University of Strathclyde

AN INVESTIGATION INTO THE LIGHT INACTIVATION OF MEDICALLY IMPORTANT MICROORGANISMS

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ABSTRACT

Infection control is an area of increasing interest due to the failure of traditional disinfection and sterilisation methods, and of course, the significant problems associated with microbial multiple-antibiotic resistance. This study investigated light-based methods for the inactivation of a range of medically important bacteria.

Initial investigations involved the design and development of a PUV-light air-disinfection system for the control and prevention of airborne infection. This system was tested *in-situ* for its efficiency to decontaminate air in university lecture theatres. Results demonstrated an 80% reduction in the level of airborne bacterial population, with the majority of the surviving isolates being saprophytic, pigmented *Micrococcus spp.* which pose no risk to human health.

The second, and most significant, area of this study was the discovery, development and application of a visible-light treatment for the inactivation of MRSA and other medically important Gram-positive bacteria including *Clostridium*, coagulase-negative *Staphylococcus*, *Streptococcus* and *Enterococcus* species. The lethality of blue-light, and white-light containing blue-light, for these organisms was demonstrated through a series of filter studies, and identification of the causative wavelengths to within a 10 nm bandwidth allowed the selection of a more efficient high-intensity narrow-band light source, now termed HINS-light. Based on experimental data obtained from this study, it is proposed that *Staphylococcus* inactivation by blue light is brought about through singlet oxygen ($^1O_2$) generation by the photo-excitation of naturally-occurring endogenous porphyrins within the bacteria. This process has not previously been documented as a possible inactivation pathway for *Staphylococcus aureus*.

Although not as germicidally efficient as UV-light, this HINS-light system has the great advantage of being non-detrimental to human health, thus posing no problems with continuous exposure in occupied rooms, such as hospital wards. Consequently, HINS-light may prove to be an effective, non-harmful method for the control and prevention of MRSA transmission within the health-care environment.

CONTENTS

Chapter 1

INTRODUCTION

Airborne transmission of microorganisms is recognised as being a significant source of infection, with many medically important bacteria, fungi and viruses capable of dispersal via this route. Although this has been a topic of long-standing importance, over recent years there has been a notable increase in interest in indoor air-quality issues, both in an attempt to establish the physical mechanisms of airborne transmission and to develop effective means of achieving air disinfection [Jaisinghani, 1998; Cox, 1987].

The majority of human-shed organisms are commensals and pose little threat to immunocompetent individuals. There is, however, the possibility that infectious organisms such as *Mycobacterium tuberculosis*, *Neisseria meningitidis* and *Morbillivirus* are being released through droplet nuclei ($\leq$ 5 μm in diameter) that remain suspended in the air for long periods due to their size [Sleigh and Timbury, 1998]. These may subsequently cause infection in other individuals via the respiratory tract if an infectious dose is inhaled.

Although the transmission of infectious aerosols is a major concern in the hospital environment, other less well-documented situations must also be taken into consideration. In areas where large numbers of people are brought together in close proximity, such as in schools and on aeroplanes, there is the potential for the build-up of substantial airborne contamination and therefore cross-infection [CDC, 1995; Nardell *et al*, 1991]. A study by the US Environmental Protection Agency (EPA) found that levels of indoor air pollutants may be 2 to 5 times – and occasionally more than 100 times – those at the outdoor level [Cheong and Lau, 2003].

It is because of this and other indoor air-quality issues such as Sick Building Syndrome (SBS), that there is a need to develop effective air-quality control methods that may be used in densely populated indoor venues such as those mentioned above, and most importantly in hospitals and other health-care facilities.

The initial aim of this study was the development of a Pulsed Ultra-Violet (PUV) air-disinfection system and its subsequent testing on air samples from university lecture theatres. The testing in university lecture theatres followed on from work preceding the development of this system, in which the microbial quality of the air was measured before and after occupation of a room by large congregations of students.

Progression of this work led to an investigation into the photoreactivation of UV-pulsed bacterial isolates extracted from indoor air samples, but some unexpected results caused the study to proceed in a different, yet related, direction. These unexpected results established that prolonged exposure of the UV-inactivated isolates (and non-UV inactivated controls) to light in the visible region of the electromagnetic spectrum was capable of inducing inactivation. This discovery led to the use of more intense light sources, in order to achieve more rapid inactivation rates.

With the focus of the study already on the inactivation of medically important airborne species it was decided to expand the programme to include significant hospital-acquired bacterial species spread by other means, in addition to the airborne route. These included methicillin-resistant *Staphylococcus aureus* (MRSA) – the so-called *superbug*.

Globally, the health-care industry is experiencing increasing problems, both medical and financial, due to hospital-acquired infections and the emergence of a number of multi-antibiotic-resistant organisms such as MRSA and multi-drug resistant *Mycobacterium tuberculosis* [WHO, 2002(I)]. In the UK alone, hospital-acquired infections are estimated to cost the NHS about £1 billion per year, and, according to official estimates, cause approximately 100,000 infections per year, 5000 of which

result in fatalities [National Audit Office, 2000]. As a result of this there is a great need for the development of an effective disinfection method which can aid in the combat of these problematic – often fatal – infection-causing microorganisms.

The study into the effect of visible-light exposure involved different broadband light sources and a range of pathogenic Gram-positive bacteria associated with hospital-acquired infections including MRSA, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Enterococcus faecalis* and *Clostridium perfringens*. A major aspect of the study was the use of optical filters to isolate the causative wavelengths, which in turn allowed further development and enhancement of a novel visible-light-based system.

The significant outcome of this research has been the development of High-Intensity Narrow-Spectrum light, known as HINS-light, and this has provided a potentially viable solution in the on-going fight against MRSA and other hospital-acquired infections.

Overall, this study into the light inactivation of medically important microorganisms can effectively be divided into two main parts;

1. The development and testing of a pulsed ultra-violet light air-disinfection system for the control of airborne microbiological contamination, and;
2. The discovery, development and testing of a visible-light system (now known as HINS-light) for the inactivation of MRSA and a range of medically important Gram-positive bacterial species.

The following is an overview of the breakdown of the chapters and a brief description of the content of each.

Chapter 2 (Background and Literature Review) concentrates on air-quality control providing a brief background to the problems caused by poor quality indoor air, types of bioaerosols and their sampling, and methods of air-quality control, with a focus on Ultra-Violet light.

Chapter 3 (Microbiological Systems and Techniques) provides information on the microorganisms, media, equipment and microbiological methods used throughout the investigation.

Chapter 4 (Monitoring and Pulsed UV Treatment of Indoor Air) investigates the levels of airborne microbiological contamination within university lecture theatres, and also shows the results of the development of a pulsed-UV air-disinfection system and its subsequent testing on university lecture-theatre air samples.

Chapter 5 (Discovery of Visible-Light Inactivation) investigates the photoreactivation of PUV-treated bacteria isolated from air samples and the subsequent discovery that 400–500 nm visible-light has a lethal effect on the airborne bacterial isolates, in addition to other Gram-positive bacterial species.

Chapter 6 (Inactivation of Staphylococcal Species through Visible-Light Exposure) develops the discovery of the lethality of visible-light and discusses the use of more intense light sources for bacterial inactivation on surfaces and in suspensions. Results are presented that demonstrate the inactivation of a range of medically important bacteria including MRSA, and the chapter also investigates factors that may influence treatment efficacy.

Chapter 7 (Investigation into the Visible-Light Wavelength Sensitivity of Staphylococcus aureus) presents results concerning the wavelength sensitivity of visible-light inactivation and uses the results in a discussion suggesting the likely method of bacterial inactivation.

Chapter 8 (The Process of Visible-Light Inactivation for Staphylococcus aureus) examines and discusses the visible-light inactivation process, and consists essentially of two parts. Part I investigates the role of oxygen in the visible-light inactivation process. Results demonstrating the effects of oxygen depletion and enhancement are presented, and help to determine the nature of the mechanism occurring within the light-exposed staphylococci. Part II is an in-depth discussion into the inactivation process itself and provides evidence in support of the mechanism of bacterial inactivation proposed in Chapter 7.

Chapter 9 (405 nm LED Light Source) focuses on the use of an improved light source for bacterial inactivation and presents results that demonstrate its increased efficacy for inactivation of MRSA and other bacterial species.

Chapter 10 (General Conclusions and Recommendations for Further Work) discusses the results obtained throughout the study and proposes potential applications for this disinfection system within the clinical environment. Recommendations for further developments to the inactivation technology and areas of future work are also discussed.

Chapter 2

BACKGROUND AND LITERATURE REVIEW

2.0 GENERAL

This literature review focuses on indoor air quality problems, with much of the emphasis being on its importance in the health-care environment. Also reviewed are methods for the sampling and control of bioaerosols leading to an in-depth discussion regarding the nature, effects and applications of ultraviolet light disinfection. Due to the unforeseen change in direction of the experimental work, this Literature Review and Chapter 4 should be viewed together, with the relevant background information for the work in Chapters 5 through 9 being discussed in the background and discussion sections of these chapters.

2.1 INDOOR AIR QUALITY

Indoor air quality control is an increasingly important area in the prevention of infection due to the air around us being a constant source of bacterial, fungal and viral agents. Although to the healthy population these do not pose a great threat because of the low numbers of pathogenic organisms present and our functional immune systems, these airborne microorganisms can be of considerable risk to certain groups of the population: mainly immunocompromised individuals [Gerberding, 1998]. Due to the immunocompromised being the major 'high risk' group, the hospital environment is the obvious situation where the assurance of high quality air is crucial.

It is not only hospital and health-care environments which must maintain high standards of air quality, but with the emergence of 'Sick-Building Syndrome', it has

become evident that poor air quality in the home and workplace can also have adverse effects on immunocompetent individuals [US EPA, 1991].

The importance of good indoor air quality in these situations and other areas of public interest are discussed in detail in this section.

2.1.1 The Hospital Environment

Indoor air quality is of utmost importance in the hospital environment, where the hospital should be regarded as a potential hazard due to the congregation of large numbers of infectious patients coughing, sneezing and shedding their causative organisms into the surrounding environment where they can be transferred to other, already ill patients, potentially further complicating their condition. The acquisition of infection from a source within the hospital is known as a nosocomial or hospital-acquired infection, and is defined by the World Health Organization (WHO) as "an infection acquired in hospital by a patient who was admitted for a reason other than that infection" [WHO, 2002(II)].

Good indoor air quality is most important to immunocompromised patients in hospitals because of their debilitated immune status, which is a result of either treatment or natural disease progression. Because of these underlying conditions patients are particularly predisposed to airborne and respiratory tract infections [Parham, 2000]. Immunocompromised patients are kept in hospital during, and for a length of time after, treatment and must be kept in as sterile an environment as possible so as to reduce the risk of possible infection and complications. If good air quality and hygiene procedures are not met, then the patients are at increased risk of infection by pathogens from other hospital patients that have accumulated and been subsequently transferred in the air supply. There is also the added risk that pathogens acquired from nosocomial sources could possess antibiotic resistance, which will make their treatment and elimination difficult; for example, methicillin resistant *Staphylococcus aureus* (MRSA) [WHO, 2002(I)].

Hospital-acquired infections – airborne and otherwise – are discussed in more depth in Chapter 6.

2.1.2 Sick Building Syndrome

Good quality indoor air is not only important in areas with susceptible individuals, but in all buildings, particularly in the workplace and the home since it is estimated that 90% of an individual's time is spent indoors [Kowalski and Bahnfleth, 1998]. Some well-identified illnesses such as Legionnaires disease, asthma, hypersensitivity pneumonitis and humidifier fever are directly traced to specific building problems – hence the term Building-Related Illness (BRI) [US EPA, 1995]. These are usually treatable but can have serious health risks, particularly Legionnaires disease which is fatal in 10-15% of cases [HPA, 2004].

In other cases occupants within a building may begin to experience symptoms not fitting with any specific illness and untraceable to any specific source. This is termed Sick-Building Syndrome (SBS). Symptoms are varied and may include any number of the following: sneezing; runny or stuffy nose; burning or dry mucous membranes of the throat/nose/eyes; headache; dizziness; fatigue or lethargy; nausea; irritability; forgetfulness. The symptoms usually affect the individual only while they are in the building and tend to diminish after vacation [US EPA, 1995; US EPA, 1991].

2.1.3 Public Health Issues

In addition to the workplace and in the home, indoor air in other public areas is an issue of growing importance. Large groups of people regularly congregate in enclosed areas, and outbreaks of illnesses such as measles, tuberculosis and influenza, initiated by airborne transmission of the infectious organisms, have been documented to occur in schools [Markowitz *et al*, 1989; Gustafson *et al*, 1987; Riley *et al*, 1978], prisons [Awofeso *et al*, 2001; Valway *et al*, 1994], sports arenas [Ehresmann *et al*, 1995], residential homes [Loeb *et al*, 2000] and sheltered housing [Nolan *et al*, 1991]. In addition to this, an outbreak of gastroenteritis caused by airborne transmission of Norwalk-like virus (usually transmitted via the faecal-oral

route or through direct contact with vomitus) has been reported in a school [Marks *et al*, 2003]. This highlights the potential for airborne transmission of organisms other than those traditionally transmitted through the air.

Due to the increasingly regular use of long-haul flights, aeroplanes are another situation where individuals are enclosed within a small space. Confined spaces, prolonged exposure times, limited ventilation and re-circulating air are all risk factors, common with air travel, which promote the spread of respiratory infections, although studies have shown that transmission is most likely to occur when seated in close contact to the infectious individual [Leder and Newman, 2005]. Studies carried out by the Centres for Disease Control and Prevention (CDC) showed the successful transmission of tuberculosis on board aircraft, with one case showing *Mycobacterium tuberculosis* transmission from a passenger with active tuberculosis to four other passengers [CDC, 1995]. Outbreaks of influenza, measles and smallpox (now eradicated) on board aircraft have also been suggested [Leder and Newman, 2005].

With it being suggested that the critical factor for successful airborne transmission of infectious agents on board aircraft is close proximity to the infectious source, it is possible that transmission could occur during transportation by other means. In support of this, TB transmission has also been reported as a result of bus, train and ship travel [Leder and Newman, 2005].

The Severe Acute Respiratory Syndrome (SARS) virus outbreak of 2003, transmissible via contaminated air and water droplets, highlighted the threat of airborne infections and their potentially rapid transmission – not just from individual to individual but from country to country [IDSA, 2005; Tong, 2005]. The rapid spread of the SARS virus is thought to have been facilitated by its airborne transmission on board aircraft [Leder and Newman, 2005; Olsen *et al*, 2003]. This again highlights the importance of maintaining good air quality on aeroplanes.

The influenza virus is a highly infectious and threatening airborne organism which produces annual epidemics. In the United states alone, annual influenza epidemics

result in an average of 114,000 hospitalisations and more than 36,000 fatalities [Bridges *et al*, 2003]. More recently, there has been growing concern about the spread of avian influenza. Originating in Asia, the virus has quickly spread through birds and there is concern that the antigenic shift of this virus will enable easy human-to-human transmission resulting in an influenza pandemic [CDC, 2006].

Another more political issue which has raised concerns regarding air quality control is the threat of bioterrorism. The case in October 2001 where letters containing *Bacillus anthracis* spores were sent to a number of Government buildings resulted in contamination of the post offices, mail rooms and recipient buildings and the subsequent development of either cutaneous or inhalation anthrax infections of 23 individuals – five of which were fatal. In response to this, a number of manufacturers have developed materials or equipment to kill *B. anthracis* and its spores [Spotts Whitney *et al*, 2003]. Methods of defensive filtration for buildings against other potential biological warfare agents such as botulism, plague, smallpox, tularaemia and haemorrhagic viruses have also been suggested [Miller, 2002].

2.2 BIOAEROSOLS

Bioaerosols are "airborne particles, large molecules or volatile compounds that are living, contain living organisms or were released from living organisms", as defined by The American Conference of Governmental Industrial Hygienists (ACGIH). The focus of this study is on bioaerosols of microbiological origin.

2.2.1 Airborne Transmission of Infection

Bioaerosols derive from a range of sources and as a result, they can vary widely in composition. Although generally harmless, some bioaerosol particles can potentially induce toxic, allergic or infectious responses in humans. Table 2.1 gives examples of some typical aerosols of biological origin. Pollen and dander aerosols are non-microbiological and exposure to these is generally harmless, but they act as allergens

to sensitive individuals and prolonged exposure may even induce allergic reactions in non-allergic individuals [Kowalski and Bahnfleth, 2002].

TABLE 2.1 *Examples of airborne contaminants of biological origin, their typical sources and approximate sizes [Adapted from Kowalski and Bahnfleth, 2002]*

BIOAEROSOL	SOURCE	SIZE (μm)
Pollen	Outdoor air (seasonal)	10-100
Animal dander	Skin cells or organic matter from cats, dogs, mice, dust mites, cockroaches or other animals	1-100 (with a mean of 7-20)
Fungal & bacterial spores	Normally originate outdoors, although can be generated indoors if conditions support their growth	1-20
Bacteria	Pathogenic bacteria tend to originate from human or animal sources; opportunistic bacteria tend to be from environmental sources	0.2-2
Viruses	Human and animal sources	0.01-0.3

The size of bioaerosols varies greatly depending on their nature. The smallest bioaerosols of microbiological interest are viruses, and have a size of approximately 0.01 μm. Bacteria and spores are larger in size being approximately 1 and 10 μm respectively. In general, bioaerosols are <25 μm, any larger and the particle will not remain suspended in air for long due to gravitational forces. Generally microorganisms are present in the air as part of larger particles, for example on shed skin cells, in dust, or droplet nuclei which, due to their small size (≤ 5 μm), remain suspended in air for long periods of time [Cox, 1987]. Droplet nuclei are the typical route for transmission of contagious aerosols [Sleigh and Timbury, 1998].

The most common methods for the expulsion of infectious droplet nuclei into the environment are talking, coughing and sneezing. Air released by these actions contains material such as saliva/mucus, mucosal cells and viable microorganisms, the

number of which will increase during infection. The distribution of these particles in expired air depends on factors such as the velocity of the expired air, secretion viscosity, presence of respiratory infection, and whether released via the nose or mouth [Cox, 1987].

Sneezing is the most effective mode for the generation of airborne particles. It generates particles from both the mouth and the nose and can result in the release of approximately 1 million droplets with a diameter of up to 100 μm (predominantly saliva from the buccal cavity), and tens of thousands of droplet nuclei which can disperse up to three metres away. Coughing results in less airborne particles with one cough potentially releasing approximately 3000 droplet nuclei. Talking is less effective in aerosol dispersal compared to sneezing and coughing, and produces only a few droplets per word, although talking for 5 minutes can result in the release of up to 3000 droplet nuclei [Cox, 1987]. Respiratory pathogens tend to induce nasopharyngeal irritation in colonised hosts, resulting in excess sneezing and coughing and the subsequent release and aerosolisation of large amounts of bioaerosols [Kowalski and Bahnfleth, 1998]. Infected hosts who release large amounts of airborne pathogens, either via respiratory secretions or on shed skin squames, are termed 'dispersers' [Ayliffe *et al*, 2001].

Once suspended in the atmosphere, the aerodynamic behaviour of all aerosols is determined by gravitation, turbulence, diffusion and electromagnetism. Large droplets expelled during coughing and sneezing mainly diffuse as a result of gravitational settling within minutes. Smaller, micron-sized particles rapidly evaporate to droplet nuclei close to the size of the individual microorganism and then spread by diffusion and air currents. These small particles may remain suspended for hours but over time they lose viability due to dehydration [Kowalski and Bahnfleth, 1998]. This water can only be replaced on transfer to another aqueous environment or during host infection. The movement of these water molecules is dependent on temperature and relative humidity and this is common to all bacterial aerosols [Cox, 1987].

2.2.2 Microorganisms Transmitted via the Airborne Route

Inhalation is the major route of exposure to airborne infectious organisms and there are a number of ways by which these microorganisms may become suspended in the air where they await inhalation by an unsuspecting host – the three main routes being human, dust and aerosolised fluids [Ayliffe *et al*, 2001]. Figure 2.1 summarises these routes, giving examples of sources and typical microorganisms transmitted by these routes. Most microorganisms in dust tend to be environmental in origin and only disease-causing if the infected host has debilitated immune status or the organism has breeched the skin barrier through open wounds, whereas many of the human-sourced organisms are contagious [Kowalski and Bahnfleth, 2002].

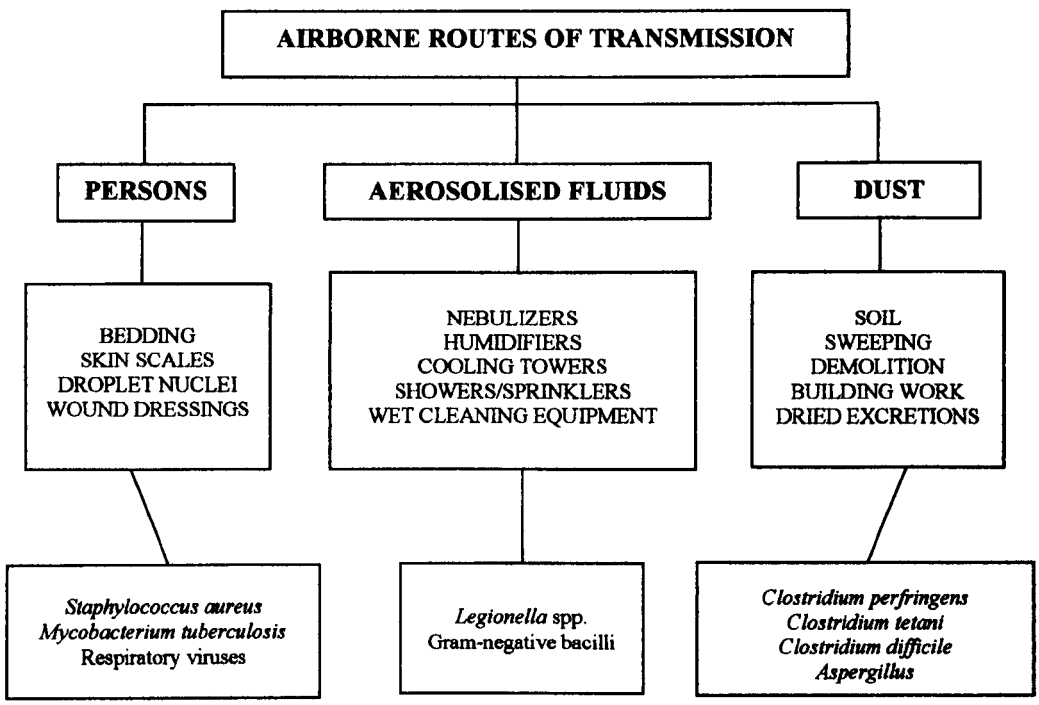

FIGURE 2.1 *Summary of the main routes of airborne transmission and typical microorganisms dispersed via these routes [Ayliffe et al, 2001]*

The majority of organisms capable of airborne transmission are respiratory pathogens but non-respiratory pathogens can also become airborne and cause infections of the skin and eyes, nosocomial infections of open wounds and also contamination of surgical equipment leading to potential patient wound/blood contamination [Kowalski and Bahnfleth, 1998].

