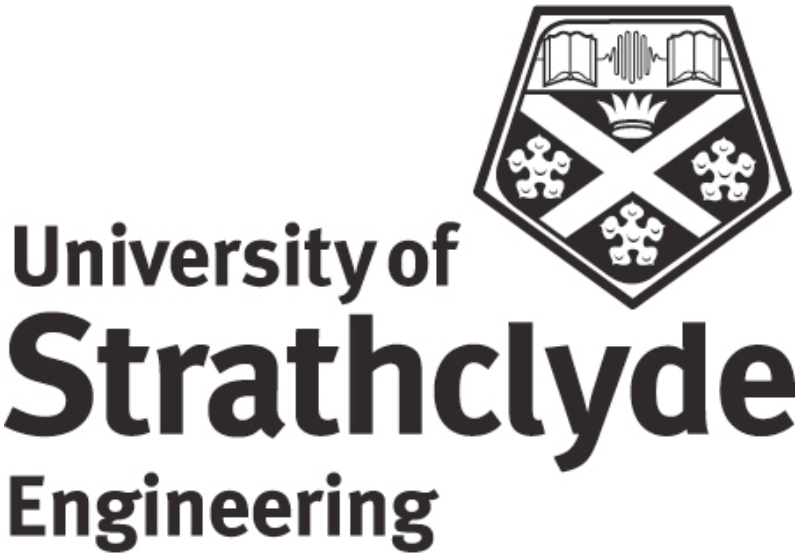

AN INVESTIGATION INTO THE USE OF HIGH-INTENSITY NARROW SPECTRUM LIGHT AS A DECONTAMINATION TECHNOLOGY

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This thesis is a result of the author's original research. It has been composed by the author and has not been previously submitted for examination, which has lead to the award of a degree.

ABSTRACT

Control of contaminant microorganisms is important in many different settings, particularly in healthcare and food production and processing, where environmental contamination by pathogenic microorganisms can be detrimental to human health. In the UK, the number of hospital-associated infections (HAI) and food-related illnesses continues to rise. As such, alternative and complementary disinfection and decontamination technologies are constantly being sought.

This investigation examined the inactivation efficacy of High-Intensity Narrow Spectrum Light (HINS-light) on a wide range of microorganisms using LED arrays that emitted intense 405-nm light. The theorised inactivation mechanism involves 405-nm stimulation of endogenous porphyrins, ultimately leading to production of reactive oxygen species (ROS) that cause oxidative cell damage and microbial inactivation. All tested microorganisms associated with HAI and foodborne illnesses were readily inactivated by 405-nm light. It was also established, for the first time, that 405-nm light was germicidal to eukaryotic microorganisms, including *Candida albicans*, *Saccharomyces cerevisiae* and the spore-forming fungus *Aspergillus niger*. Further examination found 405-nm light induced toxic photo-product formation in Nutrient Broth. The photo-product was particularly toxic to *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA) and *Acinetobacter baumannii,* although it elicited either no effect or a bacterio-static effect in other tested bacteria.

HINS-light is known to be less germicidal than pulsed ultraviolet (PUV) light produced from a Xenon flashlamp, however this study has demonstrated that sub-lethally PUV-damaged *S. aureus* cells can undergo photoreactivation upon exposure to 300-500-nm light, with maximum effect elicited at 360-380 nm. This photoreactivation potential, that can affect the inactivation capability of UV-light decontamination technologies, was not found to be associated with HINS-light treatment. In addition, HINS-light technology has significant safety advantages over UV light thereby permitting its use in occupied environments. This study has greatly extended the range of microorganisms that have been shown to be sensitive to HINS-

light exposure, including important pathogens and food spoilage micoorganisms. The study has confirmed that HINS-light has the capability to be used for the control of a wide range of microorganisms in environmental decontamination applications, and thereby has the potential to contribute to an overall reduction in the numbers of HAI and foodborne illnesses.

CONTENTS

Chapter 1

INTRODUCTION

Control of microorganisms is important in a range of different settings. Infection control in hospitals and other healthcare establishments is of vital importance as hospital in-patients are often at particular risk of infection with potentially pathogenic and even normally commensal microorganisms, due to their reduced immune status. Bacteria present in the hospital environment are a frequent source of infection, particularly with the emergence of multi-antibiotic resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA).

The control of bacteria in food production and packaging environments is also increasingly important, owing to large-scale production and global shipping of foods. Microorganisms present in the environment can easily contaminate packaging and foods during manufacturing. Foodstuffs provide substrates for growth of microorganisms, which can lead to spoilage or foodborne illness upon ingestion.

There are many methods, technologies and preventative measures available to reduce the number of microorganisms present in both healthcare and food production environments, however novel technologies are constantly being sought for use in conjunction with currently available safety measures.

The main focus of this study was determining the inactivation capability of 405 nm high-intensity narrow-spectrum light (HINS-light) on a range of bacteria important in:

(1) Food Production and Packaging Environments: common bacterial causes of foodborne illness including *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella enterica serovar enteritidis*, *Shigella sonnei*, *Listeria monocytogenes* and *Bacillus* spp were tested for susceptibility to HINS-light.

(2) Healthcare Environment: hospital-associated pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii,* and the *Mycobacterium tuberculosis* surrogate organism *Mycobacterium terrae,* were also tested for susceptibility to HINS-light. Work in this thesis also tested a selection of MRSA strains isolated from the clinical environment. Bacterial isolates were exposed to 405-nm HINS-light in the laboratory in liquid suspensions, and their relative inactivation doses were compared.

In additional to bacterial species, work in this thesis investigated the effect of 405-nm light exposure on eukaryotic species important in human health and food spoilage including the yeasts *Candida albicans* and *Saccharomyces cerevisiae* as well as the spore-forming fungus *Aspergillus niger*.

The effect of intermittent 405-nm light exposure on *S. aureus* was investigated to determine whether inactivation was dose-dependent in the first instance. However, during exposure experiments with bacteria suspended in Nutrient Broth, it was noticed that inactivation continued to occur during periods when bacteria were not exposed to 405-nm light. Further investigation into the effects of 405-nm light on various nutritious media led to the discovery that a toxic agent was being formed upon exposure to the light, but notably, only in Nutrient Broth suspensions.

Ultraviolet (UV) light was used in an inactivation study on *S. aureus* to illustrate its germicidal ability in comparison to that of non-UV visible light sources such as 405-nm light. The photoreactivation process was also studied to highlight just one of the many problematic aspects of using UV light as a decontamination technology.

A brief overview of the contents of each chapter is listed below:

Chapter 2 (Background and Literature Review) discusses the problems associated with microbial contamination of environments e.g. in healthcare and in the food production industries. It discusses traditional methods of microbial decontamination and then focuses on novel light-based microbial decontamination technologies.

Chapter 3 (Microbiological Materials and Techniques) provides details on the various microorganisms, media, diluents, microbiological preparations and biochemical tests used in experimentation.

Chapter 4 (Investigation into the Use of Ultraviolet Light as an Inactivation Technology) investigates the use of ultraviolet light (UV) for the inactivation of *S. aureus*. The photoreactivation capability of *S. aureus* after sub-lethal UV-light treatment is also studied and discussed.

Chapter 5 (HINS-light Inactivation of Foodborne Bacterial Pathogens) investigates the bactericidal effect of High-intensity Narrow-Spectrum light (HINS-light), of peak wavelength 405 nm, on a range of food-related pathogenic microoganisms, including endospore-forming *Bacillus* species. Experimental work in this chapter examined bacterial inactivation in both liquid suspension and on surfaces.

Chapter 6 (Investigation into the HINS-light Inactivation Mechanism) undertook a range of experiments to further understand the process behind the 405-nm/blue light inactivation mechanism. During experimentation it was found that a toxic effect occurred upon of exposure of nutrient media to 405-nm light. Experiments were carried out in an attempt to determine the underlying cause of this toxicity.

Chapter 7 (HINS-light Inactivation of Yeasts and Fungi) investigates the inactivation capability of high-intensity 405-nm light on eukaryotic microbial cells. Two yeast species and a spore-forming fungal species were exposed to 405-nm light to determine whether the light was fungicidal as well as bactericidal.

Chapter 8 (The Effect of HINS-light on Bacteria Commonly Found Within the Hospital Environment) examines the bactericidal effect of 405 nm HINS-light on bacterial species commonly associated with infection in the hospital environment, including MRSA, *Acinetobacter* and *Mycobacterium*.

A concurrently run study has seen the development of a HINS-light Environmental Decontamination System, which has undergone clinical evaluation within a hospital isolation room (Maclean *et al*., 2010). Work in Chapter 8 involved isolating and identifying MRSA strains collected on contact plate samples from the clinical evaluation, and through laboratory-testing, their susceptibility to 405-nm HINS-light exposure was investigated.

Chapter 9 (Conclusions and Recommendations for Future Work) examines the significance of the findings of each chapter, and the potential applications of the HINS-light technology. Recommendations for future work to be carried out are also detailed.

Chapter 2

BACKGROUND AND LITERATURE REVIEW

2.0 GENERAL

This literature review discusses the demand for decontamination technologies in many different settings, with focus on disinfection of the clinical and food production environments. More specifically this review assesses the diverse technologies that are currently available, particularly light decontamination technologies such as ultraviolet light, photodynamic inactivation and violet/blue light.

2.1 MICROBIAL CONTAMINATION ISSUES

Microorganisms are part of almost every aspect of life on Earth. Microorganisms frequently colonise humans, animals, plants, soil and water, however there are many instances in which the presence of microorganisms can be problematic. Two instances discussed in this chapter in which microbial contamination issues can arise, are in the healthcare and food production and processing environments.

2.1.1 Healthcare

The increase in hospital-acquired infections (HAI) in recent years has been attributed to the emergence of so-called "superbugs", such as methicillin-resistant *Staphylococcus aureus* (MRSA). It is estimated that around 300,000 in-patients contract a HAI during their hospital stay, 5,000 in-patients die each year as a direct result of HAI, and in a further 15,000 in-patients, HAI is implicated as a contributory factor in their death (POST, 2005). HAIs cost the economy around £1 billion each year, however it is estimated that with improved infection control measures and use of best clinical practice savings of £150 million could be made, which could be used in other NHS resources (POST, 2005).

2.1.1.1 Antibiotic Resistance

In hospitals and other healthcare establishments the emergence of antibiotic-resistant microorganisms has proven to be problematic in the treatment of in-patients. The over-prescription of antibiotics by healthcare professionals, failure of patients to complete courses of prescribed antibiotics, and use of antibiotics in animals feeds have all exacerbated the problem of antibiotic resistance (WHO, 2002; POST, 2005). As a result many bacteria are now resistant to most available antibiotics. MRSA is the most notorious of these antibiotic-resistant bacteria, with good cause, as it is the most common cause of hospital-acquired bacteraemias (POST, 2005).

2.1.1.2 Hospital Environment

Hospitals by their very nature house sick people and ever more immunocompromised patients. This is attributed to the improvements in clinical treatment of cancers with radiotherapy and chemotherapy ablative technologies, medical breakthroughs in transplantation and the emergence of AIDS, which mean a higher proportion of in-patients are at risk of infection with common nosocomial pathogens due to their immunocompromised state.

2.1.1.3 Common Hospital Associated Microorganisms

As discussed previously, hospitals house many immunocompromised individuals that are at increased risk of infection with hospital-associated microbes. Even commensal microorganisms can cause severe and often life-threatening disease in hospital patients, and the presence of many antibiotic-resistant microorganisms in the hospital environment can complicate the treatment of disease or exacerbate the patient's current medical condition. Presence of open wounds and the use of intravenous devices, catheterisation and respiratory intubation or surgical procedures increase the likelihood of infection. Breaking of the skin barrier during surgical and other hospital procedures enables microorganisms entry into the bloodstream (WHO, 2002; POST, 2005). Once microorganisms enter the bloodstream they can multiply and establish infection in tissues and vital organs such as the heart, lungs and kidneys. Some microorganisms produce toxins that lead to disease upon entry to the host, such as *S. aureus* via staphylococcal toxins (SEA, SEB, SEC and TSST-1)

causing toxic shock syndrome and other diseases (Maisch *et al*., 2004). Some of the most common causes of hospital-acquired infections are listed in Table 2.1.

Table 2.1 *Common hospital-associated microorganisms. Adapted from WHO, 2002.*

Gram Positive Bacteria	Gram Negative Bacteria	Viruses	Parasites and Fungi
Staphylococcal spp[a]	*Escherichia coli*	Hepatitis B/C	*Giardia lamblia*
Streptococcal spp	*Proteus* spp	Respiratory Syncytical	*Cryptosporidium*
Clostridium spp	*Klebsiella* spp	Virus (RSV)	*Cryptococcus*
Mycobacterium spp	*Enterobacter* spp	Rotavirus	*neoformans*
	Pseudomonas spp	Enteroviruses	*Sarcoptes scabies*[b]
	Acinetobacter	Cytomegalovirus	*Candida albicans*
	baumannii		
	Legionella spp[b]		

[a] Includes multi-drug resistant and coagulase negative species

[b] Sporadic

2.1.1.4 Transmission

Microorganisms can be transmitted in healthcare settings in numerous ways, from normal patient/staff flora, environmental microflora (including sporadic outbreaks), foods and medical devices. Figure 2.1 depicts the transmission routes of microorganisms.

Patients and Staff

Patients are colonised with their own flora (endogenous or transient), which under normal circumstances are generally considered to be non-pathogenic. However, if the patient is immunocompromised, or microorganisms become introduced into the bloodstream, then an infection from a patient's own flora can arise (WHO, 2002). Bacterial species such as *S. aureus* and some streptococcal species are part of the normal flora frequently implicated in these types of infections. The *Enterobacteriacea* family of bacteria, particularly *Escherichia coli*, that colonise the intestinal tract of patients are frequently implicated in urinary tract infections, bacteraemias and other infections (POST, 2005). The natural gut flora can be introduced into the bloodstream and by-pass mucous membranes during the insertion of cannulae, catheters and other medical devices (WHO, 2002).

The natural microflora of healthcare staff can also be introduced to a patient during medical procedures, nursing and wound dressing. The flora of visitors can also lead to infection.

Healthcare Environment

Some microorganisms can also survive well outside of a host in the healthcare environment. Microorganisms such as *Acinetobacter* spp and *Pseudomonas* spp can survive well in damp or wet environments such as sinks and showers (WHO, 2002). Others survive in hospital linens, fixtures and fittings, appliances, floors and door handles. Microorganisms often survive in dust particles, which can easily be spread if proper cleaning does not occur (WHO, 2002). Microorganisms can also be spread in droplet nuclei (<10 μm) from coughing, sneezing and conversing (WHO, 2002). These droplet nuclei can remain in the air for several hours (Cox, 1987). There have

also been sporadic outbreaks in healthcare environments with microorganisms such as *Legionella* spp, where the source of the outbreak is frequently faulty air conditioning and water systems where *Legionella* can easily survive and become aerosolised (WHO, 2002). Another possible source of microorganisms in the healthcare environment is contaminated patient food, which can cause a food-related illness that may become a more serious infection in an immunocompromised individual (WHO, 2002).

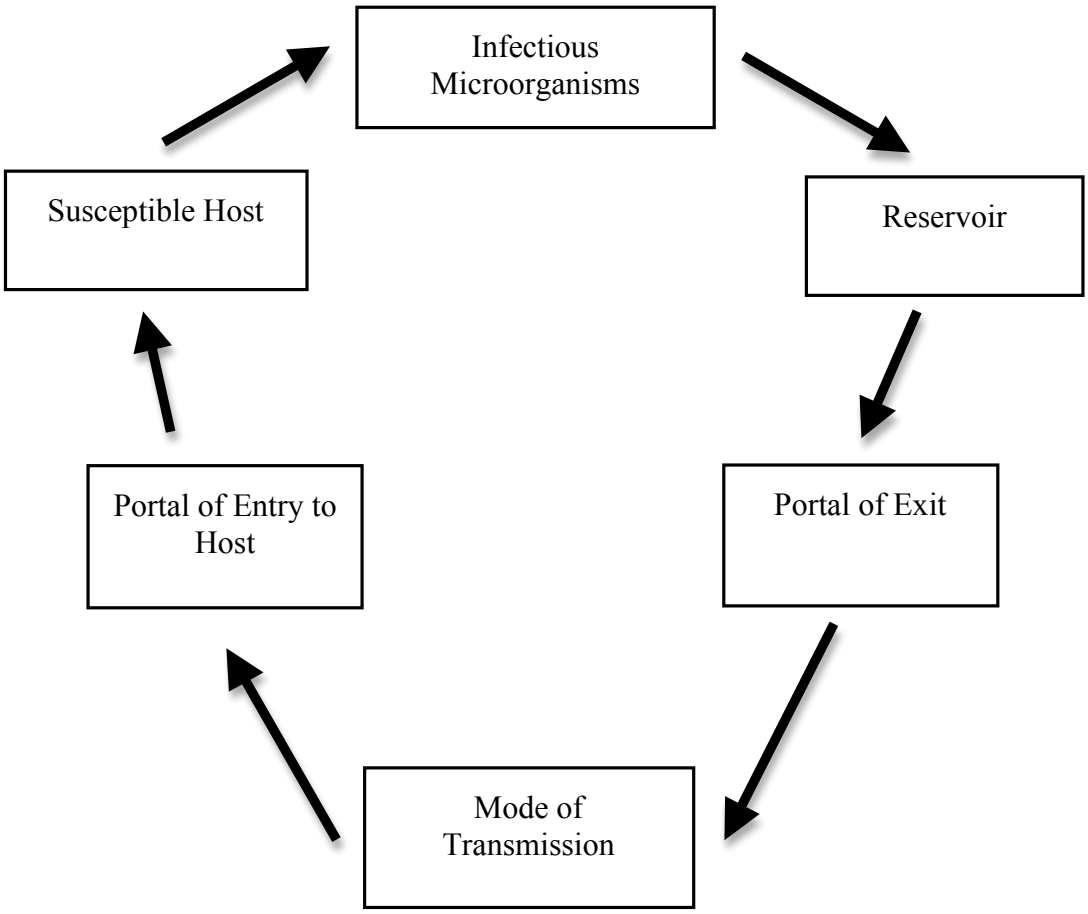

Figure 2.1 *The routes of transmission of microorganisms adapted from NHS, 2008.*

2.1.2 Food Preparation and Processing

It is important that all foods produced and sold are safe for consumption. Foods should not contain harmful levels of chemicals or microorganisms and their potentially toxic products. Microbiological safety of foods has become increasingly important in recent years. Consumer demands have changed considerably with requirements for more minimally processed fresh food products with a longer shelf life. Consumer demands have also changed owing to the increasing knowledge in terms of diet, food safety and well-being (Gould, 1995). In addition, the globalisation of food production means that we also expect the same level of food safety in foods imported from other countries (Gould, 1995). In order to maintain the nutritional and flavour qualities of foods that are desirable to consumers and maintain the microbiological safety of foods there has to be improvements in the decontamination methods currently used.

2.1.2.1 Food Spoilage

Food spoilage is defined as the breakdown of foods by microorganisms directly or in-directly as a product of the microorganism's metabolic processes (Tucker, 2008). Spoilage of foods by microorganisms can change the organoleptic qualities of foods such as the flavour and smell (Tucker, 2008). It can also negate the nutritional value and the physical appearance of the foodstuff. The microorganism may cause changes to the qualities of the food purely by using the food as an energy substrate or the microorganism may produce a by-product that affects the food, such as the production of toxic products (Tucker, 2008). A multitude of different microorganisms can cause food spoilage, some of which are listed in Table 2.2.

2.1.2.2 Foodborne Illness

Foodborne illness can arise when microorganisms are ingested in large enough numbers to cause an infection in the host –"*Food Infection"*. It can also occur when toxins produced by the microorganisms are ingested, leading to disease-"*Food Poisoning"*. Frequently microorganisms associated with food spoilage are also causative agents of foodborne illness. Some symptoms of foodborne illness include gastroenteritis, emesis, nausea, headaches, aches and pains and malaise. However,

some microorganisms cause much more severe disease such as *Clostridium botulinum* neurotoxin, which upon ingestion of a few nanograms can prove to be fatal (Tucker, 2008). Infection with *E. coli* spp, particularly O157:H7 can lead to renal failure, particularly in susceptible populations such as the elderly, young (infants) and the immunocompromised (Tucker, 2008). The number of cells required to establish an infection (infectious dose) can be very low, in many cases only a few cells need to be ingested to cause disease. Some common sources of foodborne illness are outlined in Table 2.2. In addition to the effects on human health, foodborne infections cost the economy £1.5 billion each year, which includes healthcare costs and days of work missed as a result of illness (FSA, 2009).

Mycotoxins, which are toxins produced by fungi, are also a source of food poisoning. Fungi produce mycotoxins in order to obtain a competitive advantage over other microbial species and as such are frequently produced in foodstuffs (Tucker, 2008). There are many different types of mycotoxins and some of the most important mycotoxins associated with food spoilage and foodborne illness are detailed as follows:

- *Aflatoxin* produced by many aspergilli. Aflatoxin is the most potent carcinogenic compound known to man (Bennett and Klich, 2003; Tucker, 2008).

- *Ocratoxin* produced by *Aspergillus ochraceus* and other aspergilli. It is both carcinogenic and a nephrotoxin. (Bennett and Klich, 2003; Tucker, 2008)

- *Patulin* produced by many *Penicillium* spp. It has antibiotic qualities but due to its toxic nature has not been able to be used in animals or humans (Bennett and Klich, 2003; Tucker, 2008).

- *Fumonisin* produced by *Fusarium* spp and *Alternaria alternata*. It has recently been associated with onset of some types of oesophageal cancers (Bennett and Klich, 2003).

- *Zearalenone* produced by *Fusarium graminerarum*. It is a non-steroidal oestrogen (mycoestrogen) and although not thought to be harmful to human health per se, its effects on fertility are questioned (Bennett and Klich, 2003).

- *Trichothecenes* produced by a family of over sixty fungal species, the main producer being *Fusarium* spp. Ingestion can lead to alimentary canal haemorrhaging, emesis and dermatitis (Bennett and Klich, 2003).

Table 2.2 *Common microorganisms associated with food spoilage and foodborne illness.*

Bacteria Gram Positive	**Bacteria Gram Negative**	**Fungi/Yeast**	**Viruses**	**Parasites**
Clostridium botulinum[b]	*Escherichia coli*[b]	*Botrytis* spp[a]	Hepatitis A[b]	*Trichinella spiralis*[b]
Clostridium perfringens[b]	*Salmonella* spp[b]	*Rhizopus stolonifer*[a]	Hepatitis E[b]	*Giardia lamblia*[b]
Bacillus cereus[b]	*Campylobacter jejuni*[b]	*Aspergillus* spp[ab]	Norovirus[b]	*Cryptosporidium parvum*[b]
Listeria monocytogenes[b]	*Shigella* spp[b]	*Byssochlamys* spp[ab]	Rotavirus[b]	
Staphylococcus aureus[b]	*Pseudomonas* spp[ab]	*Fusarium* spp[ab]	Enterovirus[b]	
Lactic acid bacteria[a]	*Vibrio parahaemolyticus*[b]	*Peniciillium* spp[ab]		
		Rhodotorula spp[a]		
		Zygosaccharomyces spp[a]		
		Brettanomyces spp[a]		
		Debaryomyces spp[a]		
		Candida spp[a]		
		Saccharomyces bailii[a]		

[a] Spoilage microorganism
[b] Foodborne illness related microorganism

2.1.3 Sick Building Syndrome

Microorganisms have also been implicated as a factor in "sick building syndrome" (SBS), where a person becomes ill due to the building in which they work or reside (Roston, 1997). SBS has been attributed to many factors including lighting, use of household cleaners and poor air ventilation (Bennett and Klich, 2003), however, the most frequently isolated factor associated with ill health is the presence of microorganisms, particularly fungi. The build up of dusts (including construction dusts) containing microorganisms, and the growth of moulds in damp areas such as windows, in many buildings has been associated with SBS (Bennett and Klich, 2003). Buildings, such as hospitals and other healthcare settings where immunocompromised (and ill) persons reside are at particular risk of SBS. Symptoms of SBS can include mild respiratory complaints, asthma attacks, irritations of the eyes and skin, headaches and hypersensitivity reactions (Roston, 1997). It is thought that mycotoxins produced by fungi may be a cause of such illness, however no leading cause of symptoms has been found.

2.1.4 Microbiological Safety

Microorganisms can often prove to be highly problematic in terms of their role in ill health but are also costly as previously discussed. Therefore it is vital to introduce microbiological safety measures to reduce the likelihood of disease. These include the implementation of microbiological safety legislations, increasing awareness of microbiological safety, transmission reduction and improvement, and the use of appropriate decontamination and sterilisation methods.

2.2 CURRENTLY AVAILABLE DECONTAMINATION METHODS

The methods used for inactivation of microorganisms in the hospital and the food processing and packaging environments are further discussed in this section.

2.2.1 Control of Microorganisms in the Hospital Environment

2.2.1.1 Disinfectants

In order to reduce the number of microorganisms in the environment, and also environmental cross-infection via carriage by items or persons it is necessary to use disinfectants. The disinfectants used depend on what they are going to be used on, and safety of use must always be considered.

2.2.1.2 Environmental Disinfectants

Chemical disinfectants are generally used in instances where heat inactivation of microorganisms is deemed impractical or likely to damage the equipment that is being decontaminated (NHS, 2007). Most areas in the hospital will first be cleaned with a general purpose detergent and a chlorine-containing agent such as 5% bleach (NHS, 2007). However, when decontaminating biological fluids such as blood then 1% sodium hypochlorite may be used (NHS, 2007). The decontamination of medical devices that cannot be adequately heat sterilised will be cleaned with 70% industrial methylated spirits or in the case of endoscopes, 0.26% paracetic acid solution would be used (NHS, 2007).

2.2.1.3 Antiseptics

Antiseptics can be used for skin disinfection of patients or staff. The use of 70% alcohol and/or 0.5% chlorhexidine to reduce the numbers of microorganisms on the skin is commonplace (NHS, 2007). The concentration and the specific antiseptic used depends on the site in which it will be used on the body and the procedure that being undertaken. Hand-washing in particular is seen to be one of the most effective methods of preventing the spread of microorganisms. Alcohol gels have been produced that are effective in inactivating microorganisms and are convenient as they

dry almost instantaneously and can be used wherever access to a sink is limited (NHS, 2005). Alcohol gels should always be used alongside good hand washing practices, as alcohol gels are not effective in removing visible dirt (NHS, 2005). During surgical procedures the use of 7.5% povidone-iodine is preferred as it rapidly eliminates many different microorganisms on the skin (NHS, 2007). However, compliance is a problem in hand washing, due to inconvenience and drying of the skin upon frequent hand washing/use of alcohol rubs (WHO, 2002; POST, 2005).

2.2.1.4 Sterilisation

In most instances the complete sterilisation of materials in hospitals will involve the use of heat, particularly moist heat for the sterilisation of medical devices, linens and hospital equipment (NHS, 2007). However, as described previously, this may not be possible for all devices or equipment if particularly inaccessible or heat-sensitive, although it still remains the most effective method of eliminating microorganisms.

2.2.1.5 Preventative Measures

In order to limit the transmission of microorganisms, further preventative measures have had to be put into place alongside disinfection and sterilisation. Barrier nursing of patients, the use of gloves, aprons and masks where necessary, are all effective methods in limiting the spread of microorganisms, when used correctly (POST, 2005). Closure of wards or use of specialised wards for patients carrying particularly dangerous or contagious diseases also reduces the likelihood of transmission of microorganisms to other patients. In some hospitals patients are pre-screened for MRSA, and prophylactic treatment may be administered prior to admission to limit its transmission (WHO, 2002; POST, 2005).

2.2.2 Control of Microorganisms in Food Processing and Packaging

Some of the methods used to decontaminate microorganisms in foods and food packagings described in this chapter have been used successfully for centuries. However, many of these technologies have negative effects on food qualities, and as such, novel methods have been produced in order to replace or be used in conjunction with existing microbial decontamination methods.

2.2.2.1 Heating

In order to inactivate microorganisms in foods it is necessary to heat the food product to a high temperature for a sustained period of time. This process is used routinely in the canning of foods where anaerobic conditions are ideal for the potentially life-threatening bacteria such as *C. botulinum* to thrive (Tucker, 2008). Microorganisms such as endospore-forming *Clostridium* spp and *Bacillus* spp are more difficult to inactivate than vegetative bacterial cells. Therefore, the temperatures used in thermal processing must be sufficient to inactivate bacterial endospores/toxins and prevent the formation of toxins. As *C. botulinum* is the most harmful bacterium associated with foodborne illness, it is often considered a worst case scenario and many food safety strategies use the inactivation of *C. botulinum* as a standard (Tucker, 2008).

2.2.2.2 Chilling/Freezing

Chilling of foods is a preferred method of food preservation, as it does not have negative effects on the organoleptic properties of foods (Tucker, 2008). The maintainance of below ambient temperature is considered to be chilling. Reducing the temperature of foods can limit the growth of many microorganisms, however, microorganisms such as *Listeria monocytogenes* can grow at temperatures as low as 4°C (Tucker, 2008). Nevertheless, refrigeration is a standard method of limiting the growth of many microorganisms.

The freezing of foods is a long-term preservation technique that like chilling maintains many of the organoleptic and nutritional qualities of foods. The reduction in temperature significantly limits the ability of microorganisms to grow, even more so than chilling (Tucker, 2008). Also, as available water in the food freezes, microbial deterioration of the food is less likely than in high water available foods (Tucker, 2008).

However, both chilling and freezing of foods can be problematic upon thawing as those microorganisms present upon chilling/freezing may remain viable and begin to grow in the increased temperature environment (Tucker, 2008). Therefore, the

heating of foods after thawing must be sufficient to inactivate any remaining microorganisms. This presents a particular problem in ready-to-eat foods.

2.2.2.3 Chemical Preservative Agents

For hundreds of years salt and acids have been used as methods of "curing" or preserving foods. Most microorganisms grow in a narrow pH range and as such are sensitive to acidic conditions, limiting the growth and possibly even destroying microbial cells (Tucker, 2008). Salting of foods disrupts the osmolarity of microbial cells on the foodstuff, causing cells to rupture. Today, modern preservatives such as organic acids, sulfites, nitrates, nitrites and ethylenediamine-tetraacetic acid (EDTA) are used to stress microbial cells and preserve foods (Rangan and Barceloux, 2009). However, there has been some concern about the effects of many of these food additives, also known as E numbers. Some studies have claimed that these additives are potentially toxic and even carcinogenic (Rangan and Barceloux, 2009).

2.2.2.4 Modified Atmosphere Packaging

Modified atmosphere packaging (MAP) is a relatively new technology used to minimise the growth of microorganisms in packaged foods that involves the removal of air from the packaging, which is then replaced with a modified gas mix of known composition. The mix may be similar to the content of air including oxygen, nitrogen and carbon dioxide in different quantities but may also contain inert gases such as argon and carbon monoxide (Tucker, 2008). The mix of gas is highly dependent on the foods used and the suspected microbial contamination. Carbon dioxide can be antimicrobial and is commonly used to reduce the oxygen content in the modified atmosphere for many oxygen-requiring microorganisms, however microaerophilic and anaerobic microorganisms may thrive under such conditions. It has been noted that common foodborne illness related microorganisms such as *L. monocytogenes* remain largely unaffected by MAP (Tucker, 2008). In addition, different plastic films can be used in MAP, depending on the food being packaged many of which are microwaveable (Gould, 1995).

2.2.2.5 Novel Antimicrobial Technologies

Irradiation

Irradiation of foods using gamma rays, X-rays and electron beams has been proven to be very effective in inactivating many microorganisms (Tucker, 2008). Both gamma rays and X-rays are short wavelength photons that can penetrate relatively easily into solid foods. In general electron beams that emit β particles are less penetrative than gamma rays and X rays and they have less effect on the nutritional properties of foods. However, many dairy products and high-fat foods are prone to producing an off-odour or undergoing oxidation upon exposure to ionising irradiation (Gould, 1995). Poor public understanding of these technologies, which are frequently confused with radioactivity, hinders the use of ionising technologies for decontamination of foods (Tucker, 2008).

Ohmic Heating

Ohmic heating also known as electrical resistance heating, involves an alternating current being passed through foods. This method relies on the principle that foods have a natural electric resistance, allowing food to become heated upon application of an electrical current via electrodes (Tucker, 2008). Foods can be heated more quickly than in conventional heating technologies and as a result foods retain more of their original textural and organoleptic qualities than in processes such as canning (Tucker, 2008). However, in order to heat uniformly the components of the food must have similar electrical conductivity and foods that are not ionically loaded such as fats and oils are not suitable for processing in this way (Gould, 1995).

High-pressure Processing

High-pressure processing is generally considered a viable alternative to many thermal technologies. Foods are placed under a high pressure of 100-600 MPa, which is usually produced using water, at room temperature for around 10 minutes (Gould, 1995). Foods that cannot be decontaminated using heat treatment such as shellfish, fruits and vegetables can be treated using high-pressure processing (Tucker, 2008). High-pressure processing has little adverse effect on food nutritional

qualities, however it has been known to have effects on textural qualities. At present the cost of the technology is considerably greater than other available technologies and it has a limited capacity in terms of the amount of food that can be treated, thereby limiting throughput (Gould, 1995).

Pulsed Electric Fields

In pulsed electric field (PEF) processing, foods are placed between two electrodes and a high voltage field (20-80 kV/cm) is produced across the electrodes (Mosqueda-Melagar *et al*., 2008). The voltage alters the transmembrane potential of microorganisms, causing the cell membrane to rupture which leads to cell death. The treatment times required in many food applications may be less than a second in duration (Mosqueda-Melagar *et al*., 2008). This technique is also an alternative to thermal processing, as it does not negatively alter sensory properties of foods. However, larger pulses can cause heat production (Mosqueda-Melagar *et al*., 2008). Similarly to ohmic heating processing, the electrical conductivity of foods plays an important role in food treatment. High conductivity foods reduce the peak electrical field that can be applied across the chamber, limiting the anti-microbial effectiveness of the treatment (Mosqueda-Melagar *et al.*, 2008).

2.2.2.6 Hurdle Effect

Whether contaminating microorganisms within foodstuffs can grow enough to cause food spoilage or foodborne illness is dependent on many factors, or hurdles (Gould, 1995). Common hurdles include pH, water activity, redox potential and preservatives present in foods, as well as any technologies used to inactivate or reduce microorganisms such as heat treatment or MAP (Gould, 1995; Tucker, 2008). The hurdles should be sufficient enough to limit or stop microorganisms growing in the food. However, the critical levels of each hurdle must be examined thoroughly in order for this method of preservation to be effective (Gould, 1995). As consumers are now demanding fresher, minimally processed foods, it is likely that hurdle methods will be used more frequently.

2.2.3 Need for New Decontamination Methods

There are many different methods available to control the numbers of microorganisms in the hospital and food processing environments. However, no method can be used in all situations, as many are not 100% effective and some technologies are not feasible for use in certain situations. The use of multiple methods to control the microorganisms is likely to become even more commonplace and as such complementary technologies are required.

2.3 LIGHT DECONTAMINATION TECHNOLOGIES

The use of light technologies for the inactivation of microorganisms itself is not novel. However, due to the resistance of many microorganisms to antibiotics, disinfectants and other antimicrobials, alternative decontamination methods are constantly being sought.

2.4 ULTRAVIOLET LIGHT

Light is transmitted in discrete bundles of energy called photons, which have both frequency and wavelength (Bolton, 2001). Ultraviolet (UV) light wavelengths are divided into 3 different categories according to their effects on human skin:

UV-A	**UV-B**	**UV-C**
λ= 315-400 nm	λ= 280-315 nm	λ=200-280 nm

UV-A wavelengths are associated with “tanning” or melanin production in skin, UV-B is associated with burning and melanomas, and UV-C is the highly-germicidal part of UV light that can cause DNA mutations and skin cancers (Bolton, 2001).

2.4.1 UV Inactivation Mechanism

UV light is effective for inactivating a range of microorganisms including bacteria, fungi, viruses and protozoa (Bank *et al*., 1990; Shin *et al*., 2001; Jun *et al*., 2003 and Eischeid *et al*., 2009). The part of the spectrum optimal for the inactivation of microorganisms is UV-C. UV-C is particularly germicidal as microorganism cell

contents particularly DNA, RNA and proteins absorb UV-C light with optimal absorption at 250-260 nm (Block, 1991).

The absorbed light can cause 2 main types of lesions in DNA, cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts ((6-4) PP) (Sinha and Häder, 2002). In CPD and (6-4) PP formation two adjacent pyrimidines in DNA/RNA become covalently cross-linked. In CPDs this causes the formation of a 4-membered cyclobutyl ring (Sinha and Häder, 2002). Both lesions involve pyrimidine bases and thymine is frequently associated with these UV-induced mutagenic lesions (thymine dimers) as is cytosine, though to a much lesser extent (Sinha and Häder, 2002). The production ratio of these mutagenic lesions upon exposure to UV light is around 3:1, CPD:(6-4) PP. CPD and (6-4) PP lesions cause kinks in DNA or RNA of 7-9° and 44°, respectively (Thoma, 1999). These UV-induced lesions stop transcription and replication of nucleic acids or cause the misreading of DNA/RNA by polymerase enzymes, leading to mutation and cell death.

2.4.2 UV Repair Mechanisms

Microorganisms have evolved repair mechanisms that excise mutagenic lesions through both light-independent and light-dependent mechanisms known as dark repair and photoreactivation, respectively.

2.4.2.1 Dark Repair

There are two types of dark repair- base excision repair (BER) and nucleotide excision repair (NER). These mechanisms cannot directly reverse the DNA mutations caused by UV light, however they are capable of excising lesions and replacing excised DNA/RNA.

BER utilises DNA glycosylase in conjunction with apurinic/apyrimidinic endonuclease/lyase and phosphodiesterase to remove UV-affected bases (Sinha and Häder, 2002). The gap in the nucleic acid is then filled by DNA/RNA polymerase and sealed with ligase (Sinha and Häder, 2002).

NER is a highly conserved repair mechanism amongst almost all microorganisms. The proteins UvrA, UvrB, UvrC and UvrD are involved in the mutagenic repair process (Goosen and Moolenaar, 2008). UvrA scans the nucleic acid looking for any mutagenic lesions, and upon finding a lesion the UvrB protein binds tightly to the region and UvrA dissociates (Goosen and Moolenaar, 2008). UvrC then associates with the lesion/UvrB complex and excises the mutated oligonucleotide unit (Goosen and Moolenaar, 2008). UvrD (Helicase II) removes the oligonucleotide, and polymerase I and ligase restore the nucleic acid to its pre-lesion state (Goosen and Moolenaar, 2008).

2.4.2.2 Photoreactivation

The repair process termed photoreactivation requires energy in the form of light to activate an enzyme called photolyase that can repair damage caused by UV light. Not all microorganisms possess the photolyase enzyme (Table 2.3), however it is likely that it has been lost by microorganisms that are not frequently exposed to UV-light in the form of solar radiation.

Table 2.3 *Examples of photolyase enzyme distrubtion amongst different organisms. Adapted from Sancar, 1994.*

	Presence of Photolyase	**Absence of Photolyase**
Bacteria	*Escherichia coli* *Bacillus firmus*	*Haemophilus influenzae* *Bacillus subtilis*
Eucarya	*Saccharomyces cerevisiae* *Monodelphus domesticus*	*Schizosaccharomyces pombe* *Homo sapiens*
Archaea	*Methanobacterium thermoautotrophicum*	*Methanococcus vannelii*

DNA photolyases are flavin-dependent repair enzymes, which can be one of two classes known as folate and deazaflavin (Sancar, 1994). Photolyases contain two chromophores, which have absorption maxima of ~380 nm and ~440 nm respectively (Sinha and Häder, 2002). Upon exposure to light of wavelength 300-500 nm the photoreactivation process can occur. Specific molecules within cells, known as chromophores, absorb light energy enabling it to interact with other biological molecules in order to produce a chemical reaction (Young, 1997). Chromophore molecules have very specific absorption spectra, however each will have their own range of specific absorption wavelengths (Young, 1997). One of the chromophores present in photolyase is a blue light-harvesting antenna that absorbs photons of blue light and transfers the excitational energy to the flavin-adenine dinucleotide chromophore (Sancar, 2000). The excited state chromophore transfers an electron to the covalent dimer bond, producing a pyrimidine dimer anion (Sinha and Häder, 2002)(Figure 2.2). The anion will rearrange to form the correct bond, returning the dimer to its original monomeric state (Sancar, 2000). Once a photolyase enzyme has bound to a mutagenic lesion its photoreactivation efficiency is high, with approximately one dimer split into its original monomeric form for every photon of blue light absorbed (Sinha and Häder, 2002).

The kinetics of photoreactivation are characteristic of Michaelis-Menten reactions as shown in Equation 2.1 (Sancar, 2000):

$$E + S \underset{k_2}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_p I = k_3} E + P \qquad [2.1]$$

Where, E is the photolyase enzyme, S is the substrate (the lesion), ES is the enzyme-substrate complex, P is the product (monomers), k_1 is the rate at which the enzyme finds and attaches to the lesion, k_2 is the enzyme dissociation constant, I is the intensity of light, k_p is the rate of photolysis and k_3 (k_pI) is the rate of photolysis which is affected directly by light intensity (Sancar, 2000). The rate of converting an enzyme-substrate complex to enzyme + products (monomers) is limited by k_3, as

photolyase will bind quickly to a lesion, however it requires light energy for action. (Sancar, 2000).

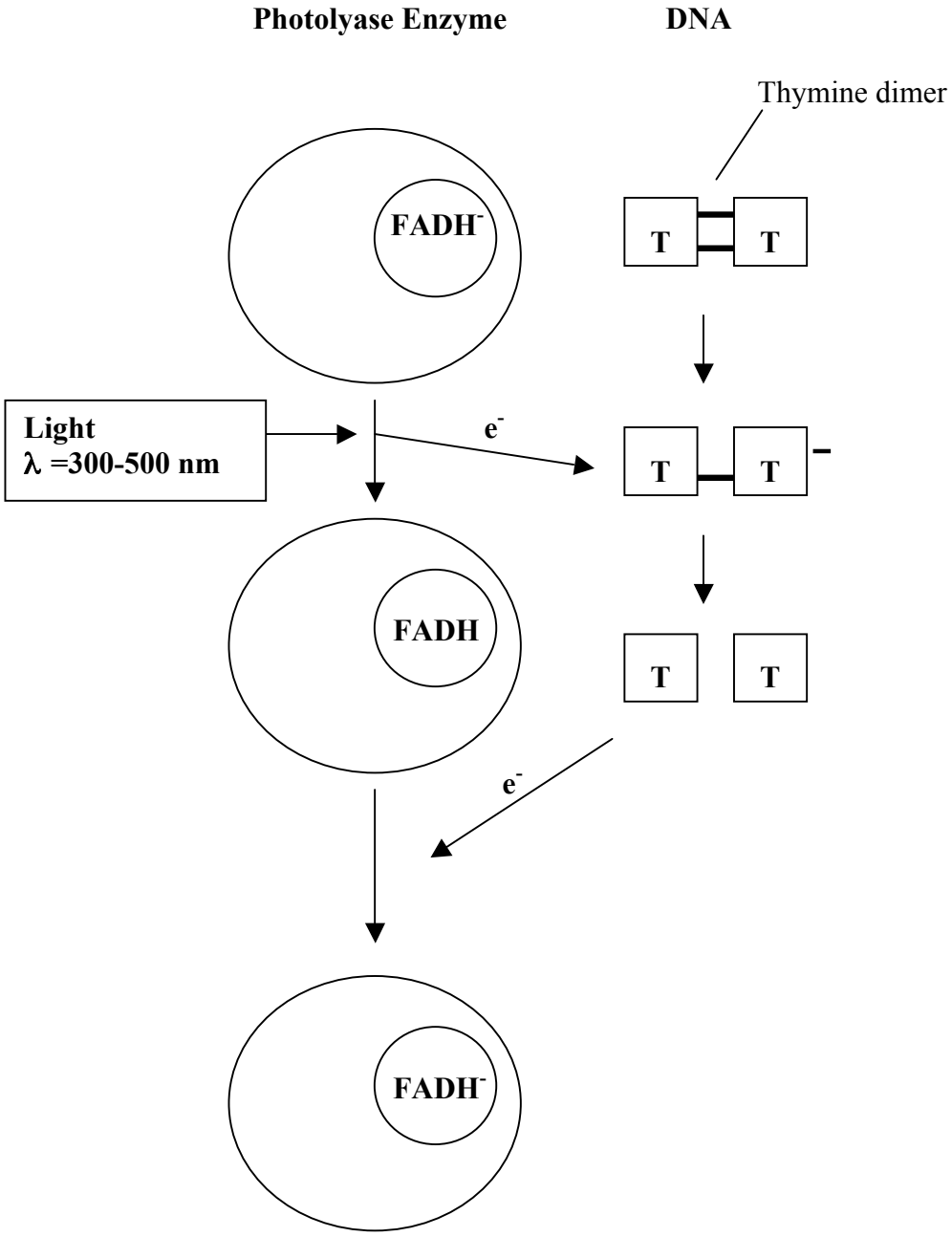

Figure 2.2 *The photoreactivation repair process of UV-induced thymine-dimers using the photolyase enzyme and FADH co-factor complex.*

2.4.3 Uses of UV Light For Disinfection Applications

UV light is highly antimicrobial and as such, UV light technologies have been developed for a variety of decontamination applications. UV light has been found to be particularly useful in the decontamination of water, air, surfaces and foods.

Water

UV light has been successful for the inactivation a range of microorganisms associated with contaminated water including *Giardia* spp and *Cryptosporidium* spp (Shin *et al*., 2001; Mofidi *et al*., 2002). No by-products are produced as a result of UV light exposure of water and as such it has not been favoured for disinfection of wastewater supplies, where the flow of water allows possible contamination to occur further downstream (EPA, 1999). As UV light cannot penetrate well into opaque liquids its usefulness as a water disinfection technology is limited (EPA, 1999).

Air

UV light technology has more recently been applied to air ventilation/filtration systems as an added measure to reduce the microbial load present in the air. UV in combination with high efficiency particulate absorbing (HEPA) filters have been introduced in settings where microorganisms may pose a particular health risk, or where person may be exposed to contaminated air for long periods of time in order to reduce the likelihood of sick building syndromes (Roston, 1997; Kowalski and Bahnfleth, 2002).

Surfaces

Surfaces can also be disinfected using UV light technologies. However, UV light has low penetrating power and therefore textured surfaces, chopping boards and natural materials such as woods may not be adequately disinfected. In addition, UV light has negative effects on some types of plastics, causing them to become brittle (Andrady *et al*., 1998).

Foods

UV light irradiation is useful on foods that have uniform, flat surfaces such as steaks and other cuts of meat (Stermer *et al*., 1987). Minced meats and other irregular foods cannot be thoroughly decontaminated using UV light, again due to its poor penetration ability (Block, 1991). Although very little detrimental effects to food qualities are seen when utilising UV light as a decontamination technology (Stermer *et al*., 1987).

2.4.4 Ultraviolet Light Sources

UV light has traditionally been applied in a continuous manner for the inactivation of microorganisms. The total germicidal dose of UV light required for complete inactivation varies for each microorganism. The dosage of light received by the microorganisms (D, expressed as mJ cm^{-2}) depends on the light exposure duration (t, expressed in seconds) and the intensity of the UV light source (I, expressed in mW cm^{-2}), as shown in Equation 2.2 (Wang, 2005):

$$D = It \qquad [2.2]$$

2.4.4.1 Mercury Lamps

Mercury (Hg) lamps are a traditional source of monochromatic UV radiation. Electrical arcs are produced in UV-transmitting tubes, usually quartz, containing a mixture of elemental mercury (Hg) and an inert gas (Block, 1991). Electrodes at either end of the tube produce a voltage across the Hg-gas mix causing photon release (Block, 1991).

Low-pressure mercury lamps have a partial pressure of ~10^{-3} mbar and a total vapour pressure of ~2 mbar (Block, 1991). They produce several emission lines in the visible and UV parts of the electromagnetic spectrum. The most germicidal is the emission line at 254 nm and the line produced at 185 nm is seen to be of little germicidal importance. Medium-pressure mercury lamps have an internal pressure of 2-5 bar and they produce a polychromatic spectrum (Block, 1991).

2.4.4.2 Excimer Lamps

In excimer lamps an electrical potential is produced across a dielectric barrier, containing a rare gaseous mixture (Block, 1991). Excimer lamps produce monochromatic emissions, and the gas mix that is used in the lamp determines the specific output (tunable) (Block, 1991).

2.4.4.3 Xenon Pulsed UV Lamps

Xenon-filled flashlamps produce polychromatic light from the UV to the Infrared region, although the output is rich in germicidal UV-C wavelengths (Block, 1991). Xenon flashlamps release intense, short-duration pulses of light, which cannot be achieved through continuous UV light exposures (Maclean *et al*., 2008c). UV light flashes are generated using storage of large amounts of energy, which is then rapidly dissipated using a high voltage (Maclean *et al*., 2008c). Owing to the rapid production of high peak power pulses of UV-rich light, Xenon flashlamps are ideal for use in disinfection processes.

Table 2.4 compares the attributes of some of the available UV light sources.

Table 2.4 *Characteristics of UV light sources. Adapted from Block, 1991.*

UV Light Source	Electrical Efficiency (%)	Lamp Surface Temperature (°C)	Spectral Emission
Low-Pressure Hg Lamp	50	40	Monochromatic 253.7 nm
Medium-Pressure Hg Lamp	15-30	400-1000	Polychromatic 200-300 nm
Excimer Lamp	10-35	Ambient	Monochromatic Tunable
Pulsed Xenon Lamp	15-20	-	Polychromatic 200-300 nm

2.4.5 Benefits and Limitations of UV Light Technologies

UV light is effective for reducing microbial numbers in water, air and on some surfaces. Pulsed UV light sources such as Xenon flashlamps, provide a particularly rapid means of microbial inactivation.

Although not considered to be a thermal technology, there can be heat build up in the use of continuous and pulsed UV light technologies, which may be synergistic in the inactivation of microorganisms (Rowan *et al*., 1999). UV light is also particularly harmful to human skin and eyes and as such must be used in enclosed environments. UV light is not useful in processes designed to inactivate opaque liquids or aggregates of microorganisms due to its poor penetrating ability (EPA, 1999).

UV lamps emitting polychromatic light must take into account that the UV-A emissions of such lamps can cause concomitant photoreactivation to occur, impeding UV light inactivation (Maclean *et al*., 2008c).

2.5 PHOTODYNAMIC INACTIVATION USING LIGHT AND PHOTOSENSITISERS

Photodynamic inactivation (PDI) is a process in which a photosensitiser molecule, often a non-toxic dye, becomes excited upon absorption of UV/visible light, resulting in electron transfer and production of reactive species. These reactive species are capable of oxidising vital cell components and can eventually lead to cell death (Hamblin and Hasan, 2004). In PDI the targets of the phototoxic agent are usually microorganisms, however PDI has alternative uses in medicine and is often also termed photodynamic therapy (PDT). Although the effects of PDI have been known for over 100 years the introduction of antibiotics in the 1940s meant interest in the use of alternative therapies such as PDI faded out (Maisch *et al*., 2004). However, with the emergence of antibiotic-resistant microorganisms coupled with the lack of new antibiotics, interest in PDI and PDT has re-emerged and these alternative therapies are been researched once more.

2.5.1 PDI Inactivation Mechanism

Photosensitisers (chromophores) absorb light of a particular wavelength, which causes excitation of the photosensitiser molecule to the singlet state (Maisch *et al*., 2004). The photosensitiser molecule can then undergo intersystem crossing to the triplet state that is lower energy but is longer-lived than the singlet state (Hamblin and Hasan, 2004). The triplet state can then react in two different ways known as the Type I and Type II pathways (Figure 2.3). Both of these pathways require the presence of oxygen (Maisch *et al*., 2004).

The Type I pathway involves electron-transfer from the photosensitiser molecule in its triplet state to a substrate with subsequent production of radical ions (Figure 2.4)(Maisch *et al*., 2004). These radical ions can then interact with molecular oxygen (O_2) to produce various toxic products such as superoxide, hydrogen peroxide and hydroxyl ions (Maisch *et al*., 2004). The Type II pathway involves the direct interaction of the triplet state photosensitiser molecule with O_2 (Figure 2.4). Electron-transfer from the photosensitiser causes the production of singlet oxygen ($^1O_2$), which is a highly toxic substance capable of oxidising many cell components such as proteins, lipids, cell membranes and even nucleic acids (Maisch *et al*., 2004).

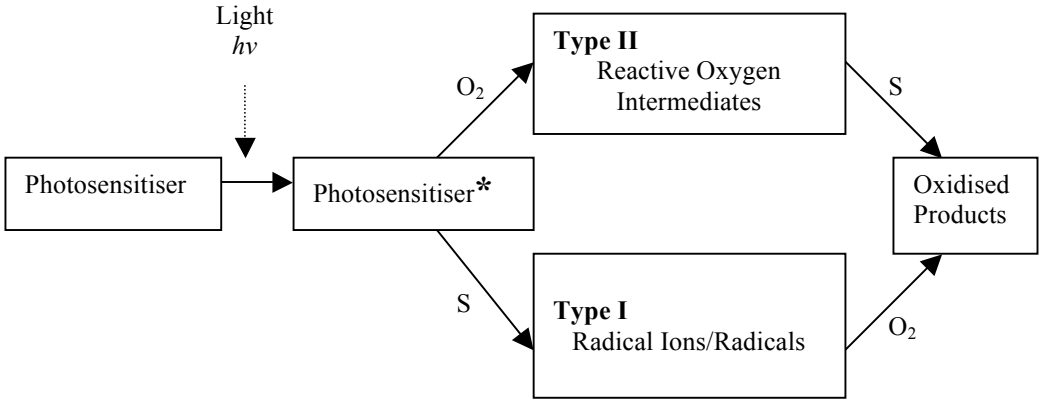

Figure 2.3 *Type I and Type II photosensitisation pathways. Interactions with triplet state photosensitiser (*) and either an organic substrate (S) or molecular oxygen (O_2) leads to production of oxidised cell products. Adapted from Maisch et al., 2004.*

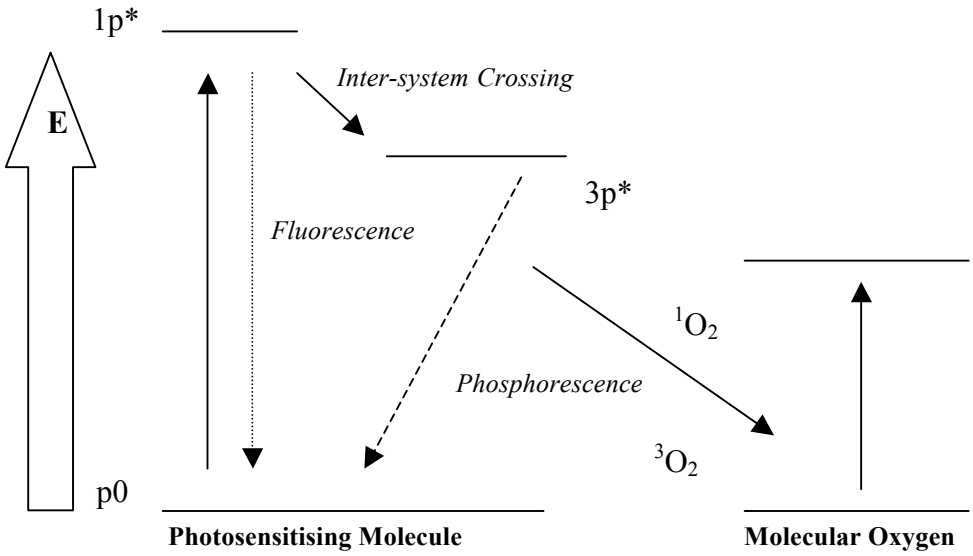

Figure 2.4 *The effect of light on photosensitising molecules (Type I and II) and molecular oxygen (Type II). Adapted from Lukšienė and Zukauskas,, 2009.*

In order for the photosensitiser to cause microbial cell damage, the photosensitising molecule must be taken up and accumulated in the target cell prior to light exposure. This is relatively easy in Gram positive microorganisms as they only possess a 40-80 nm peptidoglycan wall that contains a very small number of proteins and lipids (Maisch *et al*., 2004). Gram negative bacteria also possess this peptidoglycan wall but also have another membrane outside of the cell wall that contains negatively charged lipopolysaccharides and proteins. Only molecules smaller than 1 kDa can pass through porin channels in the cell membrane of Gram negative bacteria meaning the uptake of many photosensitising molecules is either poor or not possible (Maisch *et al*., 2004). To aid the crossing of this membrane, molecules such as nonapeptide polymyxin and Tris-EDTA, which destabilise the outer membrane of Gram negative bacteria, are frequently used (Hamblin and Hasan, 2004). Photosensitisers with an overall positive charge, meso-substituted cationic porphyrins and water soluble zinc phthalocyanines have been found to be capable of traversing the cell membrane of Gram negative bacteria without the addition of membrane destabiliser molecules (Hamblin and Hasan, 2004; Maisch *et al*., 2004; Nitzan *et al*., 2004).

Photosensitiser molecules have unique absorption spectra, therefore the wavelength of light required by the photosensitiser molecule to induce phototoxic reactions is also an important factor to consider in PDI and PDT applications. There are a variety of molecules that are described today as "photosensitisers". These include porphyrin molecules, porphyrin pre-cursor molecules such as δ-aminolevulinic acid (ALA) but also phthalocyanines, chlorines, pheophorbides, phenothiazines, porphycenes and many others (Hamblin and Hasan, 2004; Lukšienė, 2005*)*. Common porphyrin and chlorine derivatives tend to have a high absorption peak at around 400 nm known as the soret band (Goldoni, 2002). However, this is highly variable across the many different photosensitisers currently in use. The photosensitisers used to inactivate a range of different microorganisms are shown in Table 2.5.

Table 2.5 *Examples of photosensitisers used to inactivate microorganisms in PDI. Adapted from Hamblin and Hasan, 2004 and Lukšienė, 2005.*

Microorganism	Photosensitiser Used For PDI
Gram Negative Bacteria	
Escherichia coli	Hematoporphyrin, cationic hydrophilic porphyrin, "photosens", methylene blue, toluidine blue, ALA, zinc pyrimidiniumphthalocyanine, cationic, neutral and anionic tetraphenylporphyrins, thiazines, xanthenes, acridines, phenazines
Acinetobacter baumannii	Cationic hydrophilic porphyrin, tetra (4-methyl pyridyl) porphyrin
Propionibacterium acnes	Methylene blue
Pseudomonas aeruginosa	"Photosens", zinc pyridiniumphthalocyanine, methylene blue, toluidine blue
Helicobacter pylori	Hematoporphyrin derivative
Campylobacter rectus	Chlorin e6, toluidine blue
Gram Positive Bacteria	
Staphylococcus aureus	Methylene blue, "photosens", toluidine blue, hematoporphyrin, malachite green isothiocyanate, cationic, neutral and anionic tetraphenyl porphyrins, rose bengal
Methicillin-resistant *S. aureus*	Methylene blue and derivatives
Staphylococcus epidermidis	Methylene blue, "photosens"
Streptococcus pyogenes	Methylene blue
Enterococcus faecalis	Methylene blue, toluidine blue
Bacillus subtilis	Hematoporphyrin derivative
Fungi and Yeasts	
Aspergillus fumigatus	Green 2W
Sachharomyces cerevisiae	Glycosyl porphyrins, hematoporphyrin
Candida albicans	Rose Bengal, zinc phthalocyanine
Viruses	
Human Immunodeficiency Virus (HIV)	Methylene blue, hypocrellin, hypericin, rose bengal
Herpes Simplex Virus (HSV)	Merocyanine 540, sapphyrins, hematoporphyrin derivative
Influenza Virus	Hypericin, rose bengal

2.5.2 Applications of PDI/PDT

As previously discussed, photosensitisers absorb light photons of a particular wavelength resulting in the production of toxic species capable of inactivating a range of microorganisms. Owing to this germicidal ability the use of PDI as a treatment for infections is being investigated (Maisch *et al*., 2004).

PDI has been examined as a possible means of inactivating bacterial pathogens of the oral cavity frequently associated with periodontal disease. Rovaldi *et al*. (2000) demonstrated the inactivating effect of a chlorine e6-pentalysine conjugate photosensitiser used in conjunction with a 662-nm laser light on oral pathogens including *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Actinomyces viscosus* and others.

PDI has been highlighted as a possible solution to topical antibiotic treatment of wound infections. Orenstein *et al*. (1998) and Hamblin *et al.* (2002) investigated the use of deuteroporphyrin-hemin complex (exposed to incandescent light) and a poly-lysine/chlorine e6 conjugate (exposed to 660-nm laser light) in animal model wound infections, respectively. They found that both *S. aureus* and *Escherichia coli* were readily inactivated and Orenstein *et al*. (1998) even reported that the photosensitiser was lethal to *S. aureus* prior to exposure to light.

In addition to the uses of PDI in wound treatments, the process has been applied to other problematic skin conditions. The effect of PDI treatment of Impetigo contagiosa, caused primarily by streptococcal infection has also been investigated (Maisch *et al*., 2004). Zeina *et al*. (2002) found that *Streptococcus pyogenes* was readily inactivated upon application of methylene blue and exposure to 400-700 nm filtered light. Many studies have focused on the use of PDI in inactivating acne bacteria. Application of topical photosensitiser 5-aminolevulinic acid (ALA) has proven to be particularly effective in reducing the number of acne lesions caused by bacteria and reducing formation of new acne lesions (Itoh *et al*., 2001).

A rapidly emerging field in PDT is the treatment of cancers. Whereby a specific photosensitiser is preferentially absorbed into malignant tissues and light is applied to the affected area, resulting in ROS production and cell death (Patrice, 2003). Currently only Photofrin® is licensed for use in clinical applications of PDT and has been used to successfully treat cancers of the oesophagus, lungs, bladder, cervix and stomach (Moor, 2000).

2.5.3 Light Sources

The light source that is utilised in PDT has a bearing on the clinical outcome as different wavelengths of light can penetrate to different tissue depths (Figure 2.5), for example light of 630 nm can penetrate 2-3 mm into tissues whereas light at longer wavelengths of 700-800 nm can penetrate deeper into tissues, 5-6 mm (Patrice, 2003). The energy of light photons is inversely proportional to the wavelength (λ) utilised, therefore the light used in PDT/PDI must be sufficient enough to penetrate tissues and but must also have enough energy to stimulate the production of singlet oxygen (Patrice, 2003). As such, many clinical applications of PDT operate at 630-700 nm as wavelengths below 600 nm are absorbed by blood cells and wavelengths above 800 nm have lower energy and cannot stimulate a cytotoxic response in cells (Patrice, 2003).

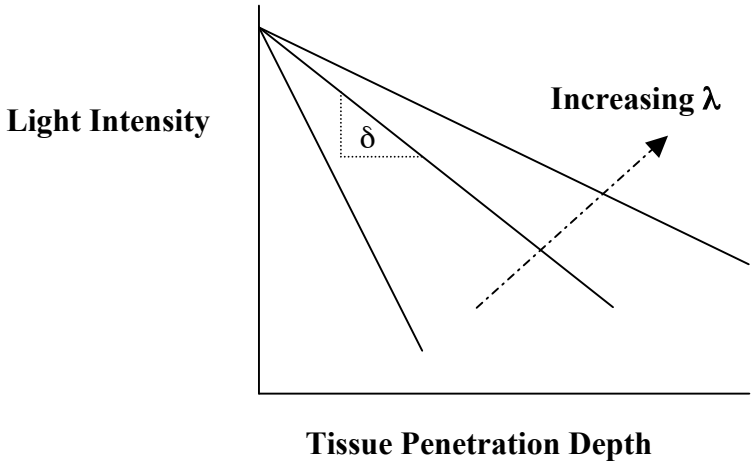

Figure 2.5 *Graphic representation of the depth of penetration of light into tissue upon increasing wavelength and light intensity. The most effective light intensity/penetration depth is highlighted with δ. Adapted from Patrice, 2003.*

Lasers

Lasers are capable of emitting light of a very narrow bandwidth and are seen to be highly efficient light sources. Argon lasers are commonly utilised in PDT/PDI as they can emit high intensity light (1 W cm^{-2}) at narrow bandwidths to stimulate photosensitiser molecules (Brancaleon and Moseley, 2002).

Metal vapour lasers are also frequently used, however more commonly in a pulsed manner (10-50 ns pulse width) (Brancaleon and Moseley, 2002). These lasers give a slightly broader bandwidth than argon lasers, although the light intensity emitted by the metal vapour laser is of the order of hundreds of mW (Brancaleon and Moseley, 2002).

Solid state lasers such as neodymium-doped yttrium aluminium garnet (Nd:YAG) lasers can also be used in a pulsed manner also and are more recent additions to PDT/PDI. They emit sub-nanosecond pulses, which can have very high repetition rates (Brancaleon and Moseley, 2002). Nd:YAG lasers emit in the infrared region 1064 nm, however the laser can undergo frequency doubling to give lines at 532 nm and even 266 nm (UV) (Brancaleon and Moseley, 2002).

In general, although lasers can be extremely efficient in stimulating the photosensitiser molecule they are expensive pieces of equipment and are often cumbersome and not highly portable which may not be useful in some PDT/PDI treatments. Lasers can often be coupled with optical fibres to produce light sources that can be used in previously inaccessible areas such as the lungs and stomach (Brancaleon and Moseley, 2002). However, they also need to be frequently maintained and require specialist knowledge in order to operate safely (Brancaleon and Moseley, 2002).

