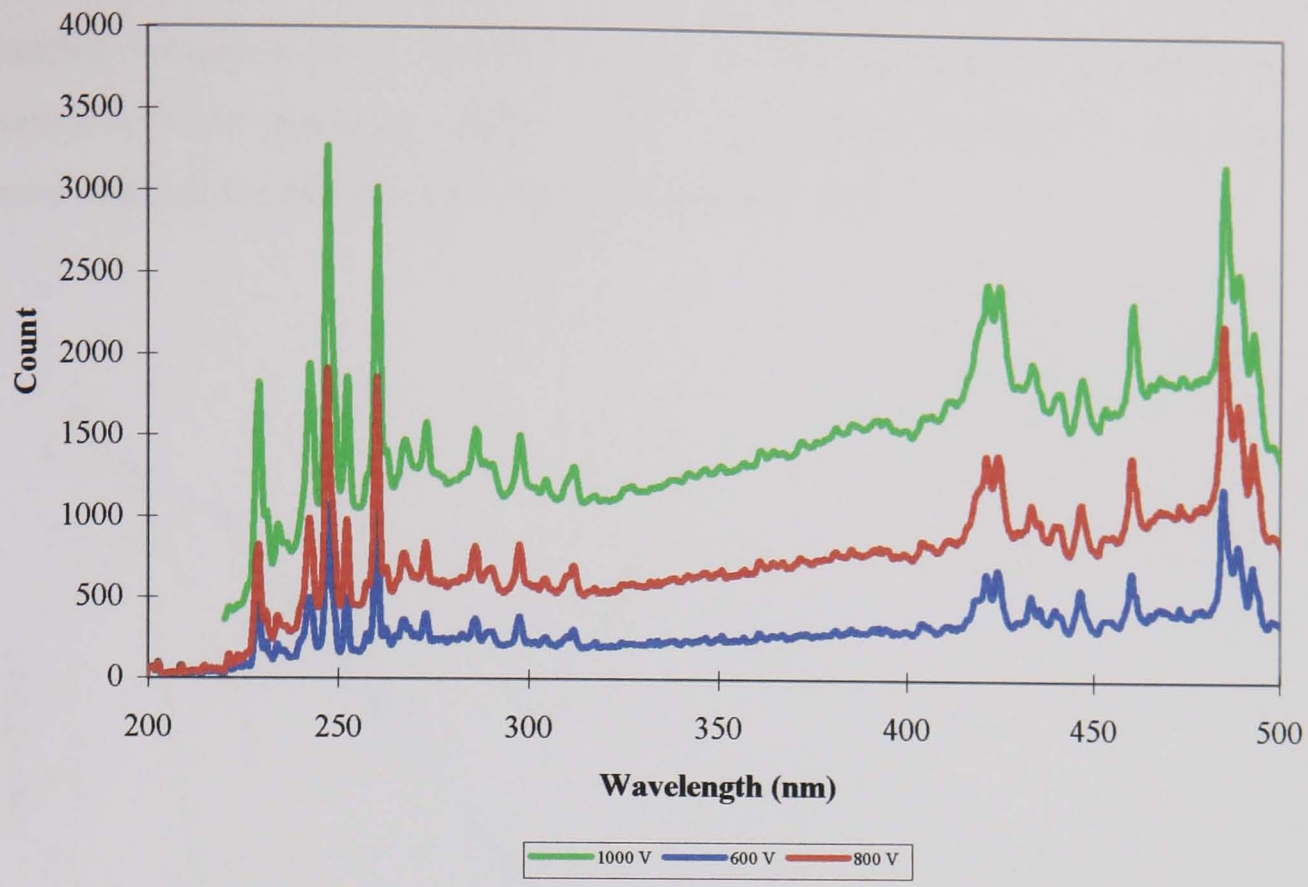


Figure 4.5.1.1 Emission spectra from the light source when operating at 600 V, 800 V and 1000 V.

From the emission spectra for each of the charging voltages three distinct peaks can be observed in the UV-C region at 230, 248 and 260 nm (desirable wavelengths for UV disinfection) When the charging voltage is set at 600 V these three UV peaks give relative counts of approximately 500, 1070 and 1025 respectively. When the voltage is increased to 800 V however the counts at 230, 248 and 260 nm increases to 823, 1910 and 1854 respectively. When the voltage is further increased to 1000 V the counts obtained at these wavelengths are 1821, 3271 and 3021, showing that as the Voltage increases the UV content of the germicidal wavelengths increases. This indicates that the intensity of light in this wavelength region follows a near linear relationship with the electrical energy dissipated in the flash lamp.

For the inactivation experiment, 10^8 $CFUml^{-1}$ of *E.coli* was prepared as described in section 3.8. 20 ml samples were then treated with 2, 5, 10, 20 and 40 UV-rich light

pulses in triplicate, with the operating voltage set at 600 V (E = 7.2 J) and the pulse repetition frequency set at 1 pulse per second. The experimental procedure was then repeated with the operating voltage set at 800 V and then at 1000 V. The inactivation results obtained for the tests are shown in Figure 4.5.1.2.

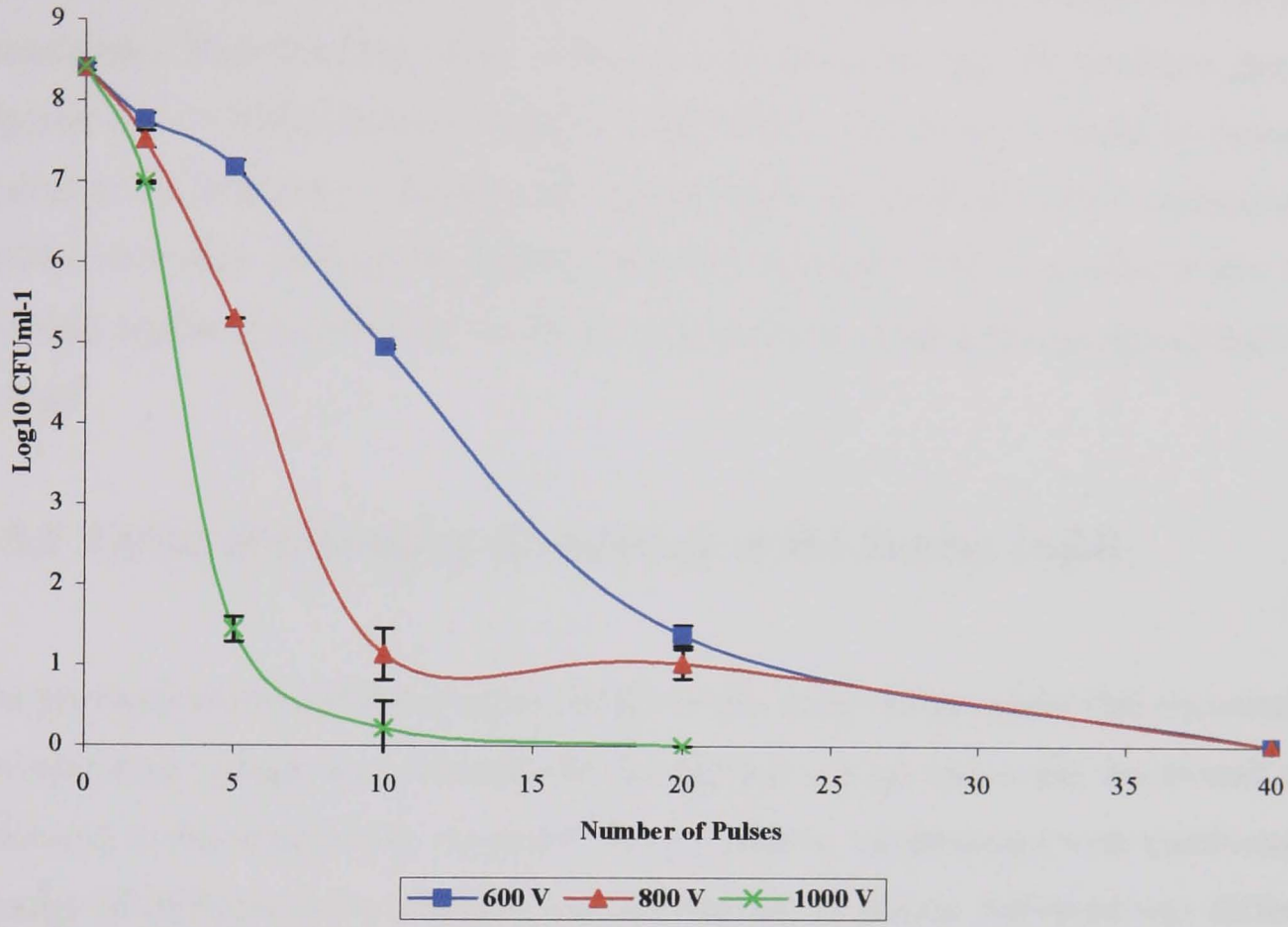


Figure 4.5.1.2 A comparison of the inactivation results achieved for the pulsed UV-rich light treatment of stationary phase *E. coli*, when the system is operating at 600, 800 and 1000 V.

For the operating voltage of 600 V a steady decrease in the bacterial population is observed and after 40 pulses complete inactivation is achieved, resulting in an overall 8.4 Log reduction. When the operating voltage is increased to 800 V, the inactivation rate increases significantly during the first 10 pulses and then starts to tail off before complete inactivation occurs after 40 pulses. When the operating voltage is set to 1000 V (maximum), microbial inactivation occurs extremely rapidly, with a 7 Log reduction

occurring after only 5 UV-rich light pulses. After this, the inactivation rate slows slightly but still undergoes a steady decrease until complete inactivation is achieved between 10 and 20 UV-rich light pulses.

From these results it is shown that as the voltage is increased, the inactivation rate increases significantly. This is primarily due to the increase in emission, of germicidal wavelengths from the flash lamp, which can be observed from the emission spectra in Figures 4.5.1.1 These results therefore demonstrate that it makes sense to operate the system at the maximum voltage as this significantly increases the UV-C content of light emitted therefore causing the greatest microbial damage. The downside of this from a practical application however would be the expense of running the system at such a high voltage.

4.5.2 Effect of Changing the Delivery of the Energy Input

The previous section of this chapter looked at the inactivation results that occurred when the operating voltage was changed. As the applied voltage increased, the overall energy delivered to the sample also increased. In this section, experiments were conducted with a range of different pulse energies, but the number of pulses delivered was different in each set of tests so that the **overall** energy delivered to each sample remained the same.

From the experimental results already reported, it can be seen that adequate levels of inactivation have been achieved by using 15 x 20 J pulses, which resulted in an overall energy of 300 J being delivered to the sample. It was therefore decided to treat different samples with an overall energy of 300 J, which was achieved by decreasing the pulse energy and increasing the pulse number accordingly.

The voltage needed to provide the different pulse energies was calculated using the equation given in section 4.5. In the first experiment carried out *E. coli*, which had a population size of approximately 10^9 $CFUml^{-1}$, was prepared as described in section

3.7.1. Aliquots of 20 ml were pipetted into Petri dishes and treated with the desired number of UV-rich light pulses (depending on the pulse energy). Table 4.5.2.1 shows the inactivation results obtained and their standard deviation. In addition, the different pulse energies and voltages used and the number of pulses needed to provide an overall energy of 300 J are shown.

Energy Input/Pulse (J)	**No of Pulses**	**Overall Energy Input** (J)	**Voltage** (V)	**Initial Concn** (cfu/ml)	**Final Concn** (cfu/ml)	**Log Reduction**	**Standard Deviation** (+/-)
5	60	300	500	1.1×10^9	388	6.45	0.033
10	30	300	707	1.1×10^9	927	6.07	0.04
15	20	300	866	1.1×10^9	295	6.57	0.014
20	15	300	1000	1.1×10^9	92	7.08	0.042

Table 4.5.2.1 Inactivation results achieved using UV- rich light pulses, with different energies, to treat a 10^9 $CFUml^{-1}$ stationary phase population of *E. coli*. The overall energy input was kept the same by increasing the number of pulses as the energy was decreased.

The results displayed in Table 4.5.2.1 show that the different energy pulses produce only slight differences in the final inactivation levels achieved. The experimental procedure was then repeated for a 10^8 $CFUml^{-1}$ population of *E. coli* to see if a similar result is obtained with a lower population. The results from these trials are shown in Table 4.5.2.2.

Energy Input/Pulse (J)	No of Pulses	Overall Energy Input (J)	Voltage (V)	Initial Concn (cfu/ml)	Final Concn (cfu/ml)	Log Reduction	Standard Deviation (+/-)
5	60	300	500	3.1×10^8	37	6.92	0.014
10	30	300	707	3.1×10^8	0	8.49	0
15	20	300	866	3.1×10^8	0	8.49	0
20	15	300	1000	3.1×10^8	0	8.49	0

Table 4.5.2.2 Inactivation results achieved using UV- rich light pulses, with different energies, to treat a 10^8 $CFUml^{-1}$ stationary phase population of *E. coli*. The overall energy input was kept the same by increasing the number of pulses as the energy was decreased.

This second set of results show that pulses with a very low energy input (5 J) may produce lower levels of inactivation. However results obtained from the higher energy inputs of 10 J, 15 J and 20 J all show the same amount of inactivation after receiving a total energy input of 300 J, indicating that the nature of the delivery of energy does not play a major role.

The way in which energy is delivered to the samples does not appear to be too important. If the overall energy delivered to a sample is the same then the overall emission of germicidal wavelength will be the same resulting in similar amounts of microbial damage being produced. This study has therefore demonstrated that low energy pulses achieve decreased inactivation but if the pulse number is increased so that the overall energy is the same, no significant difference in inactivation levels is observed. This highlights the flexibility of the pulsed UV light system because if the

system cannot be operated at the maximum voltage, for example due to high running costs, then all that is required is to use a higher number of lower energy pulses.

4.6 Effect of Varying the Pulse Repetition Frequency (PRF)

In all of the experiments reported so far, the Pulse Repetition Frequency (PRF) has been 1 pulse per second (1 Hz). The pulsed light system however can be adjusted so that the pulses can be delivered over the range 0.1 to10 pulses per second. An increase or decrease in the pulse frequency may be a crucial factor in achieving larger amounts of microbial inactivation; therefore it was decided to carry out a series of experiments where the frequency of pulses was altered.

In the first experiment, *E. coli* with a concentration of 2.1×10^9 $CFUml^{-1}$ was prepared, as described in section 3.7.1. It was decided to treat samples with 20 pulses at 1000 V (20 J) and use the following pulse frequencies: 0.01 Hz (1 pulse every 100 seconds), 0.1 Hz (1 pulse every 10 seconds), 1 Hz (1 pulse per second), 3 Hz (3 pulses per second) and 5 Hz (5 pulses per second). To produce these frequencies, each pulse at 0.01 and 0.1 Hz were triggered manually and the pulse intervals were timed using a stopwatch. For the higher 1, 3 and 5 Hz frequencies, a pulse generator was connected to the external trigger input at the back of the pulsed light system and this was used to produce the desired pulse frequencies. Once the 20 ml samples of the microorganism were treated, they were enumerated as described in section 3.7.3. The Log_{10} reductions after 20 pulses of the five different frequencies were calculated. The inactivation levels and standard deviation are shown in Figure 4.6.1.

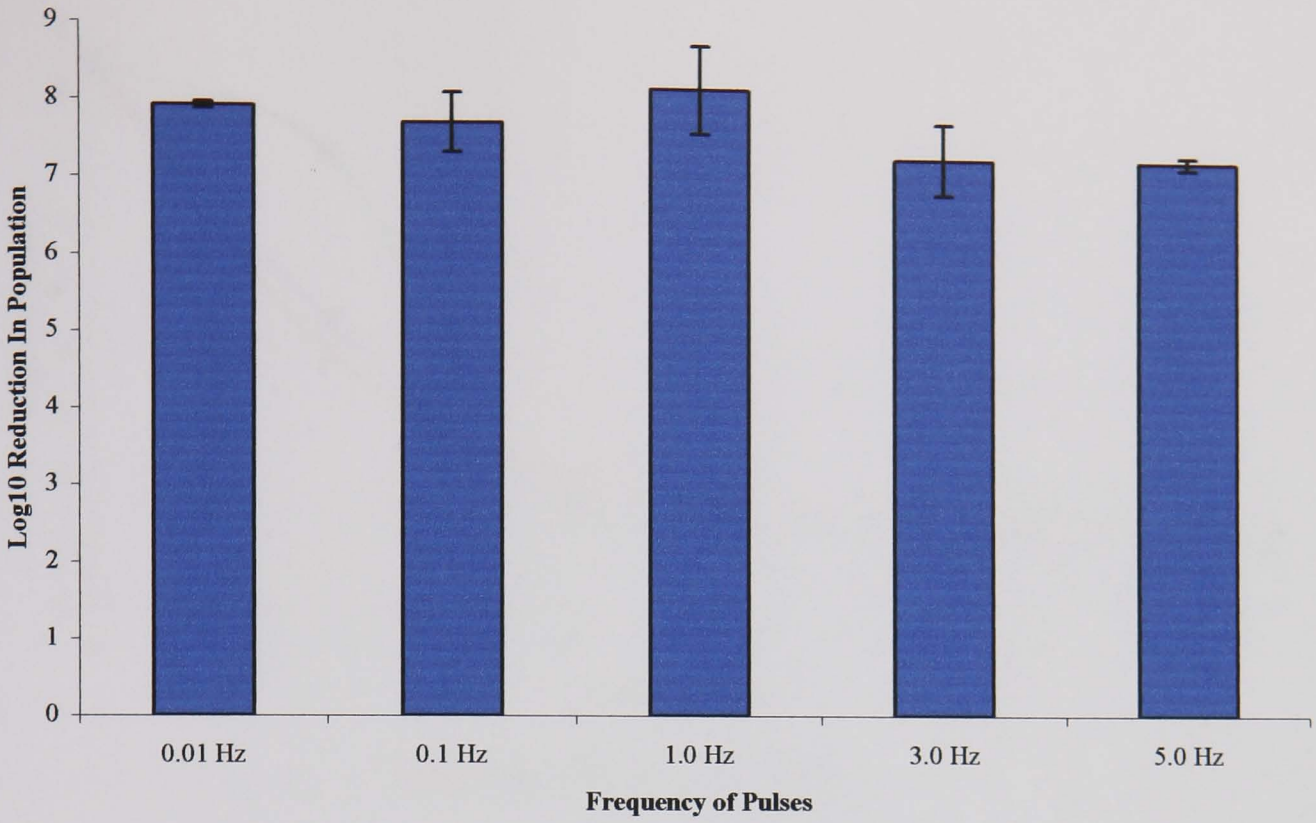


Figure 4.6.1 Log_{10} reduction of 2.1 x 10^9 $CFUml^{-1}$ stationary phase population of *E. coli* after treatment with 20 UV-rich light pulses, which were delivered at different frequencies (0.01, 0.1, 1, 3 & 5 Hz).

It can be observed from Figure 4.6.1 that there appears to be no significant difference in the Log_{10} reductions obtained for the five different frequencies. The 3 and 5 Hz pulses are shown to give a 7.3 Log_{10} reduction, the 0.01 and 0.1 Hz pulses are shown to give a 7.9 Log_{10} reduction and the 1 Hz pulses give an 8.1 Log_{10} reduction.

A further experiment to investigate the effect of pulse frequency on inactivation was undertaken. Here, 20 ml samples of a 10^8 $CFUml^{-1}$ population of *E. coli* were treated with 3, 6, 12 and 15 UV-rich light pulses. Frequencies of 0.1, 1 and 3 Hz were used to observe the effect of frequency over a pulse range. The results from these trials are shown in Figure 4.6.3.

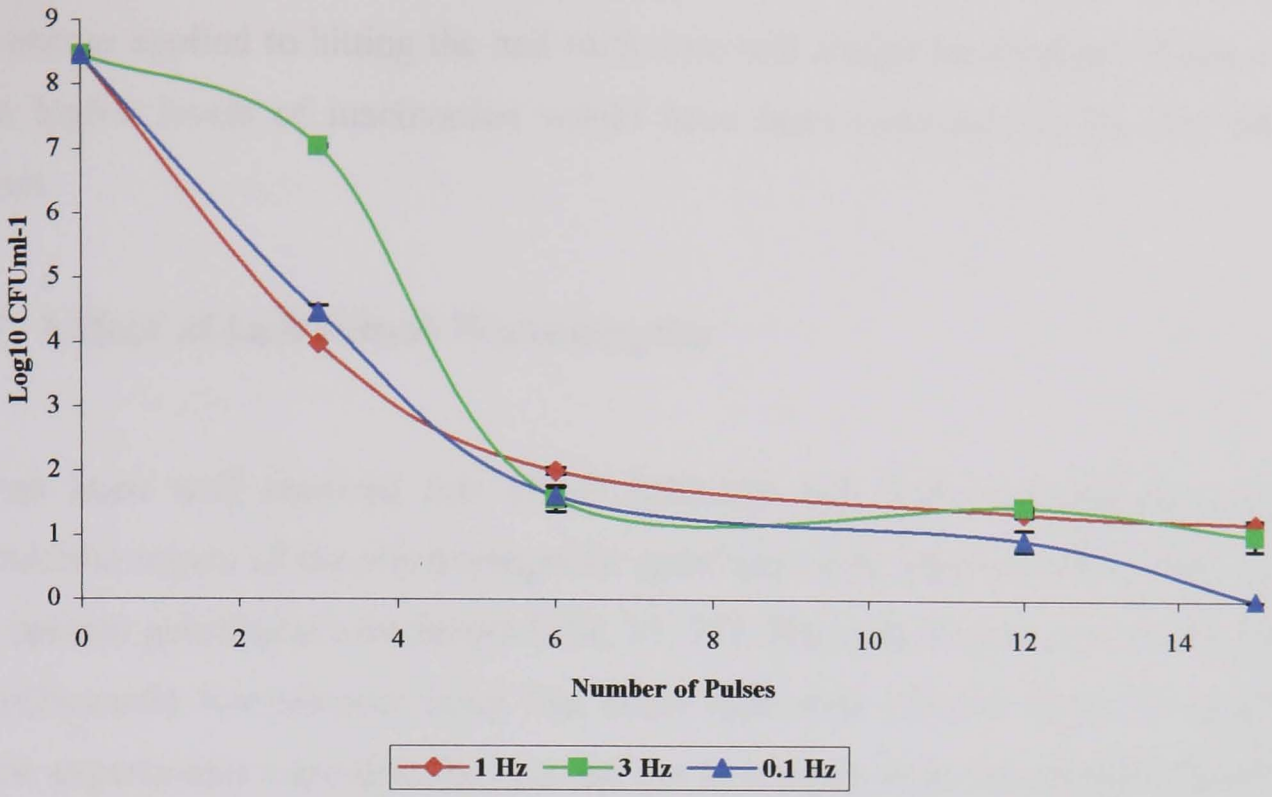


Figure 4.6.3 Inactivation results of *E. coli* after treatment with 3, 6, 12 & 15 UV-rich light pulses, which were delivered at different frequencies (0.1, 1 & 3 Hz).

The inactivation curves for all three pulse frequencies are very similar. The inactivation curves exhibit a rapid period of initial inactivation, with the rate starting to tail off after 6 UV-rich light pulses. The results for the 3 Hz pulses show a slow decrease in inactivation after 4 pulses, but this is probably just an experimental artefact rather than the effect of frequency used.

In the initial experiment using 20 UV-rich light pulses, it was found that using the higher pulse frequencies of 3 Hz and 5 Hz produced slightly lower levels of microbial inactivation than the lower frequencies, although this is not thought to be significant. In the second experiment carried out, there was also no significant difference between the inactivation rates. Both sets of results therefore do not support the suggestion that lower pulse frequencies deliver pulses with a higher energy per pulse [10]. The reasoning behind this can be likened with hitting a nail with a hammer. If someone hits a nail with a hammer once every 10 seconds they will soon tire and less energy will be used to hit

the nail. If however the nail is hit once a minute the individual will not tire as easily and the energy applied to hitting the nail each time will almost be identical. If this was true then higher levels of inactivation would have been expected for the low frequency pulses.

4.7 Effect of Individual Wavelengths

It has been well reported that UV-C (200-280 nm) light is considered to be the germicidal region of the electromagnetic spectrum, with many reporting that 254 nm is the optimal germicidal wavelength [110, 31, 77]. The light source used in this study is a polychromatic low-pressure lamp that emits light over a broad range of wavelengths. Some experiments were therefore carried out to identify what wavelengths emitted from this flash lamp are the most germicidal, and therefore responsible for the inactivation that occurs. To carry out these experiments the flash lamp arrangement had to be altered so that a monochromator could be attached. This allowed individual wavelengths through in 10 nm steps. The major drawback of using the monochromator is that the light pulses that pass through it have an extremely low energy (too low to measure) and for this reason a very small sample size had to be used for inactivation levels to be observed. A sample volume of 100 µl was used; therefore a special sample holder was made to hold the samples during treatment. The sample holder had a slot measuring 3 mm x 8 mm and was 6 mm deep, which was rinsed thoroughly with alcohol and sterile distilled water to ensure sterility. Because the energy of the pulses is very low, as well as using a small sample volume, a low population was prepared so that the bacterial reduction that occurs is more noticeable. *E. coli* at a concentration of 10^6 $CFUml^{-1}$ was used and prepared as described in section 3.7.1. Aliquots of 100 µl were carefully pipetted into the sample holder and treated with 100 and 300 pulses with the monochromator set at 220, 230, 240, 250 and 260 nm. After pulsing the samples were then serially diluted in 900 µl of ¼ strength ringer solution to give a 1 in 10 dilution. Diluted samples were plated out by the pour plate method as described in section 3.6.2.

After enumeration the Log reductions that occurred at each wavelength were calculated. The reductions observed after 100 pulses were very small (due to the low energy of the pulses); however larger reductions could be observed after 300 pulses. Data for this test is shown in Figure 4.7.1. It can be observed from this graph that very little inactivation occurs at 220 nm (0.4 Log reduction). However as the wavelength of the pulses is increased, the amount of inactivation starts to steadily increase up until 250 nm (2.5 Log reduction). After this, the amount of inactivation observed starts to decrease at 260 nm (1.9 Log reduction). From the results it therefore appears that the maximum levels of inactivation occur between 250 and 254 nm.

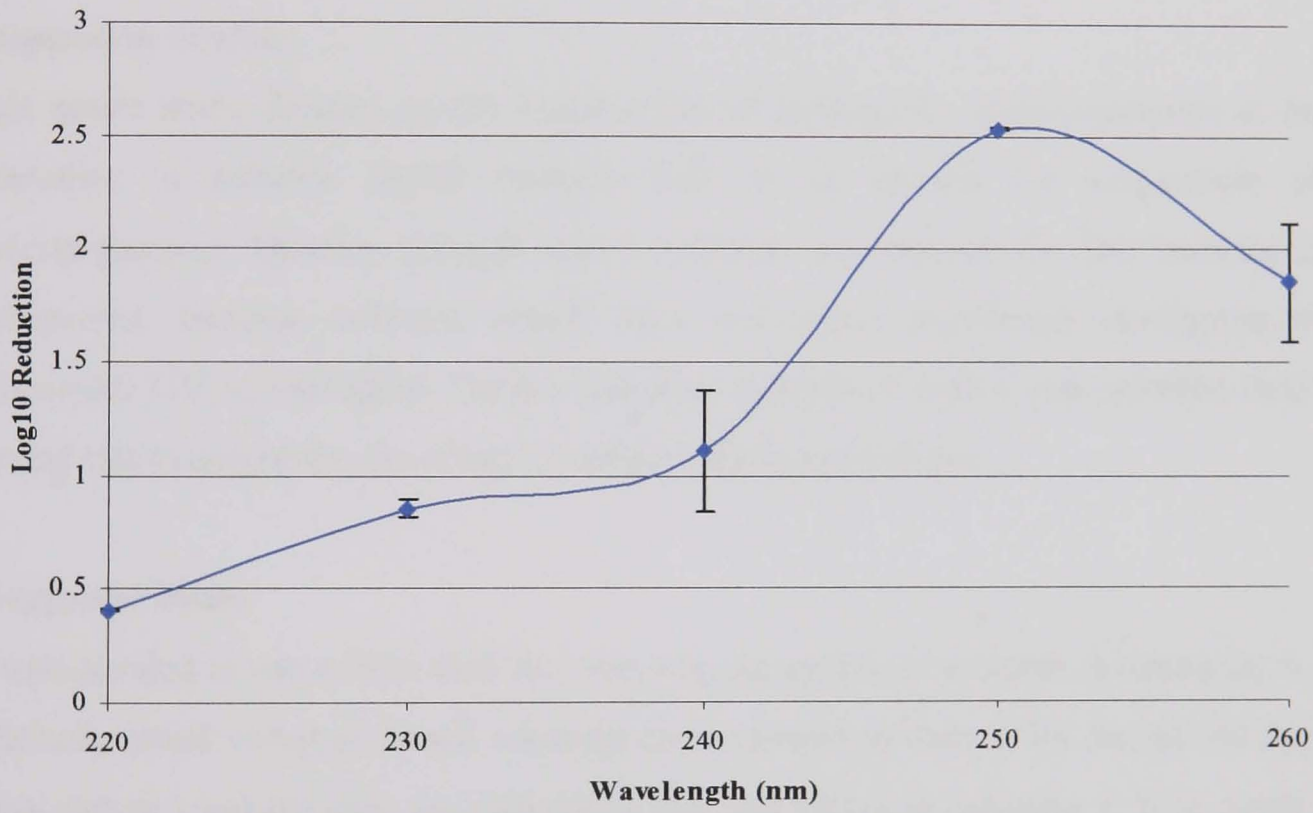


Figure 4.7.1 Log reductions in bacterial population after 300 UV-rich light pulses over the wavelength range 220-260 nm.

It has been suggested that for continuous sources the most germicidal wavelengths range from 250 nm to 260 nm. This study found that the highest levels of bacterial inactivation occur at approximately 250 nm which falls within this range. When the absorption

spectrum for DNA is considered, as in Figure 2.6.1, it can be observed that although maximum levels of UV absorption do not occur at this wavelength, absorption of UV at 250 nm by DNA is relatively high therefore high levels of microbial inactivation should occur.

4.8 Suggested Parameters

In this chapter, different electrical and biological parameters were investigated so that optimisation of the pulsed light system could be accomplished. Once the most important factors were established, a suitable protocol could be developed which could be applied to all experiments undertaken throughout the course of this investigation.

Suspension Media:

This entire study focuses on the inactivation of pathogenic microorganisms in liquids. Therefore, a suitable liquid medium had to be chosen for suspension of the microorganisms. Quarter strength ringer solution was chosen for this because it is a transparent, isotonic solution which does not cause significant absorption of the germicidal UV wavelengths. Once a suitable suspension media was selected tests were carried out to determine the effect of sample depth and volume.

Treatment Dish:

It was decided to use a Petri dish for carrying out sample treatments because (a) it holds relatively small volumes (small volumes allow numerous tests to be carried out from the same culture) and (b) they are disposable (the test cell with reflector at base needs to be sterilised between tests and does not produce significantly higher levels of inactivation). Another advantage is that the sides of a Petri dish are low which enables photons of light being emitted from the lamp at wide angles to reach the sample. It was decided not to use the dish which aided reflection of the UV back into the sample, because the difference in inactivation rates was not that large.

Sample Volume:

A relatively small sample volume was required so that numerous tests could be carried out from the same culture. A volume of 20 ml was therefore decided upon, as this is the smallest volume, which can be used in a Petri dish without causing attenuation of the UV. The depth of a 20 ml sample in a Petri dish is 3.28 mm.

Operating Voltage:

From the experiments carried out investigating the effect of charging voltage, it is considered appropriate to operate the system at its maximum voltage of 1000 V, so that high-energy pulses of high germicidal wavelengths are emitted.

Pulse Repetition Frequency:

From the tests carried out, there appeared to be no significant effect of pulse frequency on the inactivation performance therefore 1 Hz was chosen, simply because this represented a rate at which tests could be readily carried out

Wavelengths:

Individual wavelengths were not selected for use in this investigation, as the method by which they were produced resulted in the pulse energy being extremely small. All work therefore was carried out with broad spectrum (polychromatic) light pulses.

Chapter 5

INACTIVATION OF PROBLEMATIC BACTERIA

5.1 General

It is generally thought that the inactivation of a microorganism is influenced by a number of microorganism-related factors that are independent of the treatment technology itself. These include the type of microorganism (genus, species, and strain) and the growth stage of the microorganism (lag, exponential or stationary).Using the pulsed UV-rich light system with the parameters as described in Chapter 4 experiments were, conducted with a variety of bacterial species commonly associated with food- and water-borne disease. A range of experiments was carried out to observe any differences in the susceptibility of each microorganism to UV treatment, and to determine how the properties of each microorganism may render it more or less susceptible to UV irradiation.

The bacteria used for the study were:

- *Escherichia coli*
- Various *Campylobacter* species

} Common faecal contaminants of drinking water.

- *Pseudomonas aeruginosa* – A relatively new water pathogen [19] and is part of a group of environmental bacteria that are able to grow in water distribution systems.
- *Salmonella enteritidis* – A foodborne pathogen associated with foods containing eggs or poultry.
- *Listeria monocytogenes* – A foodborne pathogen which can grow at refrigeration temperatures and can contaminate meat and milk products.
- *Bacillus megaterium* – A bacterium responsible for many nosocomial infections due to contamination of enteral feeds and infant milk formulas.

Work was also carried out on water samples obtained from private water supplies, which were contaminated by bacteria from the external environment. The data from this work will be useful should the pulsed UV-rich light system be developed further for use in potable water disinfection.

5.2 Inactivation Differences between Gram-Negative and-Gram Positive Bacteria

Bacteria can generally be divided into two types, namely Gram-positive and Gram-negative, with this division being based on the structure of the cell wall of the microorganism. The initial experiments on UV inactivation were designed to determine any differences in the behaviour of the two types. Figure 5.2.1 shows the structures of the cell wall for Gram-negative and Gram-positive microorganisms.

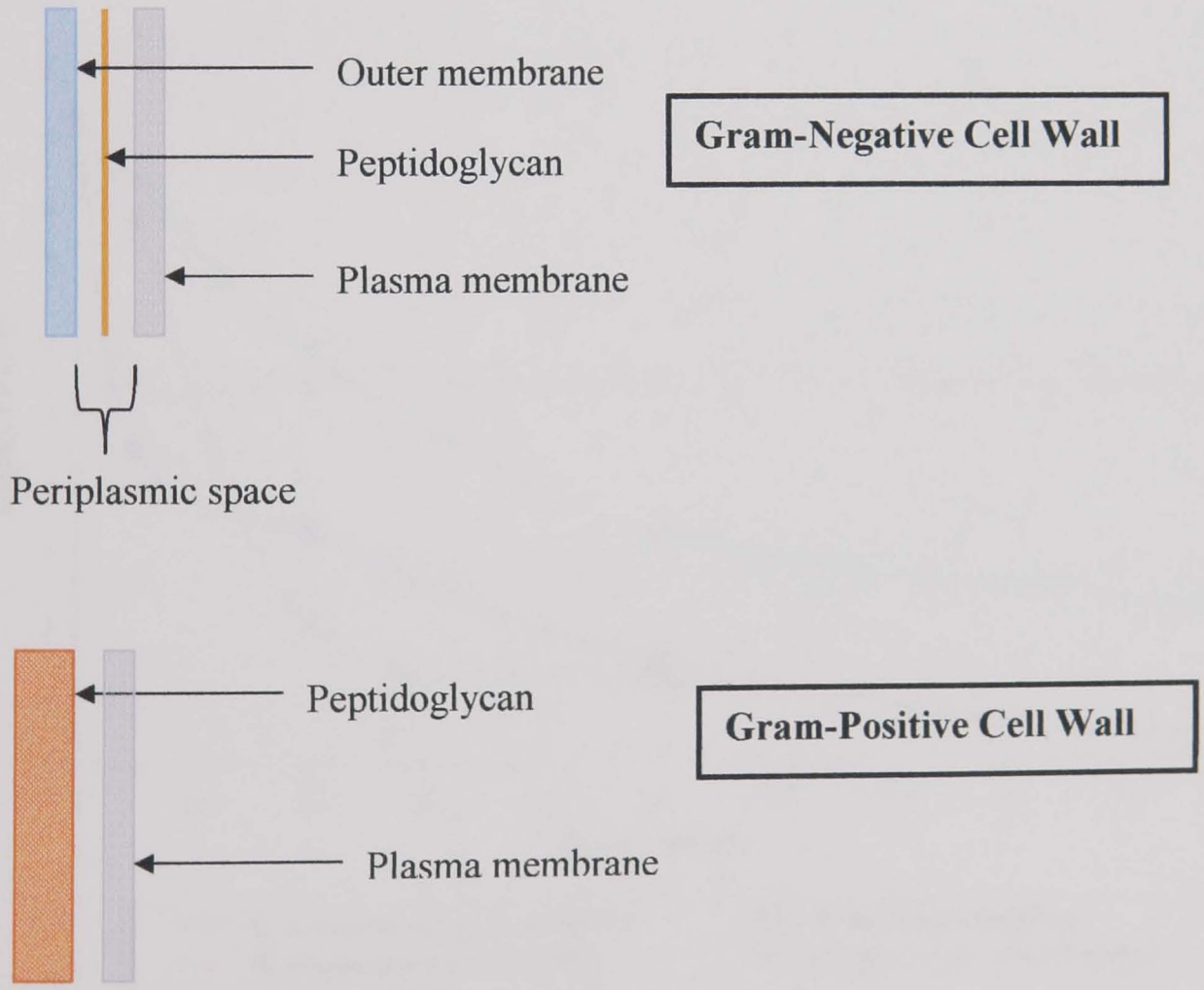


Figure 5.2.1 Gram-negative and Gram-positive cell wall structures.

The wall of a Gram-positive bacterium has a thick layer of peptidoglycan (20 to 80 nm), lying outside the plasma membrane. The Gram-negative cell wall is more complex. It has a 1-3 nm peptidoglycan layer surrounded by a 7 to 8 nm thick outer membrane. A gap called the periplasmic space exists between the plasma membrane and outer membrane of Gram-negative bacteria, and this gap contains many proteins and enzymes [20].

In the first experiment *Escherichia coli* and *Pseudomonas aeruginosa* (Gram negative) and *Listeria monocytogenes* and *Bacillus megaterium* (Gram positive) were cultured and prepared as described in Section 3.7.1. All cultures were re-suspended in equal volumes of diluent so that the populations remained undiluted. For each microorganism sample, volumes of 20 ml were treated with 5, 20, 50 and 100 light pulses using the same procedure as described in Section 3.7.2. The surviving population and the standard deviation are shown in the graph of Figure 5.2.1.

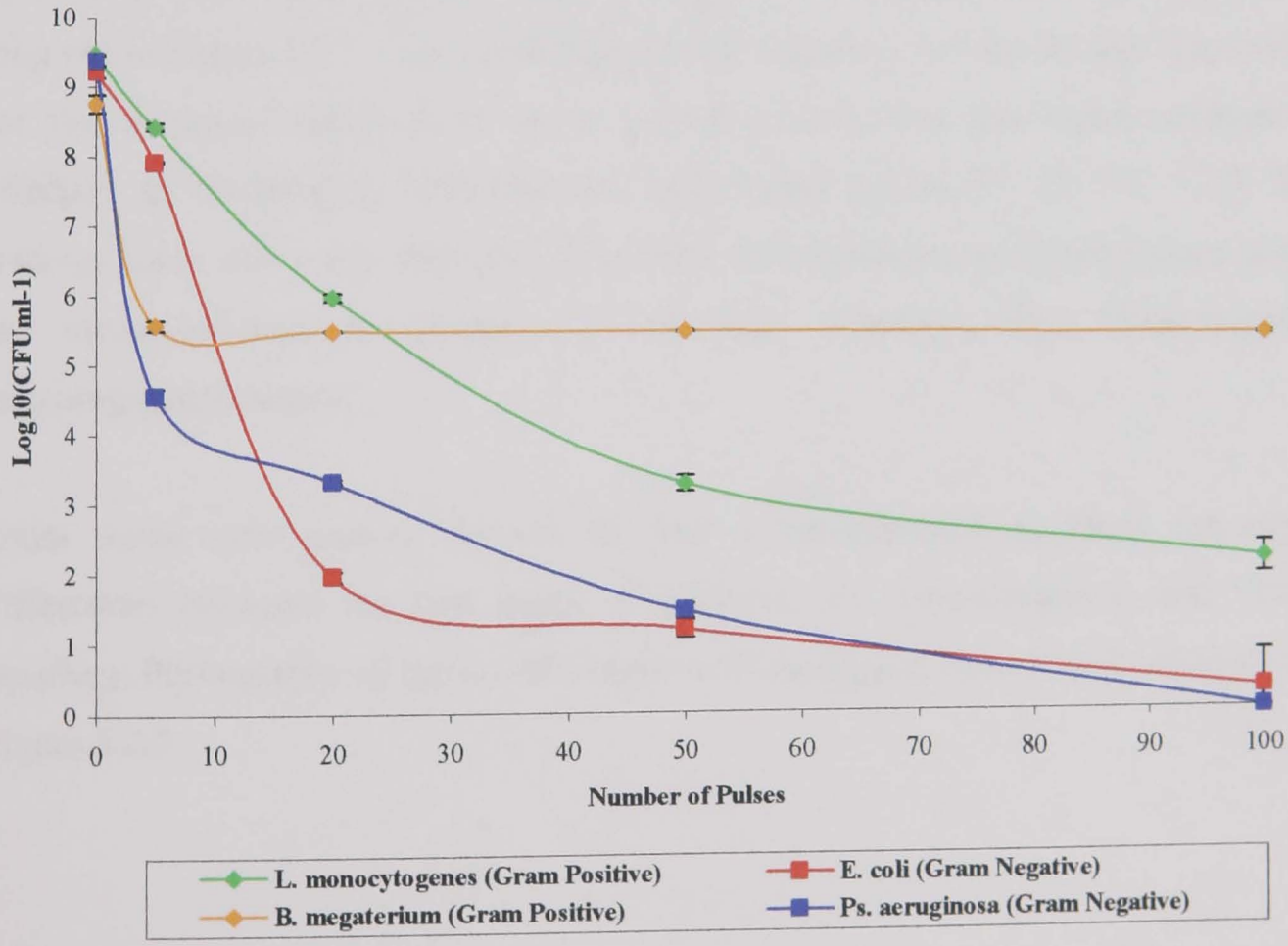


Figure 5.2.1 Populations of stationary phase *L. monocytogenes* and *B. megaterium* (Gram Positive) and stationary phase *E. coli* and *Ps. aeruginosa* (Gram Negative) after treatment with 5, 20, 50 and 100 UV-rich light pulses.

Figure 5.2.1 shows that the initial inactivation rate of *E. coli* occurs relatively rapidly with an impressive 7 Log_{10} reduction occurring after 20 UV-rich light pulses. After 20 pulses, the inactivation rate slows significantly. Complete inactivation had still not occurred following 100 pulses. The inactivation rate of *P. aeruginosa* (Gram-positive) is also very quick with a 4.82 Log_{10} reduction occurring after only 5 pulses, indicating this is more susceptible than *E. coli* at this low pulse dose level. After the initial decrease observed on the inactivation curve, the rate of inactivation slows down with inactivation occurring more slowly, with complete inactivation taking place after 100 light pulses (9.46 Log_{10} reduction). Inactivation of *L. monocytogenes* (Gram-positive) takes place less rapidly with only a 3.5 Log reduction occurring after 20 pulses. After 100 pulses, substantially more colony forming units remained for *L. monocytogenes* than for the two Gram-negative microorganisms. Inactivation results for *B. megaterium* show an initial rapid decrease in bacterial population, with a 3.18 Log_{10} reduction after 5 UV-rich light pulses. After this initial exponential decrease observed in the inactivation curve, there is little further significant reduction in numbers even after treatment with 100 light pulses, which is represented by the long tail in Figure 5.2.1. It is not thought that the presence of spores was responsible for the decreased inactivation, as it is well known that ultraviolet radiation is effective at inactivating both bacterial and fungal spores [9, 22, 51, 135]. The findings from this study therefore show that Gram-positive microorganisms tested are more resistant to pulsed UV-rich light treatment than Gram-negative microorganisms tested.

Gram stains were carried out on all four microorganisms to show the visual differences between the two types of bacteria (i.e. Gram-negative and Gram-positive). Photographs of these were taken with the digital camera and are shown in Figure 5.2.2.

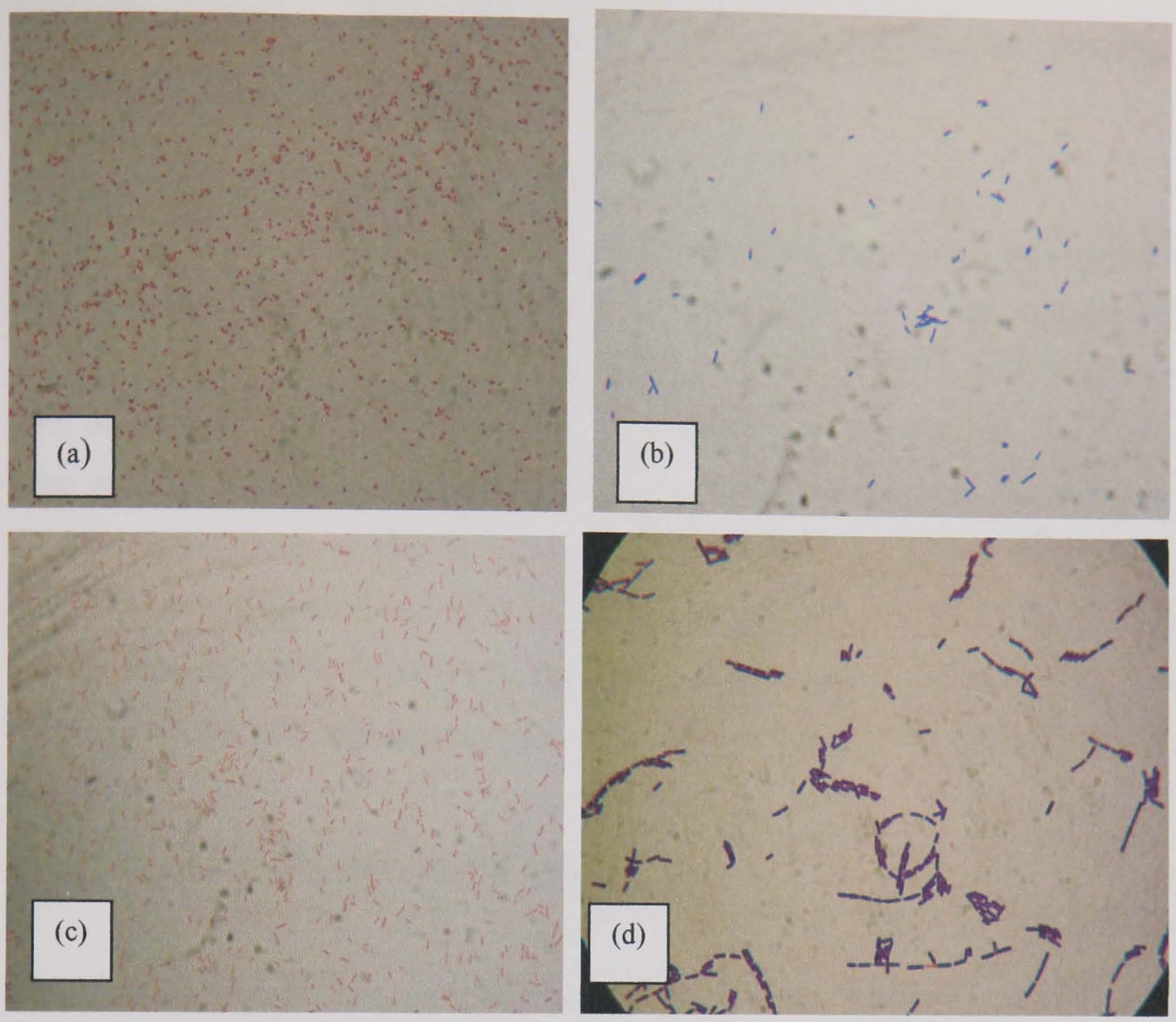


Figure 5.2.2 Gram stains of: (a) *E. coli*, (b) *L. monocytogenes*, (c) *Ps. aeruginosa* and (d) *B. megaterium*.