Although airborne transmission is the principal mode of transmission, respiratory pathogens can also transmit via direct contact with fomites, which are inanimate objects that become contaminated with pathogenic bacteria and promote the spread of infection [Wilson, 2001; Kowalski and Bahnfleth, 1998].

Table 2.2 shows examples of some of the most significant pathogenic airborne microorganisms and their typical disease manifestations. Since airborne transmission within the health-care environment is of utmost importance, also noted on the table is whether the organisms either primarily cause or are commonly implicated in nosocomial infections [Kowalski and Bahnfleth, 1998; Murray *et al*, 1998].

Most of the airborne organisms are harmless to healthy individuals unless an infectious dose is inhaled. For example the infectious dose of *Mycobacterium tuberculosis*, the infectious agent of TB, is only 1-10 bacilli cells which are transferred between hosts in only 1-3 droplet nuclei [Riley, 1961]. For immunocompromised patients **all** microorganisms are potentially pathogenic.

Bacterial and viral airborne microorganisms are more pathogenic in terms of initiating respiratory infections. Fungal infection is not usually considered a problem to immunocompetent individuals, although long-term exposure to fungi/fungal spores may induce hypersensitivity or respiratory problems in otherwise healthy individuals [Fischer and Dott, 2003]. The fungi included in Table 2.2 are only really a major threat to immunocompromised patients because such patients have debilitated immune status. In an immunocompromised host, fungal infection is invasive and often fatal, unless diagnosed early and treated with aggressive anti-fungal medication. The major invasive fungal infection is aspergillosis, which is caused by a number of species including *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. In hospitals, construction work, with the resulting increased dust load, is a factor which has been linked to increased rates of airborne *Aspergillus* and nosocomial aspergillosis [Curtis *et al*, 2005].

TABLE 2.2 *Common bacterial, fungal and viral airborne pathogens [Kowalski and Bahnfleth, 1998;Murray et al, 1998].*

AIRBORNE PATHOGEN	TYPICAL DISEASE MANIFESTATIONS
BACTERIA	
Bacillus anthracis	Anthrax
Bordetella pertussis *	Whooping cough
Chlamydia pneumoniae #	Pneumonia, bronchitis
Corynebacteria diphtheria *	Diphtheria
Haemophilus influenzae *	Pneumonia, meningitis, bacteraemia
Klebsiella pneumoniae *	Opportunistic infections
Legionella pneumophila	Legionnaires disease, Pontiac fever
Mycoplasma pneumoniae *	Pneumonia
Mycobacterium tuberculosis #	Tuberculosis (TB)
Neisseria meningitidis *	Meningitis
Pseudomonas aeruginosa *	Opportunistic infections
Staphylococcus aureus *	Opportunistic infections
Streptococcus pneumoniae *	Pneumonia, otitis media, meningitis, bacteraemia
Streptococcus pyogenes *	Scarlet fever, bacteraemia, necrotizing fascitis
Yersinia pestis	Pneumonic plague
VIRUS	
Adenovirus	Colds
Coronavirus	Colds, severe acute respiratory syndrome (SARS)
Coxsackievirus	Colds
Filovirus	Marburg & Ebola viruses causing haemorrhagic fever
Morbillivirus #	Measles
Orthomyxoviridae #	Influenza
Parainfluenza	Influenza
Paramyxovirus #	Mumps
Respiratory Syncytial Virus #	Pneumonia
Rhinovirus	Colds
Togavirus #	Rubella
Variola Poxvirus	Smallpox (now extinct)
Varicella-zoster virus #	Chickenpox
PROTOZOA	
Pneumocystis carinii *	Pneumocystosis
FUNGI	
Aspergillus spp #.	Aspergillosis
Coccidioides immitis #	Coccidioidomycosis
Cryptococcus neoformans *	Cryptococcosis
Histoplasma capsulatum #	Histoplasmosis

* Primarily nosocomial # Commonly nosocomial

2.2.3 How Microbial Bioaerosols Infect Humans

In relation to human health, bioaerosol deposition is determined by the anatomy of the respiratory tract as well as the aerodynamic diameter of the aerosol.

The respiratory tract can be separated into three distinct regions, each of which allows deposition of different sized particles: the mouth and nasopharynx; the larynx, trachea and bronchi; and the terminal respiratory bronchioles. The first two regions together are commonly referred to as the upper respiratory tract, with the third region termed the lower respiratory tract. Approximately 100% of particles in the 20 μm range are deposited in the mouth and nasopharynx (1^{st} region) by impaction, but capture efficiency decreases with decreasing particle size. These smaller particles continue on to the conducting passages of the larynx, trachea and bronchi (2^{nd} region) and this region traps particles with diameters between 2 μm and 20 μm by sedimentation. Any small particles that have not become entrained by this point follow the airflow to the terminal bronchioles (3^{rd} region). This region feeds into the alveolar sacs which are responsible for oxygen exchange within the lungs. Particles of around 1 μm to 4 μm, with an optimum of 2 μm, are deposited here by diffusion [Morris *et al*, 2000; CCOHS, 1999].

2.3 MICROBIAL AIR SAMPLING

To monitor the problem of airborne contamination there are a number of methods available for the extraction and subsequent analysis of airborne microorganisms. The objective of all methods is to remove the microorganisms from the air into another environment from which they can be more easily isolated and identified by *ex-situ* methods [DEFRA, 2000]. In addition to providing counts of microbial airborne populations, air sampling may be used for a number of other purposes including identifying potential sources of infection, monitoring cleaning procedures and identifying breakdown in ventilation/HEPA filtration systems [Morris *et al*, 2000].

Since bioaerosols have a diversity of shapes, including spherical, dodecahedral, needle-like and flakes, most sampling methods rely on the aerodynamic diameter (d_{ae}) of the bioaerosol. The d_{ae} refers to an aerosols behaviour, rather than its linear diameter and is defined as "the diameter of a sphere of density (ρ_0= 1 g cm^{-3}) which settles through air with a velocity equal to that of the actual particle under consideration" [Morris *et al*, 2000]. Due to airborne particles losing and absorbing moisture in response to atmospheric humidity, their size is subject to change and as a result, the d_{ae} of a species falls within a range rather than having a specified value [Morris *et al*, 2000].

Although a variety of methods are available to quantitatively determine the number of viable microorganisms in air, no single method is deemed suitable for collection and analysis of all types of bioaerosol, and no standardised protocols exist. This section describes available methods and also discusses their benefits and drawbacks.

2.3.1 Sedimentation

Sedimentation, or settle plates, as this method is more commonly known, is the most primitive of the air sampling methods. It involves the placement of uncovered agar plates in a variety of positions around a sampling location on which microbe-containing particles settle by gravity. This method is non-volumetric and passive, and by far the cheapest and easiest method available [DEFRA, 2000]. Larger particles settle more rapidly (causing their over-representation), and the method is also inefficient for collection of smaller particles as air turbulence around the plate can cause their continuous re-suspension (causing their under-representation) [Morris *et al*, 2000]. The sedimentation method is therefore considered best as a pre-screening tool or for use in conjunction with other air sampling methods, but it should not be relied upon to provide accurate results representative of the sampled environment.

2.3.2 Impaction

The impaction method of air sampling involves the collection of microorganisms by aspirating air in a laminar flow pattern with sufficient velocity to impact organisms

onto an agar surface. Due to their higher mass, the microorganisms become impacted on the agar surface while the rest of the air flows around the plate and exits the sampler [Cherwell Laboratories Limited, 2002]. A variety of impaction air samplers is available, all working by the same general principle. The main types are discussed below.

Sieve-Impaction

Sieve-impaction samplers draw air in at a fixed speed for a variable time through a perforated plate, directly under which is an agar plate. The influx of air results in the deposition of microorganisms on the agar surface [Cherwell Laboratories Limited, 2002], as illustrated in Figure 2.2. There are a number of sieve-impaction samplers available on the market, ranging from the single-stage impactor just described, to multi-stage (or cascade) impactors. Cascade impactors may have up to 6 stages of perforated plates and agar plates with the perforations in each plate becoming gradually smaller. This enables larger particles to deposit on the first stage while the smallest deposit on the last stage, thus allowing information about particle size to be obtained in addition to the airborne population counts [Andersen Instruments Incorporated].

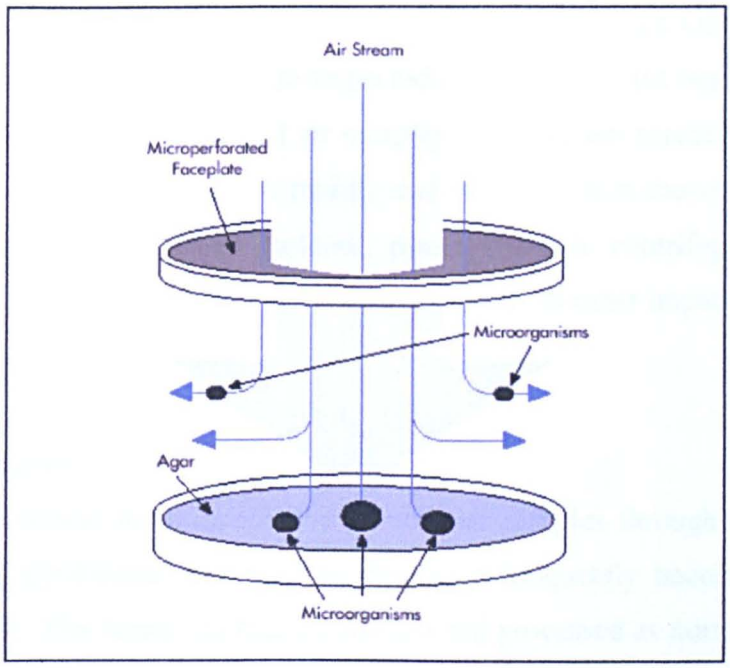

FIGURE 2.2 *Sieve-impaction sampling method [Millipore Corporation, 2003]*

The use of sieve impactors allows the sampling of large air volumes in relatively short time periods; for example, the SAS super-180 model (Cherwell Laboratories, UK) can sample 180 litres in one minute [Cherwell Laboratories Limited, 2002]. A consequential problem of this, if using extended sampling periods, is that dessication of the agar capture media may occur leading to unfavourable growing conditions for any impacted microorganisms.

Slit-to-Agar Impaction

With slit-to-agar impaction samplers, a known volume of air is drawn in through a slit opening. The sampled air is then directed at a large agar plate rotating on a turntable at a specified speed, and the impacted microorganisms are spatially separated on the plate due to its rotation. A unique feature of this sampling technique is that it provides a time-based analysis [Buddemyer, 2005]. As with the sieve-impaction samplers, desiccation of the agar plate may be a problem when sampling large volumes of air. Due to their use of high flow rates they are also inefficient for the sampling of smaller particles [Buddemyer, 2005].

Centrifugal-Impaction

The principle for microbial collection by this method is centrifugation. This involves the creation of a vortex and particles with sufficient inertia are then forced out of the air-stream by centrifugal force and are impacted onto a semi-solid medium, usually agar [Buddemyer, 2005]. Centrifugal air samplers have the advantage that they can sample large volumes of air relatively rapidly and have also been shown to have high recovery rates compared to other methods, possibly due to centrifugal impaction being a less stressful isolation procedure when compared to other impaction methods and impingement [DEFRA, 2000].

2.3.3 Impingement

Impingement extraction methods involve passing air samples through a volume of liquid, with any particulate matter within the air subsequently becoming trapped within this liquid. The liquid can then be filtered and processed as normal [DEFRA, 2000; Buddemyer, 2005].

Two different types of impinger exist – low velocity and high velocity. Low-velocity models are suitable for extracting only particles >5 μm in diameter and are therefore not used for the extraction of single bacterial cells from the air. For isolation of smaller particles, a high-velocity impinger is required. These extract particles of >1 μm, but due to the shear force exerted on the bioaerosols during sampling, destruction of some vegetative cells is to be expected [DEFRA, 2000].

2.3.4 Gelatine membrane filtration

This sampling method involves passing a set volume of air through a gelatine membrane filter, approximately 300 μm thick. Microorganisms in the airflow attach to the surface and also become entangled within the porous filter. After sampling, the filter is placed on a 90 mm Petri dish and then dissolves, allowing the captured microorganisms to be cultured on the agar as normal [Buddemyer, 2005]. This method has benefits over the others in that it has an absolute retention rate and can also be used for the capture of airborne viruses. In addition to this, with the filter having a composition of 50% water, desiccation is not viewed as a problem as it is in most other sampling procedures [Buddemyer, 2005].

2.3.5 Electrostatic Precipitation

Although the most commonly used extraction methods employed – impaction and impingement – are efficient, their use of high-velocity air imparts an additional stress upon the collected microorganisms. As a result of this, a new method called electrostatic precipitation (the principle of which is more commonly seen in air-cleaning applications [US EPA, 1990]) has been developed for air sampling in an attempt to reduce stress. The method operates by imparting an electrostatic charge on incoming particles and these are then collected on a rotating disc of opposite charge. An advantage of this sampler type is that it has a high throughput of up to 1000 l/min, but the system is difficult to operate [DEFRA, 2000].

Table 2.3 gives examples of commercially available air-sampler models for each of the extraction methods.

TABLE 2.3 *Commercially available air samplers and their manufacturers*

SAMPLING METHOD	COMMERCIAL SAMPLER	MANUFACTURER
SIEVE IMPACTION	Andersen 6-stage Sampler	Andersen Instruments Inc., USA
	SAS Super-180 Sampler	Cherwell Laboratories Ltd., UK
	M Air T Tester	Millipore Corporation, USA
SLIT-TO-AGAR IMPACTION	New Brunswick STA Sampler	New Brunswick Scientific, USA
	Mattson Garvin Sampler	Mattson Garvin C/O, USA
CENTRIFUGAL IMPACTION	RCS Plus Centrifugal Sampler	Folex-Biotest-Schluessner Inc., USA
IMPINGEMENT	SKC Biosampler	SKC Inc., USA
	All-Glass Impinger 30 (AGI-30)	Ace Glass Inc., USA
MEMBRANE FILTRATION	Gelman Membrane Filter Sampler	Gelman Sciences Inc., USA
ELECTROSTATIC PRECIPITATION	LVS Sampler	Sci-Med Environmental Systems, USA

2.3.6 Factors Affecting Air Sampling Methods and Performances

Ideally, when collecting bioaerosols the total number collected should be representative of the airborne population. Commercially available devices have differing sampling parameters, including inlet efficiency, cut size (the size below which most microbes are not removed from the air stream), the amount of dehydration during and after collection, impact velocity and surface density of collected microorganisms (leading to masking of resultant colony-forming units) [Stewart *et al*, 1995]. These parameters may affect sample collection efficiency and microbial recovery, thus causing disparity between measurements from different air sampling devices in the same environment [Stewart *et al*, 1995].

Sampler design has traditionally been influenced by a desire to mirror the particle retention and deposition characteristics of the respiratory tract. But due to air samplers having a continuous flow rate and the air flow into the lungs being neither continuous nor at a constant rate, they do not provide good replication of the

respiratory tract [Morris *et al*, 2000]. Pasquarella *et al* [2000] proposed the passive sedimentation sampling method to be the most effective in this respect, since no high velocity forces – as applied with the active sampling methods – are involved, and they "give the measurement of the harmful part of the airborne population which falls on to a critical surface in a given time". For this reason they also suggest that this method would be most suitable for estimating particle deposition onto wounds since it replicates the exact deposition method that would be experienced in normal situations.

A further issue which may influence the total airborne viable microbial counts isolated using any of the air sampling methods is the *viable but nonculturable* (VBNC) phenomenon. VBNC bacteria have lost their culturability but still exhibit metabolic activity, and it has been suggested that this state is induced by environmental stresses [McDougald *et al*, 1998]. Due to the stresses experienced by the organism during aerosolisation or prolonged suspension, or injury imposed as a result of high-velocity impact onto the agar collection surface, it is likely that a number of organisms are damaged, and this leads to underestimated counts.

The fact that these VBNC pathogens can persist and produce toxins in the environment, while not being culturable – therefore undetectable by standard methods – is a significant public-health concern [McDougald *et al*, 1998].

2.4 INDOOR AIR QUALITY CONTROL

No standards exist for acceptable levels of microbial contamination in indoor air due to the infectivity of microorganisms being species-dependent. The normal concentration range of indoor air allergens is 100 – 1000 CFU/m^3, and so levels below 100 CFU/m^3 are considered to be a realistic target, but in general, pathogenic bacteria and viruses, particularly contagious pathogens, are considered to have no safe limits [Kowalski and Bahnfleth, 2002]. Therefore, most of the focus is on providing adequate ventilation to buildings/rooms. Conversely, due to the

importance of high quality air in hospital operating theatres, specific guidelines are in place for both conventional and ultra-clean theatres [Holton *et al*, 1990], and these are summarised in Table 2.4. In addition to these levels, it has been stated that in an empty theatre, the bacterial concentration should be less than 35 CFU/m^3 and should include less than 1 CFU of *Staphylococcus aureus* or *Clostridium perfringens* in 30 m^3 [Holton *et al*, 1990].

TABLE 2.4 *Guidelines for bacterial counts within operating theatres [Holton et al, 1990]*

SAMPLING PERIOD	BACTERIAL LEVELS IN OPERATING THEATRES	
	CONVENTIONAL	ULTRA-CLEAN
EMPTY THEATRE	<35 CFU/m^3	<0.5 CFU/m^3
DURING OPERATION	<180 CFU/m^3	<10 CFU/m^3

In 2004, the American Society of Heating, Refrigeration and Air Conditioning Engineers (ASHRAE) published a standard entitled *Ventilation for Acceptable Indoor Air Quality* (ASHRAE Standard 62.1) and the purpose of this was to "specify minimum ventilation rates and indoor air quality that will be acceptable to human occupants and are intended to minimise the potential for adverse effects". In this they define acceptable indoor air quality as being "air in which there are no known contaminants at harmful concentrations as determined by cognisant authorities and with a substantial majority (> 80%) of the people exposed not expressing dissatisfaction" [ASHRAE Standard 62.1, 2004]. The upkeep of good quality air is of importance, particularly in high-risk areas such as hospitals, and there are a number of methods employed within buildings for this purpose and these will be discussed in this section.

2.4.1 Air Quality Control Methods

All buildings have ventilation systems. Some depend on natural ventilation but large buildings in temperate climates depend on mechanical-heating, ventilation and air-conditioning (HVAC) systems [Nardell, 1998].

There are four main categories of mechanical control which limit the spread of allergens and airborne pathogens within indoor air – although in most cases they are found to be interdependent. The four categories are summarised in Figure 2.3 and will be discussed in more detail in the following sections.

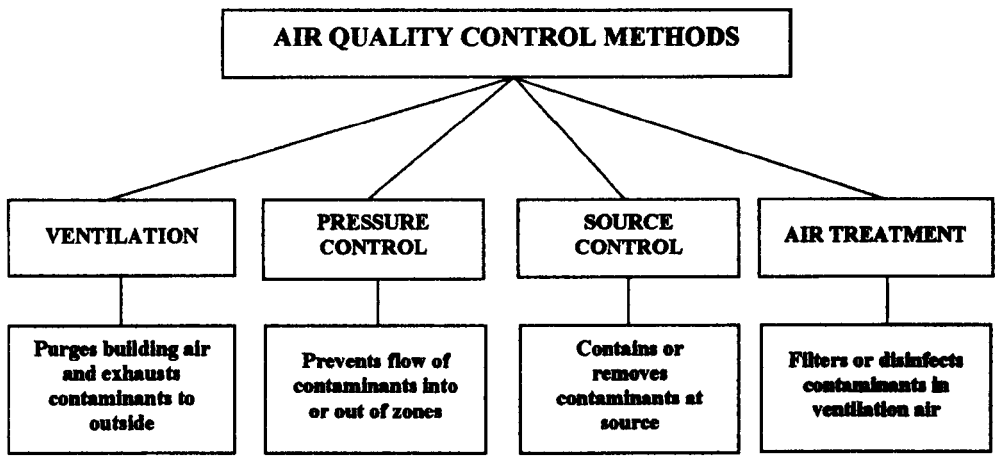

FIGURE 2.3 *Air quality control methods [Kowalski et al, 2002]*

2.4.2 Ventilation

Ventilation can be described using two interrelated units – room/building air changes per hour (ACH) and ventilation rate (l/s) per occupant, and ventilation rates should be proportional to the occupancy of the room [Nardell, 1998]. There are a number of mechanical ventilation systems employed in buildings whose principle function is to introduce 'fresh' air into the room.

Dilution ventilation (also known as purge ventilation) draws in outdoor air, mixes it with room air and then exhausts an equal quantity of indoor air [Kowalski *et al*, 2002]. This method of mixing and exhaust results in a decrease in airborne contaminants. The volume of outdoor air drawn into the system depends on the type of building in which it is installed. Higher intakes of air (hence higher ACH rates) are used in buildings estimated to have higher contamination levels [Kowalski and Bahnfleth, 2002]. In high risk areas where prevention of infection is crucial; for

example, hospital operating theatres, typical air change rates are 20 air changes per hour, with airflow from clean to dirty areas [Holton *et al*, 1990].

Alternatively, vertical displacement ventilation introduces low-velocity air through floor-level vents. As a result of being warmed on contact with room occupants, this air then rises, taking any airborne contaminants up and out of the breathing zone and is then exhausted through ceiling vents. The benefit of this non-mixing method is that air contaminants are less likely to re-circulate back to the breathing zone [Nardell, 1998].

Ultra-clean environments are created using laminar ventilation systems. They are recommended for use in operating theatres during orthopaedic surgery, particularly prosthetic hip and knee replacements. They work by directing a laminar flow of filtered air over the operating table and use over 600 air changes per hour [Wilson, 2001].

2.4.3 Air Treatment

Air treatment within HVAC systems can be classified into two main methods: air filtration and air disinfection.

2.4.3.1 Air Filtration

Filtration is a form of air treatment commonly used within ventilation systems. The effectiveness of a filter against airborne contaminants depends on a number of factors including the filter characteristics, the size and type of airborne particles and the velocity of the air [Kowalski and Bahnfleth, 2002]. Differing filters are available for use within ventilation systems and are selected depending on the level of protection required.

MERV Filters

Filters were originally categorised by a percentage efficiency rating not based on particle size, and therefore not actually providing crucial information about efficiency with smaller, respirable particles. As a result, ASHRAE and the air-filter

industry developed a standardised rating system of a **minimum efficiency reporting value (MERV)** from 1 to 16 for each type of air filter. The basic principle is that the higher the MERV rating the more efficient the filter. Table 2.5 gives information on MERV-rated filters and their efficiencies.