Wavelength Filtered Lamps

Many different high power lamps such as Xenon arc lamps, metal halide lamps, fluorescent lamps and tungsten filament quartz halogen lamps can be used for PDT/PDI in conjunction with wavelength filters (Brancaleon and Moseley, 2002; Calin and Parasca, 2009). As these lamps emit broadband light it is necessary to limit the effects of UV (mutational) and infrared (heat/hyperthermia) due to their effects on the skin (Brancaleon and Moseley, 2002). Lasers require the use of a single photosensitiser as they emit narrow monochromatic light, however more than one photosensitiser can be used when using lamps in PDT/PDI due to their polychromatic nature. The upkeep of lamps is also less complicated and expensive than that of lasers (Brancaleon and Moseley, 2002).

Light Emitting Diodes

Light Emitting Diodes (LEDs) can be used in LED arrays to emit a range of wavelengths from UV-A to near Infrared (Power Output of up to 150 mW cm^{-2}) (Brancaleon and Moseley, 2002). LEDs are highly portable and are relatively inexpensive compared to other light sources currently available for use in PDT/PDI (Brancaleon and Moseley, 2002). They require no specialist knowledge to operate and could be applied to many different clinical applications of PDT/PDI.

2.5.4 Benefits and Limitations of PDI

PDI has been shown to be effective in the inactivation of microorganisms in many therapeutic instances. With the introduction of novel light sources such as LED arrays and optical fibres it has been possible to treat lesions more efficiently than before, even previously light-inaccessible areas, allowing the specific infected site to be targeted with the photosensitiser and light, giving dual specificity. It has also been suggested that PDI might offer specific advantages over the use of antibiotics and other drug treatments, as it is highly unlikely that microorganisms could develop a similar resistance to PDI as is seen in antibiotic and drug treatments (Maisch *et al*., 2004).

PDI treatments can be complicated as all microorganisms are susceptible to different types of photosensitisers, whose action can only be induced upon exposure to specific wavelengths of light. Therefore this knowledge is essential for effective treatment. In addition phototoxicity of the normal tissues surrounding the treated area is a common side effect of PDI (Patrice, 2003). As PDI is an emerging clinical procedure it is likely that many healthcare specialists will not be trained in its use and as a result may be reluctant to use the technology (Patrice, 2003).

2.6 BLUE LIGHT PDI

During PDI studies it was theorised that microorganisms that contain a large amount of endogenous intracellular porphyrin molecules might be susceptible to light inactivation without the addition of exogenous photosensitisers, due to the fact that porphyrin derivatives such as hematoporphyrin are used commonly as photosensitisers.

2.6.1 Porphyrins

Porphyrins are molecules that are present naturally in many organisms. They are responsible for many different biological processes such as oxygen transport, electron transport, photosynthesis and pigmentation (Goldoni, 2002). The basic structure of porphyrins consists of 4 tetrapyrrolic subunits linked by methane bridges

and 4 nitrogen atoms (Figure 2.6) (Goldoni, 2002). This framework is also the most basic porphyrin, known as porphine.

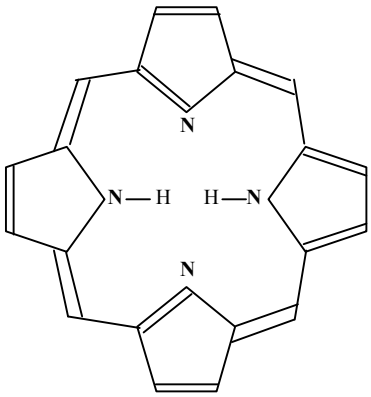

Figure 2.6 *Basic structure of the simplest porphyrin molecule, porphine.*

The porphyrin molecule is capable of binding many metal ions including zinc, iron, copper, cobalt and nickel, an example of which is haemoglobin a porphyrin used to transport oxygen in blood (Goldoni, 2002). Porphyrin molecules are able to absorb photons of light and absorb extremely well at ~400 nm (Soret band) as well as weakly absorbing light at 450-700 nm (Q bands), although the precise absorption maxima can be altered slightly by other constituents of porphyrins such as the bound metal ions (Goldoni, 2002). Metal-bound porphyrins cannot be used in PDI as the metal moiety quenches any singlet oxygen produced upon photo-stimulation (McCarthy and Weissleder, 2007). This process is further described in Section 2.6.2.

The levels and types of porphyrins present in microorganisms are thought to be organism-specific. *Propionibacterium acnes* has been shown to have predominantly coproporphyrin and protoporphyrin IX (PpIX) (Cunliffe and Goulden, 2000; Ashkenazi *et al*., 2003). The black oral pigmented bacteria associated with periodontal disease have varying porphyrin content consisting manily of PpIX and varying quantities of coproporphyrin, uroporphyrin and heptacarboxyl porphyrin (Soukos *et al*., 2005). *Staphylococcus aureus* contains coproporphyrin and

uroporphyrin and *E. coli* contains coproporphyrin, uroporphyrin and PpIX (Nitzan and Kauffman, 1999; Soukos *et al*., 2005).

2.6.2 Inactivation Mechanism

The mechanism for inactivation of microorganisms using light is theorised to be similar to that of PDT/PDI. Endogenous porphyrin molecules absorb light and become excited and enter the triplet state (Figure 2.4) (Cunliffe and Goulden, 2000). Once in the triplet state the reaction is thought to be predominantly that of the Type II reaction pathway (Figure 2.4), whereby the triplet state porphyrin molecule interacts with oxygen to produce singlet oxygen and other reactive oxygen species (ROS) (Cunliffe and Goulden, 2000). The singlet oxygen oxidises the microbial cell components, causing cell death.

2.6.3 Previous Inactivation Studies

In comparison to PDI studies relatively little research has been completed on the germicidal effects of singlet oxygen production by light-stimulated endogenous porphyrins. Studies have often been limited to those microorganisms containing relatively high levels of porphyrins such as those microorganisms involved in acne or highly pigmented oral bacteria.

Many of the available PDI investigations utilising endogenous porphyrin-produced ROS have concentrated on dermatological treatments. Such as, Papageorgiou *et al*. (2000) which used blue light of peak 415 nm and a mix of blue and red light of wavelength peaks 415 nm and 660 nm respectively to treat acne vulgaris. They found a marked improvement in the severity of the acne particularly when using the mixed light, which was thought to be due to the antibacterial effects of blue light and the anti-inflammatory effects of the red light. A study by Kawada *et al*. (2002) demonstrated the bactericidal effect of 415-nm light *in vitro* and *in vitro* in acne sufferers. *Propionibacterium acnes*, isolated from treated patients, was inactivated by exposure to this light source however, *Staphylococcus epidermidis* was not affected. Similarly, Ashkenazi *et al*. (2003) demonstrated that *P. acnes* was readily inactivated using 407-420-nm light.

Another use for PDI of bacteria through endogenous porphyrin-produced ROS is in the inactivation of microorganisms that cause periodontal disease. Soukos *et al*. (2005) demonstrated the effectiveness of broadband light of 380-520 nm for inactivating a range of oral black-pigmented bacteria (*Prevotella* spp and *Porphyromonas* spp), which are a common cause of human periodontal disease. As most of these bacteria inhabit the mouth in biofilms (plaque), Henry *et al*. (1996) exposed biofilms of *Prevotella* spp and *Porphyromonas* spp to an argon laser of wavelength 488-514 nm to successfully reduce biofilm growth without exogenous photosensitisers. Feuerstein *et al*. (2005) also demonstrated the effectiveness of different light sources of wavelengths from 400-500 nm, similar to the wavelengths currently used in dental curing units, to successfully inactivate *Porphyromonas gingivalis* and *Fusobacterium nucleatum*.

Very few studies into the germicidal effect of light on microorganisms outwith dermatology and dentistry applications have been undertaken. However, Hamblin *et al*. (2005) demonstrated the germicidal effectiveness of blue/visible light on *Helicobacter pylori*, a cause of gastric ulcerations, using a broadband light source in conjunction with optical filters, concluding that optimum inactivation occurred at 400 nm.

Research has been conducted on *S. aureus* and MRSA, presumably due to the current issues of antibiotic resistance in hospitals. Guffey and Wilborn (2006) found that 470-nm light could inactivate *Pseudomonas aeruginosa* and *S. aureus*. Interestingly they found 470-nm light was not capable of inactivating *P. acnes*. Enwemeka *et al*. (2009) also demonstrated the inactivating effect of 470-nm light on *S. aureus* and MRSA strains using 470-nm light. Lipovsky *et al*. (2009) established the effectiveness of 400-800-nm light in reducing bacterial numbers of two different *S. aureus* strains.

2.6.4 405-nm Light

After the success of treatment using light of around 400-420 nm to inactivate microorganisms, specific wavelengths of light were targeted to achieve greater inactivation. Maclean *et al.* (2008b) used a broadband white light source in conjunction with a range of optical filters to identify the wavelengths of light that were optimal for inactivation in *S. aureus*. The wavelengths found to be optimal in the inactivation of *S. aureus* was 405 (±10) nm.

A further study by Maclean *et al*. (2009) demonstrated the antibacterial effects of 405-nm light not only on *S. aureus* but also on a range of different medically-relevant microorganisms. Similarly Guffey and Wilborn (2006) reported the germicidal effect of 405-nm light using a superluminous diode (SLD) array on *S. aureus* and *Pseudomonas aeruginosa*. They found the 405-nm wavelength to be bactericidal but found no inactivating effect on *P. acnes*. Other similar studies by Maclean (2006), Maclean *et al*. (2009) and Enwemeka *et al*. (2008) found 405-nm light to be particularly effective in inactivating MRSA bacterial strains *in vitro*. The inactivation achieved upon 405-nm light stimulation of a range of bacteria is presented in Table 2.6.

405-nm light has been shown to be bactericidal, however its ability to inactivate microorganisms other than bacteria has not been demonstrated to date. There have been limited numbers of bacteria studied, with most research focusing on hospital-associated pathogenic bacteria. The use of 405-nm light to stimulate endogenous porphyrin-induced ROS has several operational advantages over conventional PDI with exogenous photosensitisers but has yet to be as extensively tested.

Therefore the scope of this current investigation is to determine the effectiveness of 405-nm light in other important bacteria, yeasts and fungi and determine its possible usefulness as an environmental decontamination technology.

Table 2.6 *Inactivation of a range of bacteria using 405-nm light sources.*

Microorganism	Light Source	Log Reduction	Dose (J cm^{-2})	Reference
Pseudomonas aeruginosa	405-nm SLD Array	1.31	10	Guffey and Wilborn (2006)
Staphylococcus aureus	405-nm SLD Array	0.91	15	Guffey and Wilborn (2006)
Propionibacterium acnes	405-nm SLD Array	Stimulatory	15	Guffey and Wilborn (2006)
Staphylococcus aureus	405-nm LED Array	5.0	36	Maclean (2006)
Methicillin-resistant *S. aureus* (MRSA)	405-nm LED Array	5.0	45	Maclean (2006)
Staphylococcus epidermidis	405-nm LED Array	4.6	42	Maclean *et al*., (2009)
Clostridium perfringens	405-nm LED Array	4.4	45	Maclean (2006)
Streptococcus pyogenes	405-nm LED Array	5.0	54	Maclean (2006)
Acinetobacter baumannii	405-nm LED Array	4.2	108	Maclean *et al*., (2006)
Proteus vulgaris	405-nm LED Array	4.7	144	Maclean *et al*., (2009)
Pseudomonas aeruginosa	405-nm LED Array	4.2	180	Maclean *et al*., (2009)
Klebsiella pneumoniae	405-nm LED Array	3.9	180	Maclean *et al*., (2009)
Escherichia coli	405-nm LED Array	3.1	180	Maclean *et al*., (2009)
Enterococcus faecalis	405-nm LED Array	2.6	216	Maclean *et al*., (2009)

SLD= Super Luminous Diode
LED= Light Emitting Diode

Chapter 3

MICROBIOLOGICAL MATERIALS AND TECHNIQUES

3.0 GENERAL

This chapter details the various microorganisms, media, diluents and techniques that were used for the duration of this study. The light systems and inactivation methodologies used in this study are detailed in subsequent chapters.

3.1 MICROORGANISMS

This section details the bacterial, yeast and fungal strains used in the study, as well as their various culture requirements.

3.1.1 Microorganism Strains

The strains of microorganisms used are listed in Table 3.1. Cultures were obtained from the National Collection of Type Cultures (NCTC) (Colindale, UK), the Laboratorium voor Microbiologie, Universiteit Gent (LMG) and Mycotheque de l'Universite catholique de Louvain (MUCL) (LMG and MUCL are both part of The Belgian Co-ordinated Collections of Microorganisms (BCCM)). Isolates used included methicillin-resistant *Staphylococcus aureus* (MRSA) 16a, and a multi-drug resistant *Acinetobacter baumannii* isolate, both obtained from Glasgow Royal Infirmary (Scotland, UK), *Bacillus megaterium* B17/97 isolated from a septic human finger and *Bacillus subtilis* isolated from Aptamil™ Infant Formula.

3.1.2 Culture and Maintenance of Microorganisms

Microbial strains obtained from culture banks were reconstituted from their dried state into the appropriate broth medium for growth. Once it was ensured that cultures were viable and pure, they were transferred to Microbank™ beads (ProLab Diagnostics, UK) and kept at –70°C. Beads could then be taken from the

Microbank™ collection and streaked onto an agar plate and incubated (the media, temperature and incubation period dependant on the microorganism). Once grown on the agar plate, the microorganism was then subcultured onto an agar slope and refrigerated. This slope was used as a regular inoculum source for experimental work. All slopes were re-streaked onto the appropriate agar and Gram stained in order to check the strain purity under a light microscope, at least every 4 weeks.

The *Aspergillus niger* (MUCL 38993) culture used in this study was live when purchased. Spores were taken from the live culture and streaked onto malt extract agar (MEA) plates, and after checking the purity of the culture, the spores were subcultured onto MEA slopes and refrigerated until experimental use.

To culture microorganisms for experimental use, a loopful of the microorganism being investigated was inoculated into 100 ml of a broth growth medium and incubated under rotary conditions (125 rpm). The media, temperature and time of incubation used were dependent on the microorganism being cultured, as detailed in Table 3.2.

When culturing *A. niger*, a loopful of spores was taken from the refrigerated culture and subcultured onto a MEA slope. This was incubated at 30°C for 5 days until heavy spore production was observed.

Table 3.1 *Strains of microorganisms used.*

Microorganism	**Strain**
Staphylococcus aureus	NCTC 4135
Shigella sonnei	NCTC 12984
Salmonella enteritidis	NCTC 4444
Campylobacter jejuni	LMG 8841
Bacillus cereus	NCTC 11143
Listeria monocytogenes	NCTC 11994
Bacillus subtilis	Isolate from Aptamil™ Infant Formula
Bacillus megaterium	B17/97 Isolate from septic human finger
Mycobacterium terrae	LMG 10394
Enterococcus faecalis	NCTC 00775
Methicillin Resistant *Staphylococcus aureus*	16a (Glasgow Royal Infirmary)
Escherichia coli	NCTC 9001
Escherichia coli O157:H7	NCTC 12900
Klebsiella pneumoniae	NCTC 9633
Pseudomonas aeruginosa	NCTC 9009
Acinetobacter baumannii	NCTC 12156
Acinetobacter baumannii	Multi drug resistant isolate (Glasgow Royal Infirmary)
Saccharomyces cerevisiae	MUCL 28749
Candida albicans	MUCL 29903
Aspergillus niger	MUCL 38993

Table 3.2 *Growth requirements of microorganisms used.*

Microorganism	Growth Medium	Incubation Time	Temperature (°C)
S. aureus	Nutrient Broth/Agar	18-24 hours	37
Sh. sonnei	Nutrient Broth/Agar	18-24 hours	37
Salm. enteritidis	Nutrient Broth/Agar	18-24 hours	37
C. jejuni	Blood Agar	48 hours	42
L. monocytogenes	Tryptone Soya Broth/ Agar	18-24 hours	37
B. cereus	Nutrient Broth/Agar	18-24/72 hours	37
B. subtilis	Nutrient Broth/Agar	72 hours	37
B. megaterium	Nutrient Broth/Agar	72 hours	37
M. terrae	7H9 Broth + ADC enrichment media/ 7H10 Agar + OADC enrichment media	14 Days in broth for growth/ 7 days on agar plates to count	37
E. faecalis	Nutrient Broth/Agar	18-24 hours	37
MRSA 16A	Nutrient Broth/Agar	18-24 hours	37
E. coli strains	Nutrient Broth/Agar	18-24 hours	37
K. pneumoniae	Nutrient Broth/Agar	18-24 hours	37
Ps. aeruginosa	Nutrient Broth/Agar	18-24 hours	37
A. baumannii strains	Nutrient Broth/Agar	18-24 hours	37
S. cerevisiae	Malt Extract Broth/Agar	18-24 hours in broth/ 48 hours on agar plates to count	30
C. albicans	Malt Extract + Yeast Extract Broth/Agar	18-24 hours in broth/ 48 hours on agar plates to count	37
A. niger	Malt Extract Broth/Agar	5 days on slope for use/ 3 days on agar plates to count	30

3.2 MEDIA

Media was prepared by measuring the dried media to the quantity stated by the manufacturer, and dissolving in distilled water. The media was sterilised by autoclaving at 121°C for 15 minutes, unless stated otherwise. Molten agar was cooled in a water bath at 48°C prior to preparing agar plates and slopes.

3.2.1 Agars and Broths

Broths

NUTRIENT BROTH (Oxoid Ltd, UK) [CM0001]- 13 g/L

TRYPTONE SOYA BROTH (Oxoid Ltd, UK) [CM0129]- 30 g/L

BRAIN HEART INFUSION BROTH (Oxoid Ltd, UK) [CM0225]- 37 g/L

NUTRIENT BROTH No. 2 (Oxoid Ltd, UK) [CM0067]- 25 g/L

MALT EXTRACT BROTH (Oxoid Ltd, UK) [CM0057]- 20 g/L; sterilised at 121°C for 10 minutes.

MIDDLEBROOK 7H9 BROTH (Becton Dickinson and Company, USA) [217310]- 4.7 g of medium was dissolved in 900 ml of distilled water, containing 2 ml of glycerol (Fisher Scientific Ltd, UK) and then sterilised at 121°C for 10 minutes. When the agar cooled to 45°C, 100 ml of Middlebrook ADC enrichment medium (Becton Dickinson and Company, USA) [211887] was added aseptically.

Agars

NUTRIENT AGAR (Oxoid Ltd, UK) [CM0003]- 28 g/L

TRYPTONE SOYA AGAR (Oxoid Ltd, UK) [CM0131]- 40 g/L

BRAIN HEART INFUSION AGAR (Oxoid Ltd, UK) [CM0225]- 37 g/L

As for Brain Heart Infusion Broth but with the addition of 1% w/w Agar Bacteriological (Agar No.1) [LP011].

NUTRIENT AGAR No. 2 (Oxoid Ltd, UK) [CM0067]- 25 g/L; as for Nutrient Broth No. 2 but with the addition of 1% w/w Agar Bacteriological (Agar No.1) (Oxoid Ltd, UK) [LP011].

MALT EXTRACT AGAR (Oxoid Ltd, UK) [CM0059]- 50 g/L; sterilised at 121°C for 10 minutes.

MIDDLEBROOK 7H10 AGAR (Becton Dickinson and Company, USA) [262710]- 19 g of medium was dissolved in 900 ml of distilled water, containing 5 ml of glycerol (Fisher Scientific Ltd, UK) and was sterilised at 121°C for 10 minutes. When the agar cooled to 50-55°C, 100 ml of Middlebrook OADC enrichment media (Becton Dickinson and Company, USA) [211886] was added aseptically.

BLOOD AGAR (Oxoid Ltd, UK) [CM0055]- 40 g/L;

When the blood agar base had cooled to 45°C, defibrillated horse blood (Oxoid, Ltd) [SR0050C] was added aseptically to make a blood concentration of 7%.

MULLER HINTON AGAR (Oxoid Ltd, UK) [CM0337]- 38 g/L

BAIRD PARKER EYT AGAR T/V CONTACT PLATE (Cherwell Laboratories, UK) [101170]- purchased pre-poured from Cherwell Laboratories Ltd.

3.2.2 Diluents and Reagents

PHOSPHATE BUFFERED SALINE (Oxoid Ltd, UK) [BR0014G]- 1 tablet in 100 ml distilled water. The buffer was dispensed into 100 ml volumes for re-suspension of bacterial pellet, or 9 ml volumes for serial dilutions using a bottletop-dispenser (VITLAB, Germany). The dispensed phosphate buffered saline was autoclave sterilised at 115°C for 10 minutes.

GRAM STAINING REAGENTS: Crystal violet, Lugol's iodine, Ethanol and Safronin.

OXIDASE STRIPS (Oxoid Ltd, UK)

CATALASE (Sigma, UK)

API STAPH (bioMérieux, UK)

3.3 ENUMERATION OF MICROORGANISMS

When undertaking microbial inactivation studies it is essential to know what the population density of the microorganism was prior to and after treatment, thereby allowing the achieved reduction in microbial population to be calculated. To enumerate microorganisms, samples taken before and after treatment were plated onto agar plates and incubated at the optimum incubation temperature and time required by the microorganism for growth (Table 3.2). When microbial samples were expected to contain a high number of microorganisms they were diluted prior to plating. After plate incubation, the number of microorganisms present was determined by counting the number of colonies present on the plate. This was recorded as colony forming units per millilitre (CFU ml^{-1}), not number of cells as it is possible that colonies could arise from more than one cell.

The dilution and plating methods used to enumerate microorganisms in the study are detailed in this section.

3.3.1 Serial Dilutions

After incubation, broths were centrifuged at 3939 × g for 10 minutes. The resultant microbial pellet was then resuspended in 100 ml of phosphate buffered saline (PBS) (pH= 7.3). A 1 ml volume of the microbial sample was added to 9 ml PBS to make a 10^{-1} CFU ml^{-1} dilution. The pipette tip was then discarded. The diluted sample was vortexed using a Whirly mixer (FisherBrand, UK) in order to achieve a homogenous suspension. Using a fresh pipette tip a further 1 ml was taken from the 10^{-1} CFU ml^{-1} diluted sample and added to a fresh 9 ml of PBS giving a 10^{-2} CFU ml^{-1} dilution, and so on until the dilution required for experimental use was reached. In order for the concentrations to be correct it is vital that a fresh pipette tip is used each time to minimise carry-over of microorganisms.

3.3.2 Techniques For Plating and Enumerating Microorganisms

In order to enumerate microbial samples before and after light treatment, a number of different plating methods, described in the following section, were required depending on the number of microorganisms that were expected to survive light treatment.

3.3.2.1 Spiral Plate Method

A WASP 2 spiral plater (Don Whitley Scientific, UK), shown in Figure 3.1, was used to produce spiral plates by dispensing a 50 μl liquid sample onto the surface of an agar plate in a logarithmic Archimedes spiral. The sample was left to dry onto the agar plate before incubation. After incubation, colonies were counted either manually using a colony counter, or using a PC with ACOLYTE software (Version 1.19). When using the manual counting technique, the agar plate was placed on a colony counter (Stuart Scientific, UK), with a counting grid then placed over the agar plate. The number of CFUs in sections of the grid were then counted, each section corresponding to a known deposited volume of sample, and the microorganism count in CFU ml^{-1} was calculated by reference to the supplied data sheets. The ACOLYTE software automatically calculates the CFU ml^{-1} when given a plate to count. In both cases, any dilutions used must be taken into account when recording the number of CFU ml^{-1} in a particular sample.

Figure 3.1 *WASP 2 Spiral Plater and Vacuum Pump.*

3.3.2.2 Spread Plate Method

As with the spiral plates, spread plates could also be prepared by the WASP 2 spiral plater, by dispensing 100 µl of sample of microorganism onto an agar plate in a linear manner. Spread plates were also prepared manually by pipetting 100 µl of a sample onto an agar plate and spreading over the agar surface using a sterile plastic L-shaped spreader. Samples were left to dry onto the agar prior to incubation. After incubation, the entire plate was counted and multiplied by a factor of ten in order to ascertain the microorganism count in CFU ml^{-1}.

3.3.2.3 Pour Plate Method

When samples were expected to have less than ~2-Log_{10} CFU ml^{-1}, the pour plate method was used. In the pour plate method, 1 ml of a sample of microorganism was pipetted into a sterile 90 mm Petri dish. This was then covered with ~20 ml of sterile molten agar (microorganism dependent) cooled to around 45°C. The plate was then swirled 10 times in a clockwise and 10 times in an anticlockwise manner to evenly disperse the microorganism throughout the plate. This was then left to set prior to

incubation. As 1 ml of sample was used the entire plate was counted and directly corresponded to the number of CFU ml^{-1}.

3.4 STATISTICAL ANALYSIS

In light exposure experiments, data points show the standard deviation and significant differences calculated from results and are depicted throughout this study using an asterisk (*). Significant differences were calculated at the 95% confidence interval using ANOVA (one-way) with MINITAB software release 15.

3.5 OTHER EQUIPMENT

Throughout this investigation it has been necessary to use many pieces of equipment that are outlined in the following section.

Sterilisation of Media, Diluents and Microbiological Wastes

- Kestrel automatic autoclave (LTE Scientific, UK) and a bench-top autoclave (Dixons Surgical Instruments Ltd, UK).

Distilled Water

- Merit 400 Distil (LTE Scientific, UK) provided water free of impurities for preparation of media and diluents.

Measurement of Solid Media and Reagents

- OHAUS Navigator and OHAUS Adventurer digital balances (both OHAUS, Switzerland).

Incubation of Microorganisms

- A rotary shaker incubator (New Brunswick Scientific, UK), with adjustable temperature settings, was used to culture microorganisms. The number of revolutions per minute (rpm) could also be adjusted. The rotation of the

incubator ensured that the culture was aerated, ensuring optimum conditions for the growth of the microorganism.

- Fan-assisted incubators (LTE Scientific, UK) with adjustable temperature settings were used for the cultivation of microorganisms on agar plates. The temperature setting and incubation time required were dependent on the microorganism being studied. Incubation allowed the growth of colonies on plates for counting.

Centrifuges

- A Heraeus Labofuge 400R (Kendro Laboratory Products, Germany) was used in conjunction with 50 ml volume centrifuge tubes (Nunc, Denmark) to spin down microorganisms from their broth growth media at 3939 × g for 10 minutes. After this time the supernatant could be removed to leave the pellet of the microorganism. This pellet could then be suspended in PBS for experimental use.
- A Sciquip 1-15K centrifuge (Sigma, UK) was used in conjunction with 1.5 ml Eppendorf tubes to centrifuge small volumes of microbial suspensions. In this investigation it was used for the Penicillin Binding Protein 2' latex agglutination test (Oxoid, UK), and as such was spun at 1500 × g for 5 minutes.

Pipettes

- Gilson pipettes (Scientific Laboratoey Supplies, UK); 100 µl, 1 ml, 5 ml and 10 ml were used with sterile pipette tips (Greiner, UK) for the aseptic transfer of liquids including diluents and microbial suspensions.

Refrigerators

- Lee Medical refrigerators (Lee Medical, UK), maintained at 4°C, were used for the storage of microbial cultures on agar slopes and agar plates. They were also used for storage of reagents and test kits that required refrigeration.

Water Bath

- A Grant water bath (Grant Instruments, UK) was used to keep molten agar at around 48°C. The water bath has an adjustable heat setting, which was adjusted to 80°C for heat treatment of bacterial spores, and 95°C for the Penicillin Binding Protein 2' (PBP 2') latex agglutination test.

Magnetic Stirrers

- An MSH basic magnetic stirrer (Scientific Laboratory Supplies, UK) was used in conjunction with a 7 × 2 magnetic follower (Fisher Scientific, UK) to ensure the constant agitation of samples during experimentation.

Spectrophotometer

- A Biomate 5 UV-Visible Spectrophotometer (Thermo Spectronic, UK) was used to attain the absorbance and transmission readings of samples, including bacterial suspensions, broths and diluents. This could be done for a single wavelength or could be completed as a scan of wavelengths ranging from the ultraviolet (UV) to the infrared part of the spectrum. When investigating UV wavelengths it was necessary to use quartz cuvettes in order to allow the transmission of UV light through the sample. When not investigating UV wavelengths it as possible to use a plastic cuvette.

Microscope

- A Nikon Eclipse E400 Light Microscope (Nikon Instruments, UK) was used to check purity of microbial cultures. It was also used in this study to count spore suspensions using an Improved Neubauer Hemacytometer to ensure the correct dilution was being used in experimentation. A Nikon Coolpix 4500 digital camera was attached to the light microscope to photograph microscope preparations.

Camera

- A Sony Cyber-shot DSC-T2 (Sony, UK) was used to take pictures of equipment and agar plates that are presented in this thesis

Anaerobic Jars

- Plastic anaerobic jars (Oxoid, UK) were used with CampyGen™ sachets (Oxoid, UK) (1 sachet/jar) to create an artificial atmosphere for the growth of microaerophilic organisms, such as *Campylobacter jejuni* used in this study.

3.6 MICROBIOLOGICAL TESTS

In order to identify the microorganism being studied and check for purity of cultures it was necessary to perform some routine microbiological tests.

3.6.1 Gram Stain

The Gram stain is used to identify the two different types of bacteria- Gram-positive and Gram-negative. This is commonly used as a first step in the identification process of any bacteria. The preparation and staining procedures are outlined in the following section.

Preparation of Smear For Staining

- 1 or 2 colonies of the microorganism were picked from an agar plate and emulsified in a drop of water onto a clean microscope slide. Alternatively a loopful of microorganism cultured in broth could be placed onto a clean microscope slide. In both cases, the microorganism must be 24 hours old in order to minimise the risk of a Gram-variable result.
- The film of microorganism was allowed to dry completely onto the slide and was fixed to the slide by passing through a blue flame of a Bunsen Burner 3 or 4 times.
- The slide was then allowed to cool on a pair of metal rods placed over a sink prior to the staining procedure.

Gram Staining Procedure

- The fixed film of microorganism was flooded with Crystal Violet for 30 seconds.
- This was drained off and rinsed with Lugol's Iodine, and was covered with fresh Lugol's Iodine solution for 1 minute.
- After 1 minute has passed the Lugol's Iodine was drained off and rinsed with alcohol for 15 seconds until the violet colour had completely drained from the slide.
- The alcohol was then drained off and the slide was rinsed gently with tap water.
- The slide was then covered with the counter-stain Safronin for 30 seconds.
- The Safronin was washed off gently with tap water after the allocated time and dried on blotting paper.
- The stained preparation was then viewed under the oil-immersion lens on the microscope using 1 drop of immersion oil on the slide.

Gram-positive bacteria will appear deep purple under the oil-immersion lens as it retains the Crystal Violet stain in its thick peptidoglycan layer even after decolourisation. Gram-negative bacteria are stained pink by the Safronin counter-stain as the Crystal Violet stain is decolourised. This is because Gram negative-bacteria have a double membrane constituting a thin peptidoglycan layer, which is enclosed in an outer layer that has high lipid content. This high lipid content layer allows the ethanol to decolourise the Crystal Violet Stain in the peptidoglycan wall.

3.6.2 Catalase

The catalase test is a test to detect the catalase enzyme that is present in cytochromes. Most aerobic and facultative anaerobic bacteria possess this enzyme; therefore it can be used as a tool for identifying bacteria. A few millilitres of hydrogen peroxide (H_2O_2) were pipetted onto an agar slope containing the bacteria. If the microorganism is catalase positive, the H_2O_2 is broken down into hydrogen gas (producing bubbles) and oxygen (O_2). If when the H_2O_2 is added no bubbles of gas are produced, then the bacterial sample is catalase negative.

3.6.3 Oxidase

Many different bacteria produce cytochrome oxidase and as such this can be used as a presumptive test in bacterial identification. The oxidase test is performed using Oxidase Detection Strips (Oxoid) that are impregnated with NNN'N' tetramethyl -p-phenylene-diamine dihydrochloride. The strip was touched onto a colony on an agar plate and observed after 5 seconds. If the NNN'N' tetramethyl -p- phenylene-diamine dihydrochloride has been oxidised by the bacteria then indophenol blue will be formed; a blue/purple colour is a positive reaction. If no colour change is observed this is considered to be a negative reaction.

3.6.4 API Staph Identification Test

The API Staph is an identification kit containing many different miniaturised biochemical tests in a single test strip capable of differentiating different the genera of *Staphylococcus*, *Micrococcus* and *Kocuria*. The API Staph test kit contains 20 different microtubules with dehydrated substrates. Before the API Staph test kit is used it is important to carry out Gram stains and Catalase and Oxidase tests to ensure the correct identification kit is being used.

For preparation of the API Staph test, an incubation box was filled with 5 ml of distilled water to create a humid environment in order to prevent test kits drying out in the incubation step. A bacterial suspension was prepared in 6 ml of API Staph medium to 0.5 McFarland standard using a young bacterial culture (16-24 hours). The inoculated medium was then pipetted using a sterile Pasteur pipette into each of

the microtubules filling up only to the tubule portion. The tests ADH and URE were filled to the cupules with mineral oil to ensure anaerobiosis. The test kit was then incubated at 37°C for 24 hours.

After incubation, reagents were added to 3 of the tubes in the test kit; VP test-VP1 and VP2 reagents, NIT test- NIT1 and NIT 2 reagents and PAL test- ZYM A and ZYM B reagents. After 10 minutes the results of the tests could be read and interpreted using a reference provided with the test kits [APPENDIX A]. Tests are separated in to 7 groups of 3 on the results sheet provided with the test kits, each with a value of 1, 2 or 4. The addition of each positive result in a group allows a numerical profile to be produced and referenced using the API database.

Chapter 4

INVESTIGATION INTO THE USE OF ULTRAVIOLET LIGHT AS AN INACTIVATION TECHNOLOGY

4.0 GENERAL

Part I of this chapter focuses on the strong bactericidal capability of pulsed ultraviolet-rich (PUV) light on *Staphylococcus aureus*. Two methods are used, one of which allows some quantitative conclusions to be reached regarding the efficiency of UV light for bacterial inactivation. Part II deals with the process of photoreactivation, whereby UV-inactivated bacteria may be repaired by light of appropriate wavelengths. The dependence of photoreactivation on wavelength is examined for *S. aureus*.

PART I -PHOTOINACTIVATION

4.1 BACKGROUND

Staphylococcus aureus is a Gram positive bacterium that commonly colonises the skin, particularly the anterior nares. Around 20% of healthy individuals carry the organism and 60% of people will be intermittent carriers of *S. aureus* in their lifetime (Kluytmans *et al*., 1997). *Staphylococcus aureus* infections can cause serious illness such as endocarditis and bacteraemia, which are difficult to treat due to the increasing prevalence of antibiotic resistant strains such as methicillin resistant *S. aureus* (MRSA). Therefore novel technologies for decontamination of the environment (particularly air and surfaces) and medical devices are being sought.

UV light is a very effective decontamination technology capable of achieving high $\log_{10}$ reductions of bacteria, viruses, protozoa and fungi (Bank *et al*., 1990; Shin *et al*., 2001; Jun *et al*., 2003; Eischeid *et al*., 2009). UV light has been used traditionally in a continuous manner with particularly efficient decontamination

achieved in non-opaque liquids and on surfaces (Elmnasser *et al*., 2007). More recently pulse-power technology has been applied to UV light in order to provide a means of producing high energy, short duration UV-rich light pulses. The use of pulsed UV-rich (PUV) light technologies means that many microorganisms can be inactivated in only a few seconds. The inactivation achieved is primarily induced by UV damage (UV-B and UV-C 220-320 nm) to DNA. Microbial DNA absorbs UV light causing the formation of cyclobutane pyrimidine dimers (CPD), and less frequently, 6-4 photoproducts (6-4 PP) (Sinha and Häder, 2002). These mutations cause bends in the DNA helix, distorting the helix and inhibiting the ability of DNA polymerase enzymes to transcribe and translate DNA, and this, in turn, halts DNA replication and ultimately leads to cell death (Sinha and Häder, 2002).

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Culture

Staphylococcus aureus NCTC 4135 was inoculated into a flask containing 100 ml of Nutrient Broth (Oxoid, UK) and incubated at 37°C for 18 hours under rotary conditions (125 rpm). The broth was then centrifuged at 3939 × g for 10 minutes and the resultant pellet formed was re-suspended in phosphate buffered saline (PBS) (Oxoid, UK) as described in Chapter 3, Section 3.1-3.2. The bacterial suspension was then diluted to the appropriate starting population for experimental use.

4.2.2 Photoinactivation

PUV-rich light was generated using a solid-state pulsed power source (Samtech Ltd, Glasgow, UK) (Figure 4.1) based upon a 1 kV capacitive discharge circuit. The source was used to transfer stored electrical energy to a low-pressure (450 torr) Xenon-filled flashlamp in short duration (30 μs), using high peak-power (1 MW) with an exponentially decaying waveform. The energy per pulse is around 20 J when operating at peak voltage (1 kV). The lamp emits over a wavelength range from the ultraviolet to the infrared with high UV content. The spectrum of Figure 4.2 highlights the important UV wavelengths (a UV grating was used to obtain the

spectrum). The flashlamp was enclosed in PVC housing. Photoinactivation of bacterial suspensions was carried out using two different methods; A and B.

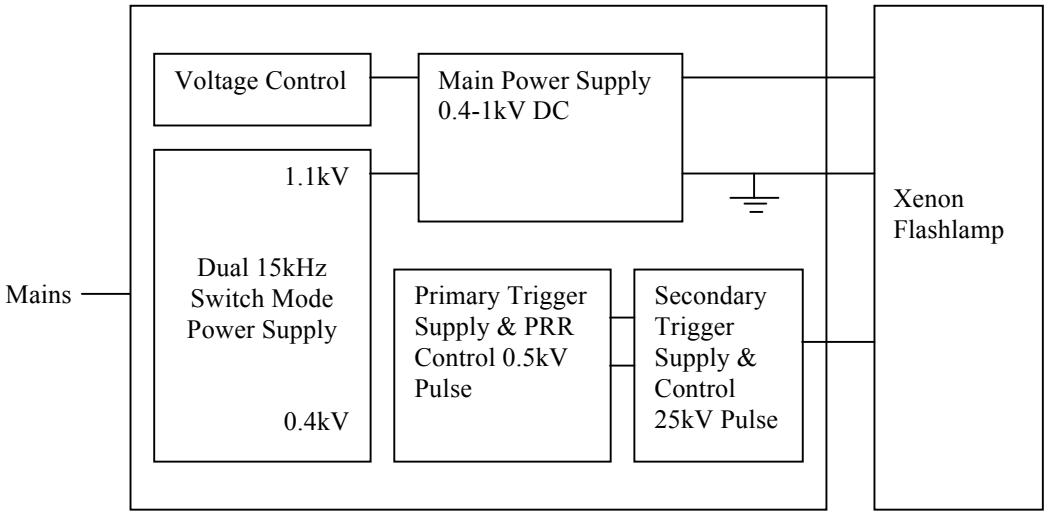

Figure 4.1 *The major components of the pulsed UV generator.*

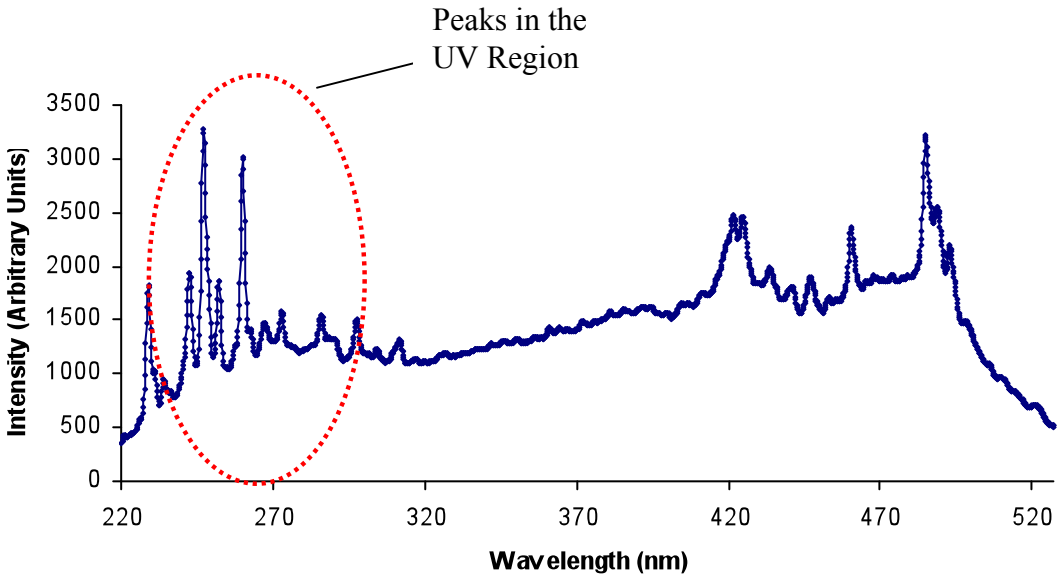

Figure 4.2 *Emission spectrum of the Xenon flashlamp. The flashlamp also emits into the infra-red region - this is not shown since a UV grating was used to emphasise the important UV wavelengths.*

In method A, the full spectral output of the flashlamp was used to irradiate the bacterial sample, while in method B, the spectrum of the light irradiating the bacterial samples was limited to a narrow wavelength range, namely 260 (±10) nm, using a narrow-band interference filter. Method A was used to demonstrate the efficacy of the pulsed UV source for photoinactivation of *S. aureus*, whereas method B was used to provide quantitative information on the efficiency of the photoinactivation process for *S. aureus*.

For method A, samples were contained in a 90 mm Petri dish (without lid). The platform supporting the dish was adjustable and was set in such a way that the Petri dish was 80 mm directly below the flashlamp (Figure 4.3). A 20 ml volume of the bacterial suspension at a known population density (3, 5 or 7-Log_{10} CFU ml^{-1}) was pipetted into a sterile 90 mm Petri dish (giving a liquid depth of 3.3 mm) using aseptic techniques, after which the Petri dish was placed on the platform inside the pulsed light system and exposed to a set number of UV-rich light pulses. This was repeated for increasing pulse numbers using a different 20 ml bacterial suspension sample each time.

In order to minimise the risk of occurrence of photoreactivation after exposure to UV light, all handling of UV-damaged bacteria was completed in a dark room illuminated with a Kaiser Spectral 590 Safelight (Figure 4.4), consisting of an array of 590-nm LEDs (590 nm is outside the photoreactivation wavelength range; see Chapter 2, Section 2.4.2.2). Samples were transferred from the Petri dishes to 30-ml sterile, plastic universals immediately following UV light treatment and wrapped in aluminium foil as a further precaution in preventing photoreactivation.

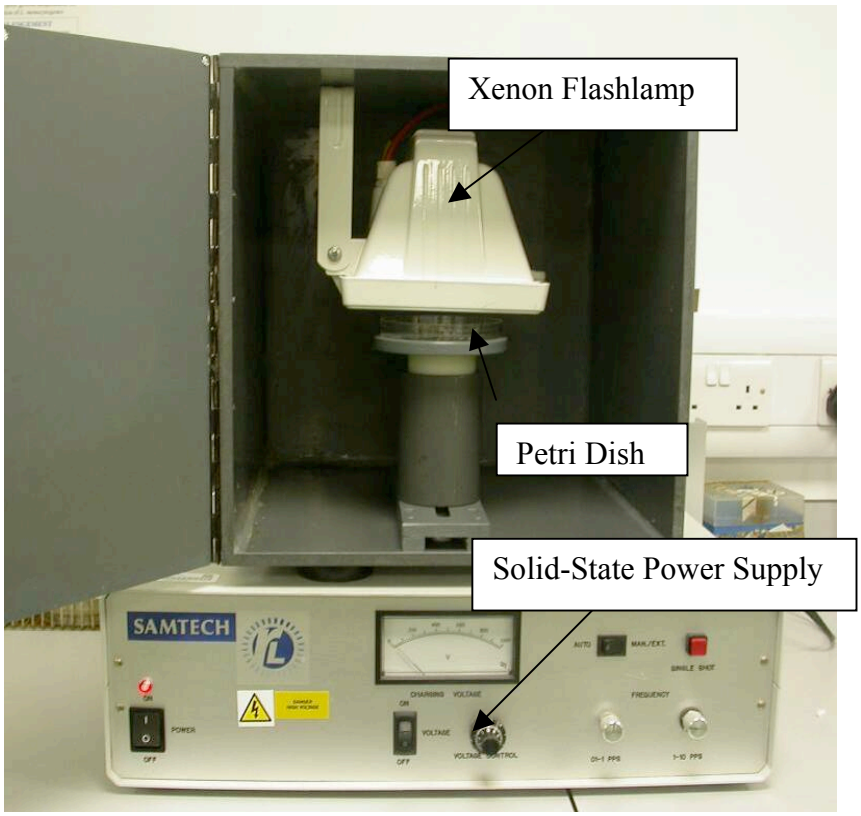

Figure 4.3 *Xenon flashlamp and solid-state pulsed power source.*

In method B, the spectrum of the light irradiating the bacterial samples was limited to the narrow wavelength range of 260 (±10) nm, using a narrow-band interference filter. This filter was used to isolate a band of UV light in the wavelength region close to the optimum wavelength for UV inactivation of Gram positive bacteria (Wang *et al.,* 2005). Here the flashlamp system was modified to hold 12-well multi-well plates (NUNC, Denmark) giving a sample depth of 7.2 mm (well diameter 23 mm). A 3-ml volume of *S. aureus* bacterial suspension of 6-Log_{10} CFU ml^{-1} was pipetted into a central well of a multi-well plate. A black PVC cylinder was placed around the well to prevent stray light entering the well, and the 260 (±10) nm band pass filter was placed on top of the well. The distance from the light source to the surface of the liquid test sample was 50 mm. Samples were exposed to increasing pulse numbers using a different 3 ml bacterial suspension sample each time and were completed in triplicate.

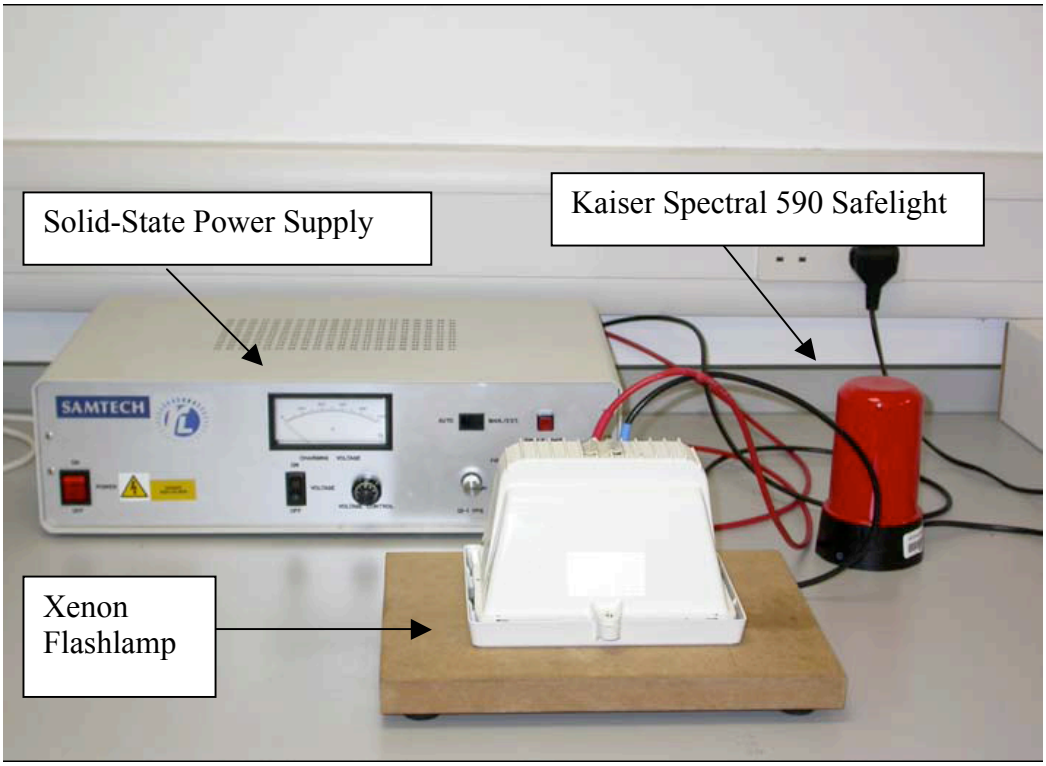

Figure 4.4 *Xenon flashlamp modified to hold 12 well multi-well plates. Also shown, a solid-state pulsed power source and Kaiser Spectral 590 Safelight.*

4.2.3 Plating and Enumeration

Both test and control samples in the pulsed-light inactivation and photoreactivation experiments were plated onto Nutrient Agar as described in Chapter 3, Section 3.3.2. Plates were then incubated at 37°C for 24 hours and plates were enumerated and recorded as $\log_{10}$ CFU ml^{-1}. Error bars indicate standard deviation and asterisks indicate statistical significant differences between values at the 95% confidence limit.

4.3 RESULTS OF PHOTOINACTIVATION

PUV light inactivation of *S. aureus* was completed over a range of population densities. The results for method A (full spectrum of flashlamp) are displayed graphically in Figures 4.5 and 4.6. Figure 4.5 shows the inactivation of 3, 5 and 7-

Log_{10} CFU ml^{-1} populations, which were inactivated completely by 2, 4 and 6 pulses respectively. Figure 4.6 demonstrates that a 9-Log_{10} CFU ml^{-1} population of *S. aureus* was reduced by around 7-log_{10} CFU ml^{-1} at 50 pulses. There appears to be little change in inactivation from around 35 pulses onwards. All PUV-exposed samples were statistically significantly different to their respective starting control populations (Figures 4.5-4.8). The results for method B (narrow band of UV around 260 nm) are displayed in Figures 4.7 and 4.8. Around 225 pulses were required for inactivation of a 7-Log_{10} CFU ml^{-1} population of *S. aureus*. However, some tailing occurs at this higher population density.

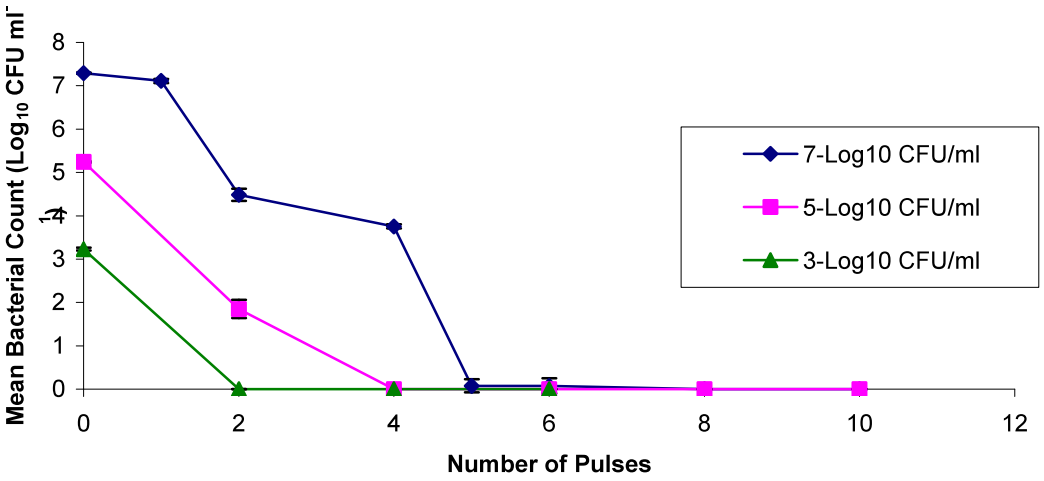

Figure 4.5. *Broadband PUV inactivation of* ***S. aureus*** *for three different starting population densities; 3, 5 and 7-Log_{10} CFU ml^{-1}.*

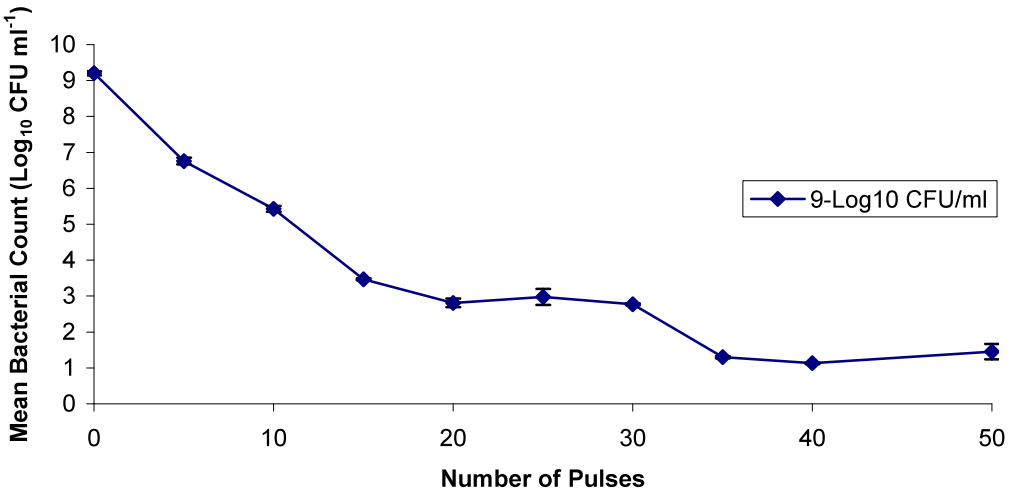

Figure 4.6 *Broadband PUV inactivation of* ***S. aureus*** *at a 9-Log$_{10}$ CFU ml^{-1} starting population density.*

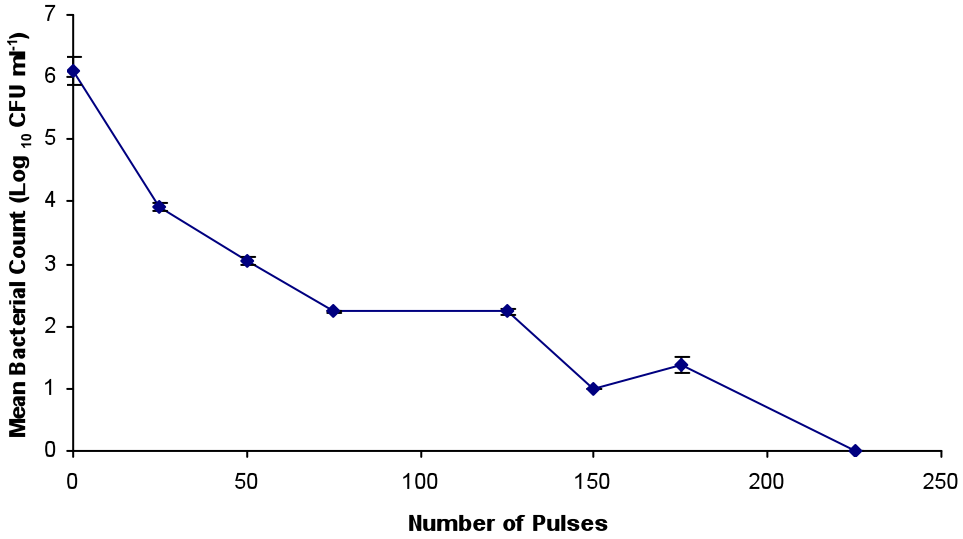

Figure 4.7. *260 (±10) nm PUV inactivation of* ***S. aureus*** *in liquid suspension.*

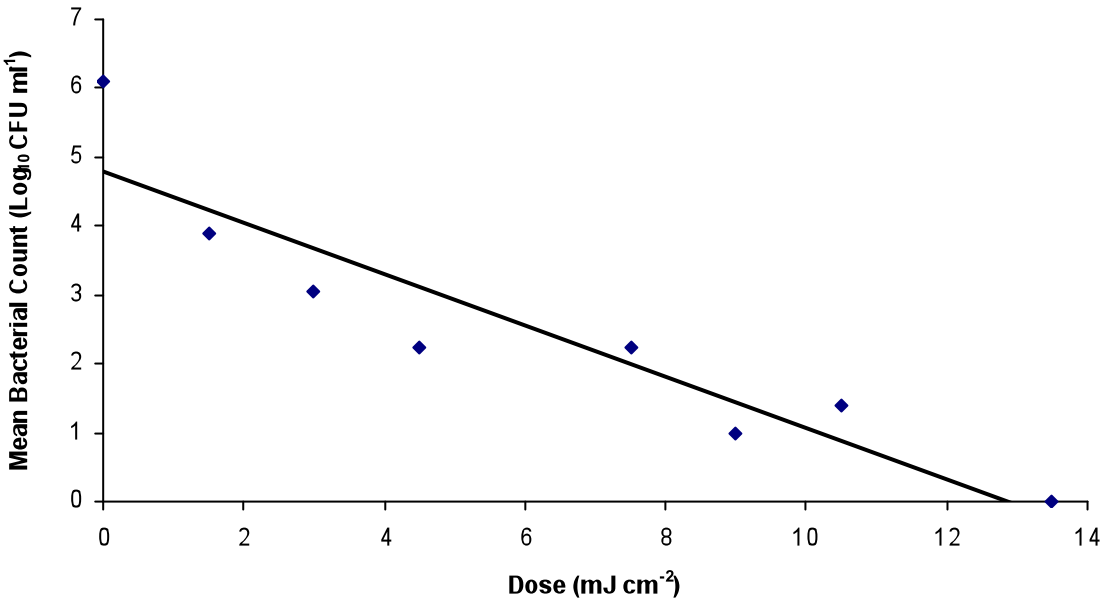

Figure 4.8 *260 (±10) nm PUV inactivation of* ***S. aureus*** *in liquid suspension, with mean bacterial count shown as a function of dose.*

4.4 DISCUSSION OF PHOTOINACTIVATION RESULTS

4.4.1 Method A

The use of the full spectral output of a Xenon flashlamp with high UV content has proved to be very effective in inactivating *S. aureus* in liquid suspension, even at high population densities as is seen in Figures 4.5 and 4.6. At populations of 7-$\log_{10}$ CFU ml^{-1} and higher UV light struggles to penetrate opaque liquids and therefore it takes an increased number of pulses to inactivate the bacteria. Another issue when inactivating bacteria at high population densities is that cells can form aggregates and clumps meaning they can be "in shadow" and protected from the damaging effects of UV light causing tailing to occur (Elmnasser *et al*., 2007). This tailing effect shown in Figure 4.6 has been reported in may UV light studies including a study by Pennell *et al.* (2008) in which they discussed tailing to most likely be due to shielding or phenotypic resistance within the bacterial population being exposed. Continuous

agitation of PUV-exposed samples might have reduced the tailing effect, but this is not possible in the current experimental design.

It is difficult to compare the inactivation achieved in this study with that achieved in the studies of others as each system is unique and often inactivation is described in terms of time of exposure rather than number of pulses given. Very few UV inactivation studies have been completed for *S. aureus* as many investigations have been associated with water decontamination. However, a study by Rowan *et al*. (1999) used two different Heraeus Noblelight XAP series light sources to irradiate seeded agar plates, one providing a high UV content source using a clear quartz-filled tube and one providing a low UV content source with a cerium-doped quartz envelope (restricting the UV region) – both were filled with Xenon to a pressure of 59 kPa. They tested many important food-related bacteria including *S. aureus*. They found when using either 100 or 200 pulses of low UV light around a 1-$\log_{10}$ CFU ml^{-1} reduction was achieved, whereas when using 100 or 200 pulses of high UV content light reductions of 4 and 5-$\log_{10}$ CFU ml^{-1} were achieved respectively. They also commented that in general Gram positive bacteria were more resistant to UV-induced damage than Gram negative bacteria. It has been proposed by Anderson *et al*. (2000) that differences in the compositions of the cell walls between Gram negative bacteria may be responsible for differences in inactivation achieved but also note that perhaps Gram positive organisms are exposed more often to solar UV and thus have more UV resistance than Gram negative organisms. Chang *et al*. (1985) used a nearly collimated beam of continuous UV light to inactivate liquid suspensions of *S. aureus* and other pathogenic organisms and found that a dose of 5-10 mW S^{-1} cm^{-2} was capable of inactivating 99.9% of *S. aureus*. A further study by McKillip *et al.* (1998) used a 254-nm emitting continuous UV light to inactivate 10^6 CFU ml^{-1} broth cultures of *S. aureus* in Petri dishes for a period of 2.5 hours. A 100 ml volume of this broth was placed into fresh brain heart infusion broth to grow over 48 hours at 37°C. They found no growth in the broths after this time. The methods used by McKillip *et al*. (1998) are not quantitative as they only assessed turbidity in the broth culture. It is possible that bacteria may in fact be present in the broth in small enough numbers so as to not significantly change the turbidity of the broth. As

they did not vary the UV dosages applied to the *S. aureus* suspensions it is not known whether the bacteria required 2.5 hours irradiation for inactivation or whether they required much less time. Hence, this study by McKillip *et al*. (1998) only demonstrates that *S. aureus* can be inactivated by continuous UV light and does not provide useful information on the light dosages required for inactivation.

4.4.2 Method B

The advantage of this method of inactivation is that it allows a quantitative measure of the degree of inactivation (germicidal efficiency) and thus comparison with values of this quantity obtained for other bacteria. When exposing *S. aureus* to 260 (±10) nm filtered light, the photoinactivation achieved is much less than that achieved using PUV-rich light alone (Figure 4.6), although wavelengths of around 260 nm are considered to be optimal for inactivating bacteria. Clearly, this is due to the total dose of UV-inactivating light received by the bacteria through the filter being significantly lower than the dose received in the absence of the filter. Not only does the filter isolate only a small fraction of the UV light from the pulsed source, but the peak transmission of the filter is only 18%. Using an identical experimental arrangement to that used in this study, Lani (2007) measured the energy transmitted per pulse through the 260 (±10) nm filter as 0.06 mJ cm^{-2}, compared to 20 J cm^{-2} per pulse over the whole spectrum of the flashlamp. Using this information, along with the data in Figure 4.7, it is possible to obtain a value for the germicidal efficiency of UV light at a wavelength of 260 nm for inactivation of *S. aureus.* By re-plotting the graph of Figure 4.7 with the number of pulses replaced by dose (mJ cm^{-2}) on the *x*-axis, the germicidal efficiency in units of $\log_{10}(N/N_0)$ per mJ cm^{-2} can be determined from the slope of the graph (line of best fit), which is shown in Figure 4.8.

The germicidal efficiency of *S. aureus* at 260 nm was thus calculated to be 0.45 ± 0.02 $\log_{10}$ (N/N_0) per mJ cm^{-2}. Lani (2007) found the Gram positive bacterium *Listeria monocytogenes* to have a germicidal efficiency value of 0.26 ± 0.02 $\log_{10}$ (N/N_0) per mJ cm^{-2} and the Gram negative bacterium *Escherichia coli* to have a value of 0.38 ± 0.03 $\log_{10}$ (N/N_0) per mJ cm^{-2}, both at 260 nm. Wang *et al.* (2005) have

measured the germicidal efficiency of *E. coli* over the UV wavelength range 230 - 300 nm; their value at 260 nm being 0.40 $\log_{10}$ (N/N_0) per mJ cm^{-2}. These results suggest that *S. aureus* is similar to *E. coli* in terms of its susceptibility to pulsed 260– nm light, whereas *L. monocytogenes* appears to be more resistant to inactivation at this wavelength.

Many investigations into UV light inactivation have argued that not only DNA damage occurs in the bacterial cells but also other damage occurs from the use of broadband light sources containing UV-A and visible light. Takeshita *et al*. (2003) reported that heat produced from some broadband UV systems is capable of causing photothermal effects on microorganisms. They found that heat produced from UV light treatment could cause super heating of cell contents, production of steam within the cell and subsequently disruption of the cell membrane. Oguma *et al*. (2002) suggested UV-A wavelengths (320-400 nm) could also cause photosensitive molecules in bacteria to produce radicals capable of damaging bacterial cells either lethally or sub-lethally. A study by Mori *et al*. (2007) used a 365-nm UV-A LED array to inactivate *E. coli* by approximately 4-$\log_{10}$ CFU ml^{-1} at a dose of 54 J cm^{-2}. This further demonstrates that UV-A is capable of causing significant damage to bacterial cells. Oguma *et al*. (2002) also noted that damage could occur in proteins and enzymes that contain unsaturated bonds as these absorb UV-B and UV-C. It is highly likely that the inactivating effects of broadband light pulses are due to both photochemical and photothermal effects simultaneously.

PART II- PHOTOREACTIVATION

4.5 BACKGROUND

Treatment of bacteria with light containing UV has limitations, as many bacteria possess mechanisms to repair UV-damaged DNA. Some bacteria possess DNA repair mechanisms such as nucleotide excision repair (NER) and base excision repair (BER), commonly grouped under the term "dark repair" as they are processes that do not require energy from light to repair mutations (Sinha and Häder, 2002). The most notable DNA repair process, however, is a light-dependent mechanism termed photoreactivation (Kelner, 1949). Photoreactivation is a process in which enzymes called photolyases scan DNA at regular intervals and bind strongly to any lesions that are encountered (Sancar, 1994). The enzyme requires energy from light of around 300 - 500 nm in order to initiate the photoreactivation process (Sancar, 1994). Photons of light are absorbed by the photolyase chromophore molecules, which cause the excitation and electron donation of an FAD co-factor antennae complex (Sancar, 2000). The electrons are donated to the dimer molecules, which subsequently return to their monomeric states, allowing correct base pairs to re-form. Photoreactivation is a highly efficient process, which requires only one photon of light to split a single dimer molecule (Sinha and Häder, 2002).