The Gram-negative rods of *E. coli* and *Ps. aeruginosa* are stained pink and can be observed in Figure 5.2.2 (a) and (c) and the purple stained Gram-positive rods of *L. monocytogenes* and *B. megaterium* can be observed in Figure 5.2.2 (b) and (d). From the photographs it can be observed that the Gram-positive rods are much longer than the Gram-negative rods suggesting that possibly the size of a microorganism can account for its sensitivity/resistance to the pulsed UV light treatment. Of all four microorganisms studied, *B. megaterium* is the largest (2 x 6 μm, according to "Bergy's Manual of Determinative Bacteriology" [111]). If size is a contributing factor then this may explain why this microorganism is so resistant to the treatment.

5.3 Pulsed Light Inactivation of Bacteria In Different Phases of Growth

When cultures of many bacterial species are grown in standard laboratory media, the microorganisms grow exponentially until the conditions no longer support rapid growth, and the cells then enter a stationary phase [60]. In this section *Escherichia coli*, *Salmonella enteritidis*, *Listeria monocytogenes* and *Pseudomonas aeruginosa* were studied to observe inactivation differences when in each of these phases of growth. The growth curves for each microorganism were prepared in order to determine how long each bacterium spends in the two phases. These curves allowed two appropriate growth times (corresponding to the exponential and stationary states) to be chosen for each bacterium, in order to obtain samples for the inactivation study.

5.3.1 Preparation of Growth Curves

To obtain the growth curves for the four microorganisms, suitable inoculums had to be prepared. Each microorganism was inoculated into a suitable broth, which was incubated at 37 °C for 18 hours. From each broth, 1 ml was removed and diluted 3-fold to give an approximate concentration of 10^6 CFUml^{-1}. From this dilution 100 µl (inoculum) was added to a 200 ml broth, providing a concentration of around 10^2-10^3 CFUml^{-1}. These broths were then incubated at 37 °C and samples were removed every hour for enumeration over a 12 hour period. Additional samples were also removed after 28 and 30 hours of growth. In addition to enumerating each sample, optical density measurements were made using the Spectrophotometer to observe the differences in turbidity as the population size increases. The growth and optical density curves for *E. coli*, *S. enteritidis*, *L. monocytogenes* and *Ps. aeruginosa* are shown in Figures 5.3.1.1, 5.3.1.2, 5.3.1.3 and 5.3.1.4 respectively.

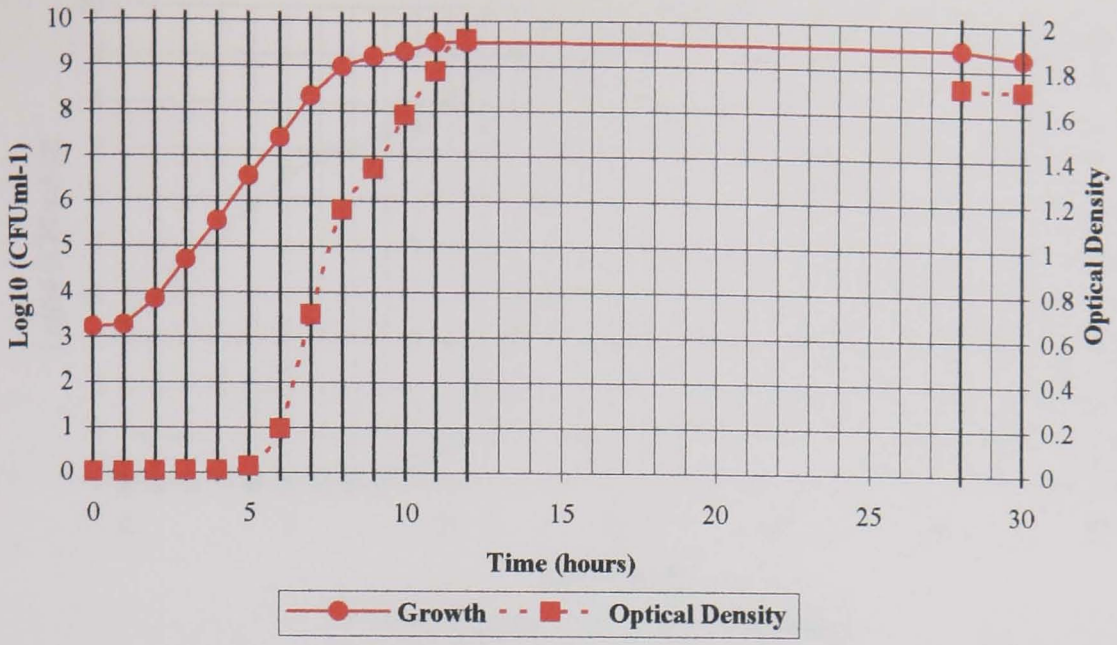


Figure 5.3.1.1 A 30-hour growth curve and corresponding optical-density curve for *Escherichia coli.*

Each of the growth curves for *E. coli* and *S. enteritidis* in Figure 5.3.1.1 and 5.3.1.2 show that an exponential period of growth occurs between 1 and 8 hours, after which growth levels off with each of the microorganisms entering the stationary phase after about 11 hours. After 28-30 hours the death phase appears to commence.

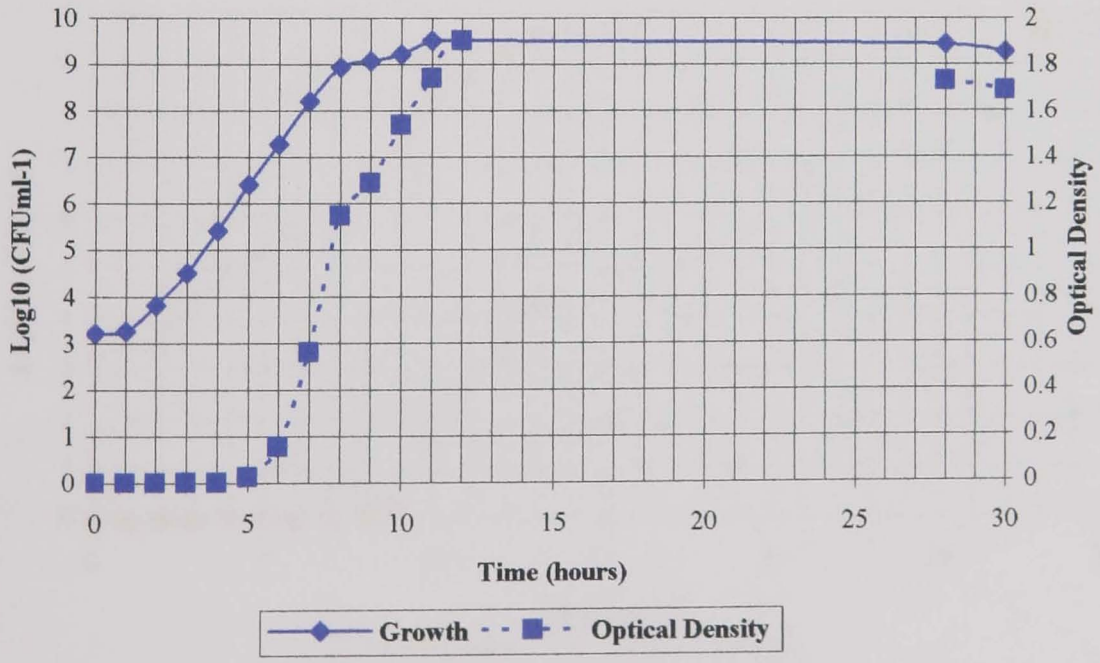


Figure 5.3.1.2 A 30 hour growth curve and corresponding optical-density curve for *Salmonella enteritidis.*

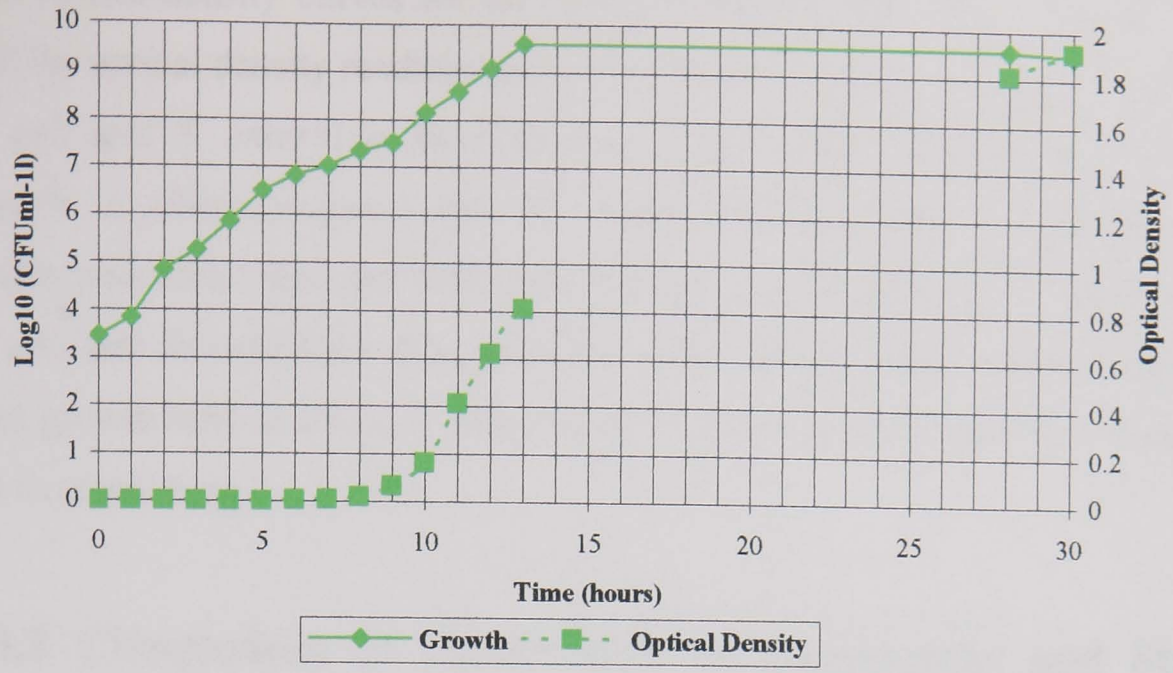


Figure 5.3.1.3 A 30 hour growth curve and corresponding optical-density curve for *Listeria monocytogenes*.

Each of the growth curves of *L. monocytogenes* and *Ps. aeruginosa* in Figures 5.3.1.3 and 5.3.1.4 shows an exponential period of growth occurs between 0 and 13 hours. In each case, the microorganism enters the stationary phase where it remains for up to 28-30 hours of growth when they thereafter enter death phase.

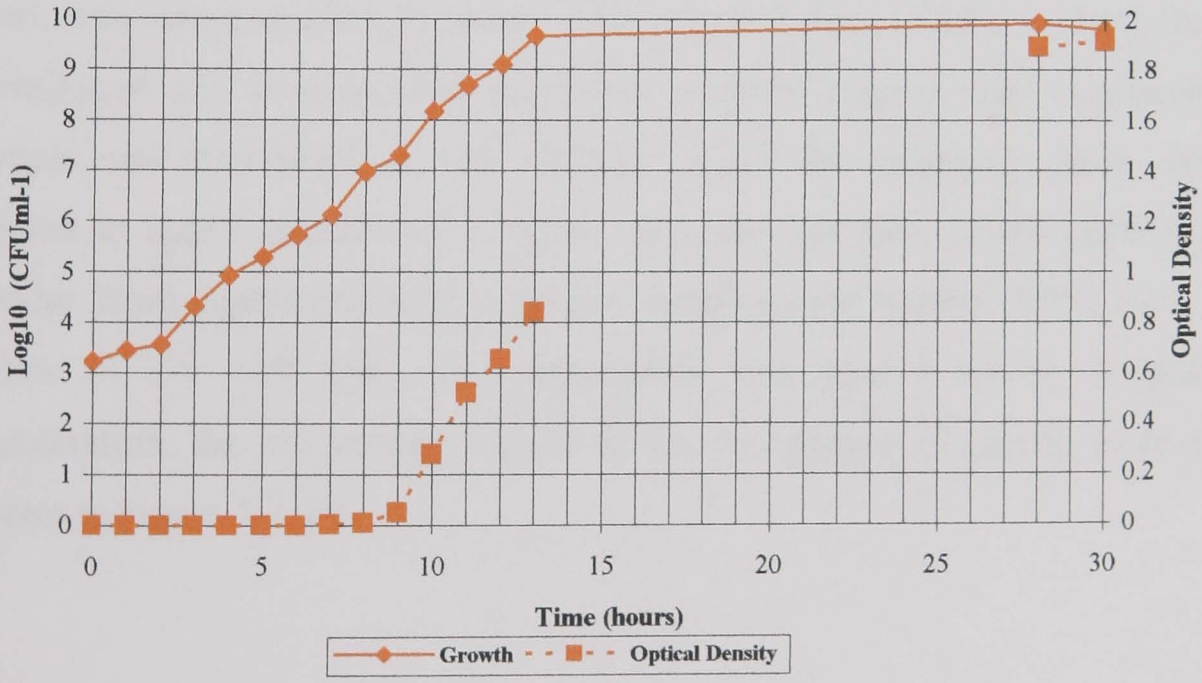


Figure 5.3.1.4 A 30 hour growth curve and corresponding optical-density curve for *Pseudomonas aeruginosa*.

The optical-density curves for all four microorganisms follow a similar pattern in that the optical density readings increase exponentially as exponential growth occurs. *E. coli* and *S. enteritidis* have similar optical-density readings at each Log_{10} of growth. *L. monocytogenes* and *Ps. aeruginosa* also have similar optical-density readings to each other, but both these microorganisms have much lower values than *E. coli* and *S. enteritidis*. The optical-density readings can be used in combination with growth time to estimate what phase of growth each of the four microorganisms are in at any time.

5.3.2 Comparison of Inactivation In Exponential and Stationary Phase

For the investigation of *E.* coli, a 100 ml broth was inoculated and incubated at 37 °C under rotary conditions. A 1 ml sample of this broth was then removed and diluted 3 fold to give an approximate concentration of 10^6 $CFUml^{-1}$. From this dilution, 100 µl (inoculum) was added to 2 x 200 ml broths (as for the growth curves). The two broths were then incubated at 37 °C, and for the exponential phase samples, one broth was removed after 6 hours, and for stationary phase samples, the remaining broth was removed after 18 hours. After removal from incubation both broths were centrifuged and re-suspended in quarter strength ringers. The exponential phase sample was maintained at ~10^7 $CFUml^{-1}$ while the stationary phase sample was diluted to approximately 10^7 $CFUml^{-1}$ in order that both initial populations were similar. Both exponential and stationary samples were treated with 2, 4, 6, 8 and 10 pulses of UV rich light. The experiment was carried out in triplicate. After enumeration, the inactivation curves of the two phases of growth were plotted as shown in Figure 5.3.2.1.

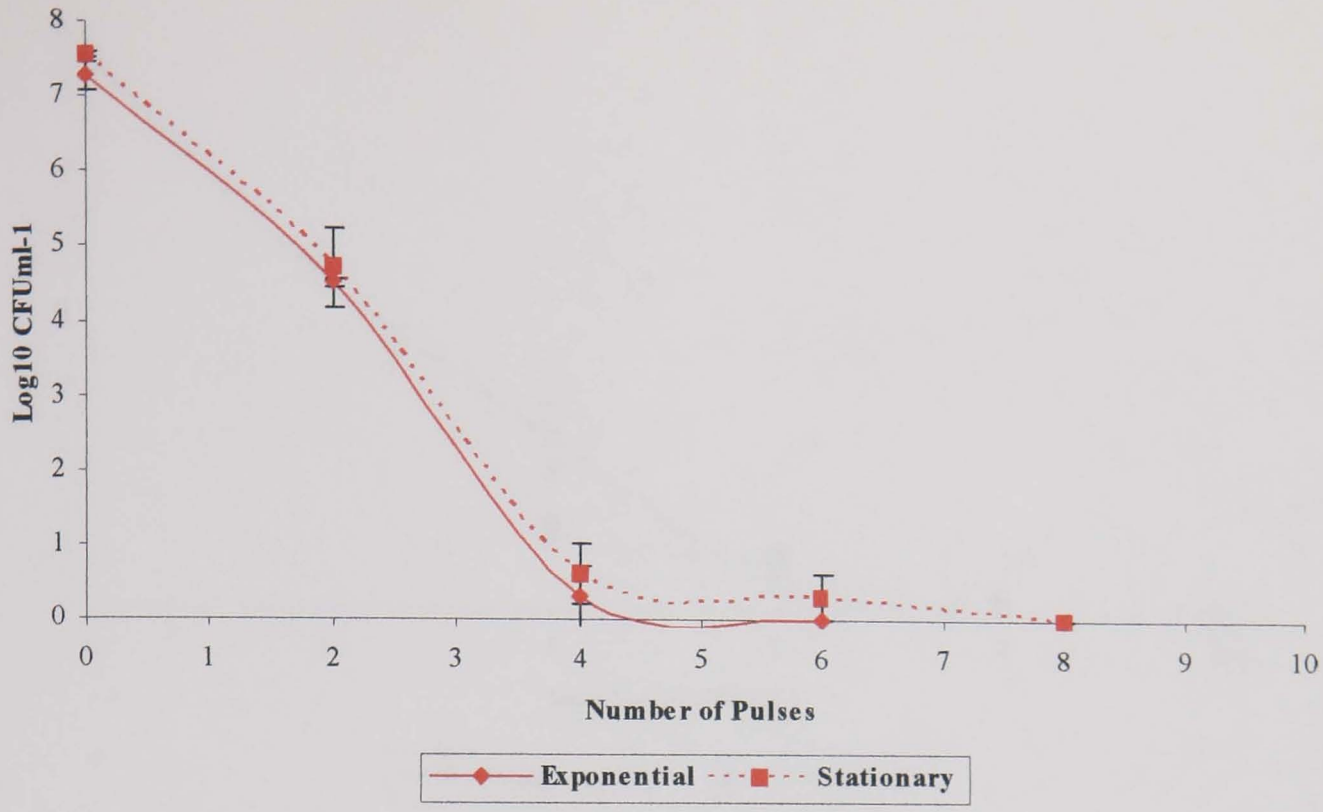


Figure 5.3.2.1 Inactivation curves for *E. coli*, when grown to exponential and stationary phase, after treatment with a range of UV-rich light pulses.

Within the levels of experimental uncertainty, the fall in population with increasing number of UV pulses is observed to be the same for both samples, indicating that for *E. coli* the phase of the growth phase is not important for pulsed UV inactivation. This suggests that the changes that *E. coli* undergoes upon entry to stationary phase do not interfere with its susceptibility to pulsed UV treatment.

Because *Salmonella enteritidis* follows a similar growth curve to *E. coli*, *S. enteritidis* samples for exponential and stationary phase were taken at the time intervals of 6 and 18 hours respectively. The same experimental procedure was followed as for *E. coli* and the resultant inactivation curves for the exponential and stationary phases are shown in Figure 5.3.2.2.

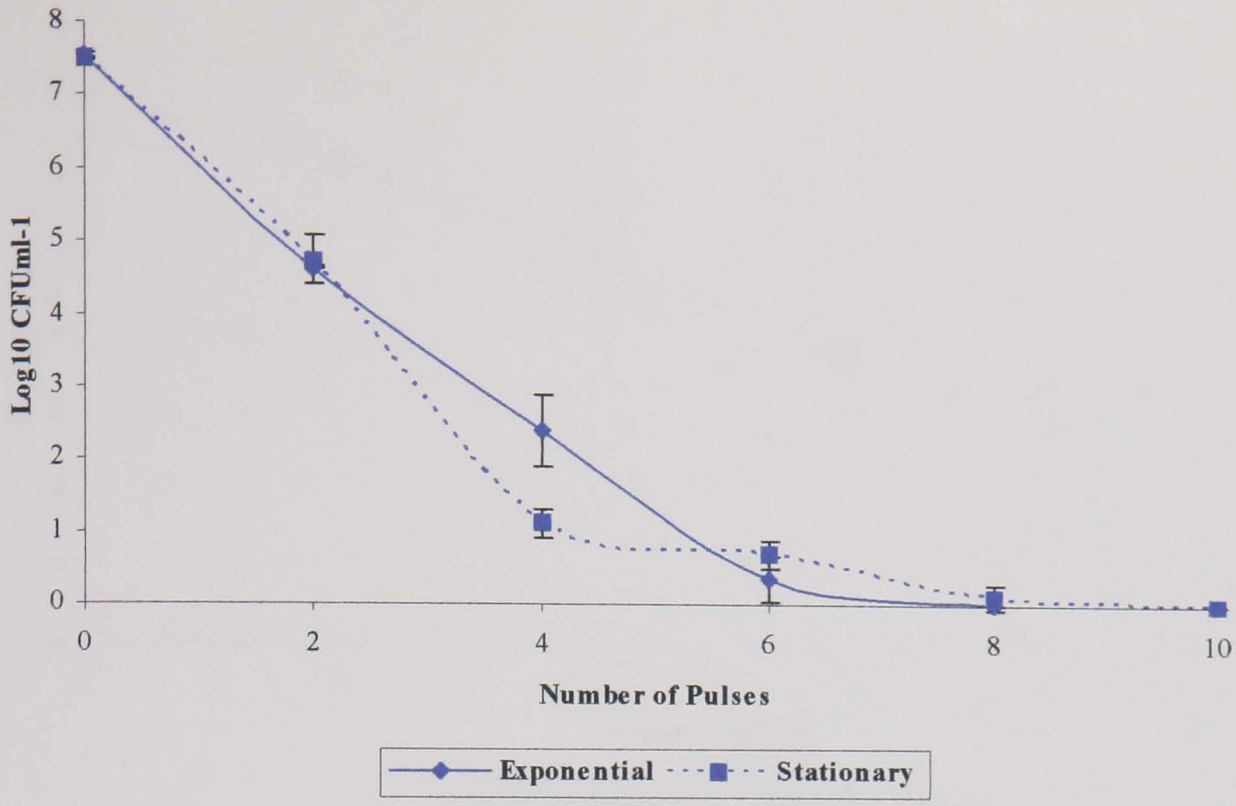


Figure 5.3.2.2 Inactivation curves for *S. enteritidis*, when grown to exponential and stationary phase, after treatment with a range of UV-rich light pulses.

Although small differences exist between the two inactivation curves for *S. enteritidis*, these are not considered significant due to the low concentrations, and it appears that, as for *E. coli* the UV-light inactivation of *S. enteritidis* is independent of phase of growth.

Knowing the growth curve for *Listeria monocytogenes*, the exponential phase and stationary phase samples were taken at 7 hours and 18 hours respectively. Using the same experimental procedure as for the first two bacteria, Figure 5.3.2.3 was obtained following treatment.

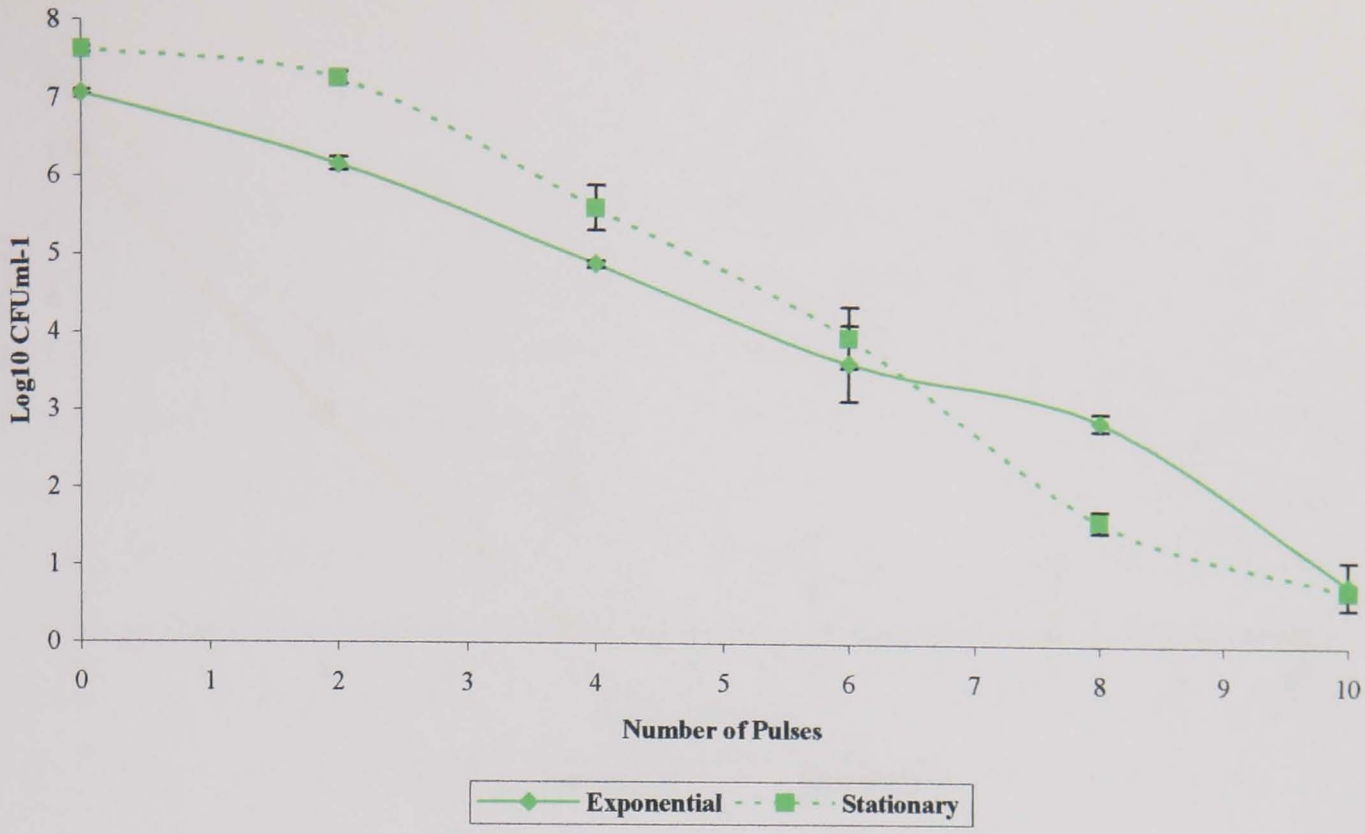


Figure 5.3.2.3 Inactivation curves for *L. monocytogenes*, when grown to exponential and stationary phase, after treatment with a range of UV-rich light pulses.

Listeria monocytogenes has already been shown to be a more resistant microorganism than the previous two organisms that were studied. However, once again the inactivation curves for the two growth phases show similar structures and trends. The behaviour of the inactivation curve for a sample of *L. monocytogenes* also appears therefore to be largely independent of the choice of growth phase.

The inactivation of *Pseudomonas aeruginosa* was studied using the same procedure as for the previous three bacteria, with samples taken after 7.5 and 18 hours. The treated samples from exponential and stationary phase yielded the inactivation curves shown in Figure 5.3.2.4.

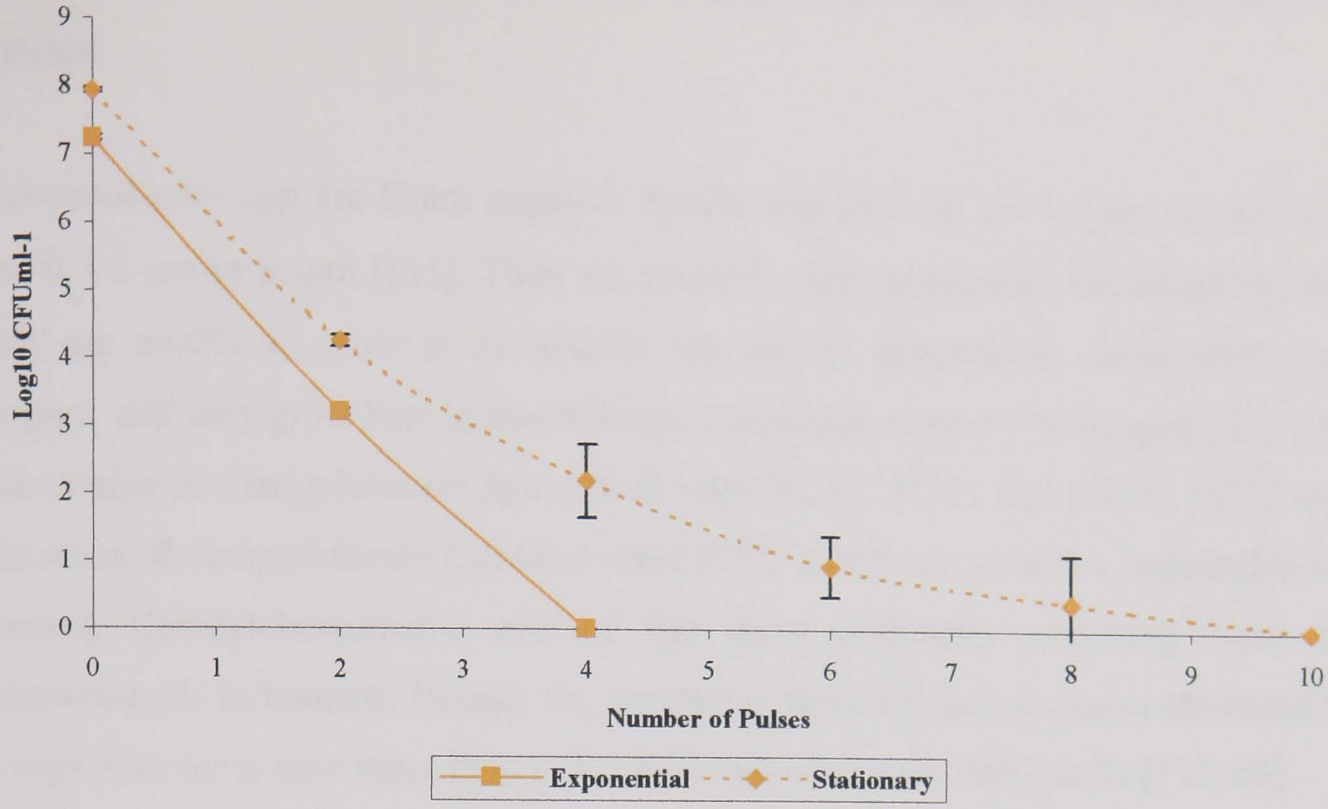


Figure 5.3.2.4 Inactivation curves for *Ps. aeruginosa*, when grown to exponential and stationary phase, after treatment with a range of UV-rich light pulses.

Here, there is a significant difference in the behaviour of the two samples for *Ps. aeruginosa.* Inactivation is more rapid for the exponential phase sample, with total inactivation taking place following 4 UV-rich light pulses compared to 10 pulses for the stationary phase sample. The reason for the decreased sensitivity of *Ps. aeruginosa* in stationary phase may be the ability of the microorganism to produce a polysaccharide slime layer. This layer will not start to be produce until the organism enters stationary phase, and it will undoubtedly absorb some of the pulsed light therefore producing lower levels of inactivation. The significance of this result needs to be borne in mind when preparing samples of *Ps. aeruginosa* for UV inactivation studies.

5.4 Inactivation of *Campylobacter* Species

The sensitivity of a microorganism to UV irradiation can vary within the same species. This variation was examined for three different types of *Campylobacter.* Two different strains of *C. jejuni* were used to observe the variations from strain to

strain and one strain of *C. coli* was used to observe the variations between bacterial species.

Campylobacter spp are Gram negative motile rods that are 0.2-0.5 μm in diameter and 0.5-5 μm in length [111]. They are generally microaerophillic which means that they are unable to grow in or tolerate the normal atmospheric concentration of oxygen, and they grow best in atmospheres containing around 5 % oxygen [60]. The two strains of *Campylobacter jejuni* used were NCTC 11351 and NCTC 11322 and the strain of *Campylobacter coli* used was NCTC 11366. All three are responsible for causing Campylobacteriosis, one of the most frequently occurring types of gastroenteritis in humans. Indeed, the number of cases of gastroenteritis attributed to *Campylobacter* is now more than triple that associated with *Salmonella* [112, 60].

Campylobacter is a relatively slow growing microorganism compared with the microorganisms discussed earlier. Before any UV inactivation experiments were carried out, a growth study was made of one of the strains (*C. jejuni* 11351) to determine the time taken to obtain a stationary phase culture. The growth curve was obtained using the procedure outlined previously (section 5.3.1). The resultant growth curve for *C. jejuni* 11351 is shown in Figure 5.4.1. The exponential phase occurs over the first 15 hours after which the microorganism enters the stationary phase. Hence for the UV inactivation studies of *Campylobacter*, samples were taken after 30 hours of growth to ensure a mid-stationary phase culture.

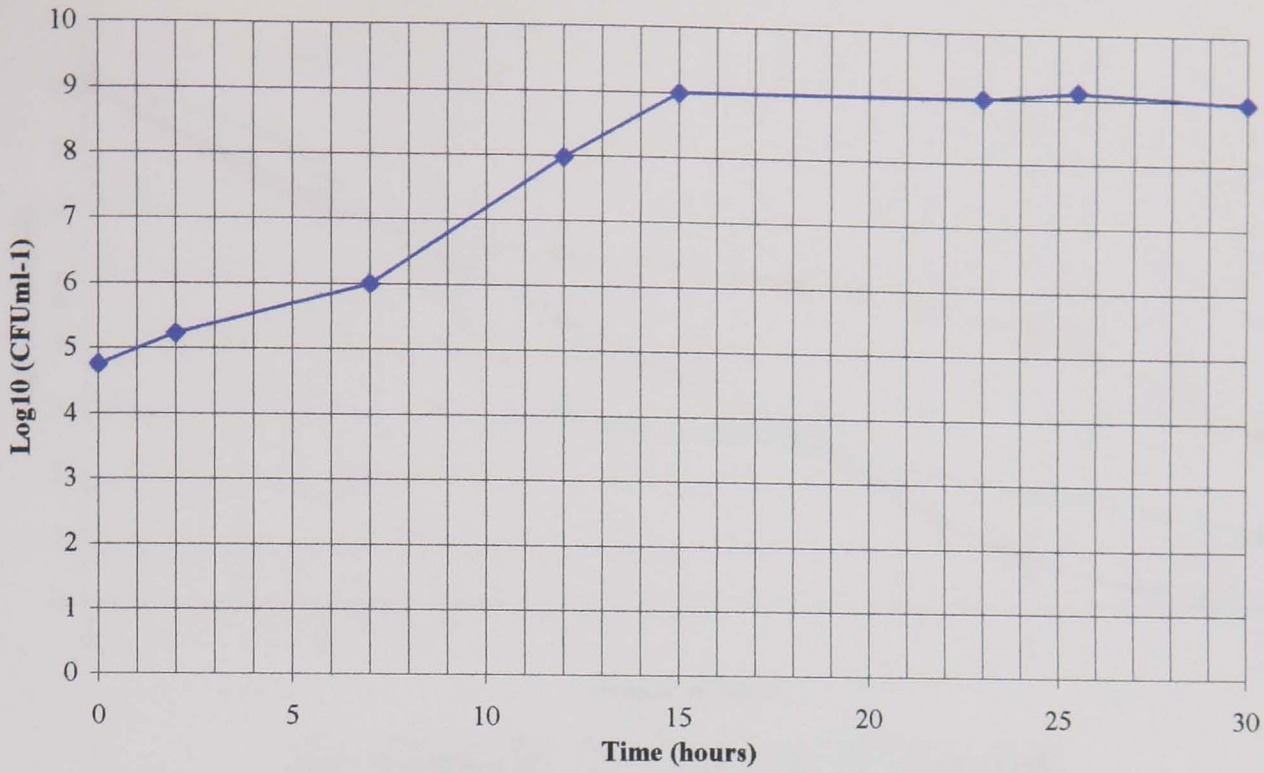


Figure 5.4.1. 30 hour growth curve of *Campylobacter jejuni* 11351.

For *C. jejuni* 11351, *C. jejuni* 11322 and *C. coli* 11366, the procedure for sample preparation and inactivation measurement was as follows: The bacteria were grown in Brucella broth, at 37 °C, for 30 hours. Agitation was not used during growth because the bacteria are considered to be microaerophilic and grow best in atmospheres containing around 5- 10 % oxygen. After 30 hours growth, the microorganism was centrifuged and re-suspended in quarter strength ringer. 20 ml samples were then treated with 1, 2, 3, 4 and 5 light pulses as described in section 3.7.2. All samples were then enumerated and the plates were put into an anaerobic jar containing a CampyGen sachet. The jar was then incubated at 37 °C for 48 hours to allow colonies to develop.

Inactivation curves were prepared from the data for the three different *Campylobacter* species and these are displayed in Figure 5.4.2. The results show that *Campylobacter jejuni* (11351) is extremely sensitive to the pulsed light treatment. The population decreases constantly as the pulse number increases, and complete inactivation is achieved after only 5 light pulses, giving an overall reduction of 8 Log_{10}.

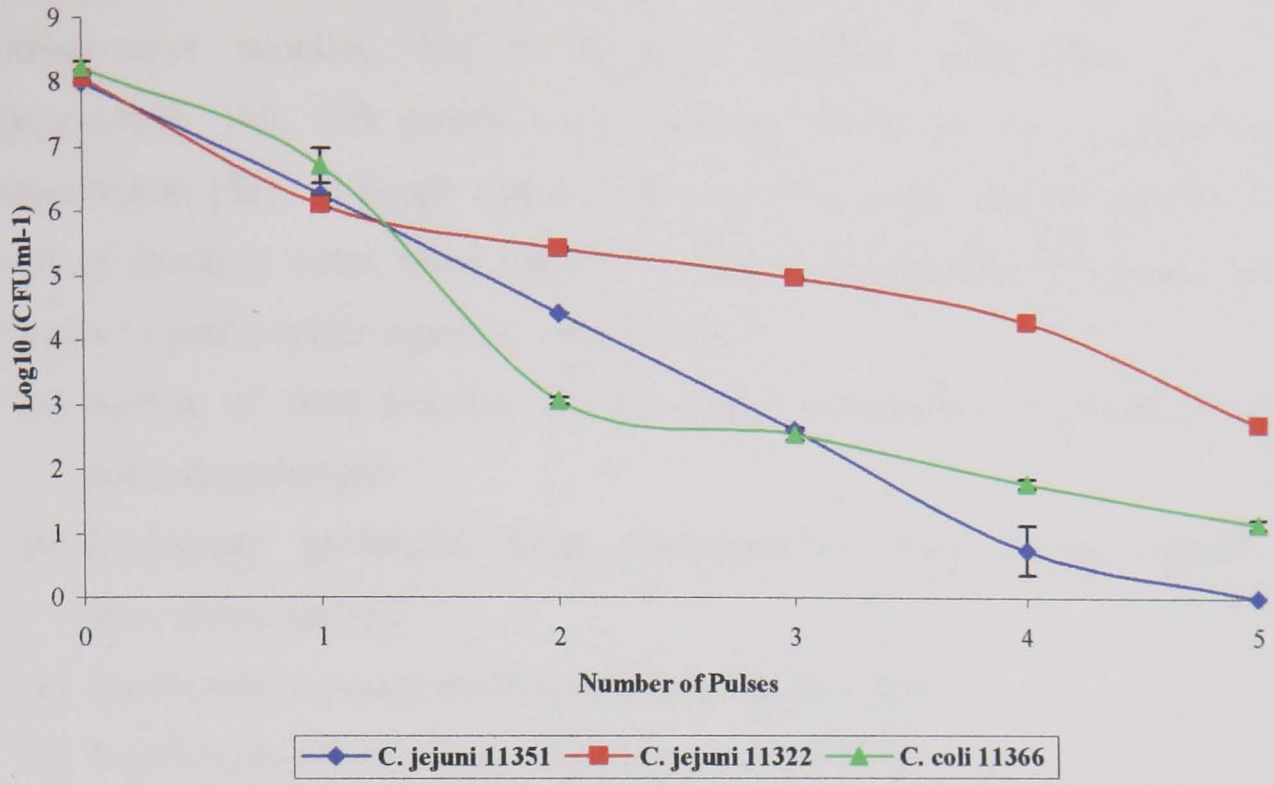


Figure 5.4.2 Inactivation curves obtained for stationary phase *C. jejuni* (11351), *C. jejuni* (11322) and *C. coli* (11366), after treatment with 1, 2, 3, 4 and 5 UV-rich light pulses.

UV inactivation of *Campylobacter jejuni* (11322) appears to be less efficient than for *C. jejuni* (11351), with around a 5 Log_{10} reduction following 5 UV-rich light pulses. *Campylobacter coli* (11366) appears to behave more like *C. jejuni* (11351) than *C. jejuni* (11322). There is no apparent reason why the two strains of *C. jejuni* should show such different susceptibilities to the pulsed UV light treatment. It is possible however that the *C. jejuni* 11351 strain may be a hybrid strain and therefore behaves more like the *C. coli* strain.

5.5 Inactivation of Bacteria Present In Private Water Samples

So far, the studies reported in this chapter have focussed on the inactivation of bacteria in laboratory media (Quarter strength ringer solution). The remainder of the chapter reports on an investigation into the inactivation of bacteria in drinking-water samples.

The literature review (Chapter 2) discusses the problems of contamination of public drinking-water supplies, but in England, Scotland and Wales, there are approximately 140, 000 private water supplies which are more susceptible to contamination [82]. Although many of these private water sources provide a safe supply of drinking water, there are certain risks of contamination that generally do not apply to public water supplies. These include:

(a) Access of farm animals to the source catchments, wellhead or spring collecting chamber
(b) Inadequate protection from contamination from surface runoff and agricultural activity
(c) Inadequate or poorly maintained treatment facilities
(d) Possible proximity of private sewage systems [82].

A pilot study was carried out on private water samples. Upon collection, these samples were examined for the presence of bacterial contamination. Once the number of contaminants was obtained, the samples were treated with UV-rich light pulses to look at the effectiveness of the treatment process with environmentally grown microorganisms as opposed to laboratory grown.

5.5.1 Total Viable Counts (TVC) of Water Samples

Five 500 ml samples were obtained from a private drinking water supply in Stirlingshire, Scotland. Two 100 ml volumes of each sample were poured into sterile Duran Bottles. From one bottle a 1 ml sample was removed and un-diluted neat and 1 in 10 dilutions were plated out by the pour-plate method. These plates were incubated at 25 °C for 72 hours. From the other bottle the same procedure was carried out except that the plates were incubated at 37 °C for 48 hours. The results showing the number of viable cells present in each sample, when incubated under the different conditions are shown in Figure 5.5.1.1.

	Total Viable Counts ($CFUml^{-1}$)	
Sample	**After 72 h incubation at 25 °C**	**After 48 h incubation at 37 °C**
1	11	0
2	78	4
3	770	46
4	330	0
5	320	130

Figure 5.5.1.1 Number of viable cells present in the different water samples after incubation at (a) 25 °C for 72 hours and (b) 37 °C for 48 hours.

All five water samples contain some degree of bacterial contamination, with sample 5 showing the highest level of contamination. The microorganisms grown at 25 °C will be normal drinking-water flora, which are considered harmless to human health. The microorganisms, which have grown at 37 °C, could possibly be pathogenic.

5.5.2 Analysis of Water for the Presence of Coliforms

To try and identify whether any of these potential pathogens were coliforms, the multiple-tube method was used as described in section 3.8.2. A positive result following incubation is indicated by a change in colour from purple to yellow (acid production) with or without the production of gas. See Figure 5.5.2.1. The number of positive tubes recorded is shown in Table 5.5.2.1.

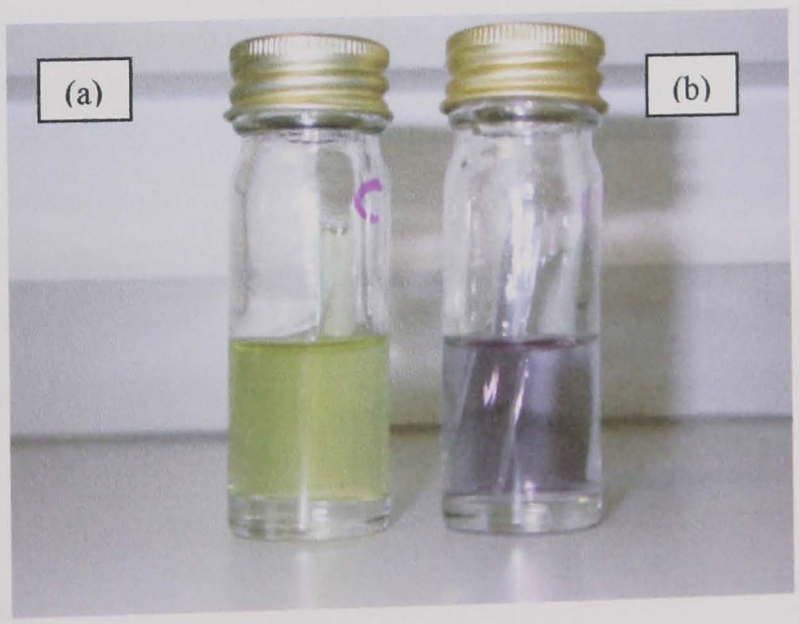


Figure 5.5.2.1 Photograph showing (a) a positive MMGM bottle and (b) a negative MMGM bottle

	MMGM Bottles					
Sample Number	**50 ml**	**10 ml**	**10 ml**	**10 ml**	**10 ml**	**10 ml**
(1)	-	-	-	-	-	-
(2)	++	+	+	-	-	-
(3)	++	+	-	-	-	-
(4)	-	-	-	-	-	-
(5)	++	++	++	++	++	+

Key
++ Acid and gas produced
+ Acid produced
- No acid or gas

Table 5.5.2.1 Number of bottles of MMGM showing positive and negative results.

The numbers of bottles showing positive results were added and the MPN of coliforms per 100 ml was obtained from a table of most probable numbers (Appendix A). These results are tabilised in Figure 5.5.2.2.