TABLE 2.5 *Minimum efficiency reporting values of filters [ASHRAE Standard 52.2, 1999]*

MERV RATING	EFFICIENCY RANGE		
	PSE at 0.3 – 1.0 μm	**PSE at 1.0 – 3.0 μm**	**PSE at 3.0 – 10.0 μm**
MERV 1	-	-	< 20%
MERV 2	-	-	< 20%
MERV 3	-	-	< 20%
MERV 4	-	-	< 20%
MERV 5	-	-	20 – 34.9%
MERV 6	-	-	35 – 49.9%
MERV 7	-	-	50 – 69.9%
MERV 8	-	-	70 – 84.9%
MERV 9	-	< 50%	≥ 85%
MERV 10	-	50 – 64.9%	≥ 85%
MERV 11	-	65 – 79.9%	≥ 85%
MERV 12	-	80 – 89.9%	≥ 90%
MERV 13	< 75%	≥ 90%	≥ 90%
MERV 14	75 – 84.9%	≥ 90%	≥ 90%
MERV 15	85 – 94.9%	≥ 90%	≥ 90%
MERV 16	≥ 95%	≥ 95%	≥ 95%

MERV: minimum efficiency reporting value

PSE: particle size efficiency

HEPA Filters

High Efficiency Particulate Air (HEPA) filters remove at least 99.97% of all particles 0.3 μm or larger in diameter when operated at design air velocity [Kowalski *et al*, 2002]. HEPA filters are not MERV-rated as they exceed the ASHRAE test protocol, and they are the only type of filter tested and confirmed to meet a specific efficiency at a specific particle size. Because of this efficiency they are termed 'absolute filters' and their main applications are in operating rooms, TB isolation rooms and

pharmaceutical clean rooms [Kowalski *et al*, 1999]. Although considered highly effective, a study by Kowalski and Bahnfleth [2002], has shown that airborne pathogens – a number of which are implicated in nosocomial infections – can penetrate the HEPA filter, thus emphasising the need for additional air treatments such as ultra-violet germicidal irradiation (UVGI).

A common problem with filters is that over time, the build-up of particulate matter affects the performance of the ventilation system. In addition to this, there is the danger that if conditions are right, trapped microorganisms – most likely bacterial and fungal spores – will multiply and shed back into the air stream [Jaisinghani, 1998]. To counter this problem antimicrobial filters (impregnated with fungicides and bactericides) have been developed although there has been concern that they may produce carcinogenic vapours which will be carried in the air stream [Kowalski and Bahnfleth, 2002; Jaisinghani, 1998].

2.4.3.2 Air Disinfection

Air disinfection methods within HVAC systems can be used alone or in combination with filtration. Their main benefit is that when used alongside filters, they kill trapped microorganisms, thus preventing any further growth and possible contamination of the air stream. Ultra-violet germicidal irradiation (UVGI) is the most used method and will be discussed in depth in Section 2.4.6, due to this investigation initially focusing on UV light air treatment technologies. Alternative methods of air disinfection include ionising electrically-enhanced filtration [Jaisinghani, 1998], photocatalytic oxidation, ionisation, and electrostatic filtration [Kowalski and Bahnfleth, 2002; Kowalski *et al*, 2002].

2.4.4 Pressure Control

The principle of pressurisation control is to ensure airflow is in a specific direction and two types of control exist, negative-pressure and positive-pressure ventilation. With negative-pressure ventilation, air flows into a room to ensure contamination does not escape. This is enabled by exhausting room air at a 10% higher rate than it enters, with the room ventilation making up the imbalance by continuously drawing

air in from outside the room via a specified gap under the door [Nardell, 1998]. The contaminated exhaust air is then either treated before re-circulation or removal, or exhausted directly to the outside air [Kowalski *et al*, 2002]. This type of ventilation is also known as source control (discussed further in Section 2.4.5).

Conversely, positive-pressure ventilation enables air to flow out of a room ensuring no contamination enters – a good example being an operating theatre (Figure 2.4). In this case, air being drawn into the room is first treated to remove any contamination [Ayliffe *et al*, 2001; Wilson, 2001]. This method is also referred to as plenum ventilation and protective isolation. Immunosuppressed patients such as those in oncology, transplant and HIV wards must be kept in this 'protective isolation' ventilation, as they cannot tolerate any level of ambient bacteria or fungal spores, since these could initiate potentially fatal infections.

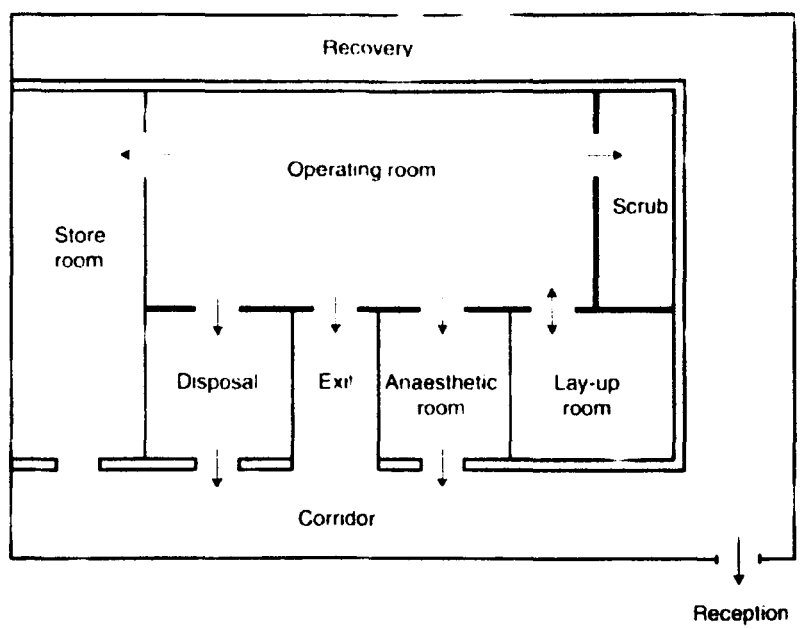

FIGURE 2.4 *An example of positive-pressure ventilation in situ is that of an operating theatre. Air moves from the cleanest areas to the least clean areas. Arrows indicate direction of airflow [Wilson, 2001]*

2.4.5 Source Control

Source control is a method employed principally in the health-care environment, and a good example of this is in hospital isolation rooms. These rooms are maintained at

negative pressure to prevent escape of infectious organisms. Exhaust air is treated, usually by HEPA-filtration or by the combination of a HEPA filter and UVGI, ensuring no infectious contaminants are re-circulated throughout the building [Nardell, 1998]. Source isolation wards are ideally suited for patients with tuberculosis, viral respiratory infections, eczema (causing excess skin shedding) and patients likely to disperse large numbers of multi-drug resistant organisms, for example MRSA [Ayliffe *et al*, 2001].

2.4.6 Ultra-Violet Germicidal Irradiation (UVGI)

As briefly mentioned in section 2.4.3.2, UVGI is an effective method of air disinfection. It has been used in various capacities for more than 100 years and is now becoming increasingly used in clinical and industrial situations [Kowalski and Bahnfleth, 2000]. UVGI for air disinfection is currently employed either in ventilating ducts, or as an upper air disinfection system [Kowalski and Bahnfleth, 2002].

In a typical ventilation-duct UVGI system, the UV lamp is situated within a duct, crossing the air stream. Airborne microorganisms passing through this section of the duct are exposed to UV light, both directly from the lamp and indirectly from duct-reflected light [Nardell, 1998]. UVGI can be used either alone or in combination with filters, usually HEPA filters. Both methods of deployment have advantages: when used alone, UVGI provides much less airflow resistance through the ventilation system, and as a combination treatment microorganisms trapped on the filter are destroyed, thus preventing further growth as well as disinfecting the passing air. The HEPA-UVGI combination system appears to be highly effective and this is thought to be due to the fact that UVGI has greater success against smaller microbes such as bacteria and viruses while the HEPA filter easily deals with the larger, more resistant spores [Kowalski and Bahnfleth, 2002]. For this reason, the HEPA-UVGI combination system is considered the ideal system for use in ventilation ducts [Kowalski and Bahnfleth, 2002].

UVGI and/or HEPA filtration are predominantly placed in ventilation ducts to sterilise re-circulated air. Their use is rare in outside air supply ducts; the exception being where the supply air is for areas inhabited by immunosuppressed individuals (for example HIV clinics), for whom exposure to relatively harmless organisms could be lethal [Kowalski and Bahnfleth, 2002; Kowalski and Bahnfleth, 2000].

Upper-room air disinfection is considered to be an ideal use for UVGI. It is a passive system independent of room ventilation rates – which is both an advantage and a limitation, since system efficacy is greatly dependent on the rate of air exchange between the lower and upper room volumes. Microbial air decontamination is effected by the passive upward movement of air through a UV beam, installed at a ceiling height of at least 2.5 metres. These systems are silent (unlike filtered ventilation systems), inexpensive, and are ideal for disinfection of large areas such as waiting areas and homeless shelters where high air change rates are hard to achieve [Nardell, 1998].

UV lamps have also been used directly in infectious-disease isolation rooms. These lamps are mounted on the ceiling or wall with shielding to protect room occupants but this shielding reduces the efficiency of the UV irradiation technology [Jaisinghani, 1998].

2.5 ULTRA-VIOLET LIGHT

In addition to being very effective for the treatment of contaminated air, UV has applications for surface and water sterilisation [Xenon Corporation, 2002; Dunn *et al*, 1998; Dunn *et al*, 1995]. Its efficacy as an air-quality-control measure – and a water and surface disinfectant – is accredited to the well-known biocidal properties of UV-light [Block, 1991]. With the initial focus of this study being on the development and application of a pulsed UV-light air-disinfection system, this section will discuss the physical properties and nature of UV light and UV-light sources.

2.5.1 Nature of UV Light

UV light is a region of the electromagnetic spectrum of wavelengths longer than X-rays but shorter than visible radiation, and is approximately 100 nm to 400 nm. The UV-light wavelengths of the electromagnetic spectrum are further subdivided into long-wave (UV-A), medium-wave (UV-B), short-wave (UV-C) and vacuum-UV radiation, based on their effects (direct and indirect) on tissue – more specifically, human skin tissue [Bolton, 2001].

UV-A wavelengths – often referred to as blacklight – are the most penetrating but least damaging form of ultraviolet radiation. Exposure can cause changes in the skin that lead to sun tanning [Bolton, 2001] and prolonged exposure to bright light has also been shown to induce optical damage [WHO, 2006]. UV-B wavelengths cause erythema (sunburn) and have been implicated as the cause of skin cancer [Bolton, 2001; Medical Ecology Organisation, 2004]. The UV-C range is extremely dangerous due to its shorter, more energetic wavelengths. It is absorbed by nucleic acids and proteins and can lead to mutations, cancer and/or cell death. This region is also known as the germicidal region because of its efficiency for inactivating microorganisms, and as a result, it is these wavelengths which are exploited by UV-light sources used for disinfection [Block, 1991].

Vacuum UV (VUV) – it is only transmissible through vacuum – is absorbed by almost all substances including air and water, and does not therefore affect human health [Bolton, 2001].

In addition to these detrimental effects, UV light, more specifically UV-A, has medical applications such as for the treatment of skin complaints including psoriasis, and the treatment of Vitamin D deficiency in skin cells [WHO, 2006].

The complete UV spectrum is present in natural sunlight but, due to absorption, UV-A wavelengths are the predominant wavelengths reaching the earth's surface. UV-B wavelengths are mostly absorbed by stratospheric ozone but increased levels are now reaching the earth's surface due to the hole in the ozone layer, and it is this increase

in UV-B which is thought to be the cause of increasing skin cancer rates. The UV-C range is not found on earth due to it being absorbed by ozone and oxygen in the earth's atmosphere [Sinha and Häder, 2002].

2.5.2 UV Light Sources

Although all ultraviolet light wavelengths induce detrimental changes in living organisms ranging from prokaryotes to eukaryotes, plants, animals and humans, exposure to UV-C light is by far the most dangerous, and the reasons for this will be discussed in Section 2.6. Since this shortwave UV light is not naturally present in the sunlight spectrum at the surface of the earth, it is of little consequence in environmental situations, but the effectiveness of UV-C light for the inactivation of microorganisms has led to the development of a variety of UV-light sources for use in disinfection technologies. These include low and medium-pressure mercury arcs, excimer lamps and flashlamps, each of which will be reviewed in this section.

2.5.2.1 Mercury Arcs

The traditional light source used in UV-based disinfection systems is the mercury (Hg) arc lamp. This lamp is composed of a quartz tube with electrodes at either end and contains a small amount of elemental mercury and an inert gas, usually argon. A voltage is established across the electrodes to generate an electrical arc. The optical emission from the arc depends on the gas pressure [Block, 1991]. Two different forms of mercury lamp have been widely used for UV-disinfection – low-pressure mercury arcs and medium-pressure mercury arcs.

Low-pressure Mercury Arc

For a low-pressure (<10 torr) mercury arc, the mercury atoms are raised to excited states producing very narrow emission lines with the principal output emission being at 253.7 nm [Bolton, 2001]. Around 40% of the electrical input power is converted to 254 nm light. This UV wavelength efficiently inactivates a wide range of Gram-positive and Gram-negative bacteria.

Medium-pressure Mercury Arc

Medium-pressure mercury arcs are characterised by a polychromatic output. When the mercury is excited at an increased pressure (~1000 torr), the narrow-line, monochromatic output emission (seen with the low-pressure lamps) broadens to produce a continuum while retaining strong line emission [Bolton, 2001; McDonald *et al*, 2000]. Medium-pressure lamps are less energy efficient than their low-pressure counterparts but they produce a higher intensity of UV-C light and therefore can apply the required UV dosage in a reduced irradiation time [UV Light Technology Limited, 2004]. Due to the increased output emission of these lamps they have been favoured over low-pressure lamps for their use in some UV disinfection systems, in particular large-volume treatment systems, but with this benefit there are also the disadvantages of high temperatures, higher costs and reduced lamp efficiency [Block, 1991].

A comparison of the spectral outputs of low and medium-pressure mercury lamps is shown in Figure 2.5.

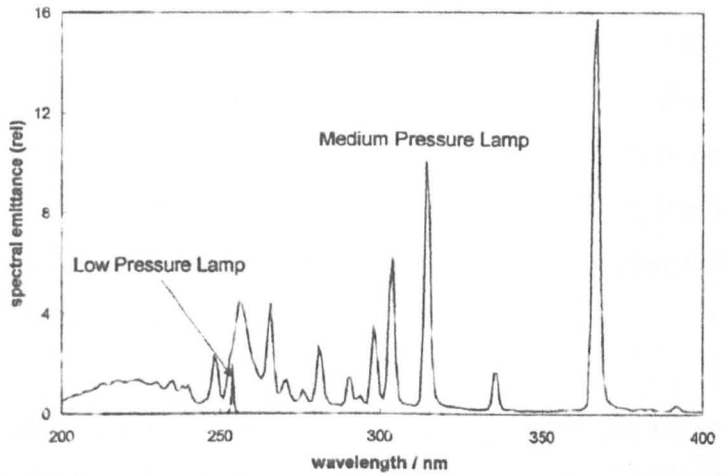

FIGURE 2.5 *Comparison of the emission spectra from low-pressure and medium-pressure mercury-arc lamps [Bolton, 2001]*

2.5.2.2 Excimer Lamps

An excimer (*excited dimer*) is a short-lived molecule that bonds two molecules in an electronic excited state: on decay to the ground state, the excimer dissociates. Excimer lamps are tunable monochromatic light sources that produce UV radiation by applying a voltage to a mixture of gases contained in a discharge gap with a dielectric barrier between the electrodes [Bolton, 2001; Block, 1991]. This voltage excites the gaseous molecules to form dimers that then dissociate and release photons of light energy of a specific wavelength depending on the excimer used. Common excimer lamps are Xe_2 with output emission at 172 nm, KrCl (222 nm), Cl_2 (259 nm), XeCl (308 nm) and I_2 (342 nm) [Bolton, 2001]. The major advantage of excimer lamps is their low thermal output and surface temperature. This allows for their use with heat-sensitive substrates [Block, 1991].

2.5.2.3 Flashlamps

Flashlamps, like continuous arc lamps, are gas-filled quartz tubes with electrodes at either end, but in this case the gas is Xenon. Flashlamps release light energy in intense pulses rather than continuously. This pulsed-power approach enables high peak powers to be produced at a level unattainable using continuous excitation [MacGregor *et al*, 1998]. The generation of these light pulses involves the storage of large amounts of energy and the subsequent dissipation of the energy using high voltage over a very short time, usually microseconds. Pulsed-power technology produces pulses of high intensity polychromatic light, 20,000 times the intensity of sunlight [Dunn *et al*, 1995]. With Xenon gas, the emission output is rich in germicidal UV-C wavelengths, as shown in Figure 2.6, making it suitable for use in disinfection systems, unlike continuous Xenon lamps, which have little UV-C output [Xenon Corporation, 2002].

For their use in disinfection systems the pulse repetition rate of xenon flashlamps will vary depending on factors such as microbial load and sample volume/size, but typically ranges from 1 – 30 pulses per second (pps) [Block, 1991].

Due to the commercial requirement for ever-improving disinfection technologies, pulsed ultraviolet (PUV) light is fast becoming the method of choice – the major attraction being its capability to dissipate high peak powers over very short time periods, resulting in rapid inactivation and treatment rates [Xenon Corporation, 2006].

A summary of the main characteristics of all the described UV light sources is shown in Table 2.6.

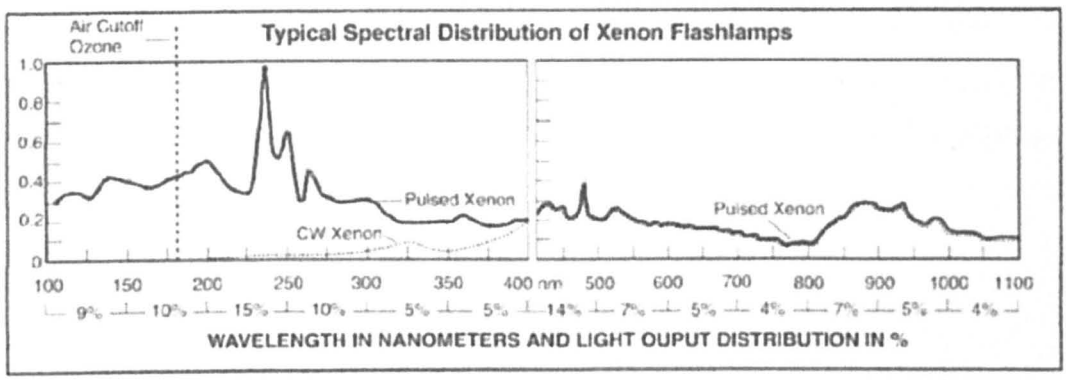

FIGURE 2.6 *Typical spectra of pulsed and continuous wave xenon flashlamps [Xenon Corporation, 2002]*

TABLE 2.6 *Summary of UV sources and their basic characteristics*

	RADIATION SOURCE			
	LOW-PRESSURE Hg ARC	**MEDIUM-PRESSURE Hg ARC**	**EXCIMER**	**FLASHLAMP**
UV EMISSION SPECTRUM	Monochromatic	Polychromatic	Monochromatic	Polychromatic
UV-C WAVELENGTH	254 nm	200 – 300 nm	Tunable	200 – 300 nm
ELECTRICAL EFFICIENCY	30 – 50 %	15 – 30%	10 – 35%	15 – 20%
LAMP SURFACE TEMP.	40°C	400 – 1000°C	Ambient	-
DEPLOYMENT METHOD	Continuous	Continuous	Continuous	Pulsed

2.6 EFFECT OF UV LIGHT ON MICROORGANISMS

Exposure to UV light causes changes within microorganisms at the molecular level, with these changes having potentially lethal results. This section discusses UV light-induced microbial damage, repair mechanisms possessed by microorganisms and factors affecting microbial sensitivity to UV light.

2.6.1 UV-mediated Damage to Microorganisms

The damage caused by the UV radiation corresponds to its photon energy. The amount of energy carried by each photon is proportional to its frequency. Therefore as the wavelength of radiation increases the energy of the photon decreases. The energy carried by a photon is known as a quantum of energy (E) and is given by the frequency of light (f) multiplied by Planck's constant ($h = 6.63 \times 10^{-34}$ J s) [Block, 1991]:

$$\mathbf{E = h f}$$

This means that short-wave UV-C photons carry more energy than medium-wave UV-B photons, which in turn are more energetic than long-wave UV-A photons – as is shown in Table 2.7 [Bolton, 2001].

When looking at the bond energies of typical bonds found within biomolecules (shown in Table 2.8) it can be seen that they coincide with the photon energies of UV wavelengths, more specifically wavelengths of less than 320 nm. This means that VUV, UV-C and UV-B photons have sufficient energy to induce reactions within biomolecules. Absorption of a VUV photon causes the breakage of one of more bonds due to the high energy content of the photons, 598 – 1196 kJ/mole [Bolton, 2001], but since VUV (100 – 200 nm) is absorbed by almost all substances these high energy photons are unable to be used in germicidal systems. Therefore wavelengths between 200 and 320 nm (UV-C and UV-B) are considered germicidal, although as determined earlier, UV-C wavelengths between 240 – 260 nm are the most effective for inactivation.

TABLE 2.7 *UV wavelengths and their corresponding photon energies*

UV RADIATION	CHARACTERISTIC WAVELENGTH (nm)	CHARACTERISTIC PHOTON ENERGY (kJ/mole)
C	200 – 280	427 – 598
B	280 – 315	380 – 427
A	315 – 380	299 – 380

TABLE 2.8 *Bond energies and their corresponding wavelengths [Block, 1991]*

BOND	TYPICAL BOND ENERGY (kJ/mole)	CORRESPONDING WAVELENGTH (nm)
O–H	460	260
C–H	410	290
N–H	390	310
C–O	370	320
C≡C	830	140
C=C	620	190
C=N	850	140
C=O	740	160
C=N	600	200

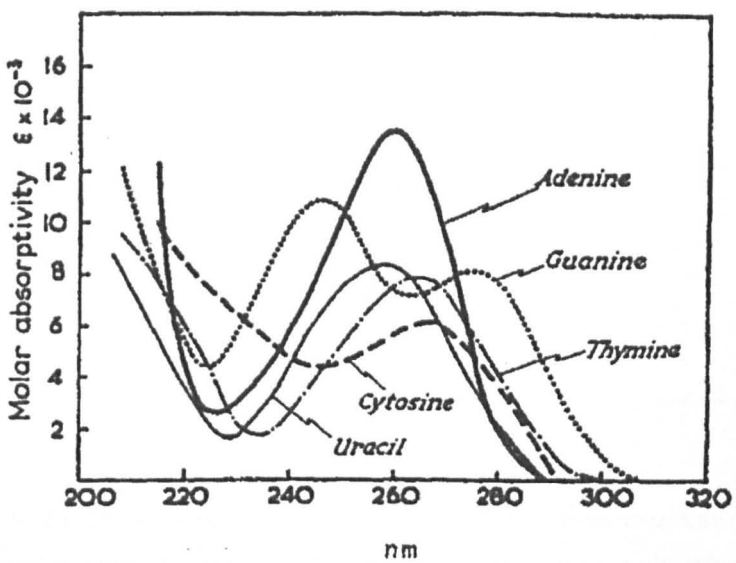

FIGURE 2.7 *Absorbance spectra as a function of wavelength of nucleic acids. Adenine, guanine, cytosine and thymine are components of DNA whereas in RNA, thymine bases are substituted for uracil [Block, 1991]*

The major cellular target for the mutagenic effects of UV radiation is genomic DNA. This is due to the nucleotide base components of DNA and RNA having peak absorbencies between 240 – 280 nm, as shown in Figure 2.7 [Block, 1991].