Photoreactivation capability has the potential to considerably reduce the usefulness of UV-light as a bacterial decontamination technology.

In this work, photoreactivation of *S. aureus* was examined experimentally by first inactivating bacterial samples with PUV light, as described in Part I, and then exposing the inactivated samples to light within the wavelength range 320-450 nm. Previous studies have shown that the 320-450 nm wavelength range is where photoreactivation is most likely to occur, although microorganism-dependent (Kelner, 1949; Lani, 2007). Wavelength dependence of the photoreactivation process was obtained through the use of a white-light source and narrow-band optical filters.

4.6 MATERIALS AND METHODS

4.6.1 Preparation of Culture

Staphylococcus aureus was cultured and prepared for treatment as described in Section 4.2.1.

4.6.2 Light Source

A spotlight source (Lightningcure LC5, Hamamatsu Photonics UK, Ltd) (Figure 4.9) with a 150 W Xenon broadband white light lamp (emission spectrum showing photoreactivation wavelengths only, Figure 4.10) was used in conjunction with a range of commercially available (L.O.T.-Oriel Ltd, UK) narrow-band pass filters (320-450 nm, full-wave at half-maximum (FWHM) of 10 nm) [APPENDIX B] to elucidate the photoreactivation spectrum for *S. aureus*.

4.6.3 Measurement Procedures

Inactivation

The *S. aureus* suspension (initial population density 7-Log_{10} CFU ml^{-1}) was exposed to pulses of UV-rich light (as described in Section 4.2.2) until a surviving population of around 1-2-Log_{10} CFU ml^{-1} remained.

Photoreactivation

Under dark conditions, 3 ml of the UV-inactivated bacterial suspension was pipetted aseptically into a central well of a 12-well multi-well dish. A black PVC cylinder was placed around the well to prevent stray light entering the well, and a short band-pass filter was placed on top of the well. The light source was positioned 50 mm directly above the liquid sample surface and the sample was exposed for a chosen time to filtered light from the Xenon source.

A Radiant Power Meter (model 70260, L.O.T.-Oriel Ltd, UK) in conjunction with a photodiode detector was used to obtain measurements of the irradiance (mW cm^{-2}) of the light transmitted through the filters from the Xenon lamp. In order to quantify

the experiments on photoreactivation, it is necessary to measure the amount of light energy to which the inactivated samples are exposed through each filter. The energy density (J cm^{-2}) or dose of light is calculated from the product of the irradiance (mW cm^{-2}), as measured using the Radiant Power Meter, and the exposure time (s). For each dose applied to an inactivated sample of bacteria, the level of photoreactivation is taken as the difference between the bacterial numbers in CFU ml^{-1} after the dose is applied (N_{pr}) and the bacterial numbers before the dose is applied (N).

4.7 EXPERIMENTAL RESULTS

4.7.1 Optimum Dose for Photoreactivation

Before carrying out the experiment to determine the photoreactivation spectrum, it was necessary to first determine the optimum dose, or energy density, of light for use in that experiment. It was expected that the maximum photoreactivation of *S. aureus* would occur at around 370-380 nm based on similar experiments using *L. monocytogenes* (Lani, 2007), and so 370 (±10) nm and 380 (±10) nm filters were used to determine the dose required for optimum photoreactivation. For each of the two wavelength ranges investigated, PUV-damaged cells of 2-Log_{10} CFU ml^{-1} population were exposed to increasing doses of light until saturation was achieved. The results are plotted in Figure 4.11, in the form of $\log_{10}$ ($N_{pr} - N$) v. Dose. The results show that saturation occurs between 1 J cm^{-2} and 1.5 J cm^{-2} for the 370 nm and 380 nm band-pass filters. Each datapoint on the Figures was repeated at least twice and represents the mean photoreactivation achieved in each case.

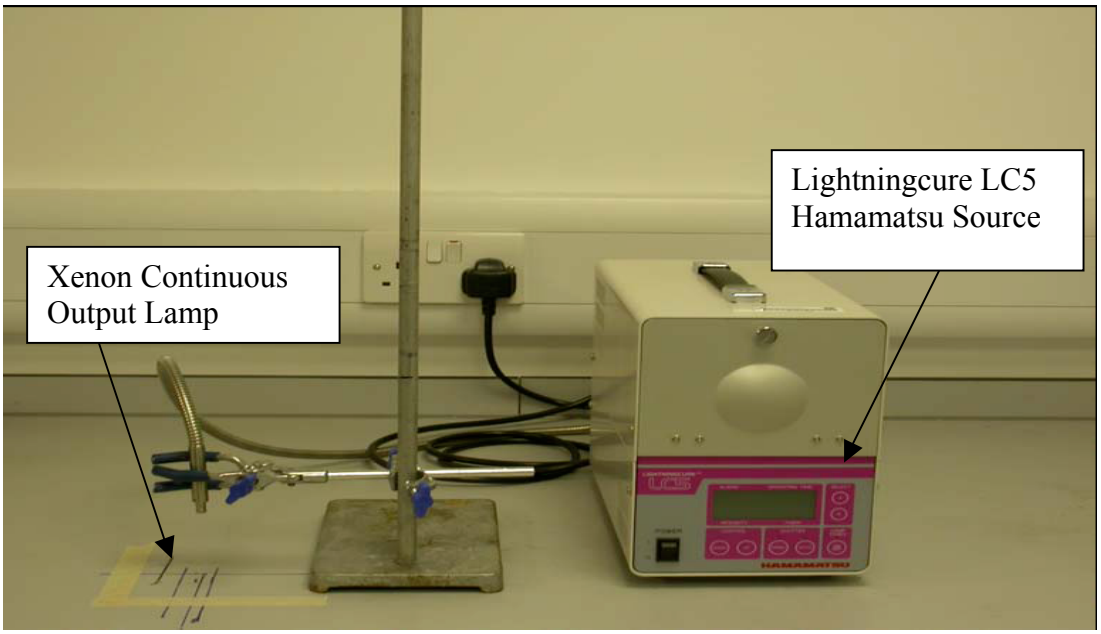

Figure 4.9 *Xenon continuous output lamp used in photoreactivation studies.*

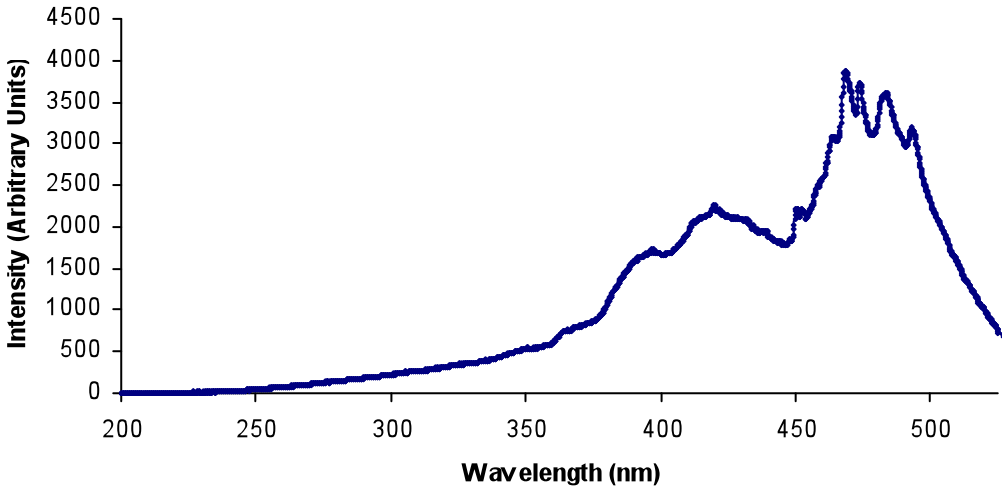

Figure 4.10 *Emission spectrum of the Hamamatsu Xenon continuous output lamp. The spectra also emits in the infrared region (not shown). A UV grating was used to demonstrate the important photoreactivation wavelengths in more detail.*

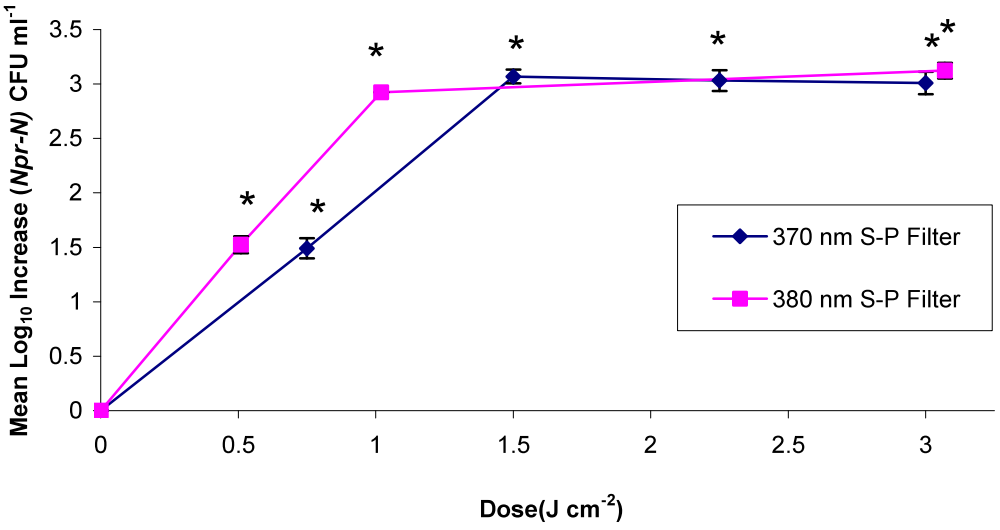

Figure 4.11 *Saturation curves of photoreactivation achieved in sub-lethally damaged* ***S. aureus*** *cells usimg 370 (±10) and 380 (±10) nm bandpass filters.*

4.7.2 Optimum Wavelengths for Photoreactivation

From the results of 4.7.1, a dose of 1.5 J cm^{-2} was chosen as the standard for the study of the degree of photoreactivation at different wavelengths over the range 320-450 nm. Each filter has a different transmission coefficient - transmission coefficients vary between around 35% and 55% - and as seen in Figure 4.10, the lamp output varies appreciably with wavelength. This means that the sample exposure time for photoreactivation is different for different wavelengths in order to obtain the standard dose of 1.5 J cm^{-2}. Table 4.1 lists these exposure times for the different wavelengths.

With N_0 as the starting population of *S. aureus* prior to exposure to PUV light, N as the number of remaining cells following PUV light exposure (inactivation) and N_{pr} as the number of cells that have undergone photoreactivation, the photoreactivation efficiency (ε) of *S. aureus* is defined as:

$$\varepsilon = (N_{pr} - N)/(No - N) \qquad [4.1]$$

The denominator of the equation is the number of cells successfully inactivated and available for photoreactivation and the numerator is the number of cells undergoing photoreactivation.

In Figure 4.12, ε is plotted as a function of wavelength. The results demonstrate that photoreactivation readily occurs in *S. aureus*, with optimum photoreactivation occurring between 360 nm and 380 nm (ε = 0.024) (all photoreactivated samples were statistically significantly different to their respective PUV-treated samples).

Table 4.1 *Light intensity and exposure times required in the band-pass filters from 320-450 nm to achieve a dose of 1.5 J cm^{-2} in each case.*

Short-pass Filter Wavelength (nm)	**Irradiance through Filter (mW cm^{-2})**	**Exposure Time**	**Dose (J cm^{-2})**
320	0.313	81m 46s	1.5
330	0.392	65m 17s	1.5
340	0.369	69m 21s	1.5
350	0.577	44m 21s	1.5
360	0.484	52m 52s	1.5
370	0.833	30m 43s	1.5
380	1.706	15m	1.5
390	4.8	5m 20s	1.5
400	5.1	5m 1s	1.5
410	5.3	4m 49s	1.5
415	5.7	4m 29s	1.5
420	6.2	4m 8s	1.5
430	6.1	4m 12s	1.5
440	7.1	3m 36s	1.5
450	7.4	3m 28s	1.5

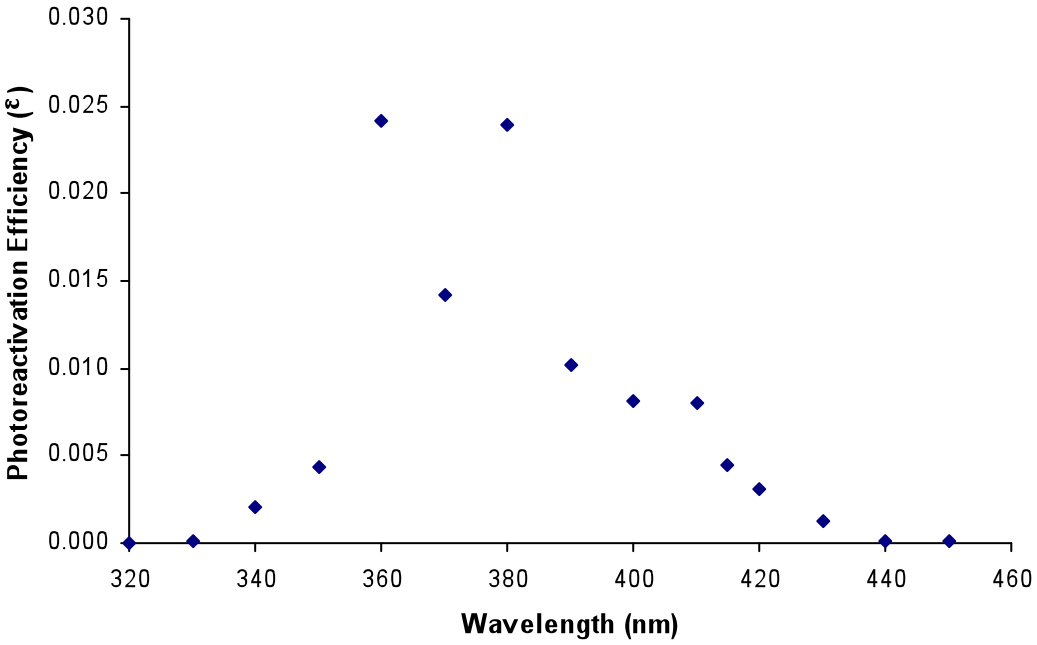

Figure 4.12 *The photoreactivation spectrum of* ***S. aureus****; photoreactivation efficiency (ε) is plotted as a function of wavelength.*

4.7.3 Dependence of Photoreactivation on Inactivation Damage

In order to investigate the effect of increasing UV damage on photoreactivation, suspensions of *S. aureus* were exposed to an increasing number of pulses of UV-rich light and then exposed to photoreactivating light of 370 (±10) nm at the standard dose of 1.5 J cm^{-2}. As shown in Figure 4.13, no cells survived more than 6 pulses and therefore no photoreactivation occurred. However, around a 2 to 3-log_{10} CFU ml^{-1} increase in bacterial numbers is achieved for 1 to 5 pulses of UV-rich light. All successfully photoreactivated samples are statistically significantly different to their respective PUV-treated samples. The results in Table 4.2 show the photoreactivation as a percentage of the original inactivated population. It demonstrates that, upon increasing the UV pulse number administered to the suspension, the degree of photoreactivation decreases.

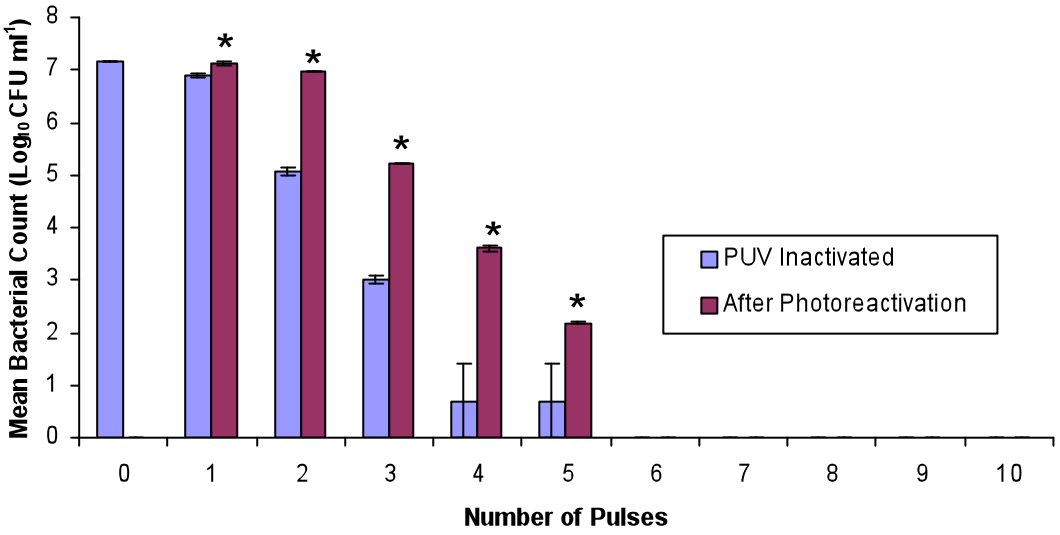

Figure 4.13 *Inactivation curve of a 10^7 CFU ml^{-1}* ***S. aureus*** *population as a function of number of UV pulses, and subsequent photoreactivation achieved following exposure to a 1.5 J cm^{-2} dose of 370 (±10) nm-light.*

Table 4.2 *The percentage photoreactivation upon exposure to 370 (±10) nm filtered light at a total dose of 1.5 J cm^{-2} following exposure to different numbers of pulses of UV-rich light. Percentage error is zero when photoreactivation data is identical.*

Pulses	**Initial Population (Log_{10} CFU ml^{-1}) (N_0)**	**Population After PUV-treatment (Log_{10} CFU ml^{-1}) (N)**	**Population After Photoreactivation (Log_{10} CFU ml^{-1}) (N_{pr})**	**Percentage Photoreactivation (%) $\varepsilon = (N_{pr}-N)/(N_0-N) \times 100$**
1	7.1 (±0.01)	6.9 (±0.05)	7.0 (±0.05)	99.12 (±19%)
2	7.2 (±0.01)	5.1 (±0.07)	6.9 (±0.01)	63.52 (±3%)
3	7.2 (±0.01)	3.0 (±0.08)	5.2 (±0.01)	1.12 (±0.05%)
4	7.2 (±0.01)	0.7 (±0.7)	3.6 (±0.07)	0.03 (±0.01%)
5	7.2 (±0.01)	0.7 (±0.7)	2.2 (±0.02)	0 (±0%)
6	7.2 (±0.01)	0 (±0.0)	0	0 (±0%)
7	7.2 (±0.01)	0 (±0.0)	0	0 (±0%)
8-10	7.2 (±0.01)	0	0	0 (±0%)

4.8 DISCUSSION OF PHOTOREACTIVATION RESULTS

The photoreactivation efficiency data of Figure 4.12 show that optimum photoreactivation occurs between 360 nm and 380 nm. The maximum photoreactivation efficiency of 0.024 corresponds to an increase in population of approximately 2.4% (of original PUV-rich light inactivated population 7-Log_{10} CFU ml^{-1}) or a 3-log_{10} CFU ml^{-1} increase. Lani (2007) inactivated *L. monocytogenes* cells using a similar Xenon flashlamp system and then exposed the cells to fluorescent light to undergo photoreactivation. Lani (2007) found that *L. monocytogenes* was capable of undergoing photoreactivation, and that saturation was reached by around 2.5-log_{10} CFU ml^{-1}. Although the work cannot be directly compared, the results are very similar to those found in this study with the use of filtered light. Although only a relatively small percentage of the UV-damaged cells undergo photoreactivation, the number of cells remaining may be enough to cause infection or spoilage. A study by Bohrerova and Linden (2006) used both medium-pressure and low-pressure lamps at different UV fluencies to determine the photoreactivation capability of *Mycobacterium terrae*. They found that the photo-repair ability was similar in each system and that this ability decreased upon increasing the UV fluence (dose). They attained optimum photoreactivation of 10.3% in *M. terrae* after 5 mJ cm^{-2} medium-pressure UV-rich light exposure. Their result is higher than that attained in this study for *S. aureus*. They also used an endonuclease sensitive site assay capable of detecting CPD dimers formed in DNA as a result of UV-light damage. The results of the study demonstrate that CPDs were produced as a result of both the medium-pressure and low-pressure lamps.

The results of Figure 4.13 and Table 4.2 show that increasing the number of pulses of PUV-rich light decreases the likelihood of photoreactivation. Table 4.2 highlights the photoreactivation capability of *S. aureus* sub-lethally damaged cells; after exposure to 1 or 2 UV-rich light pulses, a 7-Log_{10} CFU ml^{-1} population can photoreactivate 99% or 64% respectively. In a 7-Log_{10} CFU ml^{-1} population of *S. aureus*, after 5 pulses of UV-rich light, no photoreactivation of *S. aureus* cells occurred. This could be due to production of more mutagenic dimers than the

photolyase enzyme could repair within the time period. It may also be possible that after 5 pulses, free radicals produced by UV-A could have caused irreparable cell damage (Oguma *et al*., 2002). Oguma *et al*. (2002) have described that UV-B and UV-C radiation can destroy vital cell proteins, perhaps the photolyase enzyme itself, rendering the cell unable to repair any dimers formed subsequently, thus causing cell death. This result is significant in that it shows that high dose exposure to PUV-rich light reduces the possibility of photoreactivation.

Pulsed broadband light containing UV has proven to be an extremely effective decontamination technology, inactivating even large bacterial populations of *S. aureus* in fewer than ten pulses. However, reduced bactericidal effect was seen in 9-Log_{10} CFU ml^{-1} populations due to increasing opacity of the liquids being exposed. The results from this study have also shown that *S. aureus* cells that are not completely inactivated can undergo photoreactivation and return to infective numbers upon exposure to light. In addition, this study demonstrates that photoreactivation occurs optimally at wavelengths between 360 nm and 380 nm. In order to reduce the likelihood of photoreactivation it is important to establish the total doses required for inactivation of various problematic bacteria in order to prevent sub-lethal damage occurring. It may also be useful to remove photoreactivation wavelengths (UV-A) from broadband UV-rich light sources to prevent concomitant photoreactivation.

Chapter 5

HINS-LIGHT INACTIVATION OF FOODBORNE BACTERIAL PATHOGENS

5.0 BACKGROUND

Chapter 4 assessed the inactivation capability of PUV-rich light on *Staphylococcus aureus* and the subsequent photoreactivation phenomenon that can occur upon exposure to wavelengths of UV-A/Visible light. This chapter progresses to investigate a visible light-based system termed High-intensity Narrow Spectrum (HINS) light that utilises high-intensity 405-nm LED arrays to inactivate microorganisms.

The contamination of foodstuffs with microorganisms that can cause illness is a long-standing problem in the food industry with the Food Standards Agency (FSA) reporting 926,000 cases in the UK in 2007, with a cost to the economy of around £1.5 billion (FSA, 2009). This is a conservative estimate of the true cost of foodborne illness as it only reflects instances in which medical care was sought, and where positive clinical specimens identified foodborne bacteria to be the cause of the illness (Kuchenmüller *et al*., 2009). This chapter investigates the susceptibility of common foodborne pathogens to inactivation through 405-nm HINS-light exposure, and the bacteria investigated in this chapter are some of the microorganisms most frequently associated with foodborne illness throughout the world.

Campylobacter jejuni is a Gram negative, helical-shaped bacterium, around 0.2-0.5 μm in length. It is a microaerophilic bacterium requiring an atmosphere of 10% CO_2/5% O_2 in order to survive and replicate. *Campylobacter jejuni* is the most common bacterial cause of diarrhoeal disease around the world, and 82% of hospital foodborne illness admissions are related to *Campylobacter* infection (Humphrey *et al*., 2007; Zilbauer *et al*., 2008). Clinical disease ranges from gastroenteritis to more severe peripheral neuropathies such as Guillan Barré Syndrome (Humphrey *et al*.,

2007). Some of the foods implicated in causing *C. jejuni* infection are unwashed salads, and undercooked meats and poultry, with the latter being recognised as the main source of *C. jejuni* associated with foodborne illness: ~57% of store-bought chickens test positive for *C. jejuni* (Humphrey *et al*., 2007).

Shigella sonnei is a Gram negative, facultative anaerobic, non-sporulating rod and is also classified as serogroup D in relation to serological differences between O antigens in *Shigella* species (Niyogi, 2005). *Shigella sonnei* is another causative microorganism involved in diarrhoeal disease. The total number of diarrhoeal episodes thought to be due to *Shigella* species is considered to be around 165 million worldwide (Kotloff *et al*., 1999). Around 1 million cases are in developed countries; the remainder are in developing countries. Symptoms of infection include watery diarrhoea that becomes mucoid and bloody due to the production of enterotoxins that destroy the lining of the colon (Warren *et al*., 2006). Infection with *Shigella* may lead to more complicated diseases such as haemolytic uremic syndrome (HUS) (Warren *et al*., 2006). Infection is spread via the faecal-oral route, with flies known to be a large cause of the spread of *Shigella* onto foods and frequently touched surfaces (Niyogi, 2005).

Salmonella enterica subsp. *enterica* serovar *enteritidis* (formerly *Salmonella enteritidis*) is a facultative anaerobic Gram negative rod of length between 0.7-2.5 μm. *Salmonellae* infections account for 30% of all foodborne illness-associated deaths, and is the second most common bacterial cause of diarrhoeal illness (Crum, 2008). Symptoms of infection include gastroenteritis, endovascular infections and bacteremia, with the young and immuno-suppressed being particularly susceptible to infection. The most common sources of *S. enterica* serovar *enteritidis* are poultry, eggs and dairy products (Crum, 2008).

Escherichia coli is a Gram negative rod that has received a large amount of media attention owing to the numerous outbreaks of foodborne illness caused by the O157:H7 strain of *E. coli*. *Escherichia coli* O157:H7 is an enterohaemorrhagic strain of *E. coli* that causes haemorrhagic diarrhoea, kidney failure and can be potentially

life-threatening in susceptible groups such as the elderly and young children (Olsvik *et al*., 1991). *Escherichia coli* O157:H7 strains are often capable of producing toxins such as Shiga toxin or verocytotoxin that can lead to the development of HUS (Grys *et al*., 2009). Possible sources of *E. coli* are minced meats, unpasteurised milk, minimally washed vegetables such as lettuce, and contact with farm animals (Woodward *et al*., 2002).

Listeria monocytogenes is a Gram positive, non-sporulating rod-shaped facultative anaerobe. *Listeria monocytogenes* has the ability to grow at very low temperatures and has been known to survive for up to 3 months when stored at 4°C (Chan and Wiedmann, 2009). This ability allows it to grow in food commonly stored at refrigeration temperatures such as coleslaw, soft cheeses, cold meats and other so-called "ready to eat" foods. *Listeria monocytogenes* is not a problematic cause of foodborne illness to most healthy individuals; severe disease often presents in the young, elderly, immuno-suppressed and pregnant women. Symptoms of listeriosis include septicaemia, meningitis and endocarditis (Chan and Wiedmann, 2009). Pregnant females who ingest food contaminated with *L. monocytogenes* are at risk from miscarriage and stillbirth.

Bacillus cereus is a Gram positive, endospore-forming rod, and like the other members of the *Bacillus* genus, it is ubiquitous in the environment (Rowan *et al*., 2003). Owing to this, bacilli in soils can frequently contaminate cereal crops, rice and spices (Brown, 2000). The production of an endospore allows the bacterium to survive various environmental stresses, and when conditions are optimal, germinate and proliferate in many processed foodstuffs (Brown, 2000). Foodborne illness tends to occur where food has been cooked but is left to cool slowly, providing ideal growth conditions for *B. cereus* (Brown, 2000). *Bacillus cereus* can cause two types of foodborne illness: emetic and diarrhoeal. Emetic-type illness occurs when *B. cereus* has proliferated and formed toxins in foods, which are then consumed, and has an onset of 1-6 hours (Brown, 2000; Wijman *et al*., 2007). Diarrhoeal-type illness occurs when contaminated food is ingested and *B. cereus* cells produce toxins

in the small intestine, and has an onset of 8-24 hours (Brown, 2000; Wijman *et al*., 2007).

The economic burden of foodborne disease is a global issue and there is an ongoing need to augment established food safety methods such as chemical decontamination, modified atmosphere gases and irradiation, through application of hurdle technologies, and where possible introduce new approaches, to achieve effective decontamination of foodstuffs and the environments in which they are packaged and processed.

This chapter investigates the use of high-intensity 405-nm light for the inactivation of some of the most important foodborne illness-related bacteria in both liquid suspensions and on surfaces.

5.1 LIGHT SOURCE- LED ARRAYS

Throughout this investigation three different Light Emitting Diode (LED) arrays were used in the liquid based test system. The light emitted by the LED arrays was generated in the active region of an indium gallium nitride/gallium nitride (InGaN/GaN) semiconductor junction. The three LED arrays used were as follows:

- A 99-DIE LED array with a centre wavelength of 400.72 nm (Source A)
- A 99-DIE LED array of centre wavelength 400.46 nm (Source B)
- A 60-DIE LED array of centre wavelength 407.29 nm (Source C)

The output of the LED arrays have a bandwidth of ~10 nm at full width half maximum (FWHM) (Figure 5.1). These arrays were produced by OptoDiode Corp (CA, USA) and are shown in Figure 5.2.

The LED arrays were bonded to a heat sink and cooling fan, attached to minimise the temperature increase in the semiconductor junction, which in turn minimised heat transfer to the liquid samples. The LED arrays were mounted in PVC housing

designed to fit a 12-well micro-plate (NUNC, Denmark), with the array positioned directly above one of the central sample wells (Figure 5.3). The LED arrays (Sources A and C) were powered by a DC power supply (0-3 A and 0-15 V). Source B was powered by a second DC power supply (0-20 A and 0-16 V). Source A's current was set at 0.5 A (±0.05) at a voltage of 11.2 V (±0.2), to give an approximate irradiance of 10 mW cm^{-2} at the surface of the bacterial suspension (Source B was set at a current of 0.2 A (±0.05) at a voltage of 9.6 V (±0.2) to give 10 mW cm^{-2} and Source C was used at both 10 mW cm^{-2} (0.3 A (±0.5) and 9.9 V (±0.2)) and 40 mW cm^{-2} (0.8 A (±0.5) and 12.7 V (±0.2)).

Irradiance was measured using a radiant power meter (L.O.T.-Oriel Ltd, UK) in conjunction with a photodiode detector.

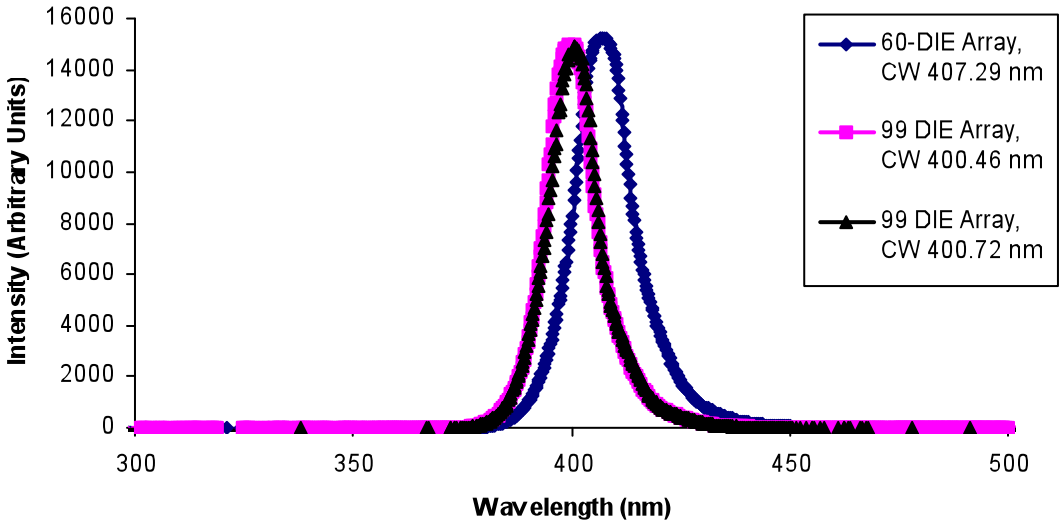

Figure 5.1 *The emission spectra of the 3 different LED arrays*

Figure 5.2 *The three 405-nm LED arrays used in this study, L-R Source A (20 × 16 mm), Source B (20 × 16 mm) and Source C (33 × 33 mm).*

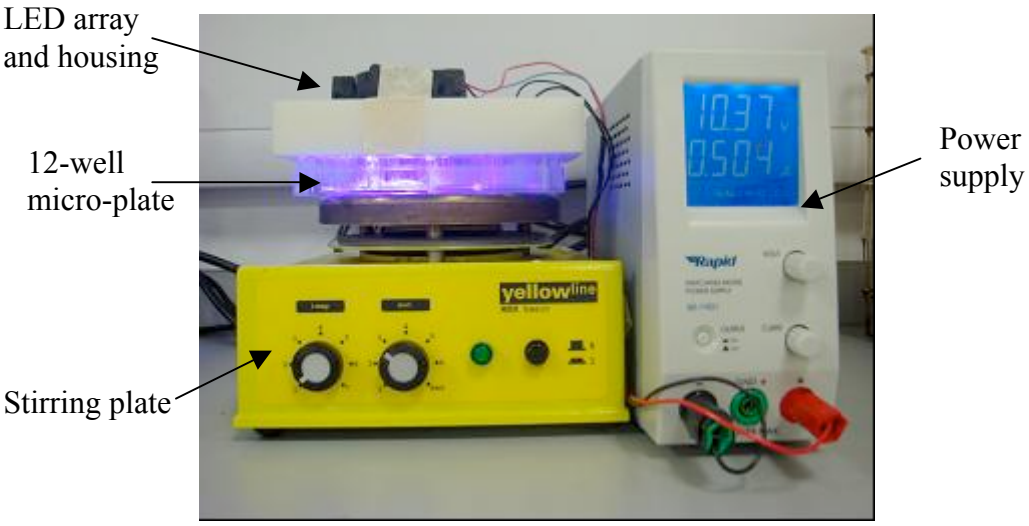

Figure 5.3 *Experimental arrangement of 405-nm LED array used in the inactivation of liquid bacterial suspensions.*

5.2 PREPARATION OF BACTERIA AND TREATMENT METHOD

5.2.1 Bacterial Preparation

The bacteria used in HINS-light exposure experiments were *Campylobacter jejuni* LMG 8841, *Shigella sonnei* NCTC 12984, *Salmonella enterica* serovar *enteritidis* NCTC 4444, *Escherichia coli* O157:H7 NCTC 12900 and *Listeria monocytogenes* NCTC 11994. Bacteria were cultured according to their growth requirements and were centrifuged at 3939 × g for 10 minutes in order to produce a bacterial pellet (see Chapter 3, Section 3.1-3.2). The pellet was re-suspended into 100 ml of PBS and diluted to the appropriate starting population for experimentation.

5.2.2 Treatment Method

A 2 ml volume of the bacterial starting population was pipetted into one of the central wells of a 12-well micro-plate giving a liquid depth of 7 mm. A micro-magnetic follower (7 × 2 mm) was placed in the well and the plate placed onto a magnetic stirrer plate for continuous agitation of the sample. The LED array (in housing) was set at the required irradiance of 10 mW cm^{-2}, and placed directly over the sample well (without plate lid). A second multi-dish was set up exactly as described but received no high-intensity 405-nm light and served as a control (with plate lid on). Test samples were exposed to increasing durations of high-intensity 405-nm light, and control samples were obtained for each time exposure investigated. It is worth noting that due to the population densities (approximately 5-Log_{10} CFU ml^{-1}) and sample depths (7 mm) used in this study, there is no measurable attenuation of light irradiance through the bacterial suspension, therefore the 10 mW cm^{-2} irradiance can be considered to be constant throughout the entire sample depth (Maclean *et al*., 2009).

Test and control samples of *Salm. enterica* serovar *enteritidis* and *Sh. sonnei* were plated onto Nutrient Agar, and *L. monocytogenes* onto Tryptone Soya Agar as described in Chapter 3, Section 3.3.2. These samples were incubated at 37ºC for 24 hours. *Campylobacter jejuni* samples were plated onto Blood Agar, placed in a gas jar containing a CampyGen™ sachet and incubated at 42ºC for 48 hours. After the

appropriate incubation period, the plates were enumerated and results reported as $\log_{10}$ CFU ml^{-1} (Chapter 3, Section 3.3.2).

5.2.3 Dose-Dependence Experiments

In order to establish if inactivation of bacteria was dose-dependent, a series of experiments were carried out. Suspensions of *L. monocytogenes* at a population density of 5-Log_{10} CFU ml^{-1} were exposed to 108 J cm^{-2} of 405-nm light from Source A applied in three different modes. In this case, three different light intensities were used: 10 mW cm^{-2} (0.5 A; 11.2 V), 20 mW cm^{-2} (0.8 A; 11.6 V) and 30 mW cm^{-2} (1.3 A; 12.4 V). In order to keep the total dose constant in each case (i.e. 108 J cm^{-2}), the sample exposure time was adjusted according to Equation 4.1 in Chapter 4 Section 4.7.3.2, where $E=P't$.

5.3 RESULTS- INACTIVATION OF FOODBORNE BACTERIA

Bacterial suspensions were exposed to 405-nm light at an irradiance of 10 mW cm^{-2} for increasing time periods; each data point represents an individual experiment that was repeated at least twice (using Source A). Figure 5.4 shows the effects of high-intensity 405-nm light exposure on *C. jejuni* and it can be seen that complete inactivation of a 5-Log_{10} CFU ml^{-1} population was achieved at a dose of 18 J cm^{-2}. With statistically significant differences between the control and 405-nm light-exposed samples occurring from a dose of 6 J cm^{-2} onwards.

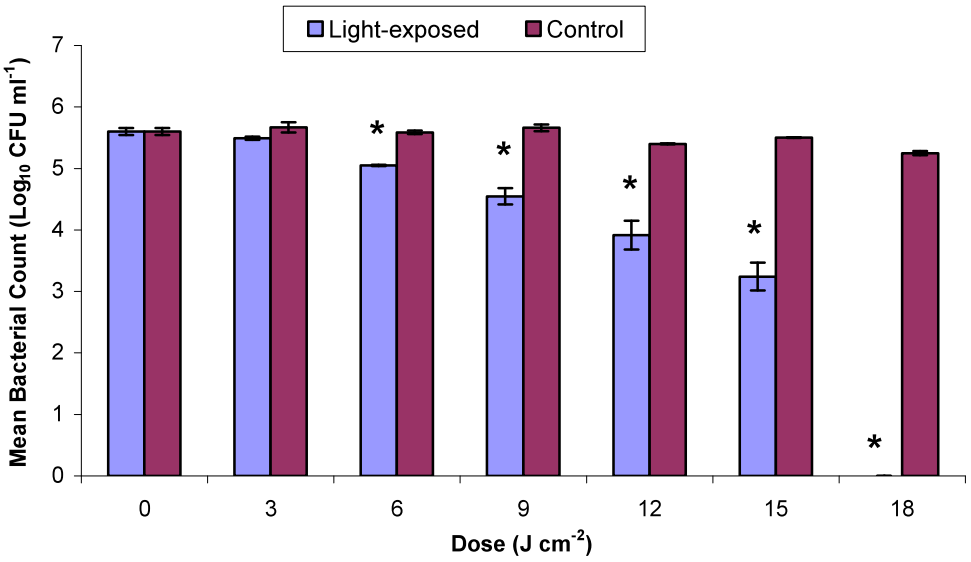

Figure 5.4 *Inactivation of* ***Campylobacter jejuni****, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 10 mW cm^{-2} using Source A.*

Figure 5.5 depicts the effect of high-intensity 405-nm light exposure on *Sh. sonnei*, and it is observed that a 5-log$_{10}$ reduction was achieved at a dose of 180 J cm^{-2}, ten times the dose required for inactivation of 5-Log$_{10}$ CFU ml^{-1} population of *C. jejuni*. Statistically significant difference between controls and light-exposed samples was seen from a dose of 36 J cm^{-2}.

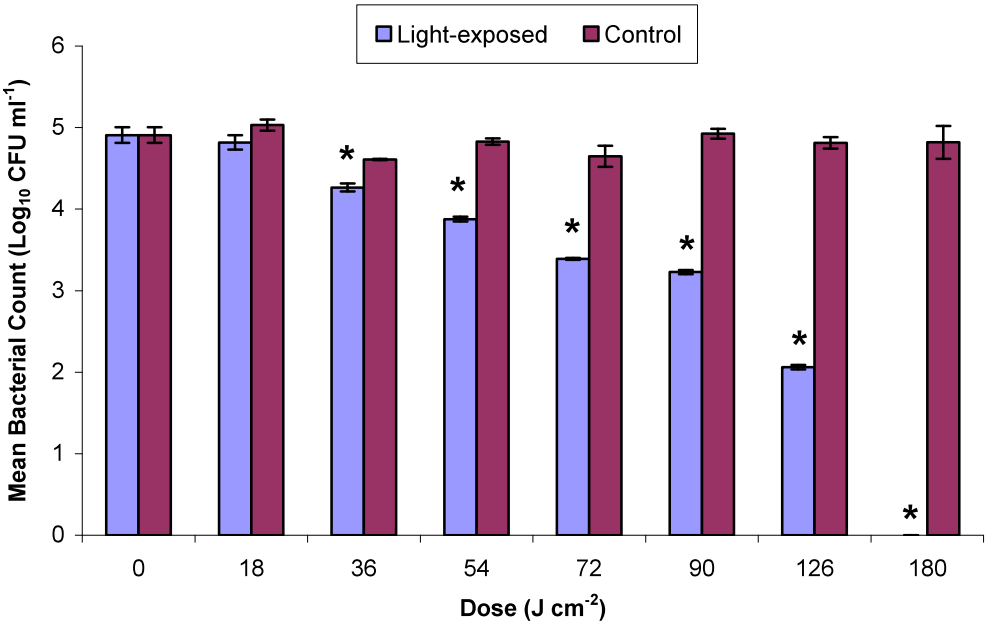

Figure 5.5 *Inactivation of **Shigella sonnei**, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 10 mW cm^{-2} using Source A.*

Exposure to high-intensity 405-nm light also had a bactericidal effect on *Salm. enterica* serovar *enteritidis*, although to a lesser extent than that observed with both *Sh. sonnei* and *C. jejuni*. Figure 5.6 demonstrates that an approximate 3-log$_{10}$ reduction of *Salm. enterica* serovar *enteritidis* was found upon application of a dose of 288 J cm$^{-2,}$ with a statistically significant difference between control and light-exposed samples occurring from a dose of 108 J cm^{-2}. At the exposure times and light intensities applied in this study, complete inactivation of *Salm. enterica* serovar *enteritidis* was not observed, although the general trend indicates that application of an increased exposure time (and consequently a higher dose) would have induced a further decrease in the *Salmonella* population.

HINS-light inactivation of *E. coli* O157:H7 is shown in Figure 5.7. A 5-log$_{10}$ reduction in bacterial numbers is seen by a dose of 288 J cm^{-2}. *E. coli* O157:H7, was more difficult to inactivate than *C. jejuni* and *Sh. sonnei*, but more readily inactivated than *Salm. enterica serovar enteritidis*. A statistically significant difference between

control samples and light-exposed samples was seen after a dose of 72 J cm^{-2} was applied to the *E. coli* O157:H7 suspensions.

All the bacteria tested were reduced by approximately 5-log_{10} CFU ml^{-1} with the exception of *Salm. enterica* serovar *enteritidis*, with each organism varying in the total dose required for complete inactivation.

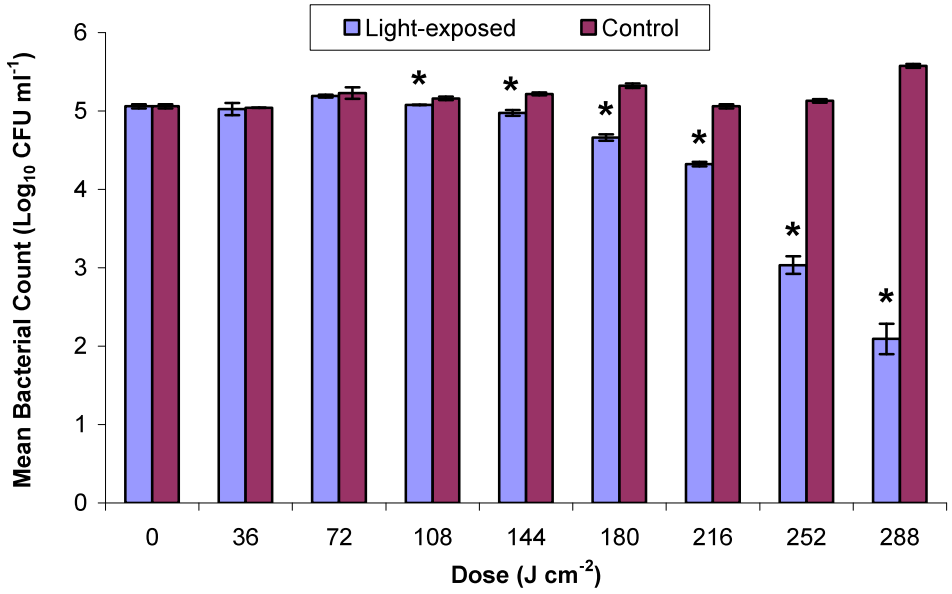

Figure 5.6 *Inactivation of **Salmonella enterica** serovar **enteritidis**, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 10 mW cm^{-2} using Source A.*

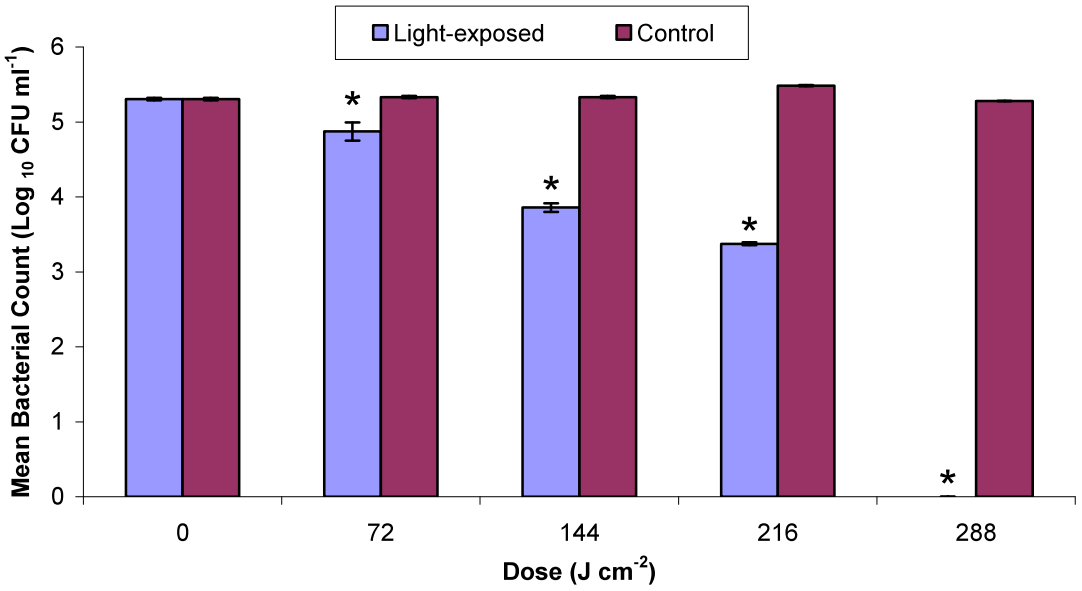

Figure 5.7 *Inactivation of* ***Escherichia coli*** *O157:H7, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 10 mW cm^{-2} using Source A.*

L. monocytogenes was also exposed to high-intensity 405-nm light and results (Figure 5.8) demonstrate that a 5-log$_{10}$ reduction was achieved after exposure to a dose of 108 J cm^{-2}. Similar to *Sh. sonnei*, a statistically significant difference between control and light-exposed samples was seen from 36 J cm^{-2} onwards.

It should also be noted that the population densities of control samples for all five bacterial species remained constant throughout this series of experiments (Figures 5.4, 5.5, 5.6, 5.7 and 5.8). This result was particularly important with relevance to the *C. jejuni* experiments as this demonstrated that there was no direct inactivation of *C. jejuni* through exposure to normal atmospheric conditions over the experimental period.

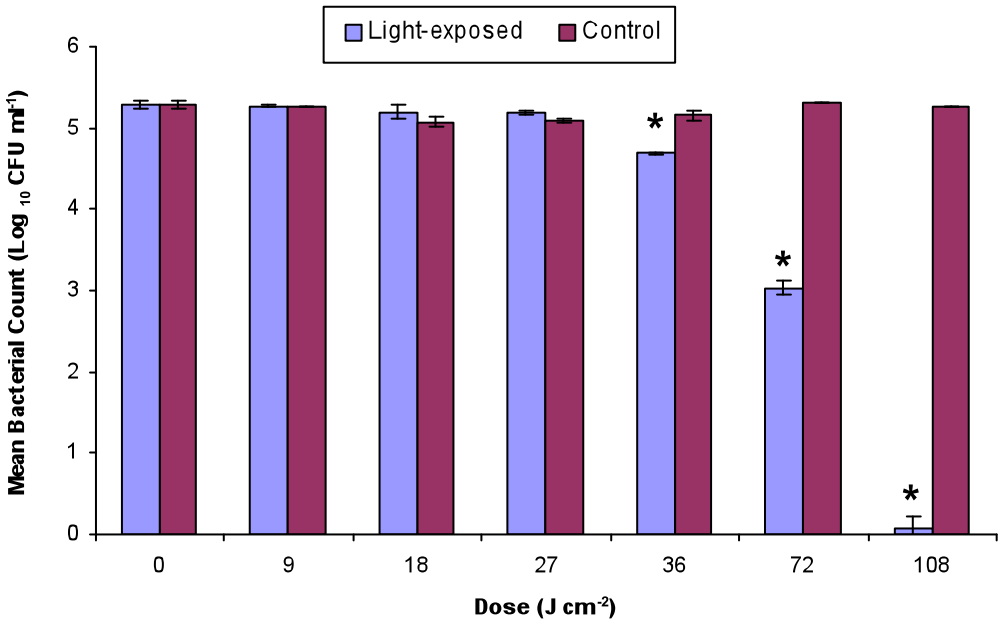

Figure 5.8 *Inactivation of* ***Listeria monocytogenes****, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 10 mW cm^{-2} using Source A.*

Table 5.1 contains the results of exposing suspensions of *L. monocytogenes* to 405-nm light, with the 108 J cm^{-2} dose being applied in 3 different ways: 10 mW cm^{-2} for 180 minutes, 20 mW cm^{-2} for 90 minutes and 30 mW cm^{-2} for 60 minutes. The results demonstrate that application of the dose, regardless of how it is applied (i.e. lower irradiance for longer exposure time or higher irradiance for shorter exposure time), yields very similar final populations, achieving an approximate 5-log$_{10}$ reduction in bacterial population. Although the final populations of *L. monocytogenes* were all statistically significantly different from the initial populations, there was no statistical difference between the final values achieved in the *L. monocytogenes* high-intensity 405-nm light exposures, regardless of how the 405-nm light dose was applied (10, 20 or 30 mW cm^{-2}).

Table 5.1 *High-intensity 405-nm light inactivation of L. monocytogenes through exposure to a dose of 108 J cm^{-2}, applied in three different ways.*

Power Density (mW cm^{-2})	Exposure Time (min)	Dose (J cm^{-2})	Initial Population, N_0 (Log$_{10}$ CFU ml^{-1})	Final Population, N (Log$_{10}$ CFU ml^{-1})
10	180	108	5.3 (±0.05)	0.07 (±0.2)*
20	90	108	5.04 (±0.07)	0 (±0.0)*
30	60	108	5.16 (±0.02)	0.18 (±0.4)*

5.3.1 Effect of Temperature on Bacterial Inactivation

In order to eliminate the possibility of inactivation being the result of heat transfer from the LED array during sample exposure, the temperature of the bacterial suspensions was monitored during experimentation. Temperature readings (taken every 15 minutes) showed that the bacterial suspensions experienced minimal temperature changes during light exposure. In the case of the longest exposure period, which was 480 minutes for *Salm. enterica* serovar *enteritidis and E. coli* O157:H7, the initial temperature of the suspension was 26^{o}C, and fluctuated ±1^{o}C throughout the exposure period. Temperatures were measured using a thermocouple (Kane May, UK) placed directly into the liquid sample.

5.4 INACTIVATION OF *BACILLUS* SPECIES

5.4.1 Bacterial Preparation

This section describes the growth and culture requirements for the *Bacillus* species investigated in this study.

Vegetative Cells

Bacillus cereus NCTC 11143 was cultured in 100 ml of Nutrient Broth at 37°C for 24 hours and subsequently centrifuged for 10 minutes at 3939 × g to produce a

bacterial pellet. The pellet was re-suspended into 100 ml PBS and diluted to the appropriate starting population for experimentation (Chapter 3, Section 3.1-3.2).

Endospores

B. cereus NCTC 11143, *B. megaterium* (B17/97 isolated from a septic human finger) and *B. subtilis* (isolated from Aptamil™ infant formula) were grown in 100 ml of Nutrient Broth supplemented with 100 μL of $MnSO_4$ at 37°C for 3 days to encourage spore production. The broths were then centrifuged for 10 minutes at 3939 × g to produce a bacterial pellet. The pellet was re-suspended into 100 ml of PBS and diluted to ~6-Log_{10} CFU ml^{-1} and heat-treated in a water bath set at 80°C for 10 minutes to inactivate any vegetative cells. The heat-treatment inactivated ~ 1-log_{10} CFU ml^{-1} of vegetative cells from the spore suspension leaving ~5-Log_{10} CFU ml^{-1} of *Bacillus* spores remaining for experimental use.

5.4.2 Treatment Method

The bacterial suspensions (vegetative and spore) were exposed to HINS-light as described in Section 5.2. Two LED arrays were used for experimentation; Source A and Source C. Source A was used at an irradiance of 10 mW cm^{-2} and Source C was used at both 10 mW cm^{-2} and 40 mW cm^{-2}.

After exposure of the vegetative cell suspensions to HINS-light, test and control samples were plated onto Nutrient Agar and incubated at 37°C for 24 hours. HINS-light-exposed spore suspensions were heat-treated prior to plating in order to remove any vegetative cells that may have germinated from spores during the exposure period. Plating and enumeration are detailed in Chapter 3, Section 3.3.

Also, for vegetative cell experiments, the final inactivation data point was repeated with heat-treatment in order to ensure remaining cells were vegetative cells and not spores.

5.4.3 Effect of Temperature/Holding Conditions on *B. cereus* Endospores

In order to determine whether temperature was part of the spore inactivation process an experiment was devised in which *B. cereus* endospore suspensions were maintained at 35°C (higher than any HINS-light exposed temperature recordings) for a maximum duration of 12 hours. Two-ml suspensions of 5-Log_{10} CFU ml^{-1} heat-treated *B. cereus* spores were pipetted into each well of a 12-well multi-dish and placed in a rotary incubator set at 85 rpm at a temperature of 35°C. After each elapsed time-point the 2 ml sample was heat-treated at 80°C for ten minutes to ensure only spores were present, i.e. no germination had occurred. This was completed in triplicate for each time point investigated.

5.5 RESULTS

5.5.1 HINS-light Inactivation of *B. cereus* Vegetative Cell Suspensions

B. cereus vegetative cells were inactivated as in Section 5.2 using Source A at 10 mW cm^{-2}. Figure 5.9 shows *B. cereus* was inactivated by almost 4-log_{10} CFU ml^{-1} at a dose of 45 J cm^{-2}. Although the control also falls slightly, a significant statistical difference between control and light-exposed samples is seen from 36 J cm^{-2}.

Figure 5.10 uses Source C at an irradiance of 10 mW cm^{-2} to determine any difference in inactivation capability between LED sources A and C. It can be seen that approximately 1-log_{10} CFU ml^{-1} of *B. cereus* vegetative cells remain (~3-log_{10} CFU ml^{-1} reduction) after exposure to 72 J cm^{-2} of HINS-light from source C. Again the control falls slightly, however a statistically significant difference between control and light-exposed samples is seen from 9 J cm^{-2} onwards.

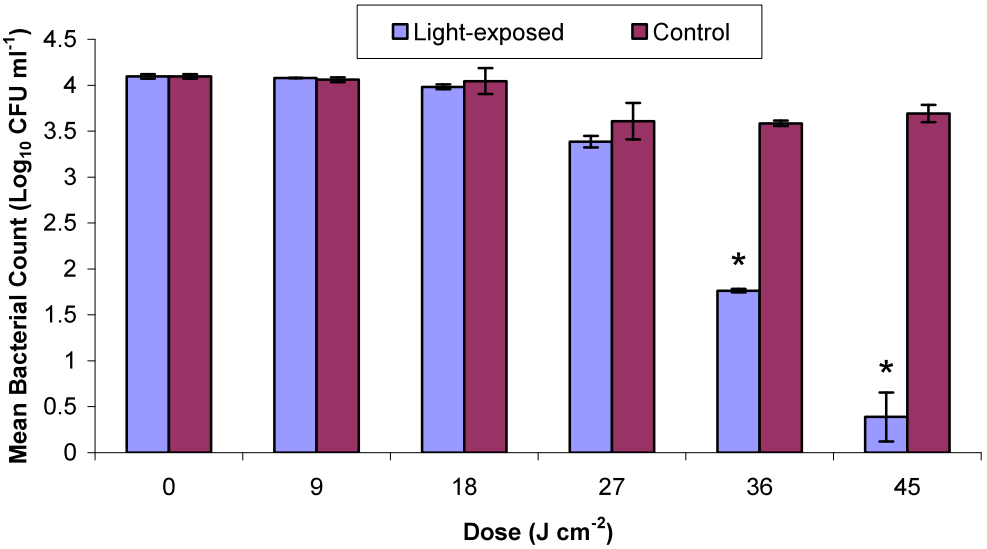

Figure 5.9 *Inactivation of **Bacillus cereus** vegetative cells, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 10 mW cm^{-2} using Source A.*

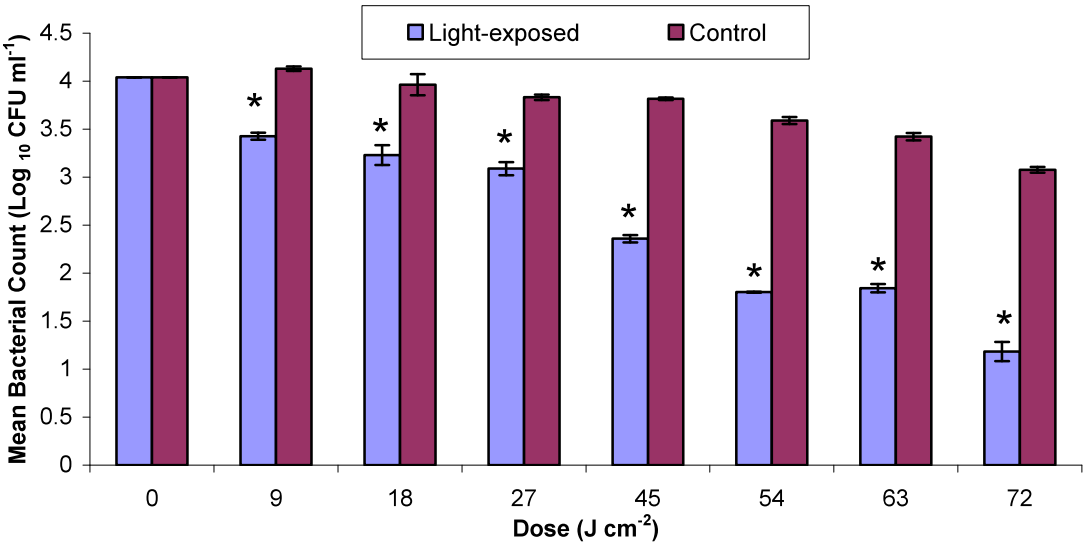

Figure 5.10 *Inactivation of **Bacillus cereus** vegetative cells, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 10 mW cm^{-2} using Source C.*

Source C was also used at a higher intensity of 40 mW cm^{-2} (see Figure 5.11) to determine whether inactivation through HINS-light exposure, was dose-dependent. Figure 5.11 shows that around a 3.5-log_{10} CFU ml^{-1} inactivation was achieved at a dose of 72 J cm^{-2}, similar to the results seen in Figure 5.10. In this case the control remained steadier than in previous experiments, possibly due to the shorter timescales used at 40 mW cm^{-2} to achieve the same total dose. Statistically significant differences between control and light-exposed samples were seen from exposure to a dose of 36 J cm^{-2} and above.

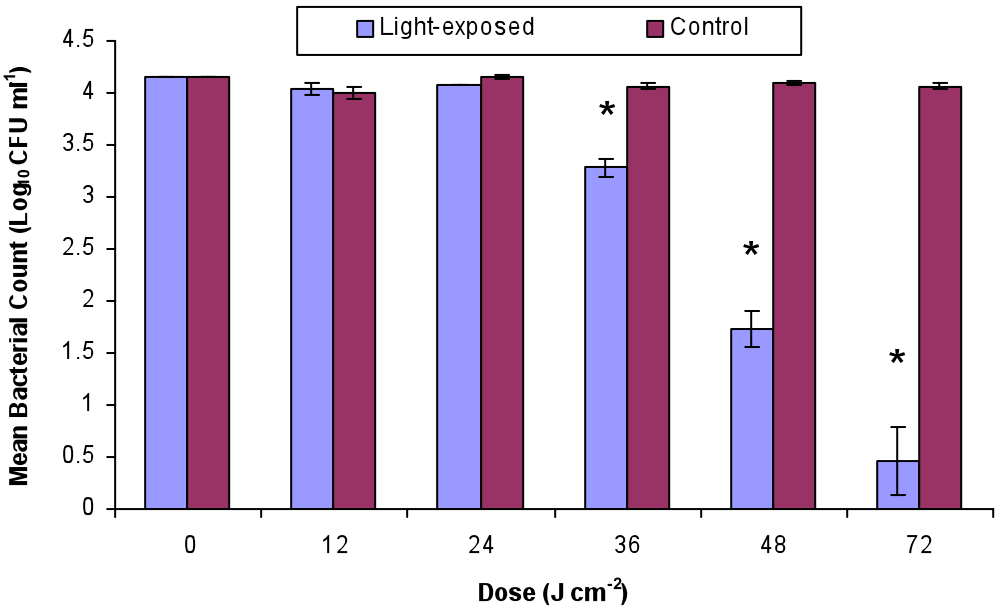

Figure 5.11 *Inactivation of **Bacillus cereus** vegetative cells, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 40 mW cm^{-2} using Source C.*

5.5.2 HINS-light Inactivation of *Bacillus* Spore Suspensions

A series of experiments were conducted using *Bacillus* species endospores exposed to high-intensity 405-nm light for a period 4 hours and 8 hours using Source C at an irradiance of 40 $mWcm^{-2}$. Three *Bacillus* species were tested: *B. cereus, B. megaterium* and *B. subtilis*. Table 5.2 depicts the light-exposed, non-exposed control and log_{10} CFU ml^{-1} reductions (all light-exposed samples were statistically significantly different to control samples) achieved for each species exposed to high intensity 405-nm light.

Figure 5.12 demonstrates the effect of temperature on *B. cereus* endospores over a 12-hour period. It can be noted that some change (statistically significantly different) in population density occured throughout the 12-hour period at 35°C, however the reduction was only around 0.5-log_{10} CFU ml^{-1}.

Table 5.2 *Comparison of the inactivation of different* ***Bacillus*** *species endospores using a 405-nm high-intensity light (Source C) at an irradiance of 40 mW* cm^{-2}.