	Number of Tubes Giving a Positive Reaction		
Sample Number	**1 x 50 ml**	**5 x 10 ml**	**MPN per 100ml**
(1)	0	0	0
(2)	1	2	5
(3)	1	1	2
(4)	0	0	0
(5)	1	5	> 18

Table 5.5.2.2 The most probable number of coliforms present according to the number of positive reactions.

Coliforms present in samples (2), (3) and (5). To try and identify the microorganisms further, bottles showing positive results were inoculated into BGLBB (see section 3.8.2 for method). After incubation, all tubes of BGLBB gave positive results at both incubation temperatures, which was indicated by the presence of gas (in the Durham tube) and turbidity. These results show that coliforms are present and the positive growth at 44 °C indicates the presence of thermotolerant organisms such as *E. coli*.

5.5.3 Pulsed-Light Treatment of Water Samples

Aliquots of 20 ml, of each water sample, were treated with 5, 10 and 20 UV-rich light pulses as described in section 2.7.2. After treatment, samples were incubated at 25 °C for 72 hours and 37 °C for 48 hours, and enumerated by the pour-plate method. The results obtained are shown in Figures 5.5.3.1 and 5.5.3.2 respectively. Figure 5.5.3.1 shows that all microorganisms in samples (1) and (4) were completely inactivated by 20 light pulses. For the other samples the populations were reduced, but not totally inactivated by 20 pulses. For the samples that were pulsed and then incubated at 37 °C (Figure 5.5.3.2), complete inactivation occurred in sample (2) with 10 pulses. For samples (3) and (5) most of the population was inactivated with 20 pulses (2 and 7 $CFUml^{-1}$ respectively remaining). The results therefore show that the bacteria present in the water sample, which grow at 48 °C, are more susceptible to the pulsed light treatment than those which grow at 37 °C. Surviving colonies were removed from the treated plates, and after Gram staining, they were found to be Gram-positive rods.

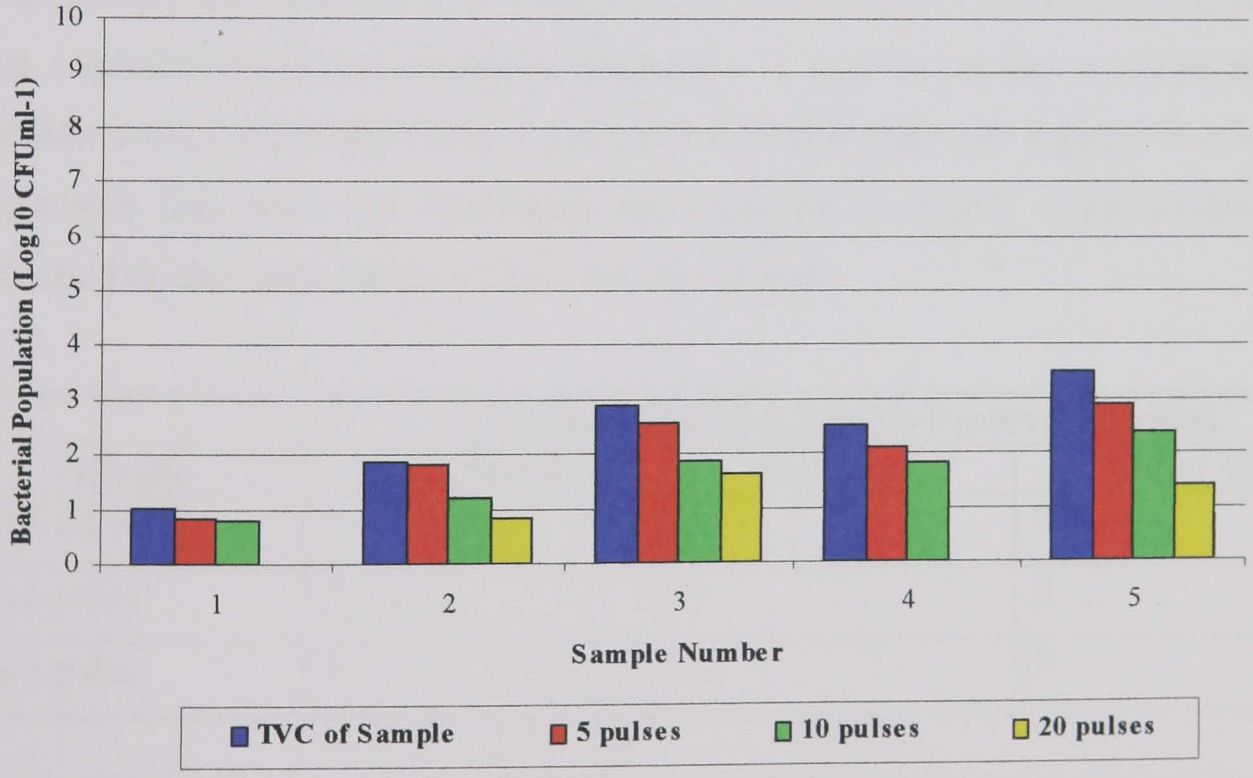


Figure 5.5.3.1 Number of viable organisms present in the various water samples after 72 hours incubation at 25 °C, and the numbers present after treatment with 5, 10 and 20 UV-rich light pulses.

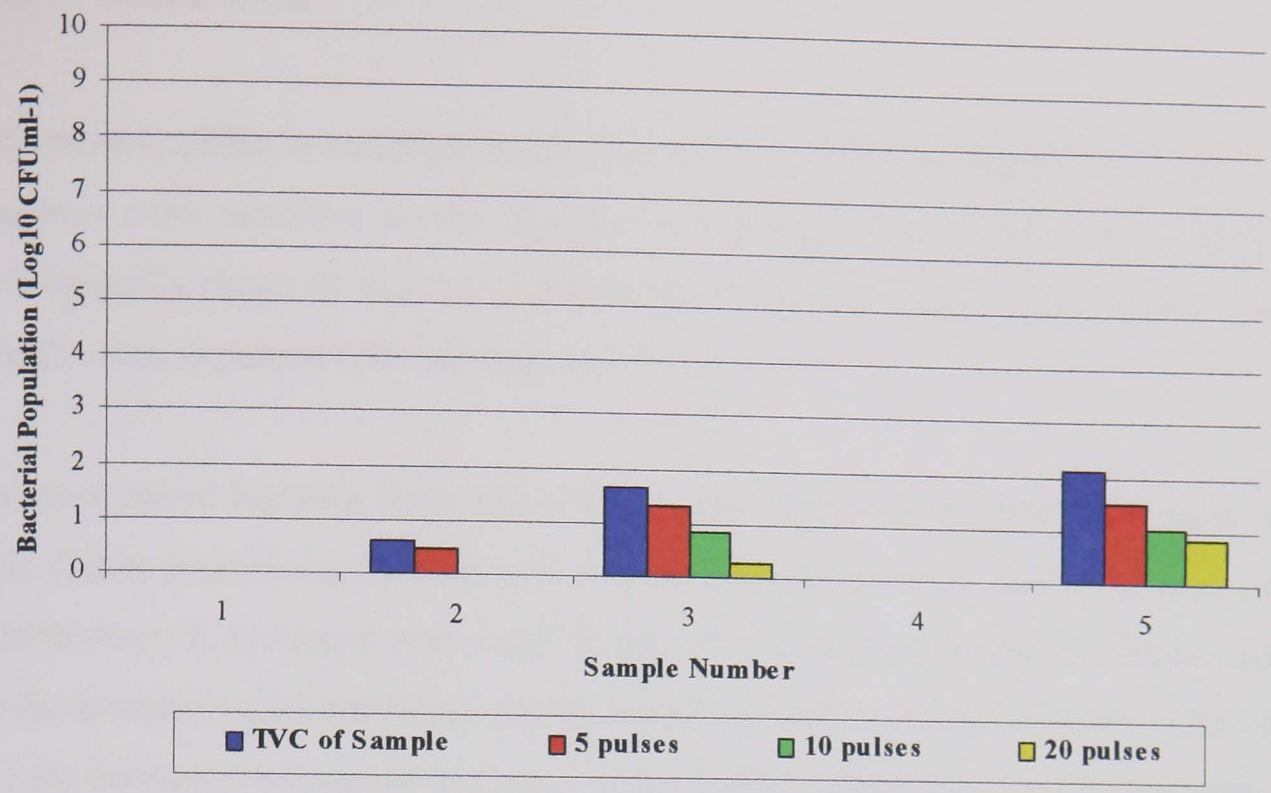


Figure 5.5.3.2 Number of viable organisms present in the various water samples after 48 hours incubation at 37 °C, and the numbers present after treatment with 5, 10 and 20 UV-rich light pulses.

The most probable number test (section 5.5.2), was carried out on the treated samples that originally contained coliforms (samples 2, 3 and 5) and this revealed that the coliforms were inactivated with 5 pulses of UV rich light (see Figure 5.5.3.3). The organisms that were not inactivated are likely to be mainly Gram-positives, as indicated by the Gram stains which were carried out.

	Number of Tubes Giving a Positive Reaction		
Sample	**1 x 50 ml**	**5 x 10 ml**	**MPN per 100ml**
(2) 5 pulse	0	0	0
(3) 5 pulse	0	0	0
(5) 5 pulse	0	0	0

Figure 5.5.3.3 The most probable number of coliforms present according to the number of positive and negative MMGM tubes.

5.6 Discussion

All bacteria differ in structure and function and it is these properties that make some bacteria more sensitive to the external environment than others. This chapter has considered a range of microorganisms and they have been found to have varying sensitivities to pulsed UV-rich light treatment.

Gram negative bacteria were found to be more sensitive to pulsed UV inactivation than Gram positive bacteria. *E. coli* and *Ps. aeruginosa* were shown to have similar sensitivities. *L. monocytogenes* and *B. megaterium* were considerably more resistant to the treatment with the latter displaying the greatest level of resistance. One reason for the increased resistance of Gram positive microorganisms, to UV treatment, may be the presence of the thick peptidoglcan wall surrounding the plasma membrane of these microorganisms (Figure 5.2.1). The peptidoglycan wall around Gram negative bacteria is much thinner which is likely to reduce the degree of UV absorption. Gram negative bacteria also have a periplasmic space between their outer membrane and their plasma membrane, and this space contains proteins involved in nutrient acquisition and in the transport of materials into the cell. [20]. The UV-rich light pulses may be responsible for causing photochemical reactions within these molecules as absorption in proteins has been shown to peak at around 280 nm [35]. If the light pulses are responsible for causing damage to these proteins, the bacterial cells may not be able to acquire nutrients, which in turn could lead to cell death and hence an increased rate of inactivation. *Bacillus megaterium* may be more resistant than other Gram positive bacteria, for example, *Listeria*, because it has a more complex surface structure. *Bacillus megaterium* synthesises a capsule composed of both polypeptide and polysaccharide which may provide the microorganism with additional protection [113]. Another reason for the germicidal efficiency varying amongst these microorganisms (in addition to cell wall structure) could be due to variations in the content of cytosine relative to thymine in their DNA [49]. The shape of the absorbance spectra for these two pyrimidine bases are different, with thymine's maximum absorbance peaking at approximately 267 nm and cytosine's peaking at 271 nm [46]. The emission spectra of this lamp would favour

photochemical changes within the thymine bases (see lamp emission spectra in Figure 3.5.1.1), as there are greater emissions at this wavelength, resulting in increased inactivation of the microorganisms with a larger content of thymine.

Because the growth condition of a microorganism can lead to structural and morphological changes, it was thought important to investigate whether any of these changes affect pulsed UV light resistance. Of the four bacteria studied in both the exponential and stationary phase of growth, only *Pseudomonas aeruginosa* displayed a difference in UV-inactivation treatment. *Pseudomonas* was shown to be much more resistant to the treatment in the stationary phase with complete inactivation occurring after 10 light pulses compared with 4 light pulses for the exponential-phase sample. The increased resistance in the stationary phase is believed to be due to the sigma factor RpoS that is the central regulator for many stationary phase induced changes. It is critical for the survival of bacterial cells in stationary phase, particularly during exposure to unfavourable conditions. The increased resistance to heat shock, oxidative, osmotic and acid stresses may be linked to the profound structural and physiological changes that occur upon entry into the stationary phase [60], and these changes such as polysaccharide slime formation may also be increasing the resistance of *Pseudomonas aeruginosa* to pulsed UV-rich light treatment. Such structural changes however do not appear to increase the resistance of the other three microorganisms tested when they move into stationary phase.

Susceptibility of different strains and species of *Campylobacter* was also investigated to observe any differences in susceptibility to the treatment. The initial experiment with *C. jejuni* (strain 11311) showed that this bacterium is extremely sensitive to the pulsed UV-rich light treatment, with a constant inactivation rate, no tailing and complete inactivation after only 5 light pulses. *Campylobacter* species generally tend to be sensitive to the extra-intestinal environment [95] and they also have previously been shown to be inactivated by UV (sunlight) in the outside environment. This was reported by Obri-Danso *et al* [94] who observed that the levels of *Campylobacter* species in surface waters were lower in summer than in winter months. This appears to be due to the combination of higher temperatures and higher UV-B radiation

levels during the summer months [94]. When *C. jejuni* (strain 11322) was treated with the pulsed UV-rich light pulses a different result was obtained. The inactivation rate was slower, with slight tailing occurring and incomplete inactivation of the bacterium, after 5 pulses. These results are somewhat surprising, as it is expected that different strains of the same species of microorganism would show similar inactivation levels. However, functions associated with iron uptake and metabolism can differ significantly between strains of *Campylobacter* [114], and so their functions such as DNA repair may also vary between strains. It has also been demonstrated that certain strains of *C. jejuni* can survive for longer periods in drinking water than others, and this was found to depend on the origin of the strain [115]. Strains of *C. jejuni* which have been isolated from the environment, for example, in streams, may be used to UV in the form of sunlight and may therefore build up some degree of resistance to UV light. When a different species of *Campylobacter* was treated (*C. coli* strain 11366), it was found to behave more like *C. jejuni* (strain 11351) than *C. jejuni* (strain 11322) but less sensitive than *C. jejuni* (strain 11351). Generally, *Campylobacter* species are relatively sensitive to pulsed UV-rich light treatment, more so than the other microorganisms tested. This could be due to the absence of RpoS, which is thought to be responsible for the failure of *C. jejuni* NCTC 11351 to induce stress resistance in the stationary phase [60]. The sensitivity of *Campylobacter* to pulsed UV-rich light treatment is surprising for a human pathogen that is exposed to many harsh conditions before it enters the body [95].

Private water supplies can readily become contaminated by a variety of means (section 5.5). Total viable counts (TVCs) were carried out on five water samples and it was found that all five contained bacteria. This may not necessarily indicate contamination in the supply, it may be due to run-off or leaves and these bacteria are not necessarily harmful. The Most Probable number test was carried out and indicated that three of the samples contained coliforms and further analysis revealed that thermotolerant bacteria such as *E. coli* might be present. The presence of these bacteria indicates possible faecal contamination of the private source. UV inactivation revealed significant reductions in the bacterial numbers and total

inactivation in some samples. Pulsed UV-rich light clearly provides an effective method of treating drinking water, and although complete inactivation did not always occur, inactivation of all the coliforms, including the faecal ones, was achieved.

Overall, in this chapter it was found that the sensitivity of different bacteria, to pulsed UV-rich light treatment, is not only dependent on the type of microorganism, but the species, strain and growth phase of each bacterium plays a major role. From the work carried out, it was found that Gram negative bacteria are generally more sensitive to pulsed UV-rich light treatment than Gram positive bacteria and out of all the microorganisms studied, *Campylobacter* was shown to be the most sensitive to the pulsed UV-rich light treatment. The most resistant bacterium was found to be *Bacillus megaterium* (Gram positive). As well as effectively inactivating laboratory-grown bacteria, the pulsed UV-rich light treatment proved successful at inactivating bacterial contaminants of private water supplies. It reduced the numbers of contaminants and accomplished complete inactivation of the potentially pathogenic microorganisms (coliforms).

Chapter 6

INACTIVATION OF PROTOZOA AND VIRUSES

6.1 General

The previous chapter focussed mainly on the inactivation of bacterial microorganisms commonly associated with food- and waterborne disease. This chapter is centred on the susceptibility of other types of microorganisms to pulsed UV-rich light treatment. The microorganisms examined and reported here are *Cryptosporidium parvum* and some viruses which are also important causes of food and waterborne illness.

6.2 Inactivation of *Cryptosporidium* Oocysts

Cryptosporidium parvum is a protozoan parasite that causes mild to severe gastroenteritis in humans and animals [103]. It is one of the most important waterborne pathogens because of its presence in wastewater and some drinking water sources and it has a high resistance to treatment processes [105]. *C. parvum* oocysts are between 4 and 6 μm in diameter and contain 4 sporozoites (infective stage of life cycle) that escape by excystation once ingested by a susceptible host (see section 2.9.3 for more information on the life cycle) [116]. Once released, the sporozoites enter the gut and cause infection. A differential interference contrast (DIC) image of *Cryptosporidium parvum* oocysts is shown in Figure 6.2.1.

Because of the nature of the microorganism and the need for special facilities due to health and safety considerations, the work could not be carried out at the University. The sensitivity of this important enteric pathogen to pulsed UV-rich light treatment was

therefore investigated, with assistance from Professor Huw Smith and his staff in the Scottish Parasite Diagnostic Laboratory at Stobhill Hospital, Glasgow.

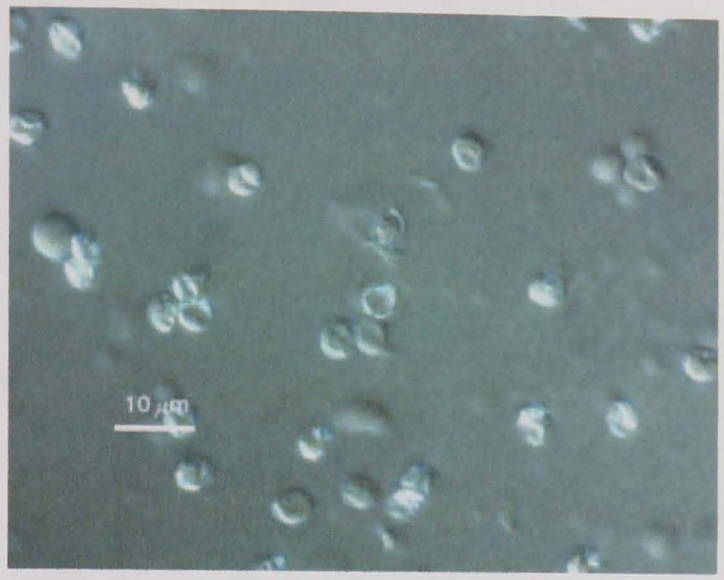


Figure 6.2.1 DIC image showing the 4 to 6 microns spheroidal oocysts of *C. parvum*. Photograph was obtained from the US Environmental Protection Agency (EPA). Photo Credit: H.D.A Lindquist, U.S. EPA

6.2.1 Preparation of the Oocysts

The *Cryptosporidium parvum* oocysts used in the experimental work were purchased from the University of Arizona. They had been collected from the faeces of deer calves that had previously been infected with oocysts. To prepare the oocysts for the pulsed light treatment they were suspended in Reverse Osmosis (RO) water so that there were approximately 1 million oocysts in each sample. RO water is sterile water which has salts and other impurities removed from it. 1.5 ml samples were then pipetted into Eppendorf tubes and placed into a pulsifier for around 5 seconds to separate any oocysts that might have formed clumps.

6.2.2 Pulsed Light Treatment of the Oocysts

The parameters of the pulsed light system were kept the same as those described in section 3.7.2 and suspensions of the oocysts were treated with 5, 25 and 50 pulses of UV radiation.

The procedure for the treatment of the *Cryptosporidium* oocysts however, had to be altered slightly from the standard pulsing procedure used for bacterial species. The reason for this is that there was a limited supply of oocysts available for the experiment. A 1.5 ml sample was therefore used and instead of using a standard-sized Petri dish for treating the samples, a 6-well 'Tissue Culture Testplate' was used to allow even distribution of the sample. Samples of the oocysts were prepared by pipetting 1.5 ml of the suspension into one of the wells. The plate was then placed inside the treatment chamber so that the well containing the sample was directly under the flashlamp. After pulsed light treatment, each sample was carefully pipetted into a sterile epindorff and the regime was carried out in triplicate.

E. coli was also treated for comparison as a reference test. The *E. coli* was grown as described in section 3.7.1 and diluted to an approximate concentration of 10^6 CFU ml^{-1}. A 1.5 ml sample of *E. coli* was treated immediately after each oocyst sample. After treatment, samples were pipetted into universals, wrapped in aluminium foil and stored in a cool box for 1-2 hours. The samples were then enumerated as described in section 3.6.

6.2.3 Enumeration of the Oocysts

For enumeration of the treated and untreated oocysts, each sample was prepared and stained before being examined at 200x magnification by Epifluorescent microscopy. To prepare each sample for staining, the oocysts were centrifuged at 13000 x g for 45 seconds. The Reverse Osmosis water was then aspirated to leave a 100 µl of sample. 1 ml of (1x) Acidified (HCl, pH 2.75) Hanks Balanced Salt Solution (HBSS) was then

added, and the samples were vortexed and stored at 37 °C for 1 hour. After the incubation period the samples underwent a washing stage to remove the acid. During the washing stage the oocysts were centrifuged at 13000 x g for 45 seconds, and the acid was aspirated off to leave a 100 µl of sample. 1ml of non-acidified HBSS was then added and the samples were vortexed. This procedure was carried out a further two times to ensure complete removal of the acid.

For the staining procedure, each sample was incubated with 50 µl of 4'-6-diamidino-2-phenylindole (DAPI) and 50 µl of Propidium Iodide (PI) at 37 °C for 1 hour. After incubation, the samples were again washed and fluorescently labelled monoclonal antibodies were added. The samples were then incubated for 30 mins followed by preparation of the slides for microscopic examination. This procedure has been published by Campbell *et al* [136].

All of the sample slides were examined using an Olympus fluorescence microscope equipped with appropriate filter blocks for visualisation of the dyes. To begin with each slide was examined under the blue filter block, where the oocysts could be observed as bright green oval shapes. A digital camera was not available at the time of the experimental work but the photograph in Figure 6.2.3.2.1 shows a similar image to what was observed.

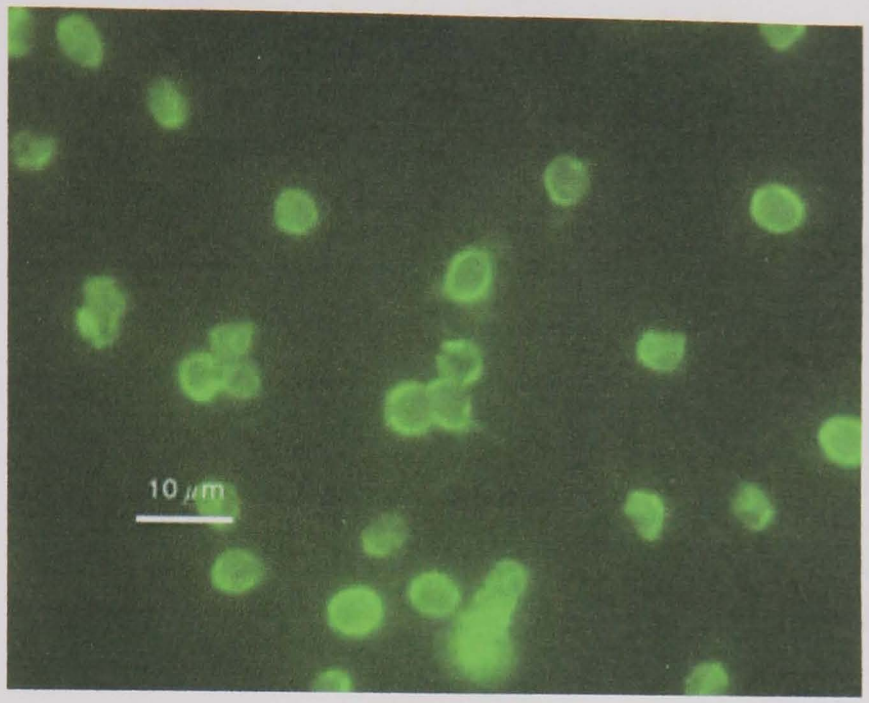


Figure 6.2.3.2.1 Immunofluorescence image of *Cryptosporidium parvum* oocysts. Photograph was obtained from the US Environmental Protection Agency (EPA). Photo Credit: H.D.A Lindquist, U.S. EPA.

For the detection of viable oocysts, the slides were observed under the UV filter block, which shows up DAPI-stained cells. Only viable oocysts allow the penetration of DAPI, which is absorbed by intact DNA, staining the sporozoites (infective stage) blue/white. In each oocyst there should be four sporozoites, each with one nucleus, or four stained nuclei. Oocysts that appear to have fewer than four stained nuclei may in fact have four nuclei, but some may not be visible in the plane of focus. Oocysts with no nuclei visible may be dead. Figure 6.2.3.2.2 shows another image similar to what was observed. The image clearly shows some oocysts with four sporozoites (viable oocysts) and oocysts with less than four sporozoites (non-viable oocysts).

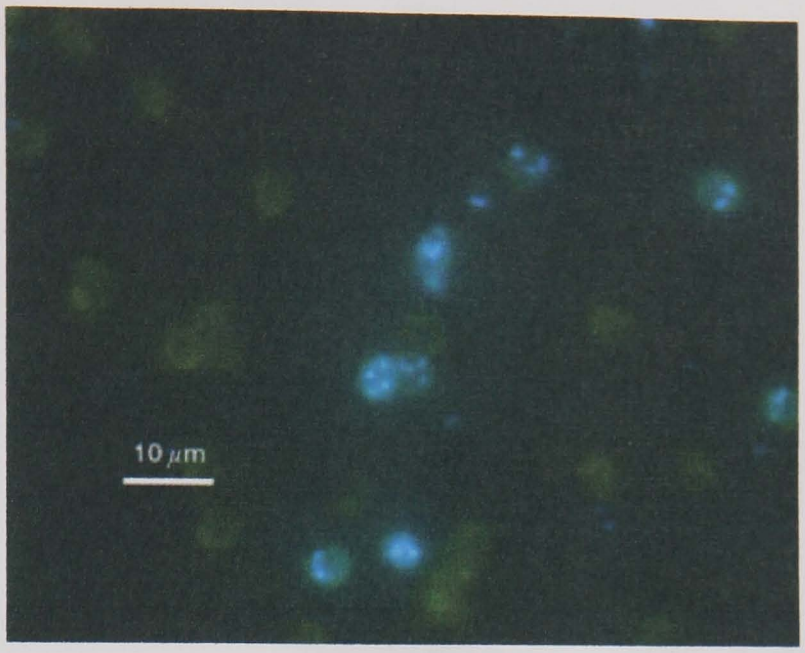


Figure 6.2.3.2.2 Fluorescence image of *C. parvum* oocysts. Photograph was obtained from the US Environmental Protection Agency (EPA). Photo Credit: H.D.A Lindquist, U.S. EPA.

To detect non-viable oocysts, the microscope was switched to the green filter block to allow observation of PI-stained cells. PI only passes through damaged cell membranes and interacts with the nucleic acids of injured or dead cells to form a bright red fluorescent complex.

The microscopic examination provided numbers of viable and non-viable cells and the results were expressed in percentage terms of non-viable oocysts.

6.2.4 Results

A large number of viable oocysts were observed for the 5-pulse sample. These were characterised by the presence of four blue sporozoites within each oocyst, and in some cases, cytoplasmic degradation was also observed. There were also a small number of non-viable oocysts present that were red/orange in colour. When the 25 and 50 pulse samples were examined, all of the oocysts showed up red/orange, indicating that all were non-viable. Figure 6.2.4.1 shows the percentage of non-viable oocysts after pulsed light

treatment. The results concerning the *E. coli* test are also expressed as percentage of non-viable microorganisms to allow a better comparison between the two, and these can also be observed in Figure 6.2.4.1.

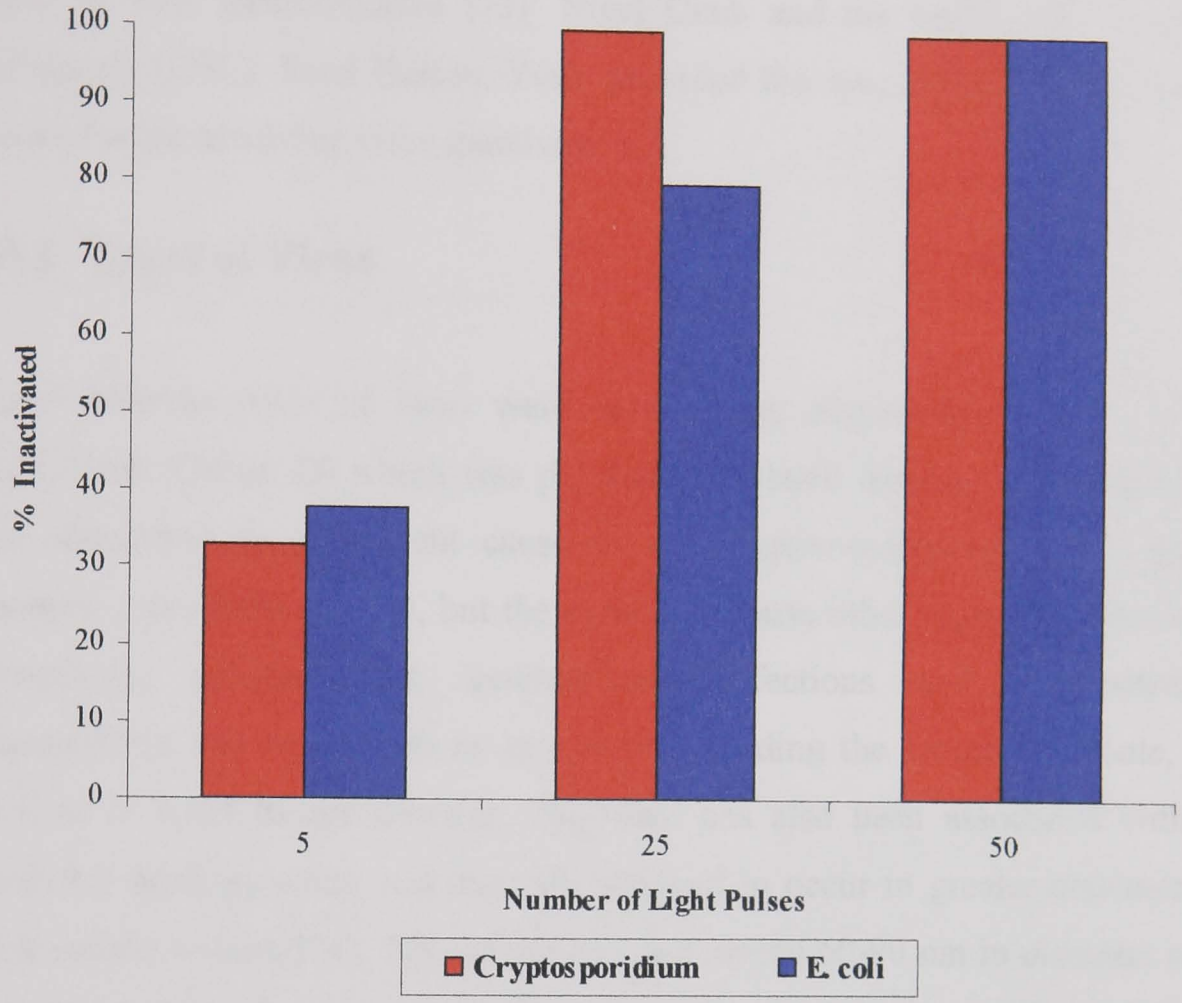


Figure 6.2.4.1 % of Inactivated *Cryptosporidium parvum* and *E. coli* after treatment of 1.5 ml samples with 5, 25 and 50 UV-rich light pulses.

It is clear from the results in Figure 6.2.4.1 that the inactivation achieved for the two microorganisms is comparatively similar. After 5 UV-rich light pulses 33 % and 38 % of *Cryptosporidium* and *E. coli* is inactivated respectively. After 25 pulses a slight difference was observed with 20 % more *Cryptosporidium* cells being inactivated, however after 50 pulses, 100 % inactivation of both microorganisms was accomplished. This means that a 6 $\log_{10}$ reduction can be achieved for both *E. coli* and *Cryptosporidium parvum* oocysts using a dose of only 50 light-pulses (Total energy = 1KJ).

6.3 Inactivation of Viruses

Viruses were another important type of microorganism that were tested for their sensitivity to UV-rich light pulses as their presence in drinking water is an important cause of viral gastroenteritis [73]. Nigel Cook and his staff at the Central Science Laboratory (CSL), Sand Hutton, York, provided the specialised facilities to enable this piece of work involving virus inactivation.

6.3.1 Types of Virus

Three different types of virus were used in the experimental work. The first was Adenovirus (Group D) which was previously isolated from a clinical (faecal) sample. The adenovirus is a frequent cause of acute upper-respiratory tract infections (for example, the common cold), but they can also cause other types of infection including pneumonia, gastroenteritis, genitourinary infections and conjunctivitis [117]. Transmission can be through several ways including the faecal-oral route, respiratory droplets or hand to eye transfer. The virus has also been associated with outbreaks involving drinking water and they are believed to occur in greater concentrations than other enteric viruses [74]. The adenovirus is between 60-90 nm in diameter and consists of a non-enveloped polyhedral capsid containing double stranded DNA [59]. It has 12 pentons that consist of a slender shaft with a globular head which are involved in the attachment of the virus particle to the host cell [117]. An illustration depicting the structural shape of the virus is shown in Figure 6.3.1.1.

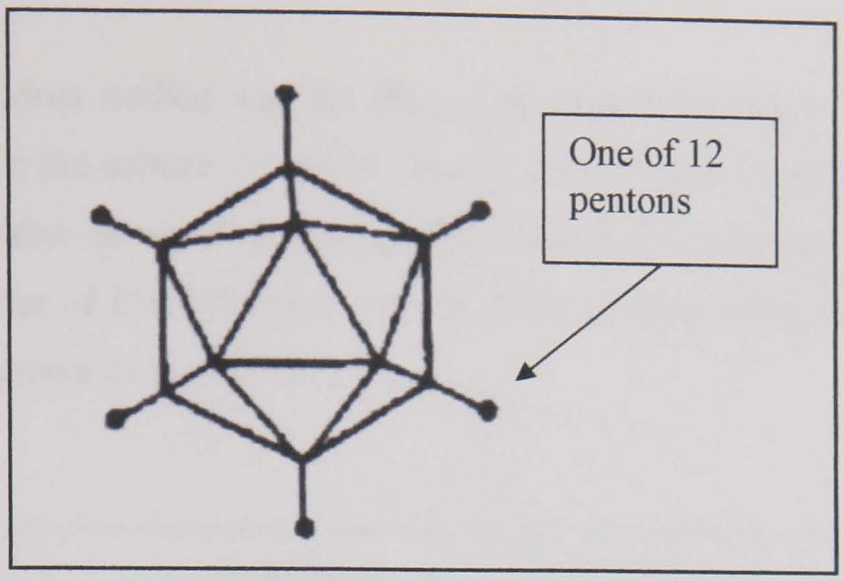


Figure 6.3.1.1 The polyhedral capsid of adenovirus, showing pentons on the surface.

An electron micrograph image of two adenoviruses is shown in Figure 6.3.1.2. The penton fibres easily become detached during preparation for electron microscopy and can be observed surrounding the outside of each virus.

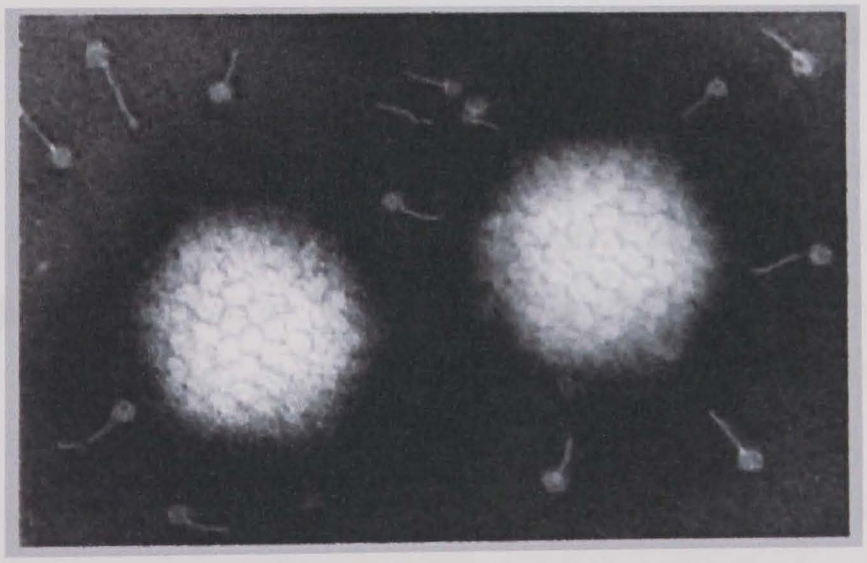


Figure 6.3.1.2 Electron micrograph of the adenovirus. Image produced by Linda Stannard, of the Department of Medical Microbiology, University of Cape Town.

The second virus studied was the Herpes Simplex Virus Type 1 (HSV-1), which was obtained from the culture collection. This is another double stranded DNA virus that is contained within an enveloped polyhedral capsid. It is a larger virus than Adenovirus with a diameter of 150-200 nm. A sketch of the virus showing its polyhedral capsid and envelope is shown in Figure 6.3.1.3.

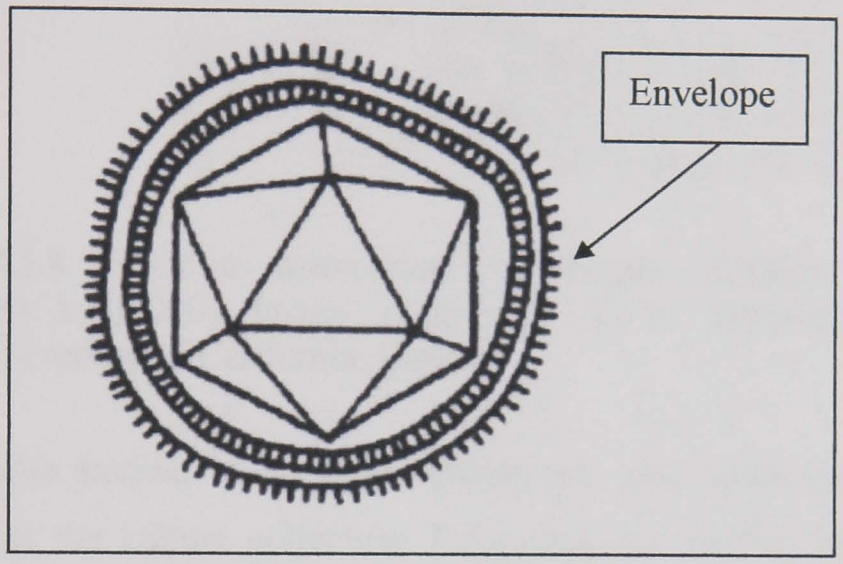


Figure 6.3.1.3 The enveloped polyhedral capsid of herpesvirus.

Transmission of HSV-1 is normally via a break in the mucus membrane of the mouth, via the eye or genitals or directly via minor abrasions in the skin. HSV-1 is primarily associated with oral and ocular lesions which, although painful, are usually resolved spontaneously [118]. The virus can re-emerge when the immune system is compromised. Although it is not responsible for food- or water-borne illness, it was studied to observe how effective UV pulsed-light treatment is on enveloped viruses. An electron micrograph image of the virus is shown in Figure 6.3.1.4

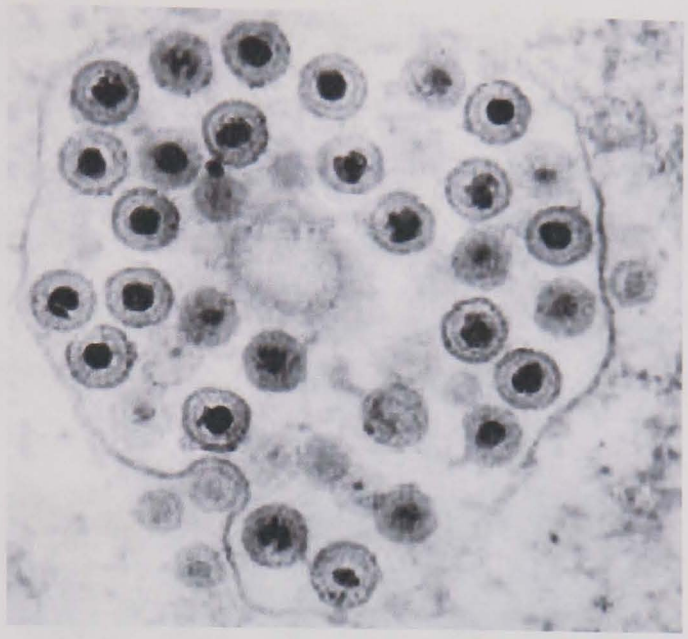


Figure 6.3.1.4 Electron micrograph of herpes simplex virus (magnification approximately x 40,000). Image produced by F. A. Murphy, School of Veterinary Medicine, University of California, Davis.

The final virus studied in the investigation was poliovirus type 1a, which was also obtained from the culture collection. Poliovirus was studied because it has the same genomic structure and gene organisation as that of hepatitis A virus (HAV). Both HAV and norovirus are leading causes of foodborne disease in the United States with norovirus being the most common cause of viral gastroenteritis in adults [119, 73]. These viruses may therefore show similar sensitivities as poliovirus to pulsed light treatment. poliovirus is a positive single stranded RNA virus with a non-enveloped polyhedral capsid and it is one of the smallest known viruses with a diameter of 20-30 nm. A drawing of the virus is shown in Figure 6.3.1.5

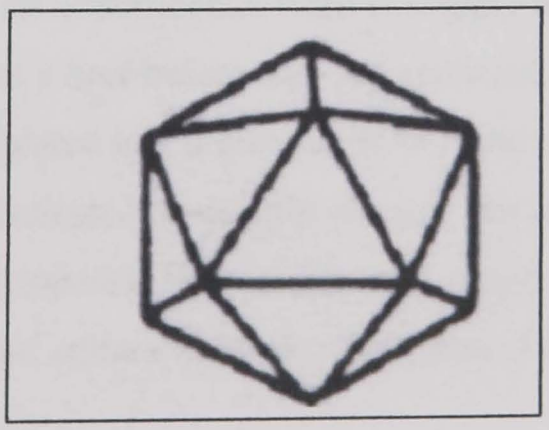


Figure 6.3.1.5 The naked polyhedral capsid of Poliovirus, which contains ssRNA.

Poliovirus causes poliomyelitis, which is an acute disease of the central nervous system (CNS) [120]. The primary site of infection is the lymphoid tissue associated with the oropharynx and gut leading to transient viraemia, following which the virus may infect the CNS [121]. The virus is generally transmitted from person to person via the faecal-oral route, but it can be transmitted indirectly by contaminated sewage or water [122]. An electron micrograph image of the virus is shown in Figure 6.3.1.6.

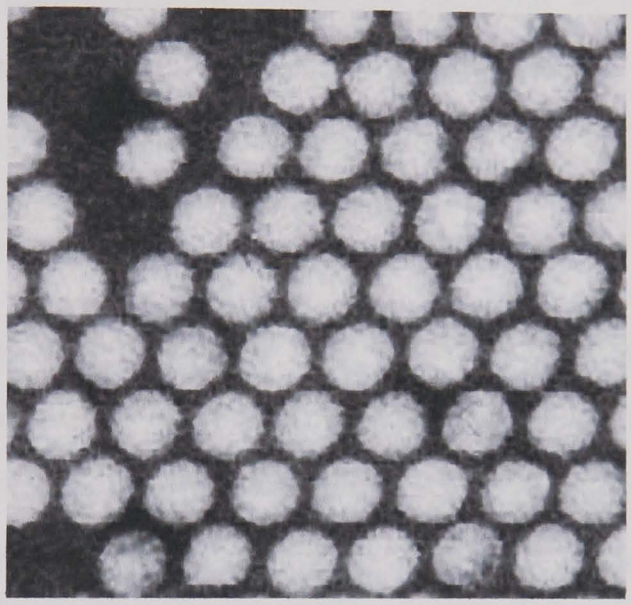


Figure 6.3.1.6 Electron micrograph image of poliovirus (magnification approximately x 200,000). Micrograph from J. Esposito, Centres for Disease Control and Prevention, Atlanta, Georgia, and F. A. Murphy, School of Veterinary Medicine, University of California, Davis.

6.3.2 Viral Growth

Propagation of viruses for experimental work is slightly more complex than culturing of bacteria. Viruses require a host before they can replicate, so therefore in the laboratory, viruses have to be inoculated into a monolayer of tissue cells for viral growth to occur. Growth of the virus is indicated by cellular changes that are visible by light microscopy. These changes, or the cytopathic effect (CPE) as it is commonly called, include swelling or shrinkage of the tissue culture cells, the formation of multinucleated giant cells, and

the production of "inclusions" (made visible by staining) in the nucleus or cytoplasm of the infected cell.

6.3.2.1 Cell Lines

In this investigation the polioviruses were propagated using MA104 cells which are from green monkey kidney (European Collection of Cell Cultures). The MA104 cell lines were routinely maintained in minimum essential medium (MEM) with Earle's modified salts, Glutamax I, 1 % non-essential amino acids, supplemented with 10 % heat-inactivated foetal calf serum and the addition of 100 Units penicillin ml^{-1}, 100 μgml^{-1} streptomycin and 2.5 μgml^{-1} Fungizone.

For the adenovirus and herpes simplex type 1 virus, Hela cells (European Collection of Cell Cultures) were used for their propagation. The Hela cell lines are from human epithelial cells and were routinely maintained in Dulbecco's modified Eagle medium (DMEM) with Glutamax I, 1 % non-essential amino acids, supplemented with 10 % heat inactivated foetal calf serum and the addition of 100 Units penicillin ml^{-1}, 100 μgml^{-1} streptomycin and 2.5 μgml^{-1} Fungizone.

All cell culture media used for the viruses is produced by Gibco and they require 5 % CO_2.

6.3.2.2 Viral Preparation

Four 225 cm^2 flasks of either MA104 or Hela cells were inoculated with the appropriate virus and incubated at 37 °C until a visible cytopathic effect (CPE) was produced, that is, when approximately 90 % of the cell monolayers had been destroyed. Once a CPE was produced, the contents of the flasks were pooled, and the cell debris was removed by centrifugation at 5,000 x g for 30 min. The supernatant was then stored at 4 °C until required for experimental work.

6.3.3 Pulsed Light Treatment of the Viruses

The protocol involving the treatment of viruses had to be altered slightly from the standard pulsing procedure used for bacterial species. The reason for this was that there was a limited volume of viruses to carry out the work with; therefore the sample volume had to be reduced to 2 ml. Therefore, instead of using a standard Petri dish for treating the samples; a 6-well tissue culture plate was used. This ensured even distribution of the sample in the well. Viral samples to be treated were pipetted into the middle two wells of the 6-well tissue culture plates, as those two wells were placed directly under the lamp when the drawer of the treatment chamber was closed. All other parameters involving the pulsed-light system were maintained the same as those described previously in section 3.7.2.