Nucleotides are the building blocks of DNA and each is composed of a phosphate group, a deoxyribose pentose sugar and either a purine or pyrimidine UV-sensitive nitrogenous base, shown in Figure 2.8. For the formation of a DNA strand, each nucleotide binds through its phosphate group to the sugar of the adjacent nucleotide. Two of these polymer strands, held together by hydrogen bonds between complimentary bases (adenine and thymine; cytosine and guanine) form the characteristic double helix structure of DNA (Figure 2.9) [Campbell, 1996].

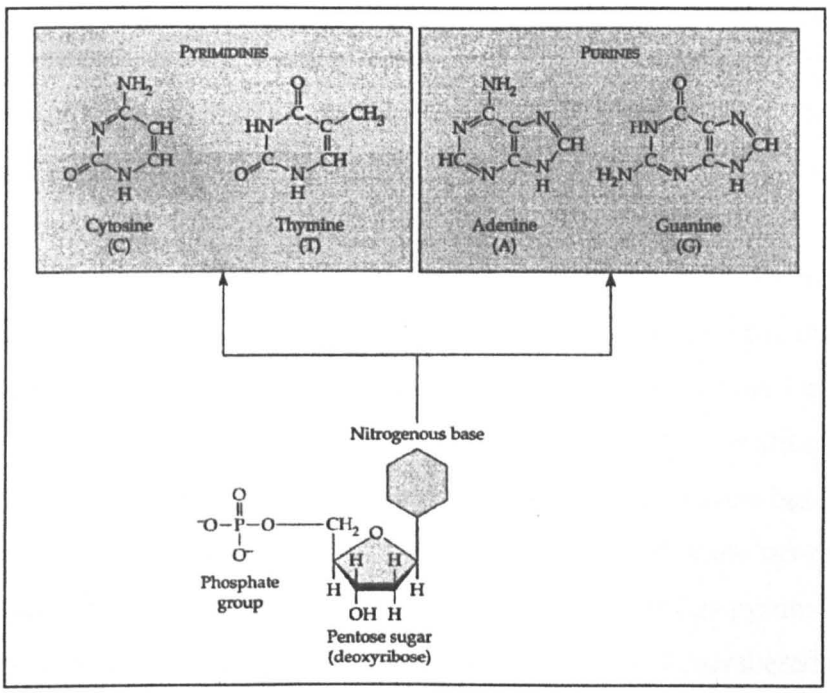

FIGURE 2.8 *Molecular structure of a nucleotide. In DNA, the nitrogenous base can be one of four possibilities: adenine, guanine, cytosine or thymine. These four bases are classified as either purines (adenine or guanine) or pyrimidines (cytosine and thymine). [Campbell, 1996]*

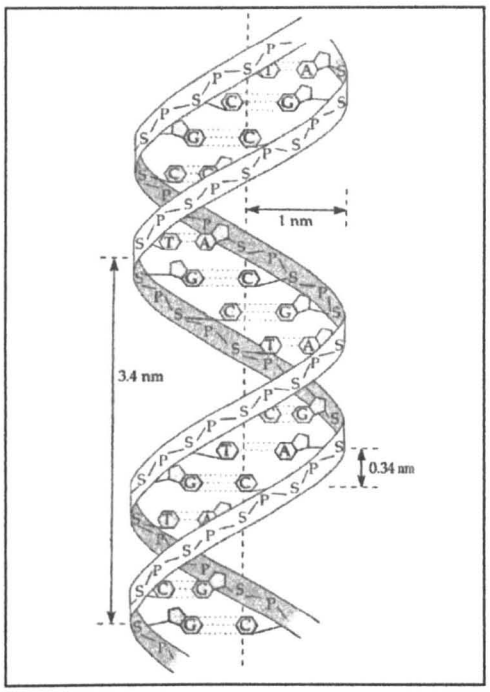

FIGURE 2.9 *The DNA double helix. This structure is composed of two DNA strands each with a sugar-phosphate backbone, held together by hydrogen bonds between complimentary base pairs. Also shown are typical dimensions of the DNA helix molecule [Campbell, 1996]*

Absorption of UV-C and UV-B photons induces direct damage to genomic DNA, with a single photon having the potential to induce a carcinogenic or even a lethal effect [Sinha and Häder, 2002]. Photon absorption ultimately causes the formation of mutagenic DNA lesions and renders microorganisms unable to further replicate.

The major types of mutagenic lesion resulting from the absorption of germicidal UV radiation are cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PP), making up approximately 75 and 25% of the DNA damage products respectively [Sinha and Häder, 2002]. Cyclobutane pyrimidine dimers are formed by the absorption of UV light by a double bond in pyrimidine bases (thymine and cytosine in DNA; cytosine and uracil in RNA). The bond of the pyrimidine base is then opened enabling reactions with neighbouring bases. If this pyrimidine is next to a second pyrimidine base they covalently bond to form a 4-membered cyclobutyl ring [Goodsell, 2001]. Thymine dimers are the most common CPD lesions formed, with cytosine dimers and mixed cytosine-thymine dimers also being produced at a lower frequency [Block, 1991].

Although not as abundant or cytotoxic as CPD lesions, 6-4PP lesions may have more mutagenic, potentially lethal consequences for the damaged cell. Rather than

resulting in the formation of a cyclobutyl ring, UV absorption causes the formation of a single bond between two carbon atoms on neighbouring pyrimidines [Goodsell, 2001]. In contrast to CPD lesions, cytosine is the pyrimidine base most frequently involved in the formation of 6-4PP lesions, either as cytosine dimers or cytosine-thymine dimers. Thymine-thymine 6-4PP lesions are rarely formed [Block, 1991]. The formation of CPD and 6-4PP lesions is shown in diagrammatic form in Figure 2.10. Although CPD and 6-4PP are the most frequently found lesions, other types of UV-mediated damage including inter- and intra-strand cross-links, pyrimidine hydrates, thymine glycols and protein-DNA cross-links are produced and contribute to cellular inactivation [Miller *et al*, 1999; Block, 1991].

UV radiation also has the potential to damage protein molecules. Compared to nucleic acids, proteins are more complex and their response to UV exposure is much more variable. A sufficient UV dose can alter the biological, antigenic or enzymatic activities of a protein but at doses capable of inactivating microorganisms through DNA lesions, most protein preparations experience only a minimal detrimental effect [Block, 1991].

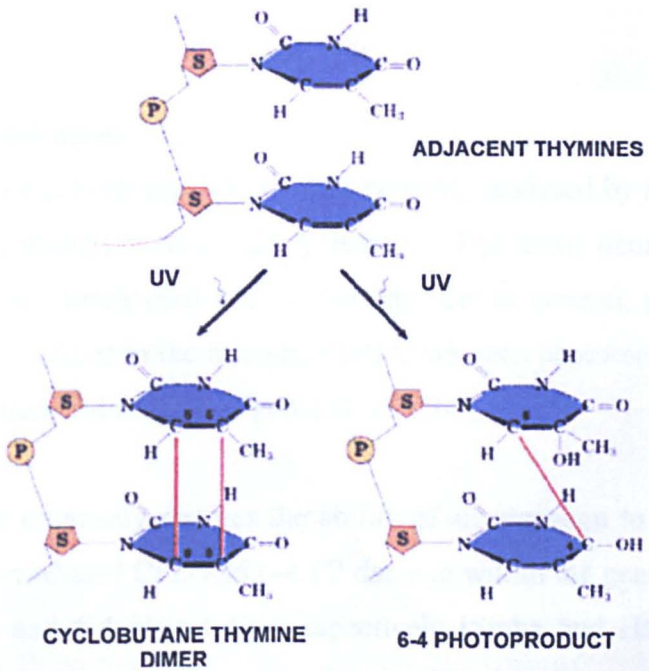

FIGURE 2.10 *CPD and 6-4PP lesions in DNA resulting from the absorption of UV-C and UV-B light by nucleotide bases [Medical Ecology Organisation, 2004]*

In addition to the DNA damage induced by UV-C and UV-B light, UV-A has the potential to inflict injury upon the microorganisms. UV-A photons induce *indirect* damage, unlike UV-C and B, which caused direct damage. UV-A causes cytotoxic effects via sensitiser molecules [Sinha and Häder, 2002]. These sensitiser molecules absorb energy that is then transferred to generate reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and, in the majority of reactions, singlet oxygen ($^1O_2$) [Oguma *et al*, 2002]. These ROS can then react with DNA to damage bases and break strands and also cause protein-DNA cross-linking [Miller *et al*, 1999].

2.6.2 Repair Mechanisms

As a result of exposure to UV radiation from natural sunlight, microorganisms have developed repair mechanisms to counteract the detrimental effect of UV light [Zimmer and Slawson, 2002]. These mechanisms will repair sub-lethal damage but are ineffectual for bacteria that have been killed outright by the UV exposure. Numerous repair pathways can exist within microorganisms but they are generally classified into dark-repair and light-dependent repair, the latter more commonly referred to as *photoreactivation.*

2.6.2.1 Photoreactivation

Photoreactivation is a light-dependent repair process, catalysed by a single enzyme – photolyase – that directly reverses DNA damage. The terms photoreactivation and photorepair are sometimes used interchangeably, but in general, photorepair is the repair of the DNA lesions in the microbial DNA, whereas photoreactivation refers to the recovery of microbial activity [Oguma *et al*, 2001].

Photoreactivation ultimately restores the ability of an organism to replicate through repair of the UV-mediated CPD and 6-4 PP damage within the genomic DNA, using CPD photolyase and 6-4 photolyase, respectively [Sinha and Häder, 2002]. For successful photorepair, exposure to specific wavelengths of long-wave UV (UV-A) and visible radiation between approximately 300 nm and 500 nm are required [Zimmer and Slawson, 2002].

DNA photolyases are thought to continuously scan the genome for lesions and when encountered they become tightly bound to the located lesion. The photorepair process is then initiated by the absorption of photons by the chromophores of the photolyase. This in turn causes the excitation and subsequent electron donation of a co-factor ($FADH^-$), which splits the pyrimidine dimers, returning them to their monomeric form. Photoreactivation is a highly efficient process with one dimer split for every blue-light photon absorbed [Sinha and Häder, 2002; Block, 1991].

Due to the requirement for UV-A in the photoreactivating process, the use of these wavelengths has been termed *concomitant photoreactivation* because of the fact that these wavelengths themselves have the potential to cause oxidative damage to the microbial cell [Oguma *et al* 2002].

2.6.2.2 Dark-Repair Mechanisms

There are a number of dark-repair mechanisms for the repair of UV-induced genomic lesions but the most important and widespread of these is *Nucleotide Excision Repair* (NER). This process has been identified in almost all organisms although the ability to dark-repair varies between species [Oguma *et al*, 2001]. Dark-repair of microorganisms does not directly reverse DNA damage, as photorepair does, but employs numerous proteins and enzymes to remove DNA lesions and subsequently replace the excised lesions with new, undamaged nucleotides [Sinha and Häder, 2002; Zimmer and Slawson, 2002].

Other dark-repair mechanisms include *SOS-error prone repair*, *post-replication recombinational repair* and, in regions of DNA, where for some reason traditional repair methods are unable to occur, *lesion bypass* occurs in order to promote cell survival [Sinha and Häder, 2002; Miller *et al*, 1999].

2.6.3 Microbial Inactivation by UV Light

As already determined, the effect of UV irradiance on microorganisms is dependent on the amount of UV energy absorbed (Section 2.6.1) and this energy can be applied over either a long period with low power – as with continuous UV sources – or a

short period with high power, using a pulsed source (Section 2.5.2.3). Microbial inactivation is dependent on the applied UV dosage, defined as the product of radiation intensity and exposure time [Block, 1991]:

$$\textbf{UV dose} = \mathbf{I\,t}$$

where I is the intensity (mW/cm^2) and t is the exposure time (s). The unit of UV dose is therefore mJ/cm^2.

Microorganisms vary in their response to UV irradiation and a study by Chang *et al* [1985], which used a collimated beam for exposure at 254 nm, found that the most susceptible organisms are Gram-negative bacteria followed by Gram-positive bacteria, viruses, spores and protozoa. Although most studies generally support this ranking in UV susceptibility, it has also been demonstrated that there can be differences in UV sensitivity among members within the microbial groups and even differences between species and strains of the same genus [Hinjen *et al*, 2006; Block, 1991]. Recent studies have also proved the protozoan parasites *Cryptosporidium* and *Giardia* to be much more sensitive to UV light than first thought [Block, 1991].

In addition to the differences in susceptibility between organisms, a number of factors affect the sensitivity of microorganisms to UV light. These include:

- inoculum size; with a larger inoculum size requiring a greater dose to achieve the same lethality [Lamont, 2005]
- the turbidity, colour and nature of the suspending medium; due to the limited penetrating ability of UV light
- clumping of particulates causing the shielding of microorganisms; resulting in a 'tailing effect' [Lani *et al*, 2006; Blatchley III *et al*, 2001]
- bacterial growth phase; increased sensitivity is shown when bacteria are in the exponential phase of growth and lower sensitivity when exposed in the stationary phase [Hinjen *et al*, 2006].

As already stated, the extent of UV damage experienced by the microorganisms is influenced by the applied dose. If low, sub-lethal doses are applied the microorganisms may be induced into an active but non-viable state and can then undergo effective repair mechanisms to regain viability. For the microbes to be irreversibly damaged a high enough UV dose must be delivered, thereby generating thymine dimers in quantities too numerous to be repaired [Blatchley III *et al*, 2001].

TABLE 2.10 *Examples of some typical UV doses required for the inactivation of a range of microorganisms using continuous UV sources*

ORGANISM	DOSE (mJ/cm^2)	LOG_{10} REDUCTION	REFERENCE
BACTERIA			
Escherichia coli	10	5.2	Zimmer & Slawson, 2002
	10	5	Chang *et al*, 1995
	8	3.5	Sommer *et al*, 1996
Salmonella typhi	9	5	Chang *et al*, 1995
Shigella sonnei	10	5	Chang *et al*, 1995
Staphylococcus aureus	10	4	Chang *et al*, 1995
Streptococcus faecalis	12	4	Chang *et al*, 1995
BACTERIAL SPORES			
Bacillus subtilis	78	4	Chang *et al*, 1995
	50	3	Sommer *et al*, 1996
	60	4	Nicolson & Galeano, 2003
Bacillus anthracis	60	3.5	Nicolson & Galeano, 2003
VIRUSES			
Poliovirus	20	3	Chang *et al*, 1995
Rotavirus	25	3	Chang *et al*, 1995
S. aureus phage	30	3.5	Sommer *et al*, 1996
PROTOZOA			
Acanthamoeba cysts	70	2	Chang *et al*, 1995
Cryptosporidium parvum oocysts	3	3	Shin *et al*, 2001
Encephalitozoon intestinalis spores	3	2	Huffman *et al*, 2002

Tables 2.10 gives examples of microorganisms inactivated through exposure to continuous UV irradiation. Differences in inactivation dose for the same organism between studies are due to experimental parameter variations (for example, exposure distance, initial inoculum size, differences in lamp output intensity).

For inactivation by PUV-illumination, material is treated with intense, short duration pulses of broad-spectrum white light, which is rich in germicidal UV-C wavelengths. Material to be treated is exposed to pulses, each with a dose in the range of approximately 0.01 to 50 J/cm^2. The applied pulses can range in duration from 1 μs to 0.1 s, and are applied with a pulse repetition rate (PRR) of typically 1 to 20 pulses per second, resulting in the extremely rapid inactivation characteristic of this PUV technology [US FDA, 2000].

Research using PUV-light is relatively new in comparison to that with continuous UV-light, but its potential as a rapid sterilisation technology has led to considerable interest in its industrial and commercial applications. A number of published studies demonstrate the efficacy of PUV-light for the inactivation of bacteria, fungi, viruses, protozoa and yeasts [Lani *et al*, 2006; Lamont, 2005; Wang *et al*, 2002; Anderson *et al*, 2000; Rowen *et al*, 1999; MacGregor *et al*, 1998]. Results from these studies state the pulse number and associated $\log_{10}$ reduction, but in general, data regarding the dose are not stated, therefore preventing comparison with other studies – but still emphasising the potential for rapid inactivation. Factors such as different exposure distance greatly affect the dose required for microbial inactivation, and this may explain the range of pulse numbers required for inactivation.

A number of other studies have been published highlighting the efficacy of PUV illumination. A patent filed by Dunn et al [1988] claimed a range of organisms including *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Saccharomyces cerevisiae* could be inactivated by exposure to 1 to 35 pulses of light, with doses ranging from 1-2 J/cm^2. McDonald *et al* [2000] demonstrated that *Bacillus subtilis* spores could be inactivated, both in aqueous suspension and on surfaces, with a dose of 44 mJ/cm^2 resulting in a 5.5-$\log_{10}$ reduction, and an

8 mJ/cm^2 dose a 3-log_{10} reduction, respectively. A study by Takeshita *et al* [2003] showed that a 5-log_{10} reduction of *Saccharomyces cerevisiae* could be achieved using a dose of 3.5 J/cm^2 (5 light pulses).

Due to the interest in the use of PUV irradiation for applications, such as food treatment (thus extending shelf-life) or sterilisation of packaging, studies have also been carried out using a range of food-stuffs and materials rather than laboratory media, with very successful results [US FDA, 2000].

Chapter 3

MICROBIOLOGICAL SYSTEMS AND TECHNIQUES

3.0 GENERAL

This chapter details the microorganisms, culture media and equipment used throughout the research.

3.1 MICROORGANISMS

This section details the bacterial strains used throughout the experimental work and describes the procedures involved in the culturing and maintenance of these strains.

3.1.1 Bacterial Strains

The bacterial strains used are listed in Table 3.1. Cultures were obtained from the National Collection of Type Cultures, NCTC, (Collindale, UK), the Laboratorium voor Microbiologie, Universiteit Gent, LMG, (a section of The Belgian Co-ordinated Collections of Micro-organisms, BCCM) and MicroBiologics Inc. (Minnesota, USA). A clinical isolate was also obtained from Glasgow Royal Infirmary. A range of *Staphylococcus aureus* strains was used, two methicillin susceptible (MSSA), and two methicillin resistant (MRSA). A hospital-acquired MRSA isolate was included so as to investigate the effectiveness of the developed disinfection technologies on an actual epidemic strain.

3.1.2 Culturing and Maintaining Bacterial Cultures

Upon receipt of a bacterial strain from the culture collection, the bacteria must be reconstituted, by inoculation into an appropriate broth, and put onto Microbank™ beads (ProLab Diagnostics) for storage at minus 70°C. For regular use of a bacterial strain, an inoculated bead was removed from the Microbank system and streaked

onto an agar plate – the choice of medium being determined by the organism being cultured. The streaked plate was incubated at an appropriate temperature and duration before being sub-cultured and incubated on an agar slope. This slope was stored at 4°C and used as a regular source of inoculum. The bacterial strain was re-streaked onto a fresh agar slope every 4-5 weeks and its purity checked by Gram staining (section 3.6.1) and visual identification under the microscope.

Culturing of a bacterial strain for experimental use involved aseptically extracting a loopful of organism from the agar slope (stored at 4°C) and inoculating 100 ml nutrient broth. This broth was then incubated at 37°C for 18 hours to provide a population of approximately 10^9 colony-forming units per millilitre (CFU/ml). The inoculated broth was then centrifuged and diluted to the population required.

TABLE 3.1 *Bacterial strains used*

MICROORGANISM	SOURCE	COLLECTION NUMBER	ADDITIONAL COMMENTS
Staphylococcus aureus	NCTC	4135	n/a
Staphylococcus aureus	LMG	8064	n/a
Staphylococcus aureus	LMG	15975	Methicillin & oxacillin resistant
Staphylococcus aureus	Clinical isolate from a wound (Royal Infirmary, Glasgow)	16a	Methicillin resistant
Staphylococcus epidermidis	NCTC	7944	Reclassified as *Staphylococcus hyicus*
Escherichia coli	NCTC	9001	n/a
Streptococcus pyogenes	NCTC	8198	n/a
Enterococcus faecalis	University Microbiology Teaching Labs	Unknown	n/a
Clostridium perfringens	MicroBioLogics Inc	13124	n/a

3.2 MEDIA

Culture media were prepared as required by dissolving the appropriate weights in distilled water. Sterilisation of the media was then achieved by autoclaving at 121°C for 15 minutes (unless otherwise stated).

3.2.1 Broths and Agars

Broths

- NUTRIENT BROTH [CM1] (Oxoid Ltd., Basingstoke, UK) 13 g/L
- BRAIN HEART INFUSION BROTH [CM225B] (Oxoid Ltd.) 37 g/L
- THIOGLYCOLLATE MEDIUM U.S.P. [CM173] (Oxoid Ltd.) 29.5 g/L

Agars

- BLOOD AGAR [CM055B] (Oxoid Ltd.) 40 g/ L
  After cooling, defibrinated horse blood [SR0050C] (Oxoid Ltd.) was added to make a 7% blood concentration. (35 ml blood : 500 ml agar)
- BRAIN HEART INFUSION AGAR (Oxoid Ltd.)
  As for BHI broth (37 g/L), but with the addition of 1% AGAR BACTERIOLOGICAL (AGAR No. 1) [LP011B] (Oxoid Ltd.)
- CHOCOLATE BLOOD AGAR (Oxoid Ltd.) 40 g/ L
  As Blood Agar, except steamed for a few minutes after addition of blood.
- MILK AGAR [CM0021B] (Oxoid Ltd.) 24 g/ L
- NUTRIENT AGAR [CM0003B] (Oxoid Ltd.) 28 g/ L
- THIOGLYCOLLATE AGAR (Oxoid Ltd.)
  As for Thioglycollate broth (29.5 g/L), but with the addition of 1% AGAR BACTERIOLOGICAL (AGAR No. 1) (Oxoid Ltd.)
- TRYPTONE SOYA AGAR [CM0131B] (Oxoid Ltd.) 40 g/ L

After autoclaving, the molten agar was allowed to cool to 48°C, and was maintained at this temperature in a water bath until poured into either 90 mm single vent Petri dishes or Universal tubes to prepare agar plates and agar slopes, respectively.