Microorganism	Exposure Time / Dose	Non-exposed population (N_0)	Light-exposed population (N)	Log_{10} Reduction (N_0/N)
B. cereus	4 h / 0.58 kJ cm^{-2}	4.34 (±0.4)	3.32 (±0.6)	1.02*
	8 h / 1.15 kJ cm^{-2}	4.34 (±0.03)	2.09 (±1.5)	2.25*
B. megaterium	4 h / 0.58 kJ cm^{-2}	5.70 (±0.1)	4.83 (±0.3)	0.87*
	8 h / 1.15 kJ cm^{-2}	5.13 (±0.05)	3.29 (±0.04)	1.84*
B. subtilis	4 h / 0.58 kJ cm^{-2}	5.57 (±0.05)	4.99 (±0.3)	0.58*
	8 h / 1.15 kJ cm^{-2}	5.48 (±0.02)	3.77 (±0.2)	1.71*

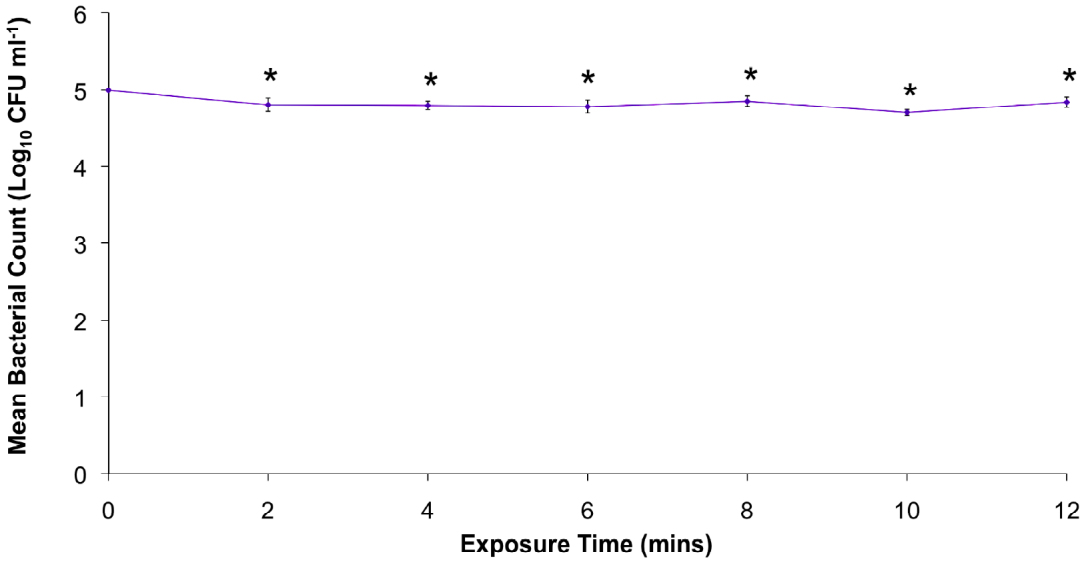

Figure 5.12 *Changes in **B. cereus** endospore count over a 12-hour period when spores are suspended in PBS at 35°C.*

5.6 GERMICIDAL EFFICIENCY

The inactivation capability of 405-nm light can be quantified as the germicidal efficiency (η), defined as the $\log_{10}$ reduction of a bacterial population by inactivation per unit dose in Joules per square centimetre (Wang *et al*., 2005):

$$\eta = \log_{10}(N / N_0) J cm^{-2} \qquad [5.1]$$

Where N_0 = the bacterial starting population, and N = the final bacterial population after exposure. Table 5.3 details the inactivation parameters for all the foodborne illness-related bacteria investigated following exposure to high-intensity 405-nm light. Uncertainty of the mean germicidal efficiency value was determined by calculating the germicidal efficiencies for the lowest and highest $\log_{10}$ reductions achieved at the final light dose applied for each microorganism, and the difference expressed as a percentage of the mean value.

Table 5.3 *Log_{10} reduction and germicidal efficiency values (including percentage uncertainty) for inactivation of the investigated pathogenic foodborne bacteria upon exposure to 405-nm high-intensity light (using LED Source A).*

Organism	Initial Population N_0 (Log_{10} CFU ml^{-1})	Final Population N (Log_{10} CFU ml^{-1})	Log_{10} (N/N_0) Reduction	Dose (J cm^{-2})	Germicidal Efficiency η (Log_{10} (N/N_0)/ J cm^{-2})
Campylobacter jejuni	5.60 (±0.06)	0 (±0.0)	5.60	18	0.31(±0.0%)
Shigella sonnei	4.90 (±0.09)	0 (±0.0)	4.90	180	0.03(±0.0%)
Salmonella enterica serovar *enteritidis*	5.06 (±0.02)	2.10 (±0.2)	2.96	288	0.01(±0.01%)
Escherichia coli O157:H7	5.30 (±0.02)	0 (±0.0)	5.30	288	0.02 (±0.0%)
Listeria monocytogenes	5.28 (±0.04)	0.08 (±0.2)	5.20	108	0.05 (0.0%)
Bacillus cereus (vegetative cells)	4.10 (±0.02)	0.39 (±0.3)	3.71	45	0.08(0.01%)

5.7 HINS-LIGHT INACTIVATION OF BACTERIA ON SURFACES

To investigate the inactivation effect of 405-nm light on surface adhered bacteria, a higher intensity 405-nm light emitting LED array system was utilised. Four common causative agents of food-related illness were used in this series of experiments.

5.7.1 Surface Decontamination System

In order to inactivate microorganisms rapidly on surfaces an ENFIS QUATTRO Mini Air Cooled Light Engine, which had an array of 144 violet LEDs and a centre wavelength of 407.52 nm, was used (Figure 5.13 and Figure 5.14). The light engine was attached to an LED driver, which was powered by a 48 V power supply. The light engine had an integrated heat sink and cooling fan to minimise any heat build up during experimentation.

In these experiments agar plates and inert surfaces were used as the surface onto which bacteria were seeded and subsequently exposed to 405-nm light emitted by the LED, and the test set-up is shown in Figure 5.15. Power density measurements from the LED light engine were recorded over a distance of 50 cm and a distance of 5 cm – which gave an approximate irradiance of 100 mW cm^{-2} at the centre point – was chosen for experimental use. Although the system provided high-intensity light at the centre point, it can be seen from Figure 5.16 that light irradiance decreased outwards from the centre point.

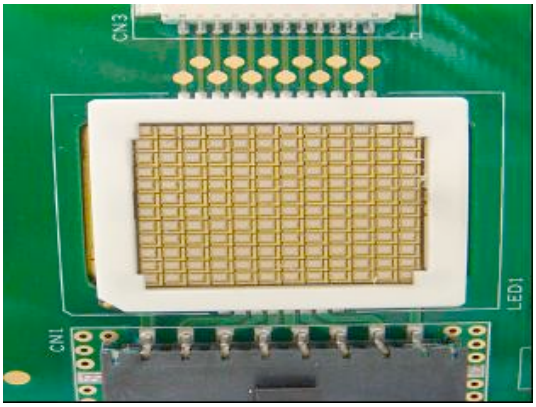

Figure 5.13 *LED array used for high intensity 405-nm light inactivation of bacteria seeded onto agar surfaces (40 mm × 40 mm).*

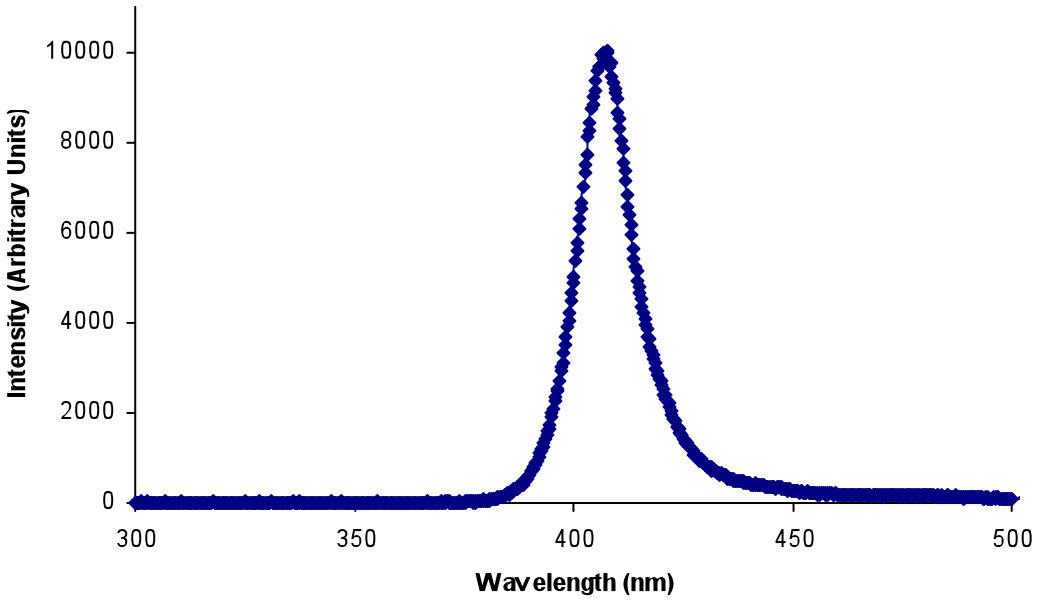

Figure 5.14 *The emission spectrum of the ENFIS QUATTRO LED array.*

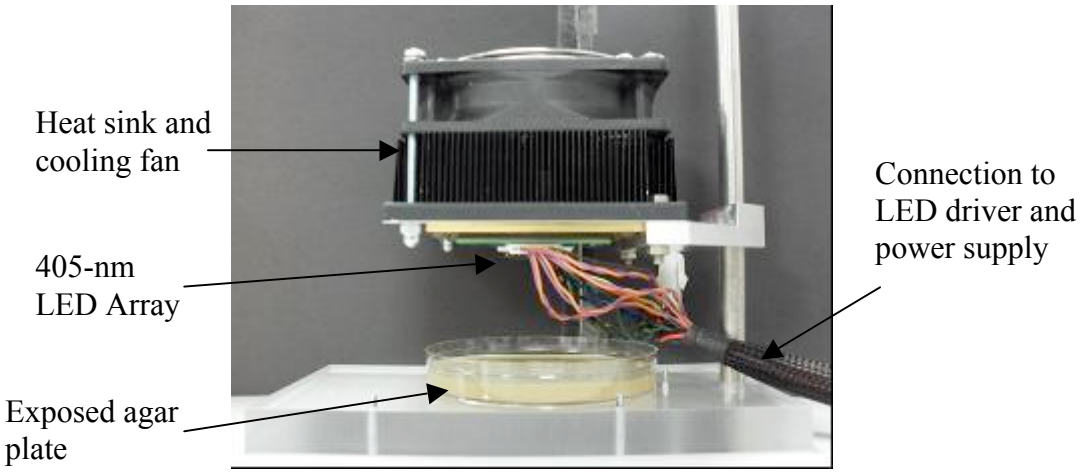

Figure 5.15 *LED array system for inactivation of bacteria on surfaces.*

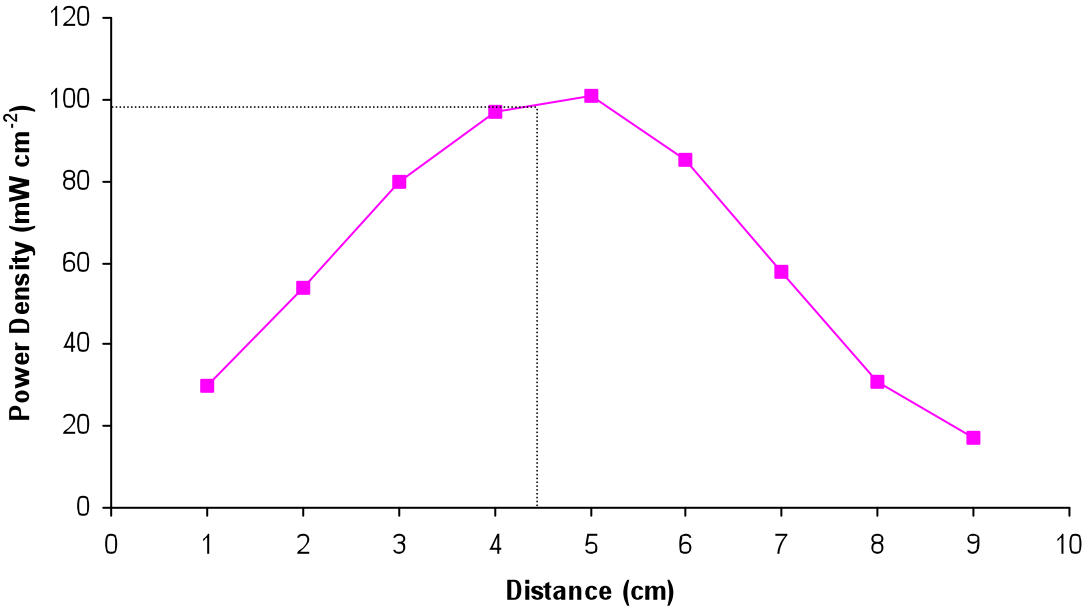

Figure 5.16 *Angular distribution of light produced by the 405-nm light engine across 9 cm distance (Petri dish diameter). Highlighting the centre point and the corresponding power density value.*

5.7.2 Bacterial Preparation

Salmonella enterica subsp. enterica serovar enteritidis NCTC 4444, *Sh. sonnei* NCTC 12984, *E. coli* O157:H7 NCTC 12900 and *L. monocytogenes* NCTC 11994 were cultured according to their growth requirements as described in Chapter 3, Section 3.1. Bacterial pellets were centrifuged and resuspended in 100 ml PBS as in Section 5.2.1. Bacterial suspensions were serially diluted to ~**3-Log$_{10}$** CFU ml^{-1} for experimental use (Chapter 3, Section 3.3.1).

5.7.3 Treatment Method

Method A: Agar Surface Exposure Experiments

Bacterial suspensions of *Salm. enterica* serovar *enteritidis* (100 μl), *E. coli* O157:H7 (200 μl), *L. monocytogenes* (200 μl) and *Sh. sonnei* (250 μl), containing approximately 2-Log$_{10}$ CFU ml^{-1} were pipetted onto the surface of Tryptone Soya Agar (TSA) plates and allowed to dry. The plates were then exposed to increasing durations of the high-intensity 405-nm light (without Petri dish lid). Non-exposed control plates, i.e. TSA plates seeded with bacteria and maintained in ambient lighting for the same duration as the exposed sample, were prepared for each 405-nm light-exposed sample.

Directly following exposure, seeded agar plates were incubated at 37ºC for 24 hours, after which time the plates were enumerated and results reported as total CFU/plate.

For a qualitative indication of the bactericidal effects of 405-nm light, a loopful of **6-**Log$_{10}$ CFU ml^{-1} bacterial suspensions of *Salm. enterica serovar enteritidis*, *E. coli* O157:H7, *L. monocytogenes* and *Sh. sonnei* were streaked in individual lines of length 6 cm (1 cm apart) onto the surface of a TSA plate. Half of each inoculum line (3 cm) was exposed to high-intensity 405-nm light, with the other half covered with aluminium foil to prevent light exposure. Plates were exposed to 15, 30 and 45 minutes of 100 mW cm^{-2} high-intensity 405-nm light. After exposure, plates were incubated at 37°C for 16 hours and photographs of each plate were taken.

Method B: Inert Surface Exposure Experiments

Bacterial suspensions of *Salm. enterica* serovar *enteritidis* and *L. monocytogenes* (100 ml) containing approximately 5-Log_{10} CFU ml^{-1} were loaded into a 6-jet Collison nebuliser (BGI Inc, USA) inside a fume cupboard. Coupons of acrylic (6 cm × 4 cm) and polyvinyl chloride (PVC) (6 cm × 4 cm) were used in these experiments to represent surfaces where bacteria might become attached and present a potential cross-contamination issue. Prior to use coupons were sterilised with ethanol (80%), and after use coupons were cleaned in 1% Virkon and rinsed thoroughly before re-use. Each coupon was held at a distance of 3 cm from the nebuliser for 15 seconds, allowing the aerosolised bacteria to deposit onto the surface of the coupon. In order to quantify the initial inoculated population, a inoculated coupon was immediately pressed directly onto a TSA plate for 5 seconds in order to recover the bacteria from the surface. To investigate the effect of 405-nm light for the inactivation of the contaminated surfaces, coupons were inoculated as described and then exposed to increasing durations of high-intensity 405-nm light, with a peak irradiance of 100 mW cm^{-2} at the centre of the surface, decreasing to 50 mW cm^{-2} at the outer edge of the surface. Following exposure, coupons were pressed onto a TSA surface for 5 seconds in order to recover the surviving bacteria from the inoculated surface. This seeding and recovery process was also carried out for non-exposed control samples. These experiments were repeated in triplicate.

Directly following exposure, agar plates were incubated at 37ºC for 24 hours, after which time the plates were enumerated and results reported as total CFU/plate.

All results in Section 5.8 are recorded as percentage inactivation in order to allow direct comparison of the inactivation achieved between non-exposed and light-exposed values for each microorganism/surface type due to the low density of inoculated bacteria used.

5.8 RESULTS

Bacterial seeded TSA plates were exposed to increasing dosages of high-intensity 405-nm light (100 mW cm^{-2}). All four bacteria investigated were inactivated by 99.3-100% following treatment with high-intensity 405-nm light (Table 5.4). Statistically significant differences of light-exposed and control samples are also highlighted in Table 5.4, using an asterisk. A total dose of 180 J cm^{-2} was required to inactivate *L. monocytogenes* by approximately 100% whereas a total dose of 270 J cm^{-2} was required to inactivate all of the other tested bacteria by 99.3-100%. Figure 5.17 shows the inactivation achieved upon exposure of *Sh. sonnei* seeded TSA plates to 405-nm light; Plate A was exposed to 180 J cm^{-2} of 405-nm light and Plate B was the unexposed control.

Figure 5.18 shows a qualitative representation of the bactericidal effect of high-intensity 405-nm light. The streaks of bacteria were light-exposed for 15, 30 and 45 minutes (Figure 5.18a, b and c respectively), and the bacterial streaks are numbered from 1-4: (1) *E. coli* O157:H7 (2) *Sh. sonnei* (3) *Salm. enterica* serovar *enteritidis* (4) *L. monocytogenes*. By 15 minutes, the light-exposed *L. monocytogenes* is showing complete inactivation, there is some inactivation of the three Gram negative bacteria by 30 minutes and by 45 minutes all four species of bacteria are inactivated.

Table 5.4 *Percentage (%) inactivation of* ***Salm. enterica*** *serovar* ***enteritidis****,* ***Sh. sonnei****,* ***E.coli*** *O157:H7 and* ***L. monocytogenes*** *on TSA surfaces using high-intensity 405-nm light (100 mW cm^{-2}).*

Exposure Time (min)	Dose (J cm^{-2})	*Salm. enterica* serovar *enteritidis*	*Sh. sonnei*	*E. coli* O157:H7	*L. monocytogenes*
		% Inactivation	% Inactivation	% Inactivation	% Inactivation
10	60	-	-	-	15.3* (SD 11.7)
15	90	11.3 (SD 6.3)	64.4* (SD 18.0)	39.4* (SD 32.4)	-
20	120	-	-	-	76.4* (SD 28.9)
30	180	91.4* (SD 11.7)	93.3* (SD 11.0)	95.7* (SD 1.6)	100* (SD 0.0[a])
45	270	100* (SD 0.0[a])	99.3* (SD 1.7)	99.8* (SD 0.7)	-

[a] All percentage uncertainty values were 0.0 % indicating there was no difference between inactivation values. SD= Standard deviation.

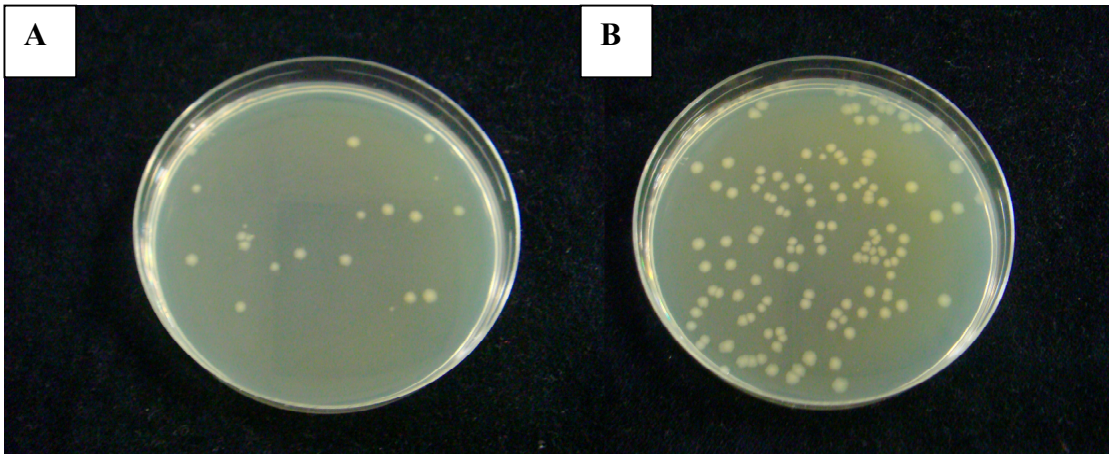

Figure 5.17 ***Sh. sonnei*** *inoculated TSA plates (A) exposed to 180 J cm^{-2} of high-intensity 405-nm light and (B) non-exposed control.*

Figure 5.18 *Qualitative representation of the bactericidal effect of high-intensity 405-nm light on (1)* ***E. coli*** *O157:H7, (2)* ***Sh. sonnei,*** *(3)* ***Salm. enterica*** *serovar* ***enteritidis*** *and (4)* ***L. monocytogenes****. Plates A, B and C demonstrate the inactivating effect of 15, 30 and 45 minutes light exposure, respectively, on streaks of foodborne pathogenic bacteria, with half of each plate being light-exposed and the other half being a non-light-exposed control.*

The results of the 405-nm light inactivation of *Salm. enterica* serovar *enteritidis* and *L. monocytogenes* seeded onto acrylic and PVC surfaces are reported in Table 5.5. Both *Salm. enterica* serovar *enteritidis* and *L. monocytogenes* are inactivated more readily on the PVC surface using high-intensity 405-nm light than on acrylic surfaces. Interestingly, the Gram negative bacterium *Salm. enterica* serovar *enteritidis* is more rapidly inactivated than *L. monocytogenes* on both surfaces. *Salm enterica* serovar *enteritidis* was inactivated by 100% on PVC with a dose ranging from 22.5 J cm^{-2} (at surface edge) to 45 J cm^{-2} (at surface centre), and 98% on acrylic with a dose ranging from 30 J cm^{-2} (at surface edge) to 60 J cm^{-2} (at surface centre). *L. monocytogenes* was inactivated by 90% on PVC with a dose ranging from 22.5 J cm^{-2} (at surface edge) to 45 J cm^{-2} (at surface centre), and only 61% on acrylic with a dose ranging from 30 J cm^{-2} (at surface edge) to 60 J cm^{-2} (at surface centre). When non-exposed control counts were compared to the counts of the initial seeded population it was found that between 70-80% of the seeded bacteria died on acrylic and PVC surfaces after 7.5-10 minutes, likely as a result of desiccation Therefore, results were reported as the percentage inactivation of light-exposed bacteria compared to the respective non-exposed control.

Table 5.5 *Percentage (%) inactivation of* ***Salm. enterica*** *serovar* ***enteritidis*** *and* ***L. monocytogenes*** *on inert surfaces using high-intensity 405-nm light (100 mW cm^{-2}).*

Surface Material	Exposure Time (min)	Peak Dose at Centre of Exposed Surface (J cm^{-2})	*Salm. enterica* serovar *enteritidis*	*L. monocytogenes*
			% Inactivation	% *Inactivation*
PVC	2.5	15	98* (SD 1.0)	78* (±SD 15.0)
	5.0	30	99* (SD 2.3)	86* (SD 25.0)
	7.5	45	100* (SD 0.0[a])	90* (SD 2.0)
Acrylic	2.5	15	93* (SD 1.0)	36 (SD 53.0)
	5.0	30	93* (SD 9.0)	39 (SD 33.0)
	7.5	45	96* (SD 5.0)	36 (SD 50.0)
	10.0	60	98* (SD 2.0)	61* (SD 36.0)

[a] All percentage uncertainty values were 0.0 % indicating there was no difference between inactivation values. SD= Standard deviation.

5.9 DISCUSSION AND CONCLUSIONS

These experiments have demonstrated that high-intensity 405-nm light has a bactericidal effect on the foodborne bacterial pathogens *C. jejuni*, *Sh. sonnei*, *Salm. enterica* serovar *enteritidis*, *E. coli* O157:H7, *L. monocytogenes* and *Bacillus* species. The most susceptible bacterium tested in liquid media was found to be *C. jejuni*, which was completely inactivated (5-$\log_{10}$ reduction) after treatment with a dose of 18 J cm^{-2}. The least susceptible organism was *Salm. enterica* serovar *enteritidis*, which showed an approximate 3-$\log_{10}$ reduction after a dose of 288 J cm^{-2}. Table 5.3 shows *C. jejuni* and *Salm. enterica* serovar *enteritidis* had the highest and lowest germicidal efficiency values, respectively. High-intensity 405-nm light was just over 30 times more effective at inactivating *C. jejuni* than *Salm. enterica* serovar *enteritidis*. It is possible that the high sensitivity of *C. jejuni* may be due to exposing this microaerophilic bacterium to 405-nm light under normal atmospheric gaseous oxygen conditions. A combined oxygen and light stress may have induced an enhanced inactivation rate in *C. jejuni*, although further work would be required to investigate this possibility.

The results from Table 5.1 demonstrate that 405-nm light inactivation of *L. monocytogenes* is dose-dependent, i.e. that by increasing the intensity of the 405-nm light the exposure time required for bacterial inactivation is reduced. It is suggested that the development of 405-nm light sources capable of emitting at greater intensities would significantly reduce the time required to inactivate bacteria and contribute to the applicability of this inactivation technology.

Figures 5.9, 5.10 and 5.11 show that *B. cereus* vegetative cells can be readily inactivated when using irradiances of both 10 mW cm^{-2} and 40 mW cm^{-2}. Results shown in Figures 5.10 and 5.11 show 3-3.5 $\log_{10}$ CFU ml^{-1} reductions in bacterial numbers when using Source C at 10 mW cm^{-2} and 40 mW cm^{-2} (Dose = 72 J cm^{-2}). This result further demonstrates that 405-nm HINS-light inactivation is a dose-dependent process. Results also demonstrate that there appears to be some difference in the capabilities of the 2 different sources used as Source A appears to be more

efficient than Source C. This variation in inactivation achieved could be due to the differences in the peak wavelengths emitted by the sources, as Source A has a peak wavelength of 400.72 nm, whereas Source C has a peak wavelength of 407.29 nm. As the type and content of endogenous bacterial porphyrins varies amongst microorganisms, so too does the optimum wavelength required for photosensitisation. In this case, light of around 400 nm appears to be more capable of stimulating endogenous porphyrin molecules than light of around 407 nm.

Table 5.2 shows the inactivation of 3 different species of *Bacillus* endospore through exposure to 40 mW cm^{-2} HINS-light for 4 and 8 hours (doses of 576 J cm^{-2} and 1.15 kJ cm^{-2} respectively). Both *B. megaterium* and *B. subtilis* have similar log_{10} reductions after exposure to 576 J cm^{-2} (0.87-log_{10} CFU ml^{-1} and 0.58-log_{10} CFU ml^{-1}, respectively) and 1.15 kJ cm^{-2} (1.84-log_{10} CFU ml^{-1} and 1.71-log_{10} CFU ml^{-1}, respectively). *B. cereus*, however, appears to be more readily inactivated achieving log_{10} reductions of 1.02-log_{10} CFU ml^{-1} and 2.25 -log_{10} CFU ml^{-1}at 4 hours and 8 hours, respectively. Variation in the inactivation rates of members of the *Bacillus* genus has previously been demonstrated in PDI studies (Demidova and Hamblin, 2005).

It should also be noted that inactivation occurs directly from 405-nm high-intensity light exposure and not from any increase in temperature arising from exposure to the LED arrays. The highest temperature recorded during HINS-light inactivation using Source A was 26°C, which is well below the optimum growth temperature for each of the microorganisms investigated in this study. Experimentally, Figure 5.12 shows that even at 35°C there is no effect of temperature on *Bacillus* endospores, therefore the bactericidal effect is not an artefact of any elevation in temperature experienced during light exposure.

Bacteria inoculated onto TSA plates were inactivated using a higher intensity 405-nm LED array with an approximate irradiance of 100 mW cm^{-2} at the centre of the agar plate. All four bacterial species tested were effectively inactivated by between 99.3-100% demonstrating that bacteria appear to be less susceptible to inactivation

when on nutritious surfaces in comparison with liquid suspension. This may be in part due to the availability of the nutrients present during exposure on agar plates that are not present during exposure whilst in PBS suspension. This nutrient effect will be further investigated in Chapter 6.

Inactivation of bacteria inoculated onto the inert surfaces PVC and acrylic have shown different kinetics to that of previous experiments on agar plates and in liquid suspensions, as *Salm. enterica* serovar *enteritidis* was found to be more susceptible to inactivation than *L. monocytogenes*. The reasons for this cannot be explained at this point, however it may be due to Gram negative bacteria being particularly susceptible to desiccation, a stress which may have increased the susceptibility of *Salm. enterica* serovar *enteritidis* to high-intensity 405-nm light (Potts, 1994). Another possible explanation is that *L. monocytogenes* attaches to PVC and acrylic more readily than *Salm. enterica* serovar *enteritidis*, meaning they are less likely to detach from the coupon surface onto the agar, affecting overall counts. In fact studies by Kim and Silva (2005) and Chen *et al*. (2008) have shown that *L. monocytogenes* has higher attachment strength (k=0.22) when seeded onto glass surfaces than both *Salmonella typhimurium* (k=0.9) and *E. coli* O157:H7 (k=1.0).

There have been an increasing number of investigations into the bactericidal effect of visible light on microorganisms. This is at least partly due to difficulties associated with microbial resistance to chemicals and antibiotics, problems that have stimulated the search for alternative methods of inactivation and decontamination. A number of studies have demonstrated the effects of high-intensity blue light on microorganisms without the use of exogenous photosensitisers. Maclean (2006) and Maclean *et al*. (2008a) identified that visible light – with optimal effect at 405 nm – was capable of inactivating *S. aureus* in liquid suspension. A further study by Maclean *et al*. (2009) demonstrated that 405-nm light could inactivate a range of medically important Gram positive and Gram negative bacteria, with results also showing that high-intensity 405-nm light had the ability to inactivate high bacterial population densities, achieving a 9-$\log_{10}$ reduction in *S. aureus* with a dose of approximately 220 J cm^{-2}. Guffey and Wilborn (2006) studied the bactericidal effect of 405-nm

light, using a super luminous diode (SLD), on *S. aureus*, *Pseudomonas aeruginosa* and *Propionibacterium acnes*. They reported successful inactivation of *S. aureus* and *Ps. aeruginosa*, however it was concluded that *P. acnes* could not be inactivated using 405-nm light, although this absence of inactivation may be due to the low dosage exposure applied in the study. An investigation by Enwemeka *et al.* (2008) studied the inactivating effect of 405-nm light, also using a SLD. They examined the effectiveness of 405-nm light on two strains of methicillin-resistant *S. aureus* (MRSA) with results showing both strains were readily inactivated by 405-nm light.

In the present study, variation was observed in the inactivation rates achieved for the bacterial species investigated, with the Gram positive *L. monocytogenes* and *B. cereus* (vegetative cells) proving to be more susceptible to 405-nm light inactivation than the Gram negative organisms tested, with the exception of *C. jejuni*. The germicidal efficiencies calculated in the present study can be compared with the 405-nm inactivation data obtained in the study by Maclean *et al.* (2009). *L. monocytogenes,* although much more readily inactivated than *Salmonella* and *Shigella*, was slightly less susceptible than the majority of the medically significant Gram positive organisms (*Staphylococcus*, *Streptococcus*, *Clostridium* species) investigated by Maclean *et al*. (2009), whereas *B. cereus* had a similar germicidal efficiency to *Streptococcus*. All Gram negative organisms tested (*Acinetobacter*, *Proteus*, *Pseudomonas*, *Klebsiella* and *Escherichia* species) by Maclean *et al*. (2009) had values of 0.04 or less, which are comparable with the values found in the present study for *Sh. sonnei*, *Salm. enterica* serovar *enteritidis* and *E. coli* O157:H7.

Reasons for the differing susceptibility of the bacterial species are as yet undetermined. Maclean *et al.* (2009) reported that, in general, the Gram positive species tested were more susceptible to 405-nm light inactivation than the Gram negative species, and it was theorised that this may be due to organism specific differences in porphyrins, and porphyrin levels, each with their own absorption maxima. Some photodynamic inactivation (PDI) studies utilising exogenous photosensitisers have suggested that physiological differences between Gram positive and Gram negative bacteria may be responsible for the variation in

inactivation rates achieved, with a study by Malik *et al.* (1992) speculating that inactivation differences may be due to the morphological differences between their respective cell membranes. Hamblin and Hasan (2004) discussed that the differences in susceptibility may also be due to the relatively small distance that singlet oxygen molecules can diffuse (~ 20 nm^3 in solution), meaning that not all singlet oxygen produced would diffuse inward to susceptible sites in the cell to cause oxidative damage. However, this may not be the same process that occurs in endogenously stimulated porphyrin singlet oxygen production, as it is likely that the sites where endogenous porphyrins are present are also the sites of cell destruction. Studies in yeast cells-studied for use in fluorescence microscopy visualisation techniques due to their larger size-have shown both the cell wall and the mitochondria to be endogenously-produced singlet oxygen targets, and it is probable that a similar process to this occurs in bacteria (Bertoloni *et al*., 1987; Strakhovskaya *et al*., 1999)

As stated previously, it is thought that high-intensity light causes endogenous porphyrin molecules to become stimulated and subsequently produce ROS, predominantly singlet oxygen ($^1O_2$). One study by Enwemeka *et al*. (2008), conflicts with this theory, suggesting that inactivation by high intensity blue light is similar to inactivation caused by UV-light. Most of the current research would seem to contradict this theory. Previous studies have shown that inactivation of bacteria with high intensity visible and blue light is an oxygen-dependent mechanism (Feuerstein *et al*. 2005; Maclean *et al*. 2008b). Results from these studies show that inactivation does not occur readily in the presence of scavengers of ROS. They also demonstrated that inactivation did not occur in anaerobic conditions, and that inactivation rates could be increased upon oxygen-enhancement. The Enwemeka *et al*. (2008) hypothesis is contradicted further by the results of a study by Schafer (1998) in which the bacterium *Deinococcus radiodurans*, which has extremely efficient DNA repair mechanisms, was successfully inactivated using photodynamic inactivation techniques, thus demonstrating that the inactivation mechanism is due to more than DNA damage.

A number of previous studies have reported that photodynamic inactivation (PDI) using exogenous photosensitising agents is effective against some of the bacterial species investigated in the present study. Romanova *et al*. (2003) demonstrated that *L. monocytogenes* could be inactivated using 660-nm LEDs in conjunction with photosensitising agents, with the most effective photosensitiser found to be tetra (N-methyl-4-pyridyl) porphine tetratosylate salt (TMPyP). Walther *et al*. (2009) used protochlorophyllide, as a photosensitiser in conjunction with a 60 W tungsten filament lamp for inactivation of *L. monocytogenes.* PDI studies have also been carried out on various *Salmonella* and *Campylobacter* species. Buchovec *et al*. (2008) showed that 5-aminolevulinic acid (ALA) used in conjunction with LEDs emitting 400-nm light could inactivate *Salm. enterica*. Rovaldi *et al*. (2000) used an e6-pentalysine conjugate and a laser of centre wavelength 662-nm to inactivate *C. rectus*. Demidova and Hamblin (2005) investigated the use of toluidine blue O (TBO) on different *Bacillus* spp in conjunction with 40 J cm^{-2} of 630-nm light and found that *B. cereus* was highly susceptible and *B. subtilis* to a lesser extent however *B. megaterium* was resistant to this PDI treatment. Demidova and Hamblin (2005) noted a large difference in susceptibilities of *Bacillus* spp to PDI treatment and also noted that vegetative cells were more rapidly inactivated than endospores. Although these results are in keeping with those found in this current study, it is difficult to compare the results of PDI studies with the results of the present study as each has many variable factors such as the light source, the peak wavelength and photosensitiser used. Although many of these PDI treatments have been shown to be effective in inactivating bacteria, the use of high-intensity 405-nm light eliminates the requirement of microorganism-specific exogenous photosensitisers.

Cross-contamination of foods, particularly in ready to eat foods (RTE), during and after processing is a major cause of food-related illness, with the organisms investigated in this chapter being some of the most notorious causative agents. Potentially, pathogenic microorganisms can be transferred from contaminated raw foods, machinery, utensils, and hands or via aerosol dispersion onto foods before packaging (Sheen, 2008). An FSA study (FSA, 2009) has noted that a high number of recorded Listeriosis cases are being presented in persons over 60 years old. One

of the factors implicated in this is that persons over 60 tend to eat more RTE, cold food products. RTE products can easily become contaminated with bacteria, as many are capable of surviving hours or even days on a variety of different surface types (Silva *et al*., 2008). There has been some progress made in reducing food-related illness, however the bacteria investigated in this study still present a considerable problem (FSA, 2009).

In contrast to the recent emergence of visible light-based inactivation methods, the use of UV-light technologies (continuous and pulsed) for food applications has been extensively studied. Bialka and Demirci (2007) studied the efficacy of PUV-light for the decontamination of raspberries and strawberries and demonstrated a reduction in the number of mixed *Salmonellae* that were seeded onto the surface of the fruits. Reductions of 3.4-$\log_{10}$ CFU ml^{-1} and 4.3 $\log_{10}$ CFU ml^{-1} were achieved in raspberries and strawberries at a dose of 72 J cm^{-2} and 64.8 J cm^{-2}, respectively. It was noted that there was a significant temperature increase during this process (from ~20°C to 80°C) causing damage to the exposed strawberries. Another limitation of PUV-light highlighted in the Bialka and Demirci (2007) study was the limited penetration of UV-light. Bialka and Demirci (2007) used a total dose of 72 J cm^{-2} to achieve a reduction of 3.4 $\log_{10}$ CFU ml^{-1} in seeded strawberries, whereas a study by Ghasemi *et al*. (2003) used a 10 pulses radiating 9 J of energy per pulse to inactivate *Salm. enterica* serovar *enteritidis* by around 5-$\log_{10}$ CFU ml^{-1} in liquid suspension, suggesting that the lower inactivation rate may have been due to the poor penetration of UV-light into fruit crevices. It is clear from these studies that both photothermal and photochemical processes are involved in pulsed UV-light inactivation and that reductions achieved in foods are significantly reduced due to the poor penetration of UV-light. In addition, exposure to UV irradiation is harmful to humans, causing ocular disease and skin cancers, and as such, must be used in a contained manner. Although high-intensity 405-nm light is not as germicidally efficient as UV-light, its ease of operation and safety-in-use present distinct advantages as a decontamination technology.

A novel method of bacterial decontamination currently being studied for potential application within the food industry is the addition of titanium dioxide (TiO_2) to food preparation surfaces and packaging. Titanium dioxide is a photocatalyst that, upon illumination with UV-A wavelengths of around 300-400 nm, causes the production of hydroxyl radicals (•OH) and ROS (Chawengkijwanich and Hayata, 2008) that oxidize cellular components, causing cell death. A study by Chawengkijwanich and Hayata (2008) found that TiO_2 coated oriented-polypropylene (OPP) seeded with *E. coli* exposed to UV-A light produced by two blacklights (λ 300-400 nm; 1 mW cm^{-2} intensity) reduced bacterial numbers by around 3-log_{10} CFU ml^{-1} after 180 minutes. However, they also noted that a 1-log_{10} CFU ml^{-1} reduction was attributable to UV-A light alone. In another study by Kühn *et al*. (2003), *E. coli* suspensions were pipetted onto TiO_2-coated "plexiglass" slides and irradiated with 2 white lights (peak emission, 356 nm). They recorded around a 7-log_{10} CFU ml^{-1} reduction within 60 minutes. They did not attribute any inactivation due to UV-A during this exposure time. These studies show significant bacterial reductions, however it has been noted that problems with aggregation of TiO_2 powders and clumping of bacteria causing shadowing (also seen in UV-light inactivation) affects the bactericidal ability of this inactivation process (Kühn *et al*., 2003).

The findings of this chapter demonstrate that high-intensity 405-nm light without the use of exogenous photosensitisers is effective for the inactivation of some of the most common bacterial causes of foodborne illness, including *C. jejuni, Sh. sonnei, Salm. enterica* serovar *enteritidis, E. coli* O157:H7*, L. monocytogenes* and *Bacillus* species. This bactericidal activity, coupled with the facts that 405-nm visible-light is significantly safer than UV-light with regards to human exposure, and that inactivation occurs without the addition of bacteria-specific photosensitisers or membrane destabilisers, indicates that 405-nm light exposure could, with further development, have potential applications in conjunction with other food safety strategies. In this context however, it must be appreciated that bacterial inactivation using visible 405-nm light is not a rapid process (in comparison with pulsed UV-light inactivation). Nevertheless, as this bactericidal light emanates from within the

visible-light part of the electromagnetic spectrum and does not contain UV wavelengths, it is much safer for operator use and exposure.

In order to fully determine the usefulness of high-intensity 405-nm light as a decontamination technology it would be necessary to devise experiments whereby microorganisms are exposed to 405-nm light on non-nutritious surfaces in biofilms in order to replicate "real" food contamination situations. It would also be useful to determine if inactivation of microorganisms is possible on food surfaces, although it is likely that the penetration of light may not be sufficient to inactivate bacteria on all areas of the exposed food as 405-nm light will be absorbed by some food products. However, further investigation will be required in order to optimise bactericidal efficiency and to develop larger scale systems for these specific decontamination applications.

Chapter 6

INVESTIGATION INTO THE HINS-LIGHT INACTIVATION MECHANISM

6.0 GENERAL

The previous chapter has demonstrated that 405-nm HINS-light has the capability to inactivate a range of food-related microorganisms including Gram positive, Gram negative and endospore-forming bacteria in liquid suspension and on agar, acrylic and PVC surfaces. In this chapter a series of experiments were carried out in order to gain a better understanding of the HINS-light inactivation mechanism.

The mechanism involved in violet/blue light inactivation of microorganisms has been theorised by many as described in Chapter 2. However, it is widely accepted that endogenous porphyrin molecules absorb blue light photons and become photo-excited in the presence of oxygen, causing the production reactive oxygen species (ROS) (Maisch *et al*., 2004). The predominant destructive component formed is thought to be singlet oxygen through the Type II pathway, which is highly reactive and causes oxidative destruction of many of the vital microbial cell components (Maisch *et al*., 2004; Luksiene and Zukauskas, 2009). Studies have shown that oxygen is required in this process and have highlighted that singlet oxygen is the chief cause of cell damage through a series of experiments using singlet oxygen scavenger solutions (Feuerstein *et al*., 2005; Maclean *et al*., 2008a). In order to assess the use of high-intensity 405-nm light in "real" applications it is vital to have a greater understanding of the inactivation mechanism.

This chapter aims to further understand the mechanism of inactivation in a number of different ways:

- Determining if inactivation of microorganisms occurs as readily in a nutritious environment as in the bacteriostatic diluent phosphate buffered saline
- Assessing the importance of 405-nm light dose and delivery method on inactivation
- Examining the direct effects of visible and 405-nm light on nutritious media in the absence of microorganisms
- Investigating whether a photorepair mechanism similar to that involved in UV light repair exists in 405-nm light-treated bacteria

For the majority of the work completed in this chapter *Staphylococcus aureus* was used as the test bacterium due to the medical importance of this organism.

6.1 INACTIVATION OF *S. AUREUS* IN DIFFERENT SUSPENSION MEDIA USING 405-nm LED ARRAY

In this series of experiments *Staphylococcus aureus* was exposed to high-intensity 405-nm light at two different irradiances, 10 mW cm^{-2} and 40mW cm^{-2} in two different suspension media: PBS and Nutrient Broth. Nutrient Broth is a general-purpose growth medium used in the cultivation of a wide range of microorganisms including *S. aureus*. It was used in this study to represent a nutritious environment.

6.1.1 Preparation of *S. aureus* for Treatment

Staphylococcus aureus NCTC 4135 was cultured in 100 ml Nutrient Broth at 37°C for 18-24 hours and then centrifuged at 3939 × g for 10 minutes in order to produce a bacterial pellet (see Chapter 3, Section 3.1-3.2). The resultant pellet was re-suspended into 100 ml PBS and diluted to the appropriate starting population for experimentation:

- *Phosphate Buffered Saline*: In experiments where *S. aureus* was exposed in PBS, bacteria were serially diluted to a population of ~5-Log_{10} CFU ml^{-1} in universals containing 9 ml of PBS.
- *Nutrient Broth*: In experiments where *S. aureus* was exposed in Nutrient Broth, bacteria were serially diluted to a population of ~5-Log_{10} CFU ml^{-1} in universals containing 9 ml of Nutrient Broth.

6.1.2 Treatment Method

Three-millilitre samples of *S. aureus* suspended in either PBS or Nutrient Broth were exposed to high-intensity 405-nm light using Source B at irradiances of 10 mW cm^{-2} and 40 mW cm^{-2} as described in Chapter 5, Section 5.2.2.

After exposure, both 405-nm light-exposed samples and control samples of *S. aureus* were plated onto Nutrient Agar as described in Chapter 3, Section 3.3.2. These samples were incubated at 37ºC for 24 hours, after which time the plates were enumerated and the results reported as CFU ml^{-1} (Chapter 3, Section 3.3.2).

6.1.3 RESULTS: HINS-light Inactivation of *S. aureus* in Suspension – A Comparison of the Effect of Nutritious and Non-nutritious Suspending Media

Figure 6.1 and Figure 6.2 depict the absorbance and transmission of light through PBS and Nutrient Broth at wavelengths from 390-410 nm respectively, highlighting the specific absorbance and transmission values obtained at 405 nm. In PBS most of the light from 390-410 nm is transmitted (~85%). In Nutrient Broth around 60% of 405-nm light is transmitted through the broth, and at wavelengths above 400 nm, absorbance of light steadily decreases. There is approximately a 25% difference between transmission of light in PBS and Nutrient Broth at 405-nm.

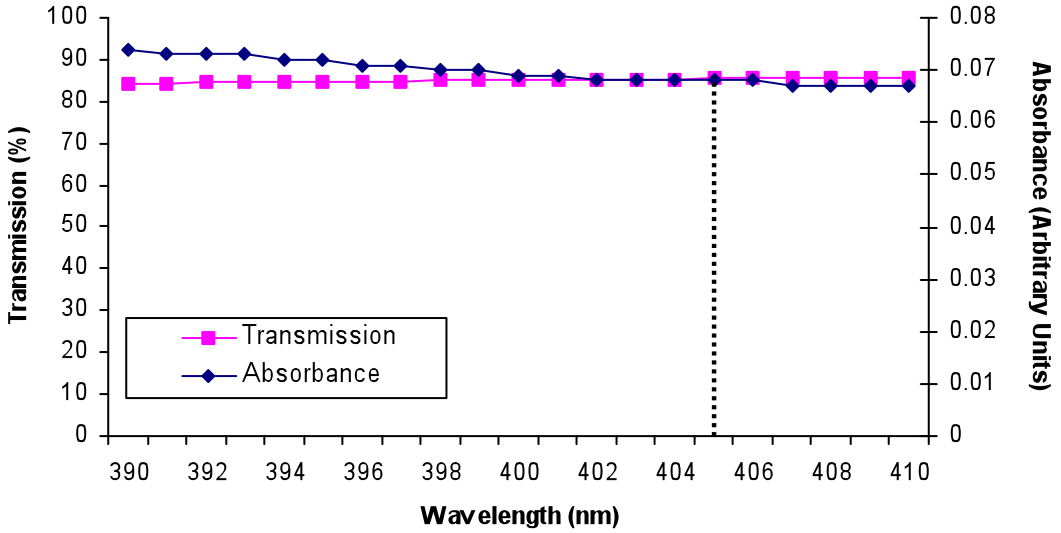

Figure 6.1 *Absorbance and transmission of light through PBS highlighting the corresponding absorbance and transmission values at 405 nm.*

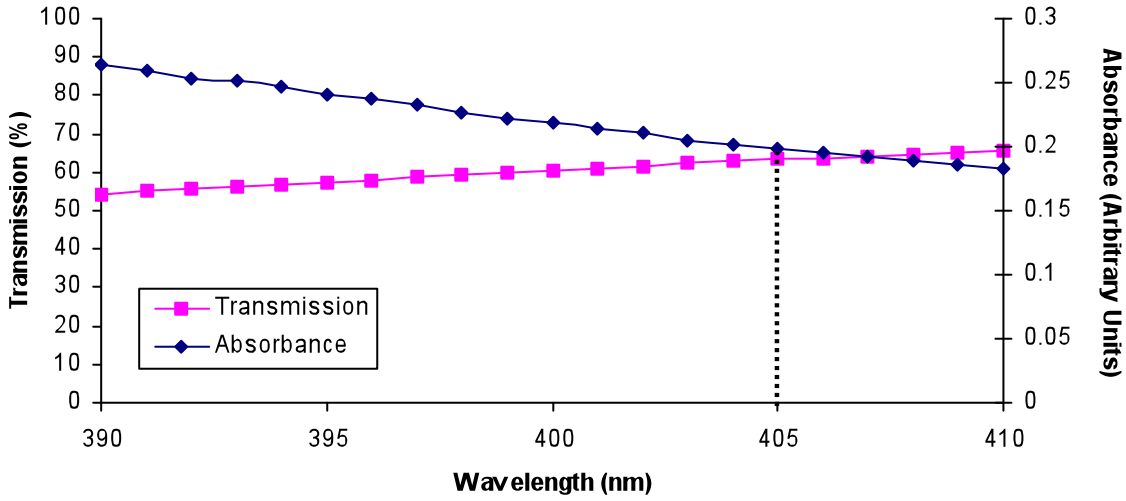

Figure 6.2 *Absorbance and transmission of light through Nutrient Broth highlighting the corresponding absorbance and transmission values at 405 nm.*

In order to determine the inactivating effect of 405-nm light on *S. aureus* in nutrient-rich environments (Nutrient Broth) it was necessary to have a comparison with a non-nutritious environment (PBS). Inactivation of *S. aureus* in these environments was completed at 10 mW cm^{-2} and 40 mW cm^{-2}.

Figure 6.3 and 6.4 show the results of the inactivation of *S. aureus* in PBS at 10 mW cm^{-2} and 40 mW cm^{-2} respectively. In each case asterisks highlight where light-exposed bacterial counts were significantly statistically different to their respective non-exposed control counts. When *S. aureus* was exposed to 405-nm light in PBS suspension at an irradiance of 10 mW cm^{-2}, a 5-log_{10} CFU ml^{-1} reduction occurred by 90 minutes (dose= 54 J cm^{-2}) and at 40 mW cm^{-2} around 5-log_{10} CFU ml^{-1} reduction occurred by 60 minutes (dose= 144 J cm^{-2}). Statistically significant differences between light-exposed and non-exposed counts were seen from 30 minutes when using 10 mW cm^{-2} (Figure 6.3), and from 15 minutes when using 40 mW cm^{-2} (Figure 6.4). The reason for the variance in the dose required for inactivation is not known however, power density measurements recorded at set current and voltages may not be exact, affecting the recorded dose. Another explanation may be that when doses are applied at high light intensity over a short exposure time microorganisms may respond differently compared to when the dose is applied at low irradiance over a longer exposure time.

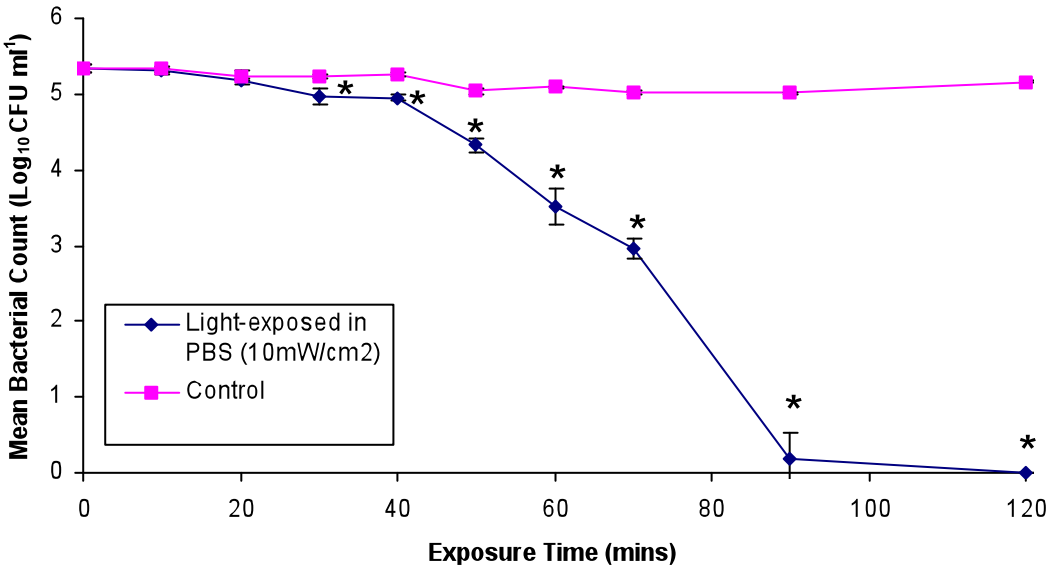

Figure 6.3 ***S. aureus*** *exposed to high-intensity 405-nm light, in PBS liquid suspension at an irradiance of 10 mW* cm^{-2}.

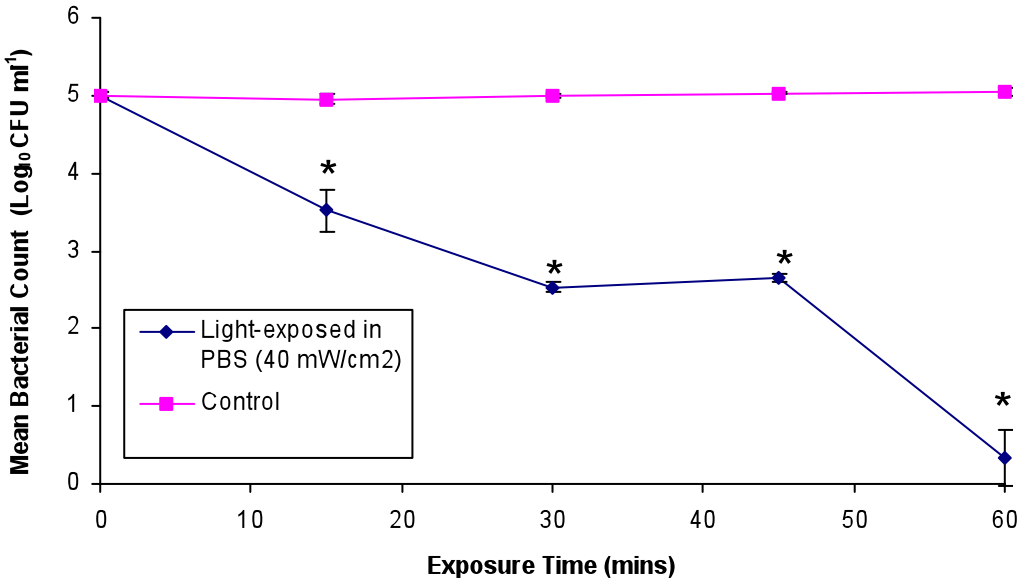

Figure 6.4 ***S. aureus*** *exposed to high-intensity 405-nm light, in PBS liquid suspension at an irradiance of 40 mW cm^{-2}.*

In Figures 6.5 and 6.6 *S. aureus* was exposed to 405-nm light in Nutrient Broth suspension at 10 mW cm^{-2} and 40 mW cm^{-2}. Figure 6.5 shows that around a 4-log$_{10}$ CFU ml^{-1} reduction is achieved at 300 minutes when using an irradiance of 10 mW cm^{-2} (dose=180 J cm^{-2}), however during this time the un-exposed *S. aureus* control has grown in the broth by around 1-log$_{10}$ CFU ml^{-1}. At an irradiance of 40 mW cm^{-2} (Figure 6.6), 90 minutes exposure to 405-nm light (dose= 216 J cm^{-2}) was required for a 5-log$_{10}$ CFU ml^{-1} inactivation. Statistically significant differences between light-exposed and non-exposed controls were seen from 30 minutes when using 10 mW cm^{-2} (Figure 6.5) and from 15 minutes when using 40 mW cm^{-2} (Figure 6.6), which is identical to doses where statistically significant differences are seen in PBS suspensions at 10 mW cm^{-2} and 40 mW cm^{-2}. Differences in inactivation kinetics between Figures 6.5 and 6.6 can be explained by the exposure times used. When bacterial cells are suspended in the nutritious media for a longer period, there is an increased likelihood of growth, or repair of damaged cells. When using an irradiance of 10 mW cm^{-2}, bacterial inactivation is at a slower rate and, as shown by the control samples, the bacteria are replicating whilst in the nutritious media: therefore at this intensity, high-intensity 405-nm light-exposed *S. aureus* suspended in Nutrient Broth

is in a dichotomy between inactivation and replication. This effect is not seen when using an irradiance of 40 mW cm^{-2} as the bacteria are being inactivated at a faster rate.

When the data of Figures 6.3 and 6.5, and Figures 6.4 and 6.6 are compared it can be seen that in general it is more difficult to inactivate *S. aureus* whilst suspended in Nutrient Broth compared to PBS, particularly at lower irradiances with longer exposure times, likely due to the existence of dichotomy between inactivation and replication rates.

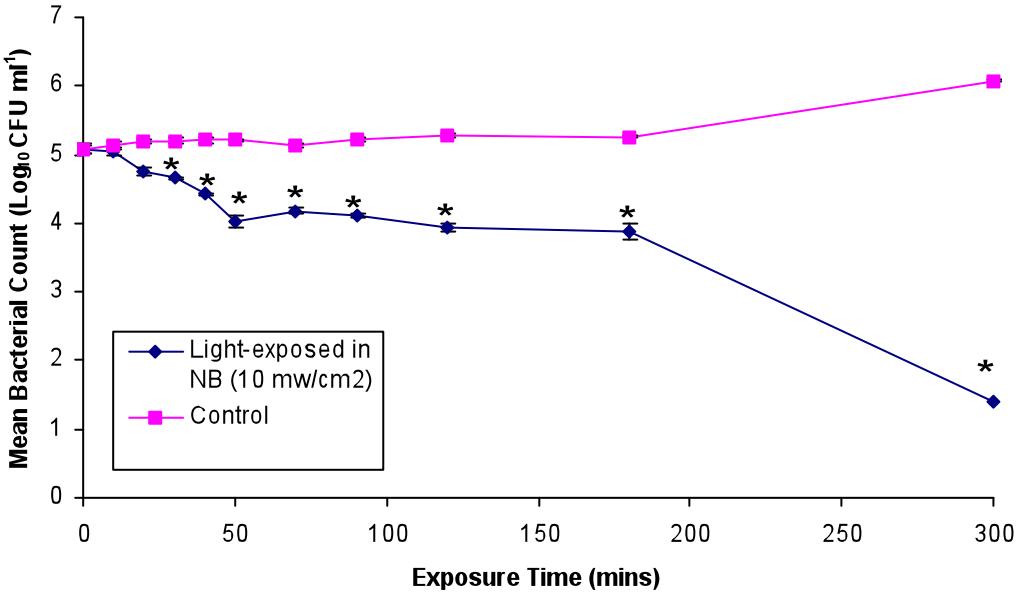

Figure 6.5 ***S. aureus*** *exposed to high-intensity 405-nm light, in Nutrient Broth liquid suspension at an irradiance of 10 mW* cm^{-2}.

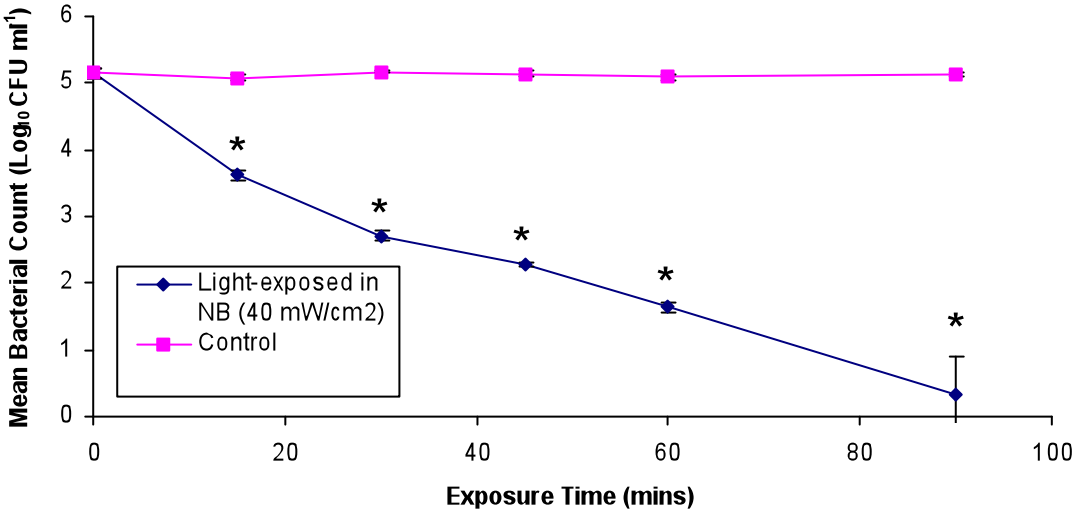

Figure 6.6 ***S. aureus*** *exposed to high-intensity 405-nm light, in Nutrient Broth liquid suspension at an irradiance of 40 mW* cm^{-2}.

6.2 INTERMITTENT 405-nm LIGHT EXPOSURE

In order to understand how application of light dose affects inactivation, a series of intermittent light exposure experiments were devised. Previous high-intensity 405-nm light exposure experiments have generally shown a sigmoidal death curve (Figure 6.7). This is where there is an initial plateau phase where no significant inactivation occurs, followed by an exponential decay/inactivation phase where microorganisms rapidly die. Occasionally some tailing of microbial inactivation occurs after the exponential decay phase. It is not fully understood why high-intensity 405-nm light inactivation has this initial phase where no inactivation occurs. However, it is likely that a build-up of oxidative molecules is required within the microorganisms before a threshold level is achieved, upon which exponential inactivation occurs in the microbial suspension.

This series of experiments set out to investigate how periods of interruption (no exposure to 405-nm light) at (i) the initial phase where little inactivation is occurring and (ii) at the exponential inactivation phase, would affect the overall inactivation achieved.

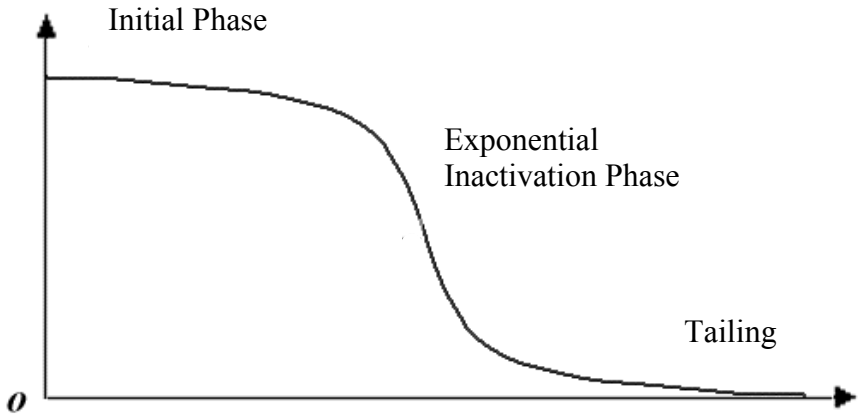

Figure 6.7 *Typical sigmoidal microbial inactivation curve highlighting the different phases of inactivation.*

6.2.1 Methodology for Intermittent 405-nm Light Treatment

Staphylococcus aureus was suspended in PBS and Nutrient Broth as described in Section 6.1.1 and exposed to high-intensity 405-nm light at 10 mW cm^{-2} and 40 mW cm^{-2} using Source B. Unlike in Section 6.1, light exposure was not continuous. Different light exposure parameters were completed to gain an understanding of how dose affects overall inactivation. The following parameters were completed:

- **Exposure with interruption during the initial plateau (i.e around the start of the inactivating dose)**

  Experiments were devised to interrupt the initial phase of inactivation to assess its effect on the overall inactivation achieved in 405-nm light-exposed *S. aureus*. *Staphylococcus aureus* suspended in PBS/Nutrient Broth was exposed to:

  - 10 minutes of either 10 mW cm^{-2} or 40 mW cm^{-2} 405-nm light exposure
  - followed by a non-exposure period of 30 minutes, before
  - further exposure to 405-nm light to give the remainder of the dose being investigated.

The results of this intermittent exposure were compared directly with results from continuous 405-nm light exposure of *S. aureus*.

- **Exposure with interruption during the exponential inactivation phase (i.e. in the middle of the inactivating dose)**

Experiments were devised to interrupt the exponential phase of inactivation to assess its effect on the overall inactivation achieved in 405-nm light-exposed *S. aureus*. High-intensity 405-nm light was only used at an intensity of 40 mW cm^{-2} in these experiments in order to reduce the overall time of experimentation. *Staphylococcus aureus* suspended in PBS/Nutrient Broth was exposed to:

- half the lethal dose normally required when exposed at an irradiance of 40 mW cm^{-2} (30 mins or 45 mins when in PBS or NB, respectively)
- followed by a non-exposure period of 6 hours, before
- further exposure to the second half of the dose (30 mins/45 mins).

The results of this intermittent exposure were compared directly with results from continuous 405-nm light exposure of *S. aureus* at light intensity of 40 mW cm^{-2}.

In Nutrient Broth suspensions this was also completed giving half the dose and then leaving the suspension for 16 hours before plating to determine if any recovery or growth had occurred during this time. This was not completed for PBS suspension as *S. aureus* began to die in suspension from upwards of 6 hours causing false results.

6.2.2 RESULTS- Intermittent HINS-light Exposure of *S. aureus*: A Comparison to Continuous HINS-light Exposure

6.2.2.1 Effect of Interrupting the Applied Dose during the Initial Phase of the Inactivation Curve

Protocol: 10 minutes exposure→ 30 minutes non-exposed→ remainder of dose

Figures 6.8 and 6.9 compare the high-intensity 405-nm light exposure of *S. aureus* suspended in PBS using continuous and intermittent exposure (10 mins exposed → 30 mins non-exposed → exposed to remainder of dose) at 10 mW cm^{-2} and 40 mW cm^{-2}, respectively. Although the same dose achieves a 5-log_{10} reduction in both continuous and intermittent exposures, with both the 10 mW cm^{-2} and 40 mW cm^{-2} irradiances continuous exposure appears to act more rapidly. However, without more data points during the exponentially-decreasing phase, it is impossible to determine the exact difference in doses required for inactivation through intermittent versus continuous exposures. A possible reason for the slightly different inactivation kinetics may be that during the 30 minutes non-exposure period some form of repair occurs, potentially explaining why intermittent and continuous exposure values at 60 min and 90 min in Figure 6.8, and 30 min in Figure 6.9 were statistically significantly different from one another.