For treatment of the viruses, viral suspensions were adjusted to approximately equal titres, except for a preliminary experiment where the viruses were subjected only to a 1 in 10 dilution. Samples of each suspension were treated by pipetting 2 ml into one of the treatment wells, and placing the plate in the flash-lamp chamber to be treated with the desired number of light pulses. Each pulsed light regime was carried out in triplicate. After treatment, the samples were stored at 4 ^{o}C until analysis.

In addition to treating the viruses, *E. coli* was also treated for comparison. The *E. coli* was cultured and prepared as described in section 3.7.1, but because the work was carried out at the CSL, no spiral plater was available and bacterial enumeration was carried out using 100 µl spread plates.

6.3.4 Enumeration of the Viruses

This was performed according to the method of Reed and Muench [138] as used by Kurdziel *et al* [138]. This was carried out by preparing ninety-six well microtitre plates

containing the desired cell lines for each virus. The cell lines were obtained from a number of 225 cm^2 flasks of confluent cells. The concentration of the resulting cell suspension was determined using a hemocytometer. The suspension of cell lines was then diluted, using the appropriate growth medium, to a concentration of 1 x 10^5 cells ml^{-1}. A 200 µl sample of this cell suspension was then dispensed into each well of the microtitre plates.

Each virus suspension was serially diluted tenfold, and 100 µl of each dilution was inoculated into a microtitre well containing either MA104 or Hela cells, depending on the virus type to be enumerated. Six wells were used per dilution. The plates were incubated for 7 days at 37 °C with 5 % CO_2, which allowed full development of CPE in all infected wells. Wells displaying CPE were counted, and the tissue-culture infectious $dose_{50}$ of the neat suspension was calculated using the method of Reed and Muench (1938) [123]. The results were expressed as tissue culture infectious units ($TCIU_{50}ml^{-1}$) ml^{-1}, by calculating the antilog and incorporating the dilution factor. This is a measure of the dilution that contains an infectious dose large enough to destroy or damage 50 % of the tissue culture cells.

6.3.5 Results

Since the sensitivity of the viruses to the UV-rich light pulses was unknown, the initial experiment involved treating the viruses with the range of pulses shown to be successful in the inactivation of bacteria. In this first experiment, 2 ml samples of 1 in 10 dilutions of polio, herpes and adenovirus suspensions were treated with 0, 1, 5, 10, 25 and 50 light pulses. Alongside each virus, 2 ml samples of 10^8 $CFUml^{-1}$ *E. coli* were treated. The initial sets of viral results obtained are shown in Figure 6.3.5.1.

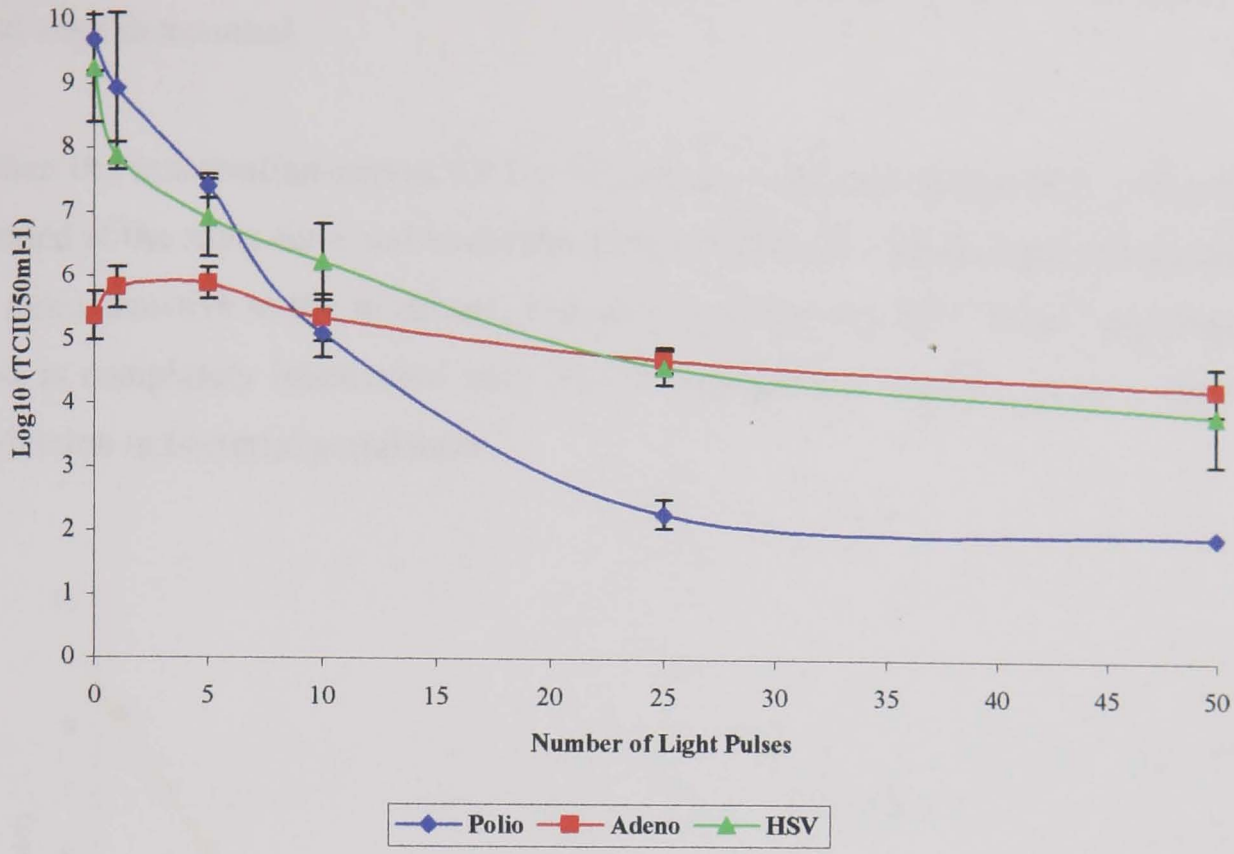


Figure 6.3.5.1 Population of polio, adeno and herpes simplex virus (HSV) remaining after treatment with a range of UV-rich light pulses.

From the results shown in Figure 6.3.5.1 it is evident that all three viruses are susceptible to the UV-rich light pulses, but they each have different sensitivities to treatment. Due to this being the initial experiment, each virus was treated as a 1 in 10 dilution of the population it had grown to. The starting population for both the polio and herpes virus were approximately the same ($\sim 10^9$ $TCIU_{50}ml^{-1}$) but unfortunately, the control plates for the adenovirus were difficult to count and the starting population could only be estimated (normally grows to $\sim 10^6$ $TCIU_{50}ml^{-1}$). The poliovirus was observed to be the most sensitive of the three viruses to the light pulses, with a 7.4 log reduction after 25 pulses and inactivation tailing off between 25 and 50 pulses. Herpes simplex virus was also observed to be quite sensitive to the treatment with a 4.6 log reduction occurring after 25 pulses. The adenovirus appeared to be the most resistant of the three viruses, but the actual log reduction could not be calculated due to the inaccuracy of the

control plates. For this reason, the results for adenovirus were not considered reliable and were discounted.

When the inactivation curves for the viruses are compared to that of *E. coli*, which was treated at the same time and under the same conditions, it can be seen that the bacterium is more sensitive to the treatment. Figure 6.3.5.2 shows a 10^8 CFUml^{-1} population of *E. coli* is completely inactivated after 50 UV light pulses, resulting in an overall 8.5 log reduction in bacterial population.

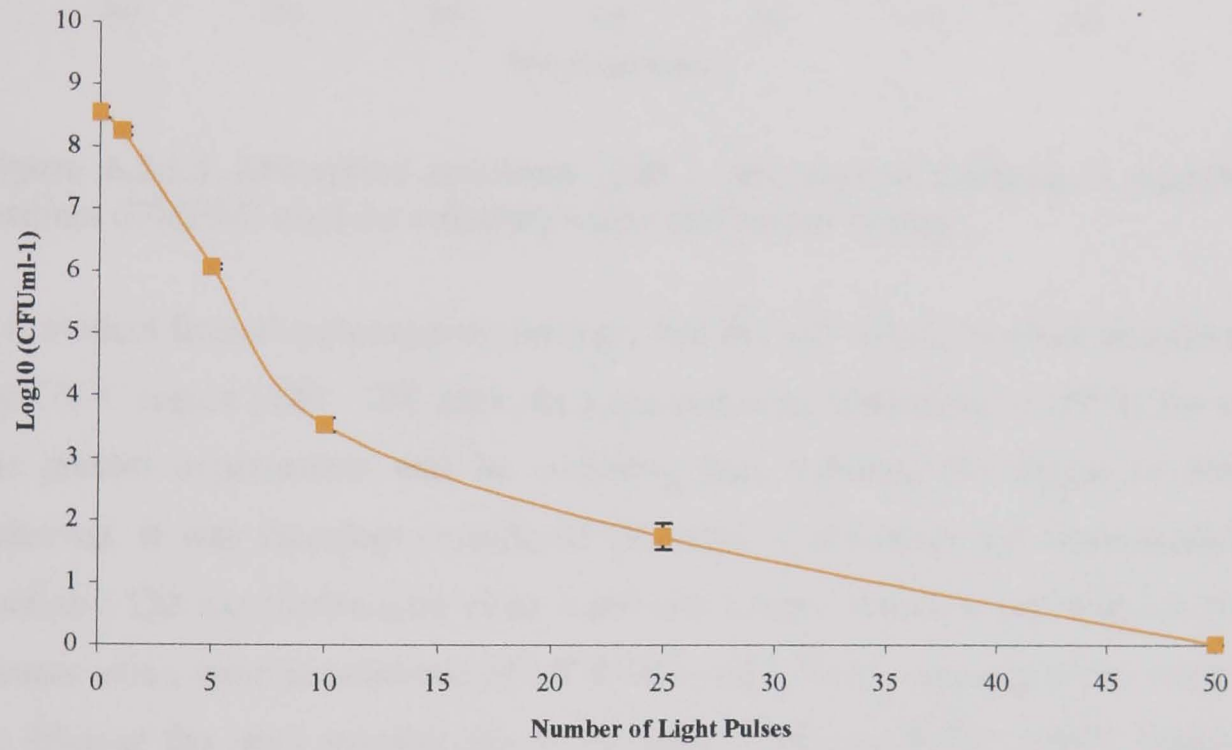


Figure 6.3.5.2 Population of stationary phase *E. coli* remaining after treatment with the same range of light pulses used for the virus inactivation.

A possible problem with the virus study concerns the stock suspension. This suspension is a pale pink colour and may absorb UV light. The pink colour is a result of remnant cell culture medium which contains a dye which acts as a pH sensor. An absorption scan was therefore made of this culture medium using a BioMate 5 Spectrophotometer (section 3.4.5), and this is shown in Figure 6.3.5.3 in order to ascertain the likelihood of UV absorption.

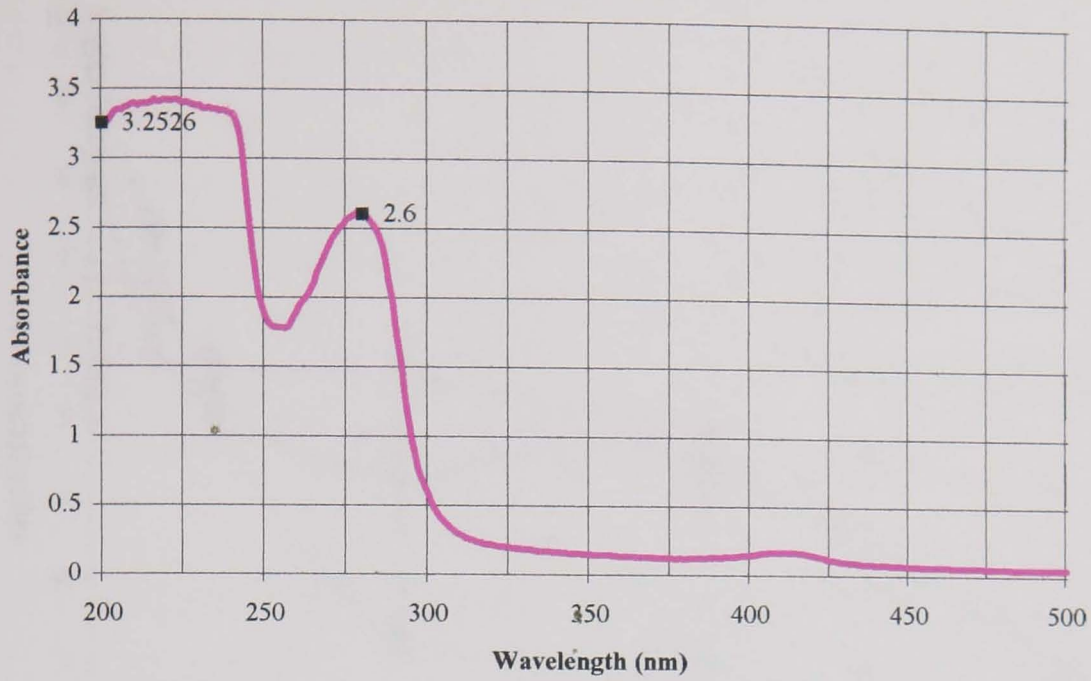


Figure 6.3.5.3 Absorption spectrum (200 – 500 nm) of Dulbecco's modified eagle medium (DMEM) used for culturing adeno and herpes viruses.

It is evident from the absorption spectrum that the cell culture medium absorbs highly in the UV-C region (200 – 280 nm). As a consequence, absorption of UV by the viruses in the present experiments will be inhibited, thus reducing the degree of inactivation achieved. It was therefore considered desirable to minimise the concentration of this medium. The experiments on virus inactivation were therefore repeated for two of the viruses using virus populations of 10^6 $TCIU_{50}$ ml^{-1}. These concentrations were obtained by diluting the stock suspensions in Phosphate-Buffered Saline (PBS), which reduces UV absorption by proteins left over from the cell culture medium. A 10^6 CFU ml^{-1} population of *E. coli* was also prepared by diluting in quarter strength ringer solution. The same protocol was followed as in the previous experiments and 0, 2, 5, 10, 25, 50 and 100 UV-rich light pulses were used. Inactivation curves for the Polio and Adeno viruses are shown in Figure 6.3.5.4.

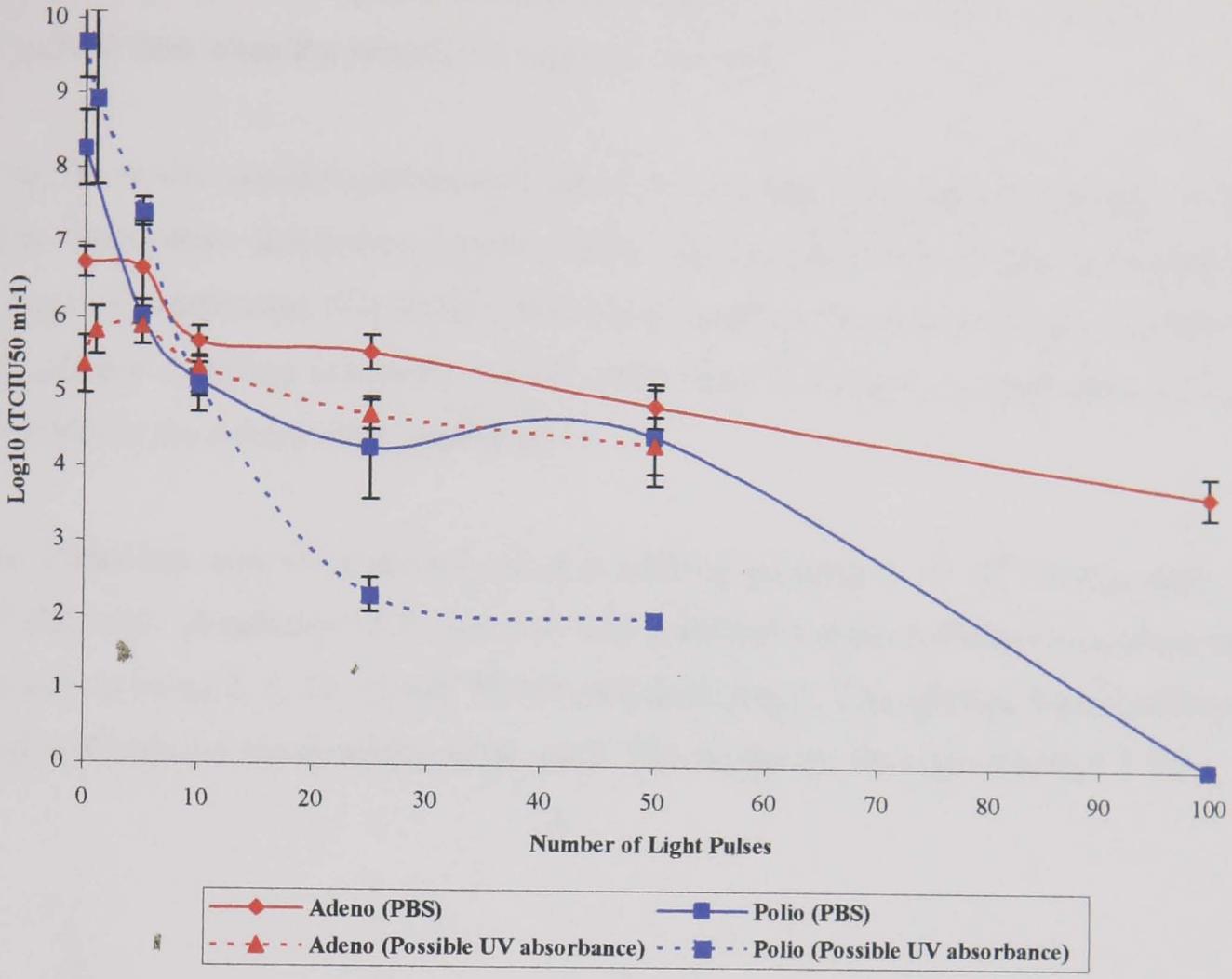


Figure 6.3.5.4 Adeno and polio virus concentration remaining after pulsed UV-rich light treatment of PBS diluted viral stock.

In addition to the inactivation results obtained for the viruses diluted in PBS, Figure 6.3.5.4 also shows the results for the previous experiment where the virus suspension medium was thought to be responsible for some UV absorption. When the two sets of results are compared it can be observed that suspending the viruses in PBS (transparent media) did not improve the inactivation kinetics of either virus. Polio virus diluted in PBS underwent a 3.8 Log reduction after 50 pulses; whereas when it was diluted in the original viral culture medium (containing pink dye) the virus underwent a 7.7 Log reduction. The difference in inactivation with the adeno virus is not so dramatic

with the original viral suspension being inactivated by approximately 1 Log more (after 50 pulses) than when the viruses are suspended in PBS.

From the initial experimental results, inactivation levels appeared to be tailing off but these results show that increasing the number of pulses (UV dose) further increases the amount of inactivation that occurs. When the number of pulses is increased to 100 (2 KJ), there is complete inactivation of the Polio virus (8.3 Log reduction) and a 4.5 Log reduction in the Adeno virus population.

The Poliovirus was re-examined using a starting population of 10^6 $TCIU_{50}$ ml^{-1}. A 10^6 CFU ml-^{1} population of *E. coli* was also prepared and the treatment procedure was carried out using 2, 5, 10, 25 and 50 UV-rich light pulses. This allowed a comparison of viral and bacterial susceptibility to be made. The results are shown in Figure 6.3.5.5.

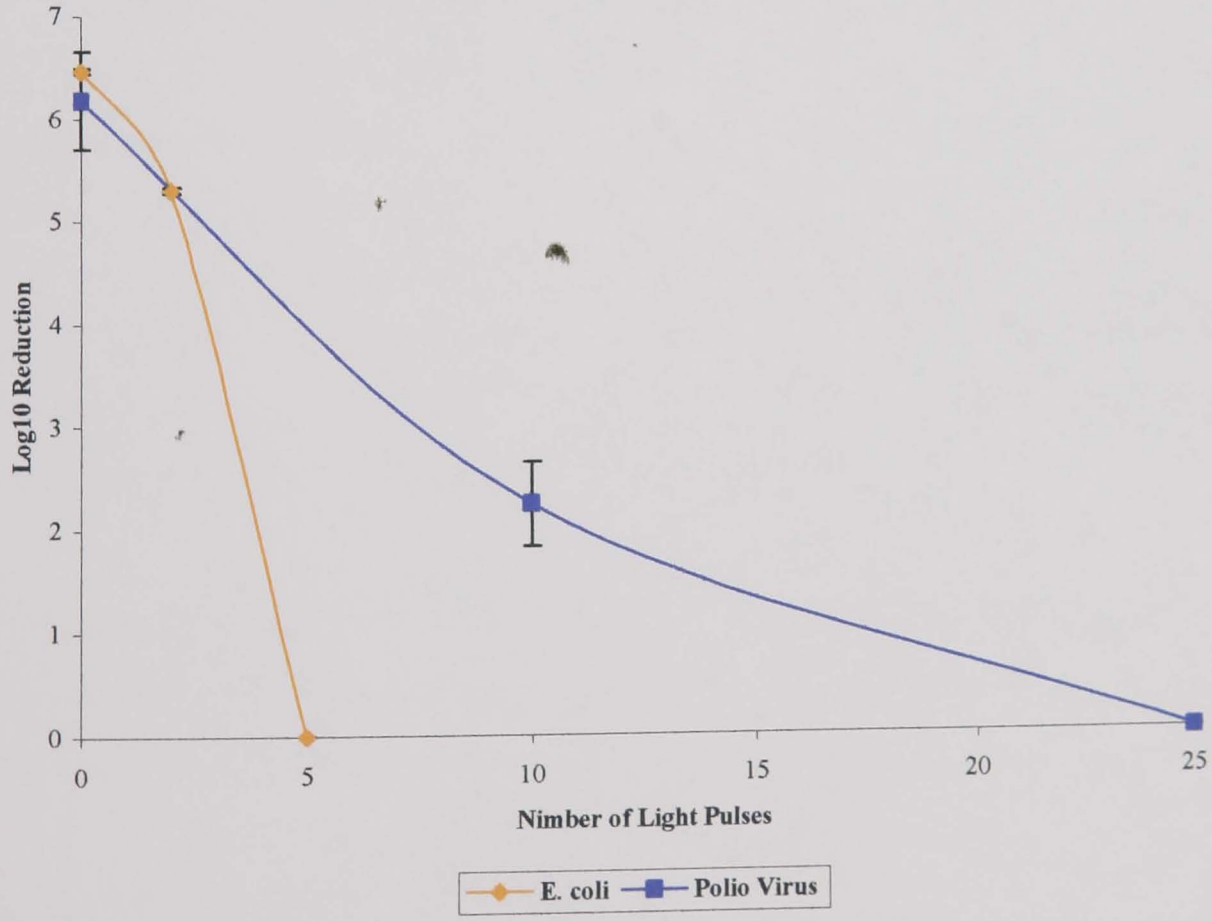


Figure 6.3.5.5 Inactivation of 10^6 populations of *E. coli* and polio virus with pulsed UV-rich light.

A 10^6 population of both types of microorganism are shown to be very susceptible to the treatment. For the Polio virus 10 pulses produced an approximate 4 Log_{10} reduction and after 25 light pulses (500 J), no infectious Poliovirus remained. Inactivation for *E. coli* on the other hand occurs much more rapidly, with as little as 5 light pulses (100 J) needed for complete inactivation of the bacterial population.

6.4 Discussion

In water purification, the use of coagulants, rapid filtration and chemical disinfection does not consistently and reliably remove *Cryptosporidium* oocysts and viruses [20]. *Cryptosporidium parvum*, an obligate enteric pathogen that can cause acute cases of gastroenteritis, and three different types of virus, all responsible for causing different types of viral infection in humans, were studied for their susceptibility to pulsed UV-rich light treatment.

The treatment of the *Cryptosporidium* parvum oocysts with pulsed UV-rich light was successful. After as little as 5 pulses (100 J) 33 % of the oocysts were rendered non-viable and after 25 pulses, 100 % were shown to be non-viable confirming that *Cryptosporidium* oocysts are very sensitive to UV radiation when it is delivered as pulses of UV-rich light. The sensitivity of the oocysts in this study is consistent with previous studies that demonstrate *C. parvum* oocysts are very susceptible to low doses of UV [98, 99, 110]. Continuous UV experiments carried out by Craik *et al* however have shown tailing characteristics on the UV inactivation curves and they have suggested that very high levels of inactivation of *C. parvum* oocysts may be difficult to achieve with UV [124]. This present investigation however has shown pulsed UV-rich light to be efficient for the inactivation of C. *parvum* oocysts at similar population levels, suggesting that pulsed UV-rich light treatment may be more effective against this pathogen than conventional continuous UV disinfection.

Cryptosporidium oocysts are also found to be equally as susceptible to pulsed UV light treatment as *E. coli*, in agreement with the findings of Oguma *et al* who reported that the oocyst wall of *C. parvum* is not any more protective against UV light than the cell wall of *E. coli* [110]. They demonstrated this with an endonuclease sensitive site assay, which can determine the number of UV-induced pyrimidine dimers in the genomic DNA. They also indicated that this might be the reason why UV treatment is effective at disinfecting *C. parvum* compared with chemical disinfectants that cannot penetrate the oocysts wall.

The findings of various researchers are not consistent in the levels of inactivation occurring [128]. Morita *et al* have suggested a 1.92 mW/cm^2 dose is needed for 4 Log_{10} reduction in oocyst infectivity [125]. Lorenzo-Lorenzo *et al* on the other hand have suggested a dose of 15000 mW/cm^2 [104]. These differences may be attributed to sensitivity variations amongst different strains of *C. parvum* as discussed by Morita et al [125]. The dose of 15000 mW/cm^2, suggested by Lorenzo-Lorenzo *et al*, will completely eliminate infectivity when oocysts are exposed to this dose for more than 150 mins [104]. Pulsed UV-rich light therefore has the advantage over continuous UV sources in that it can completely inactivate oocyst in a much shorter time-scale. This study showed that *C. parvum* oocysts could be completely inactivated (6 Log10 reduction) after only 25 seconds of treatment (25 pulses at 1 pulse per second).

It is well known that many microorganisms can recover following UV light treatment. They do this by the process known as photoreactivation (photoreactivation is explored in Chapter 8). Studies by Morita *et al* however have shown that when photoreactivation of *Cryptosporidium* occurs, the infectivity of the oocysts is not restored so that the life cycle of UV treated oocysts cannot continue once they are placed in visible light [125]. Results of photoreactivation experiments by Oguma *et al* show that UV light treatment of oocysts must produce other kinds of damage in DNA or other parts of the cell, as they also showed that the repair of pyrimidine dimers in the genomic DNA did not contribute to the recovery of infectivity of *C. parvum* [110]. Belosevic *et al* have also reported similar results and have suggested that when appropriate UV doses are used, significant and permanent inactivation of the organism may be achieved [56]. These findings are important for the use of pulsed UV-rich light treatment for water disinfection. Even if photo-repair does occur from the pulsed light treated oocysts, they will not be able to continue their life cycle and cause infection once ingested by a host.

Results from the viral studies found that they were not just as susceptible to the treatment as *Cryptosporidium parvum*. The initial results (Figure 6.3.5.1) carried out

found Polio to be very susceptible, the Herpes virus to be a bit more resistant and Adenovirus to be very resistant to the pulsed UV-rich light treatment. With the adenovirus, the starting population (titre) was much lower than for the other viruses but still demonstrated more resistance. It has been reported however that during viral replication only 10-20 % of the viral structural polypeptides are assembled into new adenovirus particles, which may explain the low titre produced by the virus in this study [73]. This may also explain the low level of inactivation obtained for adenovirus, as the structural polypeptides that are not assembled into new virus particles (90-80 %), will remain in the viral suspension and may absorb or shield the infectious adenovirus particles from the UV-rich light pulses. It has also been suggested by Gerba *et al* that Adeno virus may use host cell enzymes to repair damage in the DNA caused by UV [74]. This would mean that as the virus is being inactivated, some virions may be repairing themselves which would explain the low inactivation levels. Initially herpes simplex virus appears slightly resistant to the treatment characterised by tailing on the inactivation curve. This may be due to the presence of the envelope that surrounds the virus, which is composed of a phospholipid bilayer and proteins that may absorb some of the light pulses. Some of these proteins also protrude outward from the envelope surface, which may also allow some of the light pulses to be deflected away from the virus. Although the HSV-1 appears quite resistant, a 4.6 Log reduction was still achieved, therefore the slight resistance may be just due to the fact that a high titre of viruses were used, and shielding is occurring from other viral particles. It has also been suggested however that double stranded DNA viruses such as Adenovirus and Herpesvirus are likely to be more resistant to UV light disinfection [74], which the findings of this study using pulsed UV-rich light support. The Poliovirus however is particularly sensitive to the treatment with 7.4 Logs of inactivation occurring after 50 pulses. This increased sensitivity is probably because it is a single stranded RNA virus, which is known to be quite susceptible to UV treatment, mainly because they do not have a template strand to utilise all the repair mechanisms in the host [49]. Inactivation of Poliovirus is thought to result from a loss of function of the capsid [119]. Other causes include loss of infectivity associated with formation of photo products

(photodimer or photohydrate) or loss of function of viral genomes. At higher UV doses capsid proteins are affected and RNA-protein linkages are generated [119].

The initial concentrations of viruses were high and therefore further experiments were conducted at lower concentrations. To achieve this, viral titres were diluted in PBS which meant that any culture medium present, which may interfere with UV transmission, would be minimised. Unfortunately, results were not obtained for Herpesvirus therefore no further conclusions could be drawn for this virus type. For the other two viruses however a good set of results were obtained. From Figure 6.3.5.4 it can be observed that the PBS diluted adeno and polio virus do not appear any more sensitive to the treatment than they did in the previous experiment. The results however do show that although tailing started to occur in the initial experiment, further inactivation can be achieved by increasing the dose of UV-rich light pulses: Increasing the dose to 100 pulses (2 KJ) resulted in an approximate 8 Log_{10} reduction (complete inactivation) of poliovirus and a 3 Log_{10} reduction in adenovirus. This is consistent with other findings which have shown that adenovirus is more resistant to UV disinfection than poliovirus, with 31 mW/cm^2 and 160 mW/cm^2 needed for 99.99 % inactivation of poliovirus and adenovirus respectively [73, 74]. It is thought that the increased sensitivity of polio and other RNA viruses is down to the lack of the excision and repair mechanism in viral RNA genomes resulting in the damage not being repaired hence making the virus more susceptible [119]. Viruses such as adenovirus with double stranded genomes are less susceptible to UV inactivation since only one strand of the nucleic acid may be damaged. The undamaged strand may then serve as a template [73] and it can use host cell enzymes to repair damages in the DNA [74]. The structure of adenovirus is also more complex, as it consists of several capsid proteins and protruding protein fibres [73]. The penton projections (shown in Figure 6.3.1.1) may also cause a shadow effect or a disruption in the absorbance by viral nucleic acids [74].

An important observation that was also made was the unusual increase in the poliovirus population after 50 pulses (Figure 6.3.5.4). This is unlikely to be factual therefore an

explanation could be due to viral distribution throughout the sample. When studies were carried out with *E. coli* and poliovirus at a lower concentration of 10^6, the shape of the inactivation curves for both organisms was significantly different. *E. coli* showed rapid exponential inactivation and the poliovirus produced a sigmoidal inactivation curve which exhibited no tailing. This suggests that the high viral titres used before were responsible for the tailing observed on the previous inactivation curves.

This study on viruses has therefore demonstrated different levels of sensitivity to the pulsed UV-rich light treatment. This study has found the RNA virus, polio, to be extremely sensitive to the treatment and the DNA viruses adeno and herpes to be more resistant although still quite susceptible. It has indicated that variations in susceptibility are primarily due to the differences in viral structure, which is in agreement with previous studies, which have used continuous UV for inactivation purposes. Genomic content seems to be the biggest parameter influencing viral inactivation although other factors may also contribute. In viral inactivation, shielding or consumption of UV before reaching the nucleic acid may occur because of the presence of capsids proteins or other packaged viral proteins that are directly associated with the nucleic acid [73]. In these situations, increased pulse numbers may be required for irreparable damage, resulting in higher levels of inactivation. Other factors that can influence the effectiveness of UV radiation and may be characterised by tailing of inactivation curves (like those witnessed with the high titres of viruses) include small proteins concentrated along with viral particles in prepared viral stocks or characteristics of the architecture of viral capsids [73].

Overall this chapter has shown that pulsed UV-rich light treatment is effective against other types of microorganism other than bacterial species. Successful inactivation of the protozoan *Cryptosporidium* and a range of viruses have been accomplished. Many studies have reported encysted protozoa as being quite resistant while others have shown the major waterborne pathogen *Cryptosporidium* to be very susceptible to UV radiation (continuous) [104, 101, 103]. This study has demonstrated that *Cryptosporidium* is

extremely susceptible to pulsed UV-rich light treatment. High levels of inactivation against adenovirus, poliovirus and herpesvirus (all different structurally) have also been shown in this investigation. From all of the microorganisms studied, *Cryptosporidium* and *E. coli* were shown to have similar sensitivities to the pulsed UV-rich light treatment, closely followed by poliovirus. Herpes and adenovirus proved more resistant to the treatment but adequate inactivation levels were achieved which meet the U.S. Environmental Protection Agency (EPA) recommendations [74]. The findings of this study therefore indicate that pulsed UV-rich light treatment is extremely effective at inactivating a whole range of microorganisms including those viruses and protozoa that are commonly associated with waterborne illness.

Chapter 7

INVESTIGATION OF THE 'TAILING EFFECT'

7.1 General

A potential drawback of using ultraviolet light for disinfection purposes is that certain factors can provide protection to the microorganisms from the UV. Warriner *et al* (2000) has described three ways in which a microorganism may be protected from the UV dose: (i) A highly resistant sub-population may be present, (ii) Microorganisms may be present within clumps and thereby be shielded and/or (iii) they may be within pores/crevices of the substance being treated and be shaded [129]. As a result of these factors the microorganism may not receive the full dose of UV applied or even worse, receive none at all. A direct consequence of this is a reduction in the amount of inactivation that takes place. On inactivation curves this can be observed as a "tailing effect" where increases in UV dose can cause little further significant reduction in bacterial population. Most data on the "tailing effect" has been concerned with continuous UV systems rather than pulsed systems. However, from the extensive amount of experimental work that has been carried out in this study, it has been noticed that on occasion a "tailing effect" is observed on some of the inactivation curves. This indicates that "tailing" can be a problem with pulsed systems in addition to continuous systems. This chapter focuses on investigating the various factors that may be providing protection to the microorganisms and also what can be done to modify the experimental protocol to try to eliminate their influence.

7.2 Observation of a "Tailing Effect"

In the case of pulsed UV treatment, the "tailing effect" occurs when, after a certain number of UV-rich light pulses, no further inactivation of the microorganism is

observed. To demonstrate this effect, a standard pulsing experiment using *E. coli* was carried out. The microorganism was grown to a 10^8 CFUml^{-1} population as described in section 3.7.1. Samples of 20 ml volumes were then treated with 1-10 light pulses, as described in section 3.7.2.

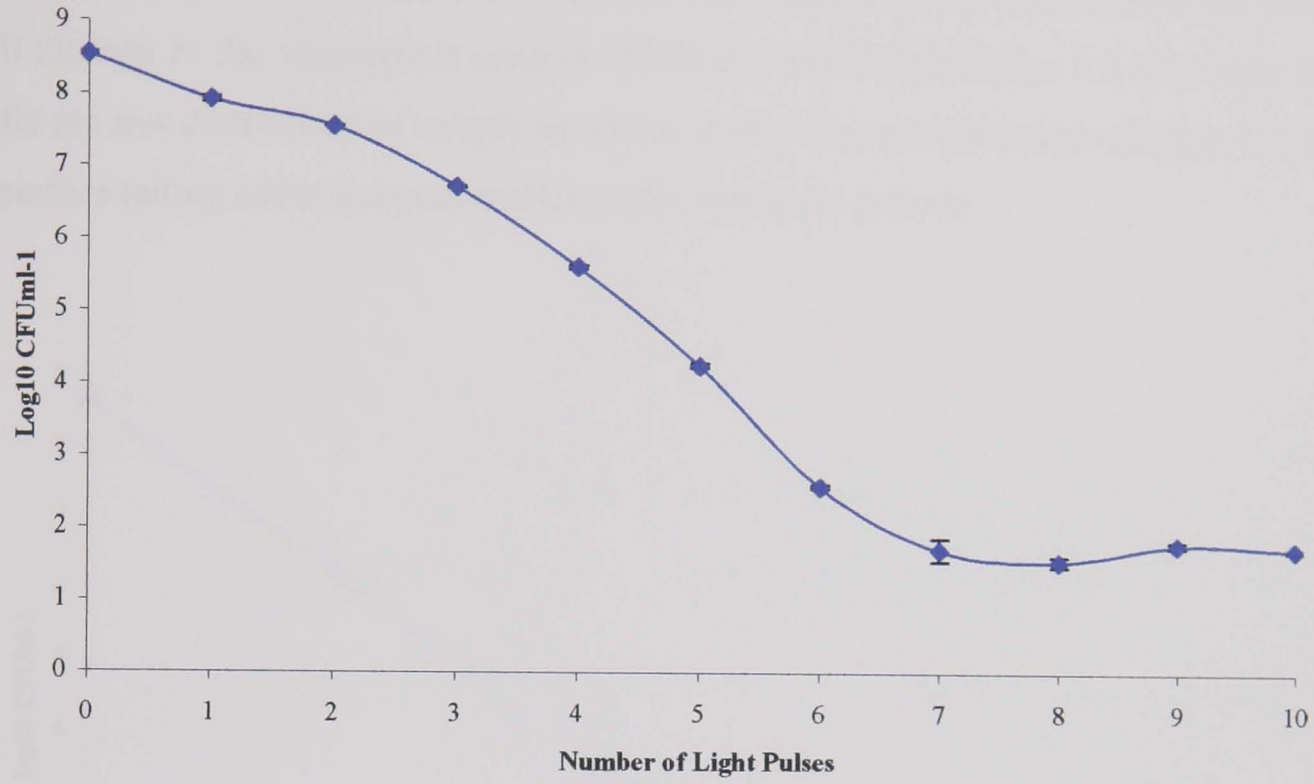


Figure 7.2.1 Inactivation curve of stationary phase *E. coli* after 1-10 UV-rich light pulses.

From the inactivation curve shown in Figure 7.2.1, an almost linear decrease (on a log scale) in bacterial population can be observed during application of 1-6 UV pulses. However, the samples that received 7-10 pulses were all inactivated to virtually the same population, and this represents a 'tailing effect'.

When the number of UV-rich light pulses was increased to 4, 8, 12, 16 and 20 a "tailing effect" could still be observed as shown in Figure 7.2.2. The results however show that on this occasion the population numbers do not start to tail off until 12 pulses, compared with 7 pulses in the previous experiment. However, the "tail" is still present and after the pulse number has been doubled to 20 and no further reduction is observed to occur. The results from this experiment, and the initial

experiment, indicate that because there is no further inactivation after a certain point, there must be something in the sample or a property of the microorganism that protects the remaining bacterial cells from receiving the light pulses. As the tailing effect is observed at different stages in the two separate experiments it suggests that the factor responsible could be due to a property of the sample suspension, since all the electrical parameters are the same. An explanation could be that there are more cell clumps in the suspension used in the first experiment (Figure 7.2.1) or that the cells are not distributed as evenly as those in the second experiment (Figure 7.2.2); therefore tailing starts to occur earlier in the treatment process.

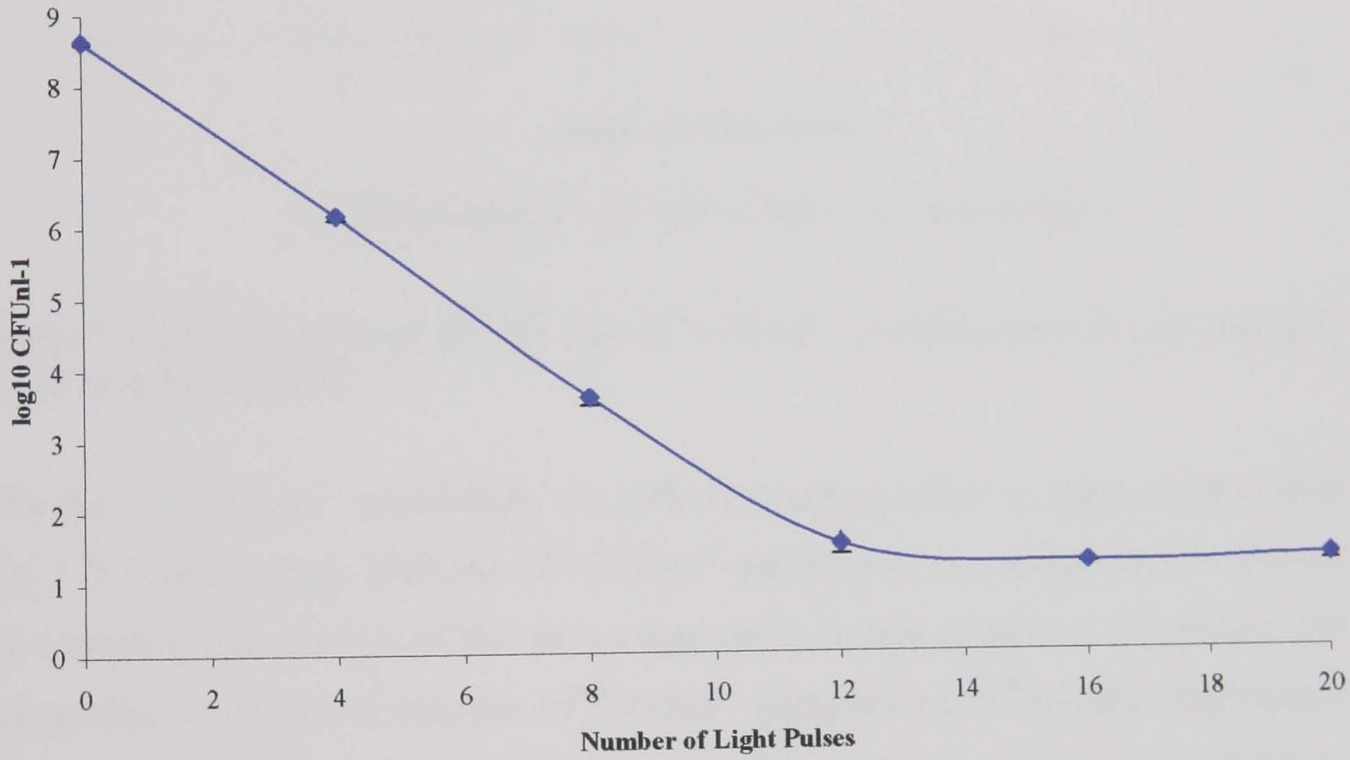


Figure 7.2.2 Inactivation curve of *E. coli* with the pulse number extended to 20 pulses.

7.3 Importance of Population Size

The effect of population density on tailing was examined using 20 ml *E. coli* samples with populations of 10^8, 10^7 and 10^6 CFUml^{-1}. In each test, samples were treated with 1-10 pulses of UV-rich light. The inactivation results obtained from these tests are presented in Figure 7.3.2.

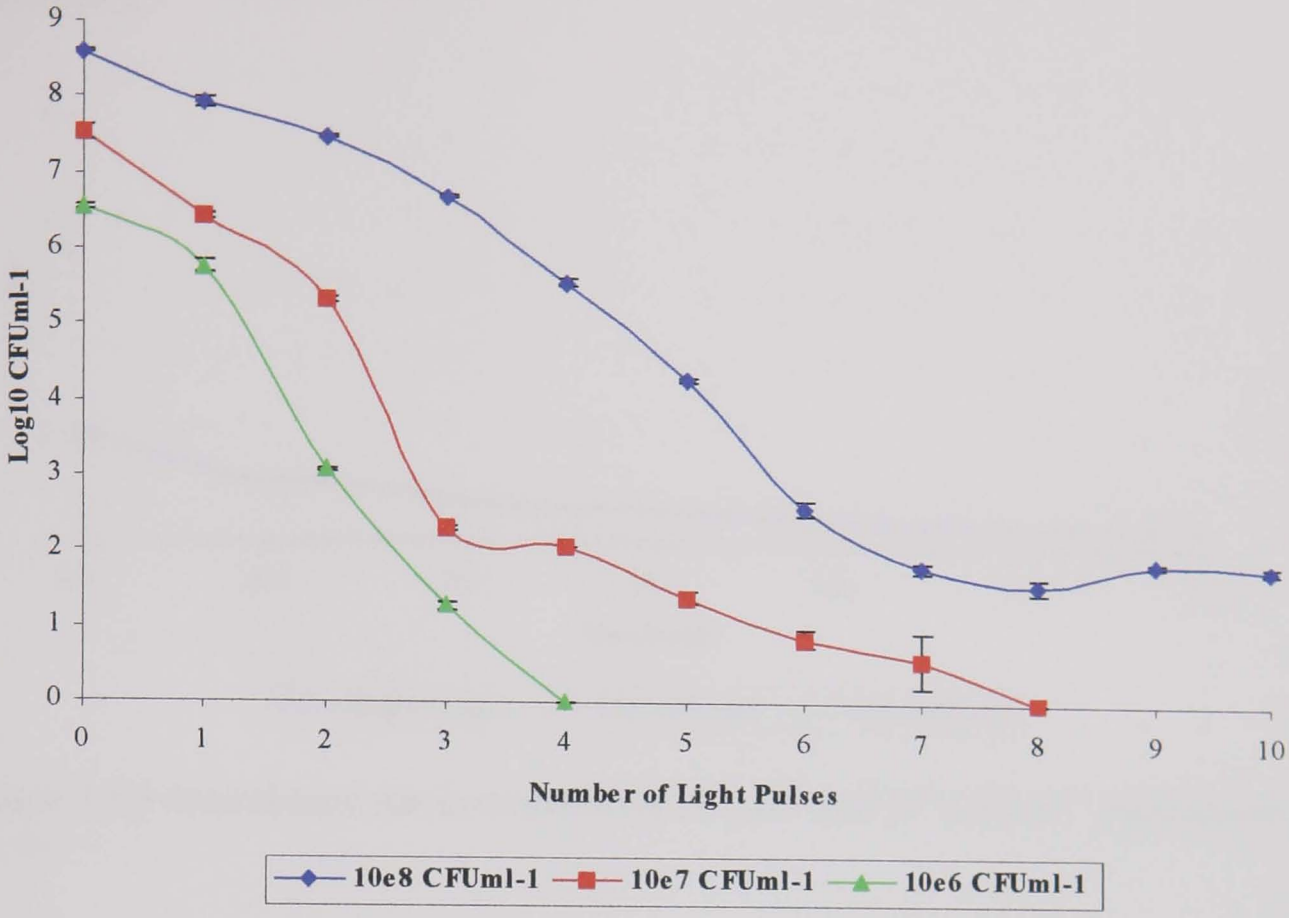


Figure 7.3.2 Inactivation of 10^8,10^7 and 10^6 CFUml^{-1} populations of *E. coli*, using 1-10 UV-rich light pulses.