3.2.2 Diluents and Reagents

- PHOSPHATE BUFFERED SALINE [BR0014G] (Oxoid Ltd.) 1/100 ml
  Dispensed into either 9 ml volumes (for use in preparation of serial dilutions) using a Brand Dispensette II Dispenser, or 100 ml volumes for bacterial pellet re-suspension. These were then autoclaved at 115°C for 10 minutes.
- HUGH & LEIFSON MEDIA (for preparation of approximately 60 test tubes)
  1 g peptone, 2.5 g sodium chloride, 0.15 g dipotassium hydrogen orthophosphate, 1.5 g agar and 2.5 ml 1% bromothymol blue solution were dissolved in 500 ml distilled water. This was autoclaved at 121°C for 15 minutes, then cooled to 50°C before adding 25 ml 20% glucose solution. The medium was then mixed and distributed into sterile test tubes.
- GRAM STAIN REAGENTS
  Crystal Violet, Lugols Iodine, Ethanol and Safronin
- OXIDASE STICKS [BR064A] (Oxoid Ltd.)
- API STAPH (BioMérieux, Basingstoke, UK)
- API 20 NE (BioMérieux)
- CATALASE (Sigma-Aldrich, Steinheim, Germany)
- ASCORBIC ACID (Sigma-Aldrich)
- 1,3-DIMETHYL-2-THIOUREA (DMTU) (Sigma-Aldrich)

3.3 BACTERIAL ENUMERATION

In bacterial inactivation studies, the bacteria must be accurately enumerated before and after treatment so as to achieve the exact decrease in population size. For enumeration, the samples must be plated onto agar and incubated at a suitable temperature and duration, usually 37°C for 24 hours. If samples are estimated to be too numerous for counting they must first be diluted before the plating procedure is carried out. After incubation, the plate count is recorded in the standard counting unit, **colony-forming units per millilitre** (CFU/ml) rather than the number of viable cells, since a colony may have arisen from more than one cell.

This section details the preparation of serial dilutions and the plating techniques used to enable accurate enumeration of the microorganisms.

3.3.1 Serial Dilutions

Serial dilutions were prepared by adding 1 ml of undiluted bacterial suspension to 9 ml phosphate buffered saline (PBS), thus giving a 10^{-1} dilution. This was then thoroughly mixed using a Whirly mixer (FisherBrand), which ensured uniform mixing of the cell suspension. 1 ml of this 10^{-1} suspension was in turn added to a further 9 ml volume of PBS – giving a 10^{-2} dilution, and so on until the desired dilution factor was achieved.

3.3.2 Plating Techniques

After light treatment of the bacterial sample, it was necessary to plate out either the neat treated sample or dilutions of this sample, depending on the expected number of surviving colonies. Three main plating methods were employed and the appropriate method was selected, again depending on the expected surviving population.

3.3.2.1 Spiral Plate Method

This is an automatic method which uses a WASP 2 spiral plater (Don Whitley Scientific Ltd., Shipley, UK), a photograph of which is shown in Figure 3.1. The spiral plater works by dispensing 50 µl of a liquid bacterial sample onto the surface of a rotating agar plate. Deposition of the sample is logarithmic, and in the shape of an Archimedes spiral (see Figure 3.2). After incubation, the colonies were enumerated either manually using a colony counter or on a PC using the ACOLYTE software package. Both methods involved centring a counting grid over the plate: each marked grid segment corresponding to a known constant volume of deposited sample, so that the number of CFU/ml in the sample could be calculated by reference to either the supplied charts (if the manual colony counter is used) or the ACOLYTE software (if using the PC and software package).

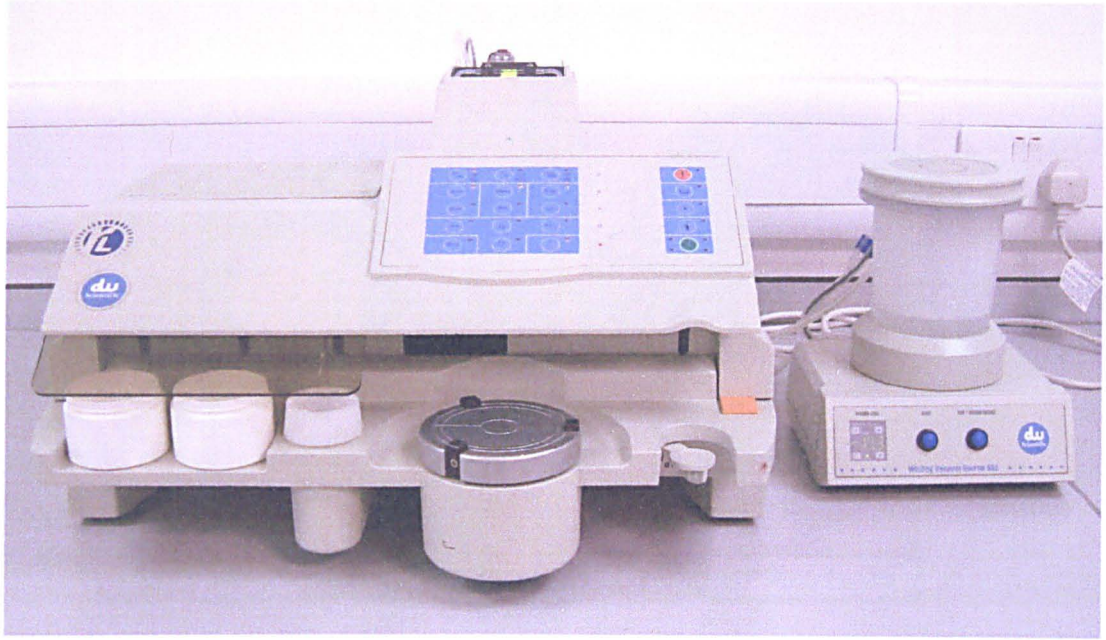

FIGURE 3.1 *WASP 2 Spiral Plater*

3.3.2.2 Spread Plate Method

Spread plates were prepared in two ways, automatically using the spiral plater or manually. When manually prepared, 100 µl of bacterial sample was pipetted onto an agar plate and, using an L-shaped spreader, evenly distributed over the surface of the plate. The plate was left until the sample had dried into the agar before incubation. Automatically prepared spread plates were done using the spiral plater set at 100 µl sample size and linear distribution mode. Figure 3.2 shows a visual comparison of logarithmic spiral plates and linear spread plates as prepared using the WASP 2 spiral plater. After incubation, a colony count of the entire plate was obtained and multiplied by ten to obtain the number of CFU/ml of sample.

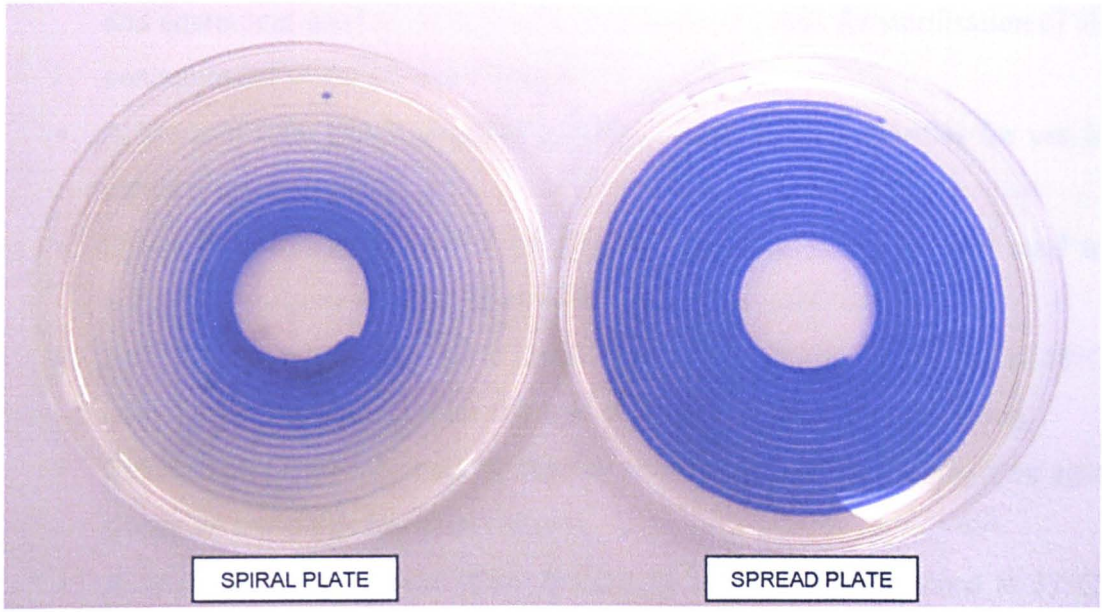

FIGURE 3.2 *Examples of plating methods of the WASP 2 spiral plater: Spiral plate – logarithmic sample distribution; Spread plate – linear sample distribution.*

3.3.2.3 Pour Plate Method

If the sample was expected to contain less than 250 CFU/ml, the pour plate method was used. For this, 1 ml of undiluted sample was pipetted into an empty sterile Petri dish. Approximately 20 ml of molten agar was then poured onto the sample and the plate gently rotated clockwise (x10) and then anticlockwise (x10), to ensure thorough mixing of the suspension. The plate was then left to solidify before incubation. Enumeration involved taking an entire plate count to obtain the number of CFU/ml of sample.

3.4 OTHER EQUIPMENT

This section gives brief descriptions of other equipment used for microbial analysis.

- A Kestrel automatic autoclave (LTE Scientific) and a bench-top autoclave (Dixons Surgical Instruments Ltd.) were used for the sterilisation of all media

and equipment used in the experimental work, and also for sterilisation of all contaminated waste prior to disposal.

- A Merit W4000 Distil provided distilled water free of impurities for use in media preparation.
- OHAUS Navigator and OHAUS Adventurer digital balances were used to accurately measure out quantities of media and chemical reagents.
- A Grant Water-bath (Scientific Laboratory Supplies) was maintained at 48°C and used to cool and preserve the agar in a molten state prior to pouring.
- Refrigerators (Lec Medical) maintained at 4°C were used for storage, on agar slopes, of the viable bacterial cultures.
- A rotary shaker incubator (New Brunswick Scientific) maintained at 37°C, was used for the overnight culture of inoculated broths. The shaking motion of the incubator ensured continual aeration of the culture, creating more optimal growth conditions compared to static incubation.
- A Heraeus Labofuge 400R Centrifuge (Kendo Laboratory Products) and 50 ml centrifuge tubes (Nunc) were used to spin down the incubated broths at 4300 rpm for 10 minutes. The resulting supernatant was then discarded and the bacterial pellet re-suspended in an appropriate diluent, usually PBS.
- A Sciquip 1-15K centrifuge (Sigma) and Eppendorf tubes were used to centrifuge smaller volumes of bacterial suspensions. Centrifuging was carried out at 7800 rpm for 5 minutes.
- Gilson Pipettes (100 µl, 1 ml, 5 ml and 10 ml) and sterile pipette tips were used for the sterile transfer of bacterial suspensions and preparation of dilutions.
- A Nikon Eclipse E400 Microscope was used to examine bacterial culture purity and cell morphology. A Nikon Coolpix 4500 digital camera could also be attached to the microscope to allow the microorganisms to be photographed.
- A Biomate 5 UV-Visible Spectrophotometer (Thermo Spectronic) was used to obtain absorbance and transmission readings of bacterial suspensions at either fixed wavelengths or over a scan of wavelengths, usually 220 nm –

700 nm. Quartz cuvettes were used for sample containment rather than the regular plastic cuvettes, as they allow the transmission of UV wavelengths.

- Incubators (LTE Scientific) set at 30°C and 37°C were used for the overnight incubation of agar plates. The incubation temperature was selected depending on the organism being incubated; for example, fungal samples are routinely incubated at 30°C and bacterial samples at 37°C. This incubation period allowed sufficient growth of the microorganism so that colony-forming units could be readily counted.
- Plastic anaerobic jars (Oxoid Ltd.) were used in conjunction with AnaeroGen sachets (Oxoid Ltd.) – 1 sachet/jar – to create anaerobic environments for the culture of anaerobic organisms.

3.5 MICROBIOLOGICAL AND BIOCHEMICAL TESTS

A selection of routine microbiological and biochemical tests was used throughout for the identification of bacterial isolates.

3.5.1 Gram stain

Staining methods were used to aid the microscopic identification of microorganisms. There are a number of staining procedures. The Gram stain allows differentiation between Gram-positive and Gram-negative bacteria – the initial step of bacterial identification.

Preparation of smear for staining

Using a wire loop, 1-2 colonies of the organism were lifted from an agar plate (or a loopful of suspension if using liquid culture) and emulsified with a drop of water on a microscope slide. After being allowed to air dry, the sample was fixed by passing the slide through a Bunsen flame a few times.

Staining procedure

The sample was covered with Crystal violet for approximately 30 seconds. The sample was drained and rinsed with Lugol's iodine, covered with fresh iodine and left for 1 minute. It was then drained and rinsed with ethanol, covered with

Safronin and left for approximately 30 seconds. Finally, the sample was rinsed with water and blot dried. It was viewed under an oil immersion lens on a microscope.

Gram-positive cells stain purple. This is due to retention of the crystal violet within the pores of the thick peptidoglycan layer of the cell wall after dehydration (caused by the alcohol washing stage) triggers closure of the pores. In contrast, Gram-negative cells have a thin peptidoglycan layer surrounded by a lipid-rich outer membrane which allows solvent passage, enabling easy removal of the crystal violet stain. The addition of the safronin causes the Gram-negative cells to stain pink.

3.5.2 Catalase Test

This biochemical test was used to determine the presence of the catalase enzyme within the bacterium. A small volume of hydrogen peroxide (~1-2 ml) was dispensed onto a nutrient agar slope of the organism. If catalase was present, the hydrogen peroxide splits into hydrogen gas and oxygen. This positive reaction was demonstrated by the formation of bubbles on the agar surface.

3.5.3 Oxidase Test

The oxidase test determines the presence of the enzyme cytochrome oxidase within bacteria. For this test, oxidase sticks, which have a reactive end impregnated with N-N-dimethyl-p-phenylenediamine oxalate, ascorbic acid and α-napthol, were used. The reactive end of the stick (which is light grey/brown in colour) was dipped into a bacterial colony on an agar plate and after 3 minutes a positive reaction is shown by a colour change to dark brown/purple. A negative reaction corresponds to no colour change.

The Gram stain, catalase and oxidase tests, along with colony morphology, are important and commonly used reactions for the preliminary identification of bacterial cultures.

3.5.4 Hugh and Leifson Test

This test was used to determine two properties of the inoculated bacterium:

- whether it was capable of fermenting glucose, and,
- whether it was an obligate aerobe, facultative anaerobe or obligate anaerobe

(The aerotolerance of non-glucose fermenters cannot be determined by this method because of the need for a colour change for result interpretation.)

The method involved inoculating the sloppy agar medium with a loopful of the organism via a stabbing method to the base of the test tube. This was then incubated at 37°C for 24 hours.

After incubation the results were analysed as follows:

- A colour change in the medium from blue/green to yellow indicated glucose fermentation. (The colour change was the result of acid production during the fermentation process.)
- If the yellow colouration was evident only at the top of the test tube, showing that the organism was only capable of growth in the oxygen-rich environment near the surface, the organism was determined to be an obligate aerobe.

  If the yellow colouration was evident throughout the length of the test tube, showing that the organism was capable of growth from the oxygen-rich surface through to the oxygen-deficient base, the organism was a facultative anaerobe.

  If the yellow colouration was only evident at the base of the test tube, showing that the organism was only capable of growth in the oxygen-deficient environment, the organism was an obligate anaerobe.

3.5.5 API Identification Tests

API Strips (BioMérieux, Basingstoke, UK) are bacterial identification systems which use miniaturised biochemical tests and a database. Each strip has a number of microtubules containing dehydrated substrates, which are reconstituted by the addition of the unknown bacterial-cell suspension. Following addition of the suspension, the strip is incubated, and it is during this period that the metabolism of

the bacterium brings about substrate colour changes – either spontaneous or following the addition of reagents – and the results of these individual tests provide identification of the microorganism through the use of the identification software.

The appropriate API strip must be selected when attempting to identify an unknown microorganism, with selection based on initial microbiological and biochemical tests such as the Gram stain and the oxidase tests.

Two types of API strips were used;

- API Staph: Used for the identification of *Staphylococcus*, *Micrococcus* and *Kocuria*
- API 20 NE: Used for the identification of Gram-negative non-Enterobacteriaceae

Chapter 4

MONITORING AND PULSED UV TREATMENT OF INDOOR AIR

4.0 GENERAL

Indoor air quality (IAQ) is an increasingly important issue in a wide variety of industrial, domestic and social settings. Although the transmission of infectious aerosols is a major concern in the hospital environment due to infections manifesting rapidly in immunocompetent individuals, other less well-documented situations must also be taken into consideration.

In areas where large numbers of people are brought together in close proximity, such as in universities/schools, cinemas and on aeroplanes, there is the potential for the build-up of substantial airborne contamination and therefore cross-infection. Although the majority of people in these areas are immunocompetent, there is still the possibility of the transfer of infectious aerosols between individuals and for these microorganisms to manifest within the individual, causing the onset of an infection.

Another reason for the importance of air quality control is the emergence of 'Sick Building Syndrome', caused by long-term exposure to allergens and pollutants, which can become a problem in workplaces and housing if appropriate air quality control measures are not implemented.

Two main areas of investigation are described in this chapter:

1. Microbial air quality within university lecture theatres, and
2. Application of a pulsed ultra-violet (PUV) light system for air disinfection

University lecture theatres were selected as suitable sampling sites within the study due to their availability throughout the university, in addition to the fact that they are regularly occupied by sizeable groups of students.

The originality of the work in this chapter involves the progression from investigating the bacterial contamination of a university lecture theatre to the development and testing of a pulsed UV disinfection treatment for the air of this lecture theatre. Results demonstrate the effectiveness of PUV light as a means of air disinfection.

4.1 SAS SUPER 180 AIR SAMPLER

As discussed in Section 2.3, there are several methods for the enumeration of airborne microorganisms and, for this research, the impaction method was determined as being the best method. There are a number of impaction air samplers on the market, all working by the same principle – that air is aspirated in a laminar flow pattern with sufficient velocity to impact organisms onto an agar surface – and the SAS Super 180 Air Sampler (Cherwell Laboratories Ltd., Bicester, UK), pictured in Figure 4.1, was selected as being the most suitable device. The reasons for this selection are discussed in section 4.2.1.

4.1.1 Selection of Air Sampler

In addition to using the preferred impaction method, the SAS Super 180 has a number of benefits over others available on the market:

- Established international standard in portable air sampling
- Portable and convenient
- Light and robust
- Provides data on programmable time between aspirations, volume of air for each aspiration, and total air volume to be sampled

- Short sampling time and high flow rate (1000 L sampled in <6 minutes and 180 L/min, respectively)
- The standard samplers detect 5-20 microns optimum

Due to the importance of air-quality issues within hospital environments, it was also important to select a method of sampling that mirrors that used within these situations. The Andersen cascade impactors are the preferred samplers employed by hospitals for environmental monitoring but are generally much less portable, and hence inconvenient for discrete sampling. The selection of the SAS Super 180 sampler allowed the impaction method to be employed but with a much more portable model.

FIGURE 4.1 *SAS Super-180 Air Sampler*

4.1.2 Use of the Air Sampler

The SAS Super-180 sampler works on the principle that air is aspirated at a fixed speed for variable time through a cover which has been machined with a series of small holes of special design (Figure 4.2). The resulting laminar airflow is directed onto the agar surface of a "Contact Plate" containing medium consistent with the microbiological examination to be performed. When the preset sampling cycle is completed, the plate is removed and incubated. The organisms are then visible to the naked eye and can be counted for an assessment of the level of contamination [Cherwell Laboratories Limited, 2002].

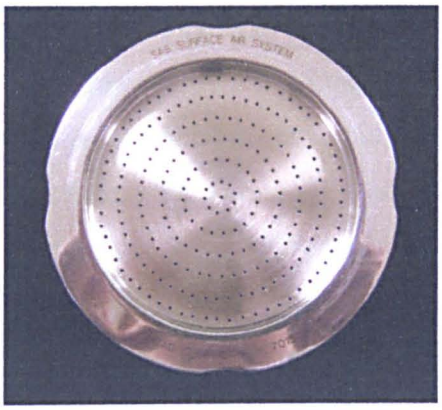

FIGURE 4.2 *Air sampler head with 219 specially machined holes*

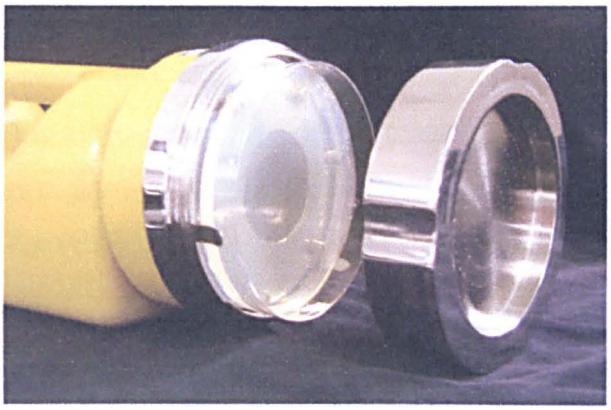

FIGURE 4.3 *Placement of contact plate/Petri dish within air sampler*

A detailed description of the method of operation and interpretation of results is discussed below.

Operation

The sampler is switched and programmed to the required sample volume/time. The sampler is positioned in the required sampling location, either free-standing or fixed on a tripod. An agar plate, containing medium selected according to the type of microorganism being detected, is placed into the sampler (see Figure 4.3). The lid of the Petri dish is removed, and immediately, the perforated head is put in place. Sampling is started.

As mentioned before, during sampling, a laminar airflow is aspirated through the perforated sampler head and, due to their weight, the airborne particles impact onto the agar surface, while the air continues to flow around the plate and exit via outlets at the base of the sampling head.

On completion of sampling, the perforated head is removed and the lid replaced on the plate. The plate is then incubated, the temperature and duration of incubation being determined by the nature of the organisms being examined. The perforated head is then cleaned by either autoclaving or using alcohol wipes.

Interpretation of Results

The number of colony-forming units (CFU) counted on the surface of the plate must be corrected for the statistical probability of multiple particles passing through the same hole. This correction is referred to as the probable count (Pr) and is found using the statistical reference tables in Appendix A.

The probable count (Pr), is then used to calculate the CFU/1000 litres of air sampled using the following equation:

$$X = \frac{Pr \times 1000}{V}$$

Where; V = volume of air sampled

Pr = probable count

X = colony-forming units per 1000 litres (=1 m^3) of air

4.2 AIR QUALITY IN UNIVERSITY LECTURE THEATRES

The aim of this section was to investigate the air quality in university lecture theatres. Sampling was carried out after different durations of consecutive lectures and in a variety of different lecture theatres.

Similar work investigating the indoor air quality of educational establishments – mainly schools – has previously been carried out, and they conclude that during occupation of the room there is a significant increase in the airborne bacterial population [Branis *et al*, 2002; Sessa *et al*, 2002], and this level is influenced by a number of environmental and structural factors [Bartlett *et al*, 2004] which will be discussed in Section 4.4.