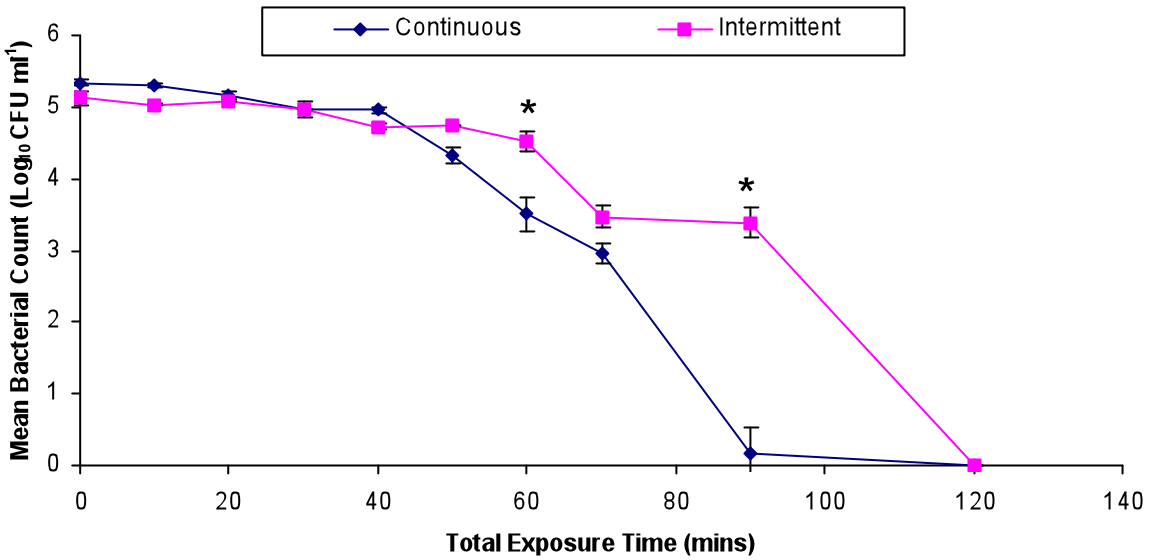

Figure 6.8 *Comparison of continuous and intermittent high-intensity 405-nm light exposure of* ***S. aureus*** *in PBS at an irradiance of 10 mW cm^{-2}. During intermittent exposure, for each data point shown (average of triplicate samples) 10 min exposure was applied, followed by a 30-min non-exposure period before application of the remaining exposure time.*

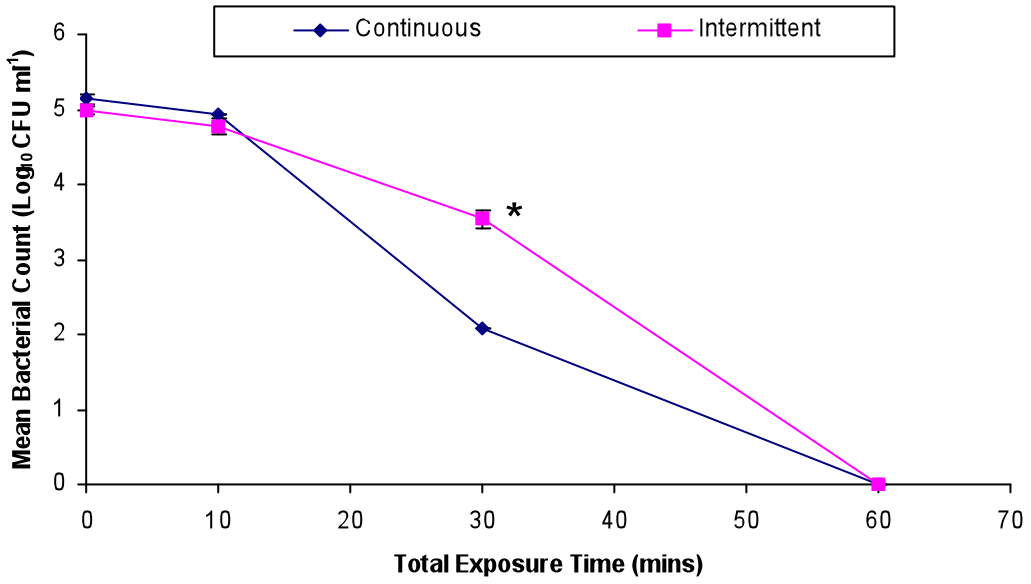

Figure 6.9 *Comparison of continuous and intermittent high-intensity 405-nm light exposure of* ***S. aureus*** *in PBS at an irradiance of 40 mW cm^{-2}. During intermittent exposure, for each data point shown (average of triplicate samples) 10 min exposure was applied, followed by a 30-min non-exposure period before application of the remaining exposure time.*

Figure 6.10 highlights the results from exposure of *S. aureus* suspended in Nutrient Broth either continuously or intermittently using 405-nm light at an irradiance of 10 mW cm^{-2}. In contrast to the previous results (Figures 6.8 and 6.9), the intermittent exposure appears to be significantly more effective for inactivating *S. aureus* than the continuous exposure (statistical significant difference from 120 minutes onwards). However, in Figure 6.11, which investigates exposure to 40 mW cm^{-2} HINS-light, both the continuous and intermittent exposures achieve a 5-log reduction after a total exposure time of 120 minutes, but the graph shows that continuous exposure of the suspension induces a significantly more rapid inactivation effect (statistically significant compared to continuous exposure from 30 minutes onwards).

In general, intermittent exposure of *S. aureus* suspended in PBS or Nutrient Broth, with a 30-minute non-exposed period near the start of the inactivation curve, results in around 5-log$_{10}$ CFU ml^{-1} inactivation at around the same dose required in

continuous exposures. However, this is not the case in Figure 6.10 as the intermittently exposed samples were more readily inactivated as statistical significant differences between intermittently and continuously light-exposed *S.aureus* were achieved from an exposure time of 120 minutes until 300 minutes.

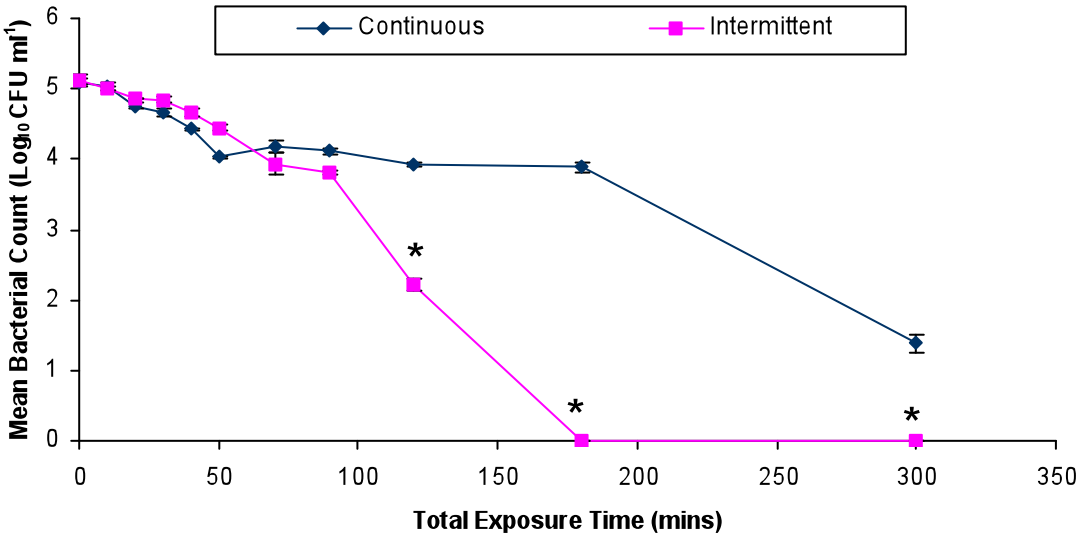

Figure 6.10 *Comparison of continuous and intermittent high-intensity 405-nm light exposure of* ***S. aureus*** *in Nutrient Broth at an irradiance of 10 mW cm^{-2}. During intermittent exposure, for each data point shown (average of triplicate samples) 10 min exposure was applied, followed by a 30-min non-exposure period before application of the remaining exposure time.*

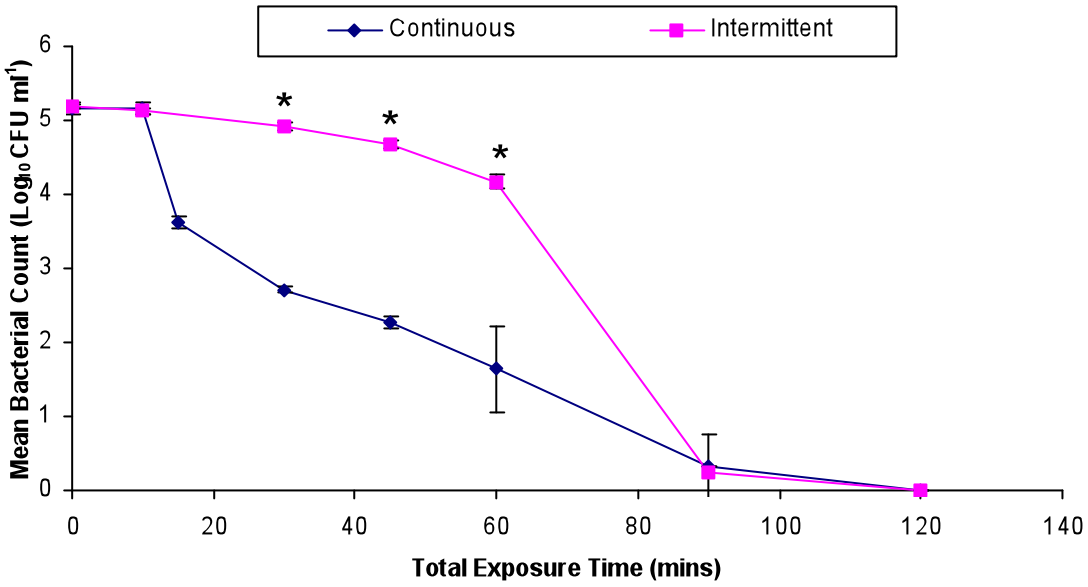

Figure 6.11 *Comparison of continuous and intermittent high-intensity 405-nm light exposure of* ***S. aureus*** *in Nutrient Broth at an irradiance of 40 mW cm^{-2}. During intermittent exposure, for each data point shown (average of triplicate samples) 10 min exposure was applied, followed by a 30-min non-exposure period before application of the remaining exposure time.*

6.2.2.2 Effect of Interrupting the Applied Dose during the Exponential-Inactivation Phase of the Inactivation Curve

Protocol: ½ Normally lethal exposure time→ 6 hours un-exposed→ remainder of exposure time

Tables 6.1 and 6.2 show the inactivation of *S. aureus* using intermittent exposure, with the break in exposure occurring during the exponentially-decreasing part of the curve. *S. aureus* suspended in PBS was exposed to half the normal lethal dose of 405-nm light (30 min at 40 mW cm^{-2}), non-exposed for 6 hours and subsequently exposed to the second half of the lethal dose 405-nm light (a further 30 min at 40 mW cm^{-2}). A period of 6 hours was chosen as previous laboratory work has shown *S. aureus* to become inactivated in PBS suspension alone after a period of 6 hours (shown in Table 6.1 control). Table 6.1 shows that overall inactivation using intermittent and continuous exposures are similar (4.5-5-log$_{10}$ CFU ml^{-1}) with both

continuous and intermittent light-exposed samples being statistically significantly different to their non-exposed control samples. Some inactivation (2-$\log_{10}$ CFU ml^{-1}) appears to have occurred during the 6 hours non-exposed to 405-nm light (statistically significantly different from non-exposed control). It is likely that sub-lethally damaged cells continue to die in PBS as it lacks nutrients, however it is also possible that there has been a product formed in the PBS suspension that continues to inactivate the suspended *S. aureus*.

The intermittent exposure of *S. aureus* in Nutrient Broth (Table 6.2) shows that the same overall dose is required for a 5-$\log_{10}$ CFU ml^{-1} reduction for both intermittently and continuously exposed *S. aureus* suspensions with both continuous and intermittent light-exposed samples being statistically significantly different to their non-exposed control samples. A 2-$\log_{10}$ CFU ml^{-1} reduction was observed after 45 min exposure, however, during the subsequent non-exposure period of 6 hours, a further 2.5-$\log_{10}$ CFU ml^{-1} reduction occurred. The reason for this is unknown, as sub-lethally damaged cells and non-damaged cells should have nutrients available for growth/repair, as the *S. aureus* in the control, which was not exposed to 405-nm light multiplied by around 3-$\log_{10}$ CFU ml^{-1}. This could indicate that an inactivating process continues to occur in the Nutrient Broth suspension when it is not being exposed. This also occurs in treated PBS solution, but not to the same degree as in the treated Nutrient Broth suspensions.

Table 6.1 *Intermittent exposures of **S. aureus** in PBS to high-intensity 405-nm light at an irradiance of 40 mW cm^{-2}. During intermittent exposure, for each data point shown (average of triplicate samples) 30 min exposure was applied, followed by a 6 hr non-exposure period before application of a further 30 min exposure. The continuous inactivation result is added for comparison.*

Population (Log_{10} CFU ml^{-1})	
Initial 5.20 (±0.01)	
Light-exposed Test Samples	**Non-Exposed Control Samples**
30 min Light-exposed	**30 min Non-exposed Control**
3.92 (±0.10)*	5.06(±0.05)
30 min Light-exposed→6 hrs Non-exposed	**6 hrs 30 min Non-exposed Control**
1.67 (0.20)*	4.91 (±0.01)
Intermittent 60 min Exposure (30 min Light-exposed→6 hrs Non-exposed→30 min Light-exposed)	**7 hrs Non-exposed Control**
0.40 (±0.50)*	4.23 (±0.07)
Continuous 60 min Exposure	**60 min Non-exposed Control**
0 (±0.0)*	5.11 (±0.03)

* Indicates a statistically significant difference between light-treated and non-exposed control samples.

Table 6.2 *Intermittent exposures of **S. aureus** to high-intensity 405-nm light in Nutrient Broth at an irradiance of 40 mW cm^{-2}. During intermittent exposure, for each data point shown (average of triplicate samples) 45 mins exposure was applied, followed by a 6 hrs non-exposure period before application of a further 45 mins exposure. The continuous inactivation result is added for comparison.*

Population (Log_{10} CFU ml^{-1})	
Initial 5.13 (±0.02)	
Light-exposed Test Samples	**Non-Exposed Control Samples**
45 min Light-exposed	**45 min Non-exposed Control**
2.91 (±0.04)*	5.13 (±0.05)
45 min Light-exposed→6 hrs Non-exposed	**6 hrs 45 min Non-exposed Control**
0.17 (±0.30)*	6.57 (±0.01)
Intermittent 90 min Exposure (45 min Light-exposed→6 hrs Non-exposed→45 min Light-exposed)	**7 hrs 30 min Non-exposed Control**
0 (±0.0)*	7.25 (±0.04)
Continuous 90 min Exposure	**90 min Non-exposed Control**
0.33 (±0.02)*	5.12 (±0.04)

* Indicates a statistically significant difference between light-treated and non-exposed control samples.

Protocol: ½ Normally Lethal Exposure→ 16 hours un-exposed

After the discovery that inactivation continued to occur during an intermittent 6-hour non-exposure period, an experiment was carried out to determine if this effect would continue over a longer timeframe (16 hours). The time period of 16 hours was completed to represent a theoretical time period, for example in a room, where lighting may be switched off overnight. These experiments were only carried out in Nutrient Broth, as *S. aureus* would not survive in PBS alone for 16 hours. Table 6.3 shows the initial population was reduced by around 3-$\log_{10}$ CFU ml^{-1} after 45 minutes 405-nm light exposure at an irradiance of 40 mW cm^{-2}, which was significantly statistically different to the non-exposed control value. After 16 hours non-exposed to the 405-nm light the remaining cells (2-$\log_{10}$ CFU ml^{-1}) were inactivated (significantly different to light-exposed test sample and non-exposed control), whilst the control, which was maintained at ambient temperature, continued to grow in the non-exposed Nutrient Broth to a population of 9-$\log_{10}$ CFU ml^{-1}.

As this effect appears to continue for some time after 405-nm light exposure it can be concluded that it is likely that some component of the Nutrient Broth itself is being adversely affected by 405-nm light.

Table 6.3 *Inactivation of* ***S. aureus*** *in Nutrient Broth with 405-nm light for 45 min, during a 6 hr non-exposure period. The continuous inactivation result is added for comparison.*

Population (Log_{10} CFU ml^{-1})	
Initial 5.03 (±0.01)	
Light-exposed Test Samples	**Non-Exposed Control Samples**
45 min Light-exposed	**45 min Non-exposed Control**
2.18 (±0.2)*	5.17 (±0.03)
45 min Light-exposed→16 hrs Non-exposed	**16 hrs 45 min Non-exposed Control**
0 (±0.0)*	9.08(±0.01)

* Indicates a statistically significant difference between light-treated and non-exposed control samples.

6.3 INVESTIGATION INTO THE EFFECT OF HINS-LIGHT ON NUTRITIOUS MEDIA

Results of the previous section have indicated that exposure to high-intensity 405-nm light may have a detrimental effect on nutritious media. To confirm that the 405-nm light adversely affects medium components, a series of experiments were conducted to:

- Assess the effect of 405-nm light-exposed Nutrient Broth on *S. aureus* populations, and determine the exposure time where Nutrient Broth becomes bactericidal.
- Examine the bactericidal effects of 405-nm light-exposed Nutrient Broth on other Gram positive and Gram negative bacteria.

6.3.1 Bacterial Preparation and Experimental Procedures

Bacterial Preparation

Staphylococcus aureus NCTC 4135, methicillin-resistant *Staphylococcus aureus* 16a (isolate from Glasgow Royal Infirmary), *Enterococcus faecalis* NCTC 00775, *Escherichia coli* NCTC 9001, *Klebsiella pneumoniae* NCTC 9633, *Pseudomonas aeruginosa* NCTC 9633 and *Acinetobacter baumannii* NCTC 12156 were cultured in 100 ml Nutrient Broth at 37°C for 18-24 hours and then centrifuged at 3939 × g for 10 minutes in order to produce a bacterial pellet (see Chapter 3, Section 3.1-3.2). The resultant pellet was re-suspended into 100 ml PBS and diluted to the appropriate starting population for experimentation.

Experimental Procedures

A 3-ml volume of sterile Nutrient Broth was exposed to 405-nm light for 45 minutes at 40 mW cm^{-2} (dose= 108 J cm^{-2}) as in Section 6.1.1. After this exposure time 100-µl volumes of a 3-Log_{10} CFU ml^{-1} population of *S. aureus* were pipetted into two wells, one containing 3-ml of 405-nm light-treated Nutrient Broth, and another containing 3-ml untreated Nutrient Broth, and incubated in a rotary shaker at 37°C

for 16 hours. After this incubation the suspensions were serially diluted as appropriate (Chapter 3, Section 3.3.1), plated onto Nutrient Agar and incubated at 37°C for 18-24 hours. To establish the initial population 100 μl of the 3-Log_{10} CFU ml^{-1} bacterial suspension was plated as manual spread plates onto Nutrient Agar plates (in triplicate) and incubated at 37°C for 18-24 hours for enumeration. Following incubation the plates were enumerated and results recorded as CFU ml^{-1}.

This procedure was repeated with the other microorganisms.

6.3.2 RESULTS

6.3.2.1 Bactericidal Effect of HINS-light Treated Nutrient Broth

Table 6.4 shows that 405-nm light-exposed Nutrient Broth is bactericidal to inoculated *S. aureus* cells. After the 16 hour incubation period, the initial 10^2 CFU ml^{-1} population inoculated into the treated broth has been inactivated, with the *S. aureus* inoculated into the untreated broth (control) continuing to multiply to around 9-log_{10} CFU ml^{-1}. There is a statistically significant difference between the *S. aureus* inoculated into treated Nutrient Broth and the untreated Nutrient Broth.

Table 6.4 *Effect of 405-nm light-treated Nutrient Broth, (NB) (108 J cm^{-2}) on a 10^2 CFU ml^{-1} population of* ***S. aureus*** *after a 16-hour incubation at 37°C.*

Microorganism	***Staphylococcus aureus***
Initial Population (Log_{10} CFU ml^{-1}) added to 405-nm- ight treated NB	1.80 (±0.1)
Population (Log_{10} CFU ml^{-1}) after 16 hours in 405-nm light-treated NB	0 (±0.0)*
Control Population (Log_{10} CFU ml^{-1}) after 16 hours in untreated NB	8.73 (±0.02)

* Indicates a statistically significant difference between light-treated and untreated control samples.

The exact exposure time/dose of 40 mW cm^{-2} light required for Nutrient Broth-induced inactivation of a 2-Log_{10} CFU ml^{-1} population was investigated by inoculating *S. aureus* into Nutrient Broth exposed to varying durations of 405-nm light. These experiments were completed exactly as in Section 6.3.1, however in this case Nutrient Broth was exposed to 10, 20, 30 and 45 minutes of 405-nm light prior to inoculation of the 2-Log_{10} CFU ml^{-1} of *S. aureus*. Figure 6.12 shows that bactericidal effects are evident from the first exposure time of 10 minutes, with maximum inactivation of the inoculated *S. aureus* seen with Nutrient Broth treated with both 30-minutes and 45-minutes. All light-exposed broth time-points investigated were statistically significantly different to the non-exposed control.

Based on these results, in all future experiments, an exposure time of 45 minutes, i.e. a dose of 108 J cm^{-2}, was used to ensure the maximum bactericidal effect was obtained.

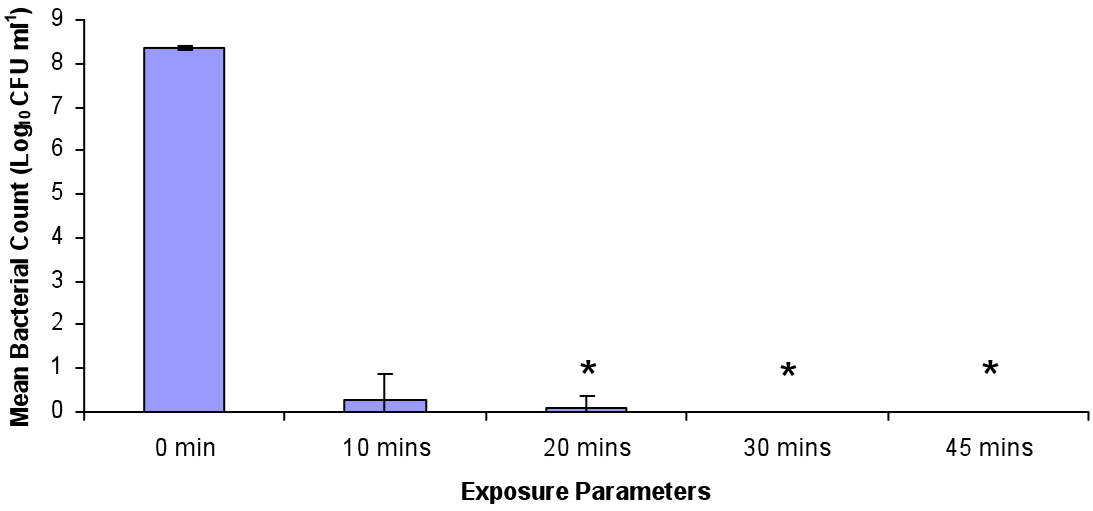

Figure 6.12 *The effect of altering the Nutrient Broth light-treatment time on levels of inoculated* ***S. aureus*** *after incubation at 37°C for 16 hours.*

In an attempt to determine the underlying cause of the bactericidal effect of 405-nm light-treated Nutrient Broth, the absorbance and transmission values of 405-nm light-treated and untreated Nutrient Broth were recorded in Figure 6.13. There is a slight difference in the absorbance and transmission of light from 390-410 nm in treated and untreated Nutrient Broth.

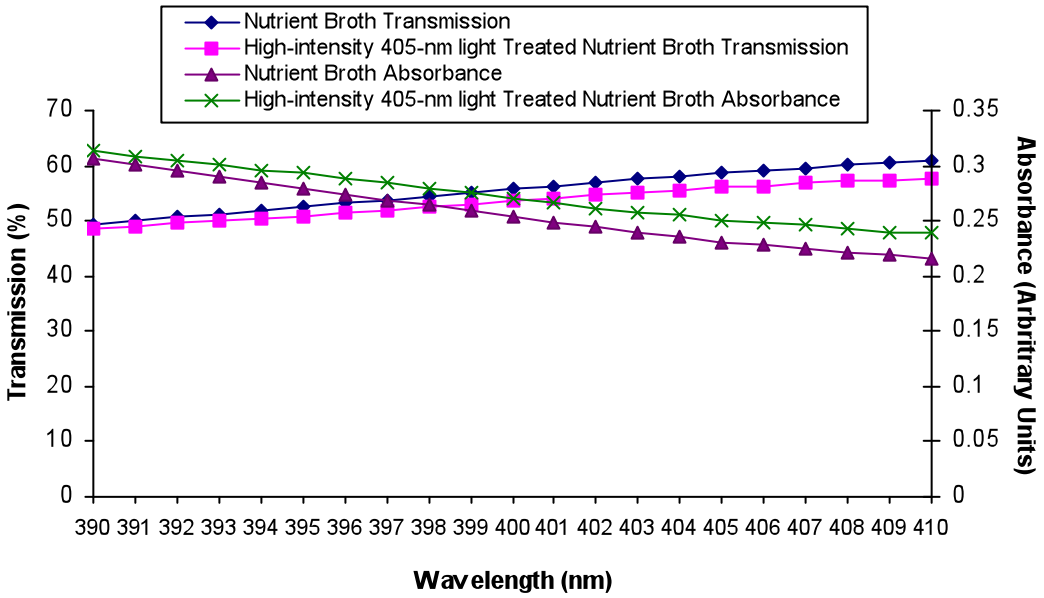

Figure 6.13 *Absorbance and transmission readings of untreated and high-intensity 405-nm light-treated Nutrient Broth (108 J cm^{-2}).*

The pH of the treated and untreated Nutrient Broth was measured using a pH 210 Microprocessor pH meter (Hanna Instruments, UK) to establish if a reaction was occurring during light exposure that might cause a pH change in the solution capable of damaging the *S. aureus* cells. It was found that untreated broth had a pH of 7.00 and 405-nm light-exposed broth had a pH of 6.84, both recorded at a temperature of 27.4°C. The difference in pH was assumed not significant enough to cause the bactericidal effects associated with 405-nm light exposure of Nutrient Broth.

Different population densities of *S. aureus* were exposed to 405-nm light-treated Nutrient Broth as in previous experiments to elucidate whether larger inoculated population densities of *S. aureus* were affected in a similar way to the 2-Log_{10} CFU

ml^{-1} population. Instead of 100 µl of a 3-Log_{10} CFU ml^{-1} being added to 3-ml of broth, as in Section 6.8, 100 µl of a 10-Log_{10}, 8-Log_{10} or a 6-Log_{10} CFU ml^{-1} was added to the treated broth – giving approximate initial populations of 9-Log_{10}, 7-Log_{10} and 5-Log_{10} CFU ml^{-1} in the treated broth, respectively. The results of Figure 6.14 show that 7-Log_{10} and 5-Log_{10} CFU ml^{-1} populations were inactivated by around 2-log_{10} CFU ml^{-1}. However, at very high population densities of around 9-Log_{10} CFU ml^{-1} this bactericidal effect does not occur. In this case a bacteriostatic effect is seen, as the control population continues to multiply and increases by around 1-log_{10} CFU ml^{-1} during the incubation time, whereas *S. aureus* neither multiplies nor is inactivated in 405-nm light-exposed broth, however there is a statistically significant difference between the light-treated and control populations. Statistically significant differences are marked with asterisks in brackets to highlight the representative untreated control and test values being compared.

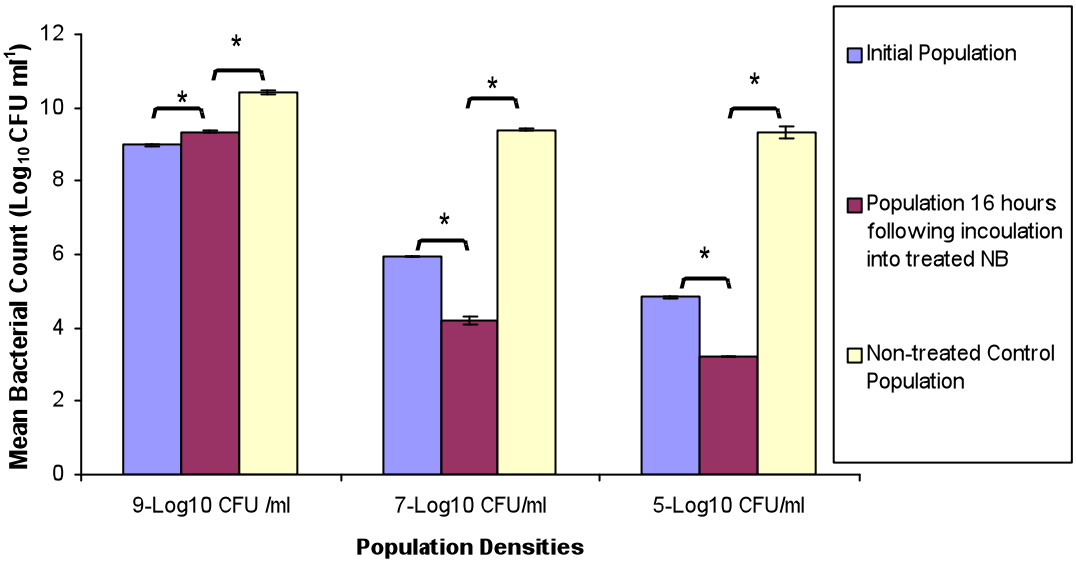

Figure 6.14 *The effect of 405-nm light-treated Nutrient Broth (NB)(108 J cm^{-2}) on different inoculum levels of* ***S. aureus*** *after a 16-hour incubation at 37°C.*

This bactericidal effect of 405-nm light-treated Nutrient Broth has been shown in *S. aureus*, however it was important to establish if different bacteria are also affected by 405-nm light-treated Nutrient Broth. A selection of Gram positive and Gram negative bacteria were cultured in Nutrient Broth at 37°C for 18-24 hours then centrifuged and serially diluted (as detailed in Section 6.3.1). Each microorganism was inoculated (100 μl of a 3-Log_{10} CFU ml^{-1}) into 3 ml of 405-nm light-treated (108 J cm^{-2}) Nutrient Broth and 3 ml of untreated Nutrient Broth (control), which were then incubated in a rotary incubator at 37°C for 16 hours. After incubation, the inoculated 405-nm light-treated and untreated control Nutrient Broth suspensions were plated and enumerated as described in Section 6.3.1

Table 6.5 demonstrates that 405-nm light-exposed broth has varying effects on different inoculated microorganisms. The most susceptible microorganisms appear to be *S. aureus*, methicillin-resistant *S. aureus* (MRSA) and *Acinetobacter baumannii* which were inactivated by ~ 2-log_{10} CFU ml^{-1}, even though bacteria in the control sample continued to multiply in the untreated broth to around a 9-Log_{10} CFU ml^{-1} population after the 16 h incubation period. In contrast, 405-nm light-treated broth appeared to have little effect on *Escherichia coli* (although statistically significant difference exists between the control and exposed samples) and *Enterococcus faecalis*, as both the untreated control and the 405-nm light-treated broths had similar levels of bacterial growth, i.e. approximately 9-Log_{10} CFU ml^{-1} after 16 hours. Although both *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were able to multiply and grow in the 405-nm light-treated media, they did not reach the same level of growth as their respective untreated controls by ~2-log_{10} CFU ml^{-1} (statistically significant difference). This suggests there is variation in the susceptibility of microorganisms to this 405-nm light induced toxic effect in Nutrient Broth, that is not wholly governed by whether a microorganism is Gram positive or Gram negative.

Table 6.5 *Effect of high-intensity 405-nm light-treated Nutrient Broth on different inoculated (~2-Log$_{10}$ CFU ml^{-1}) microorganisms.*

Microorganism	Initial Population (Log_{10} CFU ml^{-1}) added to 405nm-light treated Nutrient Broth	Population (Log_{10} CFU ml^{-1}) after 16 hours in 405nm-light-treated Nutrient Broth	Control Population (Log_{10} CFU ml^{-1}) after 16 hours in untreated Nutrient Broth
Staphylococcus aureus	1.43 (±0.08)	0 (±0.0)*	8.91 (±0.03)
Methicillin-resistant *S. aureus*	1.90 (±0.05)	0 (±0.0)*	8.08 (±0.05)
Enterococcus faecalis	1.95 (±0.02)	8.44 (±0.01)	8.47 (0.05)
Escherichia coli	1.62 (±0.02)	8.98 (±0.04)*	9.2 (±0.02)
Klebsiella pneumoniae	1.46 (±0.01)	6.25 (±0.05)*	8.47 (±0.03)
Pseudomonas aeruginosa	1.66 (±0.01)	6.89 (±0.02)*	8.95 (±0.05)
Acinetobacter baumannii	1.64 (±0.03)	0 (±0.0)*	9.03 (0.04)

* Indicates a statistically significant difference between light-treated and untreated control samples.

6.3.2.2 Investigation into Mechanism of Toxicity

In an attempt to determine what causes Nutrient Broth to become toxic to bacteria, experiments were carried out to assess how the different components of Nutrient Broth were affected by 405-nm light treatment. Again, *S. aureus* was utilised as the test organism. The experimental procedure was similar to that in Section 6.3.1 whereby a 3-ml volume of Nutrient Broth was exposed to 108 J cm^{-2} of 405-nm light at an intensity of 40 mW cm^{-2}, and 100 μl of a 3-Log$_{10}$ CFU ml^{-1} population of *S. aureus* was inoculated into the 3-ml volumes of both the light-treated and a control untreated Nutrient Broth. However, a series of experiment were completed with variations to the protocol in an attempt to elucidate the primary cause of the inactivation of *S. aureus* inoculated into 405-nm light-treated Nutrient Broth suspension.

Figure 6.15 assesses the effect of adding increasing volumes of 405-nm light-treated (108 J cm^{-2}) Nutrient Broth to fresh, untreated Nutrient Broth. This was carried out to help to elucidate whether:

- Nutrients vital to the growth of *S. aureus* were destroyed by the 405-nm light
- A toxic product was being produced

These two theories were tested by diluting out 405-nm light-treated Nutrient Broth with different volumes of fresh, non-light treated Nutrient Broth, and the addition of various different untreated components of Nutrient Broth. This addition of untreated Nutrient Broth and its components would identify if the 405-nm was targeting and possibly destroying a particular element of the Nutrient Broth vital for *S. aureus* survival or whether a toxic product was being formed in the medium.

It was discovered that addition of fresh Nutrient Broth to the light-treated Nutrient Broth did aid growth of *S. aureus* in most cases, with statistically significant differences between the untreated Nutrient Broth control and the light-treated Nutrient Broth samples from 1-ml volumes onwards. Growth in the samples containing 1.5, 2 or 2.5 ml 405-nm light-exposed Nutrient Broth was reduced by 2-7-$\log_{10}$ CFU ml^{-1}. If this were merely a nutrient depletion problem, even small quantities of un-exposed Nutrient Broth should be sufficient to allow *S. aureus* to multiply as normal.

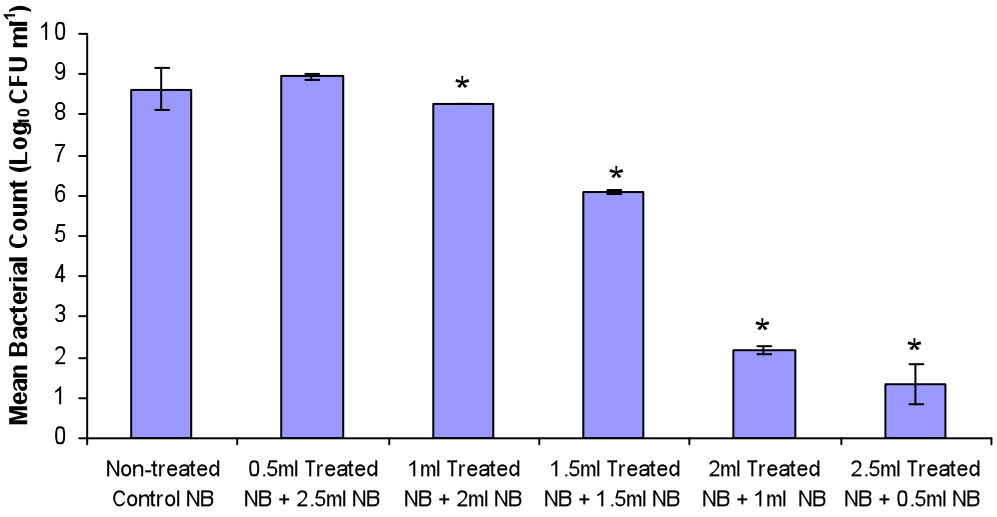

Figure 6.15 *The effect of different volumes of untreated Nutrient Broth added to 405-nm light-treated (108 J cm^{-2}) Nutrient Broth on levels of* ***S. aureus*** *after incubation at 37°C for 16 hours.*

In an attempt to pinpoint the potential source of the toxicity, three of the main components of Nutrient Broth, yeast extract (2 g/L), peptone (5 g/L) and "Lab Lemco" (1 g/L), were treated with 405-nm light at 40 mW cm^{-2} for 45 minutes (108 J cm^{-2}) in solution at the concentration present in Nutrient Broth formulations (Oxoid, UK). The 405-nm light-treated solutions were added into fresh broth inoculated with *S. aureus* as in previous experiments (Section 6.2). Figure 6.16 shows the effect of 405-nm light-treated yeast extract added to untreated Nutrient Broth on the growth of *S. aureus*. In general the 405-nm light-treated broth shows no marked reduction in bacterial growth compared to the untreated yeast extract/Nutrient Broth control. However, when 1.5 ml of 405-nm light-treated yeast extract was added to Nutrient Broth, the growth of *S. aureus* was reduced by around 2-log$_{10}$ CFU ml^{-1}, which is statistically significantly different from the respective untreated control sample containing extra yeast extract. This may mean that yeast extract is part of the cause of the photo-induced toxicity of Nutrient Broth.

Figures 6.17 and 6.18 show similar experiments carried out using peptone and Lab Lemco, which show no effect of 405-nm light on either component of Nutrient Broth on *S. aureus* growth at this applied light dose (108 J cm^{-2}).

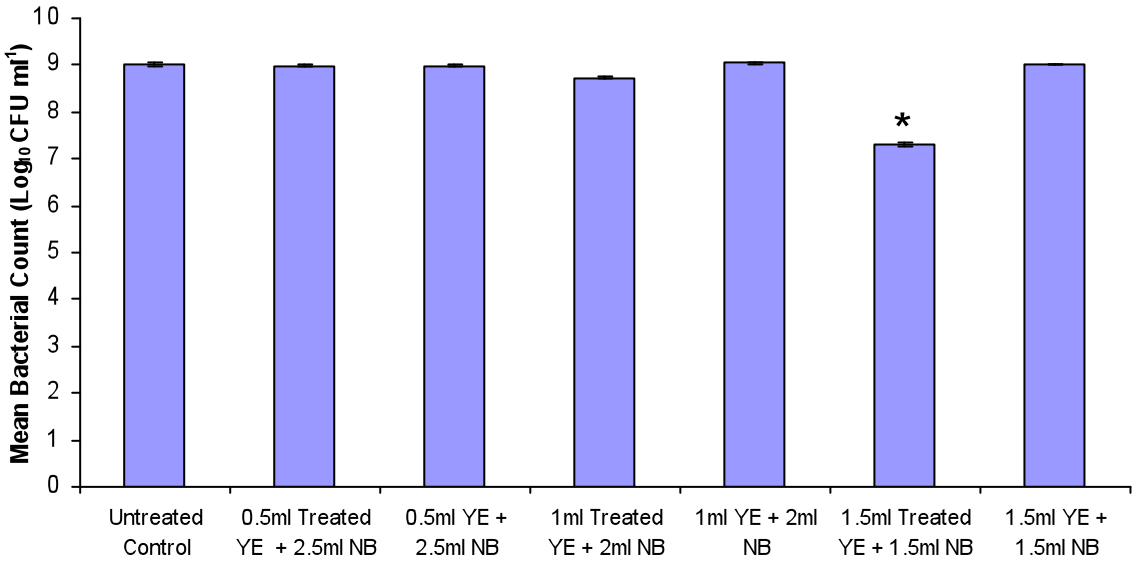

Figure 6.16 *The effect of adding different volumes of light-treated (108 J cm^{-2}) and untreated Yeast Extract (YE)(2 g/L) to Nutrient Broth (NB) on levels of* ***S. aureus*** *after incubation at 37°C for 16 hours*

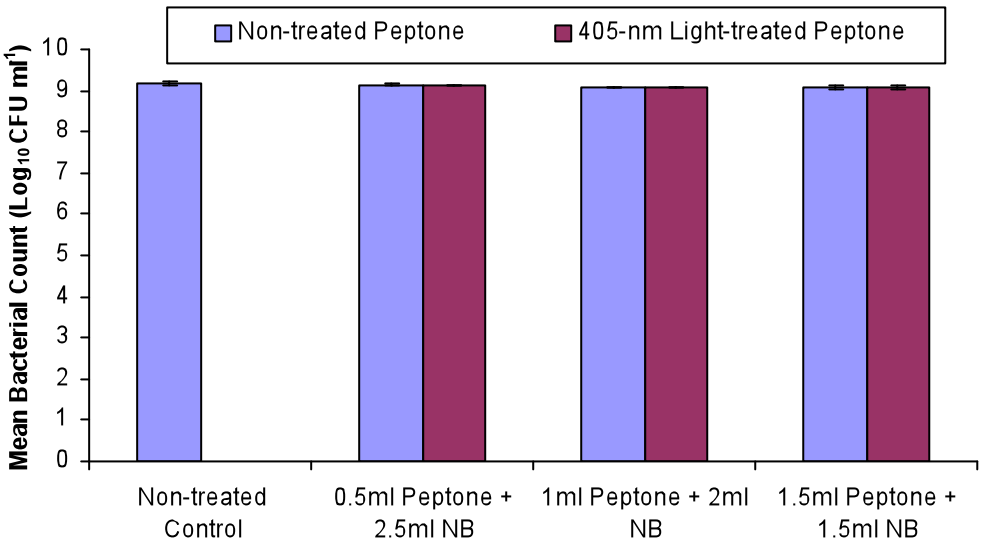

Figure 6.17 *The effect of adding different volumes of light-treated (108 J cm^{-2}) and untreated Peptone (5 g/L) to Nutrient Broth (NB) on levels of* ***S. aureus*** *after incubation at 37°C for 16 hours.*

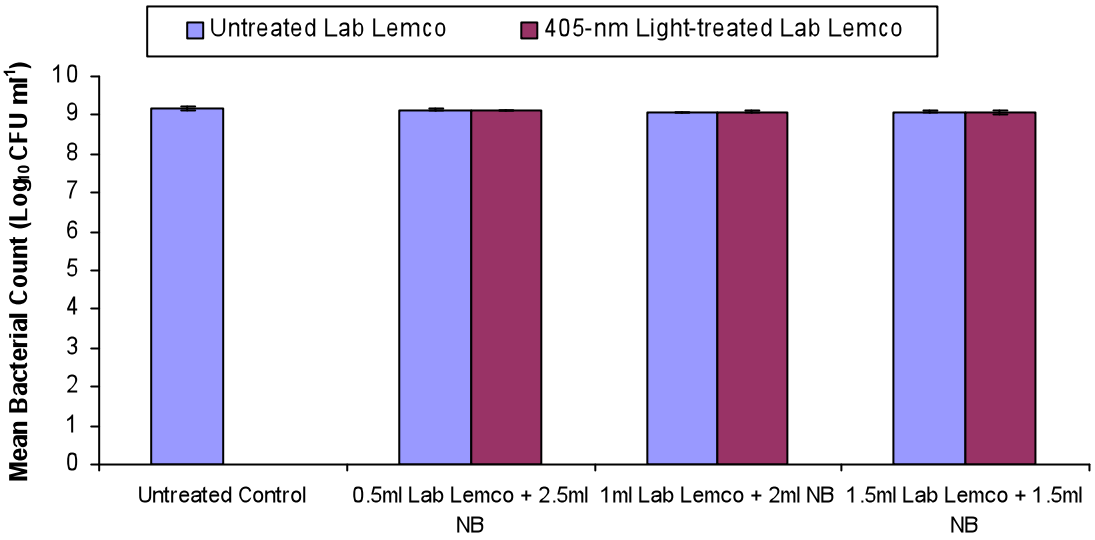

Figure 6.18 *The effect of adding different volumes of light-treated (108 J cm^{-2}) and untreated Lab Lemco (1 g/L) to Nutrient Broth (NB) on levels of* ***S. aureus*** *after incubation at 37°C for 16 hours.*

To prove that the results were not produced by dilution of nutrients in the Nutrient Broth, an experiment was completed whereby different volumes of sterile distilled water were added to untreated Nutrient Broth. The results, shown in Figure 6.19, demonstrate that even when diluted 2:1, growth of inoculated *S. aureus* was not inhibited (~9-Log_{10} CFU ml^{-1}).

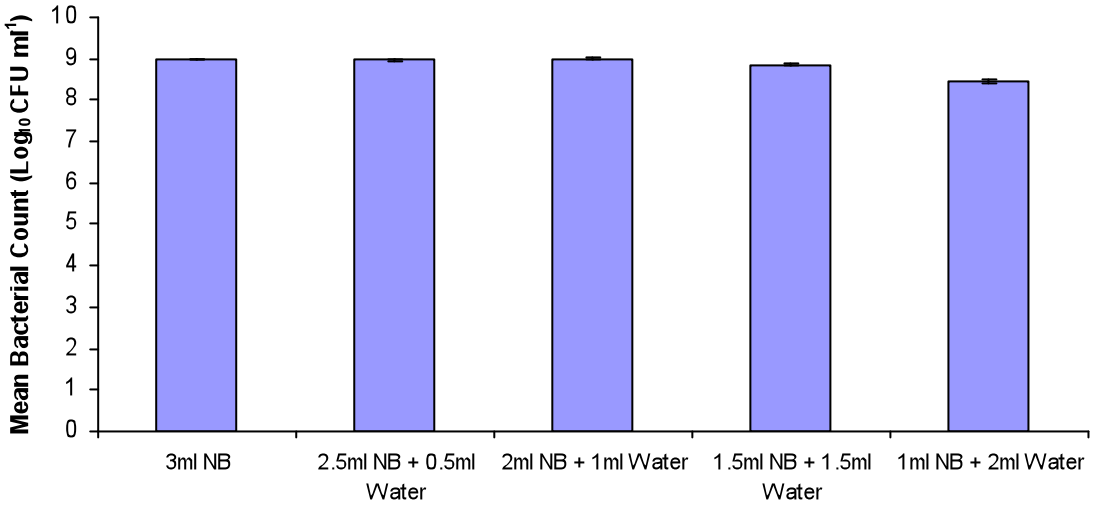

Figure 6.19 *The effect of diluting Nutrient Broth (NB) with different volumes of sterile distilled water on levels of* ***S. aureus*** *after incubation at 37°C for 16 hours.*

This study also investigated the duration of toxicity of the 405-nm light-treated Nutrient Broth. To do this, 3-ml volumes of Nutrient Broth were treated with 405-nm light as before (108 J cm^{-2}) and then stored in ambient light in a sterile universal for 24 or 48 hours after light exposure. After this time a 100 µl of a 3-Log_{10} population of *S. aureus* (~2-Log_{10} CFU ml^{-1}) was inoculated into both the 405-nm light-treated broth and an untreated Nutrient Broth (stored concurrently with the exposed sample in ambient lighting for 24 or 48 hours for consistency) and incubated in a rotary shaker at 37°C for 16 hours. After this time the Nutrient Broth/bacterial suspensions were plated onto Nutrient Agar and enumerated. Figure 6.20 results show toxicity remains after both 24 and 48 hours and no effect was seen on growth in the ambient light stored Nutrient Broth control.

Previous results (Table 6.5) showed that *E. coli* growth was not affected by Nutrient Broth, which had been treated with 108 J cm^{-2} 405-nm light. Therefore experiments increasing the exposure dose of 405-nm light on Nutrient Broth were completed using double (216 J cm^{-2}) and triple (324 J cm^{-2}) the dose that the Nutrient Broth had been previously exposed to in experiments prior to the inoculation of *E. coli* (Figure 6.21). Three-ml volumes of Nutrient Broth were exposed to either 216 J cm^{-2} or 324 J cm^{-2} of high-intensity 405-nm light at an irradiance of 40 mW cm^{-2}. A 100-µl volume of a 3-Log_{10} CFU ml^{-1} population of *E. coli* was inoculated into a 3-ml sample of untreated Nutrient Broth (serving as a control) and a 3-ml volume of the light-treated Nutrient Broth (either 216 J cm^{-2} or 324 J cm^{-2}) as in previous experiments and incubated in a rotary shaker at 37°C for 16 hours. Again the 405-nm light-exposed Nutrient Broth, even at the increased exposure doses, had no inhibitive effect on the growth of *E. coli*.

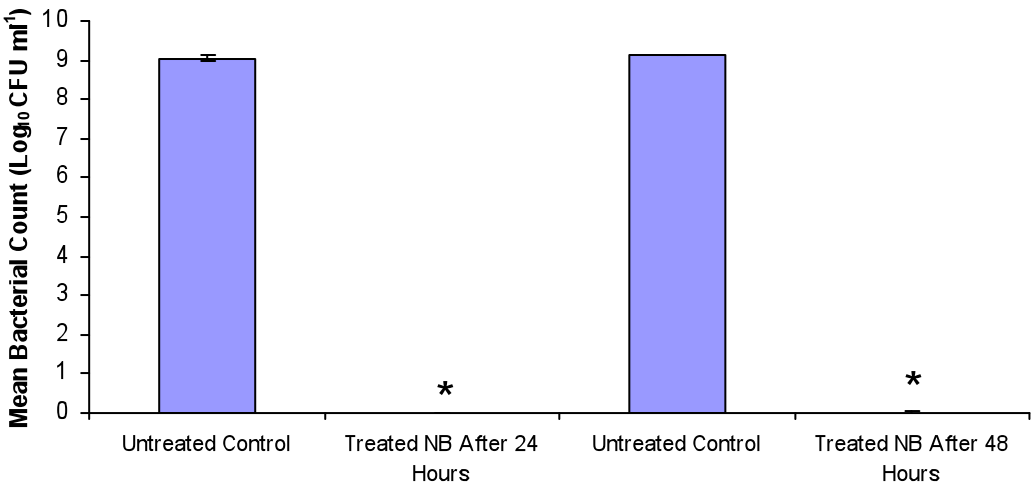

Figure 6.20 *Residual toxic effect of light-treated Nutrient Broth 24 and 48 hours after treatment on levels of* ***S. aureus*** *after incubation at 37°C for 16 hours*

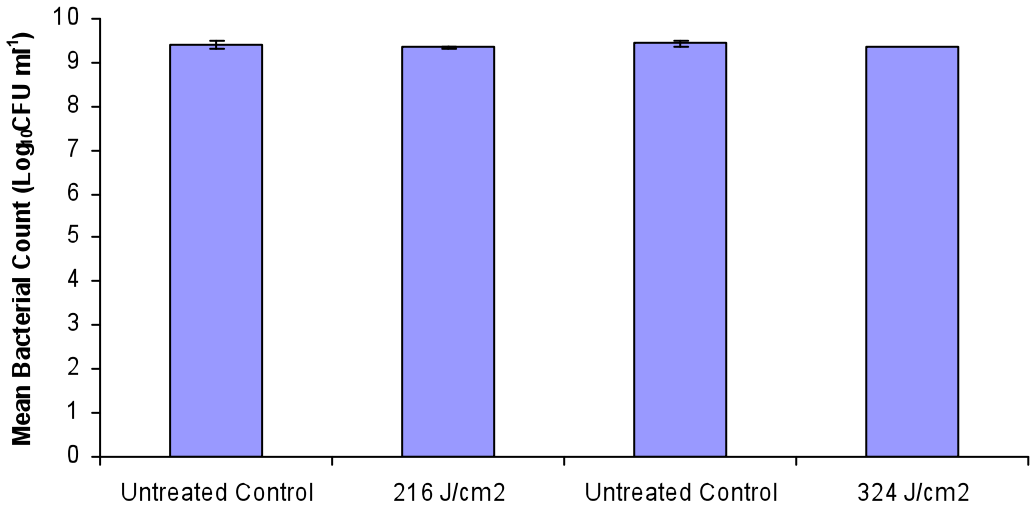

Figure 6.21 *Effect of increasing high-intensity 405-nm light-treatment dose of Nutrient Broth on levels of* ***E. coli*** *after incubation at 37°C for 16 hours.*

6.3.2.3 Effect of HINS-light on Different Nutritious Media

To assess whether the phenomenon of 405-nm induced photo-toxicity occurs in nutritious media suspensions other than Nutrient Broth, a series of experiments were carried out exactly as previous experiments (Section 6.3.2.2) using *S. aureus,* a representative Gram positive microorganism, and *E. coli,* a representative Gram negative microorganism.

Table 6.6 highlights the $\log_{10}$ CFU ml^{-1} initial population, and resulting populations after inoculation and incubation in both 405-nm light-treated and untreated control broths (all incubated at 37°C for 16 hours). In each case the broth was exposed to 108 J cm^{-2} of 405-nm light to allow comparison with previous experiments. Statistical significant differences between untreated control populations and light-treated samples are highlighted with asterisks.

Both Brain Heart Infusion Broth and Tryptone Soya Broth exposed to 108 J cm^{-2} of 405-nm light showed little negative effect on the growth of either *S. aureus* or *E. coli*. In fact *E. coli* was not as adversely affected as *S. aureus* in any of the tested broth media. *Staphylococcus aureus* growth was statistically significantly different

between light-treated and untreated controls. The growth of *S. aureus* was reduced by ~ 4-$\log_{10}$ CFU ml^{-1} in Nutrient Broth Number 2, which contains no yeast extract but has 2 g/L Lab Lemco (double the content in normal Nutrient Broth), suggesting that other components including yeast extract may be responsible for photo-toxicity. No conclusive evidence of the causative agent involved in photo-toxicity has been determined in these experiments.

Each medium suspension absorbs 405-nm light to different extents as shown in Table 6.7. Volumes of media used in experimentation (3 ml) were pipetted into a multi-well dish and set up for exposure to 405-nm as normal. Light intensity was measured using a power density meter underneath the exposed well to determine the amount light of being absorbed by the media suspension upon 405-nm light exposure. The power density measurements of 405-nm light recorded through the multi-well dish alone and with 3-ml of PBS are shown in Table 6.7 for comparison.

Both Tryptone Soya and Brain Heart Infusion Broth absorb a lot of light at 405-nm, which may be part of the reason they are not similarly affected by photo-toxicity as Nutrient Broth and Nutrient Broth Number 2.

Table 6.6 *Application of high-intensity 405-nm light (108 J cm^{-2}) to different broth-based growth media and its effect on* ***S. aureus*** *and* ***E. coli.***

Media Exposed to 405-nm light	Organism	Initial Population (Log_{10} CFU ml^{-1})	Population 16 hours after inoculation into light-exposed broth (Log_{10} CFU ml^{-1})	Untreated control population after 16 hours (Log_{10} CFU ml^{-1})
Nutrient Broth	*S. aureus*	1.43 (±0.08)	0 (±0.0)*	8.91 (±0.03)
	E. coli	1.62 (±0.02)	8.98 (±0.06)*	9.2 (±0.02)
Nutrient Broth No 2	*S. aureus*	2.11 (±0.01)	5.73 (±0.06)*	9.31 (±0.01)
	E. coli	1.84 (±0.01)	9.38 (±0.05)	9.36 (±0.08)
Tryptone Soya Broth	*S. aureus*	1.11 (±0.10)	9.32 (±0.06)	9.33 (±0.01)
	E. coli	1.10 (±0.02)	9.30 (±0.03)	9.42 (±0.06)
Brain Heart Infusion Broth	*S. aureus*	1.99 (±0.01)	7.65 (±0.02)	7.56 (±0.01)
	E. coli	1.69 (±0.04)	9.04 (±0.04)*	9.34 (±0.01)

* Indicates a statistically significant difference between light-treated and untreated control samples.

Table 6.7 *Power density measurements taken through 3 ml of each of the different broth media exposed to 40 mW cm^{-2} of 405-nm light, as measured from directly underneath the multi-well plate.*

	Power Density
Empty Multi-well Plate	19.9 mW cm^{-2}
Phosphate Buffered Saline	20.9 mW cm^{-2}
Nutrient Broth	15.6 mW cm^{-2}
Nutrient Broth No 2	14.8 mW cm^{-2}
Tryptone Soya Broth	5.7 mW cm^{-2}
Brain Heart Infusion Broth	1.3 mW cm^{-2}

6.3.2.4 Investigation into Causative Wavelengths

As it has been established that 405-nm light has a photo-toxic effect on Nutrient Broth it was necessary to resolve whether this event was only induced by 405-nm light or whether a range of wavelengths had similar effects on Nutrient Broth. Experiments exposing Nutrient Broth to different wavelengths of light using long-pass optical filters were completed to assess the effect of the exposed broth on inoculated *S. aureus* growth.

Methodology

Nutrient Broth was exposed to filtered light in multi-well plates as in previous experiments (Section 6.3.1), however, instead of using a 405-nm LED array, a spotlight Xenon broadband white light lamp set at a distance of 50 mm from the surface of the liquid sample (used in Chapter 4, Section 4.2.2) was used in conjunction with a range of commercially available long-pass filters (320-550 nm).

A 3-ml volume of Nutrient Broth was pipetted into a central well of a multi-well dish. A black PVC cylinder was placed around the well to prevent stray light entering the well, and one of the long-pass filter were placed on top of the well. The light source was positioned 50 mm directly above the liquid sample surface and the sample was exposed to the filtered light. In order to compare with previous experiments, Nutrient Broth was exposed to a dose of 108 J cm^{-2} in each case. To ensure the same dose was applied, the power density at each filter had to be measured using a power density meter and the exposure time was calculated as in Chapter 4, Equation 4.1. The power density measurements and exposure durations necessary to achieve a dose of 108 J cm^{-2} in each filter used is shown in Table 6.8. After exposure to filtered light wavelengths, *S. aureus* was inoculated into the exposed and un-exposed Nutrient Broth (37°C for 16 hours under rotary conditions) and plated and enumerated as in Section 6.3.1.

Table 6.8 *Power density measurements through each long- pass filter and the corresponding exposure time required to achieve a dose of 108 J cm^{-2}.*

Wavelength of Long-Pass Filter	Power Density Measurement Through Filter (mW cm^{-2})	Exposure Duration	Exposure Dose (J cm^{-2})
No Filter	272	6 m 37 s	108
320 nm	294	6 m 7 s	108
345 nm	287	6 m 16 s	108
375 nm	289	6 m 13 s	108
400 nm	266	6 m 47 s	108
450 nm	258	6 m 58 s	108
500 nm	220	8 m 10 s	108
550 nm	157	11 m 28 s	108

Figure 6.22 shows the effects of Nutrient Broth exposed to different wavelengths of light on the growth of inoculated *S. aureus*. In each set of wavelengths tested the bactericidal effect achieved was not as significant as that achieved with the 405 (±5) nm LED array. There appears to be some limiting effect on *S. aureus* growth when inoculated into Nutrient Broth that has been previously exposed to broadband UV-containing white light (no filter) with a reduction in growth when compared to control of ~ 4-log$_{10}$ CFU ml^{-1} and in the long-pass filters >320, >345 and >375 nm. The results of which were all significantly different to the untreated control (Figure 6.22). Nutrient Broth exposed to 320 nm, 345 nm and 375 nm filters had a growth limiting effect on inoculated *S. aureus* populations as exposed samples grew between 3-5 log$_{10}$ CFU ml^{-1} less than un-exposed control populations (Figure 6.22). This highlights that light from ~320-400 nm and broadband white light might cause similar toxic effects to 405-nm emitting LED arrays. However, 405-nm light produced from an LED array caused a more potent toxicity than had been shown in the results of Figure 6.23. Long-pass filters allow light of greater wavelength to pass through the filter therefore, 320 nm, 345 nm, 375 nm and 400 nm filters also contain 405-nm light, therefore the inactivation is most likely due to the long-pass filters and the broadband Xenon light source itself emitting some 405-nm, although not as intensely as the 405-nm LED array.

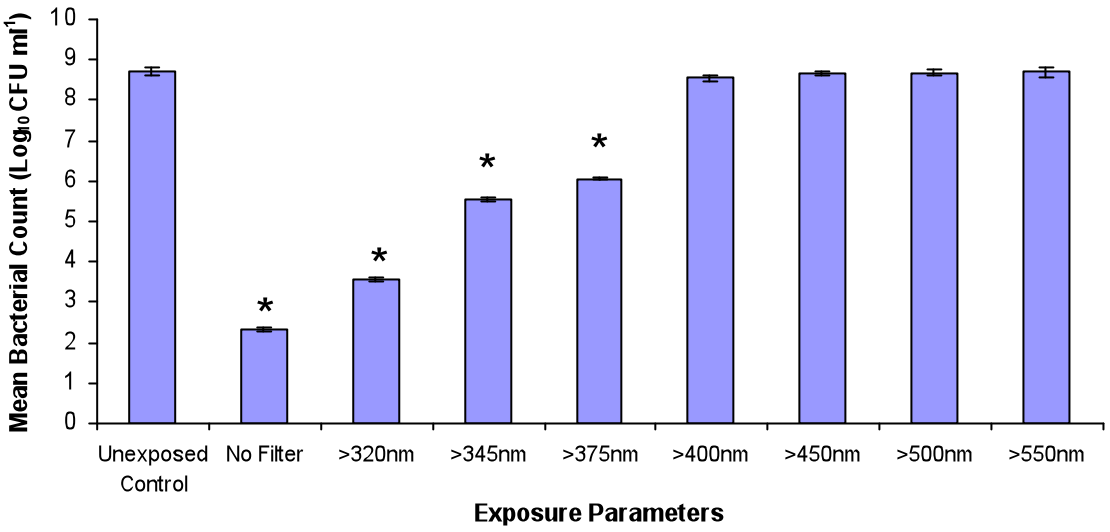

Figure 6.22 *Exposure of Nutrient Broth to different wavelengths of light through long-pass filters (108 J cm^{-2}) and its effect on levels of* ***S. aureus*** *after incubation at 37°C for 16 hours.*

6.4 REPAIR

As previously shown in Chapter 4, UV-damaged *S. aureus* cells can undergo repair when exposed to wavelengths of light from 300-500 nm, with optimum effect at 370-380 nm. At present it is not known if such a mechanism exists to repair 405-nm light damaged cells. In order to assess this, two experiments were carried out:

- UV-induced damage of *S. aureus* and subsequent light-induced repair using long-pass filters
- 405-nm induced damage of *S. aureus* and subsequent light-induced repair using long-pass filters

UV Light-induced Repair Methodology

A 7-Log$_{10}$ CFU ml^{-1} *S. aureus* PBS suspension was prepared in a 20-ml volume in a sterile 90 mm Petri dish (as in Section 6.1), which was exposed to pulses of UV-rich light using a Xenon flashlamp (as described in Chapter 4, Section 4.2.2) until a surviving population of around 2-3-Log$_{10}$ CFU ml^{-1} remained. Some of the bacterial

suspension was plated after pulsed UV light exposure to determine the number of cells inactivated using the UV light. Under dark conditions 3 ml of the UV-damaged bacterial suspension was pipetted into a central well of a multi-well dish. A black PVC cylinder was placed around the well to prevent stray light entering the well, and a long-pass filter was placed on top of the well. The light source was positioned 50 mm directly above the liquid sample surface and the sample was exposed to 55 J cm^{-2} of filtered light for the desired time period (55 J cm^{-2} was used in previous UV light experiments in Chapter 4)(Table 6.9). To achieve the same dose, light intensity through each filter was tuned using the spotlight source to ensure the required dose was achieved in a 5-minute duration (same light intensity through each filter).

405-nm-Light-induced Repair Methodology

In the 405-nm light-induced repair study a 3-ml volume of a 5-Log_{10} CFU ml^{-1} *S. aureus* PBS suspension was pipetted into a central well of a multi-well dish and was exposed to a 72 J cm^{-2} dose of high-intensity 405-nm light (30 min at 40 mW cm^{-2} using Source B) to achieve a surviving population of around 2-3-Log_{10} CFU $ml^{-1,}$ similar to the sub-lethally damaged *S. aureus* population density used in UV experiments in Chapter 4. A 1-ml volume of the exposed sample was pipetted from the well and plated onto Nutrient Agar plates as described in Chapter 3, Section 3.3.2 to determine the population before light-induced repair, the other 2 ml remained in the well and was exposed to long-pass filtered light (375-500 nm) as in "*UV-Light-induced Repair Methodology"*.