With the 10^8 CFUml^{-1} population, the extended tailing effect is again evident after only 7 UV light pulses. With the 10^7 CFUml^{-1} population, no tailing effect is present and complete inactivation of the microorganism is achieved after 8 UV pulses. No tailing effect is observed with the 10^6 CFUml^{-1} population and complete inactivation occurs with 4 pulses. The absorbance and transmittance of the three different population samples at wavelengths 200-500 nm was measured using a Spectrophotometer (section 3.4.5) to observe the variations in transmittance with population size. The results are presented in Figures 7.3.3 and 7.3.4.

Absorbance cannot be measured directly since there is no way to directly count the number of photons as they disappear (are absorbed). Transmittance is what is actually measured. If T is the percentage of light transmitted, then the absorbance is defined to be $-\log_{10} T$ absorbance units. An increase in absorbance of 1.0 corresponds to a reduction in transmittance by a factor of 10. If the absorbance is 1.0 then 10% of

the light is transmitted; if the absorbance is 2.0 only 1% of the light is transmitted, and so on.).

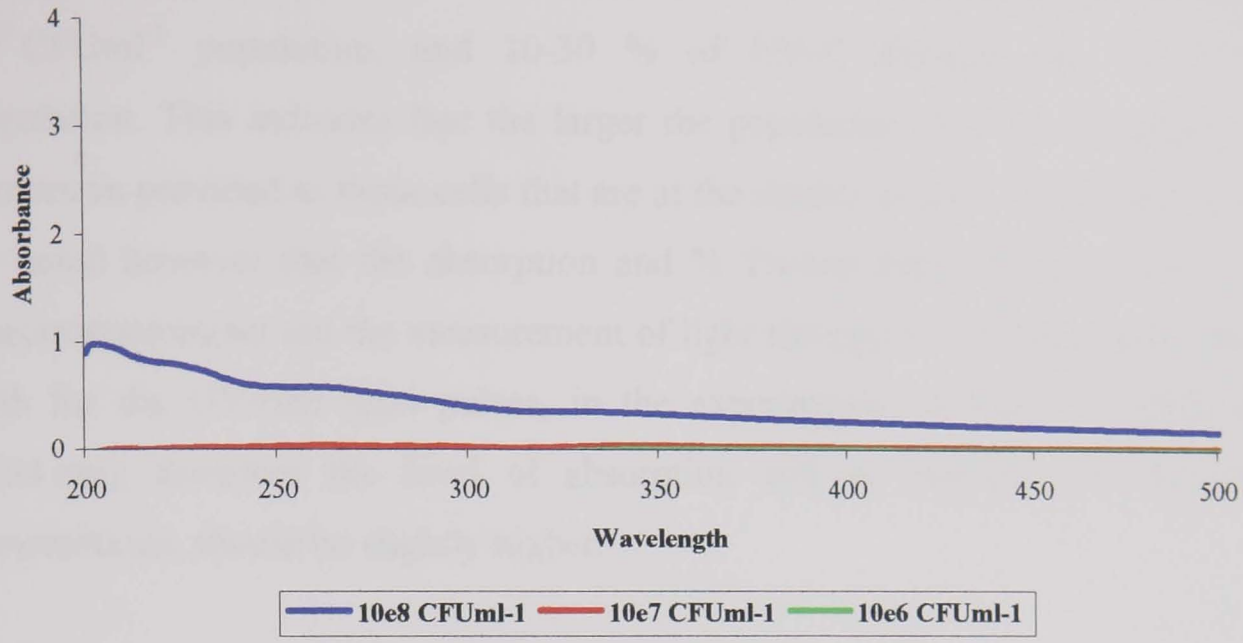


Figure 7.3.3 Absorbance measurements for 10^8, 10^7 and 10^6 CFUml^{-1} populations of *E. coli*.

From the absorbance measurements presented in Figure 7.3.3 it can be observed that there is little absorption of UV-C (200-280 nm) by the 10^7 CFUml^{-1} population of *E. coli* and there is none at all with the 10^6 CFUml^{-1} population. With the 10^8 CFUml^{-1} population however, the level of absorption is high, which will ultimately reduce the dose of UV received by the population.

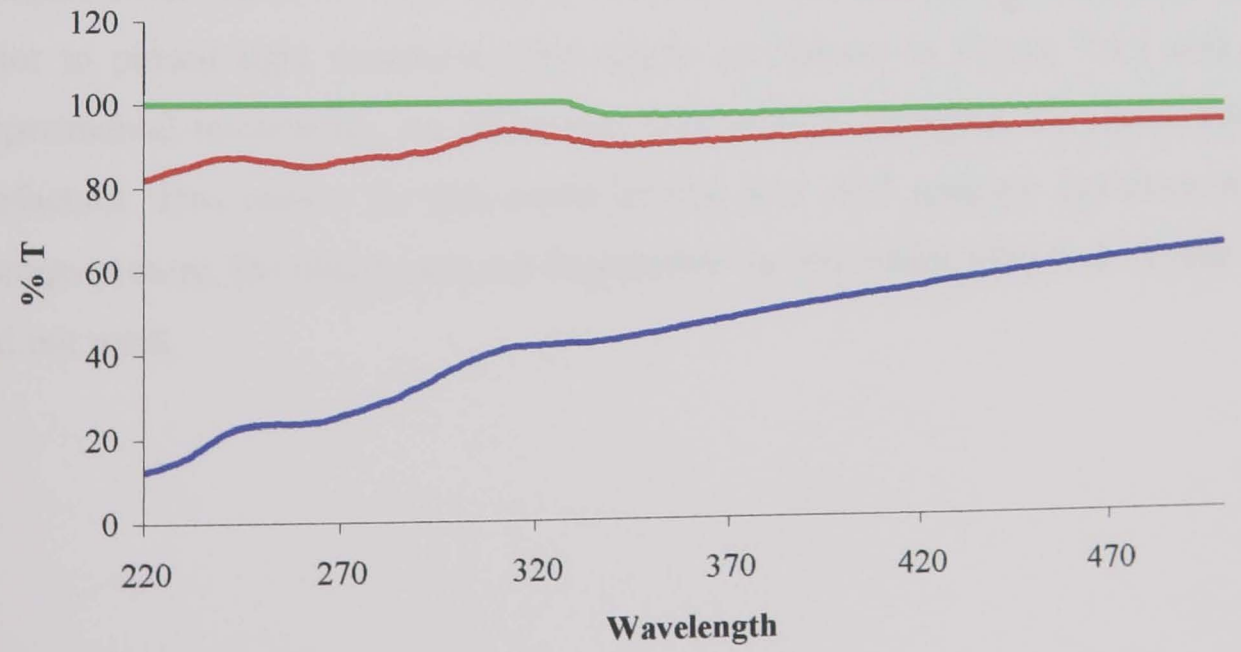


Figure 7.3.4 % Transmittance readings for 10^8, 10^7 and 10^6 CFUml^{-1} populations of *E. coli*.

In Figure 7.3.4, it can be observed that 100 % of the UV-C radiation is transmitted through the population of 10^6 $CFUml^{-1}$; 80-90 % is transmitted through the 10^7 $CFUml^{-1}$ population, and 10-30 % of UV-C through the 10^8 CFUml-1 population. This indicates that the larger the population of cells, the greater is the protection provided to those cells that are at the deeper levels of the sample. It has to be noted however that the absorption and % Transmittance readings taken by the Spectrophotometer are the measurement of light through a 1 cm light path. The light path for the UV-rich light pulses, in the experimental protocol, is much shorter (0.34 cm); therefore the level of absorption will be reduced and the level of transmittance should be slightly higher.

7.4 Effect of Addition of Tween

A factor that could affect the pulsed-light inactivation curve data is the extent to which cell clumps occur in each sample. Cell clumps are caused when the bacterial cells adhere together or when they are in very close proximity to each other. As a consequence some cells may receive protection from the UV damage. To examine this, the surfactant Tween 80 was added at two different concentrations to *E. coli* samples of the same $CFUml^{-1}$ in order to disperse any clumps of cells in the samples prior to pulsed light treatment. The results are shown in Figure 7.4.1 and within experimental uncertainty, no effect was observed on changing the concentration of surfactant. This reason for this could be due to 1 of 3 reasons: (a) there were no clumps present, (b) clumps are not responsible for the tailing effect, or (3) the Tween did not work.

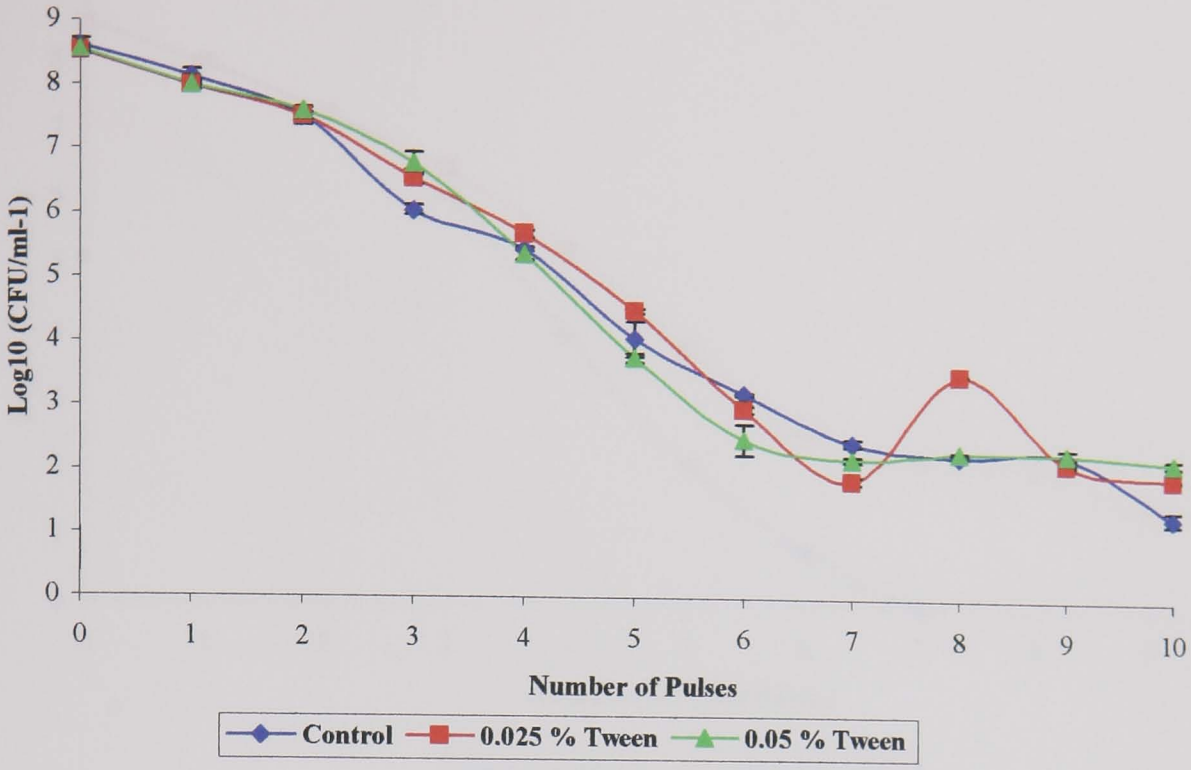


Figure 7.4.1 Effect of reducing clumping in samples, by the addition of different concentrations of a surfactant (Tween 80), prior to pulsed light treatment.

7.5 Effect of Sample Agitation

During treatment with the light pulses, the distribution of bacterial cells throughout the sample is relatively stable. This could allow some cells to be shielded from the UV light for the entire pulsing process by other cells positioned near them. Agitation of the samples should help to mix the cells such that shielding of cells is minimised. The same experimental procedure as in previous experiments was used with the addition of samples being gently shaken for approximately 10 seconds between pulses. The pulse frequency was therefore reduced from 1 pulse per second (1Hz) to 1 pulse per 10 seconds (0.1 Hz). This lower frequency was also used for a control sample, for which no agitation took place between pulses. Measurements were made on samples with population densities of 10^8, 10^7 and 10^6 $CFUml^{-1}$ for 1 to 10 UV-rich light pulses. The results for these tests are shown in Figure 7.5.1, 7.5.2 and 7.5.3 respectively.

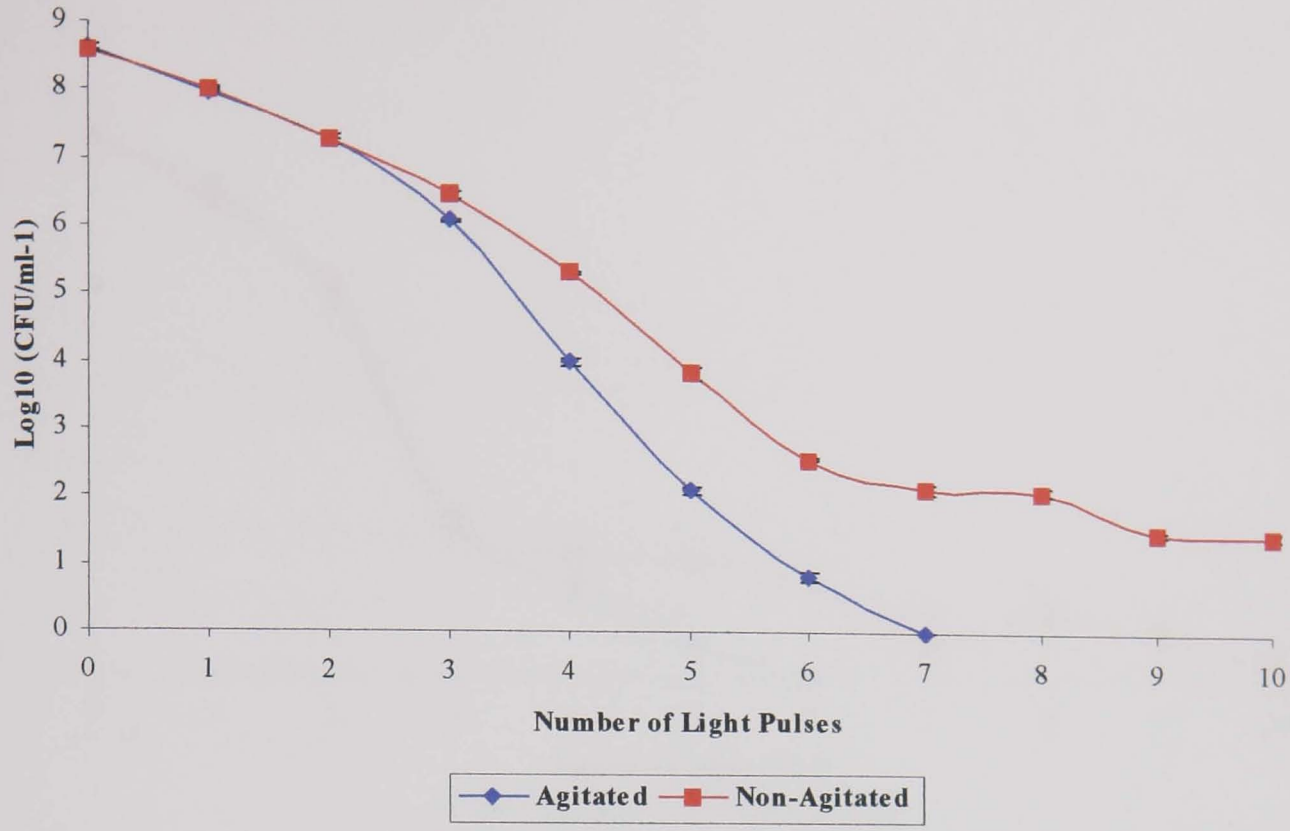


Figure 7.5.1 Inactivation curves of 10^8 CFUml^{-1} *E. coli* populations, which have received agitation and no agitation between each light pulse.

For the 10^8 CFUml^{-1} sample, Figure 7.5.1 shows that for the non-agitated control samples, the tailing effect is again present after approximately 7 UV pulses. For the agitated samples, inactivation increased more rapidly with dose and complete inactivation of the microorganisms was observed after 7 UV-rich light pulses.

The results shown in Figure 7.5.2, for the 10^7 CFUml^{-1} sample, reveal that the rate of inactivation of the agitated and un-agitated samples is almost identical over the first three pulses, following which inactivation is more rapid for the agitated sample, with complete inactivation occurring after 6 UV-rich light pulses. For the un-agitated sample, complete inactivation does not occur until after 10 UV-rich light pulses have been applied.

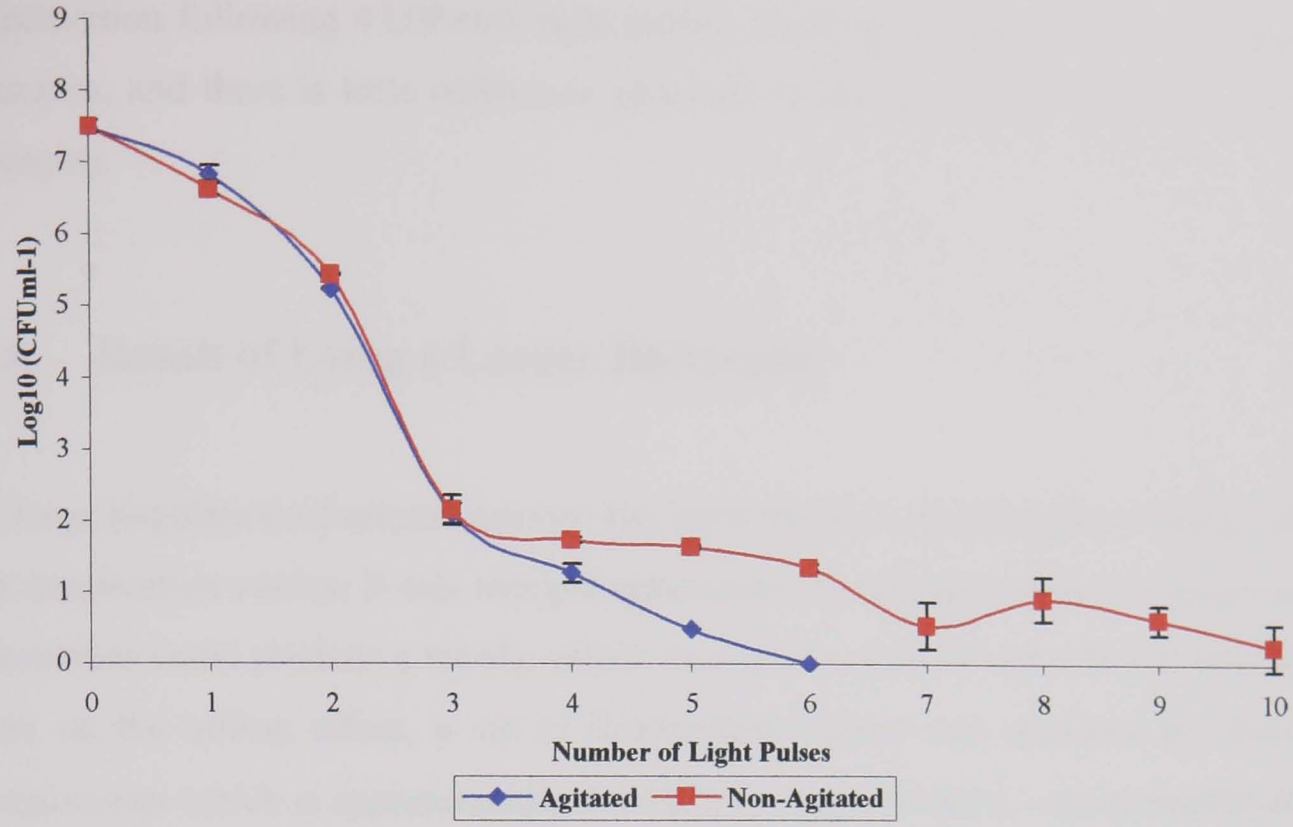


Figure 7.5.2 Inactivation curves of 10^7 CFUml^{-1} *E. coli* populations, which have received agitation and no agitation between each light pulse.

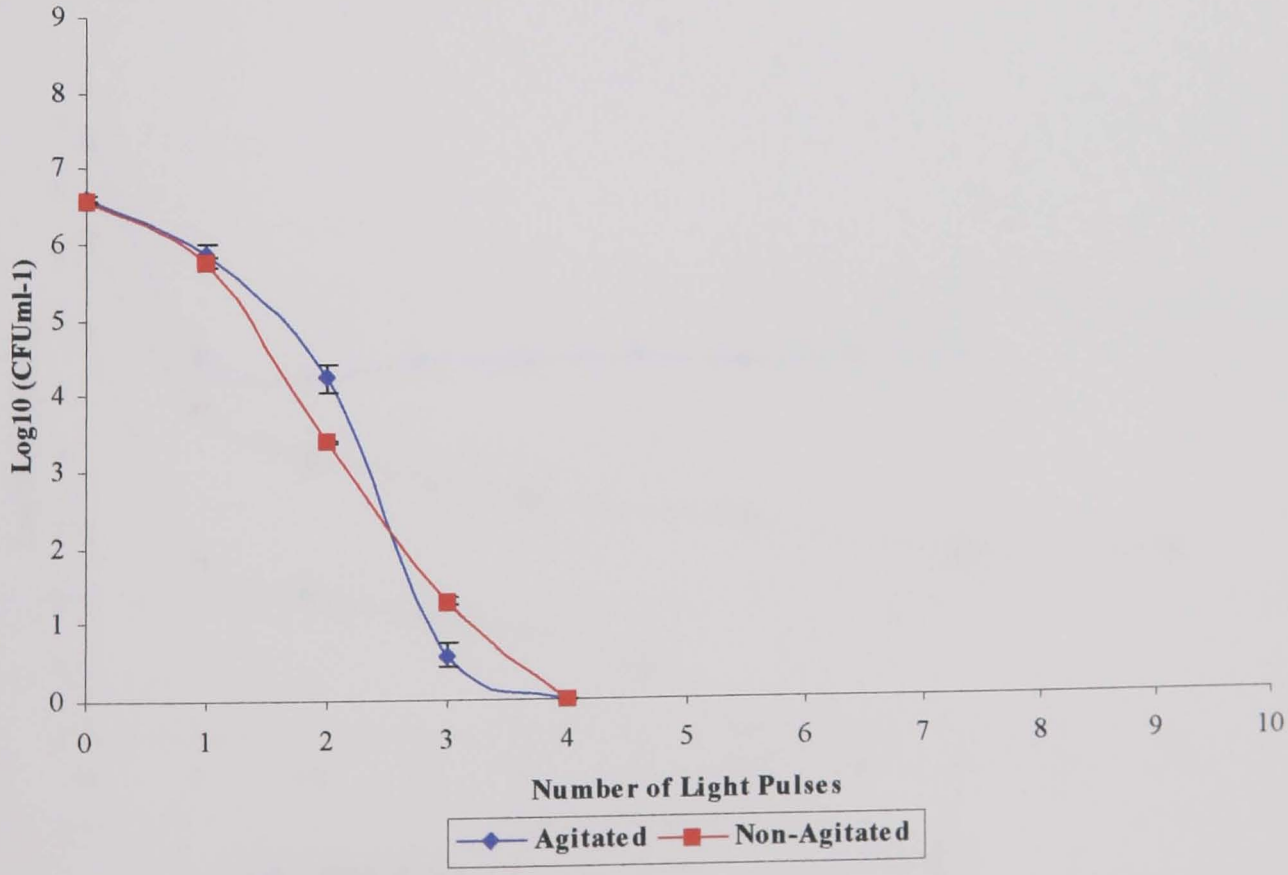


Figure 7.5.3 Inactivation curves of 10^6 CFUml^{-1} *E. coli* populations, which have received agitation and no agitation between each light pulse.

The results shown in Figure 7.5.3, for the 10^6 CFU ml^{-1} sample, show complete inactivation following 4 UV-rich light pulses, for both the agitated and un-agitated samples, and there is little difference observed in the inactivation rates for the two samples.

7.6 Result of Using a Larger Bacterium

A large population of microorganism has been shown to demonstrate a tailing effect on inactivation curves. It was thought appropriate to consider whether a larger sized bacterium could produce a similar effect. In order to examine the effect of bacterium size on the tailing effect, a set of inactivation curves was obtained for *Bacillus megaterium* which is approximately 2 x 6 μm in size whereas *E. coli* is smaller at 1.0 x 2-6 μm [111]. *B. megaterium* was cultured and re-suspended in quarter strength ringer to give population densities of 10^8,10^7 and 10^6 CFUml^{-1}. Aliquots of 20 ml of each bacterial population were then treated with a range of UV-rich light pulses. The results obtained are presented in Figure 7.6.1.

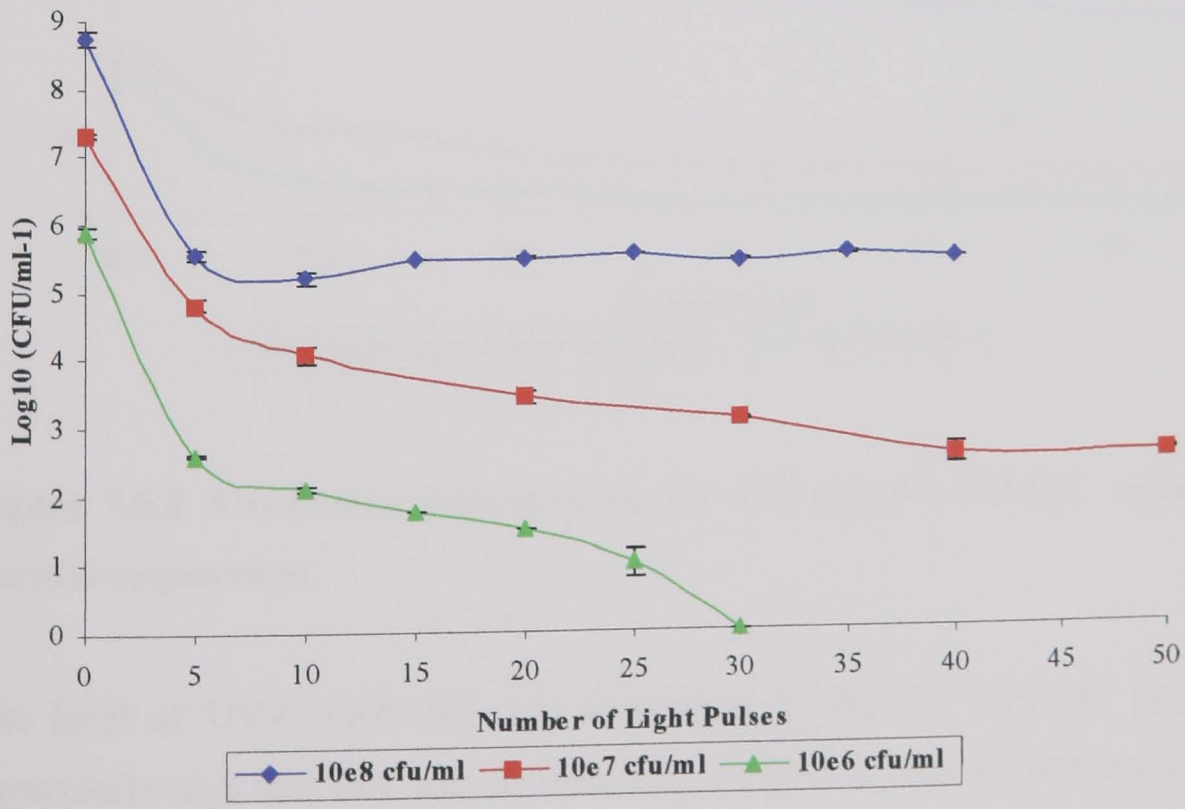


Figure 7.6.1 Inactivation of 10^8,10^7 and 10^6 CFUml^{-1} populations of *Bacillus megaterium*, using 1-10 UV-rich light pulses.

With the 10^8 CFUml^{-1} population of *B. megaterium* an overall 3.2 log reduction was achieved and a tailing effect occurred after around 6 pulses. Pulsing of the 10^7 CFUml^{-1} population achieved an overall 4.7 log reduction in the *Bacillus* population and a tailing effect can also be observed which does not start until after approximately 40 UV-rich light pulses. For the 10^6 CFUml^{-1} population no tailing effect occurs and complete inactivation occurs after 30 UV-rich light pulses.

The absorbance and transmittance of the three samples at wavelengths 200-500 nm were measured using a Spectrophotometer (section 3.4.5) as shown in Figures 7.6.2 and 7.6.3.

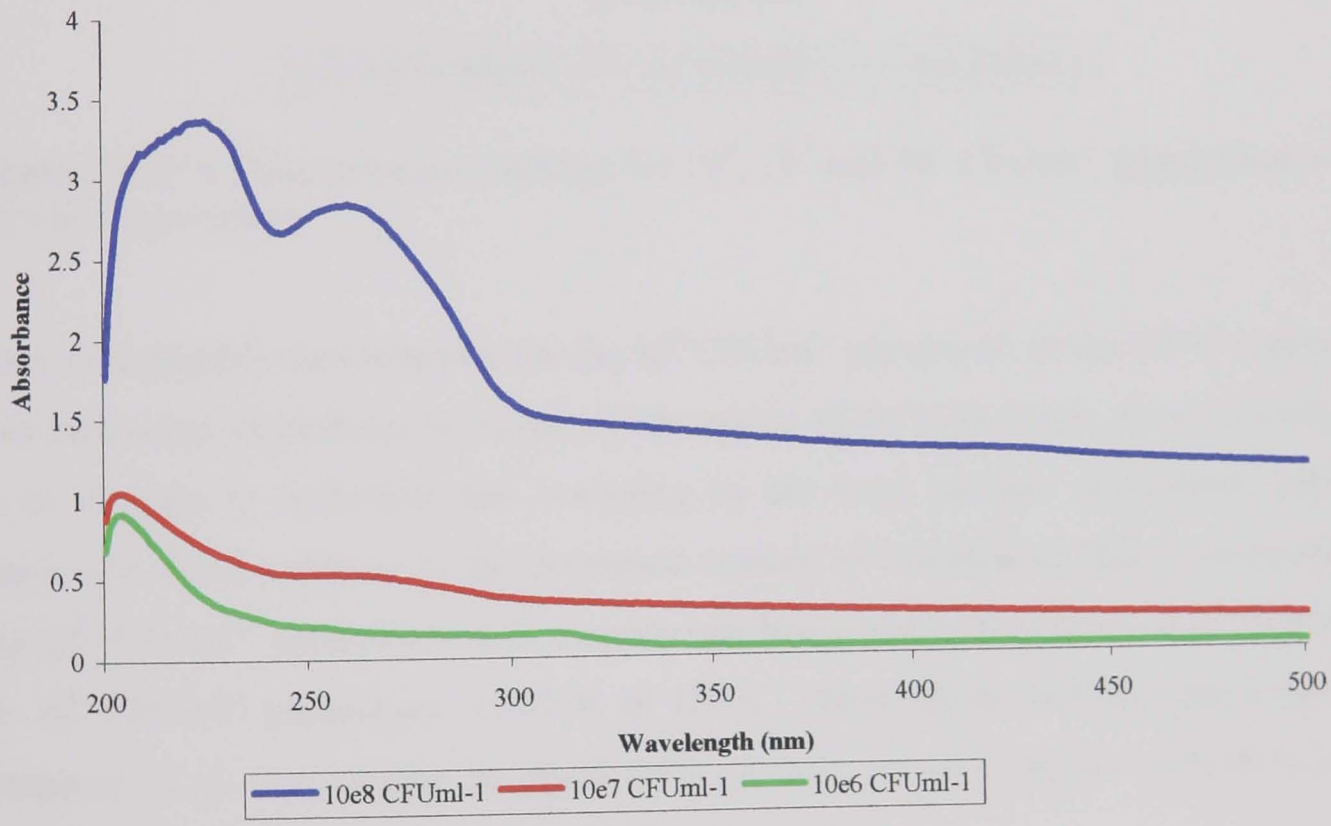


Figure 7.6.2 Absorbance measurements for 10^8 and 10^7 CFUml^{-1} populations of *Bacillus megaterium*.

The level of UV-C (200-280 nm) absorption by the 10^8 CFUml^{-1} population is particularly high and this will undoubtedly result in a reduced inactivation rate for this population. For the 10^7 CFUml^{-1} and 10^6 CFUml^{-1} populations, the level of

absorption is significantly lower, but some reduction of UV dose, does occur for both populations.

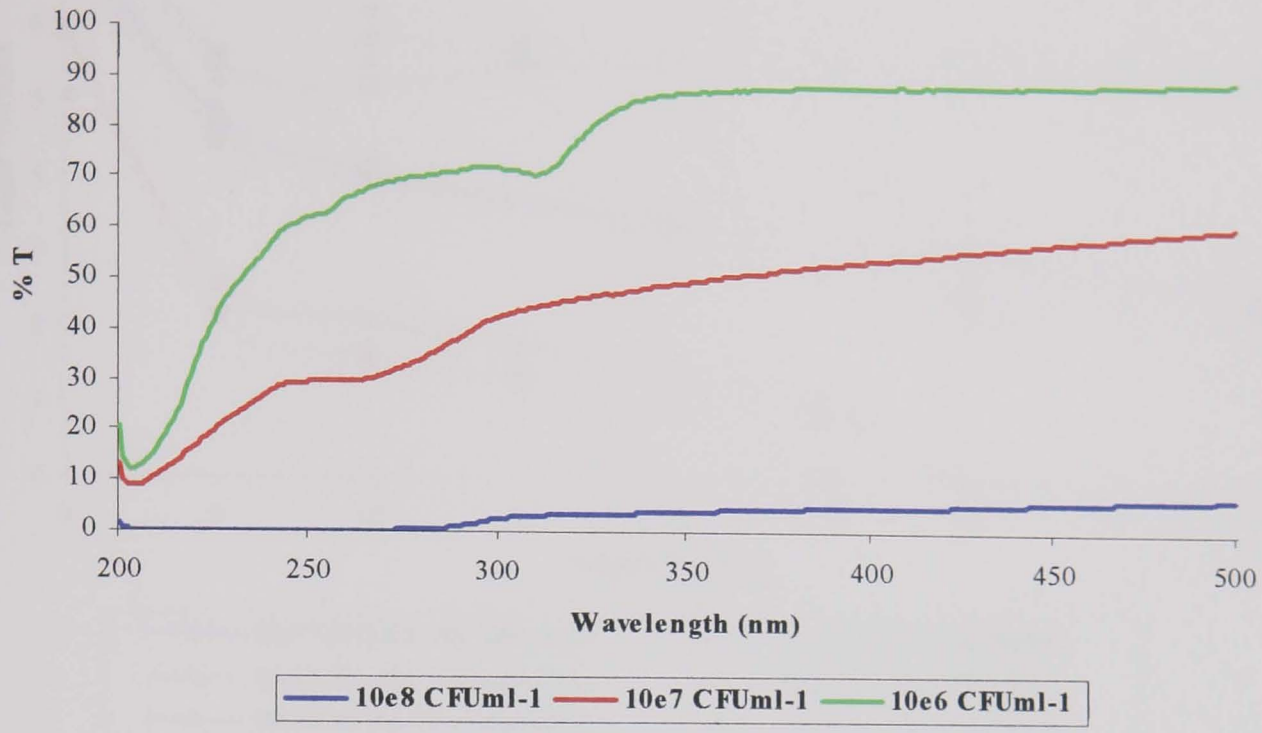


Figure 7.6.3 % Transmittance readings for 10^8, 10^7 and 10^6 CFUml^{-1} populations of *Bacillus megaterium*.

There is negligible transmittance for the 10^8 CFUml^{-1} population in the UV-C region. This is because, in addition to increased absorption of the light pulses, there will also be an increase in deflection and scattering by the large number of bacterial cells which will also contribute to the decreased amount of inactivation that is observed. The 10^7 CFUml^{-1} population of *B. megaterium* has a transmittance of 10-35 % and the 10^6 CFUml^{-1} population, 15-70 % of UV-C. These results indicate that (when compared to *E. coli*) larger bacterial cells produce an even greater reduction in transmittance, even with low population density levels.

The effect of continuous agitation on inactivation of *B. megaterium* was examined using the same experimental procedure as described in section 7.5. The inactivation results for the agitated and un-agitated *Bacillus* samples are shown in Figure 7.6.4.

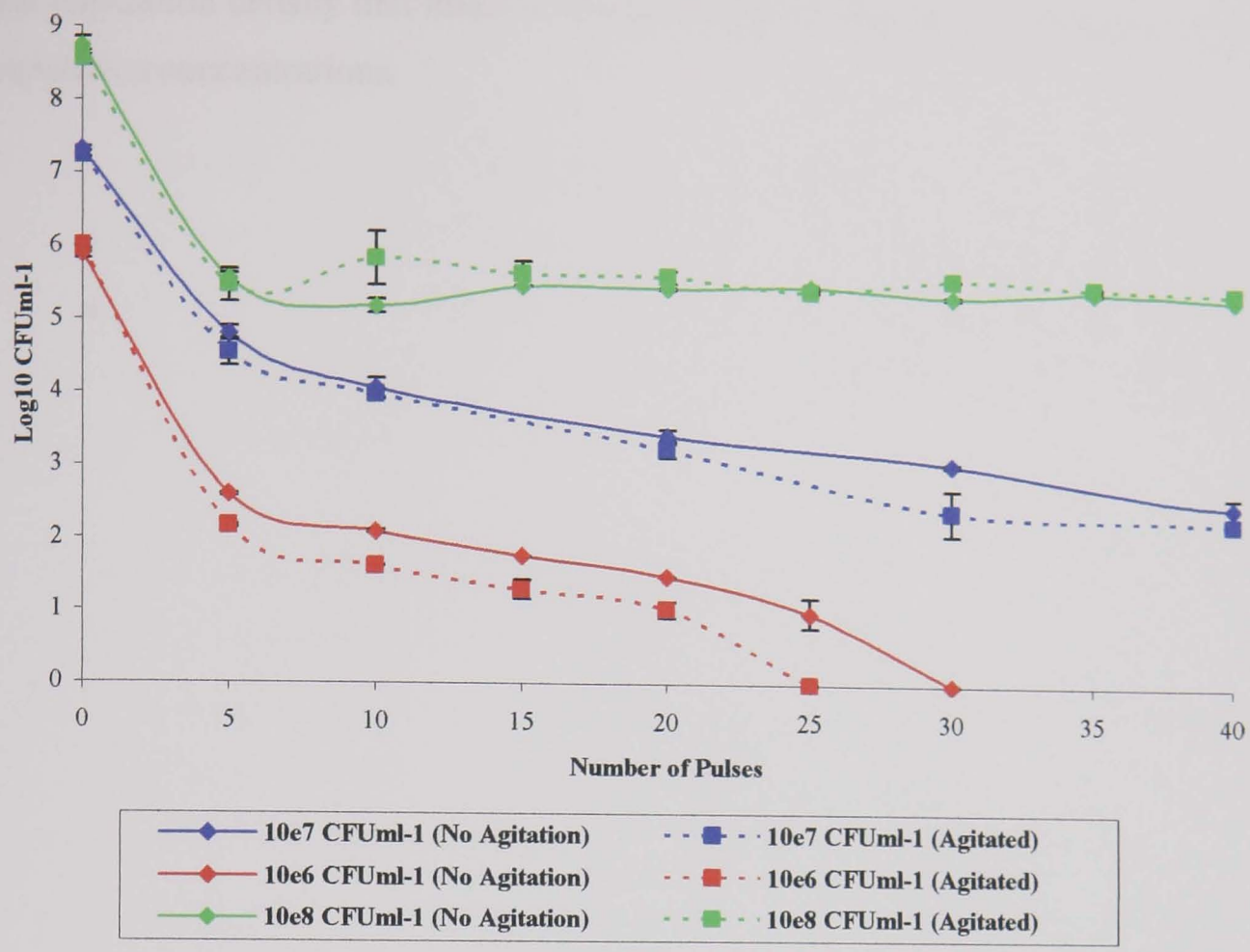


Figure 7.6.4 Inactivation curves of 10^8, 10^7 and 10^6 CFUml^{-1} *B. megaterium* populations, which have received agitation and no agitation between each light pulse.

The results of Figure 7.6.4 show that for the 10^8 CFUml^{-1} population, agitation of the samples between each pulse does not make any significant difference to the inactivation results achieved. It was also found that the agitation did not reduce the tailing effect on the inactivation curve. This could be because the high population size and the larger bacterium all occupy more space in the sample, so when the sample is agitated the cells are not able to disperse as well, as the *E. coli* cells did when agitation was introduced in the previous experiments. For the 10^7 CFUml^{-1} population slightly, more inactivation took place with agitation (0.6 Log_{10}), but the tailing effect was still present and complete inactivation was not achieved. With the 10^6 CFUml^{-1} population no tailing effect was observed for either the agitated or non agitated samples. The agitated samples were however completely inactivated using a lower number of pulses (25 compared with 30 pulses for the non agitated samples). This shows that inactivation of larger microorganisms occurs quite rapidly at low population levels with no tailing. This therefore suggests that it is the organism's size

and population density that make it less susceptible to pulsed UV treatment at higher population concentrations.

7.7 Discussion

The results presented in this chapter have investigated the problem of bacterial cells receiving protection from the UV rich light pulses and how this in turn can affect the inactivation kinetics. The problem had been detected during several experiments throughout the course of this investigation and is characterised by the appearance of a "tailing effect" on inactivation curves.

In the initial experiment (Figure 7.2.2) investigating the tailing effect, where the pulse number was increased, no further levels of inactivation occurred which agrees with other published work where tailing is shown to occur at high UV doses [135]. The results demonstrate that increasing the dose/pulse number does not increase the inactivation kinetics; therefore the 'tailing' must be caused by either a property of the microorganism or of the sample. When samples of large and small population sizes were used, very noticeable differences were observed between the inactivation curves. With the large population, Figure 7.3.2 (10^8 CFUml^{-1}), rapid initial inactivation was observed followed by slower inactivation rates, resulting in a tailing effect. In other words after a certain number of pulses, little further inactivation takes place. This is thought to be likely due to the cell distribution where the cells that make up the "tail" may have been shielded, from the UV rich light, by other cells allowing them to survive. These results can be compared with the lower population density results, also in Figure 7.3.2 (10^7 CFUml^{-1} and 10^6 CFUml^{-1}), where no tailing occurs. It is thought that the lack of tailing at low population densities is due to there being less cells present in the sample to cause shielding. If shielding occurs, it will protect the bacterial cells in two ways. It will either protect some cells from receiving any UV or else protect bacterial cells from receiving a sufficient dose to complete inactivation. With a large population of bacteria, many cells will overlap, providing partial or complete protection to the cells below. With a lower population (Log order less), however, the cells will have more space therefore less overlapping will occur. Another suggested reason for the observed tailing could be due to bacterial cells being present as a highly resistant sub-population as described by Warriner *et al* [129]. If a sub-population were present however, then this would have been expected

to be present at lower population densities as well. Resistant sub-populations therefore do not appear to be contributing to the tailing effect in this study, as tailing was not observed when treating low populations of bacteria.

Measures were taken to try and reduce the amount of shielding, and hence reduce the tailing effect, which occurs. An initial experiment involved using a surfactant (Figure 7.4.1), which was added to the bacterial sample before pulsing. It was thought that this might break up any bacterial clumps that may be present, therefore reducing the amount of tailing caused from bacterial shielding. The surfactant was found to have no effect whatsoever, indicating that overlapping of cells in the light path and not clumping was likely to be the main factor leading to shielding. The other measure taken to reduce shielding was to agitate the sample between the delivery of each pulse. This had a significant effect, which can be observed from the results in Figure 7.5.1 When the 10^8 $CFUml^{-1}$ population was agitated, no tailing effect took place and complete inactivation occurred. When lower populations were agitated (Figure 7.5.2), which previously had not demonstrated tailing, inactivation occurred much more rapidly. Agitation of the sample therefore ensured that the cell distribution was more uniformly exposed to UV light pulsing.

Experiments were also carried out using *Bacillus megaterium*, which is approximately 2 x 6 μm in length (~ twice the size of *E. coli*). Similar populations of this microorganism were shown to be much more resistant to the UV-rich light pulses than *E. coli* and the tailing effect was observed to occur at even lower populations. It was considered that this is because when treating *B. megaterium* at population levels similar to those used for *E. coli*, the total surface area of all microorganisms is increased by around a factor of 4. This increased surface area can therefore increase the amount of shielding, which in turn can decrease the level of inactivation. All microorganisms vary in their structure, and larger bacteria such as *B. megaterium* tend to have greater intra-cellular distances, containing UV-absorbing proteins and chromophores between the cell surface and the nucleic acid [41]. This ultimately results in a lower transmission of UV light through the cell and as a consequence of this, a larger number of cells are protected. From % Transmission

(Figure 7.6.3) measurements taken in this study, it was found that for 10^7 $CFUml^{-1}$ populations of *E. coli* and *B. megaterium*, the transmission was found to be more than 3 times greater for *E. coli* (96 % compared with 30 %). When agitation was introduced with this microorganism, no reduction in the tailing was observed at all, even with the lower populations (Figure 7.6.4). This suggests that agitation only has an effect with smaller sized microorganisms where less absorption of the UV occurs. In a typical 1 micron diameter bacterium such as *E. coli*, about 85 % of the light entering the bacterium exits from the other side and can pass into an adjacent microbe [61]. In this study however, the high populations of *E. coli* did not allow the UV light to transmit through all of the microorganisms, as each time this passes through a bacterial cell, it loses energy. With *Bacillus megaterium*, the diameter of the organism is double that of *E. coli*, hence even less light is transmitted through each individual microorganism. The result therefore is a reduced level of inactivation, even at lower populations, which was demonstrated in this study.

Although this study has found that protection is primarily due to the population size and type and the size and structure of a bacterium, Blatchley *et al* [57] have suggested that bacteria aggregate as part of a natural defence mechanism for protection from the UV radiation. A study carried out by them found no signs of bacterial aggregates in un-irradiated samples but they observed aggregates in irradiated samples. What they suggested is consistent with bacterial response to other forms of stress, many of which result in aggregation or particle association [57]. The tailing that has been found to occur in this study may therefore also be due to a bacterial defence mechanism.