It was important for this study to establish the occurrence of increased bacterial contamination levels in the lecture theatres, because confirmation of this allowed the work to be extended to cover investigation of the treatment of the contaminated lecture theatre air using pulsed UV-light.

4.2.1 Different Durations

Measurements of air quality were made in a lecture theatre occupied by students over periods of 2, 3, 4 and 6 hours. A plan of this lecture theatre is shown in Figure 4.4. The lecture theatre has a volume of 400 m^3 and a seating capacity of 174, and its estimated air change rate is 12.6-air changes per hour. Samples of air were taken at the beginning of each period, at the conclusion of each period, and 30 minutes after the conclusion of each period. Throughout each 1-hour period, lecture theatre occupancy was over 75%, except during the 5-minute changeovers of personnel which took place at hourly intervals. The SAS Super-180 sampler was used to collect the bacterial air samples onto NA plates. The sampler was programmed to collect volumes of 180 litres and six replicate samples were taken at each sampling time. The NA plates were incubated at 37°C for 48 hours before enumeration. The results obtained were then used to calculate the mean CFU/m^3 of air. Hourly samples were also taken when the lecture room was unoccupied to establish typical background levels, and these were used as control data.

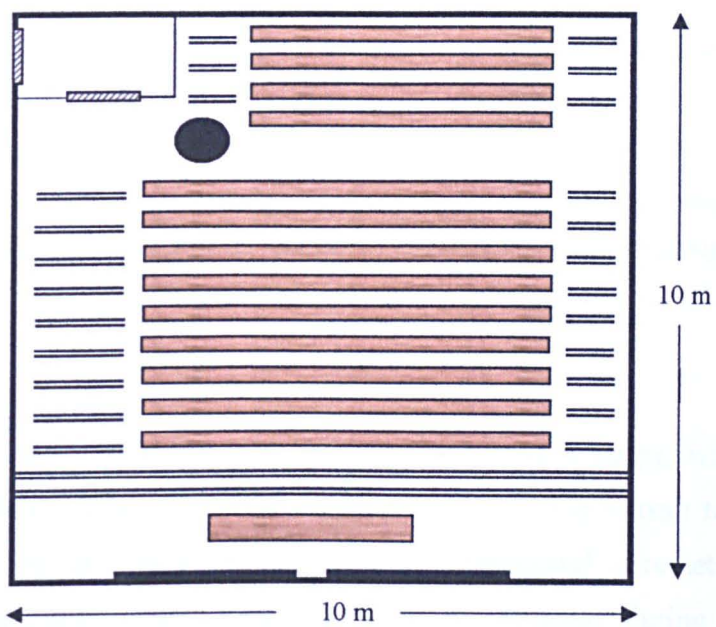

FIGURE 4.4 *Lecture theatre plan.*

(= stairs; = desks; = doors; ● = sampling position)

Figure 4.5 shows the results recorded for bacterial levels in the sampled lecture theatre, with the concentration in one case rising to 1173 CFU/m^3 after 4 hours of lectures. A general trend can be seen which shows that as the number of consecutive lecture periods increases, so to does the concentration of airborne bacteria.

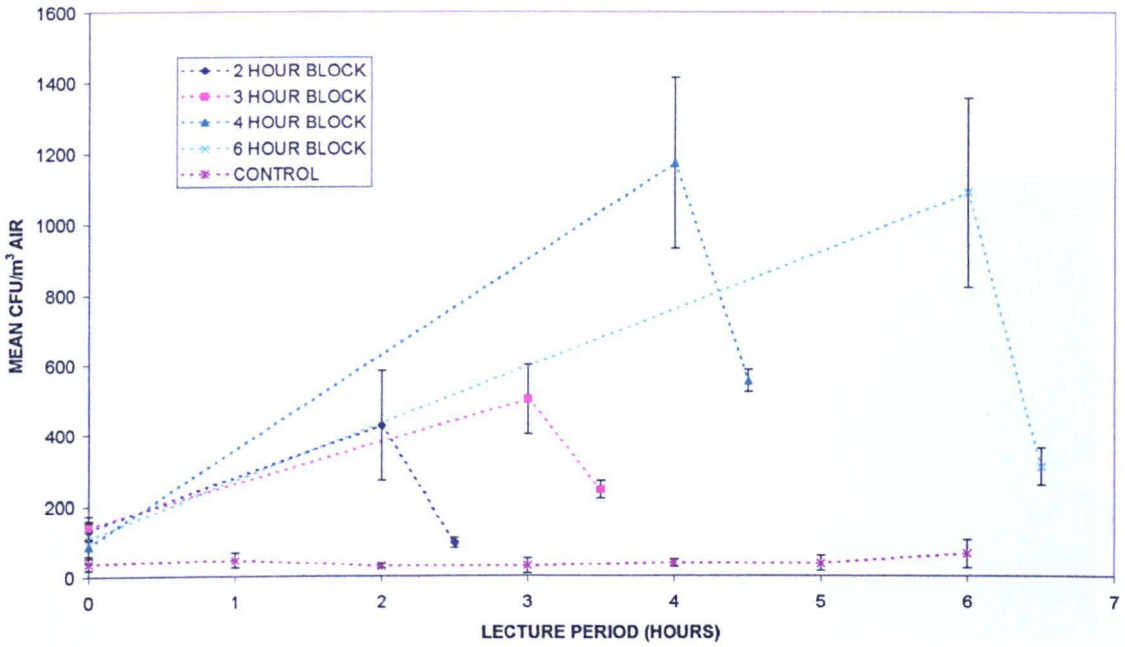

FIGURE 4.5 *Levels of airborne bacteria in a selected lecture theatre (Fig 4.7) before (0 h) and after different durations of consecutive lectures, followed by levels monitored 30 minutes after the lecture theatre was vacated.*

An exception to this is the airborne bacteria level after 6 hours, which was not significantly different from that obtained after 4 hours. The reason for this is not clear but could be due to changes in the environmental parameters or other interdependent stresses which the bacterial aerosols encounter during their launch, aerial transport and subsequent deposition.

It may be that an equilibrium concentration is eventually reached as a result of room-ventilation air changes (12.6 air changes/hour in this case).

Again, it can be seen from the graph that the standard deviations of the results are, in some cases, quite large and as stated earlier this is due to the general variation that normally occurs when sampling air volumes.

The photographs of Nutrient agar (NA) plates in Figures 4.6a and 4.6b provide a visual comparison of bacterial colony-forming units present before and after a 1-hour lecture period.

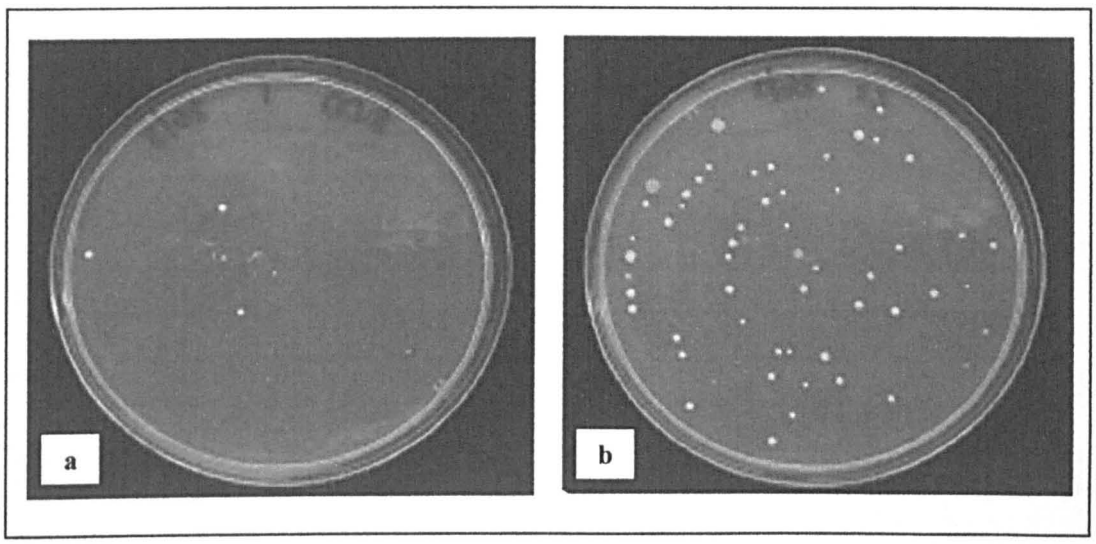

FIGURE 4.6 *A visual comparison of the colony-forming units present in 180 l of sampled air on incubated Nutrient agar plates before (a) and after (b) a 1-hour lecture.*

Another aspect that can be observed from Figure 4.5 is that during the 30-minute period after vacation of the lecture theatre, the bacterial numbers fall to values similar to, or not far above, those detected prior to the start of the lecture period. The control samples obtained from the unoccupied lecture theatre show a close to constant airborne bacteria level. Clearly, the presence of large congregations of students is the major contributor to airborne bacterial contamination in the lecture theatre.

4.2.2 Different Lecture Theatres

In addition to sampling over different lecture periods, air-quality monitoring was carried out in three different lecture theatres. The parameters of the sampled lecture theatres are shown in Table 4.1.

TABLE 4.1 *Lecture Theatre Parameters*

ROOM	CAPACITY	DIMENSIONS	VOLUME	AIR CHANGE RATE
1	252	14 x 14 m	800 m^3	9.1 ac/hr
2	350	18 x 14 m	1025 m^3	8 ac/hr
3	174	10 x 10 m	400 m^3	12.6 ac/hr

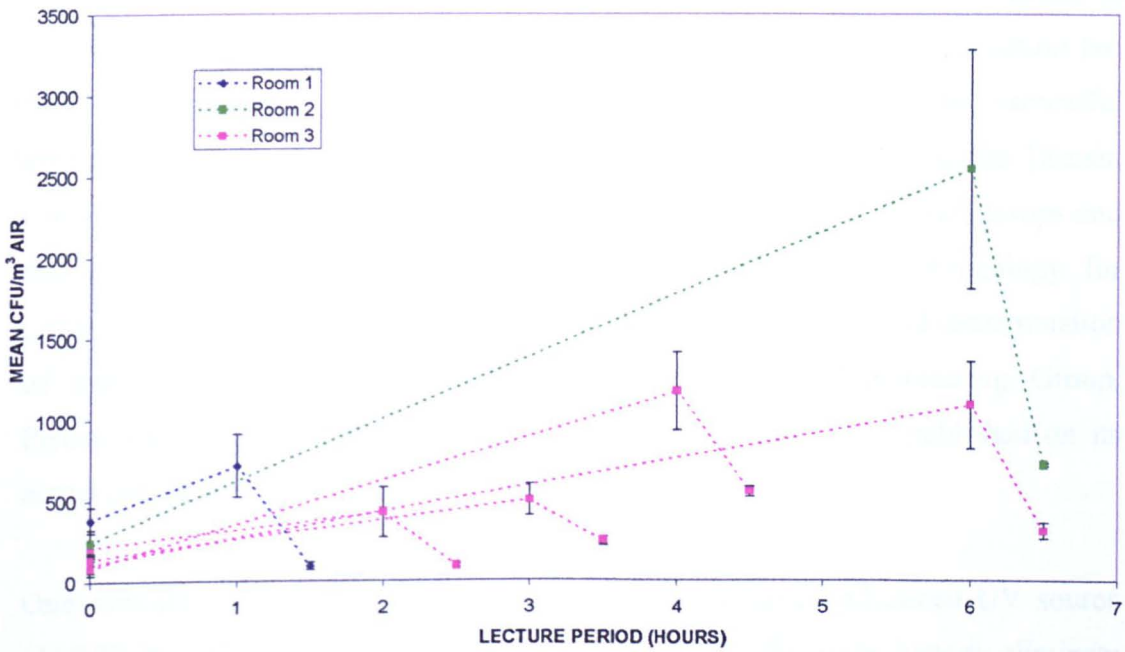

FIGURE 4.7 *Levels of airborne bacteria in different lecture theatres before (0 h) and after different durations of consecutive lectures, followed by levels monitored 30 minutes after the lecture theatre was vacated. Room 3 is the lecture theatre depicted in Figure 4.4 and its results are also observed in Figure 4.5.*

Figure 4.7 shows results obtained from sampling in the different lecture theatres. The same general trend as shown in Figure 4.5 is observed. As the duration of the lecture period increases, so too does the airborne bacterial contamination level. As can be seen from the graph, Room 2 shows particularly elevated bacterial levels in comparison to other values, with bacteria levels rising to >2500 CFU/m^3. Although this lecture theatre had the highest estimated air change rate, the reason for these high levels may be due to the larger volume and higher occupancy of the room compared to the other two.

4.3 PULSED UV LIGHT TREATMENT OF LECTURE THEATRE AIR

As discussed in Section 2.5.2.3, UV light for disinfection and sterilisation is increasingly being utilised in pulsed rather than continuous form. The benefits of rapid inactivation and treatment rates make this dissipation method very attractive to manufacturers and users alike. The use of continuous UV for air sterilisation has been in effect since 1909 [Kowalski and Bahnfleth, 2000] but the successful application of pulsed light for water, surface and packaging sterilisation [Xenon Corporation, 2006; Dunn *et al*, 1998] has led to discussions by research groups and manufacturing companies into the potential of this promising technology for providing an effective and rapid solution to the problem of microbial contamination of indoor air [Xenon Corporation, 2006; Aerobiological Engineering Group, Pennsylvania State University]. There has, however, been little published on its actual testing and application.

One available study, by Clark *et al* [2002], tested a pulsed advanced UV source (AUVS) for use within Heating Ventilation and Air Conditioning ducts to eliminate biological warfare agents. They demonstrated that this pulsed UV source was highly effective in destroying *Bacillus pumilus* spores (which are more UV resistant than anthrax spores) – with a 7-$\log_{10}$ reduction in population. This study also showed pulsed UV sources to be much more effective than continuous UV, High Efficiency Particulate Air (HEPA) filtration and Ultra-Low-Penetration-Air (ULPA) filtration.

They maintained their positive assessment by further stating that PUV has the added benefit that it destroys biological agents – including proteins which are the basis of biotoxins – rather than capturing and concentrating them.

In this study, a UV-light source and pulsed-power technology are combined to produce pulsed ultra-violet light. This application involves the storing of electrical energy in a capacitor over a relatively long period (usually milliseconds to seconds), and its subsequent dissipation over a comparatively short period – the duration of one pulse being of the order of microseconds. During each pulse, high levels of peak power are therefore generated, and treatment is effected through the application of a sufficient number of pulses. As a result of the pulsed-energy-delivery approach, the average power requirements for the system are very modest.

As discussed in Section 2.6.1, the microbial inactivation via UV-light treatment is a result of the biocidal wavelengths in the UV-region inducing DNA-based damage, including the formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6-4) photoproducts [Sinha and Häder, 2002]. These ultimately prevent the microorganism from further growth and replication.

Inactivation by PUV-light is also accredited to DNA-based damage, although a study by Wekhof [2003] suggests that pulsed illumination with UV fluxes over 2 kW/cm^2 causes, in addition to DNA damage, the rupture and disintegration of microorganisms due to the faster rate of absorption of the incident UV photons – essentially inducing a temperature rise. Wekhof also suggests that the calculated temperature rise for a treated microorganism during a single light pulse is an exponential function of the peak power, with a threshold peak existing, below which sterilisation is due only to the germicidal UV-C, and above which, all UV light contributes to rapid heating of the microorganisms in excess of 130°C – ultimately causing overheating and rupture.

The aim of this section was to investigate the use of a pulsed UV-rich light system for the treatment of contaminated lecture-theatre air samples. Included are details of

the pulsed light system, experimental techniques and subsequent identification of the bacterial isolates.

4.3.1 Pulsed Ultra-Violet Light Air Disinfection System

Figure 4.8 shows the main components of the pulsed UV-rich-light air-disinfection system in both photographic and diagrammatic form. The air sampler is used to draw air into the system via the inlet holes. This air passes along the L-shaped pipe while being exposed to pulses of UV-rich light (provided by the pulse generator and Xenon flashlamp), before impacting onto the NA sampling medium contained within the head of the sampler. The air then exits the system via the outlets below the air sampler head. Also, the L-shaped pipe has an inner lining of aluminium foil along the active length to increase reflectance, and the bend section is coated with matt black paint to prevent UV rays reflecting onto the sampling medium and thereby distorting the results.

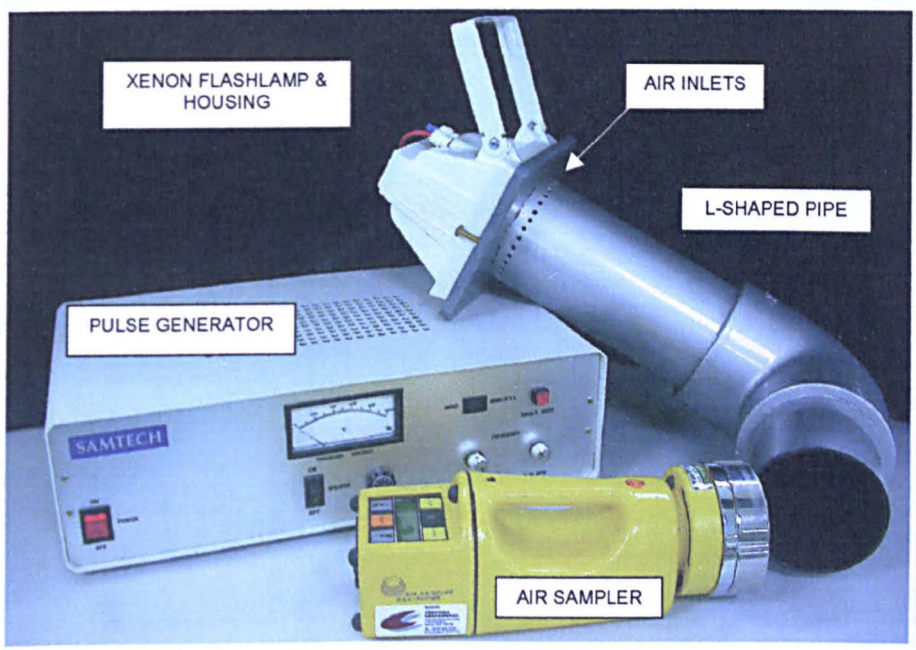

FIGURE 4.8a *Components of the Pulsed UV-rich light Air Disinfection System*

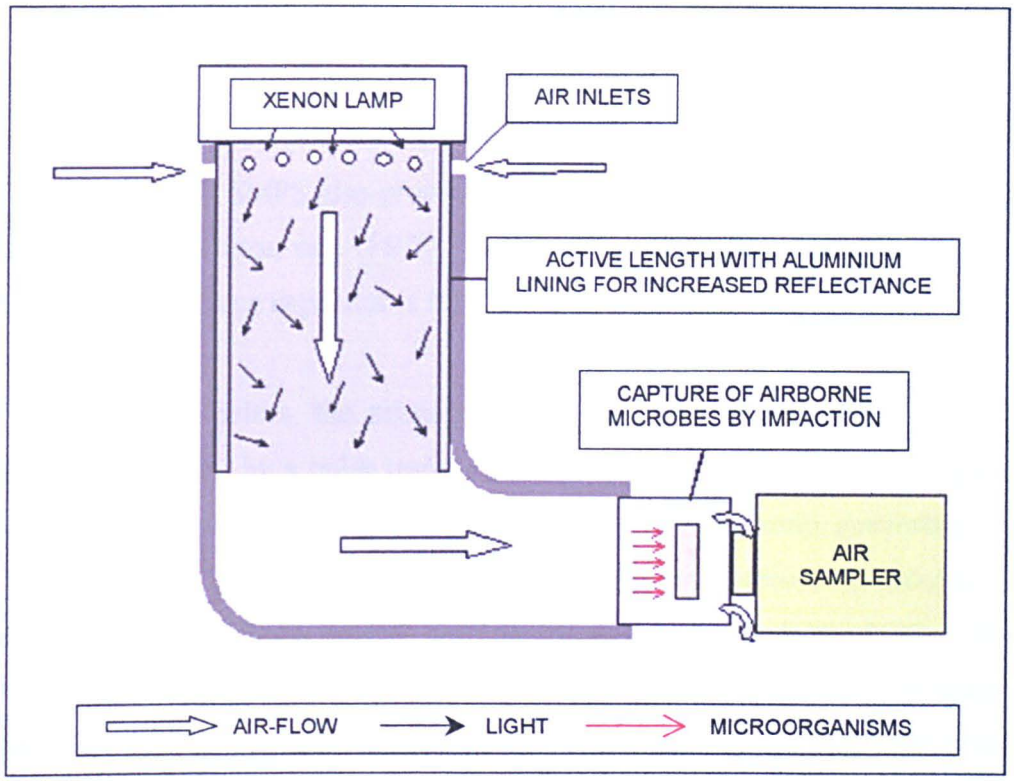

FIGURE 4.8b *Schematic diagram of the Pulsed UV-rich light Air Disinfection System. Highlighted in this diagram are the air-inlets, the aluminium lining of the active length of the system and the agar plate in the sampler head for the impaction of microorganisms.*

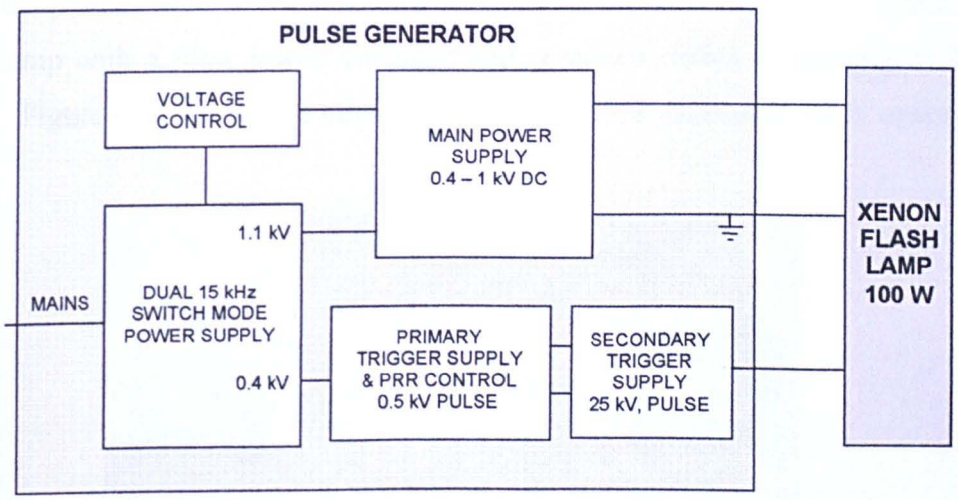

FIGURE 4.9 *Schematic diagram of the main components of the pulse UV generator and flashlamp*

A schematic diagram of the high-voltage pulse generator, which allows the supply voltage and pulse frequency to be controlled, is shown in Figure 4.9. A 15 kHz switch-mode power supply (SMPS) drives two major components of the pulse generator. It supplies 1.1 kV to charge a 40 μF storage capacitor within the main power supply. The SMPS also provides 0.4 kV to charge the primary trigger circuit and the pulse repetition rate (PRR) control. The voltage control switches off the SMPS once the storage capacitor is fully charged.