Plating and Enumeration

Bacterial samples exposed to both 405-nm and UV-light, respectively were plated onto Nutrient Agar as were bacterial samples obtained after exposure to the different long-pass filters and control samples. Control samples after UV or 405-nm light inactivation were either plated directly or left on a bench for the duration of the long-pass wavelength exposures (5 minutes) to enumerate the levels of repair at each wavelength tested. Plates were incubated at 37°C for 18-24 hours after which plates were enumerated and data recorded as log_{10} CFU ml^{-1} as described in Chapter 3, Section 3.3.2.

Table 6.9 *Power density measurements of the Xenon broadband light source through each long- pass filter (altered intensity) and the corresponding exposure time required to achieve a dose of ~ 55 J cm^{-2}.*

Wavelength of Long-Pass Filter	Adjusted Power Density Measurement Through Filter (mW cm^{-2})	Exposure Duration	Exposure Dose (J cm^{-2})
375 nm	188	5 m	56
400 nm	184	5 m	55
450 nm	188	5 m	56
500 nm	188	5 m	56

Repair of UV and 405-nm Light Damage

Figure 6.23 shows the $\log_{10}$ CFU ml^{-1} increase of pulsed UV-light damaged *S. aureus* cells upon exposure to light through long-pass filters. Light-induced repair occurred using 375, 400 and 450 nm long-pass filters, and no light-induced repair occurred from 500 nm onwards. Around 2.5-$\log_{10}$ CFU ml^{-1} cells were repaired when exposed to the 375 nm and 400 nm long-pass filters and ~2-$\log_{10}$ CFU ml^{-1} of cells were repaired when using the 450 nm long-pass filter. Asterisks highlight where UV-damaged cell samples exposed to different long-pass filtered light are statistically significant different from their respective initial UV inactivated samples (data not shown).

The effect of long-pass filtered light on *S. aureus* following high-intensity 405-nm light is also shown in Figure 6.23. The only filter that saw an increase in the number of cells following exposure was the 375 nm long-pass filter. All other filters caused inactivation at a dose of 55 J cm^{-2} (maximum inactivation following second filtered light exposure was ~ 1-$\log_{10}$ CFU ml^{-1} at 500 nm). Asterisks highlight where 405-nm damaged cell samples exposed to different long-pass filtered light are statistically significantly different from their respective initial 405-nm inactivated samples (data not shown).

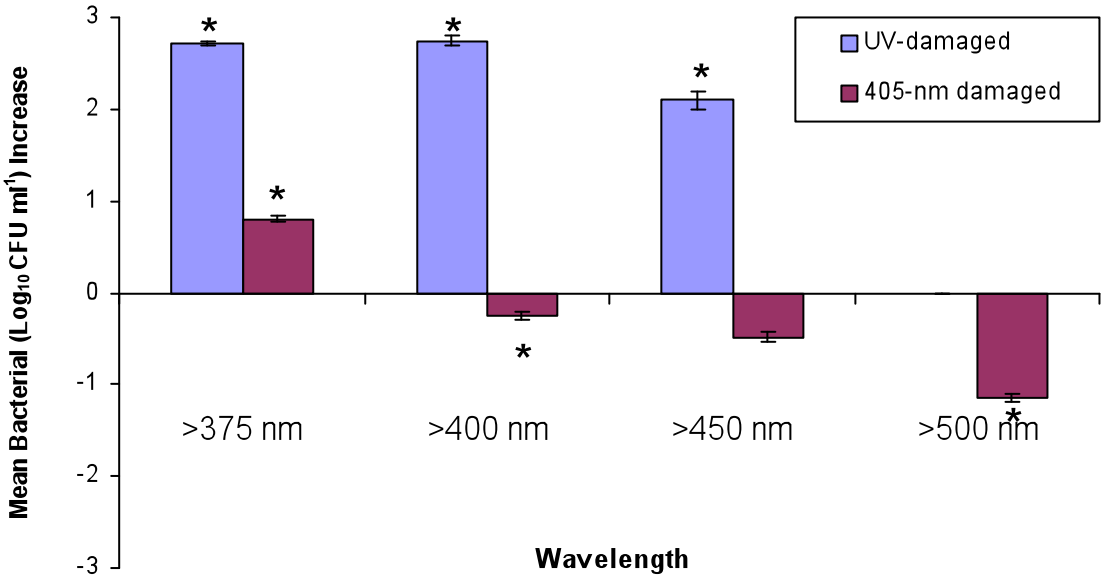

Figure 6.23 *Light-induced repair of pulsed UV light and 405-nm light in* ***S. aureus*** *using a range of long-pass filters. Repair is plotted as log$_{10}$ CFU ml^{-1} increase.*

Light-induced Repair at 370 nm

To further compare the repair ability of 405-nm induced damage with UV-induced damage, an experiment was conducted to assess the light repair capability of 405-nm cell damage at 370 nm, the optimum wavelength in *S. aureus* in the repair of UV-induced damage (Chapter 4, Section 4.6.3). The experiment was completed exactly as in Section 6.4, "*405-nm-Light-induced Repair Methodology"*. However in this case a 370 (±10) nm narrow band-pass filter was used instead of the long-pass filters. The power density of light transmitted through the filter was already known from Chapter 4, Section 4.7.2 (Table 4.1) and for comparison with previous experiments in Chapter 4, the dose of 1.5 J cm^{-2} of 370-nm light was applied to 405-nm light damaged *S. aureus* cells. As shown in Table 6.10 there appears to be around 1-log$_{10}$ CFU ml^{-1} of cells repaired upon exposure to 370 (± 10) nm light at the dose of 1.5 J cm^{-2} (Statistically significantly different to initial 405-nm damaged sample). Although not tested using the same light dose, the long-pass filter results of Figure 6.23 are very similar to that of Table 6.10, with approximately 1-log$_{10}$ CFU ml^{-1} increase in cells after exposure to light through both the 375-nm long-pass filter and 370 nm narrow-band filter. This can be compared to Figure 4.10 in Chapter 4 where

~3-$\log_{10}$ CFU ml^{-1} of *S. aureus* cells were repaired upon exposure to 370-nm (± 10) light. It is possible some form of light-repair occurs in 405-nm light damaged *S. aureus* cells, however experimental results have shown that 370-nm induced repair occurs much more readily in PUV-light damaged *S. aureus* cells.

Table 6.10 *The effect of 370 (± 10) nm filtered light (1.5 J cm^{-2}) on the repair ability of* ***S. aureus*** *after 405-nm light exposure (72 J cm^{-2}).*

Bacterial Population	**Log_{10} CFU ml^{-1}**
Initial	5.05 (±0.12)
HINS-light Inactivated	2.66 (±0.83)
Following Exposure to 370-nm Filtered Light (Log_{10} CFU ml^{-1})	3.56 (±0.21)*
Increase	0.90

6.5 DISCUSSION AND CONCLUSIONS

Other studies have noted deleterious effects of light-exposed media on bacterial growth. Webb and Lorenz (1972) described that Nutrient Agar plates (Nutrient Broth with agar added to set medium) when exposed to room lighting, reduced the plating ability of *E. coli* photoreactivation deficient mutant *BPhr*. They found that non-repair deficient *E. coli* strains were resistant to the effects of this light-induced medium toxicity, which was similar to the results obtained in this study for *E. coli*. Xu and Hurlbert (1990) found that light-exposed L-broth agar plates affected the growth of *Xenorhabdus* spp. They noted that addition of pyruvate or catalase to the medium reduced toxicity, suggesting that ROS were being produced in the medium.

Studies using mammalian culture medium have also shown similar toxic effects on mammalian cells. Stoien and Wang (1974) found that light, particularly near-UV light, exposure of Dulbecco's Modified Eagle's Medium (DMEM) on its own made the medium lethal to mammalian cells. They too suspected that this was either caused through toxic product formation or depletion of vital nutritional components in the medium. Stoien and Wang (1974) discovered that the medium contained the photo-active components riboflavin, tryptophan and tyrosine which can act as chromophores in the same manner as porphyrins and photosensitiser molecules. They found the addition of extra riboflavin, tryptophan and tyrosine offered no resistance to the toxic effects much like the effect of adding fresh Nutrient Broth to 405-nm light-exposed Nutrient Broth shown in this study. They concluded that the most likely component to be light-activated was riboflavin, which can cause the oxidation of the other photo-active components tryptophan and tyrosine. Interestingly a study by Grzelak *et al*. (2001) investigated the generation of ROS in cell culture media through exposure to light on different cell culture media including yeast extract-peptone-glucose medium. Through spin trapping they identified the main ROS involved in light activation of cell media components to be superoxide, hydrogen peroxide and singlet oxygen. They theorised that these ROS were produced through Type I and Type II photosensitisation of riboflavin. Maclean *et al*. (2009) investigated the use of 405-nm light in the inactivation of bacteria through

endogenously produced ROS in bacteria including, *S. aureus, E. coli, K. pneumoniae, E. faecalis* and *A. baumannii* and found that *S. aureus* and *A. baumannii* were most rapidly inactivated. The results of this chapter showed similar inactivation kinetics in the same bacteria upon exposure to 405-nm light-treated Nutrient Broth (*S. aureus* and *A. baumannii* were particularly sensitive). This fact coupled with evidence presented in other studies it is likely that some sort of ROS-based inactivation process is occurring.

Interestingly, some studies have shown evidence that exposure of campylobacter-specific media to light and air causes production, in the media, of toxic oxygen species such as, superoxide, hydroxyl radicals, singlet oxygen and hydrogen peroxide (Bolton *et al*., 1984; Juven and Rosenthal, 1985; Moran and Upton, 1987). The reason so much work has been completed on this bacterium is due to its high sensitivity to toxic oxygen species. As has been shown in this study, reversal of toxicity cannot occur once the ROS have been formed in the media (Bolton *et al*., 1984; Juven and Rosenthal, 1985). A study by Bolton *et al*., (1984) has shown that these toxic oxygen species are highly bactericidal to *C. jejuni* causing inactivation of >5-$\log_{10}$ CFU ml^{-1}. Studies investigating the effect of light and air on campylobacter-specific media have also found that the toxic species formed cause *Campylobacter* to shift to what was considered to be viable but non-culturable morphology (Moran and Upton, 1987). Studies have investigated the effects of different light and air-exposed media on *Campylobacter* spp growth. It was found that blood and charcoal-containing media are not subject to light-induced toxicity as any toxic oxygen species formed are quenched by detoxifying enzymes present in the media such as peroxidase, catalase and superoxide dismutase (Bolton *et al*., 1984 Moran and Upton, 1987).

These different studies show similar results to those of the present study and have proven that light and air exposure of media should be minimised in order to reduce cell inactivation through production of ROS in cell culture media. In addition, these results may also affect the general applicability of 405-nm light. Any future work in "real" situations will have to be carried out with stringent monitoring to assess the

effects of 405-nm light on other molecules, particularly if used in food settings where photo-active vitamins and amino acids may be present.

The discovery that some "repair" of *S. aureus* can occur at wavelengths ~370 nm is also interesting, however the level of this repair is far less than that achieved in UV-damaged cell light-induced repair and also occurs in a shorter spectrum of wavelengths than in UV light-induced repair mechanisms. It is not known if this is true light-induced repair, as we have not elucidated whether the photolyase enzyme was involved in the repair of oxidative damage. The damage caused by 405-nm light is thought to involve porphyrin stimulation and subsequent production of ROS that target cell membranes and organelles, not through cyclobutane pyrimidine dimer (CPD) formation in DNA as in UV-light induced damage. This makes it highly unlikely that light-induced repair is occurring, however it is possible some other form of repair occurrs, which may or may not be stimulated by 370-nm filtered light. However, the use of 405-nm light still has significant advantages for use compared to UV-light inactivation technologies.

The results of this study have shown that:

- Inactivation of *S. aureus* in a nutritious environment was generally more difficult due to the dichotomy between growth and inactivation**.**
- Intermittent light exposure had little effect on the overall inactivation achieved in *S. aureus*, either in PBS or Nutrient Broth suspensions.
- A toxic photo-product was formed in 405-nm light-exposed Nutrient Broth which was capable of inactivating *S. aureus*, MRSA and *A. baumannii*. At the levels found in this study, it had little effect on *E. coli* and *E. faecalis* growth but had a limiting effect on *K. pneumoniae*, *Ps. aeruginosa* and high inoculum levels of *S. aureus*.
- Adding extra nutrients (fresh Nutrient Broth) to 405-nm light-exposed Nutrient Broth did not reverse the effects of the toxic product, and it is likely that components of Nutrient Broth are the source of the 405-nm light-activated toxic product formation, although no conclusive evidence to the exact component responsible was found in this study.

- With the treatment levels used in this study, the toxic effect of 405-nm light on inoculated *S. aureus* was not seen with Brain Heart Infusion Broth or Tryptone Soya Broth.
- Exposure of Nutrient Broth to broadband white light, >320-nm, >345-nm and >375-nm light also produced a photo-toxic effect on *S. aureus*. However, 405-nm light produced from the LED array was the most potent inducer of photo-toxicity in Nutrient Broth.
- Exposure of 405-nm damaged *S. aureus* cells to long-pass filtered light of 375 nm appeared to induce recovery of around 1-$\log_{10}$ CFU ml^{-1} of cells. This same recovery was also shown in narrow-band pass 370 (±10) nm filtered light studies. However exposure of previously 405-nm damaged *S. aureus* cells to filtered light >400-nm only caused more inactivation and did not stimulate recovery.

Chapter 7

HINS-LIGHT INACTIVATION OF YEASTS AND MOULDS

7.0 BACKGROUND

Work in Chapter 5 has shown that 405-nm HINS-light is capable of inactivating a range of food-related bacterial pathogens. This chapter progresses this work to examine the effects of HINS-light on eukaryotic species. The inactivation of the yeast species *Saccharomyces cerevisiae* and *Candida albicans*, and the spore-forming mould species *Aspergillus niger*, was investigated, with these microorganisms being chosen as they are important in both human health and food spoilage.

Saccharomyces cerevisiae is a dimorphic yeast (Blacketer *et al.,* 1993) that is ubiquitous in the environment, and as such is frequently found on foodstuffs such as fruits, leaves and crops. It is also one of the most studied eukaryotic organisms commonly used in molecular biology, and seen by many people to be the model eukaryotic organism.

Candida albicans is a polymorphic yeast most commonly associated with human disease (Sánchez-Martínez and Pérez-Martín, 2001). The ability of *C. albicans* to switch between budding cells to a filamentous morphology has been linked to an increase in virulence. It can cause mild infections such as oral candidiasis, thrush and vaginitis, to serious and life-threatening disease, especially in the immunocompromised (Méan *et al.,* 2008; Cormick *et al.,* 2009). It gains entry to the blood stream through in-dwelling devices such as catheters, artificial ventilation or through exposed wounds and mucous membranes.

Aspergillus niger is a spore-forming fungus sometimes called “black mould”. It is also common in the environment and can cause spoilage of cereal crops and

vegetables. Aspergilli and other moulds have also been associated with "Sick Building Syndrome", where an individual becomes ill due to the building in which they reside or work in (Luksiene *et al*., 2004; Straus, 2009). This is most commonly associated with poor indoor air quality. *A. niger* is an opportunistic organism frequently associated with systemic fungal infection in hospital patients with reduced immune function, such as AIDS patients, bone marrow transplant patients and the elderly (Méan *et al.,* 2008).

7.1 LIGHT SOURCE- 405-nm LED ARRAY

In order to allow direct comparisons between experimental results for HINS-light inactivation of the yeasts and moulds with the inactivation kinetics of the tested foodborne bacteria (Chapter 5), Source A was used at the same irradiance of 10 mW cm^{-2}. Further experimentation on the yeasts and *A. niger* was completed using Source B at an irradiance of 40 mW cm^{-2} and 54 mW cm^{-2} respectively. Table 7.1 details the voltage and ampere settings required in order to achieve these desired irradiance levels (power density) levels.

Table 7.1 *Voltage and ampere settings for the DC power supplies used to achieve the various power densities with Source A and Source B.*

HINS-light Source	Power Density (mW cm^{-2})*	Voltage (V)	Amperes (A)
Source A	10	11.2(± 0.2)	0.5(± 0.05)
Source B	20	9.9(± 0.2)	0.3(± 0.05)
	40	10.9(± 0.2)	0.6(± 0.05)
	54	12.0(± 0.2)	1.0(± 0.05)

** Power density measurements taken at a distance of 2 cm from the HINS-light source*

7.2 PREPARATION AND HINS-LIGHT TREATMENT OF YEASTS

7.2.1 Yeast Preparation

Saccharomyces cerevisiae MUCL 28749 (Figure 7.1) and *Candida albicans* MUCL 29903 were inoculated into Malt Extract Broth and Malt Extract Broth with 0.1% yeast extract, respectively (Chapter 3, Section 3.1.2). After incubation for 18-24 hours at 30°C and 37°C for *S. cerevisiae* and *C. albicans* respectively, broths were centrifuged at 3939 × g for 10 minutes and the resultant cell pellet was re-suspended in 100 ml PBS resulting in a cell density of 7-Log_{10} and 8-Log_{10} CFU ml^{-1}, respectively. Yeast suspensions were serially diluted to give an approximate starting population of 5-Log_{10} CFU ml^{-1} for experimental use.

7.2.2 Yeast HINS-light Treatment Method

Saccharomyces cerevisiae and *C. albicans* HINS-light exposure experiments were carried out using Source A set to give an irradiance of 10 mW cm^{-2} at the surface of the suspension (2 cm distance). A 2-ml sample of yeast suspension at a population density of 10^5 CFU ml^{-1} was pipetted into a central well of a 12-well dish and exposed to HINS-light for increasing durations of time, as detailed in Chapter 5 Section 5.2.2. Later investigations on *S. cerevisiae* and *C. albicans* progressed to use 3 ml samples (10 mm depth) in both the control and the light-exposed wells, meaning an increased sample volume was available for plating purposes.

HINS-light exposed samples, and non-exposed control samples, of *S. cerevisiae* and *C. albicans* were plated onto Malt Extract Agar (MEA) and MEA with 0.1% Yeast Extract and incubated at 30°C and 37°C for 48 hours, respectively (Chapter 3, Section 3.1). Plates were enumerated after incubation and data was recorded as CFU ml^{-1}.

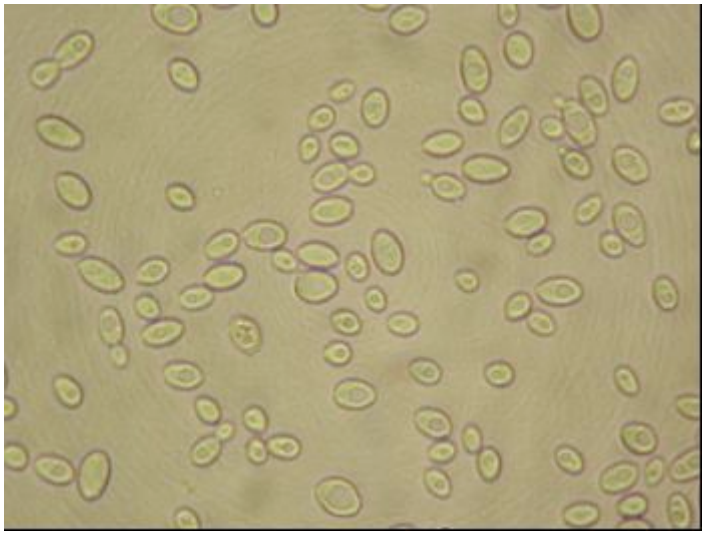

Figure 7.1 ***S. cerevisiae*** *cells viewed under a microscope using × 40 lens (400 × magnification).*

7.3 RESULTS

7.3.1 HINS-LIGHT INACTIVATION OF *SACCHAROMYCES CEREVISIAE*

The effect of HINS-light on *S. cerevisiae* was determined using two different light sources, Source A and Source B. As described in Chapter 5, Section 5.1, the two sources have slightly different peak centre wavelengths, making the inactivation results achieved using the two sources difficult to compare to one another. As both the light sources and the intensities of the HINS-light are different, germicidal efficiency values will be used to compare their inactivation capabilities.

Figure 7.2 shows an inactivation curve for *S. cerevisiae* cells in liquid suspension using 10 mW cm^{-2} HINS-light (Source A). It can be seen that a dose of 180 J cm^{-2} was required to achieve an approximate 3-$\log_{10}$ CFU ml^{-1} reduction in *S. cerevisiae* population and all light-exposed samples were statistically significantly different to their respective control populations. Figure 7.3 shows the effect of exposure of *S. cerevisiae* to a different HINS-light source (Source B) at a higher intensity, 40 mW cm^{-2}. In this instance a 5-$\log_{10}$ CFU ml^{-1} reduction in *S. cerevisiae* was achieved

using a dose of 576 J cm^{-2}, again all light-exposed samples were statistically significantly different to their respective control populations.

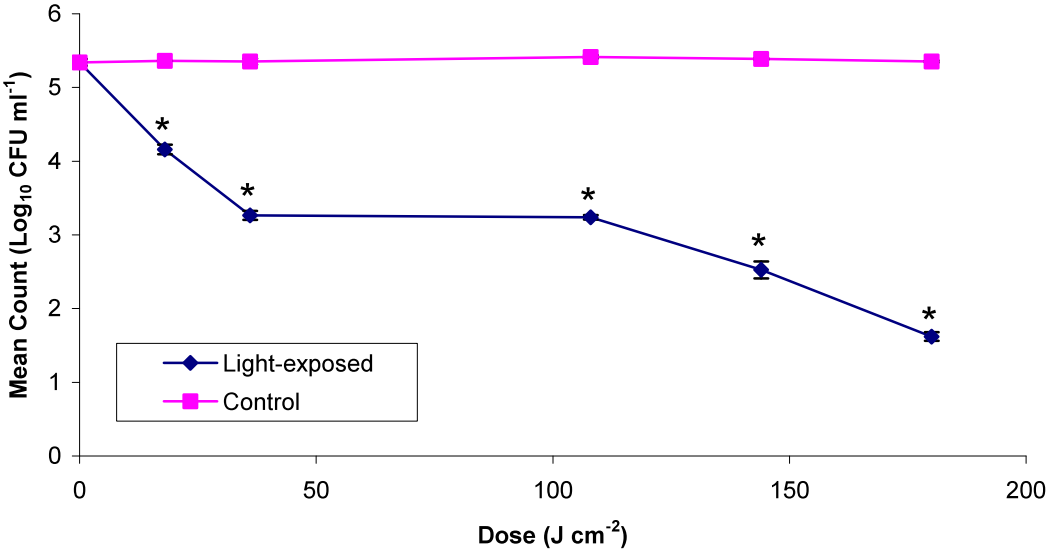

Figure 7.2 *Inactivation of **S. cerevisiae** in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 10 mW cm^{-2} using Source A.*

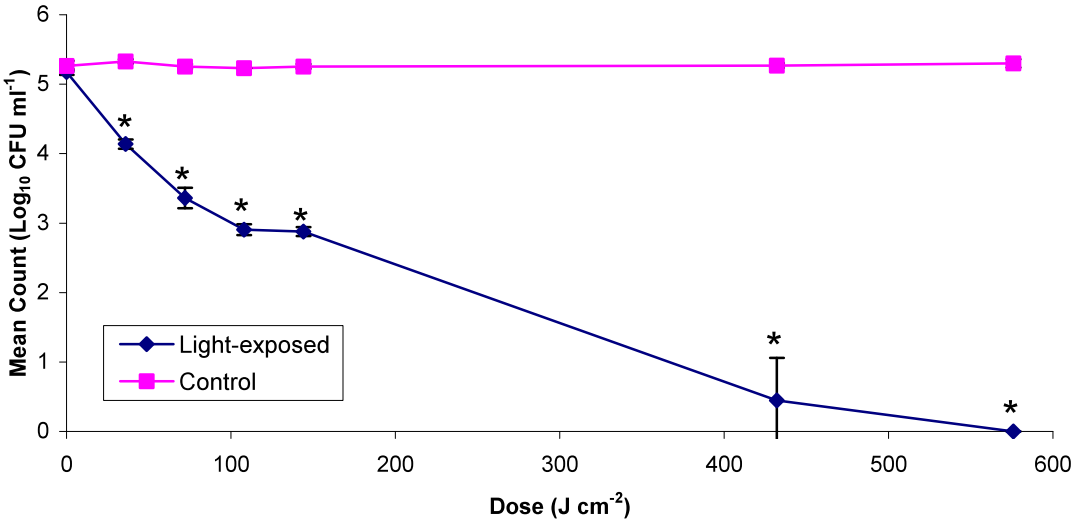

Figure 7.3 *Inactivation of **S. cerevisiae** in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 40 mW cm^{-2} using Source B.*

7.3.2 HINS-LIGHT INACTIVATION OF *CANDIDA ALBICANS*

As in the *S. cerevisiae* experiments, two different light sources and two different light intensities were used, therefore comparisons will be made using germicidal efficiency values. The inactivation curve for *C. albicans* in Figure 7.4 shows a 5-$\log_{10}$ CFU ml^{-1} reduction at a dose of 216 J cm^{-2} using Source A at 10 mW cm^{-2}, with statistically significant difference between light-exposed and control values from a dose of 72 J cm^{-2} onwards. In Figure 7.5 it can be seen that HINS-light exposure using Source B, operated at an irradiance of 40 mW cm^{-2}, yielded a 5-$\log_{10}$ CFU ml^{-1} reduction in *C. albicans* at a dose of 576 J cm^{-2}. In this instance a statistically significant difference between light-exposed and control values was only seen after a dose of 288 J cm^{-2}.

The germicidal efficiency values were calculated and are shown in Table 7.2. Data demonstrates that when using Source A at 10mW cm^{-2} both *S. cerevisiae* and *C. albicans* had the same germicidal efficiency value of 0.02. This was also found when using Source B operated at an irradiance of 40 mW cm^{-2}, with the germicidal efficiency for both organisms found to be 0.009.

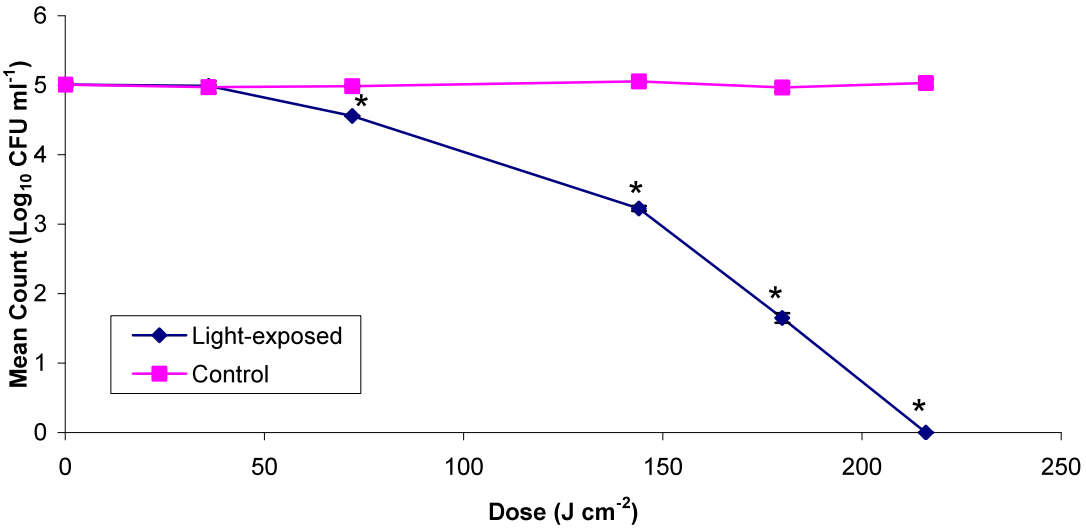

Figure 7.4 *Inactivation of* ***C. albicans*** *in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 10 mW cm^{-2} using Source A.*

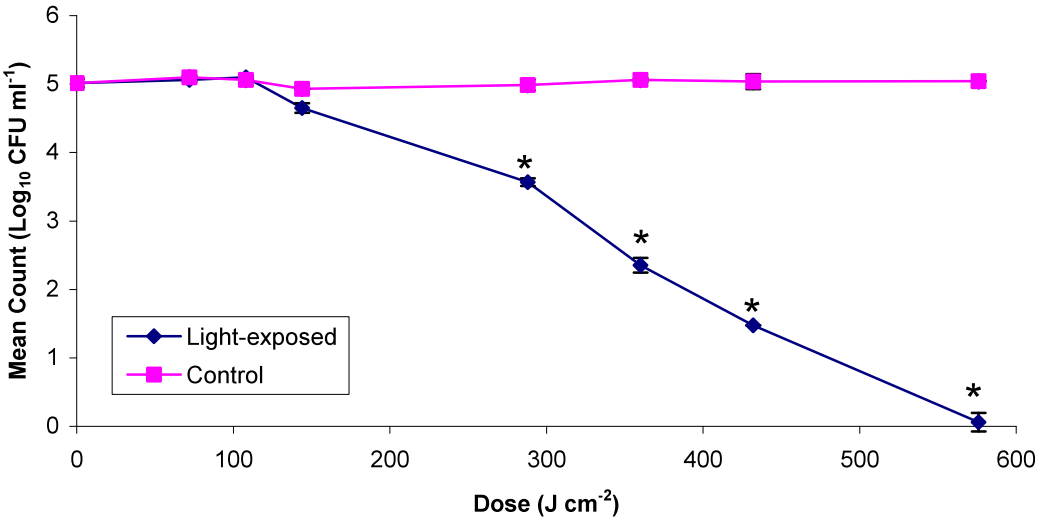

Figure 7.5 *Inactivation of* ***C. albicans*** *in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 40 mW cm^{-2} using Source B.*

Table 7.2 *Log_{10} reduction and germicidal efficiency values for the inactivation of the yeasts **S. cerevisiae** and **C. albicans** upon exposure to 405-nm high-intensity light using both Source A and B at irradiances of 10 mW cm^{-2} and 40 mW cm^{-2} respectively.*

	Organism	**Initial Population N_0 (Log_{10} CFU ml^{-1})**	**Final Population N (Log_{10} CFU ml^{-1})**	**Log_{10} (N/N_0) Reduction**	**Dose (J cm^{-2})**	**Germicidal Efficiency η (Log_{10} (N/N_0) / J cm^{-2})**
Source A @ 10 mW cm^{-2}	*S. cerevisiae*	5.34(± 0.04)	1.62(± 0.05)	3.72	180	0.02
	C. albicans	5.01(± 0.04)	0(± 0)	5.01	216	0.02
Source B @ 40 mW cm^{-2}	*S. cerevisiae*	5.18(± 0.04)	0(± 0)	5.18	576	0.009
	C. albicans	5.02(± 0.06)	0.06(± 0.1)	4.96	576	0.009

7.3.3 Cell Morphology After HINS-light Treatment

As discussed in Section 7.0, change in morphology from budding yeast to pseudo-hyphal forms has been linked with an increase in virulence in *C. albicans*. *Candida albicans* suspensions exposed to 40 mW cm^{-2} high-intensity 405-nm light, at a total dose of 576 J cm^{-2} and non-exposed samples of *C. albicans* were visualised under the × 40 lens (× 400 magnification) of a light microscope as a wet film slide, stained with methylene blue, to detect if any morphological change arose after 405-nm light exposure (Figure 7.6).

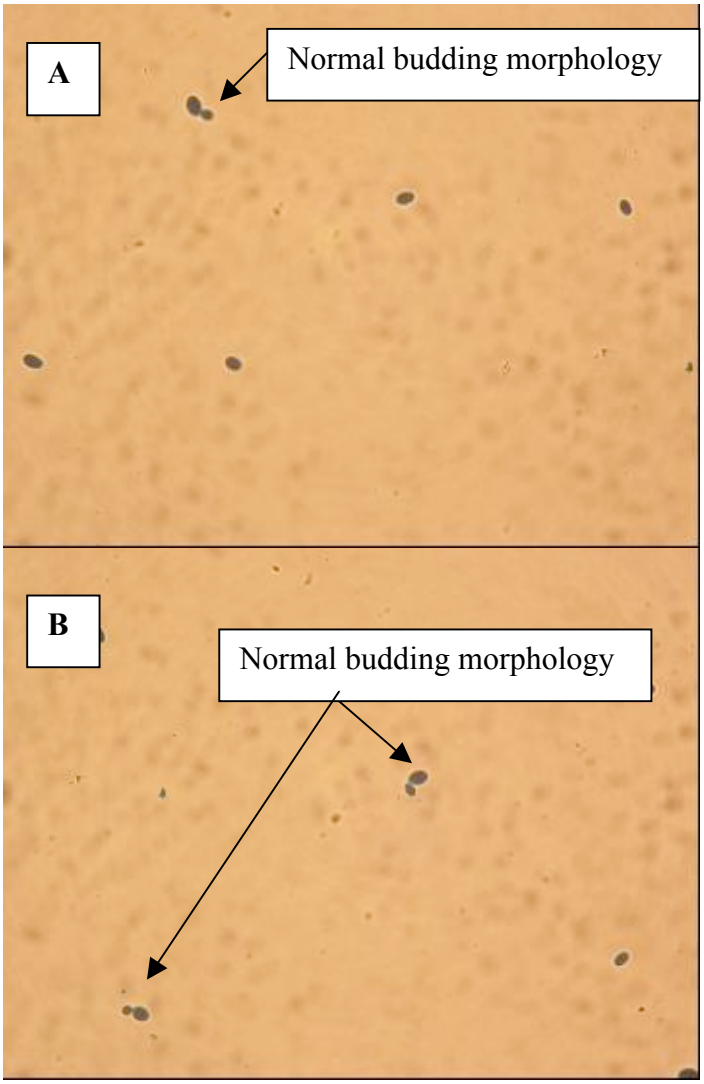

Figure 7.6 *Cell morphology of methylene blue stained* ***C. albicans*** *cells in both (A) 405-nm light-exposed and (B) non-exposed liquid suspensions visualised using a light microscope (×400 magnification).*

Figure 7.6a and Figure 7.6b show the HINS-light exposed and non-exposed control wet films, respectively. Both Figures 7.6a and Figure 7.6b show normal budding morphology of *C. albicans* cells, demonstrating that at this lethal dosage no pseudo-hyphae are formed.

7.4 PREPARATION AND HINS-LIGHT TREATMENT OF *ASPERGILLUS NIGER* SPORES

7.4.1 Spore Preparation

As described Chapter 3, Section 3.1.2, *Aspergillus niger* spores MUCL 38993 were inoculated onto MEA slopes and grown at 30°C for 5 days to produce sufficient conidial growth (spore growth) for use in experimentation. Slopes were flooded with PBS containing 1 drop of Tween 80 (added to prevent aggregation of spores) and the end of a plastic L-shaped spreader was used to gently remove spores from the surface of the agar slope. One-ml of the resultant solution was then pipetted into 9 ml of PBS before the spores were counted using an Improved Neubauer haemocytometer (Weber Scientific International, UK) to ascertain the population density of the spore suspension (Figure 7.7). Spore suspensions were then diluted to the appropriate starting solution for experimental use.

Principle of Haemocytometer

The haemocytometer is a specialised microscope slide containing a counting grid. This grid contains 25 large squares (5 × 5 squares of combined area 1mm^2 and a depth of 0.1 mm) and each of these 25 squares is further divided into 16 squares (4 × 4) as is depicted in Figure 7.8; this knowledge can be used in calculations to determine the volume of spores in the suspension.

Haemocytometer Procedure

- A specialised haemocytometer coverslip was cleaned in 70% ethanol and left to dry.
- The coverslip was moistened with breath in order to attain "Newton's Rings" upon contact with the haemocytometer microscope slide. This gives the coverslip a tight fix onto the microscope slide and ensures the correct depth of liquid in the haemocytometer for counting.
- The spore suspension was vortexed thoroughly and ~10 μl was taken up into a pipette tip. The liquid was then touched to the bottom end of the coverslip and pipetted until the entire chamber was visibly filled with liquid.
- The slide was visualised under a microscope (400 × magnification) ensuring both the spores (see Figure 7.9) and the grid lines were visible
- Five larger squares were counted and multiplied by 5 in order to obtain the number of spores in a 1mm^2 area.
- A calculation was then performed using Equation 7.1 in order to determine the spore density of the solution.

$$C = \tilde{\mathrm{N}} x 10^4 \qquad [7.1]$$

Where C is the average number of cells per ml, $\tilde{\mathrm{N}}$ is the average number of cells counted in the chamber, and 10^4 is the volume conversion factor for 1 mm^2.

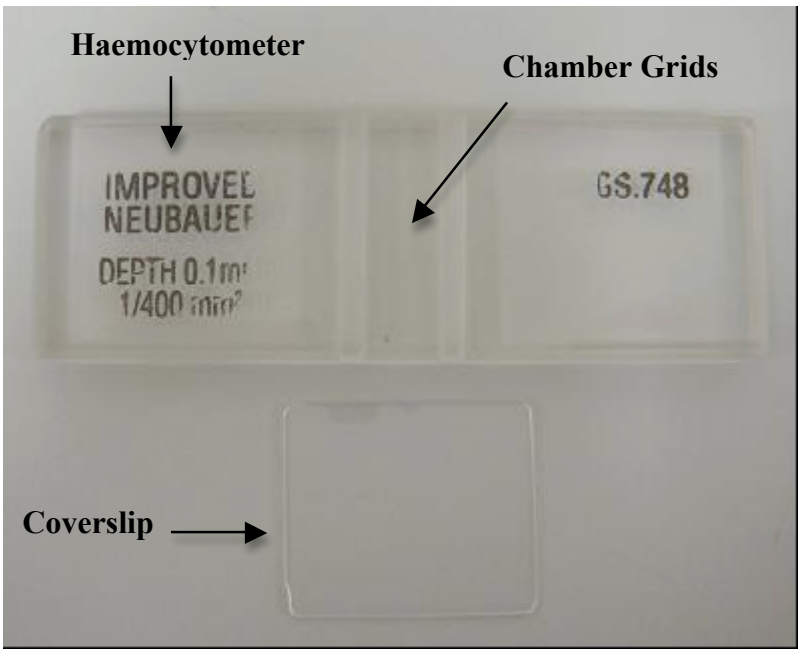

Figure 7.7 *Improved Neubauer haemocytometer and coverslip.*

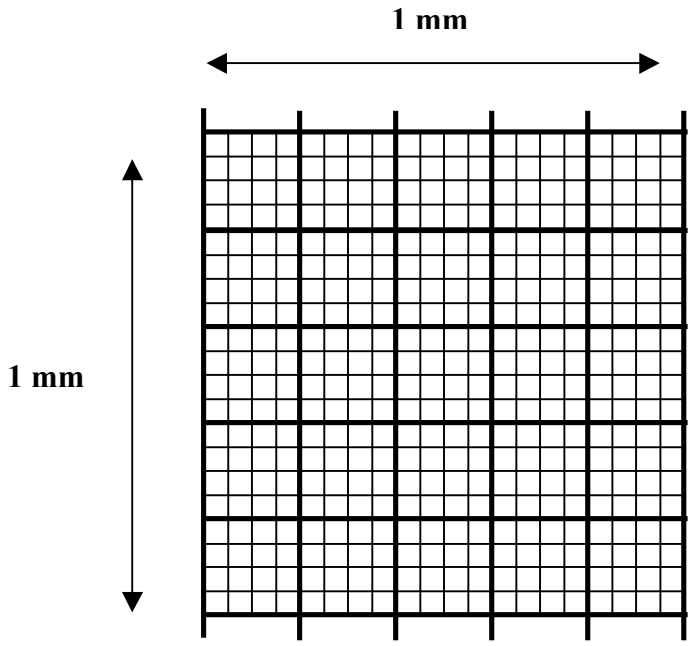

Figure 7.8 *A diagrammatic representation of the grid lines on an Improved Neubauer haemocytometer.*

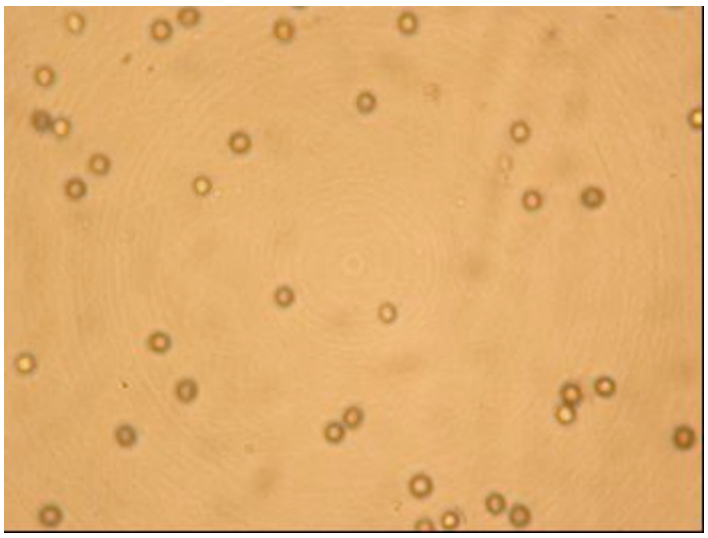

Figure 7.9 ***A. niger*** *spores viewed under a microscope using the × 40 lens (400 × magnification).*

7.4.2 Spore Treatment

Spores were exposed to HINS-light using Source B to give an approximate irradiance of 54 mW cm^{-2} at the surface of the liquid sample. A 3-ml sample of *A. niger* was exposed to HINS-light as detailed in Chapter 7, Section 7.2.2. *Aspergillus niger* spores were exposed to the higher intensity light for longer periods of time, up to 10 hours, as it was anticipated that spores would be more difficult to inactivate.

Control and light-exposed samples were plated onto MEA and incubated at 30°C for 5 days. Manual spread plates of 50 μl and 100 μl were prepared in order to aid counting, as colonies were large and often joined up at higher population densities. Colonies were enumerated and recorded as $\log_{10}$ spore forming units per millilitre (SFU ml^{-1}).

7.5 RESULTS – HINS- LIGHT INACTIVATION OF *ASPERGILLUS NIGER* SPORES

Figure 7.10 shows the results of the HINS-light exposure of *A. niger* spores. The dose required to inactivate a 5-$\log_{10}$ SFU ml^{-1} suspension of spores was 1.9 kJ cm^{-2}. A statistical significant difference between the control sample and the light-exposed sample was not seen until after 4 hours.

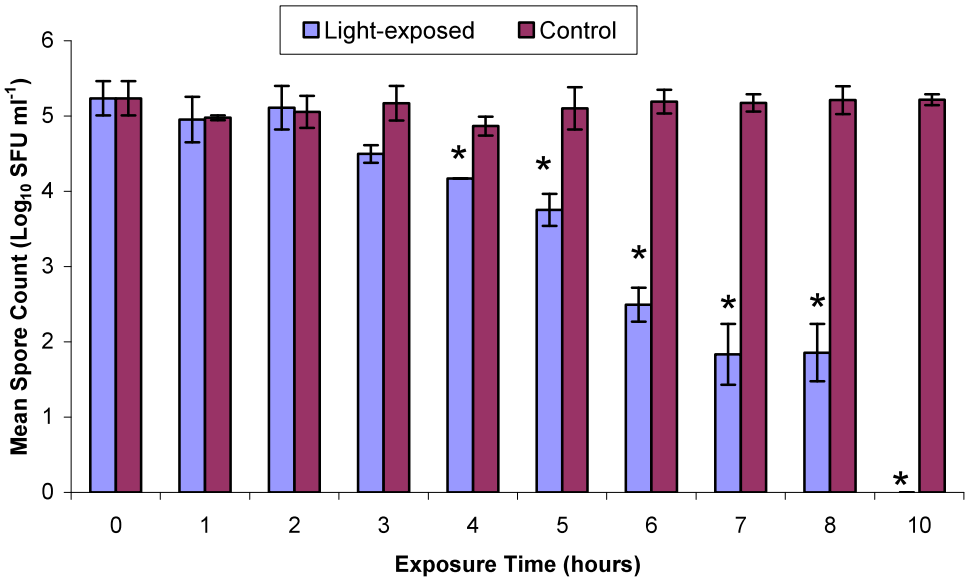

Figure 7.10 *Inactivation of **A. niger** spores in liquid suspension, using Source C at 54 mW cm^{-2}.*

The effect of dose on inactivation is shown in Figure 7.11 in which the same dose (1.2 kJ cm^{-2}) is applied in 2 different ways; dose application A which consisted of 8 hours HINS-light exposure at an irradiance of 40 mW cm^{-2} and dose application B consisting of 16 hours HINS-light exposure at an irradiance of 20 mW cm^{-2}. Both exposed samples are reduced by approximately 2-$\log_{10}$ SFU ml^{-1} and showed statistically significantly difference between their respective light-exposed and control values.

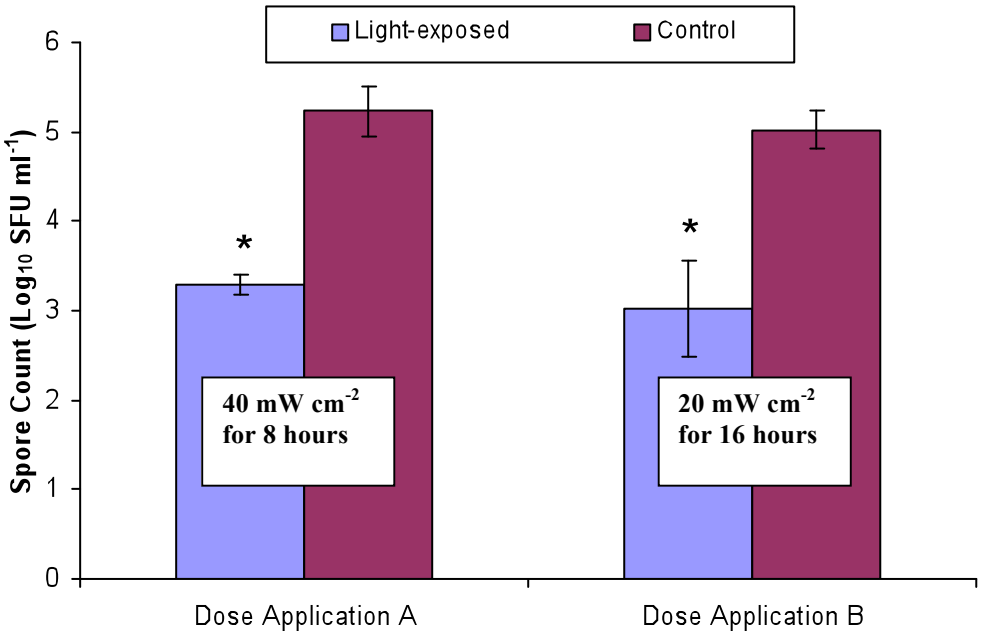

Figure 7.11 *The effect of application of the same dose (1.2 kJ cm^{-2}) in two different ways on the inactivation of* ***A. niger*** *in liquid suspension.*

During HINS-light experimentation on *A. niger* it was observed that, upon culturing, some HINS-light treated spores produced colonies that lacked conidial growth (spore growth). Figure 7.12a depicts a control plate of non light-exposed *A. niger* showing, in general, normal conidiation, although some colonies appear to lack conidia. In Figure 7.12b, plating of light-exposed *A. niger* suspensions produces colonies lacking conidia.

In order to investigate this phenomenon further, upon treating with HINS-light, colonies were counted as usual but were re-incubated at 30°C to mature for up to 10 days at optimum growth temperature to determine if spore production ability was regained. Non-exposed control sample plates were also re-incubated 30°C for 10 days to determine if lack of conidia was due to HINS-light or due to a peculiarity in the *A. niger* strain used. Figures 7.13a and 7.13b show a test plate and a control plate, respectively, after incubation at 30°C for 10 days (Note: these are not the same plates from Figure 7.12a and b). Figure 7.13a, the control plate, shows dense conidial growth present on all colonies, proving the production of colonies lacking

conidia in control plates was a temporary condition, whereas Figure 7.13b, the HINS-light exposed plate, shows some colonies that have very little or no conidial growth, however some colonies still present normal conidiation.

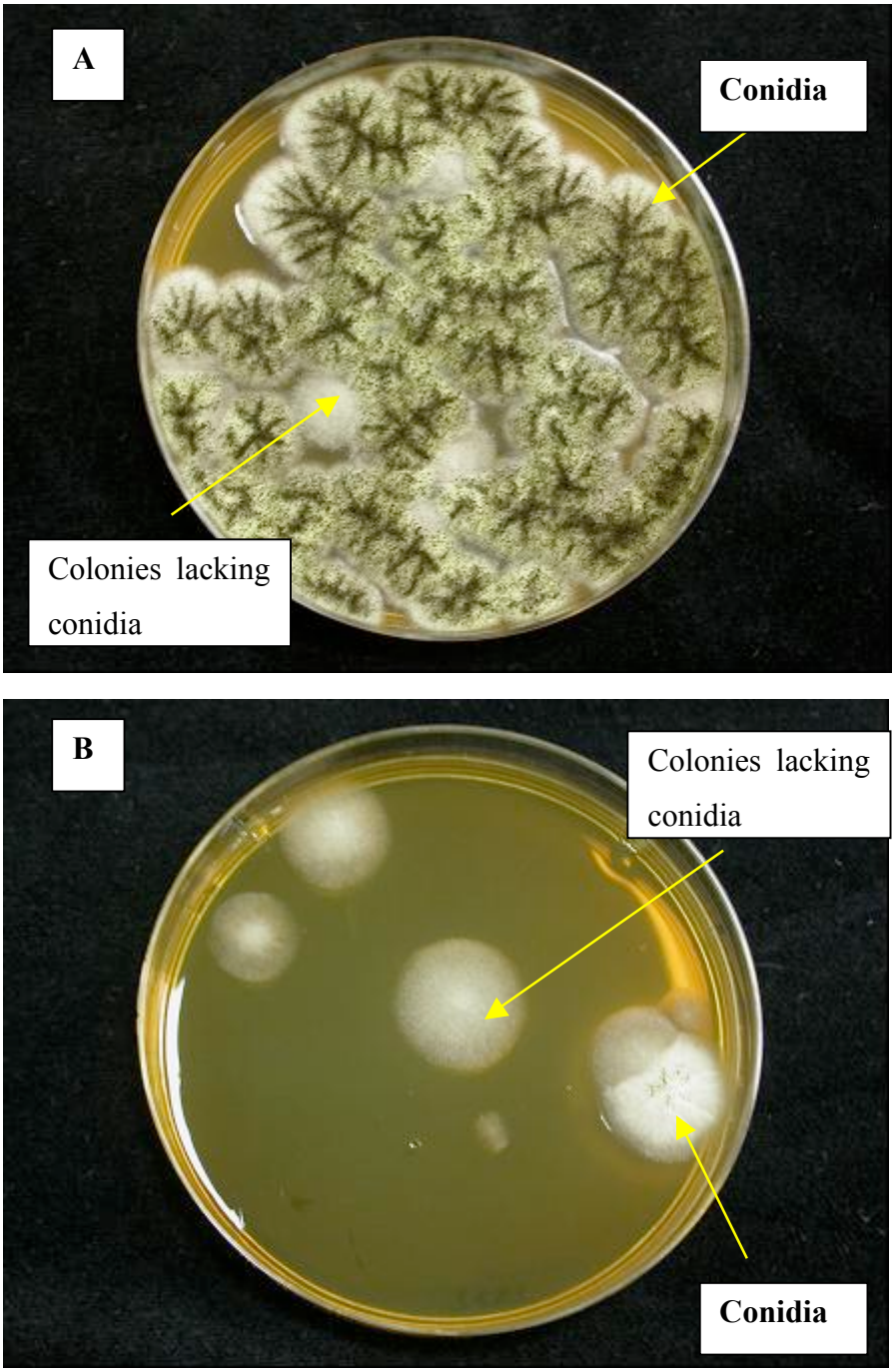

Figure 7.12 *The effect of HINS-light on **A. niger** after 3-day incubation; A is the control (non-exposed sample) and B is the HINS-light exposed sample.*

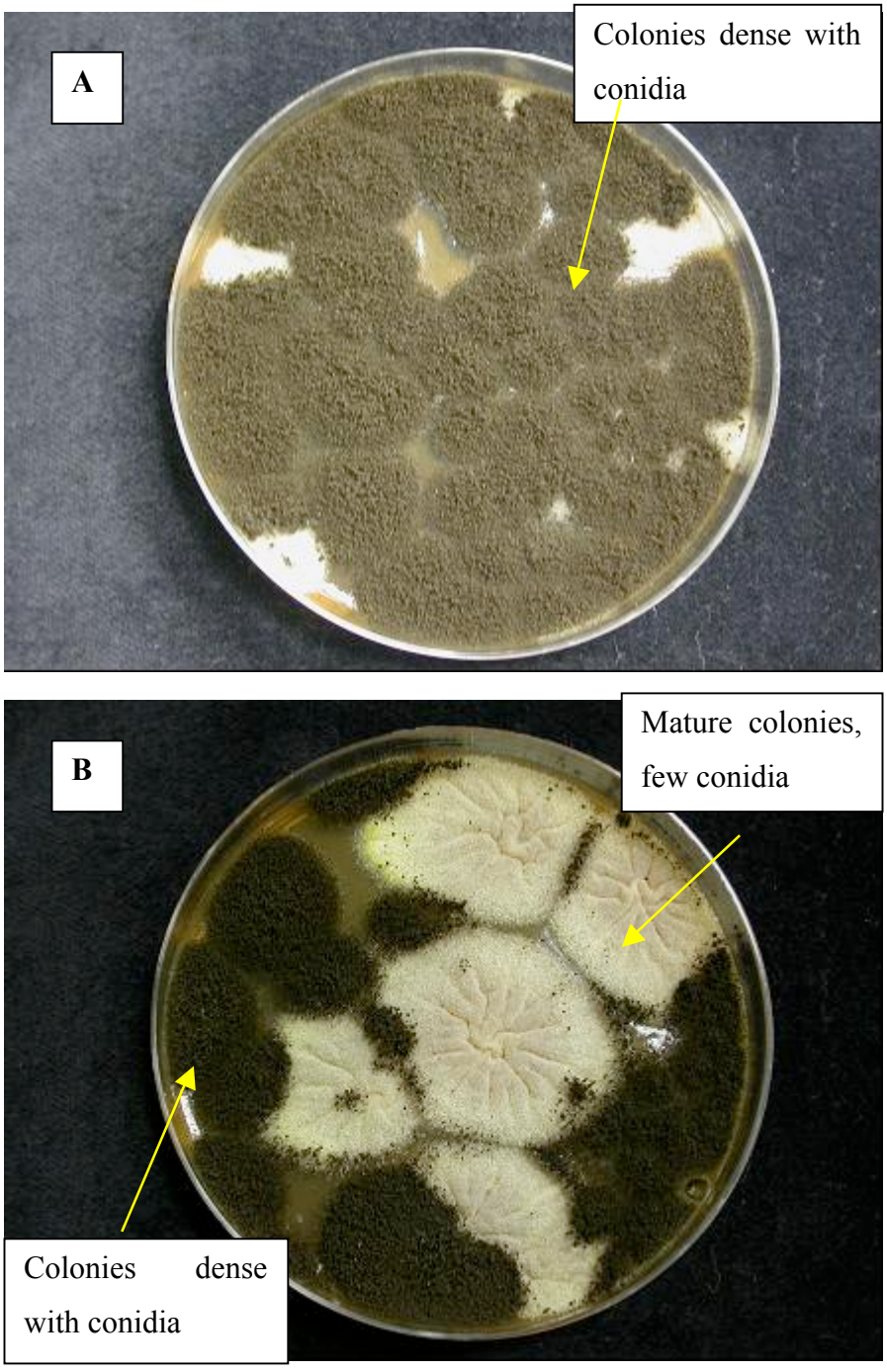

Figure 7.13 *The effect of HINS-light on conidial production of* ***A. niger*** *after 10 days; A is the (non-exposed) control and B is the HINS-light exposed sample.*

7.5.1 Temperature

As *A. niger* spores were exposed to HINS-light for a duration of 10 hours it was necessary to determine if there was any build-up of heat from the LED arrays during this exposure time. Temperature readings were taken using a thermocouple, as in Chapter 5 Section 5.3, every hour for 10 hours. There was minimal increase in temperature during this time. The temperature remained at 29°C (±1°C) during 10 hours of HINS-light exposure.

7.6 DISCUSSION AND CONCLUSIONS

Previous chapters have shown HINS-light to be bactericidal, this chapter shows it is also fungicidal as it is capable of inactivating the three fungal species *S. cerevisiae*, *C. albicans* and *A. niger*. When using Source A at an irradiance of 10 mW cm^{-2} it was found that both *S. cerevisiae* and *C. albicans* had the same germicidal efficiency value of 0.02 (Table 7.2). HINS-light caused reductions in the numbers of yeast cells by almost 4-$\log_{10}$ CFU ml^{-1} and 5-$\log_{10}$ CFU ml^{-1} at a dose of 180 J cm^{-2} and 216 J cm^{-2}, respectively. The germicidal efficiency value was similar to the Gram negative bacteria tested in Chapter 5, *Salm. enterica* serovar *enteritidis* and *Sh. sonnei* (0.01 and 0.03 respectively). This shows that yeasts are of a similar susceptibility as most of the Gram negative bacteria in this study, excluding *C. jejuni*.

The germicidal efficiency results in Table 7.2 show that *S. cerevisiae* and *C. albicans* have the same germicidal efficiency values when using both light Sources (Source A at 10 mW cm^{-2} and Source B at 40 mW cm^{-2}) meaning both yeasts are inactivated at similar rates. Overall, Source B used at an irradiance of 40 mW cm^{-2} is slightly less efficient at inactivating the yeasts *S. cerevisiae* and *C. albicans* than Source A when operating at an irradiance of 10 mW cm^{-2}. A difference of 0.011 exists between the germicidal efficiencies of the two devices; the reason for this difference in germicidal efficiency is unknown at present, however as speculated in Chapter 5, it is thought to be due to differences in the emission wavelengths of the two HINS-light sources.

Morphological appearance of *C. albicans* was assessed using a wet film slide. No morphological differences were visualised between 405-nm light-exposed cells and non-exposed cells at the light intensities and doses applied in this study. Therefore it is unlikely that exposure to 405-nm light leads to an increase in virulence commonly associated with pseudo-hyphal morphology.

Figure 7.10 shows that *A. niger* was also inactivated using HINS-light at an intensity of 54 mW cm^{-2}. Around 10 hours exposure was required at this irradiance, giving a complete dose of 1.9 kJ cm^{-2}. This is similar to the dose required for the reduction of *Bacillus* species endospores by around 2-log_{10} CFU ml^{-1} (1.15 kJ cm^{-2}) as shown in Table 5.2.

The effect of dose on the inactivation of *A. niger* was demonstrated in Figure 7.11, whereby 5-Log_{10} SFU ml^{-1} liquid samples of *A. niger* were exposed to the same dose of HINS-light (1.2 kJ cm^{-2}) applied in two different ways. In both the experiment where dose was delivered as 8 hours exposure at 40 mW cm^{-2}, and the experiment in which dose was delivered as 16 hours at 20 mW cm^{-2}, similar inactivation rates were achieved. Log_{10} SFU ml^{-1} reductions achieved in both cases were approximately 2-log_{10} SFU ml^{-1}, which corresponds with the results achieved in the dose-dependent experiment that was completed for *L. monocytogenes* in Chapter 5, Section 5.3. This provides further evidence that HINS-light inactivation is dose-dependent.

The discovery that high-intensity 405-nm exposure renders *A. niger* unable to produce conidia, demonstrated in Figure 7.12, was further investigated. Plates were incubated for a longer period of time (10 days) to allow maturation of colonies, seen in Figure 7.13. It was noted that conidia-free *A. niger* colonies failed to regain their ability to produce conidia upon further incubation. However, the colonies on the control plates that appeared to lack conidia, produced conidia after 10 days incubation showing this to be a temporary effect. A study by Hatakeyama *et al*. (2007) found that conidiation of *Aspergillus oryzae* was repressed upon exposure to white light (peaks at 430, 550 and 610 nm). Interestingly they also found that blue light completely suppressed colony formation, which supports the HINS-light

inactivation results obtained for *A. niger*. However, Zhu and Wang (2005) found that *A. niger* conidiation actually increased upon exposure to blue light, which is contrary to the results obtained in this study. It is likely that the variation in the effects of light on conidiation is dependent on the specific wavelengths and intensities of light used as well as on other factors such as the type of growth media used, however, further work would be required for confirmation of these possible effects.

A large number of fungi have blue light receptor mechanisms that are thought to play a vital role in reproduction (Donnelly *et al*., 2008). Many organisms, including fungi, monitor changes in light and temperature via blue light receptors through a process called circadian rhythm (Tisch and Schmoll, 2010). Circadian rhythm, present in all eukaryotes, is used by fungal species to optimise reproduction by using information on light and temperature to influence time of conidiation and, in some cases, to also protect conidia from harmful effects of solar UV radiation (Donnelly *et al*., 2008). For example *Neurospora crassa* has been known to produce its conidia at dusk (Tisch and Schmoll, 2010). However, it is likely that these responses to blue light are highly organism-dependent (Tisch and Schmoll, 2010).

Aspergillus niger spores are particularly difficult to inactivate, as they possess multi-layered pigmented spores containing aspergillin, a melanin-like compound (Ray and Eakin, 1975). A study by Anderson *et al*. (2000), used acetone to chemically extract this pigment from *A. niger* conidia and the light absorbance through the resultant liquid was recorded. The results from the study by Anderson *et al*. (2000) demonstrate that the aspergillin compound absorbs around 5-8 times more light in the UV-wavelengths than at 405 nm (presumably to protect against harmful UV rays present in sunlight). This suggests that *A. niger* may have less protection against light of around 405 nm, possibly explaining why it is susceptible to HINS-light inactivation.

The inactivation process involved when using blue light to inactivate yeasts and moulds is not as well established as it is in prokaryotic organisms. The proposed

process that occurs has been developed through many PDI studies. Similar to the prokaryotic mechanism, light is absorbed by porphyrin molecules causing excitation and transfer of energy. This causes the production of singlet oxygen ($^1O_2$) and other ROS, which cause damage to the fungal cells (Donnelly *et al.*, 2008). The sites of damage caused by singlet oxygen are typically the plasma membrane and mitochondria of fungi. These are likely to be where endogenous porphyrins reside in fungi as singlet oxygen can only diffuse a short distance as described in Chapter 5, (Bertoloni *et al*., 1987). In addition singlet oxygen has a relatively short life-span (10^{-6} s) and is likely to cause damage close to where it was produced (Dougherty *et al*., 1998; Kalka *et al*., 2000). A study by Böcking *et al*. (2000) demonstrated the effect of singlet oxygen on the plasma membrane of *S. cerevisiae* through PDI. They found that singlet oxygen produced by irradiated toluidine blue O reacted with a compound called ergosterol in the plasma membrane of the yeast. This subsequently caused membrane instability and production of H_2O_2, which itself is damaging to cells, thus causing greater damage to cells. Other studies by Lambrechts *et al*. (2005) on *C. albicans* and Strakhovskaya *et al*. (1999) on *S. cerevisiae* also identify mitochondria and plasma membranes to be the primary targets of damage by singlet oxygen. Donnelly *et al.* (2008) also states that there is no cellular defence in fungi against the effects of singlet oxygen, and enzymes such as superoxide dismutase and catalase designed to inactivate reactive species are inactivated by singlet oxygen. They also conclude that it is unlikely that fungi would develop resistance to the effects of singlet oxygen, as it causes no mutagenic lesions. Although data on the singlet oxygen production/inactivation mechanisms were collected from PDI studies it is highly likely that a similar process occurs in HINS-light inactivation through stimulation of endogenous porphyrins.

There are very few studies on the inactivating effect of blue light on fungi as studies have concentrated more on PDI or PDT effects on fungi. The interest in PDI/PDT inactivation of fungi has increased due to the alarming number of invasive fungal infections in hospitals, particularly amongst cancer patients, AIDS patients and other immunocompromised individuals (Méan *et al*., 2008). Anti-fungal drugs have undesirable side effects, and in addition, many fungi have become resistant to their

effects (Lambrechts *et al*., 2005). Novel anti-fungal treatments that do not produce resistant phenotypes are currently being sought (Donnelly *et al*., 2008).