The issue of protection of bacterial cells from UV is of great concern in situations outside the laboratory where the presence of particles can add to the protection from UV light. Particles present can protect microorganisms in several ways, they can completely shade microorganisms, cause scattering of the UV light or only allow limited cellular damage. Microorganisms can also be enclosed within such particles, which offer increased protection to the UV light [24]. In wastewater treatment, a potentially major application of UV disinfection, factors such as suspended solids,

and the size of the particles in the solids contribute to shielding. Microbes can be occluded within suspended solids and other microbes, such as protozoa that may be present in active sludge. Such factors would mean that these microbes may experience a lower UV dose compared to individual microbes [41]. Suspended solids also cause a decrease in UV transmittance as they absorb or scatter the UV light resulting in a reduction of available light for disinfection [25]. Filtration and sedimentation of wastewater however has been shown to decrease suspended solids and decrease particle size, therefore providing less protection to microorganisms. In water disinfection, shielding of microorganisms tends to occur from dirt and other particles, which are present in the water, but these however are usually removed by chemical treatment and filtration [79]. In the treatment of foodstuffs (although UV is not widely used for this) small recesses, fissures and folds present in the surfaces of meats allow some microorganisms to avoid UV exposure [65]. It has been found that UV is more effective in reducing the bacterial counts on the surface of smooth-fleshed fish such as mackerel than on mullet, the flesh of which contains prominent ridging. This suggests that these ridges can provide shadowing of microorganisms that are present [63]. Surfaces that may be rough or porous also permit bacteria to hide from the UV thereby preventing inactivation of these microorganisms.

Overall, this study has highlighted the importance of shielding by microorganisms from UV-rich light pulses. It has been found that with large populations, and large microorganisms, shielding is the key factor in preventing complete inactivation. A range of other factors however can contribute to protection when using UV for a disinfection system outside the laboratory, and these must be seriously considered depending upon the application of the system.

Chapter 8

PHOTOREACTIVATION FOLLOWING PULSED UV EXPOSURE

8.1 General

In the introduction of this thesis (section 2.6.2), the possibility of microorganisms reactivating following exposure to ultraviolet light was discussed. There are three different processes in which a microorganism can do this, but the one which is of greatest importance in the use of UV for disinfection purposes is the light dependent mechanism, commonly termed photoreactivation. Photoreactivation uses the enzyme photolyase and energy from the wavelengths between 300 and 500 nm to directly reverse many types of DNA damage [128]. Numerous studies have been carried out on photoreactivation following exposure from both polychromatic and monochromatic UV light sources. These studies however have been concerned with conventional continuous UV light sources. The work presented in this chapter is therefore aimed at determining whether photoreactivation occurs after treatment of bacteria with a pulsed UV-rich light system. To determine whether bacteria can self repair following the pulsed UV-rich light treatment, a range of properties has been investigated including pulse number (dose), temperature, light intensity and longer detention times. The results from these investigations are discussed in the following sections.

8.2 Procedure for Photoreactivation

The microorganism used in all of the photoreactivation experiments was *E. coli*, which was cultured for 18 hours in a flask containing Nutrient broth. Once cultured the microorganism was prepared following the method described in Section 3.7.1, followed by a 1 in 10 dilution to obtain a population of 10^8 CFUml^{-1}. Afterwards a sample of the diluted *E. coli* was removed and enumerated to determine the initial starting population. The bacterial suspension was thereafter used for treatment with UV-rich light pulses.

A 20 ml sample was pipetted into a standard sized petri dish and treated with the desired number of pulses (as described in Section 3.7.2). After pulsing, a 1ml aliquot was removed and enumerated to determine the population of microorganism remaining after pulsed light treatment. This also allowed calculation of the number of bacteria which was inactivated/damaged by the treatment (i.e. the population that could potentially be reactivated by exposure to visible light). From the remainder of the pulsed light treated sample, 9 ml was pipetted into a petri dish, which was wrapped in aluminium foil. This sample would not be exposed to any visible light treatment and was therefore called the "dark repair" sample. From the remainder of the pulsed sample, a further 9 ml sample was pipetted into a Petri dish, which remained un-covered. This sample would be exposed to visible light treatment; hence it was called the "light repair" sample. Both Petri dishes were placed inside the photoreactivation light cabinet (described in section 3.4.6), and 1 ml samples were removed every hour for enumeration, to observe whether any repair had occurred. During enumeration of the dark repair samples, the diluents were wrapped in foil to keep exposure from light in the laboratory to a minimum, and therefore prevent any unwanted photoreactivation.

8.3 Effect of Visible Light on Pulsed Light Treated Bacteria

Most of the work published on photoreactivation concerns continuous UV sources [50, 52, 56, 91], initial experiments were carried out to investigate whether photoreactivation occurs when bacteria are inactivated/damaged by pulsed UV- rich light. This was the first time that such a study had been undertaken and it was important to quantify this process and its influence on pulsed light inactivation.

8.3.1 Initial Photoreactivation Experiments

20 ml samples of *E. coli* were treated with 10 UV-rich light pulses. Afterwards, the pulsed samples were split into light and dark repair samples followed by incubation under photo reactivating light for 3 hours. The results obtained are shown in Table 8.3.1.1.

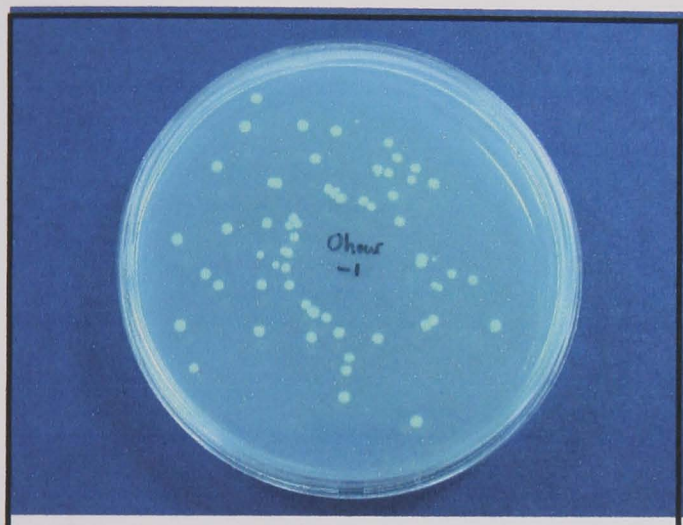
Sample	Initial Pop_n ($CFU\ ml^{-1}$)	Pop_n After 10 Pulses ($CFUml^{-1}$)	Pop_n After 1 h of Light	Pop_n After 2 h of Light	Pop_n After 3 h of Light
Light	2.65×10^8	0	0	0	0
Dark	2.65×10^8	0	0	0	0

Table 8.3.1.1 Population of *E. coli* before and after treatment with 10 UV-rich light pulses, and the numbers remaining after the pulsed population was placed under visible light for 1, 2 and 3 hours.

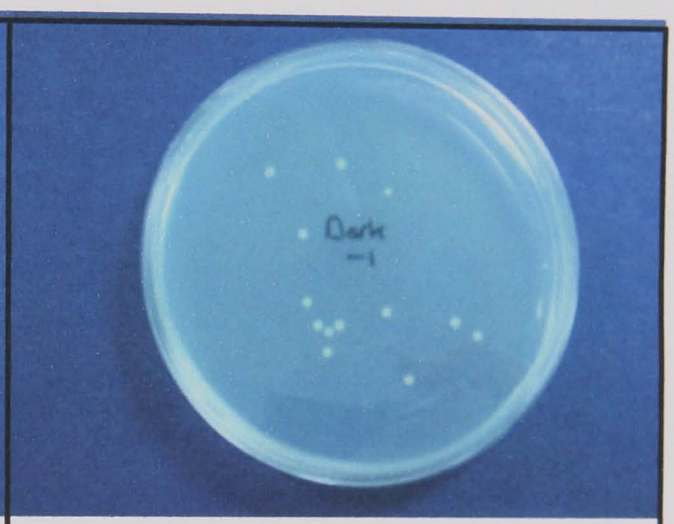
It can be observed that after 10 UV-rich light pulses, complete inactivation of the microorganism occurred. It is also shown that when the 8.42 Log_{10} of UV-damaged population is exposed to visible light for a period of 3 hours, no light repair occurred. No increase in population was demonstrated with the sample that was wrapped in foil. From these results, it therefore appears that the bacterial population is

completely inactivated and no photoreactivation is possible following pulsed UV-rich light treatment.

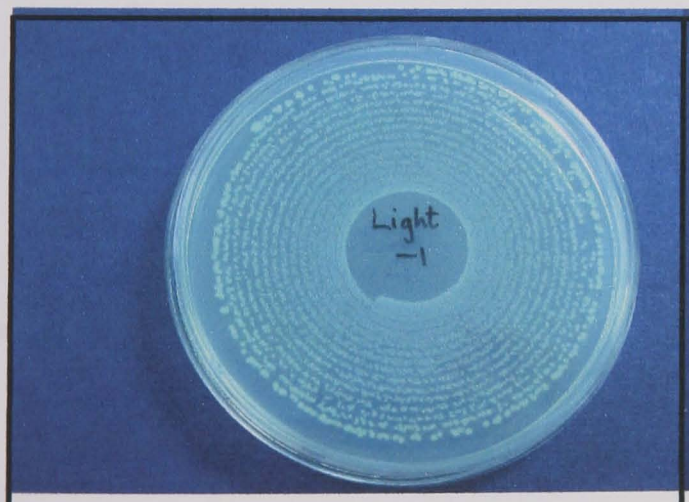
A second photoreactivation experiment was carried out using a lower number of pulses (5 UV-rich light pulses). On this occasion, when the spiral plates of each sample were observed after a 24 hour incubation period (Figure 8.3.1.1), it was obvious that a level of bacterial repair had taken place.



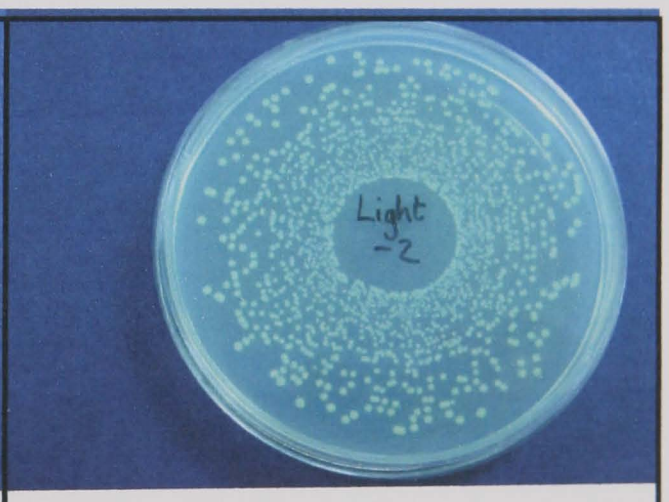
(a) 1 in 10 dilution of sample after 5 UV pulses (i.e. 0 hour Sample).



(b) 1 in 10 dilution of sample wrapped in foil for 3 hours.



(c) 1 in 10 dilution of sample after 3 hours visible light exposure.



(d) 1 in 100 dilution of sample after 3 hours visible light exposure.

Figure 8.3.1.1 Photographs showing the differences in bacterial growth when samples were exposed to light and dark conditions, after treatment with 5 UV pulses.

Plate (a) from Figure 8.3.1.1 shows survivors after pulsed UV-rich light treatment. When this is compared to plate (b), it can be observed that for the plate that was kept in the dark for 3 hours, no repair occurred. When plate (a) is compared to plate (c) however, it can be observed that photoreactivation has taken place, as represented by the significant increase in bacterial colonies. This 1 in 10 dilution had 'too many colonies to count' and the amount of repair that occurred had to be calculated from a 1 in 100 dilution, shown on plate (d). Once all the plates were counted, the graph shown in Figure 8.3.1.2 was produced to highlight the photoreactivation levels which occurred.

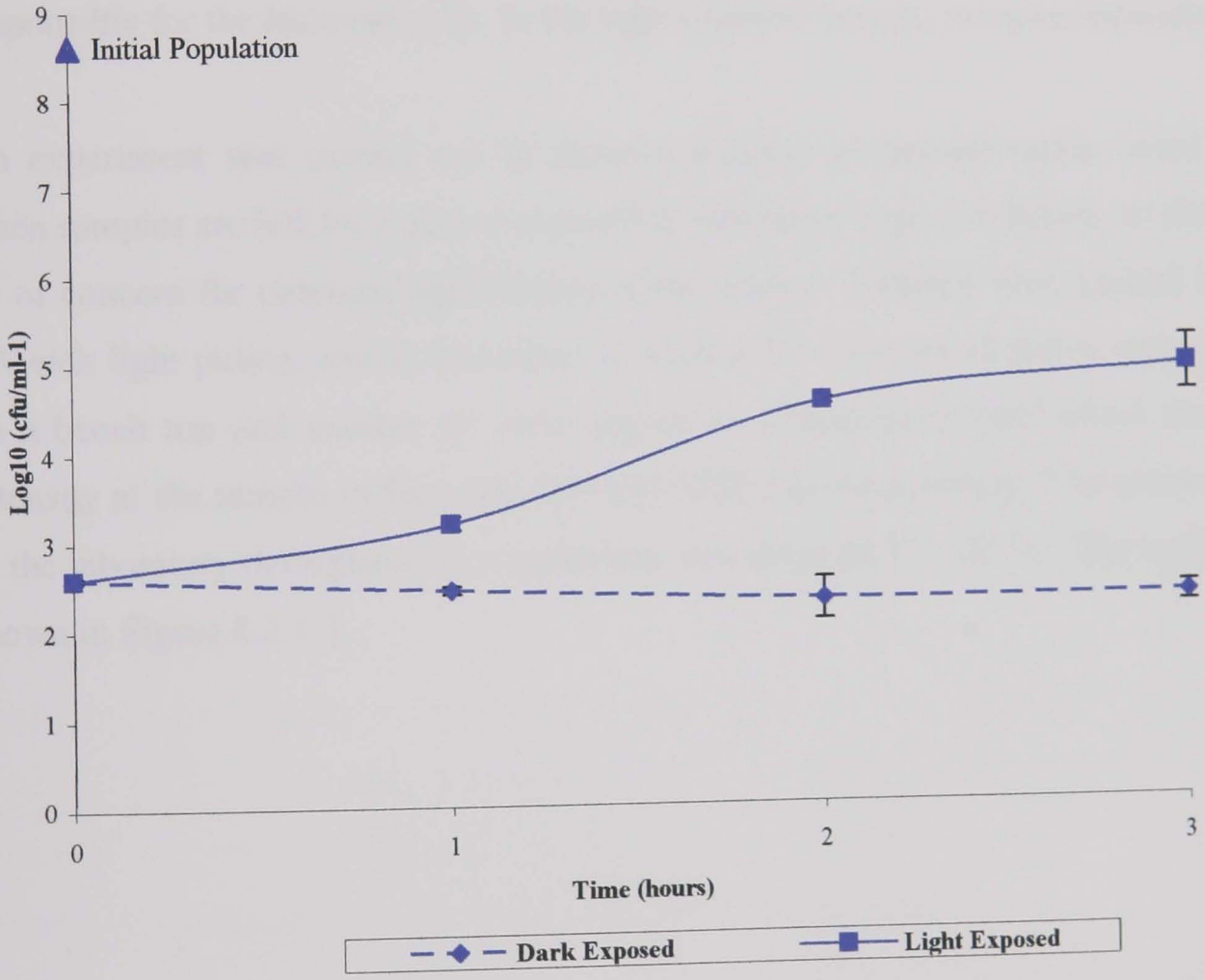


Figure 8.3.1.2 Population of stationary phase *E. coli* before and after treatment with 5 UV-rich light pulses, and the levels of repair after light and dark samples when placed under visible light for 1, 2 and 3 hours.

It can be observed that after 5 UV-rich light pulses, 4 $x10^{2}$ $CFUml^{-1}$ of the population remained from the initial population of 4.1 x 10^{8} $CFUml^{-1}$. This means

that 6 Log_{10} of the bacterial cells were inactivated by the UV pulses and could possibly undergo photoreactivation. When this population was exposed to visible light, a noticeable level of repair (0.55 Log_{10}) was observed after 1 hour. By the time the sample was incubated under visible light for 3 hours, 2.6 Log_{10} of cells had been photoreactivated. This result suggests that some of the population that was initially thought to have been inactivated was in fact only slightly damaged by the UV treatment. This damage was not large enough to prevent the bacterial cells from using their light repair mechanisms, and when exposed to visible light, their DNA could be repaired by photoreactivation. For the bacterial sample that was wrapped in foil and incubated under visible light, no repair occurred. This shows no dark repair mechanisms have taken place and also indicates that exposure to visible light is responsible for the bacterial cells, in the light exposed sample, to repair themselves.

An experiment was carried out to observe whether photoreactivation could arise when samples are left for a period exposed to laboratory light conditions, as this may be of concern for potential applications of the system. Samples were treated with 5 UV-rich light pulses, and as described in section 8.2. One set of plates were placed on a bench top and another set were placed on a laboratory shelf where the light intensity at the sample surface was 296 and 2250 Lux respectively. The temperature in the laboratory throughout the experiment was between 21 - 22 °C. The results are shown in Figure 8.3.1.3.

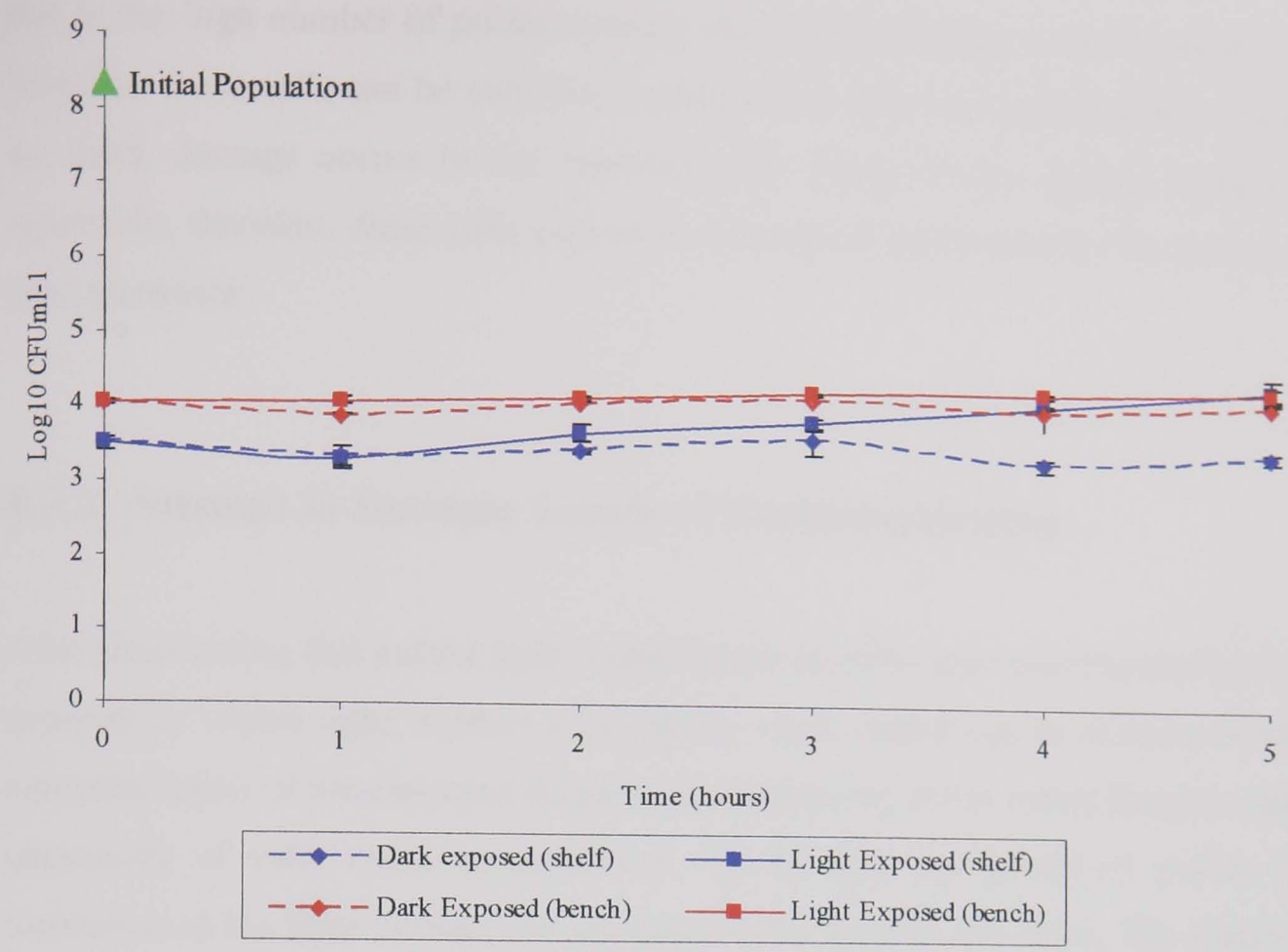


Figure 8.3.1.3 Population of stationary phase *E. coli* before and after treatment with 5 UV-rich light pulses, and the levels of repair after light and dark samples were left sitting on a bench top (296 Lux) and on a laboratory shelf at 21 °C.

For samples left on the bench top, there was no noticeable increase in the bacterial population whatsoever. When the samples were placed on a shelf however, the intensity of light was almost 8 times greater and a very slight increase in population could be observed. The total level of repair over the 5 hour period for this sample was 0.93 Log_{10}, which may increase over a longer period of time. However, these results do not appear as significant when they are compared to the short timescales required for photoreactivation to occur within the light cabinet.

Results from this initial study on photoreactivation do therefore conclude that bacterial cells treated with pulses of ultraviolet light can undergo repair following exposure to high intensities of visible light. They also show that when left at low intensities of visible light for long periods, small levels of repair can also occur. However, it was also found that if a bacterial population receives a large enough number of UV-rich pulses, then no photoreactivation occurs. This is thought to be

due to the high number of pulses causing irreversible damage to the bacterial cells, therefore these cells can be considered inactivated. When a lower number of pulses are used, damage occurs to the bacterial cells. Some of this damage however is repairable, therefore these cells cannot be considered inactivated by the pulsed UV light treatment.

8.3.2 Attempt to Increase Levels of Photoreactivation

After determining that pulsed light treated bacterial cells can repair themselves when exposed to visible light, further experiments were carried out to examine whether complete repair of the damaged population could occur, to the extent that the starting population of cells could be recovered. To do this, the period of visible light incubation in the light cabinet was increased from three to six hours. The results are shown in Figure 8.3.2.1. It can be noticed that the majority of photoreactivation occurs during the first three hours, where there is a 1.76 Log_{10} increase in bacterial population. After this period there is a gradual increase in cell numbers (0.46 Log_{10}) with the population remaining constant after 5 to 6 hours incubation under visible light. That means that out of the 1.2×10^5 $CFUml^{-1}$ cells initially damaged by the UV pulses, 1.7×10^2 $CFUml^{-1}$ cells were repaired by photoreactivation (0.14 %). Again as with the previous experiments, there was no significant change in the population size of the samples which remained in the dark.

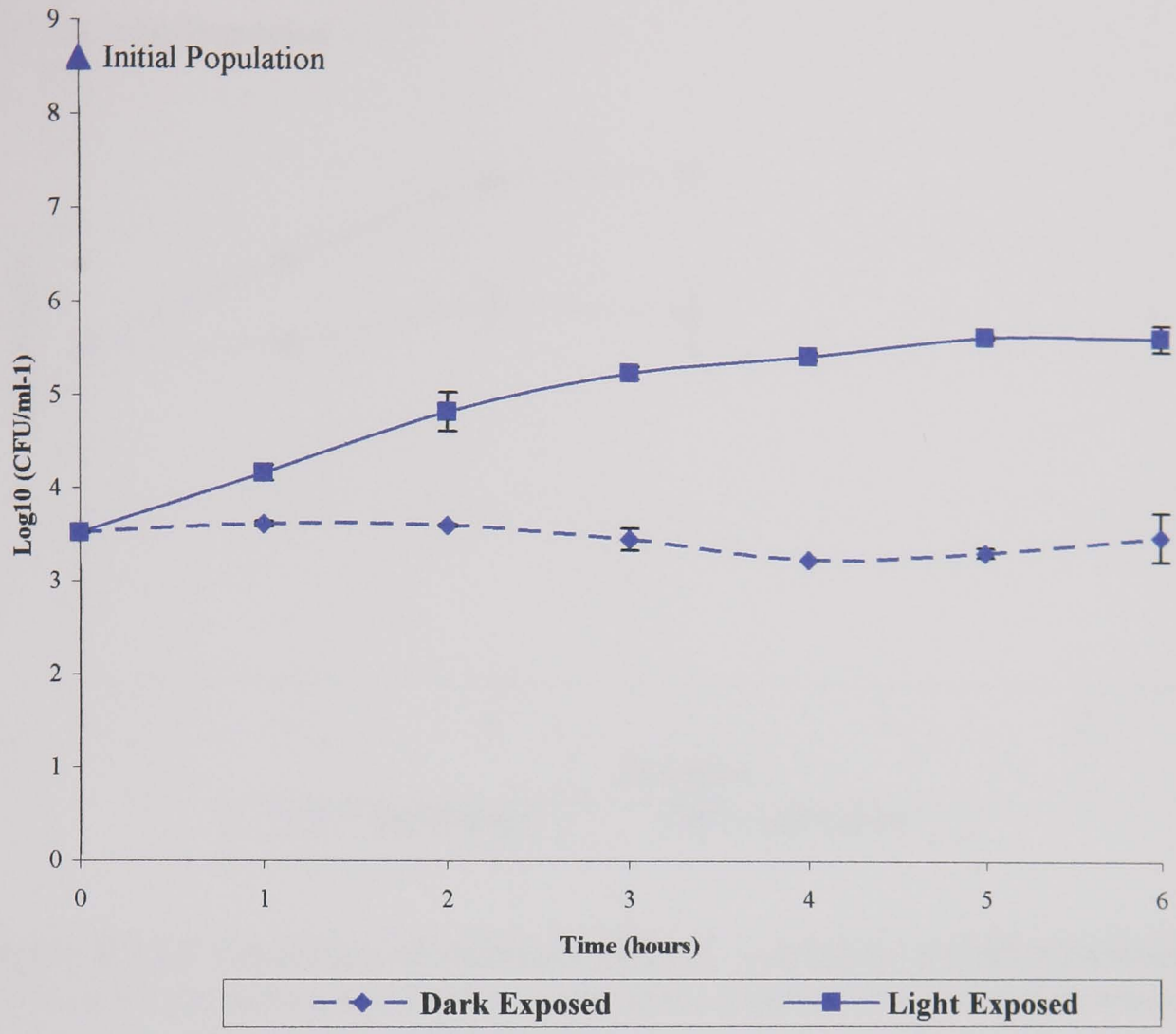


Figure 8.3.2.1 Population of stationary phase *E. coli* before and after treatment with 5 UV-rich light pulses, and the levels of repair of light and dark samples when placed under visible light for a 6 hour period.

In the prior photoreactivation experiments, the Petri dish lids were kept on during the visible light incubation period. Experiments were therefore undertaken with the lids removed to observe the effect, if any, on photoreactivation levels. The number of pulses and the period of visible light incubation were kept the same as those in the previous experiment.

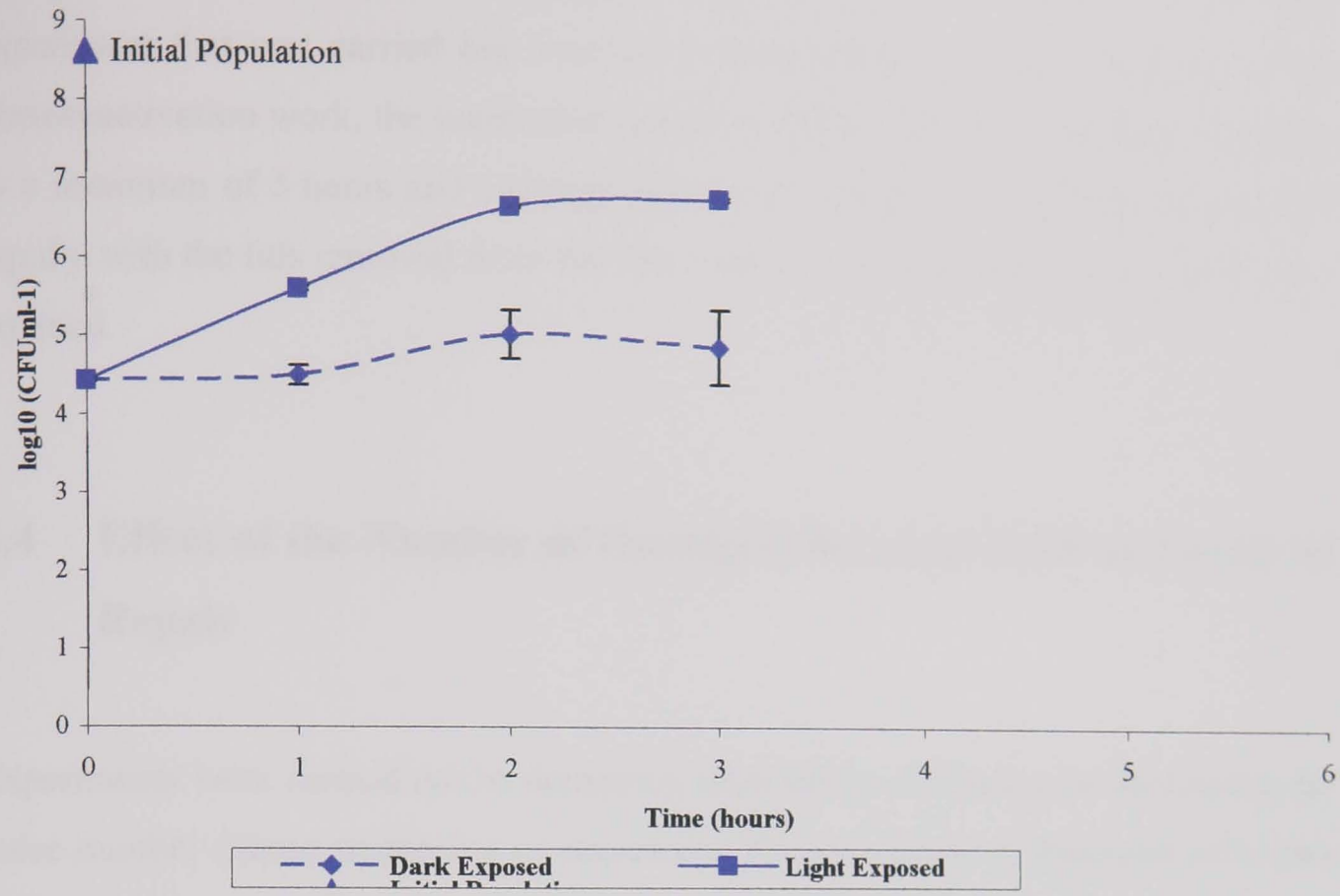


Figure 8.3.2.2 Population of stationary phase *E. coli* before and after treatment with 5 UV-rich light pulses, and the levels of repair of light and dark samples when placed in open Petri dishes under visible light.

Prior to photoreactivation, 1.5×10^4 $CFUml^{-1}$ of bacteria were inactivated by the pulses of UV. When the samples were placed under the visible light with their lids removed, 2.2×10^2 $CFUml^{-1}$ of this population was repaired (0.15 %). It can also be observed that the repair occurred over a shorter period of time (2 hours), with the amount of repair levelling off after 3 hours. The sample that remained in the dark appeared to have a slight increase in bacterial numbers but this is likely to be due to experimental uncertainty, since repair for the dark samples has not been witnessed prior to this. This is however, investigated further in this chapter. A problem that arose with leaving the lids off of the Petri dishes was that by the time the samples had been incubated for 4 hours, the remaining liquid had evaporated. As a result, the experiment could not be carried out over the 6 hour period as was intended.

Both of these experiments were aimed at investigating ways of increasing the level of photoreactivation that occurs. They were shown to repair 0.14 % and 0.15 % of UV

damaged cells, which can be compared with the 0.013 % repaired in the first experiment that was carried out over the 3 hour period. For the remainder of the photoreactivation work, the incubation period in visible light was therefore extended to a minimum of 5 hours and although photoreactivation was shown to occur more rapidly with the lids removed from the Petri dishes, for sampling reasons, these were retained.

8.4 Effect of the Number of Damaged Bacterial Cells on Levels of Repair

Experiments were carried out to determine what effect increasing or decreasing the pulse number (hence increasing or decreasing the population of damaged cells) has on levels of photoreactivation. The same procedure was carried out as in previous experiments. Using a 10^8 $CFUml^{-1}$ population of *E. coli* 3, 5 and 8 UV-rich light pulses were applied and samples were incubated under visible light for 6 hours. The results obtained are shown in Figure 8.4.1. The initial population of the samples are not shown on the graph, but they range from 3.8 – 4.0 x 10^8 $CFUml^{-1}$. For the samples that obtained 8 UV pulses, 5.7 Log_{10} of inactivation occurred and after 6 hours in visible light, 2.7 Log_{10} of this population was repaired. When the pulse number is decreased to 5 pulses, less inactivation occurred (5.1 Log_{10}) and from this population, 2.2 Log_{10} was repaired by photoreactivation. Finally, when 3 UV pulses were used, only 3.1 Log_{10} was inactivated and 1.8 Log_{10} was repaired. When the actual number of bacterial cells repaired, is observed as a percentage of the total number of cells inactivated, it is shown that the samples that obtained 8 pulses underwent less repair than those that obtained 5 and 3 pulses (Figure 8.4.2). This may be because the larger number of pulses is responsible for destroying other molecules/components, for example proteins, and when these are damaged by UV they are unable to repair themselves by photoreactivation therefore the cell dies.

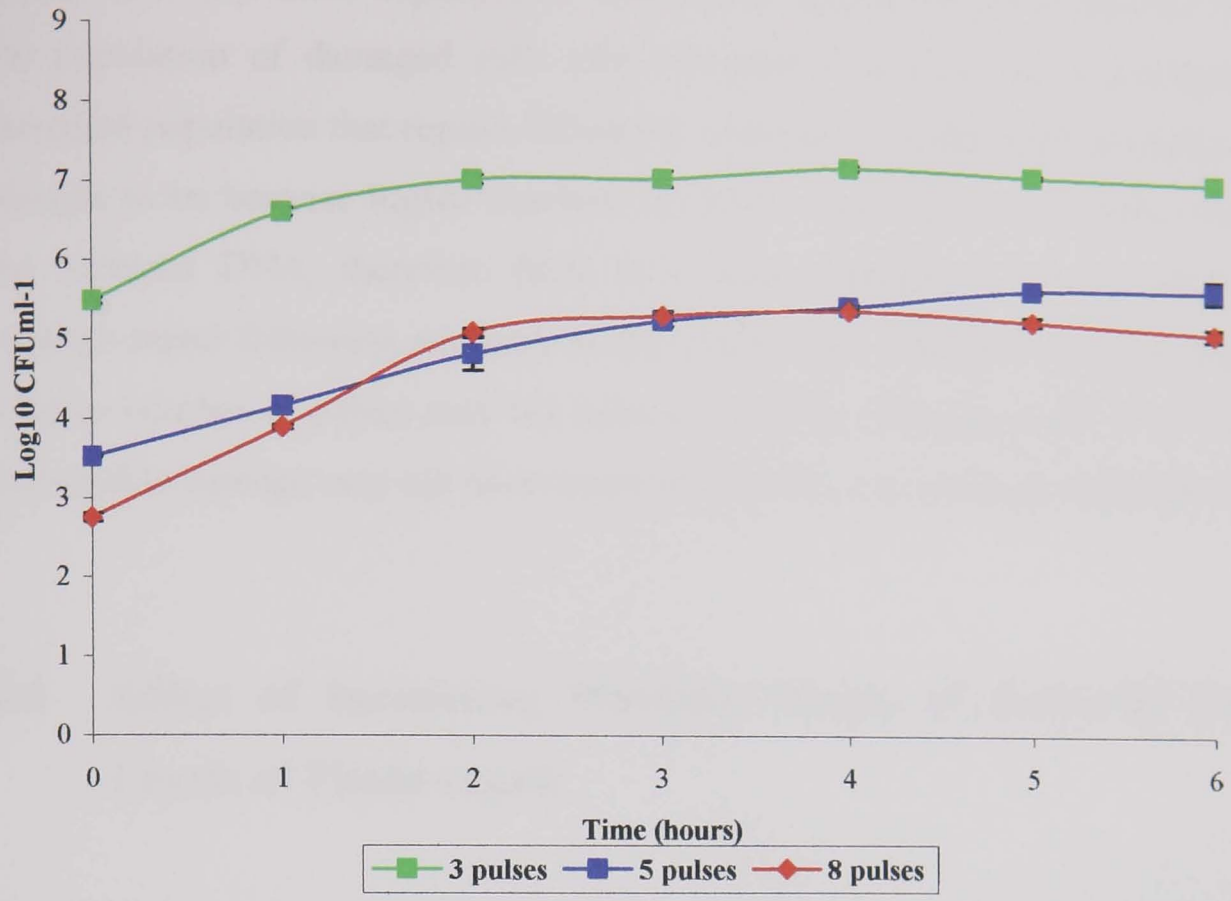


Figure 8.4.1 Levels of photoreactivation that occur after treating stationary phase *E. coli* populations with 3, 5 and 8 UV-rich light pulses.

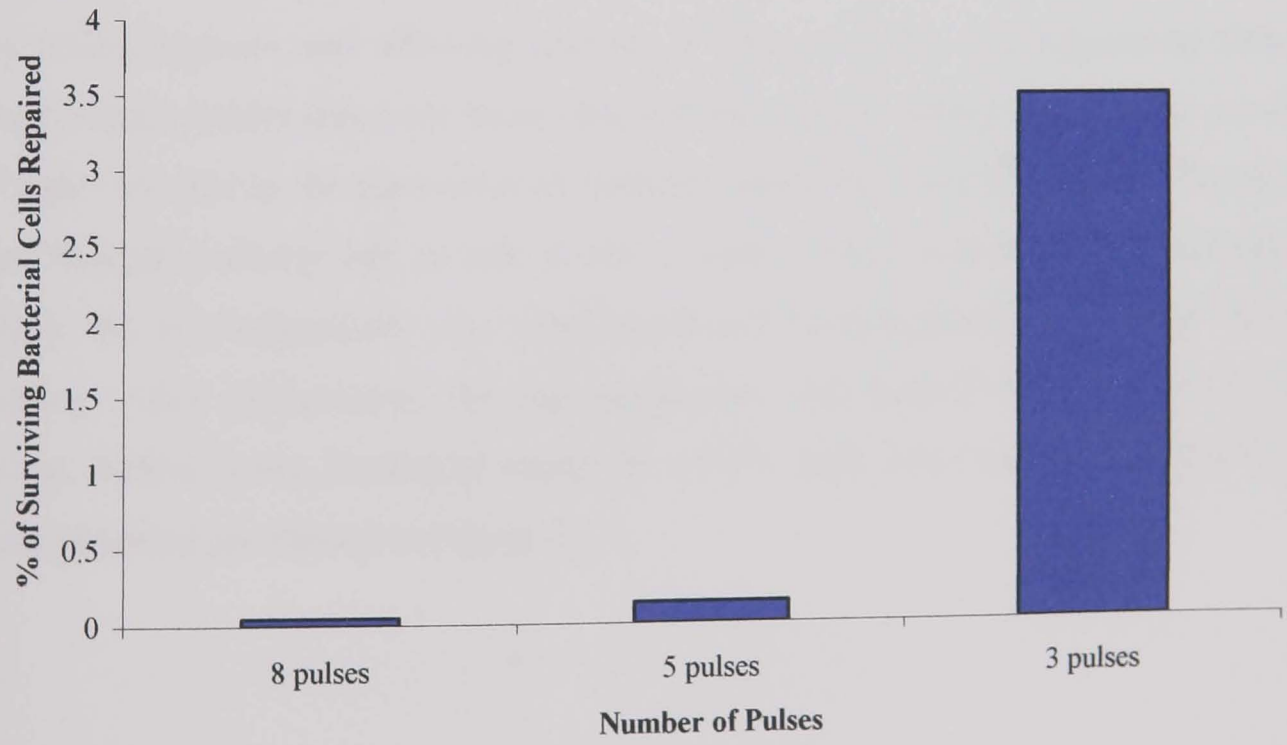


Figure 8.4.2 Percentage of *E. coli* population repaired by photoreactivation following 3, 5 and 8 UV-rich light pulses.

The results from these experiments have shown that as the pulse number increases, the population of damaged cells also increases. However, the percentage of the damaged population that repairs following exposure to visible light decreases. This is thought to be because higher numbers of pulses cause more irreversible damage to the bacterial DNA, therefore there is a large population of cells which cannot undergo repair following exposure to the visible light. Bacterial samples treated with a lower number of pulses may not have received an adequate level of UV therefore irreversible damage may not have occurred and they can undergo photoreactivation.

8.5 Effect of Increasing Washing Stages of Bacterial Cells on Levels of Photo-repair

On several occasions, a slight increase in population of the samples which were incubated in the dark has been observed (see Figure 8.3.2.2). The only explanation thought possible for this event was that after centrifuging and removing the bacteria cells from the broth, some traces of broth were being carried over into the suspension. As a consequence, the small trace of broth was providing nutrients for the microorganism and allowing growth. If this was what was happening then the photo-repair results that have been obtained may not be solely down to the presence of light, but due to the carry over of nutrients allowing bacterial growth. This theory was thought unlikely but to rule it out, a light repair experiment was carried out where the microorganism was centrifuged and re-suspended three times prior to pulsing. After preparation, the microorganism was treated with 5 UV-rich light pulses, before being incubated under the visible light for 4 hours. The results that were obtained are shown in Figure 8.5.1.

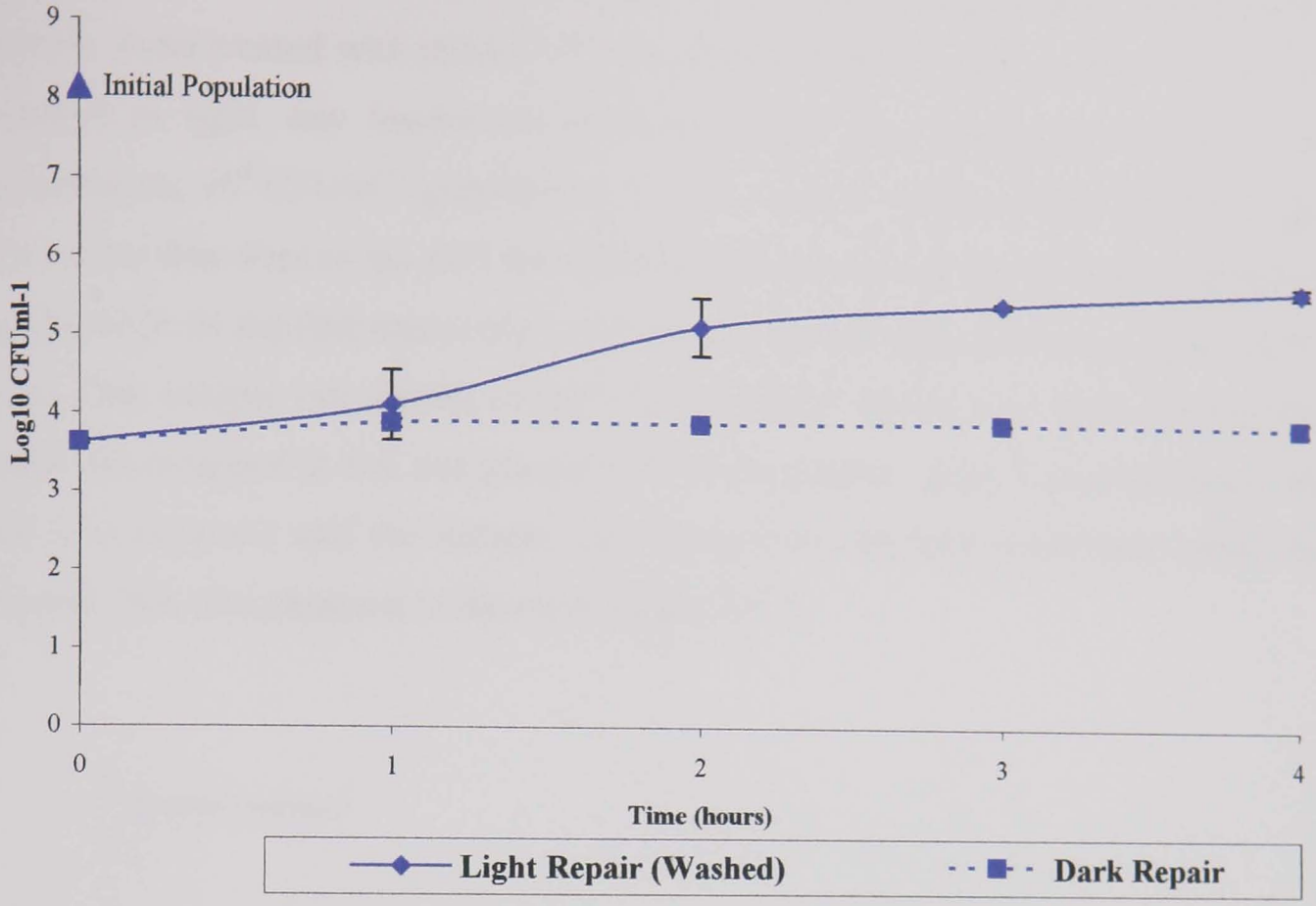


Figure 8.5.1 Level of photo-repair that occurs when stationary phase *E. coli* is washed three times prior to treatment with 5 UV-rich light pulses.

It can be observed from Figure 8.5.1 that after increasing the number of washing stages of the microorganism, to keep the carry over of broth to a minimum, photoreactivation still occurs in the samples exposed to the light. After 4 hours incubation under visible light a 2.1 Log_{10} increase in bacterial population is witnessed, which is similar to the results achieved in Figure 8.3.2.1 and Figure 8.3.2.2 where only one wash stage was carried out. No increase in population size is witnessed with the samples, which were incubated in the dark. It can therefore be concluded from this study that nutrients in the culture medium is not what is responsible for the increase in bacterial populations following pulsed UV treatment.

8.6 Importance of Delayed Visible Light Exposure

It has been established that photoreactivation occurs following pulsed light treatment. It is almost certain that it is the presence of visible light that causes this therefore some work was carried out to determine whether the process of photoreactivation can

be postponed. This would be useful to know for practical applications. For example, potable water treated with pulsed UV may be stored in the dark, but as soon as it is exposed to light, any inactivated microorganisms may reactivate. In this set of experiments, 10^8 CFUml^{-1} populations of *E. coli* were treated with 5 UV-rich light pulses and then kept in the dark for different periods of time before being exposed to visible light. In the first experiment, the sample was split after pulsing, as is normally done. One sample was placed straight into the light cabinet in a Petri dish and the other was wrapped in foil and placed in the light cabinet. After 2 hours however the foil was removed and the sample was exposed to the light conditions inside the cabinet. The data obtained is shown in Figure 8.6.1.