To generate light pulses, the primary trigger generates a 0.5 kV pulse, which is stepped up to 25 kV by a pulse transformer within the secondary trigger supply. A pulse of approximately 20 μs duration is emitted by the flashlamp, generating a peak power of 1 MW. The energy per pulse is 20 J when the system is operated at 1 kV. The pulse repetition rate (PRR) was set at 5 pulses per second (pps) for all experiments resulting in an average power consumption of 100 W. It was necessary to calculate the number of pulses that the microorganisms were subjected to during passage along the active section of the system. This was established as follows: The volume of the active section is 2.75 l, and the flow rate of the air sampler is 3 l/sec. Hence the exposure time to UV radiation for a volume of air is 0.92 s corresponding to 4 or 5 pulses, for a pulse rate of 5 pps.

The lamp used was a low pressure (450 torr), Heraeus Noblelight, xenon-filled flashlamp with a clear quartz envelope and is shown within its housing in Figure 4.10. Figure 4.11 shows the emission spectrum of the flashlamp when operating at 1 kV.

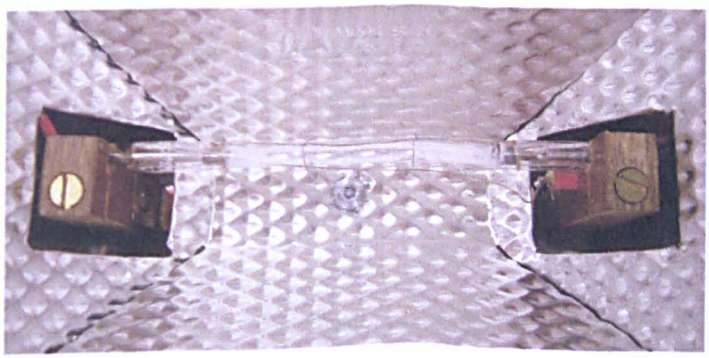

FIGURE 4.10 *Xenon flashlamp*

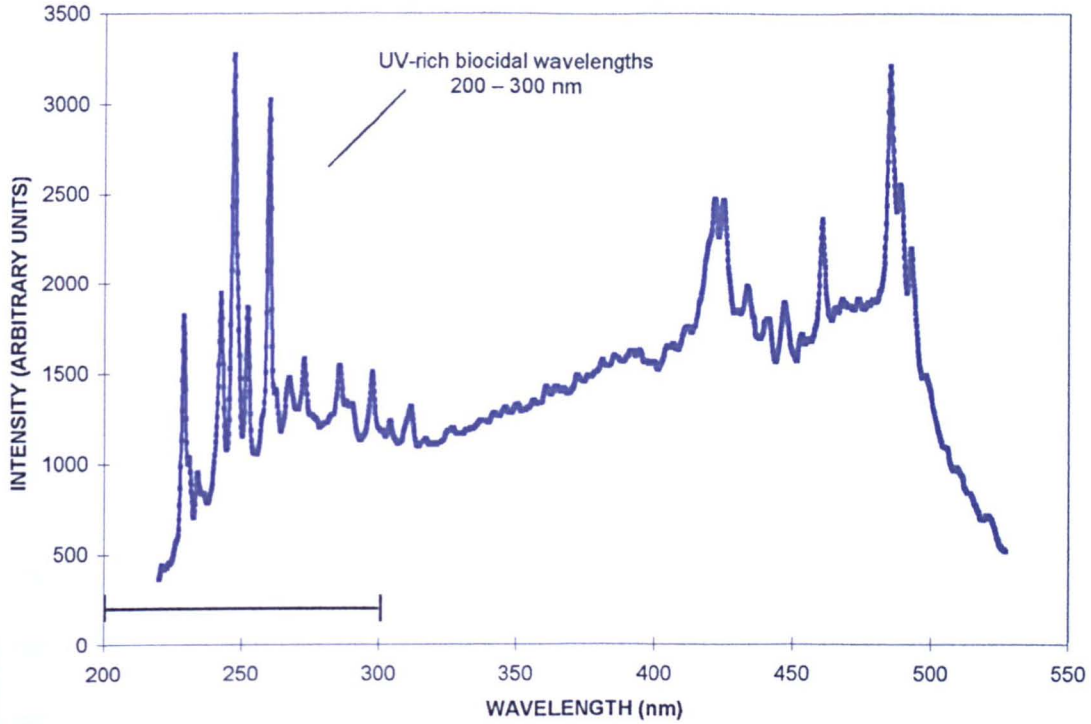

FIGURE 4.11 *Emission spectrum of Xenon flashlamp while operating at 1 kV, showing the UV-rich biocidal wavelength region.*

4.3.2 Treatment Method and Results

Immediately after the conclusion of a 1-hour lecture period, 180 litres of air were passed through the system (Figure 4.8) without samples being taken, allowing air samples representative of the surrounding environment to be drawn into the system. Triplicate air samples, each of 180 l, were then collected on NA plates; these represented non-pulsed samples. The pulsed light system was then switched on and the pulsing frequency was set at 5 pps, operating at 1 kV. 180 litres of air were again passed through the system before triplicate pulsed samples were taken. This procedure was repeated at 10 and 20 minutes later.

The results of the PUV treatment procedure are plotted in Figure 4.12. It can be seen that exposure to the pulsed-light treatment at the end of the 1-hour lecture period caused a reduction in airborne bacterial contamination, with the level decreasing from >540 CFU/1000 l to <105 CFU/1000 l. The CFU count for the second and third non-pulsed samples, which were taken at approximately 10-minute intervals,

were progressively lower – an effect that is also evident in Figures 4.5 and 4.7. This is likely the result of the absence of personnel, and consequently, the airborne bacterial population settling out of the air onto surfaces. The mean CFU counts for the pulsed air samples remain comparatively similar, ranging from 102 to 63 CFU/m^3 of air.

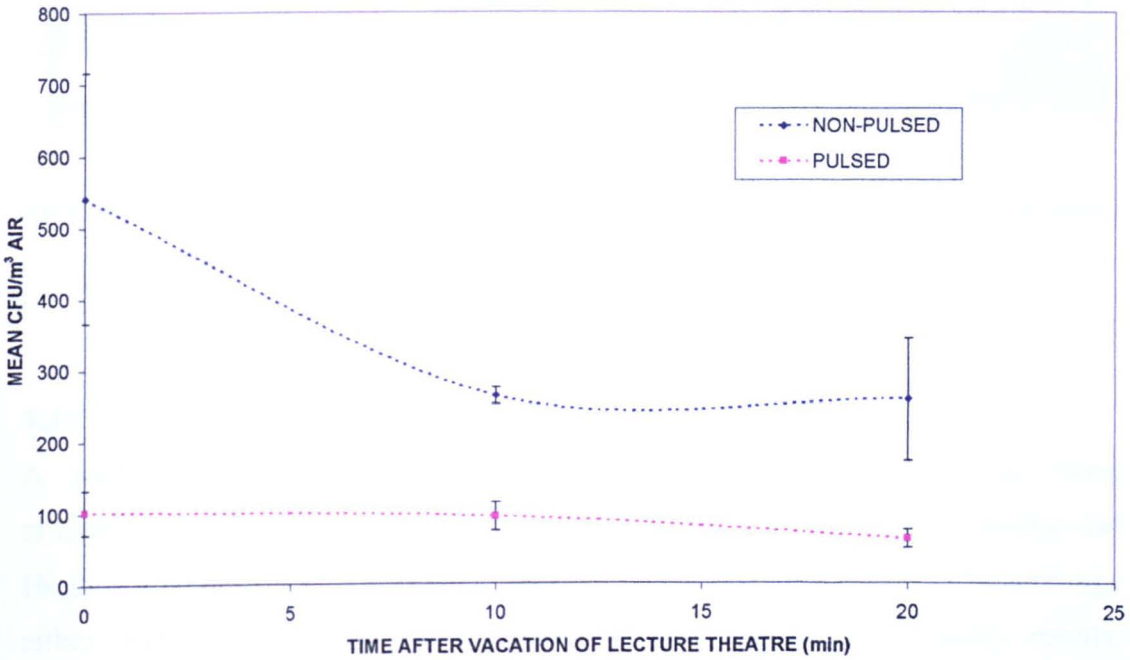

FIGURE 4.12 *Effect of pulsed light treatment on the airborne bacterial content of university lecture-room air.*

The photographs of NA plates in Figures 4.13a and 4.13b provide a visual comparison of bacterial colony-forming units present in non-pulsed and pulsed samples. It can be seen that pulsed-light treatment has significantly reduced the number of CFU/180 litres of air sampled.

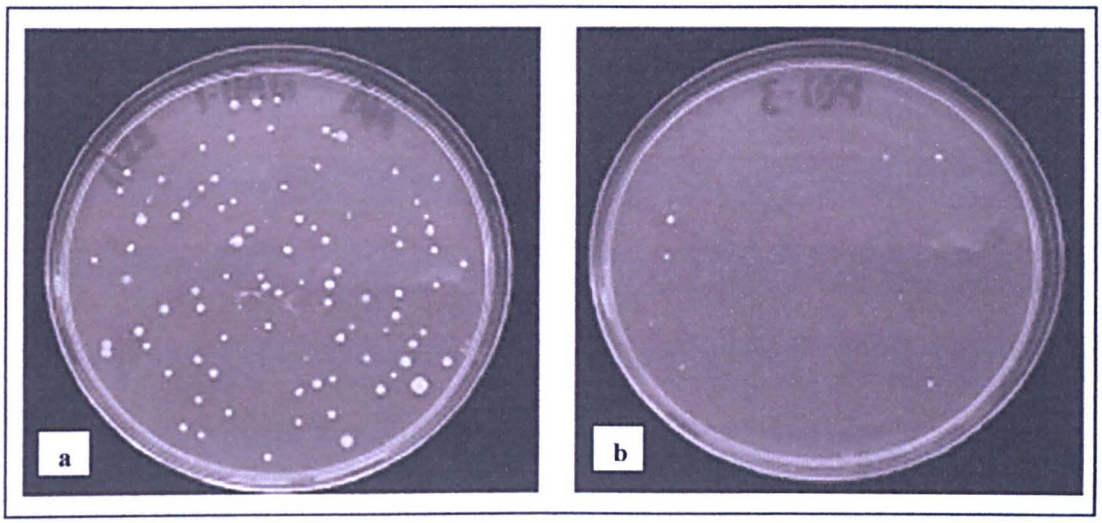

FIGURE 4.13 *A visual comparison of the colony-forming units present in non-pulsed (a) and pulsed (b) air samples on incubated Nutrient agar plates, taken after a 1-hour lecture.*

4.3.3 Microbiological Identification of Isolated Bacteria

A number of isolates from the pulsing experiments were identified using microbiological and biochemical tests including Gram staining, catalase, oxidase and Hugh & Leifson tests. For selected isolates, full identification was found through either API 20NE or API Staph kits depending upon the staining results. Identification was performed on pulsed and non-pulsed isolates to determine whether the biocidal effects of the treatment were uniform or selective against different components of the airborne microflora.

The biochemical characteristics of a range of bacterial isolates obtained from lecture-theatre air are shown in Table 4.2. The colonies were selected on the basis of size and colour in an attempt to maintain the ratio as they appeared on the sampling plates. It can be seen from the results that the majority of isolates were Gram-positive cocci, with only two Gram-negative species being detected, and both being among the non-pulsed samples. It was also found that the majority of isolates surviving pulsed-light exposure had a yellow pigmentation, whereas a larger population of cream isolates was found in the non-pulsed samples.

TABLE 4.2 *Biochemical characteristics and identification of isolates obtained from lecture-room air using a rang of biochemical tests and API Identification Strips*

	BIOCHEMICAL CHARACTERISTICS						
ISOLATE	**GRAM STAIN**	**COLONY COLOUR**	**MORPHOLOGY**	**CATALASE**	**OXIDASE**	**HUGH & LEIFSON**	**SPECIES (%ID)**
NON-PULSED							
1	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus aureus* (53.2%) [c]
2	Positive	Cream	Cocci	Positive [a]	Negative	Facultative anaerobe	*Staphylococcus lugdunensis* (90.1%) [c]
3	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus capitis* (65.0%) [c]
4	Negative	Cream	Rods	Positive	Positive	- [b]	*Sphingomonas paucimobilis* (71.7) [d]
5	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus warneri* (59.2%) [c]
6	Positive	Yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.7%) [c]
7	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus saprophyticus* (47.1%) [c, g]
8	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
9	Positive	Cream	Cocci	Positive	Negative	- [b]	- [e]
10	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
11	Positive	Cream	Cocci	Positive	Negative	- [b]	- [f]
12	Positive	Cream-yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%)
13	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus haemolyticus* (53.9%) [c, g]
14	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
15	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus epidermidis* (89.5%) [c, g]
16	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus epidermidis* (89.5%) [c]
17	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
18	Positive	Yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%) [c]
19	Positive	Cream-yellow	Cocci	Positive	Negative	- [b]	- [f]
20	Positive	Cream	Cocci	Positive	Negative	- [b]	- [f]
21	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus lugdunensis* (97.3%) [c, g]
22	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
23	Negative	Translucent	Rods	Positive [a]	Negative	- [b]	*Acinetobacter lwoffii* (59.0%) [d]
24	Positive	Yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%) [c]
25	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus saprophyticus* (47.1%) [c, g]
PULSED							
1	Positive	Yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%) [c]
2	Positive	Cream-yellow	Cocci	Positive	Negative	- [b]	- [f]

3	Positive	Cream-yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%) [c]
4	Positive	Cream	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%) [c]
5	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
6	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
7	Positive	Cream-yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%) [c]
8	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
9	Positive	Cream	Cocci	Positive	Negative	- [b]	- [f]
10	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
11	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
12	Positive	Yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.7%) [c]
13	Positive	Cream-yellow	Cocci	Positive	Negative	- [b]	- [f]
14	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
15	Positive	Pale yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%) [c]
16	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
17	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
18	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
19	Positive	Cream	Cocci	Positive	Negative	Facultative anaerobe	*Staphylococcus epidermidis* (89.5%) [c]
20	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [e]
21	Positive	Cream-yellow	Cocci	Positive	Negative	- [b]	- [f]
22	Positive	Yellow	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%) [c]
23	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
24	Positive	Yellow	Cocci	Positive	Negative	- [b]	- [f]
25	Positive	Dark cream	Cocci	Positive	Negative	- [b]	*Micrococcus spp.* (99.9%) [c]

[a] Very slight positive reaction
[b] No reaction: the microorganism does not ferment glucose
[c] Identification via API Staph kit
[d] Identification via API 20NE kit
[e] Identification not possible using API Staph kit
[f] Identification test not performed
[g] β hemolysis observed when cultured on blood agar

The Hugh and Leifson results highlight that the majority of the isolated colonies, both non-pulsed and pulsed, were non-glucose fermenters. Of the isolates obtained from the non-pulsed samples, 40% were identified as glucose-fermenting facultative anaerobes but only 4% of the isolates from the pulsed samples were found to possess these characteristics.

From the species identification tests it can be seen that a varied selection of *Staphylococcus spp.* was found within the non-pulsed samples in addition to some *Micrococcus spp.*, but in contrast, all of the identified pulsed isolates, with the exception of one, were *Micrococcus spp.* It was also noted that all of the identified *Staphylococcus spp.* were determined to be glucose-fermenting facultative anaerobes whereas none of the *Micrococcus spp.* were capable of fermenting glucose.

4.4 DISCUSSION AND CONCLUSIONS

4.4.1 Bacterial Contamination Levels

The airborne transfer of bacteria is a problem in many areas but the fact that universities and schools are regularly occupied with large numbers of people gives these situations high potential for the transfer of organisms. Childhood diseases such as measles and chickenpox are transferred primarily by the airborne route [Murray *et al*, 1998], as is *Neisseria meningitidis*, a meningitis-causing bacterium commonly associated with students [Sleigh and Timbury, 1998]. Diseases such as these reinforce the need for improved air quality in educational establishments. In addition to the transfer of pathogens such as these, asthma and SBS symptoms are commonly reported by schools [Daisey *et al*, 2003].

Air sampling in university lecture theatres has shown that airborne bacterial contamination levels can rise significantly over the period of a lecture. The values reported here correlate well with the findings of Sessa *et al* [2002] who reported ranges of 925 – 1225 CFU/m^3 in a sampled university auditorium. With a recommended threshold level of 500 CFU/m^3 in lecture theatres [Cheong and Lau, 2003], it is evident that the majority of the levels reported in this study are significantly higher – in one case 5 times higher – than this recommended level, therefore highlighting the need for improved air quality in this area.

From the low bacterial populations found on sampling the empty lecture theatre prior to occupation, it was evident that this increase in contamination is a result of the presence of personnel. This is consistent with findings in studies by Sessa *et al* [2002] and Liu *et al* [2000], where a university auditorium and school classrooms, respectively, were sampled. Both studies concluded that the occupants were the sources of bacterial contamination. This contamination may be aerosolised through human-shed organisms via skin scales and respiratory secretions, or through the re-suspension of dust containing microbes already deposited in the room, due to movement and air currents. It is highly likely that all of these mechanisms contribute to the airborne contamination.

The particle-size distribution of airborne contaminants also indicates that higher contamination results after occupation of a room. A study by Fox *et al* [2005] found that particle-size distributions in occupied and unoccupied rooms were different, with more large particles being present when occupants were present. An increase in the presence of shed skin squames was suggested as the reason for this increase in concentration of larger particles.

Because of the sampling method used, the counts obtained were only representative of the airborne levels of viable bacteria. It is likely that the actual population is higher due to the presence of viable but non-culturable (VBNC) bacteria, which are immeasurable using this method. Occupant exposure to high levels of dead cells and cell fragments is also capable of inducing hypersensitivity reactions such as respiratory distress and nasopharyngeal irritation [Liu *et al*, 2000], but these are not measurable by the impaction sampling method employed here. In order to quantify the total airborne microbial content, other studies have measured the levels of muramic acid and 3-hydroxyl fatty acids – markers for bacterial peptidoglycan on the cell surface of Gram-positive bacteria and Gram-negative bacterial lipopolysaccharide (LPS), respectively [Fox *et al*, 2005; Liu *et al*, 2000]. These give counts that include all viable and non-viable bacteria as well as bacterial cell fragments present in the sampled air.

The sampling process for this study focused only on bacterial isolates. The occupants of the lecture rooms were assumed to be healthy, and therefore fungal contamination was not considered to be of major concern. Invasive fungal infection is only considered a problem in immunocompromised hosts and is generally a hospital-based issue [Sleigh and Timbury, 1998]. In addition to this, the incubation temperature of 37°C was applied, as this selects for bacterial flora capable of growth within humans, and hence potential pathogens.

4.4.2 Environmental Parameters

The limited air-quality control measures available in situations such as the university lecture theatres investigated here are not usually a noteworthy problem since the vast

majority of occupants are healthy and not susceptible to the largely non-pathogenic airborne bacterial flora. However, it is becoming increasingly evident that poor indoor air-quality (IAQ) can have some effect on healthy occupants. Sick Building Syndrome is becoming more recognised, particularly within the workplace. Given that individuals are spending the majority of their days indoors, the importance of efficient air-quality control is high. Long-term exposure to allergens and pollutants has resulted in the development of hypersensitivity with symptoms including lethargy and headaches, as well as respiratory problems [US EPA, 1991]. Studies of student performance indicate that poor IAQ may be having a detrimental effect, leading to illness, and hence to absence from classes. Recent data are also suggesting that mental activities involving calculation, concentration and memory may be affected [US EPA, 2003].

In an attempt to reduce adverse health effects such as these, recommendations for the improvement of indoor-air quality in educational facilities were published by ASHRAE (62-1, *Ventilation Standard for Acceptable Indoor Air Quality*) and a selection of these is shown in Table 4.3.

TABLE 4.3 *Air quality recommendations for educational facilities [ASHRAE Standard 62.1, 2004]*

AREA	RECOMMENDED VENTILATION AIR CFM/PERSON	AIR FILTER EFFICIENCY
Classrooms Auditoriums Libraries Administrative areas	15	MERV 6 – 7 followed by a 2nd filter MERV 11 – 13
Toilets	20	MERV 6 – 7
Corridors Changing rooms	5	MERV 6 – 7

CFM: cubic foot of air per minute

Cleaning a room is an effective way to reduce airborne particulate levels but routine cleaning is rarely effective enough if fabrics such as carpeting, chair/couch seats and

partitions are present. These materials can absorb large amounts of dust and if disturbed can release invisible clouds of dust that are likely to contain microorganisms [Ayliffe *et al*, 2001]. Each of the university lecture theatres used in this study had carpeting and fabric-covered seating so that the movement of up to 150 occupants on entrance and exit of the lecture theatre was likely to disturb this settled dust, and thus contribute to the elevated airborne bacterial levels. Vacuuming and dry dusting of the lecture theatre during routine cleaning may also increase airborne bacteria levels. Ordinary vacuum cleaners pick up dirt and dust from the carpet, filter out the larger harmless particles and exhaust the majority of the allergenic, irritating and toxigenic particles back into the air, where they are likely to be inhaled by occupants. Dry dusting is also likely to dislodge and disperse microbe-containing dust into the room air [Ayliffe *et al*, 2001].

In hospitals, vacuuming is done using a High Efficiency Particulate Air (HEPA) filtered vacuum cleaner which filters the exhaust air ensuring no contaminants are released and suspended in the air of the room. This cleaning method is best for preventing the aerosolisation of settled microorganisms, with a 20% decrease in the number of bacteria-carrying particles, compared to other cleaning methods – the worst of which is sweeping which results in as much as a 700% increase in airborne particle concentration (shown in Table 4.4) [Ayliffe *et al*, 2001]. Damp dusting is also preferred in the hospital environment so as to avoid dispersal of microorganisms, which can potentially be inhaled or contaminate wounds [Ayliffe *et al*, 2001].

TABLE 4.4 *Typical changes in airborne bacterial counts associated with different methods of cleaning floors in hospitals [Ayliffe et al, 2001]*

CLEANING METHOD	CHANGE IN THE NUMBERS OF BACTERIA CARRYING PARTICLES IN THE AIR
Sweeping with broom	700% increase
Dust-attracting floor mop	30% increase
HEPA-filtered vacuum-cleaning	20% decrease

During the study, control over the environmental parameters of pressure, temperature and relative humidity was not possible, and so their effects on the levels of bacterial contamination within the lecture theatres could not be established. Relationships between these environmental parameters and microbial aerosols do exist although they are difficult to quantify due to a large number of interdependent factors that affect the outcome of experiments. One significant relationship which exists is associated with the hygroscopicity and physical attributes of bacterial aerosols. When bacteria are exposed to the environment, for example during sneezing, the organisms dehydrate and this water can only be replaced on transfer to another aqueous environment or during host infection. The movement of these water molecules is dependent on temperature and relative humidity and this is common to all bacterial aerosols [Cox, 1987]. To experimentally demonstrate these effects accurately, controlled environmental conditions must be applied.