Fraiken *et al*. (1996) investigated the use of visible light (400-600 nm) for the inactivation of three strains of *S. cerevisiae* (wild type and two DNA repair-deficient mutants) and *Candida guillermondii*, via a "porphyrin-type compound" that was bound to the plasma membranes of the yeasts. Upon exposure to the visible light, produced by a 1000 W mercury high-pressure lamp, reductions in cell growth of 93-94% (dose = 80 kJ cm^{-2}) and around 95% (dose= 500 kJ cm^{-2}) were achieved in *C. guillermondii* and *S. cerevisiae* (wild type) respectively (6-Log_{10} CFU ml^{-1} starting population). This is a considerably higher dose than that required in this study to inactivate *S. cerevisiae* and *C. albicans*, as 576 J cm^{-2} was the maximum dose required for inactivation of a 5-Log_{10} CFU ml^{-1} population when using Source B. They also noted that inactivation was greatly reduced when yeasts were exposed to visible light under anoxic conditions, which corresponds to the findings of Feuerstein *et al*. (2005) that concluded bacteria exposed to visible light under anaerobic conditions were not as readily inactivated as in normal environmental conditions. Maclean *et al*. (2008b) observed that bacterial reduction upon exposure to visible light was significantly increased in oxygen-enhanced conditions, suggesting that inactivation is highly oxygen-dependent. A study by Strakhovskaya *et al*. (1999) used a chelator called 2, 2'-dipyridyl that increases cellular content of protoporphyrin IX (PPIX) and its zinc complex in order to cause inactivation of *S. cerevisiae* cells upon exposure to visible light. They used a very similar set up to Fraiken *et al*. (1996) with a 1000 W high-pressure mercury lamp emitting light of 400-600nm. They exposed a 6-Log_{10} CFU ml^{-1} population of *S. cerevisiae* to light in the presence of 2, 2'-dipyridyl and also exposed cells to light without 2, 2'-dipyridyl. They found that approximately 95% reductions in yeast cell growth were obtained using ~7 J cm^{-2} and ~30 J cm^{-2} in the presence and absence of chelator, respectively. The dose required for inactivation of *S. cerevisiae* is greatly reduced in the Strakhovskaya *et al.* (1999) study compared to both this study and that of Fraiken *et al.* (1996). It is not clear why this is the case as both Fraiken *et al.* (2006) and Strakhovskaya *et al.* (1999) used the same apparatus. Another study by Oriel and Nitzan (2009)

investigated the use of δ-aminolevulinic acid (ALA), an initial metabolite required in the production of porphyrin molecules, as an enhancer for the light-induced endogenous porphyrin inactivation of *C. albicans*. *Candida albicans* was exposed to visible light of 407-420 nm (with total UV cut-off) using a metal halide lamp at an intensity of 20 mW cm^{-2}. They achieved a 1.5-log_{10} CFU ml^{-1} reduction in yeast numbers using a dose of 36 J cm^{-2} in the presence of 100 mg ml^{-1} ALA. They also performed a dark and light control in the absence of ALA and found no effect of exposure to light of 407-420 nm on *C. albicans*. This may be due to the low dosage applied, as this study and others have shown that greater light doses are required for inactivation of yeasts.

There have been few PDI studies performed on moulds such as *A. niger* and as such very little data exists on the topic. This is because PDI and PDT studies are primarily interested in the use of light for treatment of infections/wounds. Aspergilli have been known to cause opportunistic infections typically in the lungs, but the inaccessibility of this infection site may have limited the research on the photodynamic inactivation of aspergilli. This study, however, concentrates on the use of 405-nm HINS-light, without the use of exogenous photosensitiser molecules, for microbial inactivation, that could have potential for reduction of potential pathogens such as aspergilli in a variety of decontamination applications.

UV light exposure for the inactivation of fungi has been studied more extensively. This is mainly due to the interest of the food and drinks industry in reducing spoilage caused by fungal species. Anderson *et al*. (2000) studied the effects of PUV light on *Fusarium culmorum* and *A. niger*. They exposed agar plates seeded with fungi to light pulses produced by a Xenon flashlamp with high UV content resulting in around 3 or 4-log_{10} reductions in *A. niger* and *F. culmorum* respectively after 1000 pulses. They noted that the UV absorbing quality of the pigment aspergillin and clumping of spores reduced the effectiveness of PUV light. A study by Begum *et al*. (2009) treated different fungi including *A. niger* in suspensions of Tween 80 (0.03%) using a UV-C lamp emitting light at 254 nm. They found that after an exposure time of 180 seconds, a 2-log_{10} reduction in *A. niger* spores was observed at a dose of 4644

J m^{-2}. They found *A. niger* to be the most resistant to treatment with UV-C light. Takeshita *et al*. (2003) exposed *S. cerevisiae* to pulses of light produced by a quartz 340 nm wide area flashlamp. They achieved an approximate 7-log_{10} reduction after 5 pulses of light (1 pulse= 0.7 J cm^{-2}, total dose 3.5 J cm^{-2}). Some of the drawbacks when using UV light as a decontamination technology have already been noted in Chapter 4, however build up of heat, inactivation tailing, and poor penetrability into pigmented spores are all problematic in its use for the inactivation of fungal species. UV light also has to be used in a contained manner, as it is harmful to human eyes and skin, therefore lending itself to applications where people are not present.

There has been some debate in the literature as to why fungi are in general more difficult to inactivate using light decontamination technologies. This study has shown that the yeasts required similar inactivating doses to the Gram negative bacteria and that conidia of *A. niger* required similar light doses to endospore-forming *Bacillus* species. As discussed previously, many fungi are protected by carotenoid and melanin-like pigments-such as aspergillin-that absorb damaging wavelengths of light. Fungi are also protected by thick cell walls containing components such as chitin, mannan, glucan and various lipids (Donnelly *et al*., 2008). In addition to the thick outer wall, fungi have a plasma membrane separated from the cell wall by a periplasmic space. This obviously reduces the diffusion of photodynamic sensitising agents into cells, therefore limiting singlet oxygen cell damage. HINS-light has the advantage over PDI in that singlet oxygen is produced from porphyrins within the cell causing damage without having to diffuse across the cell wall and plasma membrane.

Many other studies have suggested the size of fungal cell itself and the number of cell "targets" limits the inactivating ability of singlet oxygen, as yeast cells such as *Candida* species can be between 25 and 50 times larger than bacterial cells. In addition, Zeina *et al.* (2002) discussed that, in general, fungal cells have a larger number of target sites and organelles that have to be damaged before the cell becomes completely inactivated in comparison to prokaryotic cells in a process they have termed "Target Theory".

The finding that 405-nm light causes inactivation of *S. cerevisiae*, *C. albicans* and *A. niger* is very significant however, the question arises as to whether other eukaryotic cells such as mammalian cells are also negatively affected. Although not investigated in this study Smith (2009) used 405-nm light on 3T3 cells in co-culture with *Staphylococcus epidermidis*. Smith (2009) found that although some mammalian cells were adversely affected by 405-nm light, cell viability was regained 1-2 days post-treatment. This is attributed to the extensive number of antioxidants present in mammalian cells such as superoxide dismutase, catalase, vitamins C and E and glutathione peroxidises, which neutralise ROS formed in cells (Boonstra and Post, 2004). In addition, cells also possess enzymes to repair any damage caused by ROS such as phospholipase A_2 (Boonstra and Post, 2004; Smith, 2009). Lewis *et al*., (2004) and Wataha *et al*., (2004) found that cells could be adversely affected by blue light as exposure increases the levels of ROS in keratinocytes and fibroblasts respectively. Many of these *in vitro* studies expose cells in cell culture medium, which has been shown to become toxic upon exposure to specific wavelengths of light, particularly blue light, therefore some of the toxic effect of blue light on cells may be attributable to the accumulation of toxic products in cell culture media alone. However, these are *in vitro* studies, and if blue light was used *in vivo* the results may be markedly different. Therefore further studies are required in order to demonstrate the likelihood of permanent damage to mammalian cells.

Although many PDI studies have been shown to significantly reduce fungal cell numbers, HINS-light does not require the addition of any photosensitising dyes and porphyrin-inducing chemicals, which have also been shown to have effects on mammalian cells when used *in vivo* (O'Riordan *et al*., 2005). HINS-light has been found to be capable of inactivating *S. cerevisiae, C. albicans* and *A. niger* and is as effective as many UV-light treatments which are hazardous to human health, offering greater operational advantages. Further development of the HINS-light technology could potentially lead to the development of systems for surface, air or equipment sterilisation.

Chapter 8

THE EFFECT OF HINS-LIGHT ON BACTERIA COMMONLY FOUND WITHIN THE HOSPITAL ENVIRONMENT

8.0 GENERAL

This chapter examines the germicidal effect of 405-nm HINS-light exposure on common hospital-associated bacteria when treated in liquid suspensions. This chapter also investigates the HINS-light inactivation of a number of MRSA isolates, which were collected from the hospital environment. These clinical isolates were collected as part of a concurrently run clinical trial set up to evaluate the effectiveness of a HINS-light Environmental Decontamination System (HINS-Light EDS) for reduction of environmental staphylococcal contamination.

8.1 HINS-LIGHT INACTIVATION OF COMMON HOSPITAL-ASSOCIATED BACTERIAL PATHOGENS

8.1.1 Background of Causative Microorganisms

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), a serious and potentially life-threatening disease (CDC, 2010), which commonly manifests disease in the lungs, but can also affect other vital organs including the brain. Tuberculosis patients often present with a cough (which may contain blood and sputum) that allows the microorganism to spread from person to person via airborne droplet nuclei (CDC, 2010). Some people infected with TB show no disease symptoms and are non-contagious, this is known as latent infection (CDC, 2010). However, if the individual becomes in any way immunocompromised the infection disease symptoms may manifest themselves. With the increase in immunocompromised and AIDS patients it comes as no surprise that TB infection is on the increase. Every year 2 million people worldwide die from TB infection, and a third of the world's population are infected with TB (CDC, 2010).

Since *M. tuberculosis* is a containment category 3 pathogen no studies could be conducted on this organism within the ROLEST facility as this is only categorised as a containment 2 laboratory. Instead, *Mycobacterium terrae* was used as a surrogate test organism as this would provide relevant information on the efficacy of HINS-light against a known *Mycobacterium* organism. All *Mycobacterium* species share common characteristics in that they are aerobic and generally classified as acid-fast Gram-positive bacteria due to their lack of an outer cell membrane. They possess a characteristic cell wall, thicker than in many other bacteria, which is hydrophobic, waxy, and rich in mycolic acids/mycolates. It is considered that the *Mycobacterium* cell wall makes a substantial contribution to the hardiness of this genus (Jarlier and Nikaido, 1994; Alerwick *et al.*, 2007).

Acinetobacter baumannii is known primarily for its role in healthcare infections. *Acinetobacter baumannii* is an opportunistic Gram negative pathogen that causes infections through wounds and in-dwelling devices, and can lead to serious disease such as pneumonia (Towner, 2009). It is generally spread from person to person but may also be transmitted via the environment and contact surfaces (Towner, 2009). Like MRSA it has become resistant to a wide spectrum of antibiotics and it is becoming increasingly difficult to effectively treat infections (Towner, 2009).

MRSA infections are prevalent in most healthcare settings and present a particular challenge in terms of treatment as they are often part of the individual's natural flora and environmental strains may be resistant to multiple antibiotics as discussed in Chapter 2.

There has been extensive media interest in the cleanliness of healthcare environments and the increasing rates of hospital-acquired infections (HAI). As such, many good hospital practices have been augmented, however it is necessary to produce new, efficient decontamination methods and technologies that can be used in conjunction with existing methods to significantly reduce environmental pathogenic bacteria in the healthcare setting. This section investigates the bactericidal effect of 405-nm HINS-light on these three medically significant pathogens.

8.1.2 Preparation of Bacteria and HINS-light Treatment Method

8.1.2.1 Bacterial Preparation

The bacteria used in the high-intensity 405-nm light exposure experiments were:

- MRSA 16a (a clinical isolate from Glasgow Royal Infirmary (GRI))
- *Mycobacterium terrae* LMG 10394 *
- *Acinetobacter baumannii* NCTC 12156
- Multi-drug resistant (MDR) *A. baumannii* (clinical isolate from GRI).

* As discussed previously, *M. terrae* was used as a surrogate microorganism for *M. tuberculosis* due to *M. tuberculosis* being a Hazard Group 3 microorganism not permitted for use within Category 2 Microbiology laboratories.

Bacteria were cultured according to their growth requirements; MRSA 16a and *A. baumannii* strains were cultured in Nutrient Broth at 37°C for 24 hours, and *M. terrae* was cultured in 7H9 broth containing ADC enrichment media at 37°C for 14 days (Chapter 3, Section 3.1). After incubation, broths were centrifuged at 3939 × g for 10 minutes in order to produce a bacterial pellet. The pellet was re-suspended in 100 ml PBS and serially diluted to a 5-Log_{10} CFU ml^{-1} starting population for experimental use (see Chapter 3, Section 3.2).

8.1.2.2 Ziehl-Neelsen Staining

Mycobacteria spp have a cell envelope that is markedly different to that of normal bacteria. It is composed primarily of peptidoglycan, arabinoglycan and mycolic acid, which contribute to the low permeability of molecules across the cell envelope, impeding the influence of antibiotics and other drugs (Alderwick *et al*., 2007). This envelope also makes *Mycobacteria* acid-fast, as their cell envelope is capable of withstanding the damaging effects of acid. This is a unique feature as few bacteria are acid-fast, thus providing an ideal diagnostic tool for the detection of *Mycobacteria*.

A staining technique called the Ziehl-Neelsen stain was introduced that preferentially stains acid-fast bacteria. A suspected bacterial smear is stained using carbol fuchsin then decoloursised with a solution of hydrochloric acid in ethanol; any acid-fast bacteria will remain red from carbol fuchsin as they are not affected by the acid. A counter stain is used to stain any decolourised non acid-fast bacteria in the smear.

Mycobacterium terrae was checked for purity prior to its use in experiments using the modified Ziehl-Neelsen stain. The modified Ziehl-Neelsen is a cold stain using the kinyoun method, in which heat does not have to be applied when staining. Traditional Ziehl-Neelsen staining involves heating of the staining reagents causing release of toxic phenol vapours.

A fixed film slide of *M. terrae* was prepared using the method in Chapter 3, Section 3.6.1 "*Preparation of Smear for Staining*". The Tb-colour staining kit (Merck, Germany) was used to stain the prepared slides. The kit contains:

- Tb-color carbol fuchsin solution
- Tb- color hydrochloric acid in ethanol
- Tb-color malachite green solution

- The prepared slide was flooded with solution Tb-color carbol fuchsin solution and left to stand for 5 minutes
- The stain was drained and the slide was washed with tap water until no more colour was given off from the slide
- The slide was then flooded with solution Tb-color hydrochloric acid in ethanol and left to stand for 15-30 seconds
- The stain was drained and the slide was again washed thoroughly with tap water
- Solution Tb-color malachite green solution was then applied to the slide as a counter stain for 1 minute
- Finally the stain was washed from the slide and carefully dried on blotting paper
- Slide was viewed under the oil immersion (× 100) lens.

8.1.2.3 Treatment Method

Bacterial suspensions of 3-ml volume were exposed to increasing doses of high-intensity 405-nm light at an irradiance of 40 mW cm^{-2}, using Source B as described in Chapter 7, Section 7.2.2.

Post-treatment, test and control samples of MRSA 16a, *A. baumannii* and MDR *A. baumannii* were plated onto Nutrient Agar (NA) and incubated at 37ºC for 24 hours (Chapter 3, Section 3.3.2). *Mycobacterium terrae* samples were plated onto 7H10 agar containing OADC enrichment media and incubated at 37ºC for 7 days (Chapter 3, Section 3.3.2). After the appropriate incubation period, the plates were enumerated and results reported as CFU ml^{-1} (Chapter 3, Section 3.3.2).

8.1.3 Results

8.1.3.1 Methicillin-resistant *Staphylococcus aureus*

Figure 8.1 demonstrates that when exposed to high-intensity 405-nm light, MRSA in liquid suspension was inactivated by 5-$\log_{10}$ CFU ml^{-1} at a dose of 108 J cm^{-2}. Asterisks highlight a statistically significant difference between light-exposed samples and control samples was achieved from a dose of 72 J cm^{-2} onwards.

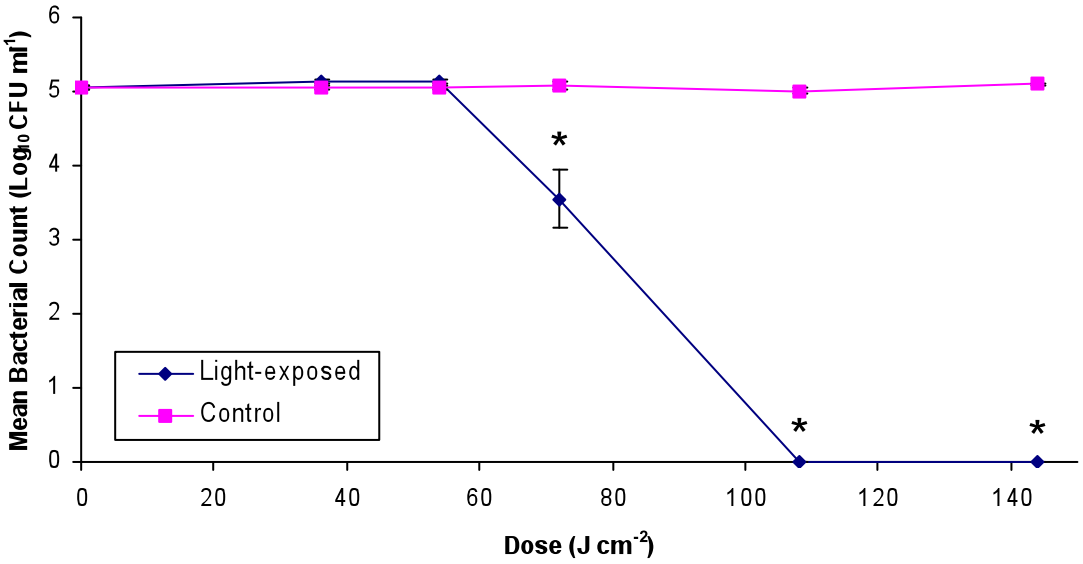

Figure 8.1 *Inactivation of MRSA, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 40 mW cm^{-2} using Source B.*

8.1.3.2 *Mycobacterium terrae*

As shown in Figure 8.2, *M. terrae* was exposed to high-intensity 405-nm light at an irradiance of 40 mW cm^{-2} and around a 5-log$_{10}$ CFU ml^{-1} reduction was achieved after exposure to a total dose of 288 J cm^{-2}. Statistically significant difference between light-exposed samples and control samples was achieved from a dose of 108 J cm^{-2} onwards.

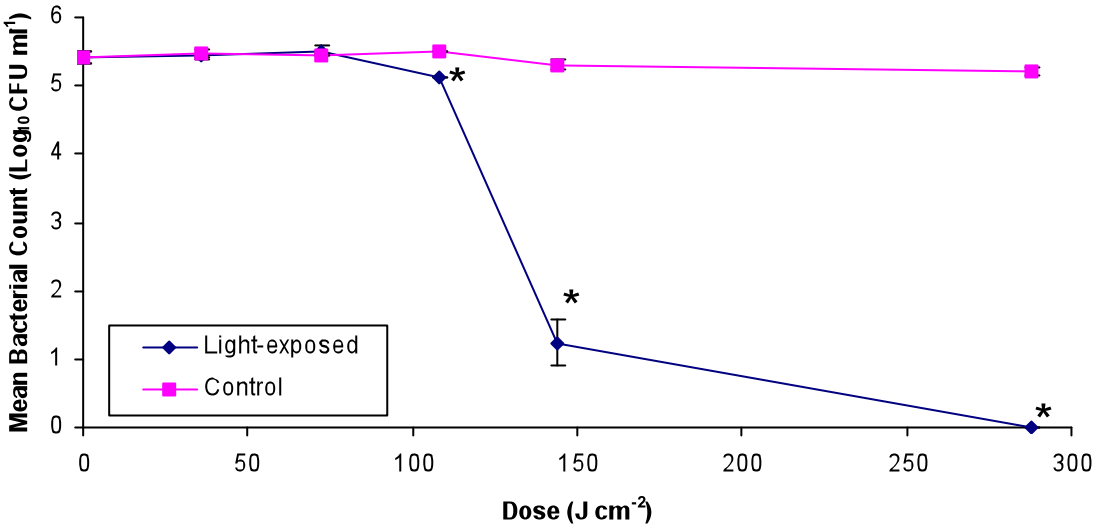

Figure 8.2 *Inactivation of* ***Mycobacterium terrae****, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 40 mW cm^{-2} using Source B.*

8.1.3.3 *Acinetobacter baumannii*

Acinetobacter baumannii was inactivated by around 3 to 3.5-log$_{10}$ CFU ml^{-1} at a dose of 144 J cm^{-2} (Figure 8.3), whereas the MDR isolate of *A. baumannii* was inactivated by 5-log$_{10}$ CFU ml^{-1} at a dose of 144 J cm^{-2} (Figure 8.4). Statistically significant difference between the light-exposed and control samples were observed from 36 J cm^{-2} and 72 J cm^{-2} in *A. baumannii* laboratory strain and the MDR *A. baumannii* strain, respectively.

The results show that 405-nm light is capable of inactivating hospital-associated pathogens including laboratory strains and MDR clinical isolates. Demonstrating for the first time that *Mycobacterium* spp can be inactivated using 405-nm light. Experimental results highlight the variation between bacterial species, as the MDR strain of *A. baumannii* was more readily inactivated than the laboratory *A. baumannii* strain.

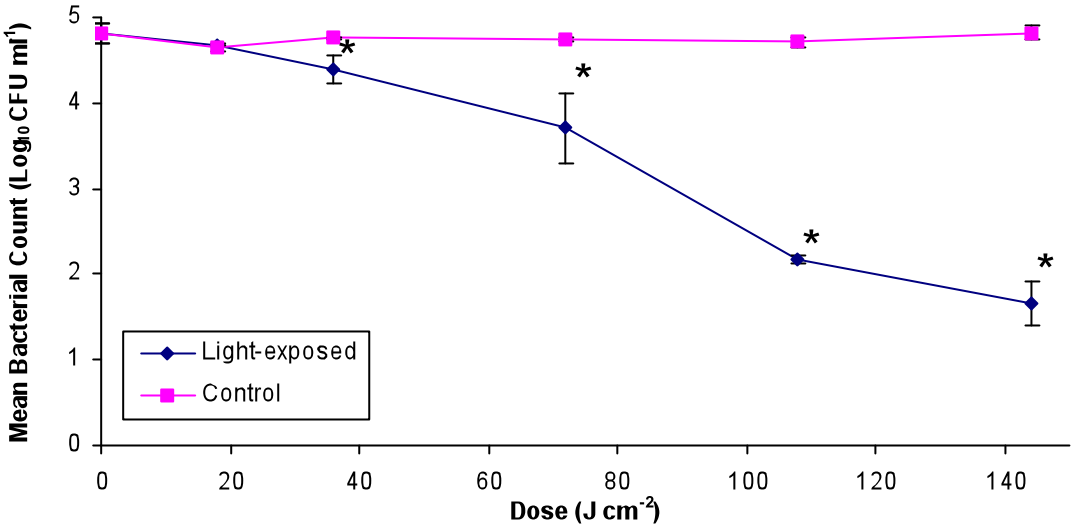

Figure 8.3 *Inactivation of **Acinetobacter baumannii**, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 40 mW cm^{-2} using Source B.*

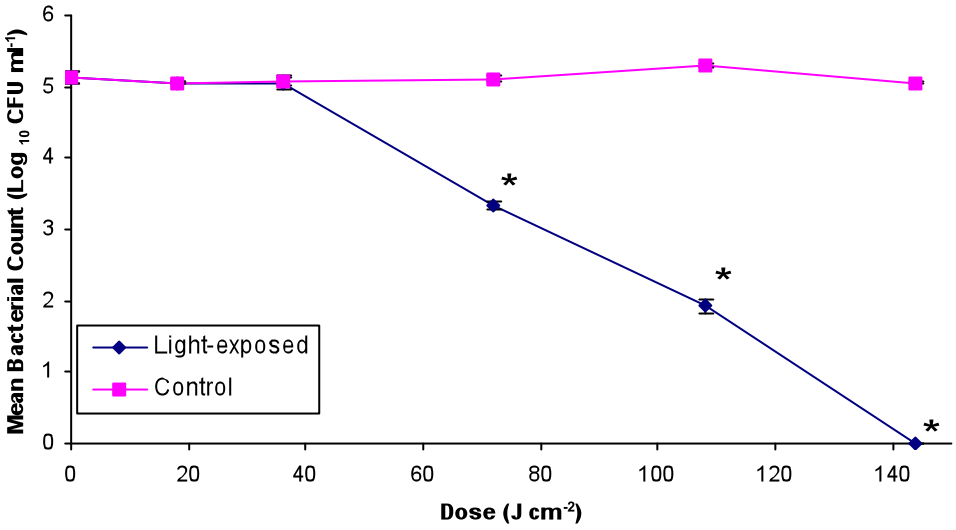

Figure 8.4 *Inactivation of MDR **Acinetobacter baumannii**, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 40 mW cm^{-2} using Source B.*

8.2 HINS-LIGHT INACTIVATION OF MRSA ISOLATED FROM THE CLINICAL ENVIRONMENT

The work in this part of the chapter looked at the effect of 405-nm HINS-light on MRSA isolates collected from environmental surfaces within a hospital isolation room.

8.2.1 Background

Work within ROLEST has lead to the development of a HINS-light Environmental Decontamination System (HINS-light EDS). These ceiling-mounted light sources provide continuous decontamination of air and contact surfaces in exposed areas.

A concurrently run study was carried out to clinically-evaluate the performance of this HINS-light EDS in a single-bed isolation room within the Burns Unit at Glasgow Royal Infirmary (GRI) (Maclean *et al*., 2010). During this clinical evaluation two HINS-light EDS sources, containing a matrix of 405-nm LEDs mixed with white LEDs (to give a predominantly white emission), were mounted in the ceiling of the isolation room, illuminating a total surface area of 10 m^2. The two HINS-light EDS sources were switched on during normal daylight hours, and were controlled using automatic timers (Maclean *et al*., 2010). Normal hospital room decontamination procedures were used by hospital cleaning staff during the studies in order to evaluate the HINS-light EDS as an additional decontamination measure to existing practices (Maclean *et al*., 2010).

The clinical evaluation assessed the decontamination ability of the HINS-light EDS on staphylococcal species including MRSA. The efficiency of the HINS-light EDS was evaluated using Baird Parker Agar (BPA) contact plating to enumerate staphylococcal bioburden on numerous frequently hand-touched sites in the isolation room before, during and after the use of the HINS-light EDS. In addition, Maclean *et al.* (2010) used culture and latex agglutination tests (Section 8.2.2.4 and 8.2.2.5) to confirm the presence of *S. aureus* and MRSA at key sites. The levels of staphylococci and MRSA strains were assessed before, during and after operation of

the HINS-light EDS and a marked reduction in bacterial levels was found to occur during its use (Maclean *et al*., 2010).

The following work was a small-scale laboratory study instigated to determine the HINS-light susceptibilities of selected clinical environmental MRSA isolates collected as part of the aforementioned clinical evaluation. This study identified MRSA isolates collected from key contact surfaces before, during and after HINS-light EDS exposure in the isolation room, and subsequently established their inactivation kinetics when exposed to HINS-light in the laboratory.

8.2.2 Isolation and Identification of Environmental MRSA Isolates for HINS-light Treatment

8.2.2.1 Sample Collection Method

Contact plates containing Baird Parker Agar (BPA) with egg yolk tellurite were used by Maclean *et al*. (2010) for environmental sampling of the key surfaces within the hospital room. BPA is selective for staphylococcal bacteria, and was chosen as it, allows presumptive identification of *S. aureus* colonies. BPA contains lithium, glycine, egg yolk and tellurite, making it selective for *S. aureus*, whilst repressing the growth of many other microorganisms. *Staphylococcus aureus* reduces tellurite in the BPA to produce grey-black, shiny colonies, ~3 mm diameter, with an opaque halo surrounding the colony (2-5 mm) after incubation at 37°C for 48 hours.

After incubation and enumeration (as per the clinical evaluation protocol), a selection of presumptive *S. aureus* colonies were subcultured onto Tryptone Soya Agar (TSA), TSA containing 5% defibrillated horse blood and NA plates/slopes and incubated at 37°C for 18-24 hours for storage and use in further testing.

8.2.2.2 Gram Staining and Microscopic Examination

After incubation for 18-24h on Nutrient Agar plates, colony morphologies were noted. Gram stains were then performed, as detailed in Chapter 3 Section 3.6.1, to determine both the Gram stain reaction and the cellular morphologies of each isolate.

8.2.2.3 Catalase and Oxidase Testing

As described in Chapter 3, Sections 3.6.2 and 3.6.3, catalase and oxidase enzymes can be used to presumptively aid identification of bacteria. *Staphylococcus aureus* is known to be catalase positive and oxidase negative, therefore isolates had to be both catalase positive and oxidase negative to undergo further identification testing. The methodology for performing the catalase and oxidase tests are outlined in Chapter 3, Section 3.6.2 and 3.6.3. For the oxidase test *Pseudomonas aeruginosa* NCTC 9009 was used as a positive control.

8.2.2.4 Staphaurex Plus™ Testing

The Remel Staphaurex Plus™ test (Remel, UK) is an agglutination test used to identify *S. aureus*. The test uses suspensions of yellow latex beads coated in fibrinogen and rabbit Immunoglobulin G (IgG), which are specific for *S. aureus* including MRSA and methicillin-susceptible *S. aureus* (MSSA) strains. When these coated beads interact with *S. aureus* rapid agglutination occurs through:

- Fibrinogen and clumping factors interacting.
- IgG binding Protein A through its Fc region.
- IgG binding surface antigens associated with *S. aureus*.

Methodology

The test kit contained:

- Test latex + Antibody
- Control latex
- Disposable reaction cards
- Disposable mixing sticks

Before testing, presumptive *S. aureus* isolates were cultured on TSA at 37°C for 24 hours. For each isolate to be tested, one drop of test latex was placed in one circle of the reaction card and one drop of the control latex was placed in another circle of the reaction card. A mixing stick was used to pick up some of the bacterial isolate from the TSA plate (around 6 colonies) and the bacterial growth was emulsified into the test latex suspension. This was repeated with a different mixing stick and fresh growth from the TSA plate for the control latex. The reaction card was then rocked gently from side to side for 30 seconds at normal reading distance. Agglutination of the test latex and lack of agglutination in the control latex indicated a positive identification of *S. aureus* (Figure 8.5). Most results are instantaneous, therefore it is vital to read the card within the 30 seconds as false positives can occur after this time period.

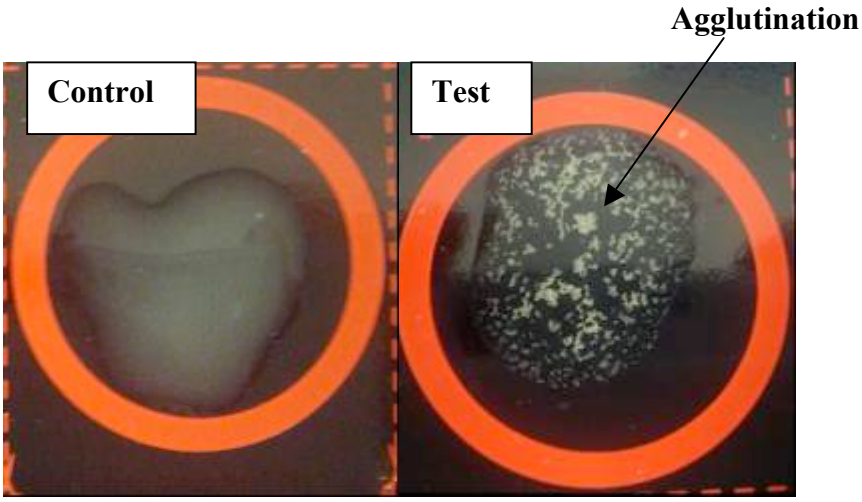

Figure 8.5 *Staphaurex Plus™ latex agglutination test showing the control and test latex results indicative of a positive* ***S. aureus*** *identification.*

8.2.2.5 Penicillin Binding Protein 2' Testing

The Penicillin Binding Protein 2' (PBP 2') test (Oxoid, UK) is used to identify methicillin-resistant strains of *S. aureus*. Like the Staphaurex Plus™ test, it uses a rapid agglutination test method. Latex beads are coated with a monoclonal antibody that is specific to the PBP 2' protein, which is encoded by the *mecA* (methicillin-resistance) gene. When the latex antibody-coated beads come into contact with MRSA agglutination occurs, which is readily visible with the naked eye.

Methodology

The test kit contained:

- Test latex coated + antibody
- Control latex
- Extraction reagents 1 and 2
- Disposable reaction cards
- Disposable mixing sticks

Isolates for testing were grown overnight on Tryptone Soya Agar (TSA) containing 5% defibrillated horse blood at 37°C for 18-24 hours prior to testing. After this time, 4 drops of extraction reagent 1 were added into a microcentrifuge tube, which was inoculated with approximately 6-Log_{10} CFU of isolate cells and vortexed to remove any bacterial aggregates. The tube was then incubated in a water bath 95°C for three minutes. The microcentrifuge tube was removed from the water bath and allowed to cool to room temperature before adding one drop of extraction reagent 2 and centrifuging at 1500 × g for 5 minutes. For each sample to be tested one drop of test latex was placed in one circle of the reaction card and one drop of the control latex was placed in another circle of the reaction card. A 50 µl volume of the supernatant was pipetted in the same circle as the test latex; this was repeated for the control latex. Disposable mixing sticks were used to mix the liquids and the cards were rocked from side to side for up to 3 minutes. Agglutination of the test latex and lack of agglutination in the control latex indicated a positive result (MRSA).

8.2.2.6 API Staph Testing

API staph test kits were used to confirm the identity of presumptive *S. aureus* isolates that had tested positive in the aforementioned tests. The methodology for the API Staph test kit is detailed in Chapter 3, Section 3.6.4.

8.2.2.7 Antibiotic Susceptibility Testing

Confirmed *S. aureus* isolates, which also gave a positive PBP 2' result (i.e. MRSA), were tested for their antibiotic susceptibility. The main antibiotic used to detect MRSA strains is not methicillin: the antibiotic of choice is usually oxacillin, owing to its increased shelf life and its ability to detect hetero-resistant strains (CDC, 2005). Oxacillin is in the same family of antibiotics as methicillin, and bacteria resistant to these antibiotics are inherently resistant to all β-lactam antibiotics (CDC, 2005).

Antibiotic susceptibility of MRSA suspected isolates was evaluated using two different methods: antibiotic disc diffusion and minimum inhibitory concentration (MIC) antibiotic strips. The disc diffusion method analyzes the susceptibility profile of several antibiotics simultaneously, whereas the MIC strip determines the lowest antibiotic concentration-in this case oxacillin-that bacteria are susceptible to. This augments the identification of clinical MRSA isolates.

Methodology

A 0.5 McFarland standard suspension was produced by inoculating a few bacterial colonies of the MRSA isolate into 9 ml PBS. A sterile cotton swab was soaked in the bacterial suspension, and the excess liquid removed by pressing the swab against the sides of the glass universal. Muller Hinton Agar plates of 4 mm depth (±0.5 mm) were then inoculated with the swab in at least three different directions to ensure full coverage of the agar plate. The plate was allowed to dry before adding either a Mastring-S™ (Mast, UK) diffusion ring of 8 antibiotic discs or M.I.C.Evaluator™ oxacillin strip (Oxoid) to the plate with sterile tweezers. An M.I.C.Evaluator™ and Mastring-S™ disc diffusion susceptibility test was carried out for each MRSA isolate, and also on the control organism *S. aureus* NCTC 4135 for reference. Plates

were then incubated at 37°C for 24 hours. After incubation, plates were interpreted as described in the following sections.

Interpretation

Disc Diffusion

Discs impregnated with a specific concentration of antibiotics, in this case a ring of antibiotics effective against many Gram positive microorganisms, was used to identify the antibiotic resistance profiles of the suspected MRSA isolates (Figure 8.6). Antibiotic impregnated discs were placed onto a lawn of bacteria and incubated at 37°C overnight. If the microorganism was susceptible to the antibiotic, a zone of inhibition was produced around the disc where bacteria were unable to grow. The diameter of this zone of inhibition is used to identify whether bacteria are resistant or susceptible to the effects of the antibiotic in each disc. Breakpoints exist in the literature that can be used to identify antibiotic susceptibility profiles (Table 8.1).

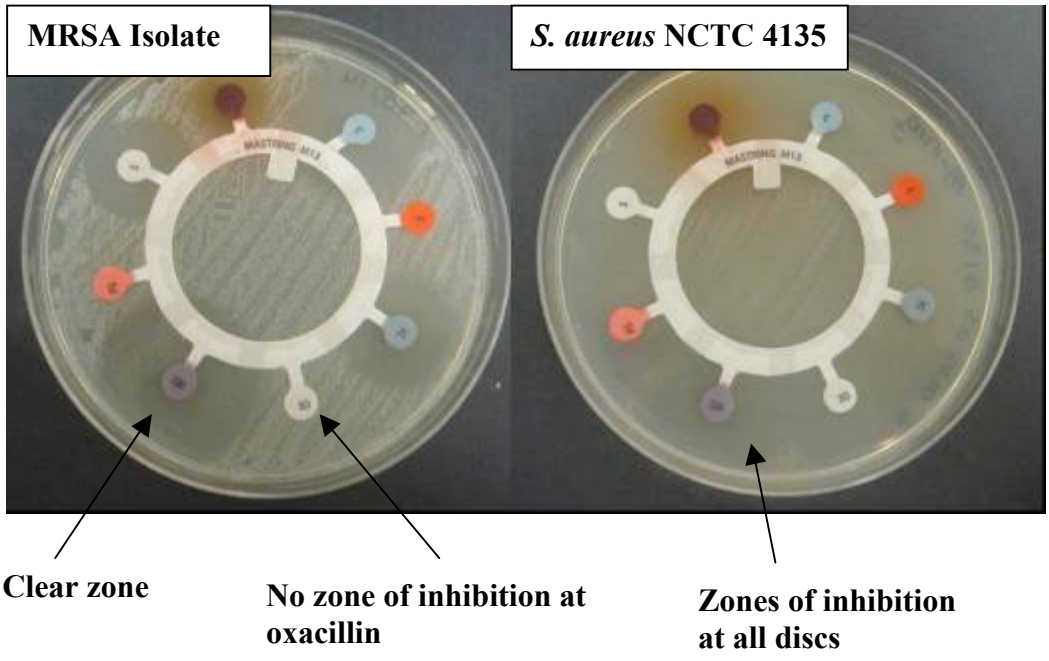

Figure 8.6 *Use of Mastring Multidisc Antibiotic susceptibility rings on bacterial lawns showing resistance patterns of both an MRSA and an MSSA strain.*

Table 8.1 *Antibiotics in the Mastring-S ™, their disc concentrations, and clearing breakpoint references for staphylococci.*

Antibiotic	Disc Concentration	Breakpoints (mm)	Reference
Chloramphenicol	25 μg	0-10 R (30 μg)[a]	Kloos *et al*., 1998
Erythromycin	5 μg	≤ 19 R ≥ 20 S	BSAC, 2010
Fusidic Acid	10 μg	≤ 29 R ≥ 30 S	BSAC, 2010
Oxacillin	5 μg	≤ 27 R, 27-34 S	SFM, 2010
Novobiocin	5 μg	10-15 R	Kloos *et al*., 1998
Penicillin G	1 Unit	≤ 24 R ≥ 25 S	BSAC, 2010
Streptomycin	10 μg	0-10 R	Kloos *et al*., 1998
Tetracycline	25 μg	0-10 R (30 μg)[a]	Kloos *et al*., 1998

[a] No reference was available for the 25 μg disc concentration of Chloramphenicol and Tetracycline used in the Mastring-S™ therefore the closest reference breakpoint was used.

MIC Strips

The minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic that inhibits the growth of a bacterium. Impregnated plastic strips containing a gradient of the antibiotic Oxacillin were placed onto a lawn of bacteria and incubated overnight at the optimum growth temperature of the microorganism, in this case 37°C for 18-24 hours (Figure 8.7). As the strip has a gradient of antibiotic, it is possible to determine the MIC for inhibition of the microorganism from the zone of inhibition surrounding the strip. Wherever this zone of inhibition stops indicates the lowest concentration (shown on the scale of the strip) capable of inhibiting the growth of the microorganism. In this case the strip was impregnated with Oxacillin with concentrations ranging from 0.015-256 μg/ml. The normal range for susceptibility in staphylococci is 0.12-0.5 μg/ml therefore any MIC achieved that is greater than 0.5 μg/ml highlights presence of Oxacillin resistance in the microorganism.

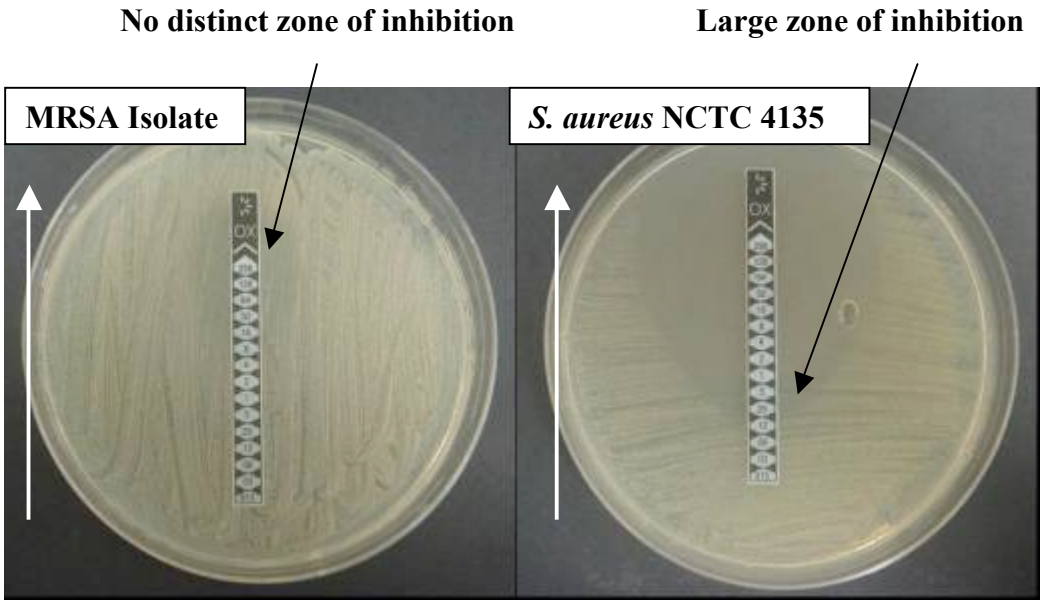

Figure 8.7 *Use of Oxoid oxacillin minimum inhibitory concentration strips on bacterial lawns showing resistance patterns of both an MRSA and an MSSA strain. The white arrow indicates the increasing antibiotic concentration of the MIC strip.*

8.3 HINS-light Exposure of Confirmed MRSA Isolated from the Hospital Isolation Room

As mentioned, the environmental MRSA isolates to be exposed to HINS-light treatment in this study were obtained from contact plate samples taken from a hospital isolation room during the clinical evaluation of a HINS-light Environmental Decontamination System (HINS-light EDS). During the clinical evaluation, contact plate samples were collected from a variety of frequently-touched surfaces (Figure 8.8) during three phases; (i) before the use of HINS-light EDS, (ii) during the use of HINS-light EDS, and (iii) after the HINS-light EDS was switched off.

The aim of this part of the study was to investigate the HINS-light susceptibility of MRSA isolates collected from the same sampling sites before, during and after the use of the HINS-light EDS. Results from the identification tests allowed the

selection of sampling sites that had detected confirmed MRSA isolates before, during and after HINS-light EDS treatment.

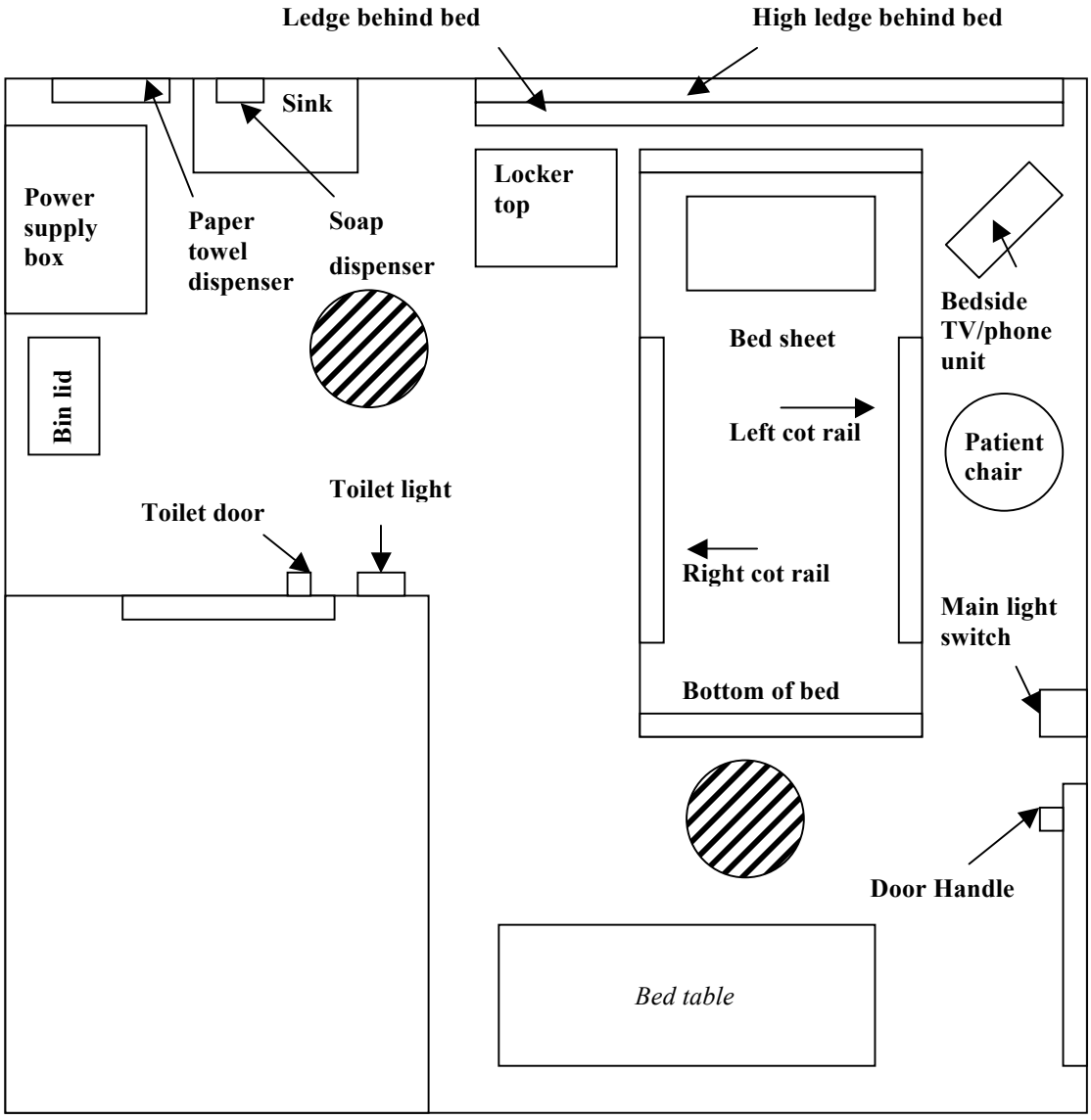

Figure 8.8 *Layout of the hospital isolation room from which the contact plate samples were collected, with labels depicting the frequently touched sampling sites within the room. Striated circles highlight the positioning of the two HINS-light EDS.*

After complete identification testing, selected isolates positively identified as MRSA were exposed to high-intensity 405-nm light, to assess the inactivation susceptibilities of different clinical MRSA strains isolated from the isolation room before during and after HINS-light EDS use.

MRSA Isolate Preparation

Confirmed MRSA isolates selected for exposure to 405-nm HINS-light were cultured in 100 ml TSB and incubated at 37°C for 24 hours. After incubation broths were centrifuged at 3939 × g for 10 minutes in order to produce a bacterial pellet. The pellet was re-suspended into 100 ml PBS and serially diluted to a 5-Log_{10} CFU ml^{-1} starting population for experimental use (see Chapter 3, Section 3.2).

HINS-light Treatment of MRSA Isolates

MRSA isolate suspensions of 3-ml volume were exposed to increasing doses of high-intensity 405-nm light at an irradiance of 40 mW cm^{-2} using Source B as described in Chapter 7, Section 7.2.2.

Test and control samples of the MRSA isolates were plated onto TSA and incubated at 37ºC for 24 hours (Chapter 3, Section 3.3.2). After incubation, the test and control plates were enumerated and results were reported as CFU ml^{-1} (Chapter 3, Section 3.3.2).

8.4 RESULTS

8.4.1 *Staphylococcus aureus* Identification

Table 8.2 shows results of the identification tests carried out on the selected presumptive *S. aureus* environmental isolates. Isolates collected from nine contact surfaces before, during and after HINS-light EDS treatment were identified through colony morphology, Gram staining, catalase and oxidase tests, Staphaurex Plus™ and PBP 2' tests. Only isolates that tested positive with Staphaurex Plus™ and PBP 2' tests were further tested using the API Staph kit. The API Staph identification percentage indicates the likelihood of the isolate tested being *S. aureus*.

From Table 8.2 it can be seen that, of the isolates tested, only two of the nine contact surfaces had confirmed MRSA strains before, during and after the use of the HINS-light EDS: these were (i) the sink and taps and (ii) the bed sheet. These six confirmed MRSA strains were retained for further testing for antibiotic susceptibility, and also for susceptibility to 405-nm HINS-light.

Table 8.2 *Isolate observations of colony morphology, cell morphology, and the results of the catalase, oxidase, Staphaurex Plus ™, PBP 2' and API Staph tests. Isolates highlighted in blue were selected for HINS-light inactivation testing.*

Site	Colony Morphology	Gram Stain	Oxidase /Catalase	Staphaurex Plus*	PBP' 2*	API Staph
Door Handle						
Before HINS	White, 1.5 mm diameter	Gram + cocci	-/+	++	++	**98.5% *S. aureus***
During HINS	White, 2 mm diameter	Gram + cocci	-/+	+	++	80.6% *S. hominus*
After HINS	White, 1 mm diameter	Gram + cocci	-/+	++	++	**91% *S. aureus***
Patient Chair						
Before HINS	White, 1 mm diameter,	Gram + cocci	-/+	++	++	95.5% *S. epidermidis*
During HINS	White, 1 mm diameter	Gram + cocci	-/+	++	++	**98.5% *S. aureus***
After HINS	White, 1 mm diameter.	Gram + cocci	-/+	++	++	**98.6% *S. aureus***
TV/ Phone						
Before HINS	White, 1.5 mm diameter	Gram + cocci	-/+	+	+	99.3% *S. xylosus*
During HINS	White, 1 mm diameter	Gram + cocci	-/+	+	++	93.5% *S. epidermidis*
After HINS	White, 1 mm diameter	Gram + cocci	-/+	++	++	**85.8% *S. aureus***
Bed Table						
Before HINS	White, 1.5 mm diameter	Gram + cocci	-/+	+	+	80.9% *S. chromogenes*
During HINS	White, 1 mm diameter	Gram + cocci	-/+	++	++	**62.6% *S. aureus***
After HINS	White, 0.5 mm diameter	Gram + cocci	-/+	++	++	**98.5% *S. aureus***
Light Switch						
Before HINS	White, 0.5 mm diameter	Gram + cocci	-/+	+	+	95.3% *S. epidermidis*
During HINS	White, 1 mm diameter	Gram + cocci	-/+	++	++	**91% *S. aureus***
After HINS	White, 1 mm diameter	Gram + cocci,	-/+	++	++	**91% *S.aureus***
Bed Sheet						
Before HINS	White, 1.5 mm diameter	Gram + cocci	-/+	++	++	**98.5% *S. aureus***
During HINS	Yellow, 1 mm diameter	Gram + cocci	-/+	++	++	**98.6% *S. aureus***
After HINS	White, 1.5 mm diameter	Gram + cocci	-/+	++	++	**66% *S. aureus***
Toilet Door Handle						
Before HINS	Suspected *Bacillus*	N/A	N/A	N/A	N/A	N/A
During HINS	White, 1.5 mm diameter	Gram + cocci	-/+	+	+	80.9% *S. chromogenes*
After HINS	White, 1 mm diameter	Gram + cocci	-/+	+	++	98.5% *S. aureus*
Bin At Window						
Before HINS	White, 1.5 mm diameter	Gram + cocci	-/+	++	+	98.4% *S. epidermidis*
During HINS	White, 1 mm diameter	Gram + cocci	-/+	+	+	93.5% *S. epidermidis*
After HINS	White, 1.5 mm diameter	Gram + cocci	-/+	+	++	*Aerococcus viridans*
Sink and Taps						
Before HINS	Yellow, 1.5 mm diameter	Gram + cocci	-/+	++	++	**98.6% *S. aureus***
During HINS	White, 0.5 mm diameter	Gram + cocci	-/+	++	++	**98.6% *S. aureus***
After HINS	Yellow, 1 mm diameter	Gram + cocci	-/+	++	++	**98.6% *S. aureus***

* In Staphaurex Plus and PBP2' tests, + = Weak positive ++ = Strong Positive

API Staph result > 90% indicates strong positive match, < 80% indicates weak match

8.4.2 Antibiotic Susceptibility Identification

The MIC of the isolates was variable; four of the six had MIC values in the range of 24-32 μg/ml, however two isolates showed high resistance to Oxacillin, with no zone of inhibition being produced - indication that a concentration of Oxacillin > than 256 μg/ml was required to inhibit their growth (Table 8.3). All isolates were resistant to Penicillin G at a concentration of 1 unit. Four of the six isolates were resistant to Erythromycin (5 μg), and four isolates were resistant to the effects of Fusidic Acid (10 μg). All six isolates were susceptible to Chloramphenicol (25 μg), Novobiocin (5 μg), Streptomycin (10 μg) and Tetracycline (25 μg). *Staphylococcus aureus* NCTC 4135, used for comparison, was susceptible to all of the antibiotics at these ranges of concentrations, and the MIC of Oxacillin was 0.12 μg/ml, which is also in the susceptible range.

Table 8.3 *Inhibition diameters (mm) and MIC concentrations (μg) of all tested MRSA isolates and* ***S. aureus*** *NCTC 4135 for comparative purposes.*

Origin of MRSA Isolates	MIC Oxacillin (μg)	Chloramphenicol (mm)	Erythromycin (mm)	Fusidic Acid (mm)	Oxacillin (mm)	Novobiocin (mm)	Penicillin G (mm)	Streptomycin (mm)	Tetracycline (mm)
Sink and Taps Before	32 ^{R}	18 ^{S}	17 ^{R}	29 ^{R}	0 ^{R}	24 ^{S}	0 ^{R}	17 ^{S}	21 ^{S}
Sink and Taps During	32 ^{R}	20 ^{S}	22 ^{S}	28 ^{R}	0 ^{R}	28 ^{S}	0 ^{R}	16 ^{S}	24 ^{S}
Sink and Taps After	32 ^{R}	17 ^{S}	18 ^{R}	30 ^{S}	0 ^{R}	23 ^{S}	0 ^{R}	16 ^{S}	21 ^{S}
Bed Sheet Before	>256 ^{R}	30 ^{S}	0 ^{R}	22 ^{R}	0 ^{R}	30 ^{S}	0 ^{R}	17 ^{S}	17 ^{S}
Bed Sheet During	24 ^{R}	24 ^{S}	23 ^{S}	30 ^{S}	0 ^{R}	22 ^{S}	0 ^{R}	20 ^{S}	26 ^{S}
Bed Sheet After	>256 ^{R}	29 ^{S}	0 ^{R}	25 ^{R}	0 ^{R}	27 ^{S}	0 ^{R}	15 ^{S}	13 ^{S}
S. aureus NCTC 4135	0.12 ^{S}	26 ^{S}	27 ^{S}	31 ^{S}	30 ^{S}	27 ^{S}	25 ^{S}	20 ^{S}	30 ^{S}

^{R} Indictes resistant phenotype ^{S} Indicates susceptible phenotype

8.4.3 HINS-light Inactivation of Clinical MRSA Isolates

All MRSA isolates from the bed sheet (Figure 8.9) were inactivated by 5-$\log_{10}$ CFU ml^{-1} at a dose between 108 J cm^{-2} and 144 J cm^{-2}. In Figure 8.10 one MRSA isolate from the sink and taps (After HINS-light EDS was switched off) was only inactivated by around 2-$\log_{10}$ CFU ml^{-1} after a dose of 144 J cm^{-2}, and the other two MRSA isolates were inactivated by around 4-4.5-$\log_{10}$ CFU ml^{-1} at the same dose. Although in general MRSA isolates required similar total doses of 405-nm light for 4-5-$\log_{10}$ CFU ml^{-1} inactivation when using Source B at an irradiance of 40 mW cm^{-2}.

In order to examine the differences in 405-nm light susceptibility of the isolates sampled before use of the HINS-light EDS room exposure and those sampled during and after HINS-light EDS room exposure, isolates were exposed to laboratory-scale 405-nm light using Source B at 40 mW cm^{-2}. Significant differences in inactivation between samples at 144 J cm^{-2} were calculated and compared at the 95% confidence interval using ANOVA (one-way). The difference between inactivation of yellow and white colonies was also examined using the same method. No statistically significant difference was found between previously exposed samples and non-HINS-light EDS exposed samples. In addition no statistically significant difference was found between the 405-nm light inactivation of yellow and white colonies. Therefore suggesting that use of HINS-light EDS does not impinge on future inactivation with 405-nm light exposure. This also implies that pigmentation has no effect on 405-nm light susceptibility in this case. However, it is possible that no statistically significant differences were found due to the low numbers of isolates investigated.

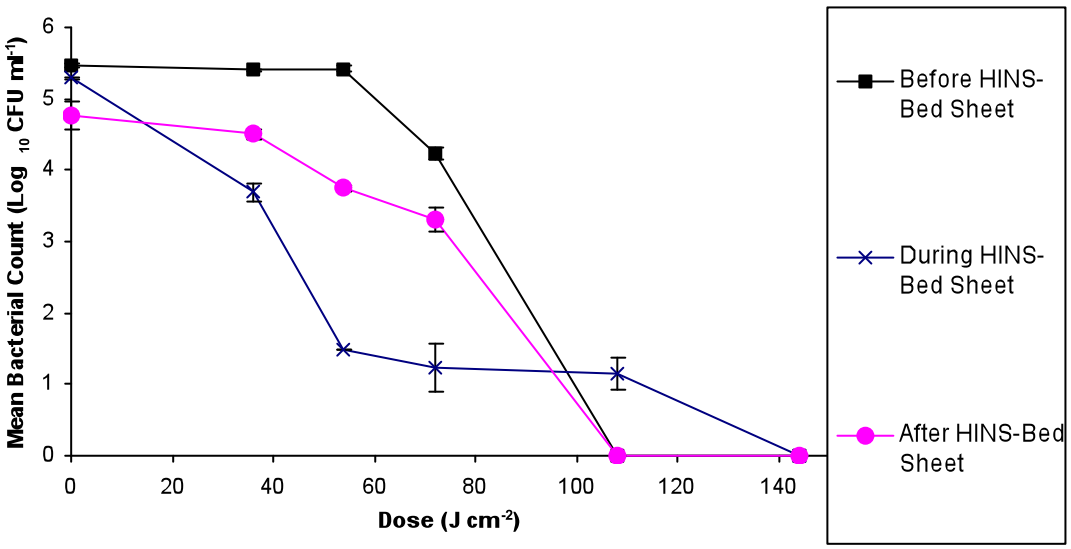

Figure 8.9 *Inactivation of 3 MRSA clinical isolates from the bed sheet, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 40 mW cm^{-2} using Source B.*

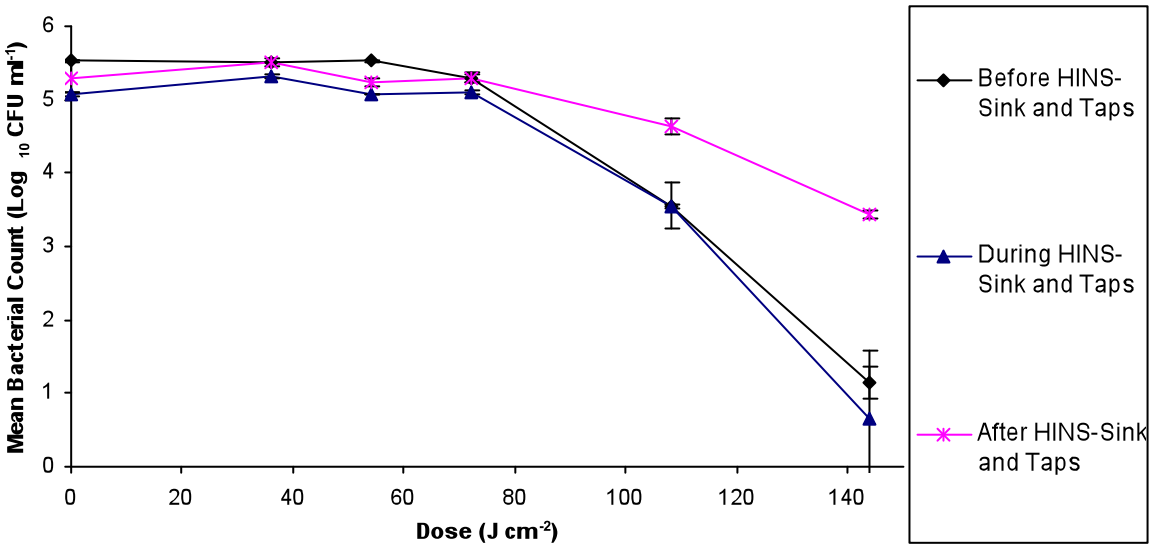

Figure 8.10 *Inactivation of MRSA clinical isolates from the sink and taps, in liquid suspension, upon exposure to 405-nm high-intensity light at an irradiance of approximately 40 mW cm^{-2} using Source B.*

8.5 DISCUSSION AND CONCLUSIONS

The results of this chapter have demonstrated that high-intensity 405-nm light is effective for the inactivation of hospital-associated bacterial pathogens – with tests on both culture collection strains and clinical isolates proving successful.

Significantly, it was found that 405-nm light inactivated *M. terrae,* by 5-$\log_{10}$ CFU ml^{-1} at a total dose of 288 J cm^{-2} as shown in Figure 8.2, meaning that other *Mycobacteria* spp such as *M. tuberculosis* might be similarly be inactivated with 405-nm light as the results of Maclean *et al*. (2009) previously demonstrated similar inactivation rates amongst members of the same genera. There was variation in the inactivation achieved between the two *A. baumannii* strains tested, with the MDR isolate of *A. baumannii* showing more sensitivity to the effects of high-intensity 405-nm light than the laboratory strain.

In this investigation, six environmental MRSA cultures isolated from contact surfaces in a hospital isolation room of the GRI and a clinical isolate from the GRI were exposed to HINS-light at an irradiance of 40 mW cm^{-2}. The majority of the environmental MRSA isolates were inactivated by 4-5 $\log_{10}$ CFU ml^{-1} by a total dose of 108-144 J cm^{-2} (Figure 8.9) and similarly, the clinical MRSA isolate tested was inactivated by around 5-$\log_{10}$ CFU ml^{-1} at a total dose of 108 J cm^{-2} (Figure 8.1). In Chapter 6 (Figure 6.3), a laboratory strain of *S. aureus* was exposed to 40 mW cm^{-2} 405-nm light and was inactivated by around 5-$\log_{10}$ CFU ml^{-1} at a total light dose of 144 J cm^{-2}, which is similar to the results of the 405-nm light-exposed environmental MRSA isolates. Environmental MRSA strains are likely to have been exposed to various "stresses", which may make them more or less resistant to the effects of 405-nm light. A study by Clements and Foster (1999) suggests *S. aureus* exposed to environmental stress, such as starvation stress, become more resistant to subsequent lethal stresses such as acid stress and oxidative stress. These results demonstrate that all *S. aureus* isolates have similar inactivation kinetics, regardless of their origin or antibiotic susceptibility patterns. However, this may not be the case in all bacterial species.

In this preliminary investigation there was no statistically significant difference found between 405-nm light-exposed environmental MRSA cultures isolated from before HINS-light EDS use, during HINS-light EDS use or after the HINS-light EDS system was turned off. Suggesting that isolates previously exposed to HINS-light were no more or less susceptible when exposed for a second time. However, it must be stressed that those MRSAs isolated after HINS-light EDS exposure may not have been present during HINS-light EDS light exposure, therefore further experiments would need to be completed in order to determine fully whether bacterial resistance to HINS-light treatment occurs. HINS-light resistance experiments could easily be completed in a laboratory, where these parameters can be kept constant.

BPA contact plates were used for the isolation of potential staphylococci from the environment. Contact plates were chosen as the preferred sampling method as they allowed simple, direct and selective sampling of key sites in the hospital room. The surface of the agar could be pressed directly onto the sampling surface as the use of BPA selectively isolated staphylococci and minimised the number of other contaminants on the agar, thus aiding counting.

The type of surface and the availability of water and nutrients also affect sampling effectiveness, for example, dry surfaces are thought to stress bacterial cells but the load of the microorganism and presence of nutrients may enhance the viability of bacteria (Scott and Bloomsfield, 1990). Different surface types affect bacterial attachment, for example, stainless steel has a rough texture allowing bacteria to reside in crevices that may be inaccessible when sampled (Kusumaningrum *et al*., 2003)**.**

The many variations in sampling techniques means that the sampling area and the needs of the study have to be thoroughly assessed prior to application. In the case of the concurrently run study by Maclean *et al*. (2010), contact plating was chosen as it has been shown to be a very effective, direct sampling technique when used in the correct circumstances (mostly flat surfaces).

In order to further identify the isolates obtained during sampling a series of tests were carried out on isolates as described in Section 8.5-6. To determine the presence of *S. aureus,* conventional culturing and standard biochemical identification tests were completed. In addition to this, the Staphaurex Plus™ direct latex agglutination test was used, which is a rapid method (incubation 24 hours and test time is around 5-10 minutes). Manufacturers Remel state the sensitivity to be 99.41% on stored MSSA cultures and 99.65% on stored MRSA cultures. However, a proportion of strains of *S. aureus* are not identified using the Staphaurex Plus™ test and other members of the *Staphylococcus* genus may give false or weak positive results.