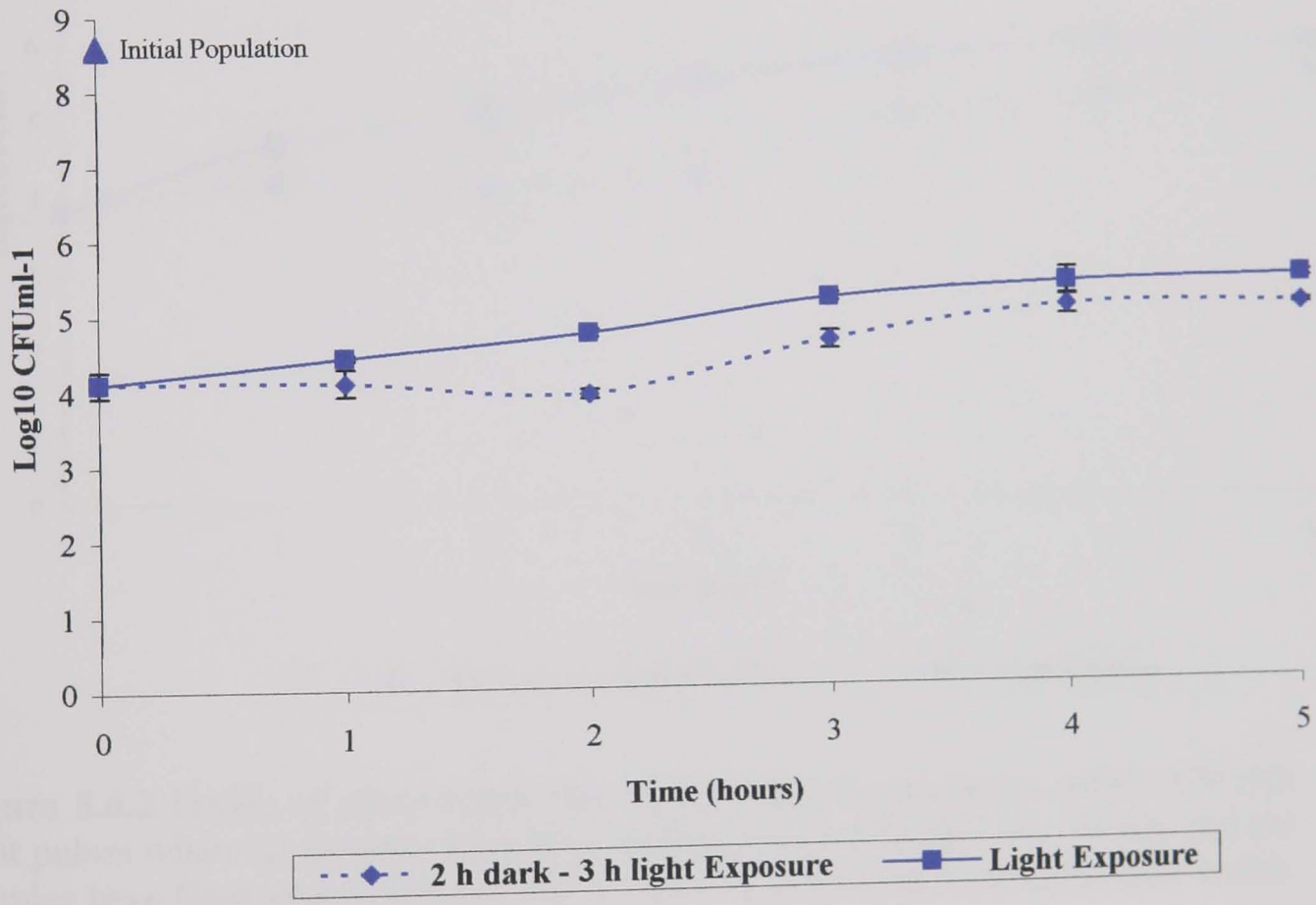


Figure 8.6.1 Levels of photo-repair that occur following treatment with 5 UV-rich light pulses when samples have been exposed to visible light for 5 hours and when samples have been kept in the dark for 2 hours before being incubated under visible light.

It can be observed from Figure 8.6.1 that 4.5 Log_{10} of the bacterial population was damaged after treatment with the UV pulses. Following 5 hours exposure to the

visible light, 1.4 Log_{10} of this damaged population was repaired. The sample that remained in the dark for 2 hours showed no increase in population for this 2 hour period. When the foil was removed from this sample and exposed to the visible light, an immediate increase in bacterial population was witnessed (0.7 Log_{10} during the first 1 hour), with a total 1.2 Log_{10} increase after a further 2 hours of light incubation. The experiment was then repeated, this time increasing the period kept in the dark to 3 hours. The results are shown in Figure 8.6.2.

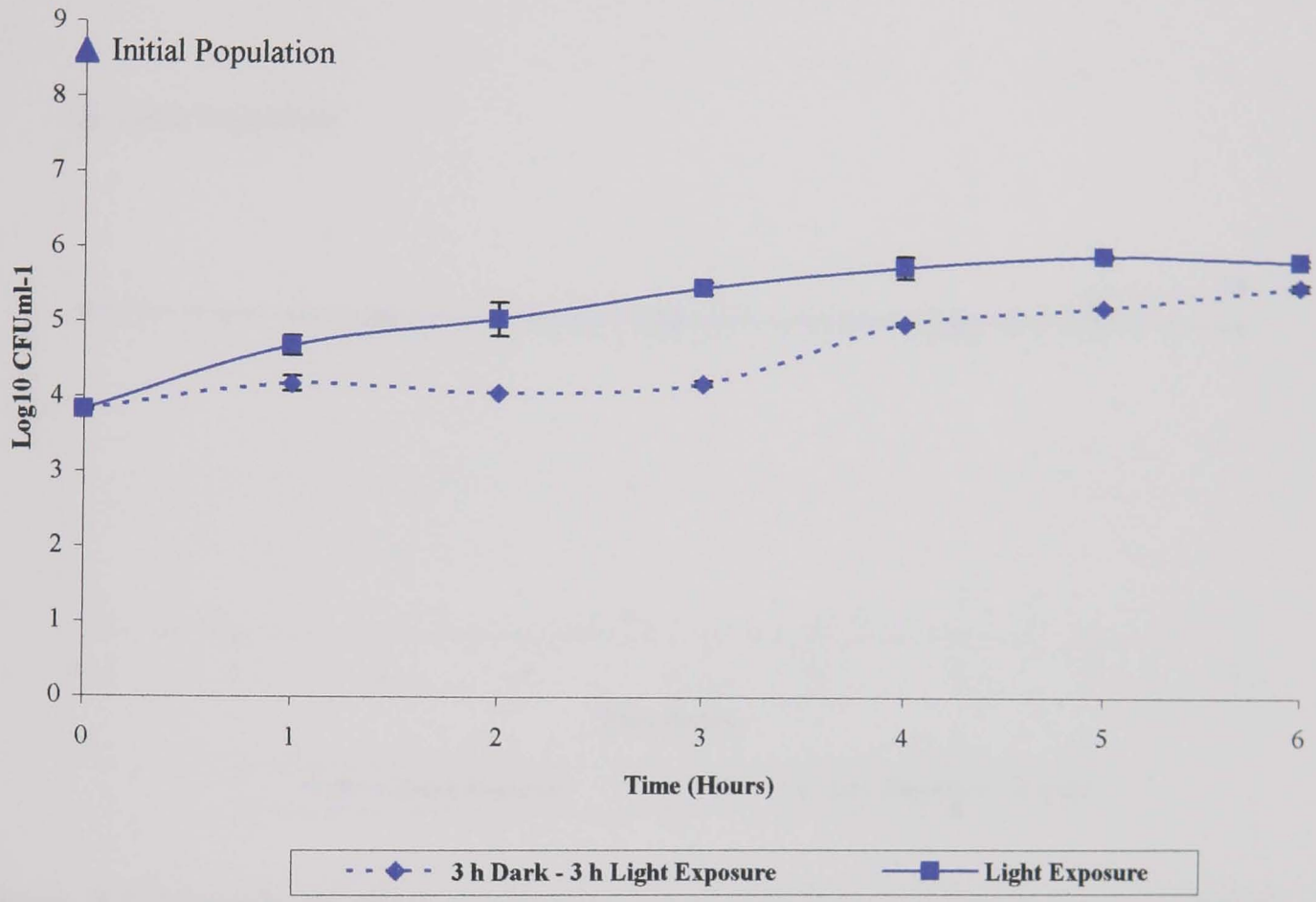


Figure 8.6.2 Levels of photo-repair that occur following treatment with 5 UV-rich light pulses when (a) samples have been exposed to visible light for 6 hours and (b) samples have been kept in the dark for 3 hours before being incubated under visible light.

From Figure 8.6.2 it can be observed that after treatment with the UV pulses, 4.8 Log_{10} of cells were damaged. Following exposure to the visible light, 2.1 Log_{10} of this population was repaired in the sample that was exposed to visible light for 6 hours. The sample maintained in the dark for 3 hours showed no significant increase in population size for this period, but the moment it was placed in the light,

photoreactivation started to occur. After 3 hours light incubation, 1.4 Log_{10} of the damaged population was repaired.

A third experiment was carried out over 8 hours, where one sample was placed in the dark for 6 hours before being exposed to visible light for 2 hours. The second sample was kept in the dark for the full 8 hours, to ensure the sample population does not increase after a prolonged period of time in the dark. Figure 8.6.3 shows the results obtained.

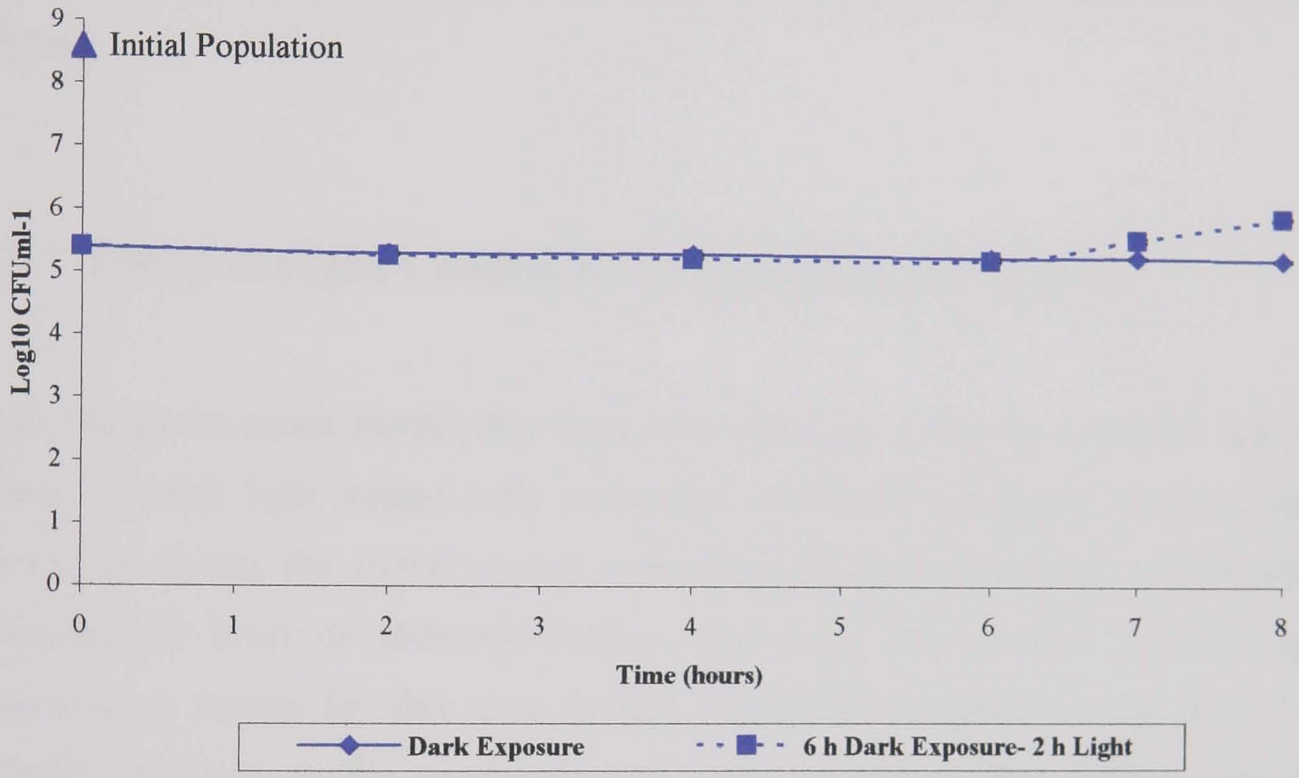


Figure 8.6.3 Levels of photo-repair that occur following treatment with 5 UV-rich light pulses when samples have been kept in the dark for 8 hours and when samples have been kept in the dark for 6 hours before being incubated under visible light for 2 hours.

Figure 8.6.3 shows that pulsed UV-rich light treated samples that have been kept in the dark for 6 hours show no increase in population size. Immediately after being exposed to visible light, the population of damaged cells starts to undergo repair with 0.7 Log_{10} increase in the damaged population occurring after 2 hours light exposure. The samples that remained in the dark for the full 8 hours showed no increase in population size.

These sets of results show that pulsed-UV treated bacteria do not have to be exposed immediately to visible light for photoreactivation to occur. They also show that the samples that have been exposed to UV pulses and then kept in the dark for a period prior to exposure to visible light undergo photoreactivation at a quicker rate. To date there has been no other work published on delaying visible light exposure after pulsed UV treatment. Work has been carried out by Groocock [139] with continuous UV however, and he has suggested that exposure to visible light must occur within 2-3 hours for photoreactivation to occur. These therefore are novel findings which are of extreme importance when considering the application of the pulsed UV system in water disinfection where treated water may be subjected to long detention times in the dark.

8.7 Effect of Light Conditions During Bacterial Growth

From the photo-repair results that have been obtained so far, it is evident that the pulsed UV-rich light treated cells are sensitive to light conditions. Ambient light conditions during the initial growth of a microorganism therefore may possibly influence the level of photoreactivation that occurs after pulsed UV-rich light treatment. A reason for this may be that enteric microorganism such as *E. coli* normally replicate in the gastrointestinal tracts of humans and animals and are therefore not exposed to light. If they are exposed to light when they are growing they may therefore experience a different rate of photoreactivation because they are more sensitive to the light. An experiment was undertaken where two 100 ml Nutrient Broths were inoculated with *E. coli*. One broth was incubated at 37 °C on a rotary shaker (166 rpm) in the dark. The other was incubated at 37 °C on a rotary shaker in the light where the intensity was 10, 780 Lux. The incubation period for both broths was 18 hours. After incubation 20 ml samples from both broths were treated with 8 and 10 pulses (a higher pulse number was used, as the strain seemed slightly more resistant in some control experiments which had been carried out). After the pulsed UV-rich light treatment, samples were prepared and put in the light cabinet for 4 hours, as described in section 8.2. The data obtained is presented in

Table 8.7.1. The results show that when each sample was treated with 8 and 10 UV-rich light pulses, relatively large levels of inactivation occurred (between 5.6 and 6.8 Log_{10} reductions were achieved). It is also shown however, that when these damaged populations were exposed to visible light for a period of 4 hours, no photoreactivation took place.

Sample	**Initial Population ($CFUml^{-1}$)**	**Population After UV-rich Light Pulses ($CFUml^{-1}$)**	**Level of Photo-repair (Log_{10} $CFUml^{-1}$)**
Light Grown (8 pulses)	2.9×10^8	1.4×10^2	NA*
Dark Grown (8 pulses)	3.4×10^8	8.2×10^2	NA*
Light Grown (10 pulses)	3.7×10^8	5.5×10^1	NA*
Dark Grown (10 pulses)	3.6×10^8	9.2×10^1	NA*

* No colonies were present on any of the photo-repair plates.

Table 8.7.1 Populations of *E.coli* grown in dark and light conditions before and after pulsed UV-rich light treatment, and the levels of photoreactivation that occurred after 4 hours incubation under visible light.

In a second experiment, a lower pulse number was used such that high levels of inactivation would not occur. The same experimental procedure was followed except 5 UV-rich light pulses were used. The results obtained for the samples that were initially grown in the light (10 780 Lux) are shown in Figure 8.7.1 and those that were grown in the dark are shown in Figure 8.7.2.

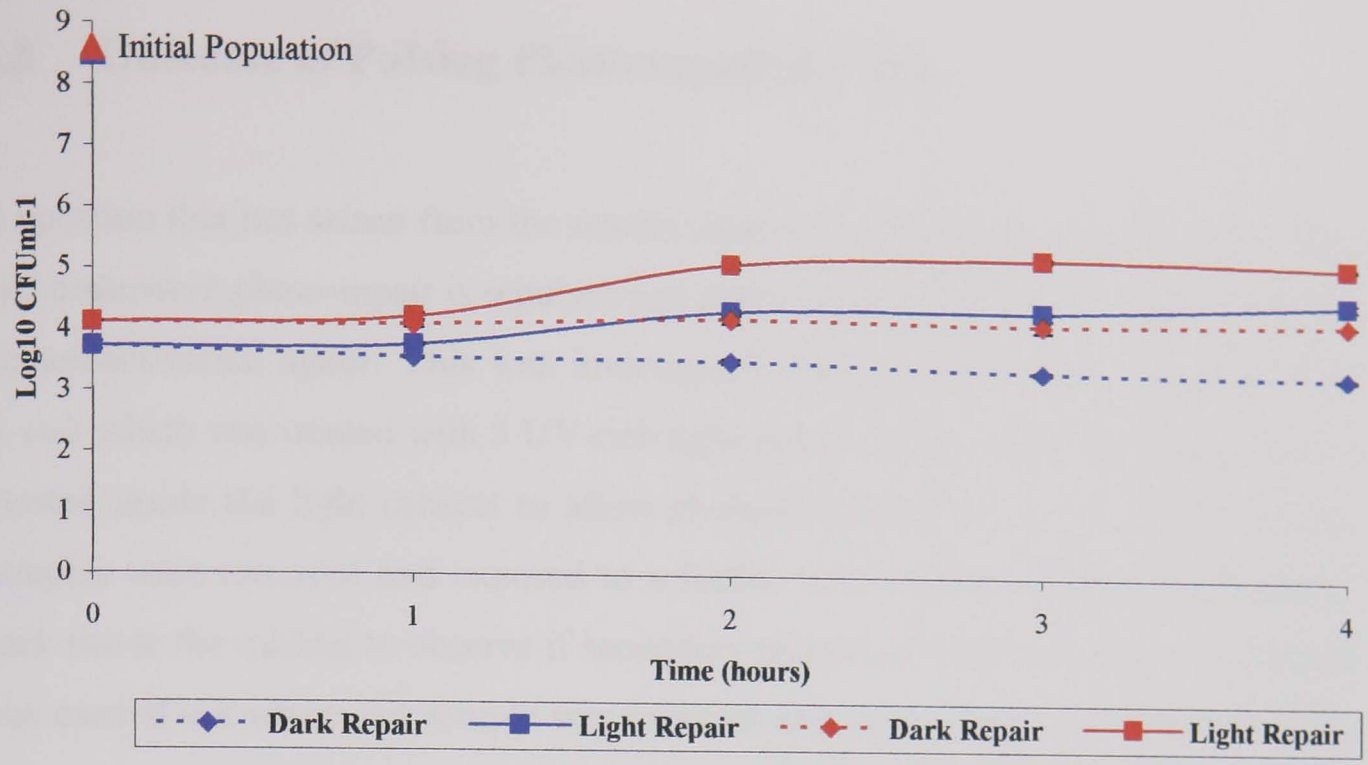


Figure 8.7.1 Amount of light and dark repair obtained from treating stationary phase *E. coli*, initially grown under light conditions and under dark conditions, with 5 UV-rich light pulses.

From the results of Figure 8.7.1, it can be seen that the *E. coli* culture predisposed to high intensity light during bacterial growth underwent a 4.8 Log_{10} reduction after treatment with 5 UV-rich light pulses. After treatment, samples that remained in the dark did not undergo photo-repair. Samples that were placed under visible light however, were shown to undergo photo-repair with 0.9 Log_{10} of the UV-damaged population undergoing photoreactivation. When the results are observed for the *E. coli* culture which was originally grown in the dark, it can be seen that 4.5 Log_{10} of microorganism was damaged after treatment with the UV-rich light pulses. When placed inside the light incubator the samples that were wrapped in foil, preventing visible light exposure, did not display any photo-repair. Samples that were exposed to the visible light however, underwent photo-repair with 1.2 Log_{10} of the damaged population being repaired. These findings therefore do not show any major differences in either Log_{10} reductions by the UV pulses, or with the levels of photoreactivation that occurs. It can be concluded therefore that light conditions during bacterial growth do not have any influence on a microorganism's ability to undergo photoreactivation following treatment.

8.8 Outcome of Pulsing Photo-repaired Cells

A question that has arisen from the results obtained in this chapter is, if a population that underwent photo-repair is repulsed and placed under visible light, will it undergo photoreactivation again? This was investigated using a 10^8 $CFUml^{-1}$ population of *E. coli* which was treated with 5 UV-rich light pulses. After treatment, samples were located inside the light cabinet to allow photoreactivation to occur. After 4 hours, samples were removed and exposed to a further 2 UV pulses, before being placed back inside the cabinet to observe if secondary photoreactivation occurred. A control was carried out where the sample was exposed only to the light for 7 hours and did not receive a second dose of light pulses. The results obtained are shown in Figure 8.8.1.

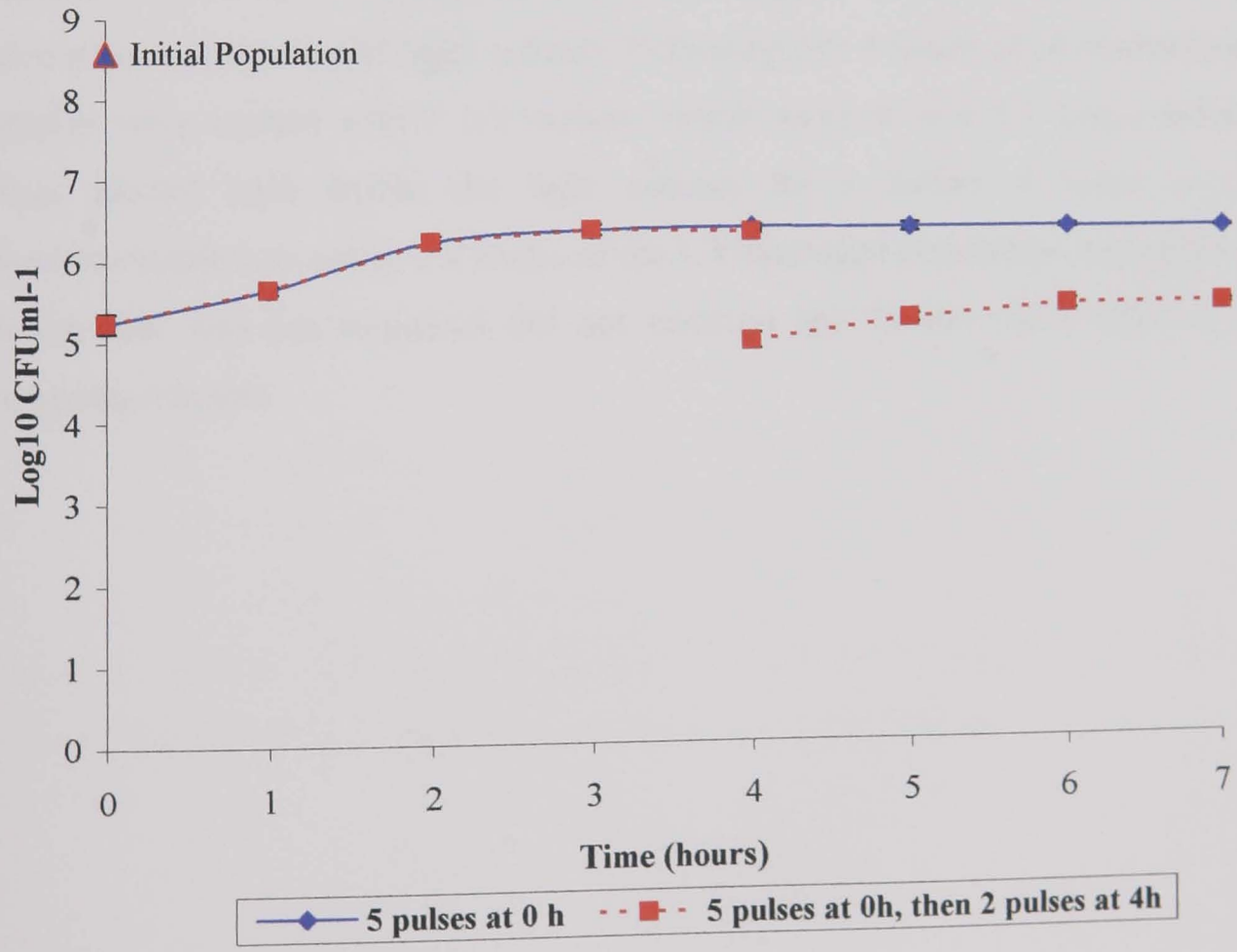


Figure 8.8.1 Photo-repair levels of *E. coli* occurring after 4 hours visible light exposure and levels that occur when this repaired population in re-pulsed with 2 pulses and placed back under photoreactivating light.

The results of the experiment investigating secondary photoreactivation (Figure 8.8.1) show that the initial level of cells damaged after 5 UV pulses was 3.4 Log_{10} followed by 1.17 Log_{10} of repair after 4 hours in the light cabinet. Following the 4 hours visible light exposure, samples were treated with 2 UV pulses and this resulted in damage to 1.36 Log_{10} of bacterial cells. After allowing this population 3 hours in the light cabinet 0.44 Log_{10} of repair occurred. The activity of the sample that was not subjected to a second set of UV pulses, after 4 hours, remained constant over a further 3 hours.

The same experimental procedure was carried out, this time increasing the second set of pulses to 3. If the photoreactivated cells received a slightly larger UV dose they may not be able to go photoreactivation again. The results are shown in Figure 8.8.2. Here it can be observed that the initial population was reduced by 3.12 Log_{10} after treatment with 5 UV-rich light pulses. 0.85 Log_{10} of photoreactivation then took place after 4 hours in the light cabinet. Following the 4 hours of photoreactivation, samples were treated with 3 UV pulses, which resulted in a 4.7 Log_{10} reduction. When placed back inside the light cabinet for a further 4 hours to allow photoreactivation to occur, 2.8 Log_{10} of the UV-damaged population took place. The sample that was not re-pulsed did not undergo any further photo-repair for the remaining 4 hours.

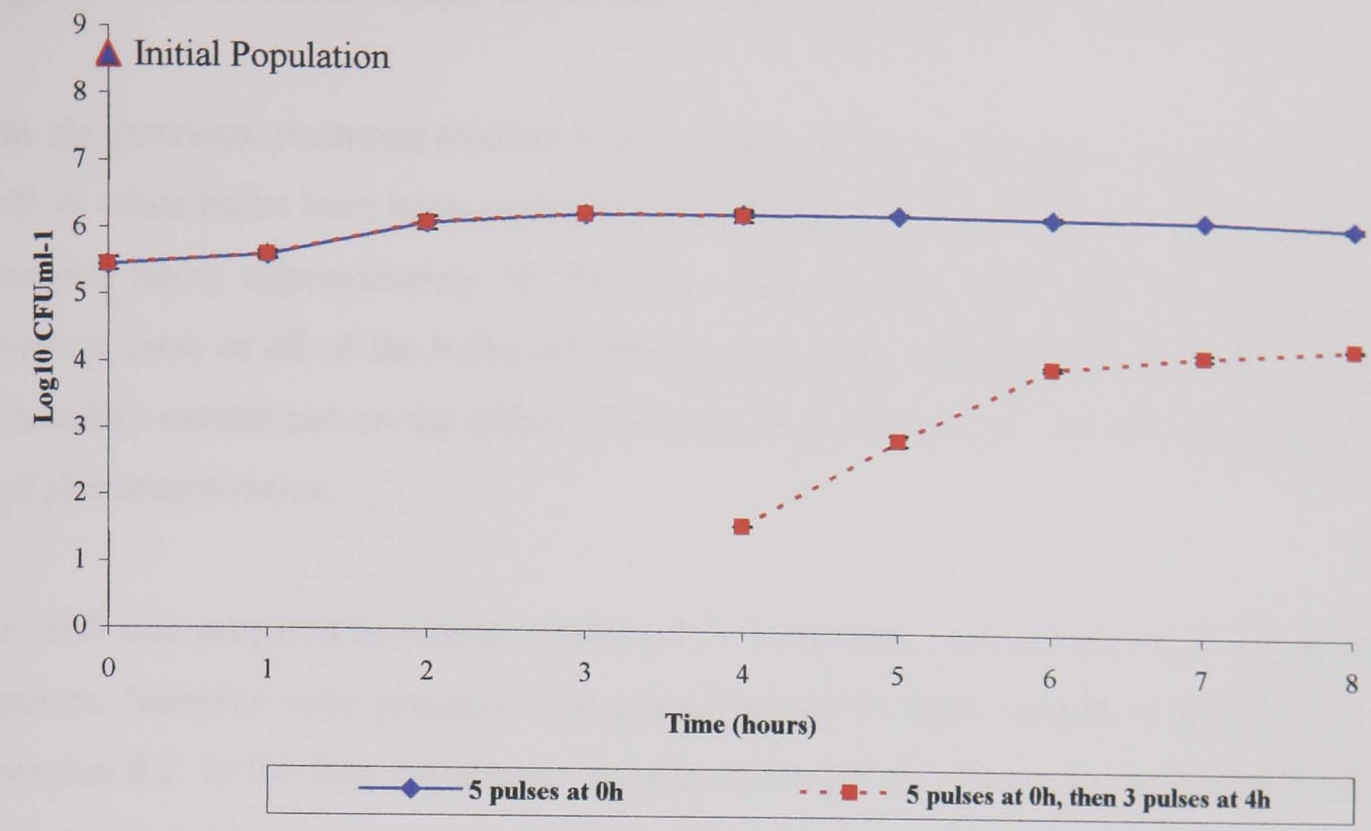


Figure 8.8.2 Photo-repair levels of *E. coli* occurring after 4 hours visible light exposure and levels that occur when this repaired population in re-pulsed with 3 pulses and placed back under photoreactivating light.

From this study it can be concluded that when a bacterial population is treated with UV pulses, a portion of the damaged population can undergo photoreactivation. After treating with a second set of pulses, a large reduction in bacterial population is observed. This time the portion of damaged cells will include photoreactivated cells and cells that were damaged after the first set of pulses. Since the cells did not undergo photoreactivation following the first set of pulses it can be safe to assume they did not undergo photoreactivation the second time around. These results therefore conclude that cells that have undergone photoreactivation can undergo photoreactivation for a second time if they are exposed to UV pulses again.

8.9 Photoreactivation from Different Intensities of Visible Light

In the previous photoreactivation experiments involving the light cabinet, all 12 x 40 W white bulbs have been switched on resulting in the light intensity at the sample surface being approximately 16 700 Lux. There is the option with the cabinet to switch some or all of the bulbs off, resulting in lower light intensities. A study was therefore carried out on the effect of varying the visible light intensity on the levels of photoreactivation.

E. coli was prepared as normal (section 3.7.1) and then treated with 3 UV-rich light pulses. Samples were prepared and placed inside the light cabinet as described in section 8.2. In the first experiment samples were left for 5 hours in the light cabinet where the light intensity was 2 920 Lux. Following the same experimental procedure other tests were carried out but with the intensity of light in the light cabinet at 6 420 Lux and 16 720 Lux. The results are shown in Figure 8.9.1.

The results obtained for the experiment carried out at 2 920 Lux show that the population before the pulsed light treatment was 1.2×10^8 $CFUml^{-1}$ and after treatment it was reduced to 3.7×10^3 $CFUml^{-1}$, meaning that 4.5 Log_{10} of the bacterial population was damaged by the UV pulses. When this damaged population was exposed to 2 920 Lux of visible light, for 5 hours, 1.1 Log_{10} of the damaged population underwent photoreactivation. In the experiment carried out at 6 420 Lux, 4.6 Log_{10} of the bacterial population was damaged and when exposed to visible light, 1.4 Log_{10} of the population was repaired. When the intensity of light in the light cabinet was 16 700 Lux there was a decrease in bacterial population of 4.6 Log_{10} after 3 light pulses. After 5 hours under the photoreactivating light 2.1 Log_{10} of the damaged population was repaired.

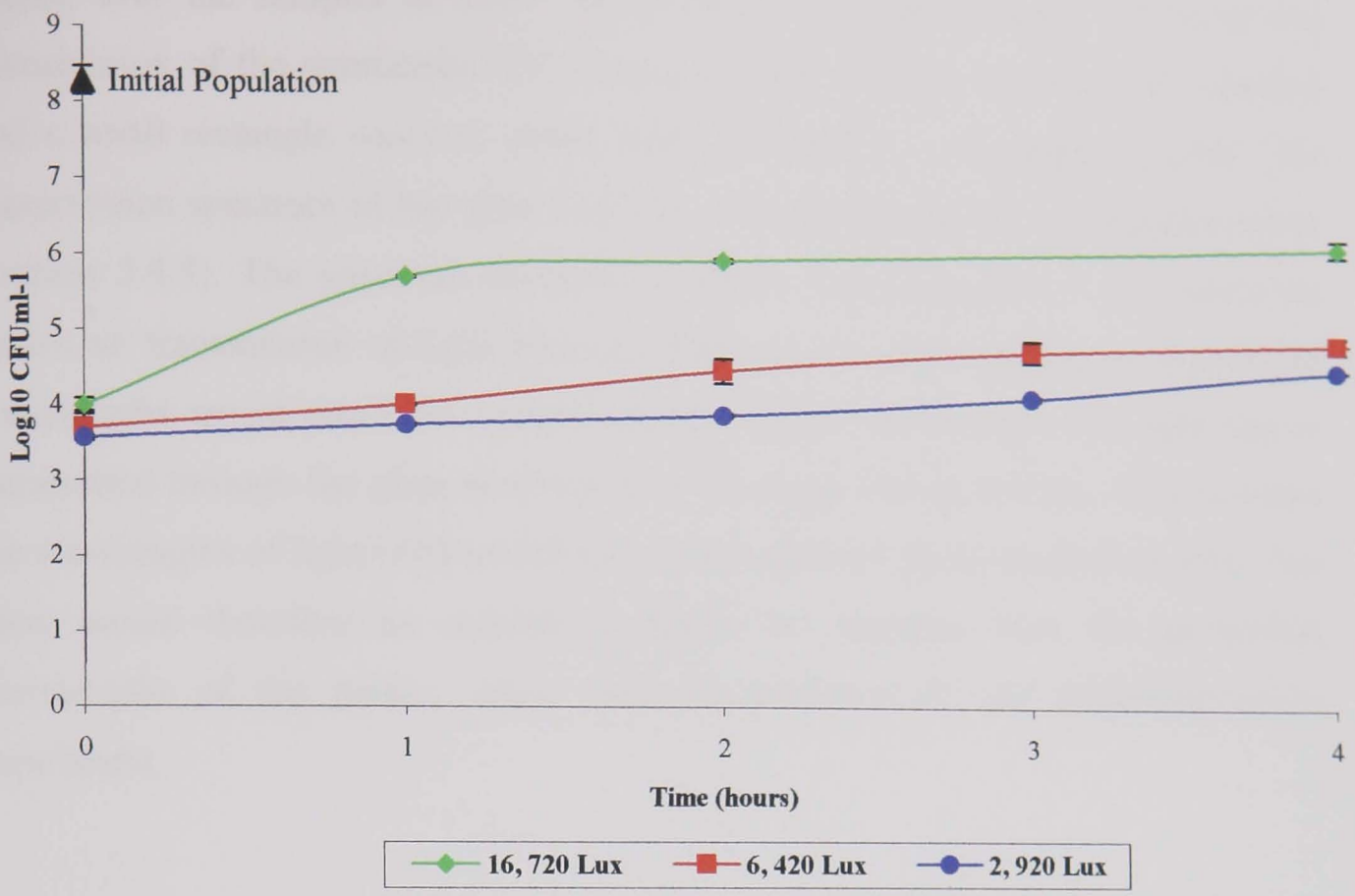


Figure 8.9.4 A comparison of levels of *E. coli* photoreactivation achieved, after exposure to 16, 700 Lux, 6, 420 Lux and 2, 920 Lux of visible light, following pulsed light treatment of *E. coli*.

There is not really a large difference in the level of repair that occurs with the samples that were incubated under 6, 420 and 2, 920 Lux. However the repair that occurs at the higher intensity of 16, 700 Lux is much greater indicating that as the intensity of photoreactivating light increases, the level of repair to the damaged cells increases.

8.10 Effect of Using Pulsed Visible Light for Photoreactivation

In all of the previous experiments, continuous white light has been used to achieve photoreactivation. This section observes whether using pulsed visible light can cause photoreactivation. To carry this out, the flash lamp used for the inactivation experiments was used to provide the flashes of white light. Firstly, a filter had to be

placed over the samples to allow pulses of visible light through, but block the transmission of the germicidal UV content of light. A piece of glass was obtained and a small rectangle was cut, which could fit inside a 1 cm quartz cuvette. The transmission spectrum of the glass was then measured using the spectrophotometer (section 3.4.5). The spectrum produced is shown in Figure 8.10.1. The spectrum shows no transmission of light between 200 and 310 nm meaning no germicidal wavelengths would reach the sample causing further inactivation. The light that is transmitted through the glass however is in the range 310 to 500 nm. This includes the wavelengths of light (310 to 480 nm) responsible for photoreactivation [50]. The glass would therefore be suitable to shield the samples from the germicidal wavelengths of the pulses, when they are produced for the photoreactivation experiment.

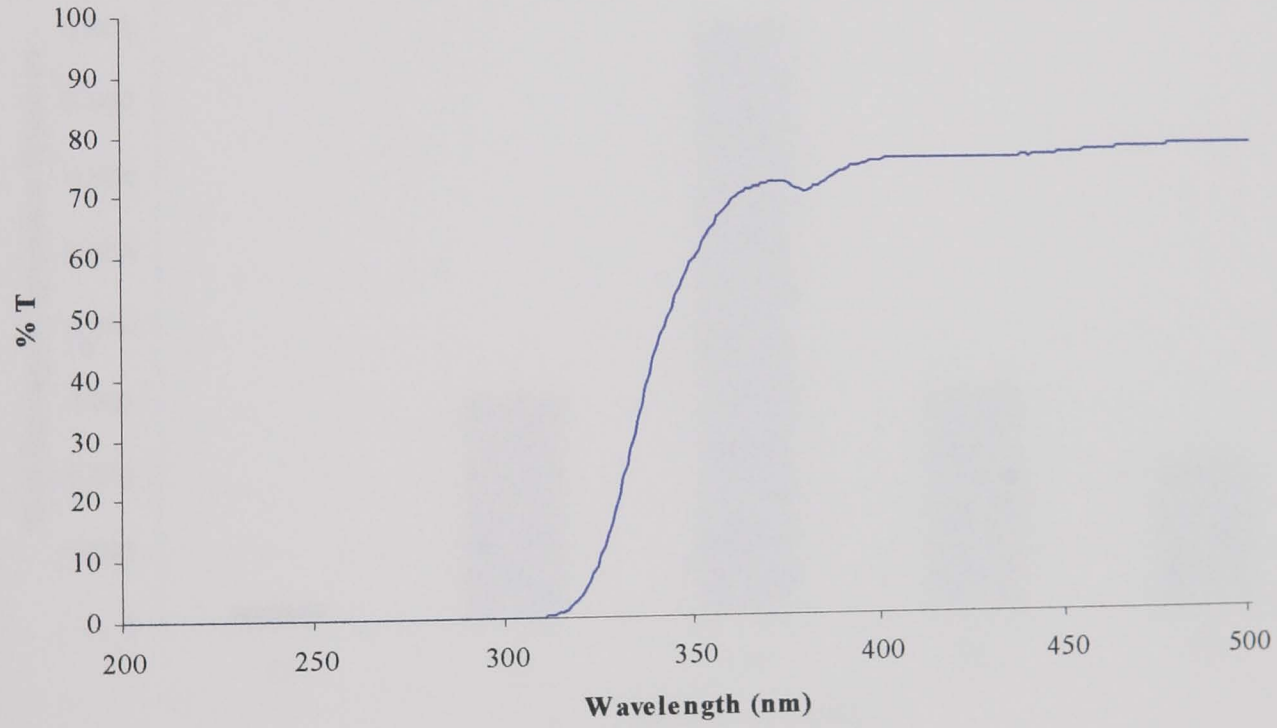


Figure 8.10.1 Transmission spectrum for the glass used to block out the ultraviolet wavelengths.

10^8 CFUml^{-1} of *E. coli* was prepared and treated with 5 UV-rich light pulses as described in section 3.7. The samples were prepared for photoreactivation as described previously (section 8.2). Samples were placed inside the pulsed UV-rich light system with glass placed over them. Each sample was then exposed to 25, 75,

100, 300 and 500 light pulses (visible light only i.e. 310 to 500 nm). The samples were then enumerated and the results obtained are shown in Figure 8.10.2. It is evident from the graph that the levels of photo-repair that have occurred are very poor. There is also no pattern in the results, as when the number of white light pulses was increased the amount of repair that occurred did not increase. It appears from these results that pulses of visible light do not cause UV damaged cells to photoreactivate. This is an important finding for commercial applications, as it would be no good if, when inactivation is taking place there is the potential that photoreactivation could occur at the same time, from the photoreactivating wavelengths that are emitted from the source. This experiment has indicated that the chances of photoreactivation happening from the pulsed light source are very low.

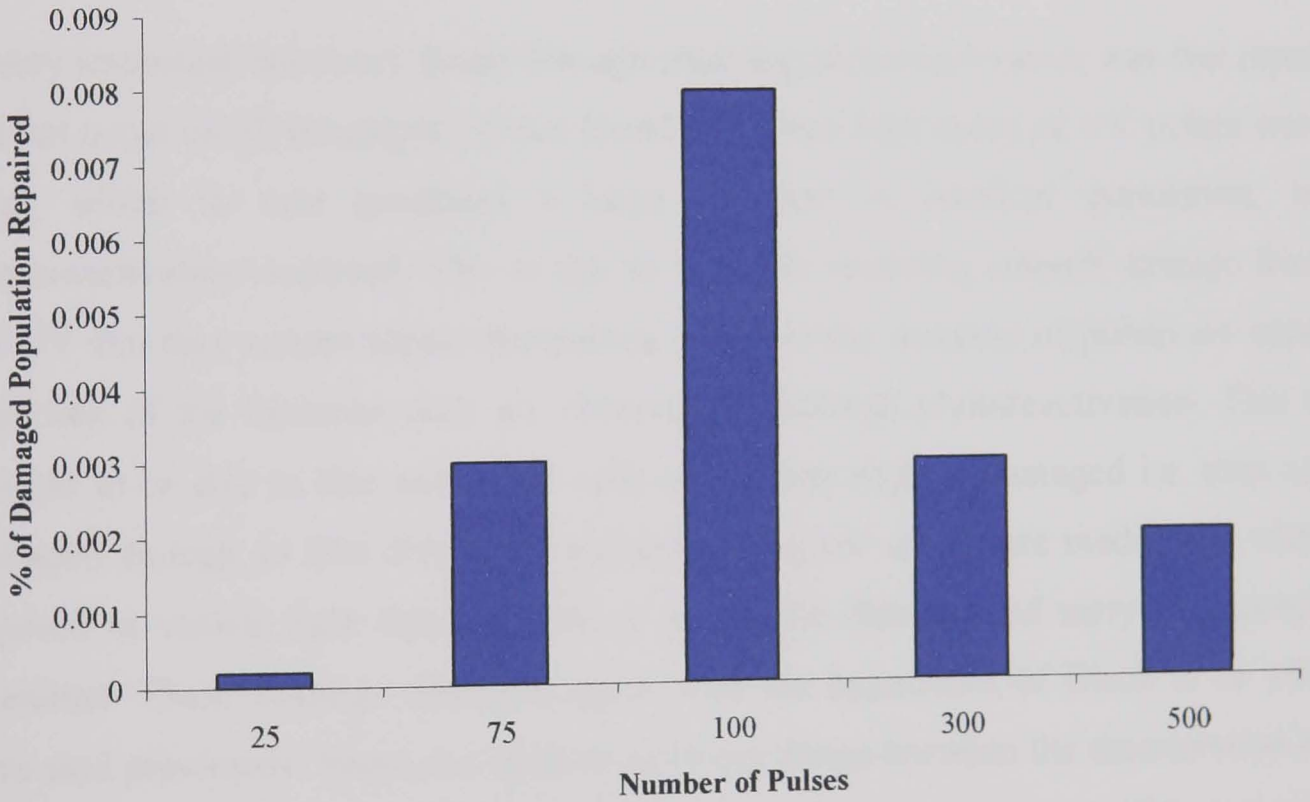


Figure 8.10.2 Percentage of damaged *E. coli* population repaired following treatment with 25, 75, 100, 300 and 500 pulses of visible light in the region 310 to 500 nm.

8.11 Discussion

Many reports have been published on photoreactivation of UV treated bacteria [20, 39, 41, 47, 52, 126]. In these instances, the light was emitted from a continuous UV source and to date there is no published work on photoreactivation following pulsed UV treatment. The work carried out and presented in this chapter has found that photoreactivation does arise when a pulsed UV source is used to treat bacteria. In all of the experiments that were carried out, no repair was found to occur with the dark exposed samples showing that, under the experimental conditions used for this study, the dark repair mechanism of *E. coli* is very ineffective after exposure to pulsed UV-rich light.

A very important discovery found through studying photoreactivation was that repair did not occur on all occasions. It was found that when high doses of UV pulses were used, which in turn produced a large decrease in bacterial population, no photoreactivation occurred. This is due to the cells receiving enough damage from the UV that they cannot repair themselves. When lower numbers of pulses are used, a portion of the bacterial cells are observed to undergo photoreactivation. This is thought to be due to this portion of cells only being slightly damaged i.e. they are damaged enough so that they can't replicate and grow on culture media, but when exposed to visible light they are able to repair the damage and carry out normal functions. These findings therefore agree with the hypothesis of Block *et al* who have said previously "there is a balance in living things between the deterioration of cellular components after exposure to UV and their biochemical repair. If the amount of damage exceeds the cell's capacity to repair this damage, the cell will die" [35]. This area of work has therefore highlighted an extremely important point which must be taken into consideration when using a pulsed UV system for disinfection purposes: A bacterial population may not necessary be inactivated by UV, because the population may have the ability to reactivate when exposed to visible light and carry on with normal metabolic functions.

The maximum level of repair obtained throughout all studies was normally observed following 3 to 5 hours of visible light incubation and the population of damaged bacterial cells was never completely repaired. These results agree with studies, using continuous UV sources, where findings have shown that the maximum level of repair occurs after 2-3 hours [52] and that exposed cells, such as *E. coli*, undergo photoreactivation to different extents, but never completely [35]. The findings from this study may therefore also indicate that other photoproducts were formed in the UV-treated cells, which could not be repaired by photoreactivation. This would be consistent with findings using continuous polychromatic light sources, which have been suggested to cause damage to other molecules within the bacterial cell [47].

Another important finding in this study was that pulsed UV-treated populations do not have to be exposed to photoreactivating light immediately for photoreactivation to occur. This finding is in disagreement with Groocock [139] who have proposed that exposure to light between 300 and 500 nm must occur within two to three hours, for photoreactivation to be encouraged. This study however found that photoreactivation still occurs when samples are postponed from exposure to visible light for up to 6 hours.

Oguma *et al* (2002) however have suggested that photoreactivation is more dependent on the time of exposure to photoreactivating light rather than the irradiance of the light [50]. This study found that the level of photoreactivation depends on both of these factors. It was found in this study that as the intensity of the photoreactivating light increased the level of photoreactivation increased. It was also found however that as the period of time samples was placed under photoreactivating light increased, then so did the level of repair that occurred. It was noticed however that the majority of repair occurred within the first 1-2 hours and no further repair was found to occur after 5 hours exposure to visible light.

Overall, this study has shown that *E. coli* can undergo photoreactivation following exposure from a polychromatic pulsed light source. This is not in agreement with the work published by Zimmer *et al* who found very limited or no photoreactivation to occur following exposure from a polychromatic light source (medium pressure) [52]. This study does however agree with the suggestion that broader wavelengths emitted by broad spectrum lamps not only damage DNA but also cause damage to other molecules, making it more difficult for cells to repair their DNA [47]. A hypothesis is that there is a synergistic effect between the various wavelengths emitted by medium pressure lamps that cause irreparable damage to the DNA; another explanation is that the repair enzymes themselves are damaged [47]. This does however explain why complete recovery of the inactivated population was never observed to occur. This study also found that high intensities of visible light are required for photoreactivation to occur from pulsed UV treated cells; therefore this system may hold an advantage over traditional UV disinfection methods where photoreactivation can occur after exposure to sunlight. It has also been suggested that on some occasions, reactivation is not seen at all above a certain UV dose [126]. This study is in agreement with this implication and has found that the elimination of photoreactivation relies upon complete inactivation of the population. Even after exposure to UV light with significant but not complete inactivation, recovery can still take place following exposure to visible light. In addition to the UV dose/pulse number, this study has shown that the intensity of photoreactivating light also correlates with the extent of reactivation. Other conditions known to influence reactivation include temperature, pH, ionic strength and nutrient levels [126].

Although not carried out in this investigation, it is generally accepted that viruses do not have the ability to repair themselves following UV disinfection, unless they are within a host cell [126] and although *Cryptosporidium* oocysts can undergo photo repair, they do not regain their infectivity [Oguma *et al*, 2001]. In addition, although many pathogenic bacteria have shown they are able to reactivate, many have shown no tendency to undergo photoreactivation [126] and others have shown certain strains can reactivate whilst others do not. For *E. coli* photoreactivation has been observed in EHEC O26 but not in EHEC O157:H7 [91]. This means that out of the

many pathogenic microorganisms destroyed by pulsed UV light treatment, only a select few may be able to undergo photoreactivation anyway.

For practical applications, it is well known that photoreactivation enables UV-inactivated microorganisms to recover and may reduce the efficacy of UV inactivation therefore disadvantaging UV disinfection methods [110]. As a consequence, the problem of photoreactivation is especially important in UV-treated wastewater after its discharge to watersheds, because UV-inactivated microorganisms would normally be exposed to sunlight, including near-UV light [110]. This study however suggests that if pulsed light systems, such as the one used in this study, were used for food/water disinfection purposes, photoreactivation would only be a problem if the product(s) being treated were kept/stored under very high visible light intensities. In drinking water applications, this system would also be very beneficial because, treated water can be subjected to long detention times prior to reaching the consumer. During this time, UV irradiated microorganisms may have the opportunity to carry out dark repair and potentially re-grow within the system [52]. This study has found that no dark repair occurs from using a pulsed UV-rich light source. Exposure to light however cannot be completely ruled out either during treatment or after the water reaches the consumers. At these times, photoreactivation may have an increased significance if cells are exposed to high visible light intensities. To conclude therefore, at relatively high doses/pulse numbers, the amount of DNA damage is possibly so substantial that the potential for photoreactivation is likely to be small for many microorganisms [126]. The ultimate goal therefore, for pulsed UV-rich light inactivation of pathogens in drinking water, is to damage DNA beyond repair so that photoreactivation cannot occur.

Chapter 9

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK

9.1 General

There are many published papers about the effectiveness of ultraviolet light sterilisation and this is now an accepted method over former chemical and heat processing [10]. New delivery techniques of ultraviolet light have led to the development of pulsed UV light systems. This thesis has been concerned with investigating the effects of UV-rich light pulses on pathogenic microorganisms. The investigation has looked into the factors effecting inactivation and has consequently came up with a suitable treatment protocol and has successfully inactivated a wide range of problematic microorganisms and has shown they all have varying sensitivities to the treatment process. In addition, the possible limitations of pulsed UV light treatment have been investigated and solutions as to how these can be overcome or kept to a minimum suggested. Unfortunately, direct comparisons could not be made with other work in the field as all published work has been concerned with continuous sources and their results are expressed in terms of dose rather than pulse number. However, overall levels of Log_{10} reduction could still be compared to determine the effectiveness of the pulsed UV-rich light system for disinfection purposes.

9.1.1 The Development of a Suitable Treatment Procedure

Fundamental to this investigation was the development of a protocol that could be used throughout the course of this study. The primary requirements were to find a suitable sample volume that was not too large therefore leading to unwanted labour

in preparation and to find an appropriate treatment cell which would allow the sample to receive the majority of the UV pulses without causing attenuation of the light. The secondary requirements were to establish the electrical parameters, which would result in the highest inactivation levels. This study found that Quarter strength Ringer solution was a suitable suspension medium for achieving optimum inactivation results. Solutions that are coloured, such as growth medium, which was used for viral suspension was found to cause attenuation of the UV-rich light pulses, therefore a decrease in inactivation was witnessed. The test cell used in this study was a Petri dish, because it could hold relatively small sample volumes at low depths. This was because it was found during this investigation that the depth of the sample is an important parameter because as the sample depth increases, the amount of inactivation that takes place decreases. It was established however that if the number of pulses the sample receives is increased, a similar level of inactivation takes place with large sample depths as it does with small sample depths therefore; matching the UV spectral output to the sterilisation objectives is an extremely important consideration. The importance of the pulse intensity applied to a sample was demonstrated in this investigation. When low energy pulses are used i.e. a low charging voltage is used, less inactivation occurs. This is because if the pulse energy is low, then so to is the optical output signifying that there are lower emissions of germicidal wavelengths entering the sample. It was however, demonstrated that if the pulse number of low energy pulses is increased so that the overall energy input is the same as that of high energy pulses, then resulting inactivation levels are similar. It was also shown that the higher the pulse frequency, the lower the amount of inactivation that occurs. The pulse frequency recommended for using with this system is 1 Hz because lower than this will be too time consuming. Finally, it was also shown that from the wavelengths emitted from the flash lamp; 250 nm was found to be the most germicidal. This study has therefore demonstrated that optimising the operating parameters of the pulsed lamp and power supply are also essential for maximum efficiency.

9.1.2 Inactivation of Pathogenic Microorganisms

Treatment of those problematic microorganisms that are commonly associated with waterborne disease was undertaken to establish the sensitivities of such microorganisms to pulsed light treatment. In the studies undertaken with different bacterial species it was found that Gram-positive bacteria are more resistant to the treatment than Gram-negative bacteria. Of the variety of bacteria tested, *Bacillus megaterium* was shown to be the most resistant and *Campylobacter jejuni* was found to be the most sensitive. It was also found that successful inactivation is not necessarily growth phase dependent, because most of the bacteria which were tested showed similar sensitivities to the treatment when in exponential and stationary phase of growth. This may therefore imply that fully established pathogens such as those found in the environment should be as susceptible to the treatment. It was also shown that different species and strains of a bacterium can show quite significant differences in their susceptibility to pulsed UV-rich light treatment, therefore, for example, just because a certain strain of *E. coli* is inactivated quite readily by pulsed UV-rich light treatment it does not necessary mean that other strains will show similar levels of inactivation.

It was also established in this investigation that as well as being extremely effective against a range of bacteria, pulsed UV-rich light treatment is extremely successful in inactivating *Cryptosporidium* oocysts and a variety of viruses. It has been suggested that in general bacteria are less resistant to UV treatment than viruses, while protozoan oocysts and cysts are regarded as the most resistant microbes [41]. This investigation has indicated however that when pulsed UV-rich light is used, protozoans such as *Cryptosporidium parvum* are as equally sensitive as bacteria such as *E. coli*, with complete inactivation of both microorganisms after just a few light pulses (500 J). In addition to these robust oocysts being very sensitive, it was found that pulsed UV-rich light could inactivate *C. parvum* oocysts in shorter time scales than conventional continuous UV systems with inactivation occurring in a matter of seconds as opposed to several hours.

This study also showed that RNA viruses such as poliovirus are just as sensitive to pulsed UV-rich light treatment as Gram negative-bacteria such as *E. coli* and C*ryptosporidium* oocysts. It did however also show that DNA viruses such as herpes and adenovirus are more resistant to the treatment. This was expected for the adenovirus but not for the herpes virus as Cameron *et al* have reported that adenovirus is more resistant to continuous UV treatment than other dsDNA viruses, such as herpes simplex type 1 [137]. The levels of inactivation of enteric viruses achieved by pulsed light treatment are adequate and meet the U.S. Environmental Protection Agency (EPA) recommendations which are: a UV light dose of 21 mW/cm^2 for a 2 Log_{10} reduction and a dose of 36 mW/cm^2 for a 3 Log_{10} reduction in enteric viruses [74]. Although it is not possible to determine the sensitivity of each and every pathogen to UV, the common mechanism of the action of UV on nucleic acids provides a high level of confidence that similar doses would be required for most pathogens likely to be encountered in drinking water [41].

It is already known that ultraviolet light is an established and increasingly popular alternative to chemicals for the disinfection of drinking water, wastewater and industrial waters of various qualities [61]. This study has however established that pulsed ultraviolet light treatment is extremely successful in the inactivation of a variety of water-borne pathogens and through the small-scale study involving private water samples, it has been demonstrated that it is an effective means of disinfecting drinking water.

9.1.3 Protection of Microorganisms From Pulsed Light

Sterilisation levels achieved with ultraviolet radiation may be reduced as a consequence of the properties of the microorganisms or of the medium the microorganisms are suspended in. As a result a tailing effect can be observed on some inactivation curves. Some of these factors were investigated and possible solutions were suggested. One of the most important factors is the population size of a microorganism. Tailing is observed with large bacterial populations but none is observed with low populations. The main reason is that shielding is provided by

other cells therefore protecting some of the microbial population. Although tailing has been shown to be a problem when sterilising liquids containing high populations of microorganisms, this should not be a problem that occurs outside the laboratory. When using the system as a sterilisation technology the products it would be treating would not expected to be contaminated with such high levels of microorganisms. therefore successful inactivation should take place, without tailing. The effect that is observed with large population sizes is probably quite similar as to what would be witnessed when treating samples with suspended solids, for example, in wastewater treatment where a large number of microbial cells could be protected by suspended particles. In addition to large population sizes and suspended particles providing protection from the light pulses it was also found that larger sized bacteria also do the same, with a greater amount of tailing observed, even at lower populations. It was found however that the introduction of sample agitation between the delivery of the pulses eliminated tailing with large bacterial population sizes. It is therefore advisable to have a treatment chamber that keeps the sample moving, so that maximum inactivation results can be achieved.

From this study it was concluded that the inactivation curve has a tailing phase due to two important factors: (i) because of experimental components such as suspended solids and microbial cell and population size that may block UV pulses, or (ii) simply due to the resistance of some of the microorganisms to the light pulses. It must be noted however that although laboratory devices, such as this one, have been developed that may destroy 100 % of waterborne bacteria, complete sterility is not necessary for production of potable water as long as it conforms to the Public Health Service's drinking water standards [35].

9.1.4 Photoreactivation

A major limitation of using ultraviolet illumination as a sterilisation technology is that the damage done to the microbial cells is reversible under certain conditions, such as in the presence of visible light (photoreactivation) or in the absence of light (dark repair). This study has found that photoreactivation can also occur with pulsed

UV light under certain intensities of visible light. An important observation made in this study was that when a population was completely inactivated or inactivated to a low number, before being exposed to visible light, no repair occurs. This signifies that the ability of bacterial cells to regain viability through photo-repair is largely dependent on the extent of the UV damage. Therefore, if a microorganism receives enough damage from pulsed UV light treatment, photo-repair will not occur. The study also showed that pulsed UV light treated populations can never be repaired to their starting population, indicating that the pulsed UV light treatment may either damage the bacterial DNA beyond repair or else damage/destroy other molecules in the cell, which cannot be repaired by exposure to photoreactivating light. This destruction of other molecules is thought to be due to the unique properties of the polychromatic wavelengths. It was also found that postponing exposure to visible light treatment does not stop photoreactivation from occurring. Another important factor which was established was that high intensities of visible light are required for photoreactivation to occur following pulsed UV light treatment. Therefore in situations outside the laboratory, photoreactivation may only occur following pulsed UV light treatment, if treated products are exposed to high light intensities.

This investigation has also found that no dark repair occurred which may be a major advantage for using this system in drinking water treatment, as water may undergo long detention periods in the dark, which would provide suitable conditions for dark repair to take place.

9.1.5 Applications and Benefits of a Pulsed Light System

Infectious diseases caused by pathogenic bacteria, viruses and protozoa or by parasites are the most common and widespread health risk associated with drinking water [81]. This study has discussed some of these important microorganisms and has shown that pulsed UV-rich light treatment is an extremely effective means of inactivating these microorganisms. Although photoreactivation has been demonstrated, it only appears to be a problem if a microorganism does not receive

enough damage (low pulse number) or if the treated population is exposed to high intensities of visible light.

Pulsed UV light has many benefits over traditional disinfection methods, such as chlorination, including no addition of toxic chemicals to the drinking water and no formation of carcinogenic and mutagenic by-products. The treatment process does not leave unpleasant tastes and odours in the treated water and it removes the need to transport, store and handle dangerous chemicals [41]. UV light also only reacts with molecules which absorb UV, which excludes a large number of microbial molecules e.g. sugar based extracellular polymers, which when degraded by chemical disinfectants can become good nutrients therefore promote microbial growth [61]. One of the most important benefits of this treatment however is its ability to inactivate protozoa and viruses which can be resistant to many chemical disinfectants e.g. Polio virus is considered to be 2 to 5 times more resistant to UV as *E. coli* but it is 40 times more resistant to chlorine than *E.coli* [41]. In addition to chemical treatment the pulsed UV light system also has many advantages over continuous UV treatment systems, the reasons being that there is no temperature build up; it is safer, quicker and more effective.

Finally, from an application perspective there are many uses, including surface decontamination, air sterilisation and disinfection of liquids (transparent). The most important application of pulsed UV-rich light treatment however, would be in the treatment of water such as drinking water and waste water. It could also be used for microbe-free water for use in breweries, wineries, soft drinks, and water bottling facilities, and in the pharmaceutical, cosmetic, food and electronic industries.

9.2 Recommendations for Further Work

The work carried out in this thesis involved the inactivation of microorganisms, suspended in liquids, by UV-rich light pulses. Because this sterilisation technology has proven highly successful, its application in the food, medical and even pharmaceutical industry could be immense. Further work should however be carried

out to look at the success of the system in the treatment of surfaces e.g. food preparation surfaces. A small-scale study on surface inactivation was carried out during this project but the work was not pursued due to difficulties in recovering microorganism from the surfaces. Before work could commence on surface inactivation it would therefore be necessary to find accurate ways of recovering microorganisms from surfaces.

In this investigation, work was carried out on a range of pathogenic microorganisms (bacteria, viruses and a protozoan), in particular those associated with waterborne disease. Fungi however are responsible for spoiling a lot of food products, and yeasts can be major causes of nosocomial infections. Studies should therefore be carried out on investigating the susceptibility of these other microorganisms to the treatment. In addition, treatment of more pathogenic microorganisms such as *E. coli* 0157:H7 should be undertaken to determine whether they are equally as vulnerable to pulsed UV-rich light treatment.

Photoreactivation work which was undertaken in this study only focussed on *E. coli* (NCTC 9001). It is therefore recommended that work should be carried out on a range of bacterial species to observe whether they are as susceptible to undergoing photoreactivation. Once determined a suitable pulse number could then be suggested which would give complete inactivation without the occurrence of photoreactivation. Photoreactivation experiments could also be carried out with *C. parvum* as to date no work has been carried out to determine whether this microorganism undergoes photo-repair following pulsed UV-rich light treatment.

This study has found that in some situations, complete inactivation of a microorganism does not occur. The population that remains will probably have received some of the UV pulses (just not enough). Therefore it would be interesting to identify whether these bacterial cells are still as pathogenic as they were prior to the pulsed UV-rich light treatment. This could be investigated by carrying out adhesion and invasion assays that determine a microorganism's ability to adhere and invade gut cells. If microorganisms cannot invade these cells, then they are more

than likely unable to initiate infection. Microorganisms such as *Bacillus megaterium* produce enterotoxins that are harmful when ingested. Work could therefore be carried out to determine whether damaged bacterial cells are still capable of enterotoxins production.

It is most likely that the principal application of Pulsed UV-rich light is the treatment of drinking water. It has already been shown that it is very effective at inactivating a whole range of microorganisms; therefore the next step would be to design a system that can treat flowing water. This aspect of work should be assigned to an engineer, but once developed there would be a range of factors warranting investigating, such as sample depth, flow rates etc. Once developed this type of system would be desirable to a lot of major industries.

Chapter 10

ACKNOWLEDGEMENTS

The author would like to thank her supervisors Professor S. J. MacGregor and Professor J. G. Anderson for their supervision, assistance and encouragement throughout the course of this study.

An extended thanks is due to Dr. Nigel Cook and Dr. Artur Rzezutka, from the Central Science Laboratory (CSL) for all of their support, work and expertise involving viruses. Many thanks are also due to Professor Huw Smith and Brian from the Parasitology Department at Stobhill Hospital for their help with the *Cryptosporidium* work undertaken.

The help of Tao Wang for all of his assistance with the engineering side of this study was very much appreciated as was that of Joe McKechie and the other technicians of the Electrical Engineering workshop, who assisted in the preparation of experimental systems.

The author would also like to thank Dave Currie for his assistance in the laboratory and fellow PhD students Alison Goudie, Karen Wall and Michelle MacLean for their encouragement.

Finally, the author would also like to thank her family and Jock for their encouragement and support throughout the duration of this PhD study.

Chapter 11

REFERENCES

1. **Egli, T., Wolfgang, K., and Meile, L.** 2002. Pathogenic microbes in water and food: changes and challenges. *FEMS Microbiology Reviews.* **26:** 111-112.
2. **Anderson, J. G., Rowan, N. J., MacGregor, S. J., Fouracre, R. A., and Farish, O.** 2000. Inactivation of food-borne enteropathogenic bacteria and spoilage fungi using pulsed light. *IEEE Trans Plasma Sci.* **28:** 83-88.
3. **Jeyamkondan, S., Jayas, D. S., and Holley, R. A.** 1999. Pulsed electric field processing of foods: a review. *J. Food Prot.* **62(9):** 1088-1096.
4. **Wall, K., MacGregor, S. J., Anderson, J. G., and Mackersie, J. W.** Evidence of nuclear membrane damage in yeast cells treated with pulsed electric fields. 26th 2004 *Power Modulator Conference Proceedings* , May 23-26, San Francisco.
5. **Hoeben, W. F. L. M.** "Pulsed corona-induced degradation of organic materials in water". Technische Universiteit Eindhoven- PhD Thesis. 2000.
6. **Wang, T., MacGregor, S. J., Anderson, J. G., Rowan, N. J., and Lamont, Y.** *Proceedings of the XIV International Conference On Gas Discharges and their Applications, Liverpool, 2nd to 6th September 2002.*
7. **Espie, S., Marsili, L., MacGregor, S. J., and Anderson, J. G.** 2001. Pulsed power inactivation of liquid-borne microorganisms. *International Conference on Pulsed Power Applications.* **B.07:** 1-6.
8. **Cairns, W. L.** "Comparing disinfection by ultraviolet light and chlorination- the implications of mechanism for practice" Produced by Trojan Technologies Inc, 3020 Gore Road, London, Ontario, Canada N5V 4T7, (http://www.trojanuv.com).

9. **Barbosoa-Canovas, Pothakamury, Palou, and Swanson.** "Nonthermal Preservation of Foods". Chapter 1 p1-7. Published by Marcel Dekker Inc, 1998.

10. "Photochemical Sterilisation by Pulsed Light" produced by Xenon Corporation (http://www.xenon-corp.com).

11. **Hassen, A., Mahroul, M., Ouzari, H., Cherif, M., Boudabous, A., Damelincourt, J. J.** 2000. UV disinfection of treated wastewater in a large-scaloe pilot plant and inactivation of selected bacteria in a laboratory UV device. *Bioresource Technology*. **74:** 141-150.

12. **Baird-Parker, A. C.** 1994. Foods and microbiological risks. *Soc. Gen. Microbiol.* **140:** 686-695.

13. "Foodborne Diseases", Factsheet produced by NIAID (http://www.niaid.nih.gov)

14. **Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F. Bresee, J. S., Shapiro, C., Griffen, P. M,. and Tauxe, R. V.** 2000. Food Related Illness and Death in the United States. *Emerg Inf Dis.* **5:** 607-625.

15. **Anderson, J. G., MacGregor, S. J., Rowan, N. J., Fouracre, R. A., and Farish, O.** 2000. Assessment of inhibitory and inactivating effects of pulsed electrotechnologies on foodborne pathogenic microorganisms. *Proceedings of the XIII international conference on gas discharge & their applications.* **1:** 5-14.

16. **Rowan, N. J., MacGregor, S. J., Anderson, J. G., Fouracre, R. A., McIlvaney, L., and Farish, O.** 1999. Pulsed light inactivation of food related microorganisms. *Appl. Environ. Microbiol.* **65(3):** 1312-1315.

17. **Mimms, C., Playfair, J., Roitt, I., Wakelin, D., and Williams, R.** "Medical Microbiology" .1998.

18. **Finlay, B. B. and Falkow, S.** 1989. Microbiol. Rev. **53:** 210-230.

19. **Szewzyk, U., Szewzyk, R., Manz, W., and Schleifer, K. H.** 2000. Microbiological safety of drinking water. *Annu. Rev. Microbiol.* **54:** 81-127.

20. **Prescott, L. M., Harley, J. P., and Klein, D. A**. "Microbiology" (3rd Ed). Wm. C. Brown Publishers.

21. **MacGregor, S. J., Rowan, N. J., McIlvaney, L., Anderson, J. G., Fouracre, R. A., and Farish, O.** 1998. Light inactivation of food-related pathogenic bacteria using a pulsed power source. *Letters App. Microbiol.* **27:** 67-70.

22. "Kinetics of Microbial Inactivation For Alternative Food Processing Technologies". June 2nd 2000. Document produced by the U.S. Food and Drug Administration, Centre for Food Safety and Applied Nutrition. (http://www.cfsan.fda.gov)

23. **McIlvaney, L., MacGregor, S. J., Anderson, J. G., Rowan, N. J., Fouracre, R. A., and Farish, O.** "Electrotechnologies for Food Pasteurisation and Sterilisation" Chapter 47 p 1-8. Published by IEE, London, 1998.

24. **Booth, I.** 1998. The bacteria strike back. *The Biochemist.* April: 8-11.

25. "UV drinking Water Treatment Consulting", produced by Bolton Photosciences Inc, (http://www.boltonuv.com)

26. **Jin-Gab, K., Ahmed, Y. E., and Sandhya, D.** 1999. Application of ozone for enhancing the microbiological safety and quality of foods: a review. *J. Food Protect.* **62(9):** 1071-1087.

27. **Cairns, W. L.** "UV Technology For Water Supply Treatment" Produced by Trojan Technologies Inc, 3020 Gore Road, London, Ontario, Canada N5V 4T7, (http://www.trojanuv.com).

28. **Espie, S.** "An investigation of plasma treatment of microorganisms in liquids". University of Strathclyde- PhD Thesis. 2002.

29. **Wall, K., Ghasemi, Z., Cameron, D., Rowan, N. J., MacGregor, S. J., and Anderson, J. G.** 2002. Inactivation of Beer and lager spoilage microorganisms using pulsed electric fields. *IUMS, The World of Microbes Conference, Paris, 27 July to 1st August, 2002* (poster presentation).

30. **McDonald, K. F., Curry, R. D., Clevenger, T. E., Brazos, B. J., Unklesbay, K., Eisenstark, A., Baker, S., Golden, J., and Morgan, R.** 2000. The development of photosensitised pulsed and continuous

ultraviolet decontamination techniques for surfaces and solutions. *Trans. Plasma Sci.* **28(1):** 89-96.

31. **Bintsis, T., Litopoulou-Tzanetaki, E., and Robinson, R. K.** 2000. Existing and potential applications of ultraviolet light in the food industry- a critical review. *J. Sci. Food. Agric.* **80:** 637-645.
32. **Ardley, N., and Matthews, R.** "Physics Today: matter, atoms, energy", 1984.
33. **Elliot, D. J.** "Ultraviolet Laser Technology And Applications" Ch 1. Published by Academic Press, Inc, 1995.
34. **Koller, L. R.** "Ultraviolet Radiation", 2nd Edition, Ch's 1, 2, 4 and 7. Published by John Wiley and Sons, Inc, 1952.
35. **Block, S. S.** "Disinfection, Sterilisation, and Preservation" (4th ed). Chapter 31 p553-565. Published by Lea & Febiger, Philadelphia, 1991.
36. **Ryder, A.** 1998. "Light Measurement Handbook". (http://www.intl-light.com/handbook)
37. Haraeus Noblelight website: http://www.noblelight.net
38. IUVA website: http://www.iuva.org
39. **Miller, R. V., Jeffery, W., Mitchell, D., and Elasri, M.** 1999. Bacterial response to ultraviolet light. *ASM News.* **65(8):** 535-541.
40. **Hartwig, M.** "Ultraviolet and Your Health" obtained from the website: http://www.arn.org.
41. **Wright, H. B., and Cairns W. L.** "Ultraviolet Light" Produced by Trojan Technologies Inc, 3020 Gore Road, London, Ontario, Canada N5V 4T7, (http://www.trojanuv.com).
42. **Bolton, J. R.** "Ultraviolet Applications Handbook" Bolton Photosciences Inc, 2001. 2nd Edition
43. **Takeshita, K., Shibato, J., Sameshima, T., Fukunaga, S., Isobe, S., and Itoh, M.** 2003. damage of yeast cells induced by pulsed light irradiation. *Inter. J. Food. Microbiol.* **85:** 151-158.
44. "Pulsed-Ultraviolet Light For Drinking Water Disinfection" obtained from the website: http://www.epri.com

45. **Otaki, M., Okuda, A., Tajima, K., Iwasaki, T., Kinoshita, S., and Ohgaki, S.** 2003. Inactivation differences of microorganisms by low pressure UV and pulsed xenon lamps. *Water Sci. Technol.* **47(3):** 185-190.

46. Tecan website: http://www.tecan.com

47. Berson UV-techniek document-"Microbial repair mechanisms after UV treatment" Obtained from the website: http://www.processingtalk.com

48. U.S. Department of Energy Human Genome Program, website: http://www.ornl.gov.hgmis

49. **Giese, N., and Darby, J.** 2000. Sensitivity of microorganisms to different wavelengths of UV light: implications on modelling of medium pressure UV systems. *Wat. Res.* **34(16):** 4007-4013.

50. **Oguma, K., Katayama, H., and Ohgaki, S.** 2002. Photoreactivation of Escherichia coli after low- or medium-pressure UV disinfection determined by an endonuclease sensitive site assay. *Appl. Environ. Microbiol.* **68(12):** 6029-6035.

51. **Rice, J. K., and Ewell, M.** 2001. Examination of peak power dependence in the UV inactivation of bacterial spores. *Appl. Environ. Microbiol.* **67(12):** 5830-5832.

52. **Zimmer, J. L., and Slawson, R. M.** 2002. Potential repair of *Escherichia coli* DNA following exposure to UV radiation from both medium- and low-pressure UV sources used in drinking water treatment. *Appl. Environ. Microbiol.* **68(7):** 3293-3299.

53. **Cleaver, J. E.** *2003.* Photoreactivation. *DNA Repair.* **2:** 629-638.

54. **Horspool, W., and Pill-Soon, S.** "A Handbook of Organic Photochemistry and Photobiology" – Ecology of UV. CRC Press, London.

55. **Sinha, R. P., and Hader, D. P.** 2002. UV-induced damage and repair: a review. *Photochem. Photobiol.* **1:** 225-236.

56. **Belosevic, M., Craik, S. A., Stafford, J. L., Neumann, N. F., Kruithof, J., and Smith, D. W.** 2001. Studies on the resistance/reactivation of *Giardia muris* cysts and *Cryptosporidium parvum* oocysts exposed to

medium-pressure ultraviolet radiation. *FEMS Microbiol. Letters.* **204:**197-203.

57. **Blatchley, E. R., Dumoutier, N., Halaby, T. N., Levi, Y., and Laine, J. M.** 2001. Bacterial responses to ultraviolet irradiation. *Wat. Sci. Tech.* **43(10):** 179-186.

58. **Chang, J. C. H., Ossoff, S. F., Lobe, D. C., Dorfman, M. H., Dumais, C. M., Qualls, R. G., and Donald Johnson, J.** 1985. UV inactivation of pathogenic and indicator microorganisms. *Appl. Environ. Microbiol.* **49(6):** 1361-1365.

59. **Bauman, R. W.** "Microbiology" Published by Pearson Benjamin Cummings. 2004.

60. **Kelly, A. F., Park, S. F., Bovill, R., and Mackey, B. M.** 2001. Survival of Campylobacter *jejuni* during stationary phase: evidence for the absence of a phenotypic stationary-phase response. *Appl. Environ. Microbiol.* **67(5):** 2248- 2254.

61. **Cairns, W. L.** "UV Technology For Water Supply Treatment" Produced by Trojan Technologies Inc, 3020 Gore Road, London, Ontario, Canada N5V 4T7, (http://www.trojanuv.com).

62. **Bank, H. L., John, J., Schmehl, M. K., and Dratch, R. J.** 1990. Bactericidal effectiveness of modulated UV light. *Appl. Environ. Microbiol.* **56:** 3888-3889.

63. **Gardner, D. W. M., and Shana, G.** 2000. Modelling UV induced inactivation of microorganisms on surfaces. *J. Food Protect.* **63(1):** 63-70.

64. "General UV Consulting", produced by Bolton Photosciences Inc, (http://www.boltonuv.com)

65. **Dunn, J., Ott, T., and Clark, W.** Sep 1995. Pulsed-light treatment of food and packaging. *Food Technology.* p95-98.

66. **McClean, J. C.** "Application of ultraviolet light for the control of airborne pathogens and spoilage organisms" Hanovia Ltd, 145 Farnham Road, Slough, Berkshire.

67. **Gosden, P. E., MacGowan, A. P., and G. C. Bannister.** 1998. Importance of air quality and related factors in the prevention of infection in orthopaedic implant surgery. *J. Hosp. Infections.* **39:** 173-180.

68. Technovation Systems Inc website: http://www.cleanroomsys.com

69. **Maclean, M., Anderson, J. G., MacGregor, S. J., and Mackersie, J. W.** The development of a pulsed UV-light air disinfection system and its application in university lecture theatres. 26th 2004 *Power Modulator Conference Proceedings* , May 23-26, San Francisco.

70. **Goswami, D. Y., Trivedi, D. M., and Block, S. S.** 1997. Photocatalytic disinfection of indoor air. *Transactions of ASME.* **119:** 92-96.

71. **El-Taweel, G. E., and Shaban, A. M.** 2001. Microbiological quality of drinking water at eight water treatment plants. *Int. J. Environ. Health Research.* **11:** 285- 290.

72. **World Health Organisation (WHO) website:** http://www.who.int. "WHO Guidelines for Drinking Water Quality" 2nd Ed. Vol. 1- Recommendations. Geneva, WHO, 1993. pp 3-29.

73. **Thurston-Enriquez, J. A., Haas, C. N., Jacangelo, J., Riley, K., and Gerba, C. P.** 2003. Inactivation of feline calcivirus and adenovirus type-40 by UV radiation. *App. Environ. Microbiol.* **69(1):** 577-582.

74. **Gerba, C. P., Gramos, D. M., and Nwachuku, N.** 2002. Comparative inactivation of enteroviruses and adenovirus 2 by UV light. *Appl. Environ. Microbiol.* **68(10):** 5167-5169.

75. Safe Water Re-use Website: http://www.safewaterreuse.org

76. **Schoenen, D.** 2002. Role of disinfectionin suppressing the spread of pathogens with drinking water: possibilities and limitations. *Water Res.* **36:** 3874-3888.

77. **Mintz, E., Bartram, J., Lochery, P., and Wegelin, M.** 2001. Not just a drop in the bucket: expanding access to point of use water treatment systems. *American J Public Health.* **91(10):** 1565- 1570.

78. United States Environmental Protection Agency, Office of Water (4606): http://www.epa.gov/safewater "National Primary Drinking Water Standards". March 2001.

79. Scottish Waterboard website: http://www.westscotlandwater.org.uk. "Your Water" Leaflet.

80. **Maier, R. M., Pepper, I. L., and Gerba, C. P.** 2000. "Environmental Microbiology", Academic Press

81. **World Health Organisation (WHO) website:** http://www.who.int. "WHO Guidelines for Drinking Water Quality" 3rd Ed.

82. **Environment Agency.** "The Microbiology of Drinking Water". 2002. Part 1- Water Quality and Public Health

83. **Butler, R. C., Lund, V., and Carlson, D. A.** 1987. Susceptibility of *Campylobacter jejuni* and *Yersina enterocolitica* to, UV radiation. *Appl. Environ. Microbiol.* **53(2):** 375-378.

84. Scottish Waterboard website: http://www.westscotlandwater.org.uk. "Your Waste Water" Leaflet.

85. **The Bathing Water Directive 76/160/EEC:** http://EUROPA.EU.INT/water/water-bathing/directeu.html

86. "UV Wastewater Treatment Consulting" produced by Bolton Photosciences Inc, (http://www.boltonuv.com)

87. **Cairns, W. L.** "Comparison of UV disinfection technologies using low intensity monochromatic and high intensity polychromatic UV lamps" Produced by Trojan Technologies Inc, 3020 Gore Road, London, Ontario, Canada N5V 4T7, (http://www.trojanuv.com).

88. Website of the National Groundwater Association: http://www.ngwa.org

89. "Microorganisms, Bacteria and Viruses" obtained from http://www.ace.orst.edu

90. Washington State Department of Health, Division of Environmental Health, Office of Drinking Water (http://www.doh.wa.gov) "Coliform Bacteria and Drinking Water Factsheet"

91. **Tosa, K., and Hirata, T.** 1999. Photoreactivation of enterohemorrhagic Escherichia coli following UV disinfection. *Wat. Res.* **33(2):** 361-366.

92. **Licence, K., Oates, K. R., Synge, B. A., and Reid, T. M. S.** 2001. An outbreak of E.coli 0157 infection with evidence of spread from animals to man through contamination of a private water supply. *Epidemiol. Infect.* **126:** 135-138.

93. **Waage, A. S., Vardund, T., Lund, V., and Kapperud, G.** 1999. Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a seminested PCR assay. *Appl. Environ. Microbiol.* **65(4):** 1636-1643.

94. **Obri-Danso, K., Paul, N., and Jones, K.** 2001. The effects of UVB and temperature on the survival of natural populations and pure cultures of *Campylobacter jejuni, Camp. Coli, Camp. Lari* and urease positive thermophilic campylobacters (UPTC) in surface waters. *J. Appl. Microbiol.* **90(2):** 256-267.

95. **Humphrey, T.** Feb 2002. *Campylobacter spp*: not quite the tender flowers we thought they were? *Microbiology Today.* **29:** 7-8.

96. Tarbiat Modares University website: http://www.modares.ac.ir

97. Centre For Disease Control and Prevention, Division of Parasitic Diseases. Website: http://www.dpd.cdc.gov

98. **Bukhari, Z., Marshall, M. M., Korich, D. G., Smith, H. V., Rosen, J., and Clancy, J. L.** 2000. Comparison of *Cryptosporidium parvum* viability and infectivity assays following ozone treatment of oocysts. *Appli. Environ. Microbiol.* **66(7):** 2972-2980.

99. **Clancy, J. L., Bukhari, Z., Hargy, T. M., Bolton, J. R., Dussert, B. W., and Marshall, M. M.** 2000. Using UV to inactivate Cryptosporidium. *J. American Water Works Assoc.* **92(9):** 97-104.

100. **Guerrant, R.** L. 1997. *Cryptosporidiosis*: An emerging, highly infectious threat. (*Synopses*) *Emerging Infectious Diseases.* **3(1):** 51-57.

101. **Slifko, T. R., Smith, H. V., and Rose, J. B.** 2000. Emerging parasite zoonoses associated with water and food. *Intern. J. Parasitology.* **30:** 1379-1393.

102. **Strickland G. T**. "Hunters Tropical Medicine", 8th edition.

103. Hanes, D. E., Worobo, R. W., Orlandi, P. A., Burr, D. H., Miliotis, M. D., Robl, M. G., Bier, J. W., Arrowood, M. J., Churey, J. J., and Jackson, G. J. 2002. Inactivation of *Cryptosporidium parvum* oocysts in fresh apple cider by UV irradiation. Appl. Envirin. Microbiol. **68(8):** 4168-4172.

104. Lorenzo- Lorenzo, M. J., Ares-Mazas, M. E., Villacorta-Martinez de Maturana, I., and Duran-Oreiro, D. 1993. Effect of ultraviolet disinfection of drinking water on the viability of *Cryptosporidium parvum* oocysts. *J. Parasitol.* **79(1):** 67-70.

105. Linden, K. G., Shin, G., and Sobsey, M. D. 2001. Comparative effectiveness of UV wavelengths for the inactivation of *Cryptosporidium parvum* oocysts in water. *Wat. Sci. Tech.* **43(12):** 171-174.

106. Tauxe, R. V. "Emerging Foodborne Diseases: An Evolving Public Health Challenge". Centres for Disease Control and Prevention, Atlanta, Gerorgia, USA.

107. Microsoft® Encarta® Online Encyclopedia. 2004. "Virus (life science)" – http://www.encarta.msn.com©

108. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition: http://www.cfsan.fda.gov. "Bad Bug Book"

109. Hepatitis website: http://www.about-hepatitis.com

110. Oguma, K., Katayama, H., Mitani, H., Morita, S., Hirata, T., and Ohgaki, S. 2001. Determination of pyrimidine dimers in *Escherichia coli* and *Cryptosporidium parvum* during UV light inactivation, photoreactivation, and dark repair. *Appl. Environ. Microbiol.* **67(10):** 4630-4637.

111. Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. " Bergy's Manual of Determinative Bacteriology", 9th Edition. 2000.

112. Rosef, O., Rettedal, G., and Lageide, L. 2001. Thermophilic campylobacters in surface water: a potential risk of campylobacteriosis. *Int. J. Environ. Health Res.* **11:** 321-327.

113. **Todar, K.** 2003. "Todars Online Book of Bacteriology", University of Wisconsin-Madison, Dept of Bacteriology. http://www.textbookofbacteriology.net

114. **Purdy, D., Buswell, C. M., Hodgson, A. E., McAlpine, K., Henderson, I., and Leach, S. A.** 2000. Characterisation of cytolethal distending toxin (CDT) mutants of *Campylobacter jejuni*. *J. Med. Microbiol.* **49:** 473-479.

115. **Cools, I., Uyttendaele, M., Caro, C., D'Haese, E., Nelis, H. J., and Debevere, J.** 2003. Survival of *Campylobacter jejuni* strains of different origin in drinking water. *J. Applied Microbiol.* **94:** 886-892.

116. **Rose, J. *B.*, Huffman, D. E., and Gennaccaro, A.** 2002. Risk and control of waterborne cryptosporidiosis. *FEMS Microbiology Reviews.* **26:** 113-123.

117. Tulane University website: http://www.tulane.edu. "Adenoviruses"

118. Tulane University website: http://www.tulane.edu. "Herpesviruses"

119. **Nuanualsuwan, S., and Cliver, D. O.** 2003. Infectivity of RNA from inactivated poliovirus. *Appl. Environ. Microbiol.* **69(3):** 1629-1632.

120. **Gutierrez-Escolano, A., Medina, F., Racaniello, V. R., and Del Angel, R. M.** 1997. Differences in the UV-crosslinking patterns of the poliovirus 5'- untranslated region with cell proteins from poliovirus-susceptible and –resistant tissues. *Virology.* **227:** 505-508.

121. Tulane University website: http://www.tulane.edu. "Picornaviruses"

122. GlaxoSmithKline-"Poliomyelitis" obtained from: http://www.worldwidevaccines.com

123. **Reed, L. J., and Muench, H.** 1938. A simple way of estimating fifty percent end points. *Am J Hyg* **27**, 493-497.

124. **Craik, S. A., Weldon, D., Finch, G. R., Bolton, J. R., and Beloseviv, M.** 2001. Inactivation of *Cryptosporidium parvum* oocysts using medium and low-pressure ultraviolet radiation. *Wat. Res.* **35(6):** 1387-1398.

125. **Morita, S., Namikoshi, A., Hirata, T., Oguma, K., Katayama, H., Ohgaki, S., Motoyama, N., and Fujiwara, M.** 2002. Efficiacy of UV Irradiation in inactivating *Cryptosporidium parvum* oocysts. *Appl. Environ. Microbiol.* **68(11):** 5387-5393.

126. **Drescher, A.** 1999. Reactivation of UV-Disabled Microbes. WaterHealth International Inc. http://www.waterhealth.com

127. **Chick, H.** 1908. An investigation of the laws of disinfection. *J. Hyg.* **8:** 92-158.

128. **Zimmer, J. L., Slawson, R. M., and Huck, P. M.** 2003. Inactivation and potential repair of *Cryptosporidium parvum* following low- and medium-pressure ultraviolet radiation. *Wat. Research.* **37:** 3517-3523.

129. **Warriner, K., Rysstad, G., Murden, A., Rumsby, P., Thomas, D.** and Waites, W. M. 2000. Inactivation of *bacillus subtilis* spores on packaging surfaces by UV excimer laser irradiation. *J. App. Micro.* **88:** 678-685.

130. "Food Safety From Farm To Table: A New Strategy For The 21st Century". February 1997. Document produced by the U.S. Food and Drug Administration, Centre for Disease Control and Prevention. (http://www.cfsan.fda.gov)

131. **Sancar A.** 1994. Structure and function of DNA photolyase. *Biochemistry.* **33(1):**2-9.

132. **Kim, S. T and Sancar A.** 1993. Photochemistry, photophysics, and mechanisms of pyrimidine dimer repair by DNA photolyase. *Photochem. Photobiol.* **57 (5):** 895-904.

133. **Cook, N and Rzezutka, A.** 2005. "Hepatitis Viruses" Emerging Foodborne Pathogens. Editors: Y. Motarjemi and M. Adams, Woodhead Publishings Ltd, Cambridge.

134. **Environmental Protection Agency.** 1999. Wastewater Technology Factsheet: Ultraviolet Disinfection. (http://www.epa.gov).

135. Dunn, J., Cooper, J. R., Salisbury, K., May, R and Leo. F. 1998. PureBright pulsed light processing and sterilisation. *Europ. J. Parenteral Sci.* 3 (4): 105-114.

136. **Campbell, A. T., Robertson, L. J and Smith, H. V.** 1992. Viability of Cryptosporidium parvum oocysts: correlation of in vitro excystation with inclusion or exclusion of fluorogenic vital dyes. *App. Env. Microbiol.* **58 (11):** 3488-3493.

137. **Cameron, K. R.** 1973. Ultraviolet irradiation of herpes simplex virus-action spectrum for survival of infectivity in relation to small-plaque effect. *J. Gen. Virol.* **18:**51-57.

138. **Kurdziel, A.S., Wilkinson, N., Langton, S. and Cook, N.** 2001. Survival of poliovirus on soft fruit and salad vegetables. *J. Food Prot.* **64**: 706 709.

139. **Groocock, N. H.** 1984. Disinfection of drinking water by ultraviolet light. *J. Institute Water Engin. & Sci. 38* **(20):** 163-172.

140. **Cox, F. E. G.** "Modern Parasitology" (2nd Ed). Blackwell Science Publishers.

Chapter 12

APPENDIX A

Table of Most Probable Numbers

Number of Tubes giving a Positive Reaction		MPN per 100 ml
1 x 50 ml	5 x 10 ml	
0	0	None found
0	1	1
0	2	2
0	3	3
0	4	4
0	5	6
1	0	1
1	1	2
1	2	5
1	3	9
1	4	15
1	5	> 18

MPN per 100 ml of sample for a 6 tube series containing 1 x 50 ml and 5 x 10 ml volumes.

* The table indicates from the various combinations of positive and negative reactions for the volumes examined in this study, the estimated number of bacteria in 100 ml of sample.

Chapter 13

APPENDIX B: LIST OF PUBLICATIONS

Throughout the course of this PhD, work was presented at international conferences and papers were submitted for publication. A list of the conferences and publications are shown below and conference abstracts and papers are included for reference in the following pages.

1. Y. Lamont, T. Wang, S. J. MacGregor, J. G. Anderson, and N. J. Rowan. "Disinfection of Drinking Water Using High Intensity Pulsed Light", 10th *International Congress on Bacteriology and Microbiology, Paris, Paper No 57-B-426, 2002.* **p221**

2. Y. Lamont, T. Wang, S. J. Macgregor, J. G. Anderson and N. J. Rowan. "Inactivation of *Campylobacter* species using pulsed light", *Society For General Microbiology, 152nd Meeting, University of Edinburgh, Paper FdBev 03, 2003.* **p222**

3. Y. Lamont, S. J. MacGregor, J. G. Anderson and N. J. Rowan. "Photo-Reactivation Responses Of *E. coli* Following Exposure To Pulsed Light", *Electromed 2003, June 11-13, San Antonio, Texas. [A54].* **p223**

4. Wang, T., MacGregor, S. J., Anderson, J. G., Rowan, N. J., and Lamont. Y. "Inactivation of pathogenic microorganisms using pulsed UV illumination and its applications in water disinfection", *Published In Proceedings of the XIV International Conference On Gas Discharges and their Applications. Liverpool, 2nd to 6th September 2002.* **p224**

5. Ghasemi, Z., Macgregor, S. J., Anderson, J. G., and Lamont, Y. "Development of an integrated solid- state generator for light inactivation of food-related pathogenic bacteria", *Published in Measurement Science and Technology*. 2003, **14:** N26-N32. **p228**

6. Y. Lamont, S. J. MacGregor , J. G. Anderson and R. A. Fouracre. "Effect Of Visible Light Exposure On *E. coli* Treated With Pulsed UV-Rich Light" *26th 2004Power Modulator Conference Proceedings , May 23-26, San Francisco.* **p235**

7. Lamont, Y., Rzezutka, A ., Anderson, J.G., Macgregor, S.J.,Deppe, C. and Cook, N. 2005. Pulsed UV-light inactivation of poliovirus and adenovirus – *Submitted to Applied and Environmental Microbiology. Awaiting publication.* **p239**

MISSING PAGES
REMOVED ON
INSTRUCTION
FROM THE
UNIVERSITY