Similarly when further identifying MRSA strains a latex agglutination test, PBP2' test was completed which utilises a monoclonal antibody capable of detecting the presence of the *mecA* gene. Detection of the *mecA* gene is seen to be the most reliable method of identifying MRSA strains. It is also a rapid test (incubation 24 hours and test time is around 15-20 minutes) with a sensitivity of 98.5% according to manufacturers Oxoid. Again cross-reaction with other staphylococci such as coagulase negative staphylococci may occur, therefore it is important to complete primary identification using biochemical tests prior to completing a PBP2' test (French, 2009).

MRSA chromogenic agars and PCR-based methods are alternative methods for identifying MRSA strains. Both methods have strengths and weaknesses as chromogenic agars will require culturing as in other methods, however the culture medium substrate undergoes a colour change upon growth of MRSA colonies aiding their identification. Although some non-specific growth of microorganisms exhibit similar colour changes, complicating identification. PCR methods can be rapid in identifying MRSA strains, however occasionally PCR amplifies incorrectly due to non-specific amplification. The PCR methods currently available and their antibiotic resistance identification abilities do not offer significant advantages over existing technologies and it is therefore impossible to rationalise the cost of purchasing and running of such PCR machines (French, 2009). In many instances antibiotic susceptibility tests include the disc diffusion or MIC method in addition to

agglutination, culture- or molecular-based testing, however these methods do not detect the presence of the *mecA* gene and may be less reliable if used as the sole MRSA testing method (Velasco *et al*., 2005).

The concurrently run study by Maclean *et al.* (2010) has shown 405-nm light to be capable of reducing the environmental levels of staphylococcal bacteria in the clinical setting in an occupied isolation room by around 60% when used alongside normal room cleaning procedures. The results of this current study has proven the principal that 405-nm light is also capable of inactivating *M. terrae* and *A. baumannii,* therefore HINS-light EDS will similarly be useful in the reduction of environmental *Mycobacteria* and *Acinetobacter* species. This study has shown the germicidal ability of 405-nm light against selected hospital-associated bacteria demonstrating the vast potential applications for the use of 405-nm light as a decontamination technology. *Mycobacterium* spp are spread in airborne droplets and in order to effectively reduce the rates of infection in hospitals and other settings, some studies have investigated the use of air filtration units and UV lamps that continually filter and inactivate microorganisms in the air. Escombe *et al*. (2009) studied the effects of upper room UV lighting and negative air ionization on aerosolized *M. tuberculosis* and the subsequent incidence of TB infection in guinea pigs. Escombe *et al*. (2009) found that UV lights reduced the incidence of TB infections by 70%. Although Escombe *et al*., (2009) state that UV levels were not harmful at lower room levels, UV poses a significant health risk when utilised in this manner due to its effects not only the bacterial DNA but also human DNA. The Escombe *et al.* (2009) study does not discuss the possibility of concomitant photoreactivation of UV-induced DNA damage in bacteria, which may further hinder this decontamination process. Peccia and Hernandez (2001) have shown that photoreactivation readily occurs in *Mycobacterium bovis* and also note that a threshold inactivation dose of UV light must be applied in order to prevent photoreactivation after UV exposure, which may be difficult to ensure in air decontamination.

The environment and surfaces in general provide a constant reservoir for infection, particularly in the hospital environment. Given favourable environmental conditions bacteria can live on surfaces anywhere from hours to years (Kramer *et al*., 2006). In fact *M. tuberculosis* can survive up to four months on dry surfaces and *A. baumannii* and *S. aureus* (including MRSA) can survive even longer, remaining viable for up to 5 months and 7 months on dry surfaces, respectively. Although there are many conflicting reports as to how microorganisms are affected by environmental variables, it is generally thought that low temperature, high humidity, presence of nutrients and inoculum level are important in increasing bacterial survival (Kramer *et al*., 2006). In hospital environments, frequently hand-touched sites such as door handles, taps, computer keyboards and many other sites are the most commonly contaminated areas (Kramer *et al*., 2006; Lu *et al*., 2009). In addition, contaminated hands also present a re-contamination issue, particularly as hand hygiene compliance rates in hospitals is often as low as 50% (Kampf and Kramer, 2004).

Another route of infection with *Mycobacterial* spp and other hospital-associated pathogens is through the use of contaminated medical devices such as the bronchoscope, which has been reported to sporadically cause outbreaks of TB through formation of viable but non-culturable morphologies even after thorough washing (Werner *et al*., 2001; Kramer *et al*., 2006). UV boxes have been produced to decontaminate and prevent re-contamination of such housed medical devices and surgical tools. One drawback of using UV light in this manner is that UV light can degrade many materials, particularly plastics; meaning medical devices may not last as long (Andrady *et al*., 1998). Many of these medical devices are extremely expensive; therefore replacement methods of decontamination are being sought. As shown in Chapter 5, high-intensity 405-nm light can be used to effectively reduce bacterial numbers on surfaces in a short time period when used at increased intensities, and with further investigation, may be of use for the decontamination of medical devices such as bronchoscopes, blood pressure cuffs, stethoscopes and endoscopes in housings similar to that used in UV-device decontamination without the harmful side effects associated with UV wavelength exposure (Schroeder *et al*., 2009).

As previously discussed high intensity 405-nm light offers significant operational advantages over UV-light based environmental decontamination technologies and has been shown to be effective in the inactivation of many hospital-associated pathogenic bacteria including MRSA, *A. baumannii* and *Mycobacteria* amongst others (Maclean *et al*., 2009). The HINS-light EDS has also been shown to significantly reduce the numbers of *S. aureus* in the hospital environment at many frequently touched sites (Maclean *et al.,* 2010). It is highly possible that HINS-light when used in conjunction with other decontamination policies and procedures could significantly reduce the numbers of the bacterial reservoir in the healthcare environment and decrease the incidence of HAI.

Chapter 9

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

9.0 GENERAL

This study investigated the use of high-intensity 405-nm light for the inactivation of a range of microorganisms important in human health and disease. The study also utilised a pulsed ultraviolet (PUV) light system to compare the efficacy of 405-nm light and UV light technologies for bacterial inactivation. The findings of these investigations are concluded in this chapter. Suggestions for future work that could facilitate a greater understanding of the inactivation mechanism associated with 405-nm light treatment of microorganisms as well as the wide range of potential applications of this decontamination process are also considered in this chapter.

9.1 CONCLUSIONS

9.1.1 The Use of Ultraviolet Light as an Inactivation Technology

In this investigation pulsed ultraviolet (PUV) light generated from a Xenon-flashlamp was found to be highly germicidal to *Staphylococcus aureus* in liquid suspensions, although this inactivation was greatly reduced in bacterial suspensions with a population density greater than 7-Log_{10} CFU ml^{-1}. *Staphylococcus aureus* was found to be particularly efficient in photo-repairing UV-damage upon exposure to optimum photoreactivation light wavelengths of around 370 nm. Experiments carried out on *S. aureus* revealed a threshold UV light dose, application of which causes such severe UV-damage that no subsequent photoreactivation can occur. Knowledge of such UV light dose thresholds is required for the effective use of this inactivation technology in decontamination applications, in order to prevent concomitant photoreactivation.

9.1.2 HINS-light Inactivation of Foodborne Bacterial Pathogens

This study set out to examine the effect of high-intensity 405-nm light, emitted from LED arrays, on a range of important bacterial causes of food-related illness in liquid suspensions. This work built upon and significantly expanded the work of previous studies in the field, which have established the bactericidal effect of blue light on medically-important bacteria. All *Campylobacter*, *Salmonella*, *Shigella*, *Escherichia* and *Listeria* bacterial species tested were susceptible to inactivation through 405-nm light exposure. Importantly, this bactericidal effect has also proven to be effective for the inactivation of both vegetative and spore forms of *Bacillus* species. The inactivation achieved with each microorganism was highly variable, but in general, Gram positive bacteria were more readily inactivated than Gram negative bacteria.

The inactivation capability of a higher intensity 405-nm LED light source (~100 mW cm^{-2}) was examined for the rapid inactivation of bacteria seeded onto a range of nutritious and non-nutritious surfaces. Agar surfaces were used to represent surfaces where nutrients were readily available to the microorganism, and PVC and acrylic surfaces were used to represent non-nutritious surfaces, indicative of "real" contamination issues in the food preparatory and processing environments. Bacterial contamination of nutritious surfaces presents a particular challenge in that bacteria have nutrients available for growth and possibly repair. Although inactivation of seeded bacteria on nutritious surfaces required higher overall light-exposures than on seeded inert non-nutritious surfaces, significant bacterial reductions were achieved in both instances.

9.1.3 Investigation of the 405-nm Light Inactivation Mechanism

A series of experiments were developed to further elucidate the 405-nm light inactivation mechanism. Inactivation of *S. aureus* in nutritious liquid broth medium revealed a complex relationship between bacterial inactivation and bacterial growth in the 405-nm light-exposed media suspensions. The study also demonstrated that in general a similar overall dose is required for *S. aureus* inactivation when using both continuous and intermittent 405-nm light exposures, highlighting that inactivation is not necessarily related to how the dosage of light was applied. However it was

during these intermittent experiments that the toxic effect of 405-nm light on Nutrient Broth itself was noticed.

In an attempt to understand the mechanism behind this toxic product formation, experiments were carried out with the aim of determining the cause and/or probable source of inactivation, although no conclusive evidence of the source of toxicity was found. However, many studies into the effects of visible light on cell culture media have shown similar toxic effects, which have been widely attributed to the production of reactive oxygen species (ROS) through the photosensitisation of riboflavin. It was established that ~300-400 nm filtered light from a broadband lamp was capable of inducing Nutrient Broth toxicity, although not as effectively as 405-nm light from an LED array at the same light dosage.

As it was not known whether repair of 405-nm light induced damage was possible, light-induced repair experiments involving exposure of 405-nm sub-lethally damaged *S. aureus* cells to varying wavelengths of light from 370-500 nm were completed. The results of these studies were compared directly to an identical experiment exposing PUV-damaged *S. aureus* cells to 370-500-nm light. Experiments showed some repair occurred in 405-nm light damaged cells at wavelengths around 370 nm, although not to the level achieved in PUV-damaged cells. It is not certain whether repair systems exist in *S. aureus* cells that can be "switched on" upon exposure to 405-nm light, similar to the photoreactivation process. Experiments designed to assess the damage caused to bacterial cells upon exposure to 405-nm light would need to be devised in order to determine whether DNA damage and subsequently any form of DNA repair was occurring in this instance.

These results emphasise the complicated nature of the inactivation mechanism of 405-nm light and the effect of other contributing factors such as the availability of nutrients and light dose delivery.

9.1.4 HINS-light Inactivation of Yeasts and Moulds

Having shown high-intensity 405-nm light to be effective for inactivation of prokaryotic species, the inactivation of eukaryotic species, namely yeasts and moulds, was investigated. The yeasts *Saccharomyces cerevisiae* and *Candida albicans* and the spores (conidia) of the mould *Aspergillus niger* were exposed to 405-nm light in liquid suspension. It was found that *A. niger* spores were particularly resistant to 405-nm light, therefore higher doses of light were applied to achieve similar inactivation levels to those of previous bacterial experimental results. During experimentation it was also noticed that 405-nm light induced some adverse effect on conidia production in *A. niger*, although the mechanism of this effect was not elucidated in this investigation.

9.1.5 HINS-light Inactivation of Hospital-associated Bacterial Pathogens

Bacteria commonly associated with hospital-acquired infections (HAIs) including MRSA, *Acinetobacter* spp and *Mycobacteria* spp were found to be susceptible to inactivation using 405-nm light whilst in liquid suspension.

A ROLEST study, which was run concurrently with this investigation, involved the development and testing of a HINS-light Environmental Decontamination System (HINS-light EDS) in hospital isolation rooms for the reduction of environmental bacteria, particularly staphylococci (Maclean *et al*., 2010). Bacterial levels on frequently hand-touched key sites in isolation rooms were sampled (using contact agar plates) in the presence and absence of HINS-light EDS treatment and the study showed that use of HINS-light EDS successfully reduced levels of environmental bacteria.

Bacterial isolates collected as part of the above-mentioned clinical evaluation were used in this investigation in a small-scale study. The isolates underwent traditional culturing, biochemical and antibiotic susceptibility testing in order to recover positive MRSA isolates. Six MRSA isolates were obtained from two key sampling sites within the isolation room (bed sheet and sink and taps) from before, during and after the use of the HINS-light EDS. These clinical MRSA isolates were exposed to

405-nm light in liquid suspension to determine the relative susceptibilities of those isolates not previously exposed to 405-nm light with previously exposed isolates. There was no significant difference recorded between the susceptibilities of the MRSA isolates. However, in order to state explicitly that no difference exists in previously 405-nm exposed bacteria more isolates would need to be tested.

9.2 FUTURE WORK

9.2.1 Mechanism of HINS-light Inactivation and Possible Toxic Product Formation

It will be useful to identify the source of the bactericidal toxicity found as a result of 405-nm light exposure of Nutrient Broth. Although such medium-induced toxicity has been speculated by some to be caused by riboflavin oxidation of tryptophan and tyrosine and subsequent production of highly ROS, this would need to be proven to also be occurring in Nutrient Broth. Spin trapping studies could be used to detect and identify the presence of any ROS produced upon 405-nm light exposure (Grzelak *et al*., 2001). In addition, scavenger solutions that quench ROS, as described by Feuerstein *et al*. (2005), could be added to 405-nm light-exposed Nutrient Broth to determine if the toxic species could be quenched enough to allow inoculated bacteria to grow. In order to assess the levels of riboflavin and other photo-sensitive molecules, synchronous fluorescence spectroscopy could be used to enable the levels of these molecules to be defined before and after 405-nm light exposure of Nutrient Broth (Garcia *et al*., 2001).

9.2.2 Decontamination and Clinical Uses of HINS-light

As previously discussed, clinical evaluation of a HINS-light EDS system has been successful in reducing staphylococcal bacterial levels in the environment of a hospital isolation room (Maclean *et al*., 2010). It is likely that both air and surface 405-nm light inactivation of bacteria occurs in this instance, highlighting the potential of 405-nm light as an air decontamination technology. Instances in which air quality control is of vital importance such as clean rooms, high-dependency wards and operating theatres use High Efficiency Particulate Absorbing (HEPA) air filters to reduce the microbial load present in the air. Many HEPA filters use UV light to further aid filtration/inactivation of microorganisms, however it is likely that such devices could also be installed with 405-nm LEDs to achieve a bactericidal effect. Although UV light is much more bactericidal than high-intensity 405-nm light exposure the 405-nm light decontamination units would be cheaper to run in the long term due to the longer life-span of LEDs as well as reduced material degradation compared to UV light producing systems.

As *Mycobacterium terrae* has been shown to be successfully inactivated in laboratory studies using high-intensity 405-nm light it would be interesting to determine whether HINS-light environmental decontamination systems (HINS-light EDS) could be trialled in Tuberculosis wards with a view to reduce airborne mycobacterial contamination in a similar manner to that of the Maclean *et al*., (2010) study. As discussed in Chapter 8, UV light decontamination systems, although successful in the reduction of airborne *Mycobacterium* spp, have to be used in a contained manner. HINS-light EDS can be used more ubiquitously than UV light systems, in the presence of both patients and staff, as 405-nm light exposure is not harmful to humans.

Microorganisms often survive in the environment by forming protective biofilms. Biofilms are a protective polysaccharide layer that allows bacteria to adhere to surfaces, particularly in moist/wet areas such as sinks, bathrooms and shower cubicles; areas commonly found in hospital isolation rooms. Formation of protective biofilms could potentially complicate the inactivation of microorganisms in the

environment. Therefore, in order to definitively assess the potential of 405-nm light for use in certain decontamination applications laboratory-based experiments would need to demonstrate whether 405-nm light is capable of penetrating biofilms enough to inactivate the bacteria within them.

The bactericidal effectiveness of 405-nm light could potentially be harnessed in the decontamination of medical devices as described in Chapter 8. Its safety-in-use in terms of human exposure would provide a huge advantage over existing UV technologies designed to reduce bacterial contamination. High-intensity 405-nm light is also less damaging to plastics, rubber compounds and a wide range of other materials, than UV light, which can cause serious degradation of sensitive structural materials thereby reducing the life-span of certain medical instruments.

Owing to existing studies depicting the positive clinical outcomes of blue light reduction of acne lesions it is likely that 405-nm light could have applications in the treatment of other problematic skin conditions as well as with wound infection and healing. With the discovery that 405-nm light is capable of inactivating yeasts and moulds this light technology could possibly be harnessed as an alternative therapy for treatment of opportunistic fungal infections such as Candidiasis, Aspergillosis and Cryptococcosis, which are prevalent in the immunocompromised. The use of light-emitting optical fibres have proven to be effective in treatment of infections at previously light inaccessible sites such as the stomach, therefore this technology could potentially be applied to the delivery of 405-nm light for management of bacterial and fungal infections in areas that are traditionally difficult to treat.

Future investigations will also be required to determine the effect of 405-nm light on viruses. It is not yet known how 405-nm light exposure will affect viruses, however it is likely that, due to the mechanism of inactivation thought to involve the photo-excitation of endogenous porphyrins, viruses may be unaffected. However, if 405-nm light exposure was shown to be capable of inactivating viruses, the technology could be used in a variety of applications such as for the inactivation of viral particles

in a wide variety of clinical and public settings as well as for decontamination of medical products.

9.2.3 Food Safety

Investigations in the area of food safety have shown that 405-nm light is capable of inactivating a range of foodborne pathogenic bacteria in a liquid based test system and on nutritious and non-nutritious surfaces. This fundamental knowledge into the bactericidal efficiency of 405-nm light against significant foodborne pathogens will be crucial for underpinning future work on the development of practical decontamination systems. In order for the research to progress to the development of practical systems, subsequent research in the following areas would be required.

Foods

Further work should involve the seeding of foodstuffs with important foodborne bacterial pathogens (that have already been shown in the current thesis) to be susceptible to the effects of 405-nm light. Although the effects of 405-nm light have been shown, in the present study, on bacteria seeded onto nutritious agar surfaces, exposure of foods presents a particular challenge. It is likely that 405-nm light decontamination of foods would be limited to food surfaces, due to the limited penetration of 405-nm light and stringent testing would need to be completed to determine if significant inactivation could be achieved by food surface exposure. It would also be necessary to test for any potential adverse effects on the food's organoleptic and nutritional qualities. In addition, as 405-nm light was shown to induce the production of a bactericidal product in exposed Nutrient Broth, it would therefore be very important to determine whether this toxic effect would be generated within the exposed foodstuffs, and also what effect (if any) this bactericidal product would have on the food and, importantly, the consumer.

Packaging and Other Surfaces

Decontamination of food packaging using 405-nm light is likely to be more effective than decontamination of foods themselves, as 405-nm light has been shown to be highly efficient for the inactivation of common food-related microorganisms on non-nutritious surfaces such as PVC and acrylic. Reduction of microbial numbers on packaging will reduce cross-contamination of treated and untreated foods, in turn reducing numbers of food-related illnesses.

Food preparation and processing surfaces can provide an important reservoir for bacteria and constitute a source of contamination for foods and packaging. Consequently 405-nm light might have potential applications as a complementary technology to normal decontamination strategies already used in food processing and preparation areas. However, as biofilms can also form on food preparation surfaces it is necessary to verify whether high-intensity 405-nm light is able to penetrate and inactivate bacterial biofilms.

It should be noted that in order to be applicable in each of these instances, the 405-nm light technology would have to be tailored specifically to the application, i.e. for room decontamination larger systems would be required, whereas for equipment decontamination it is likely that smaller, more intense light sources would be suitable.

9.2.4 Water Treatment

As shown in this study, 405-nm light has been successful used for reducing the numbers of a range of bacteria and fungi in liquid PBS suspension. Owing to this, it is likely that 405-nm light could have some potential for water disinfection applications, in the same manner as UV light. However, as 405-nm light is not as bactericidal as UV light it is likely that high-intensity 405-nm light could not be used for inactivation of microorganisms in flowing water systems, but might be useful in stored or stagnant water decontamination applications. In order to develop high-intensity 405-nm light systems for this type of application it would be necessary to

determine if protozoa, cysts and viruses, which have not been studied to date, are susceptible to 405-nm light inactivation.

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api® Staph

IVD

Identification system for staphylococci, micrococci and related genera

SUMMARY AND EXPLANATION

API Staph is a standardized system for the identification of the genera *Staphylococcus*, *Micrococcus* and *Kocuria*, which uses miniaturized biochemical tests and a specially adapted database. The complete list of those bacteria that it is possible to identify with this system can be found in the Identification Table at the end of this package insert.

PRINCIPLE

The API Staph strip consists of 20 microtubes containing dehydrated substrates. These microtubes are inoculated with a bacterial suspension, prepared in API Staph Medium, that reconstitutes the tests. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents.
The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software.

CONTENT OF THE KIT (Kit for 25 tests)

- 25 API Staph strips
- 25 incubation boxes
- 25 ampules of API Staph Medium
- 25 result sheets
- 1 package insert

COMPOSITION

Strip

The composition of the API Staph strip is given in the Reading Table of this package insert.

Medium

API Staph Medium 6 ml	Yeast extract	0.5 g
	Bactopeptone (bovine/porcine origin)	10 g
	NaCl	5 g
	Trace elements	10 ml
	Demineralized water	qsp 1000 ml
	pH : 7.0 - 7.4	

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED

Reagents

- Mineral oil (Ref. 70 100)
- Reagents : VP 1 + VP 2 (Ref. 70 422)
  NIT 1 + NIT 2 (Ref. 70 442)
  ZYM A (Réf. 70 494)
  ZYM B (Réf. 70 493)
- McFarland Standard (Ref. 70 900)
- API Staph Analytical Profile Index (Ref. 20 590) or **api*web*** ™ identification software (Ref. 40 011) (consult bioMérieux)

Material

- Pipettes or PSIpettes
- Ampule rack
- Ampule protector
- General microbiology laboratory equipment

WARNINGS AND PRECAUTIONS

- **For *in vitro* diagnostic use and microbiological control.**
- **For professional use only.**
- This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is therefore recommended that these products be treated as potentially infectious, and handled observing the usual safety precautions (do not ingest or inhale).
- All specimens, microbial cultures and inoculated products should be considered infectious and handled appropriately. Aseptic technique and usual precautions for handling the bacterial group studied should be observed throughout this procedure. Refer to "CLSI/NCCLS M29-A, *Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*; *Approved Guideline* - Current revision". For additional handling precautions, refer to "Biosafety in Microbiological and Biomedical Laboratories - CDC/NIH - Latest edition", or to the regulations currently in use in each country.
- Do not use reagents past the expiry date.
- Before use, check that the packaging and components are intact.
- Do not use strips which have been damaged : cupules deformed, desiccant sachet open, etc.
- Open ampules carefully as follows :
  - Place the ampule in the ampule protector.
  - Hold the protected ampule in one hand in a vertical position (white plastic cap uppermost).
  - Press the cap down as far as possible.

  * *Model 1 :*
    - Cover the flattened part of the cap with the upper part of the thumb.
    - Apply thumb pressure to the base of the flattened part of the cap to snap off the top of the ampule.

  * *Model 2 :*
    - Position the thumb tip on the striated part of the cap and press forward to snap off the top of the ampule.
  - Take the ampule out of the ampule protector and put the protector aside for subsequent use.
  - Carefully remove the cap.
- The performance data presented were obtained using the procedure indicated in this package insert. Any change or modification in the procedure may affect the results.
- Interpretation of the test results should be made taking into consideration the patient history, the source of the specimen, colonial and microscopic morphology of the strain and, if necessary, the results of any other tests performed, particularly the antimicrobial susceptibility patterns.

STORAGE CONDITIONS

The strips and media should be stored at 2-8°C until the expiry date indicated on the packaging.

SPECIMENS (COLLECTION AND PREPARATION)

API Staph is not for use directly with clinical or other specimens.
The microorganisms to be identified must first be isolated on a suitable culture medium according to standard microbiological techniques.

INSTRUCTIONS FOR USE

Preparation of the strip

- Prepare an incubation box (tray and lid) and distribute about 5 ml of distilled water or demineralized water [or any water without additives or chemicals which may release gases (e.g. Cl_2, CO_2, etc.)] into the honeycombed wells of the tray to create a humid atmosphere.
- Record the strain reference on the elongated flap of the tray. (Do not record the reference on the lid as it may be misplaced during the procedure.)
- Remove the strip from its individual packaging.
- Place the strip in the incubation box.

Preparation of the inoculum

- Subculture the organism onto Columbia blood agar (or P agar) 18-24 hrs. at 36°C ± 2°C.
- Check that the strain belongs to the *Micrococcaceae* family (morphology, Gram stain, catalase etc.) and also check that the culture is pure.
- Open an ampule of API Staph Medium as indicated in the paragraph "Warnings and Precautions".
- Prepare a **homogeneous** bacterial suspension with a turbidity equivalent to 0.5 McFarland. It is recommended to use young cultures (18-24 hours old).
  This suspension must be used immediately after preparation.

Inoculation of the strip

- Using a pipette or PSIpette, fill the microtubes with the inoculated API Staph Medium. Only fill the tube portion of the microtubes, not the cupules (slightly underfill the microtubes). To avoid the formation of bubbles at the base of the tubes, tilt the strip slightly forward and place the tip of the pipette or PSIpette against the side of the cupule.
- Ensure anaerobiosis in the ADH and URE tests by filling the cupules with mineral oil to form a convex meniscus.
- Close the incubation box.
- Incubate at 36°C ± 2°C for 18-24 hours.

READING AND INTERPRETATION

Reading the strip

- After the incubation period, develop the reactions by adding 1 drop of each of the following reagents and then read all the reactions by referring to the Reading Table :
  - VP test : VP 1 and VP 2 reagents.
    Wait 10 minutes. A **violet-pink** color indicates a **positive** reaction. A **pale pink** or **light pink** color obtained after 10 minutes should be considered **negative**.
  - NIT test : NIT 1 and NIT 2 reagents.
    Wait 10 minutes. A **red** color indicates a **positive** reaction.
  - PAL test : ZYM A and ZYM B reagents.
    Wait 10 minutes. A **violet** color indicates a **positive** reaction.
- Record the results on the result sheet.

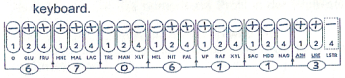
Lysostaphin resistance test

Determine resistance to lysostaphin on P agar, as indicated in the following procedure or according to the manufacturer's recommendations.
To perform the test, inoculate the surface of a P agar plate, by flooding it with a bacterial suspension (approximately 10^7 organisms/ml).
Leave to dry for 10-20 minutes at 36°C ± 2°C.
Place a drop of lysostaphin solution (200 µg/ml) on the surface of the agar.
Incubate for 18-24 hrs. at 35-37°C.
Total or partial lysis of the bacterial culture indicates susceptibility to the enzyme.
This test constitutes the 21st test of the strip. It is considered positive if resistance to lysostaphin is determined.

Interpretation

Identification is obtained with the **numerical profile**.

- Determination of the numerical profile :
  On the result sheet, the tests are separated into groups of 3 and a value of 1, 2 or 4 is indicated for each. By adding together the values corresponding to positive reactions within each group, a 7-digit profile number is obtained.
- Identification :
  This is performed using the database (V 4.1)
  * with the Analytical Profile Index :
    - Look up the numerical profile in the list of profiles.
  * with the **api*web*** ™ identification software :
    - Enter the 7-digit numerical profile manually via the keyboard.



6 706 113 *Staphylococcus epidermidis*

CONTROLE DE QUALITE

Les milieux, galeries et réactifs font l'objet de contrôles de qualité systématiques aux différentes étapes de leur fabrication. Un contrôle bactériologique des tests de la galerie est de plus réalisable par l'utilisateur avec la souche **1. *Staphylococcus xylosus* ATCC® 700404** de préférence ou l'une des souches suivantes :

2. *Staphylococcus lentus* ATCC 700403 3. *Staphylococcus capitis* ATCC 35661

ATCC : American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA.

	0	GLU	FRU	MNE	MAL	LAC	TRE	MAN	XLT	MEL	NIT	PAL	VP	RAF	XYL	SAC	MDG	NAG	ADH	URE
1.	–	+	+	+	+	+	+	+	+	–	+	+	V	–	+	+	–	+	–	+
2.	–	+	+	+	+	+	+	+	V	+	+	V	V	+	+	+	+	+	–	–
3.	–	+	+	+	–	–	–	V	–	–	+	–	+	–	–	–*	–	–	+	–

* Ce résultat peut varier en fonction du milieu de culture utilisé.

Profils obtenus après culture des souches sur gélose au sang de mouton.

Il est de la responsabilité de l'utilisateur de s'assurer que le contrôle de qualité est mis en oeuvre conformément à la législation locale en vigueur.

LIMITES DU TEST

- Le système API Staph est destiné à l'identification des espèces mentionnées dans la base de données (voir Tableau d'Identification en fin de notice), et à elles seules. Il ne peut être utilisé pour identifier d'autres microorganismes ou exclure leur présence.
- Seules des cultures pures contenant un seul type de microorganisme doivent être utilisées.

RESULTATS ATTENDUS

Se référer au Tableau d'Identification en fin de cette notice pour les résultats attendus des différentes réactions biochimiques.

PERFORMANCES

- Staphylocoques
  2104 souches de diverses origines et souches de collection appartenant aux espèces de la base de données ont été testées :
  - 92,49 % des souches ont été correctement identifiées (avec ou sans tests complémentaires).
  - 4,42 % des souches n'ont pas été identifiées.
  - 3,09 % des souches ont été mal identifiées.
- Micrococques/*Kocuria*
  171 souches de diverses origines et souches de collection appartenant aux espèces de la base de données ont été testées :
  - 87,72 % des souches ont été correctement identifiées (avec ou sans tests complémentaires).
  - 7,60 % des souches n'ont pas été identifiées.
  - 4,68 % des souches ont été mal identifiées.

ELIMINATION DES DECHETS

Eliminer les réactifs utilisés ou non utilisés ainsi que les matériels à usage unique contaminés en suivant les procédures relatives aux produits infectieux ou potentiellement infectieux.
Il incombe à chaque laboratoire de gérer les déchets et les effluents qu'il produit selon leur nature et leur dangerosité, et d'en assurer (ou faire assurer) le traitement et l'élimination selon les réglementations applicables.

TABLEAU DE LECTURE

TESTS	COMPOSANTS ACTIFS	QTE (mg/cup.)	REACTIONS / ENZYMES	RESULTAT	
				NEGATIF	POSITIF
0	Aucun		Témoin négatif	rouge	—
GLU	D-glucose	1,56	(Témoin positif) (D-GLUcose)	rouge *	jaune
FRU	D-fructose	1,4	acidification (D-FRUctose)		
MNE	D-mannose	1,4	acidification (D-ManNosE)		
MAL	D-maltose	1,4	acidification (MALtose)		
LAC	D-lactose (origine bovine)	1,4	acidification (LACtose)		
TRE	D-tréhalose	1,32	acidification (D-TREhalose)		
MAN	D-mannitol	1,36	acidification (D-MANnitol)		
XLT	xylitol	1,4	acidification (XyLiTol)		
MEL	D-mélibiose	1,32	acidification (D-MELibiose)		
				NIT 1 + NIT 2 / 10 min	
NIT	nitrate de potassium	0,08	Réduction des NITrates en nitrites	incolore-rose pâle	rouge
				ZYM A + ZYM B / 10 min	
PAL	ß-naphtyl phosphate	0,0244	Phosphatase ALcaline	jaune	violet
				VP 1 + VP 2 / 10 min	
VP	sodium pyruvate	1,904	production d'acétyl méthyl-carbinol (Voges Proskauer)	incolore-rose pâle	violet-rose
RAF	D-raffinose	1,56	acidification (RAFfinose)	rouge	jaune
XYL	D-xylose	1,4	acidification (XYLose)		
SAC	D-saccharose	1,32	acidification (SACcharose)		
MDG	méthyl-αD-glucopyranoside	1,28	acidification (Méthyl-αD-Glucopyranoside)		
NAG	N-acétyl-glucosamine	1,28	acidification (N-Acétyl-Glucosamine)		
ADH	L-arginine	1,904	Arginine DiHydrolase	jaune	orange-rouge
URE	urée	0,76	UREase	jaune	rouge-violet

Les tests d'acidification doivent être lus comparativement aux témoins négatif (0) et positif (GLU).

* Les tests MNE et XLT peuvent être oranges, lorsqu'ils sont entourés ou précédés de tests positifs. On doit alors les considérer comme négatifs.

- Les quantités indiquées peuvent être ajustées en fonction des titres des matières premières.
- Certaines cupules contiennent des composants d'origine animale, notamment des peptones.

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APPENDIX B

FILTER TRANSMISSION SPECTRA

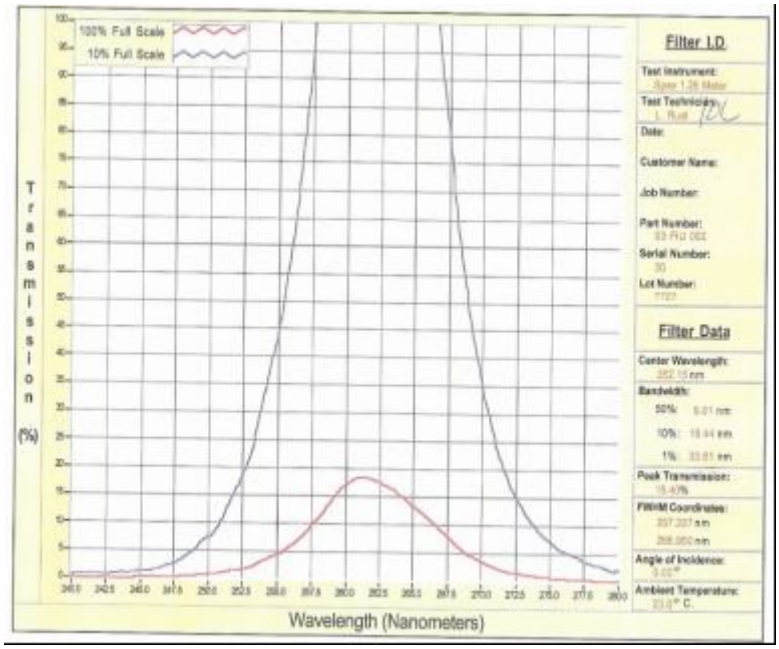

Figure B1 *260-nm bandpass filter.*

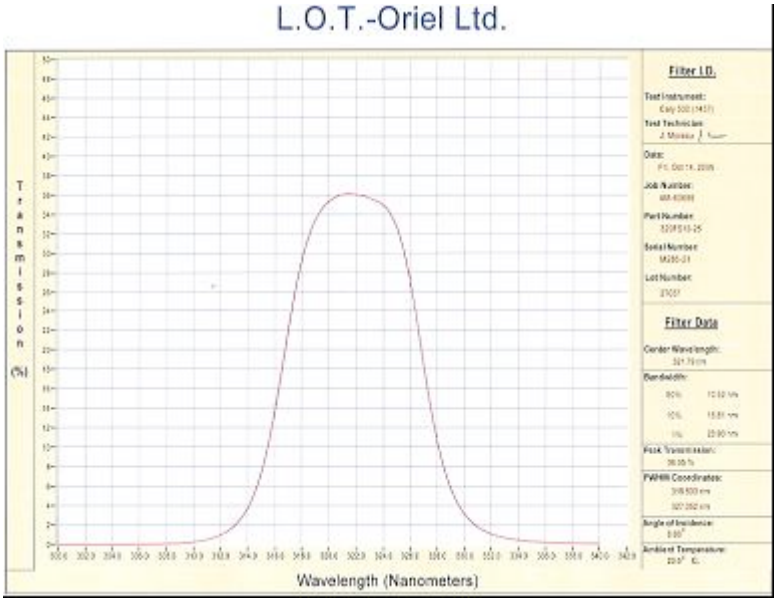

Figure B2 *320-nm bandpass filter.*

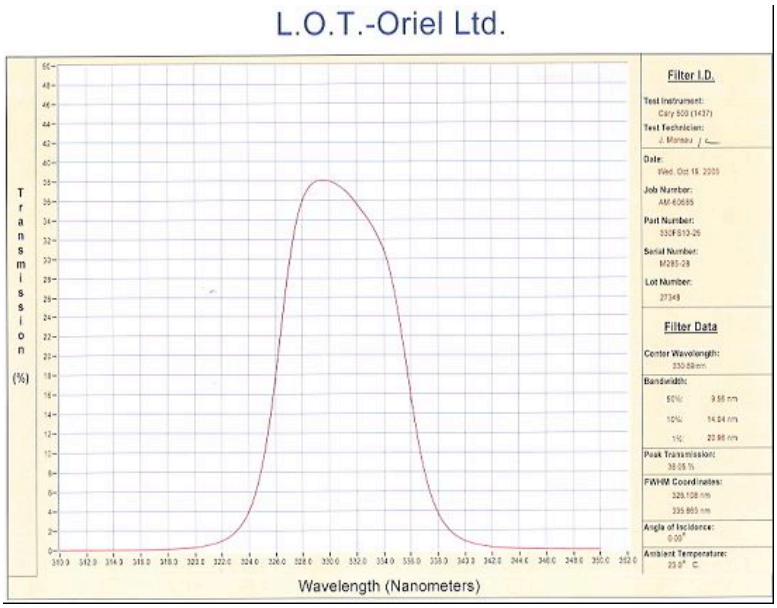

Figure B3 *330-nm bandpass filter.*

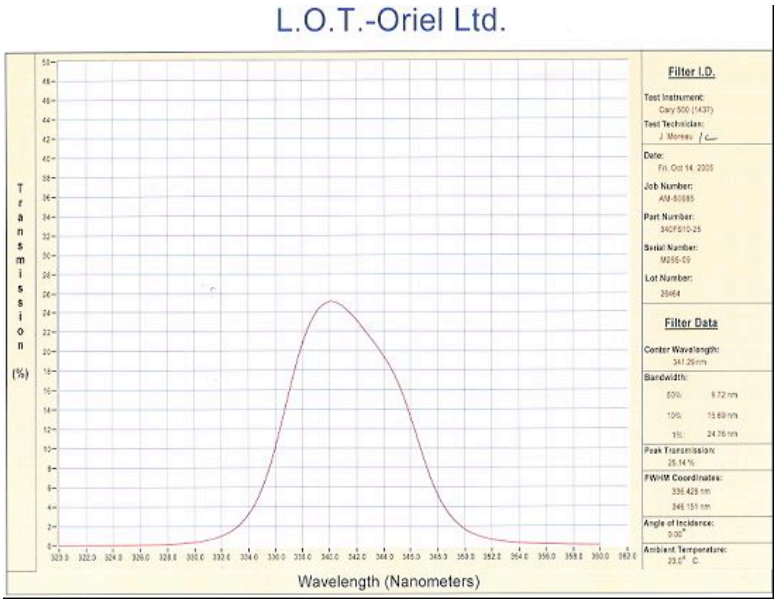

Figure B4 *340-nm bandpass filter.*

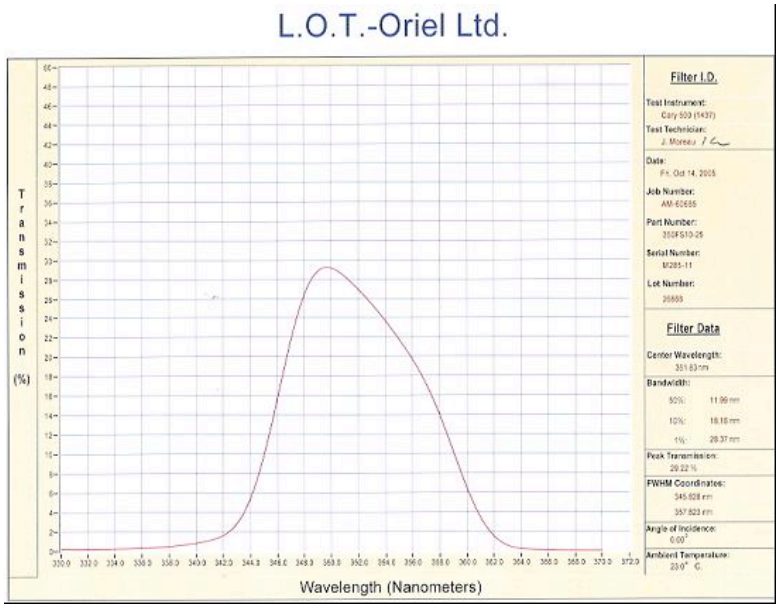

Figure B5 *350-nm bandpass filter.*

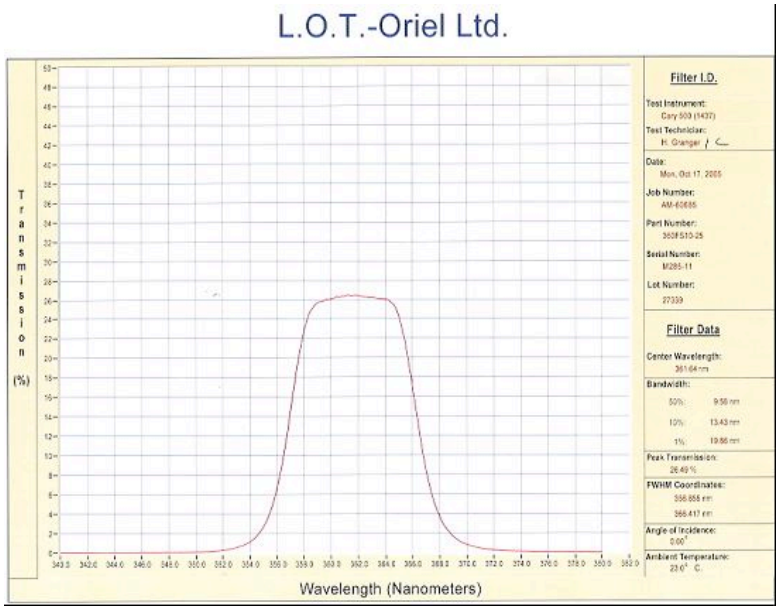

Figure B6 *360-nm bandpass filter*.

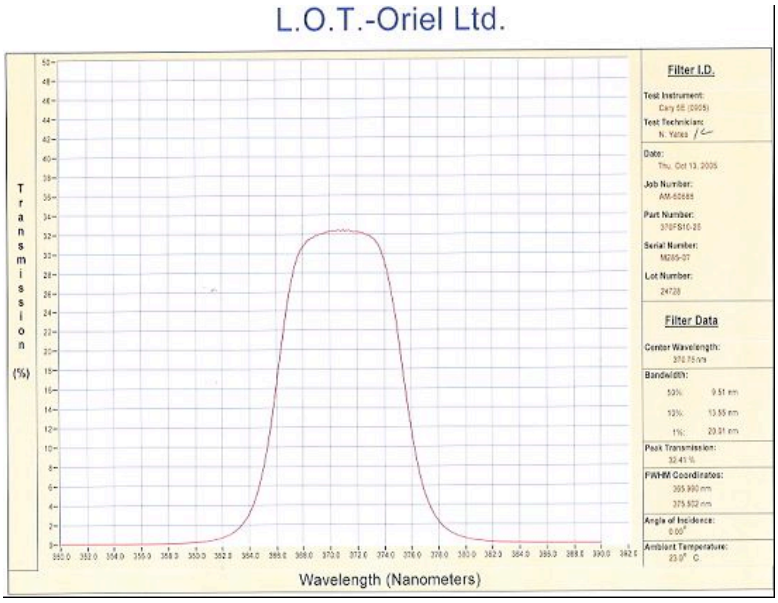

Figure B7 *370-nm bandpass filter.*

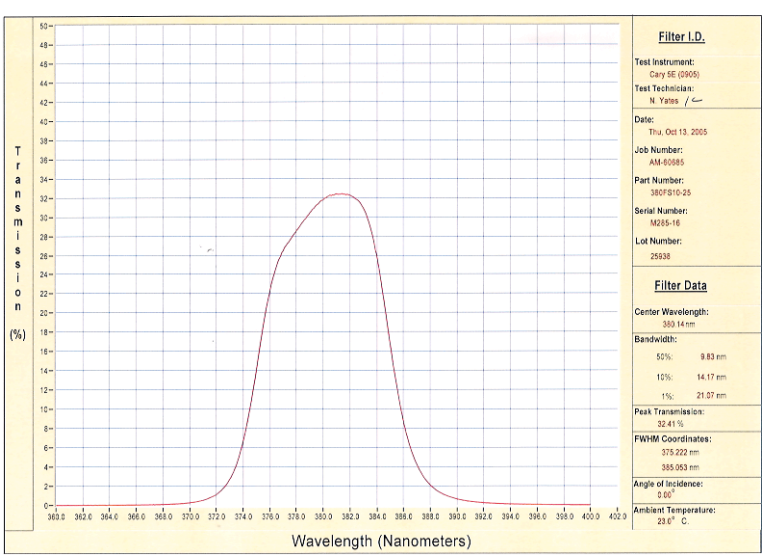

Figure B8 *380-nm bandpass filter.*

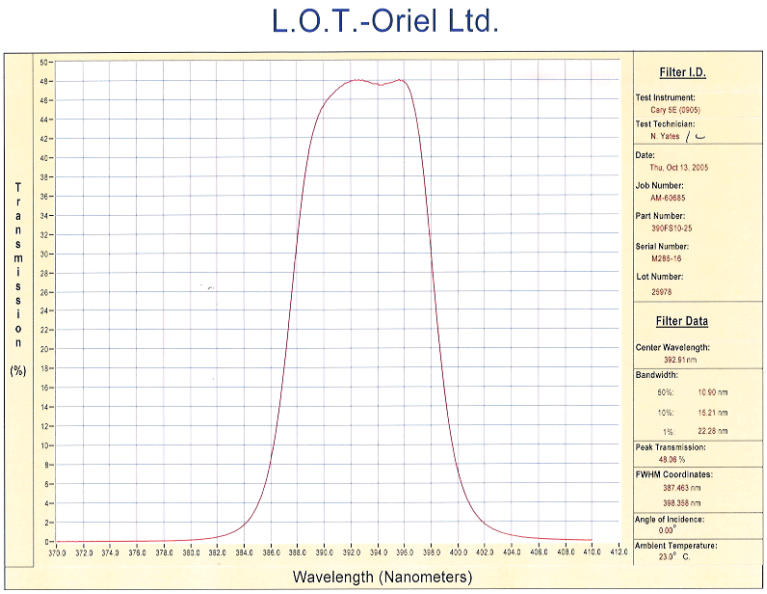

Figure B9 *390-nm bandpass filter.*

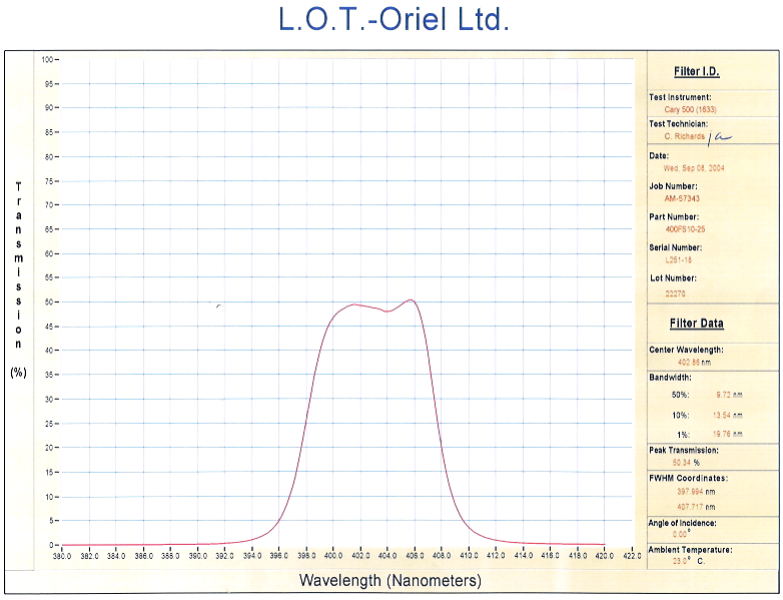

Figure B10 *400-nm bandpass filter.*

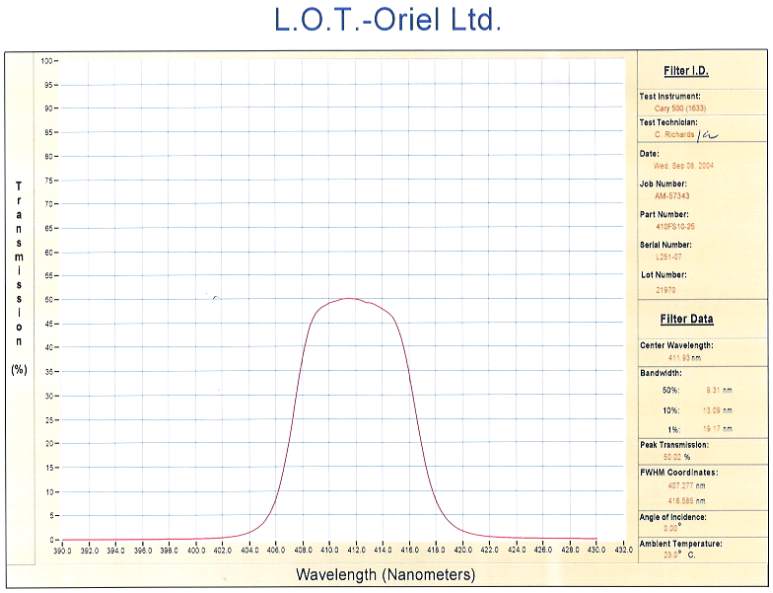

Figure B11 *410-nm bandpass filter.*

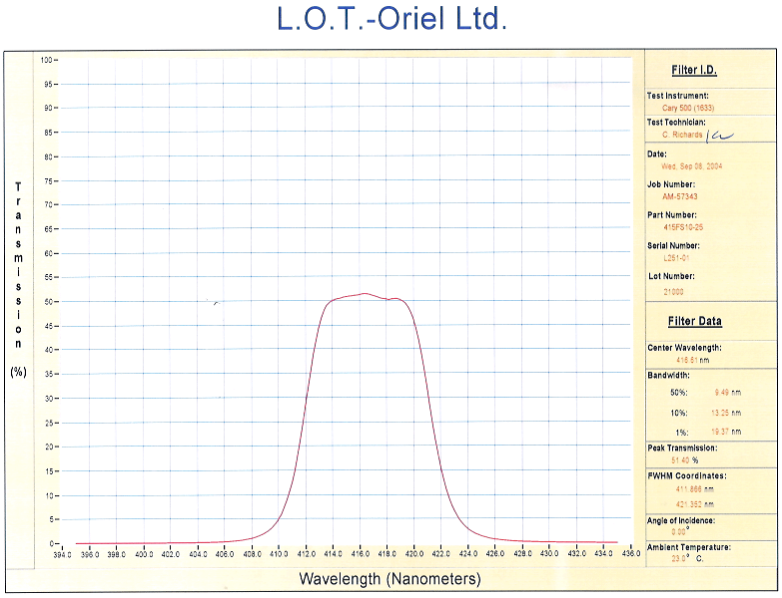

Figure B12 *415-nm bandpass filter.*

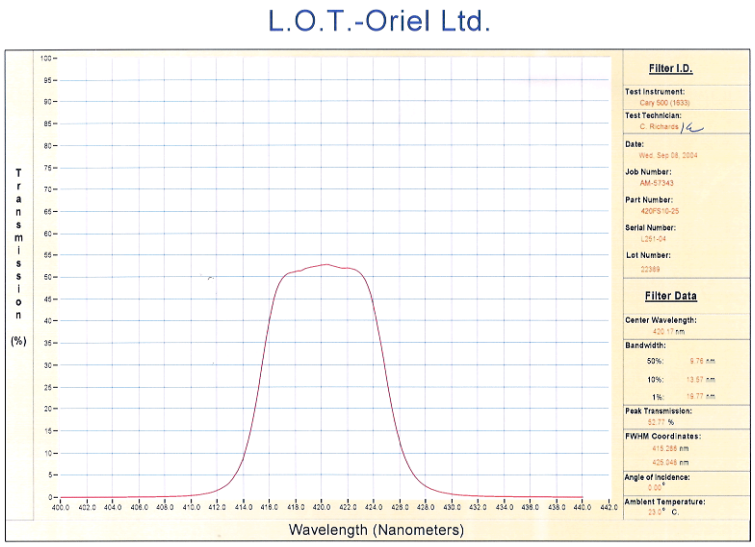

Figure B13 *420-nm bandpass filter.*

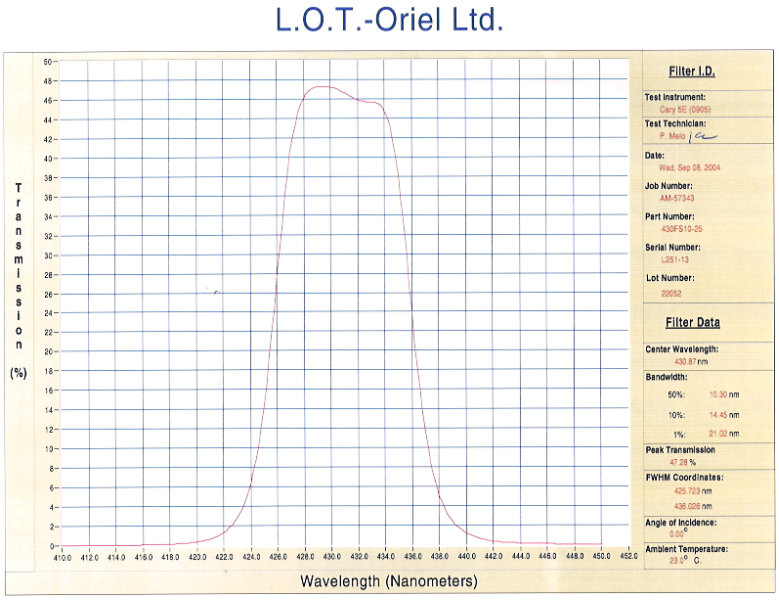

Figure B14 *430-nm bandpass filter.*

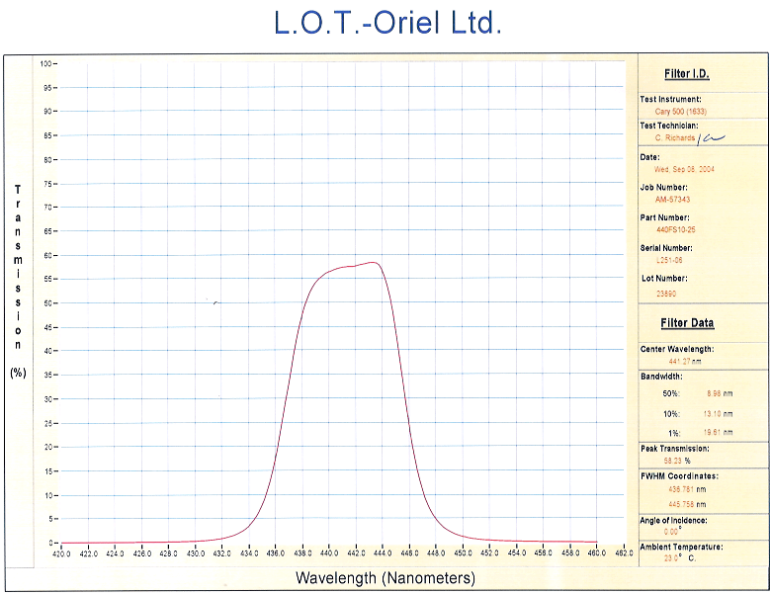

Figure B15 *440-nm bandpass filter.*

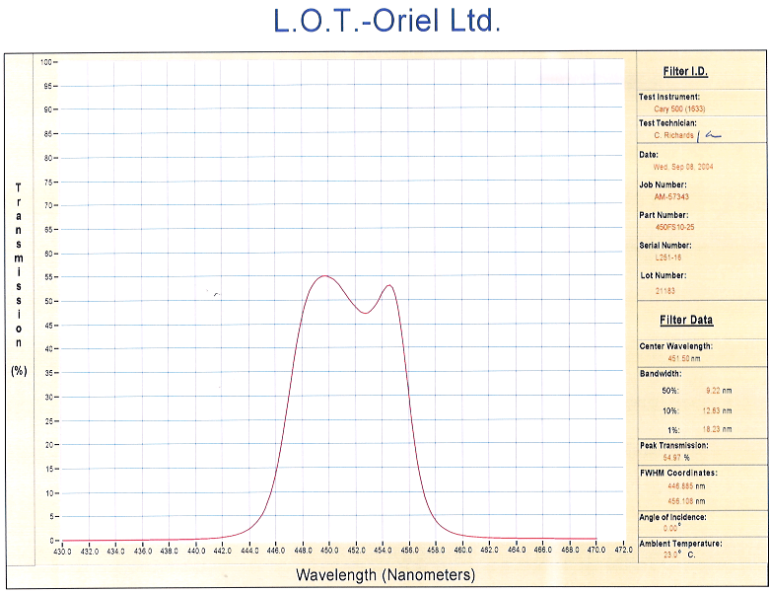

Figure B16 *450-nm bandpass filter.*

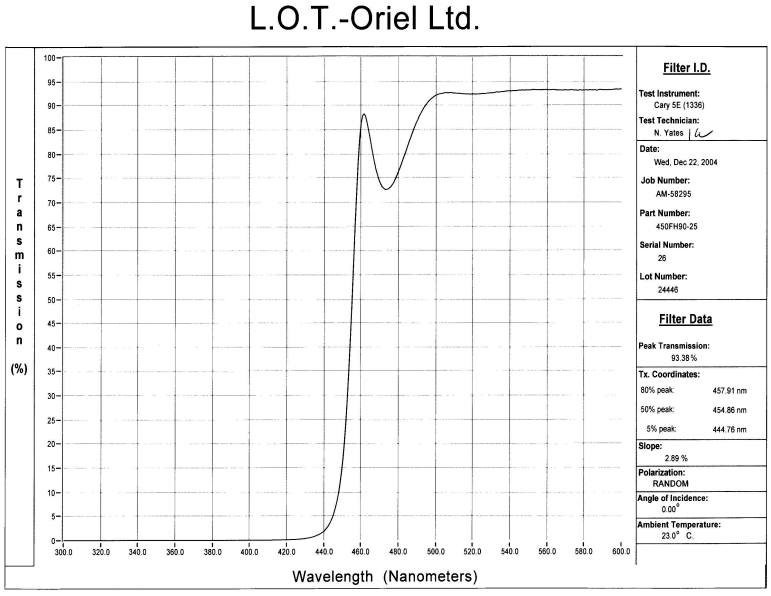

Figure B17 *450-nm long-wave pass filter.*

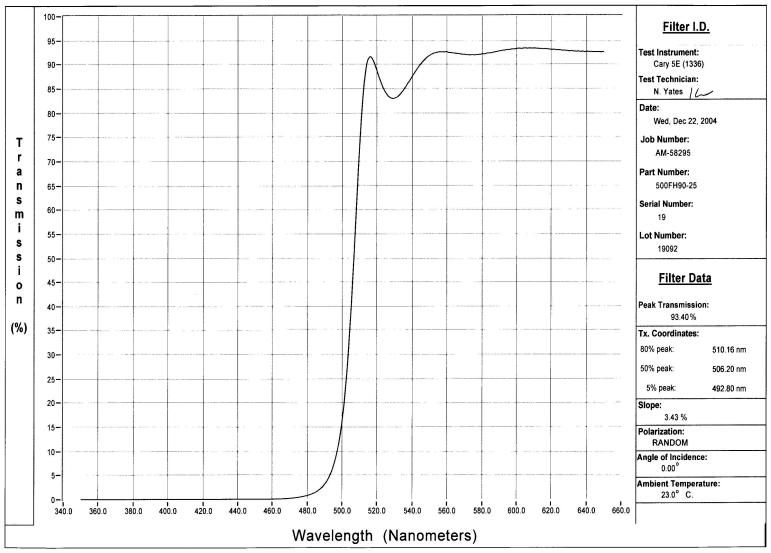

Figure B18 *500-nm long-wave pass filter.*

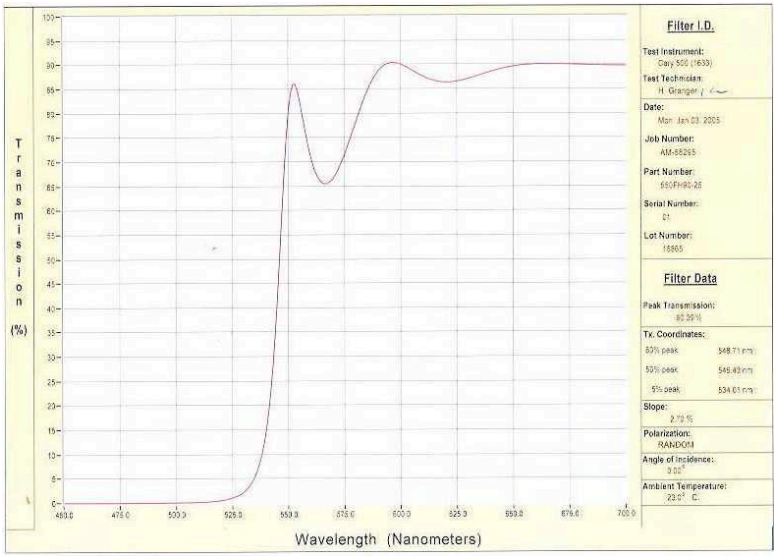

Figure B19 *550-nm long-wave pass filter.*

APPENDIX C

PUBLICATIONS AND PRESENTATIONS

- MacGregor, S.J., Anderson J.G., Maclean, M., Woolsey, G.A., Grant, M.H., Beveridge, J.R., Griffiths, S., Smith, S., Murdoch, L. and Timoshkin I. "Developments and applications in electronic sterilisation technologies". Proceedings of the XVII International Conference on Gas Discharges and their Applications, Cardiff, UK, 2008, p1-8.

- Maclean, M., Lani, M.N., Murdoch, L., MacGregor, S.J., Anderson, J.G. and Woolsey, G.A. "Photoinactivation and photoreactivation responses by bacterial pathogens after exposure to pulsed UV-light". Proceedings of the 2008 IEEE International Power Modulator Conference, Las Vegas, USA, 2008, p326-329.

- Murdoch, L.E., Maclean, M., MacGregor, S.J. and Anderson, J.G. (2010) "Inactivation of *Campylobacter jejuni* by exposure to high-intensity 405-nm visible light" Foodborne pathogens and Disease. doi:10.1089/fpd.2010.0561.

POSTER PRESENTATIONS

- McKenzie, K., Maclean, M., Murdoch, L.E., Anderson, J.G. and MacGregor S.J. "Bactericidal effect of blue high-intensity narrow-spectrum light on environmentally stressed *Staphylococcus aureus"* Society for General Microbiology Spring Meeting, Edinburgh International Conference Centre, UK, 2010.

ABSTRACT

Bacterial resistance to traditional sterilization methods is increasing, and as a result, new methods are continuously being developed for clinical and public-health applications. High-Intensity Narrow-Spectrum (HINS) light is a photodynamic inactivation technology that involves exposing micro-organisms to 405 nm blue light. Exposure to HINS-light is thought to involve the excitation of bacterial intracellular photosensitizer molecules, which subsequently results in the production of reactive oxygen species, and ultimately bacterial cell death. This study investigated the bactericidal effect of 405 nm HINS-light on environmentally stressed *Staphylococcus aureus*. Investigations involved exposing suspensions of *S. aureus* to a range of sub-lethal environmental stresses, including heat, salt, acid and starvation, and assessing how these sub-lethal stresses affected the bacterium's susceptibility to inactivation through HINS-light exposure. Results demonstrated the effectiveness of 405 nm HINS-light for the inactivation of environmentally stressed *S. aureus*, with suspensions of *S. aureus* exposed to each of the sub-lethal stress conditions in combination with 405 nm HINS-light exposure showing significant increases in inactivation rates when compared to non-stressed cells. These findings have significance for the practical application of HINS-light as a decontamination technology since overall treatment effectiveness will be increased when targeting the already environmentally stressed bacterial cells present in, for example, clinical areas and food processing environments.

ORAL PRESENTATIONS

- Murdoch, L.E., Maclean, M., MacGregor, S.J. and Anderson, J.G. "Pulsed ultraviolet light inactivation and photoreactivation of the foodborne pathogens *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli*". Society for General Microbiology Spring Meeting, Harrogate International Centre, UK, 2009.

ABSTRACT

Pulsed ultraviolet (PUV) light is a novel sterilization technology, which utilizes high peak power, applied over short time periods, resulting in rapid microbial inactivation. PUV-light is capable of killing a wide range of micro-organisms through the generation of DNA mutations, which prevent bacterial replication, rendering cells inactive. However, many bacteria possess DNA repair mechanisms capable of repairing UV-induced damage, the most notable being photoreactivation, which utilizes visible light of wavelength 300–500nm to repair the UV-induced damage.

The present study examines PUV inactivation of the foodborne pathogens *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli*, and their subsequent photoreactivation capability. Using a broadband xenon flashlamp it was found that <10 pulses of PUV-light were required to achieve a 7-log reduction in *S.aureus.* Photoreactivation of sub-lethally damaged cells was found to induce up to a 3-log increase in viable cell count, with this maximum decreasing upon increasing PUV-damage. Similar results were obtained for *L. monocytogenes* and *E. coli*.

This study has demonstrated that PUV is an effective sterilization technology that could contribute to food safety strategies. However, elucidation of the lethal doses required to completely inactivate microbial pathogens is necessary to prevent the possible subsequent photoreactivation of any remaining sub-lethally damaged cells.