In addition to environmental factors, a study of school classrooms by Bartlett *et al* [2004] found that high bacterial counts were associated with low air exchange rates, low air input and exhaust flow rates and high levels of occupant activity.

Control over environmental parameters was also not possible during the UV-pulsing experiments, and it is likely that they do have an effect on airborne bacterial inactivation. Relative humidity has a major influence on the UV-inactivation rates of airborne bacteria, with increased humidity causing decreased inactivation; the fundamental mechanism governing their response to UV exposure at differing relative humidity levels has not yet been confirmed [Peccia and Hernandez, 2001; Kowalski and Bahnfleth, 2000; Riley and Kaufman, 1972]. It has been found that air temperature has a negligible impact on the UV susceptibility of a microorganism [Kowalski and Bahnfleth, 2000].

Another environmental parameter which may affect bacterial counts – more so after pulsed UV treatment – is the level of visible-light. Sufficient exposures to wavelengths in the range 350 nm to 480 nm (typical in sunlight and artificial room lighting) will cause photoreactivation within otherwise damaged, non-viable bacteria

– essentially increasing the viable bacteria count [Oguma *et al*, 2002, Oguma *et al* 2001]. Given that when using the pulsed UV system described in this study, the treated bacteria were captured for enumeration immediately after pulsed UV exposure, this is not a problem here, but photoreactivation is likely to have a considerable effect on the airborne bacteria levels of a room using conventional UV disinfection treatments.

4.4.3 Nature of Particles

Identification of the bacterial isolates from both the pulsed and non-pulsed samples highlighted a significant factor, namely that the majority of bacteria capable of survival following pulsed-light treatment were *Micrococcus spp.* containing yellow pigmentation. This suggests that colony pigmentation may be an influencing factor for the survival of airborne bacteria when exposed to UV light. *Micrococcus spp.* are found primarily on mammalian skin and in soil, but are also commonly isolated from air and food products [Holt *et al*, 1994]. Because of their prevalence in this range of environmental situations, they generally tend to be robust organisms which can demonstrate varying degrees of UV resistance. *Micrococcus radiodurans* (now classified as *Deinococcus radiodurans*), for example, is one species that displays high resistance to UV-light treatment. This resistance is due to the capability of the organism to fully repair more than 100 double-strand breaks per chromosome without mutagenesis or lethality [Carroll *et al*, 1996]. Although *Micrococcus spp.* are common skin contaminants, they maintain a saprophytic existence so that their survival following pulsed-light illumination should not be of too great a concern.

Conversely, this pulsed UV-light treatment was found to be capable of eliminating the majority of the *Staphylococcus* species found in the air samples, and this is desirable since these bacteria are opportunistic pathogens.

4.4.4 Practical Pulsed-UV Systems

The application of UV-rich pulsed light was shown to be effective in reducing the levels of airborne bacteria within samples of air taken from the lecture theatres with a decrease of approximately 80%; 540 CFU/m^3 to 105 CFU/m^3 being observed in one

case. This 80% decrease was effected in a treatment time of 1-minute (at 5 pps), thus highlighting the rapid treatment time of the pulsed air-disinfection system.

Further research and improvements to the pulsed-light treatment are needed in order to maximise efficiency in reducing bacterial numbers, in addition to investigating effects on fungal and viral populations. Due to the flow rate of this system being controlled by the air sampler, air was drawn into the system at a rate of 180 L/min. This rate is high and could be another reason for some airborne isolates surviving PUV treatment. The contaminated air may have been drawn through the system at too rapid a rate resulting in some isolates not being subjected to sufficient UV-pulses during their rapid transit through the system. It is therefore expected that increased inactivation rates would be achieved by using lower air-flow rates. Alternatively, the use of an additional light source(s) within the system would increase the number of pulses emitted, and hence increase the probability of the airborne microorganisms being exposed. An increased pulse rate or increased pulse energy could also increase the effectiveness of this system. The pulse repetition rate used here was 5 pps with 20 J of energy per pulse – the maximum of which the system was capable. The use of a higher power pulse generator and/or flashlamp could provide these system enhancements.

A pulsed system like this could have potential use as a disinfection unit in a variety of situations. Due to the obvious rapidity of the treatment it need not be operated continuously, as with more conventional germicidal UV air-disinfection systems, but at regular intervals to treat specified volumes of air. The volume of air treated would vary between areas of application depending on the level of disinfection required. For instance, if employed in a hospital ward, air treatment levels should be high but if used in communal areas occupied by generally immunocompetent individuals, such as cinemas, the treatment volumes could be markedly smaller.

The ideal employment of this pulsed system would be either within heating ventilation and air-conditioning (HVAC) systems or as a stand-alone system. PUV systems would also be best operated in conjunction with high efficiency particulate

air (HEPA) filtration since UV treatment inactivates the microorganisms but does not remove them from the air, and non-viable organisms can go on to cause irritation in individuals due to their surface molecules inducing allergic reactions within some individuals [Liu *et al*, 2000]. From the successful results achieved in this study it is obvious that the use of PUV disinfection within HVAC systems would cause a marked reduction in airborne microbial populations, therefore reducing levels of infectious agents and potential allergens. Combination treatment using HEPA filtration and UV germicidal irradiation is considered to be the ideal air-treatment arrangement within HVAC systems [Kowalski and Bahnfleth, 2002], and the use of pulsed rather than continuous UV should greatly increase the efficiency of such systems.

The use of a PUV unit as a stand-alone system, such as that developed here, would be of major benefit in the hospital environment in situations such as isolation wards (to inactivate pathogens being continuously shed by an individual) or in specialised wards such as transplant and HIV wards (to inactivate any potential pathogens and maintain uncontaminated air levels). A stand-alone system requires careful installation. UV-light is harmful, causing skin cancer and eye damage [WHO, 2006]. The latter will be a particular problem with pulsed UV as a result of the high UV intensities involved. Strict shielding is required so that only regions of a room adjacent to walls and ceiling can be irradiated. Air circulation will however permit the whole of the air volume in the room to be exposed to some level of UV inactivation.

Pulsed-light treatment is believed to induce DNA-based damage within the bacteria (and possibly cell rupture) as they pass through the system. The major type of lesion resulting from this exposure is cyclobutane pyrimidine dimers (CPD), with pyrimidine-pyrimidone (6-4) photoproducts being produced at a lower rate [Sinha and Häder, 2002]. Other types of DNA damage also occur but to a much lesser extent [Miller *et al*, 1999]. As discussed in Section 2.6.2, some bacteria have the capability to repair this UV-induced DNA damage. Repair is carried out through mechanisms known as photoreactivation, which is light dependent, and dark repair,

which occurs in the absence of light [Oguma *et al*, 2001]. In this series of experiments the bacteria were incubated in dark conditions. Future work will investigate the extent to which photorepair can occur after airborne bacteria have been exposed to the PUV-rich light treatment.

Chapter 5

DISCOVERY OF VISIBLE-LIGHT INACTIVATION

5.0 GENERAL

As discussed in Section 2.6.2, some bacteria possess the ability to repair UV-induced nucleic acid damage. Two types of repair mechanism are believed to exist – light repair and dark repair, the former being referred to as 'photoreactivation'. This chapter follows on from the previous work and includes an initial investigation on the photoreactivation of PUV-treated air samples.

During the course of the studies of photoreactivation, however, it was observed that visible-light exposure in the light cabinet over long times produced inactivation rather than reactivation, on both UV-treated and non-treated (control) bacteria. This lethal effect was observed following an exposure time of 24 hours, and it was considered appropriate to investigate further the effect of non-UV light exposure on bacterial suspensions. Due to the focus of this study being on medically important microorganisms, *Staphylococcus aureus* strains, including MRSA, were selected for most of the experimental work. The objective of this work was to establish the extent of this visible-light inactivation and to characterise its effects.

In order to identify the range of wavelength responsible, the investigation was progressed using filters, which absorbed the ultra-violet and infrared portions of the photoreactivating light in the cabinet. This demonstrated that the visible-light portion of the electromagnetic spectrum was responsible for this inactivation, and specifically, at wavelengths between 400 and 500 nm.

The chapter details progress from the discovery that visible light has the potential to inactivate certain bacterial species – including *Staphylococcus aureus* and MRSA – to identification of the wavelength range causing inactivation.

In brief, the chapter initially focuses on the photoreactivation of pulsed-UV-treated air samples, but due to the unusual results obtained, work progressed to investigate the effect of light exposure on non-treated air samples, and finally, surface-inoculated bacteria and bacterial suspensions. The last area of study in this chapter involved the identification of the causative wavelength range and was done through the use of optical filters.

5.1 LIGHT INCUBATION CABINET

For the photoreactivation study, a Fi-totron 600 Growth cabinet (Fisons), shown in Figure 5.1a, was used as a light incubation cabinet and enabled the bacterial cultures to be exposed to light while being maintained at incubation temperature, either for short-term exposure or overnight exposure and incubation.

The light cabinet was fitted with 12 warm-white (colour temperature: 3000 K) fluorescent tubes (Phillips), each 1200 mm long x 38 mm diameter and 40 W. These were selectable at a variety of intensity levels by three switches.

The standard operating procedure was to have all 12 fluorescent bulbs set at maximum intensity and the cabinet temperature maintained at 37°C. All bacterial light exposure was carried out on a shelf 25 cm from the fluorescent lamps (see Figure 5.1b), and at this distance an illuminance of approximately 15,000 lux was recorded, using a Lutron LX-101 digital luxmeter.

Figures 5.2 and 5.3 show the total emission spectrum and the emission spectrum from 200 nm to 500nm, respectively, of the light cabinet. These, and all other spectra shown throughout this thesis, were obtained using an Ocean Optics SQ2000

fibre optic spectrometer. The spectral emission from a fluorescent lamp is made of two components; the continuum emitted by the phosphor coating and the mercury spectral lines emitted by the mercury arc used to excite the phosphor to fluorescence. The mercury lines are highlighted in Figure 5.3.

From Figure 5.3, it can be seen that the light emission of the lamps in the light cabinet begins at a wavelength of around 350 nm. This is ideal for the photorepair of UV-damaged microorganisms as the light emission contains UV-A and visible wavelengths between 350-480 nm, which are known to generate photoreactivity [Oguma *et al*, 2002, Oguma *et al* 2001], while omitting germicidal wavelengths.

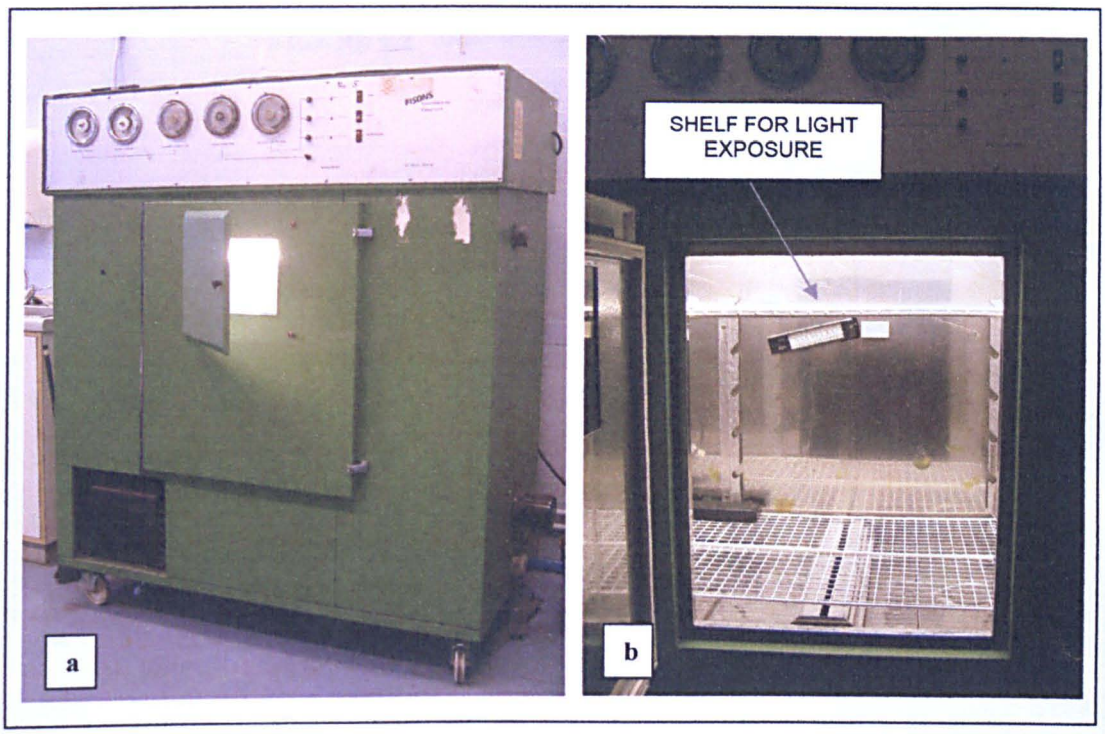

FIGURE 5.1 *(a) Light incubation cabinet and (b) interior. Highlighted is the shelf 25 cm from the light sources that was used for bacterial light exposure.*

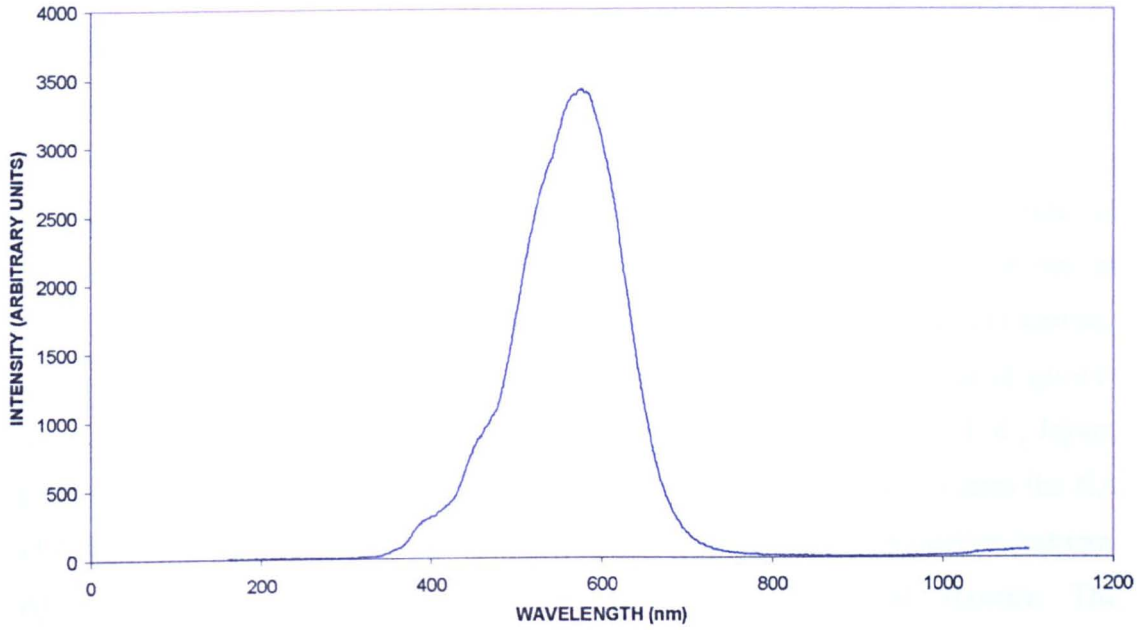

FIGURE 5.2 *Total emission spectrum of light cabinet*

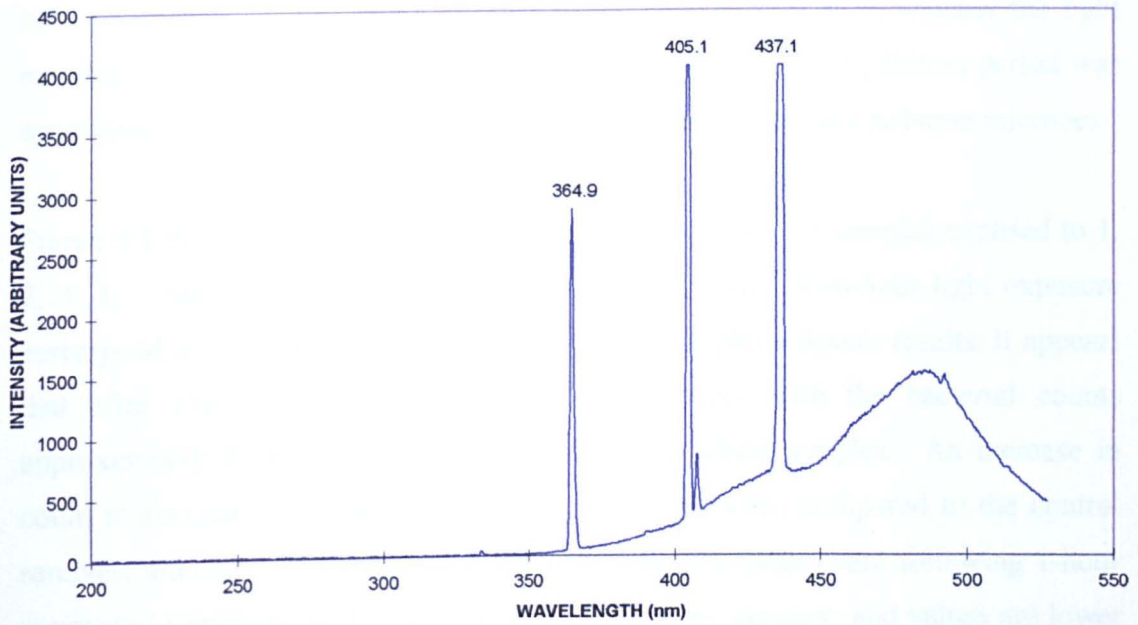

FIGURE 5.3 *Emission spectrum of light cabinet (200 – 500 nm grating). Highlighted are the values of the three mercury lines emitted by the fluorescent bulbs.*

5.2 PHOTOREACTIVATION OF PUV-TREATED AIR SAMPLES

For the study of photoreactivation of PUV-treated air samples, air samples were obtained from within a Microbiology Laboratory.

Using the PUV-light air-disinfection system (described in Section 4.3.1), samples of air, with a volume of 180 l, were treated with UV-light at a pulse repetition rate of 5 pps. Non-pulsed samples were also passed through the system and all samples were collected on Nutrient agar (NA) plates. To prevent the drying-out of growth medium, plates were then sealed with Nescofilm (Bando Chemical Ind. Ltd., Japan) prior to incubation. To investigate the photoreactivation of these air isolates the NA plates were exposed to varying durations of light in the light incubation cabinet. After exposure, plates were wrapped in foil to prevent further light exposure. The foil-wrapped plates were left in the light cabinet rather than transferred to a separate dark incubator so as to ensure environmental parameters, such as temperature and relative humidity, were identical for all samples throughout the experiment. Control samples were provided in all experiments by incubating plates in foil for the full 48-hour incubation period – these samples would provide data as to whether the light exposure induced any changes in colony growth. A 48-hour incubation period was again used to enable sufficient time for the growth of the stressed airborne microbes.

Figure 5.4 shows results for PUV-treated and non-treated air samples exposed to 1, 2, 3, 4, 5 and 48 hours of light exposure. Samples with zero-hour light exposure correspond to the control samples. Looking at the light exposure results, it appears that after 1-hour there is notable photoreactivation, with the bacterial counts approximately doubling for both pulsed and non-pulsed samples. An increase in count is also seen with the 2 and 3-hour exposures when compared to the control samples, although the figures are decreasing from the peak count following 1-hour exposure. Exposures of 4, 5 and 48 hours follow this decrease and values are lower than those for the control samples, with near-total reduction after 48-hour exposure, thereby suggesting that light exposures of 15,000 lux for more than 3 hours are

inducing some form of lethal effect upon the bacteria in both the pulsed and non-pulsed samples – an effect that outweighs any remaining effect of photoreactivity.

From Figure 5.4 it is evident that the PUV treatment was less effective than previously seen in results in Section 4.3.2 – on further inspection of the xenon lamp, it was found that this reduced efficacy was due to sputtering of electrode material onto the quartz wall of the lamp. However, a notable decrease in airborne bacterial contamination was still seen after PUV treatment.

With this light inactivation effect being clearly observed in both the PUV-treated and non-treated air samples, further experiments were carried out only on non-pulsed air samples using samples collected using the SAS Super-180 by the standard method (Section 4.1.2).

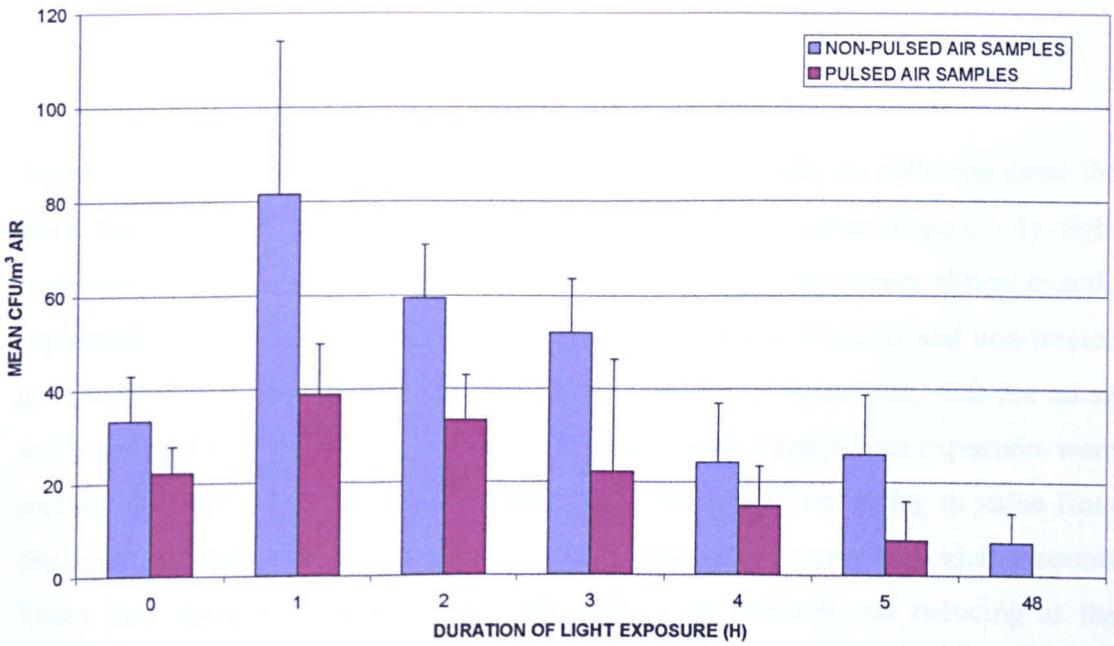

FIGURE 5.4 *Effect of 1, 2, 3, 4, 5 and 48-hour light exposure on the growth of bacteria in non-pulsed and pulsed air samples obtained from within a microbiology laboratory.*

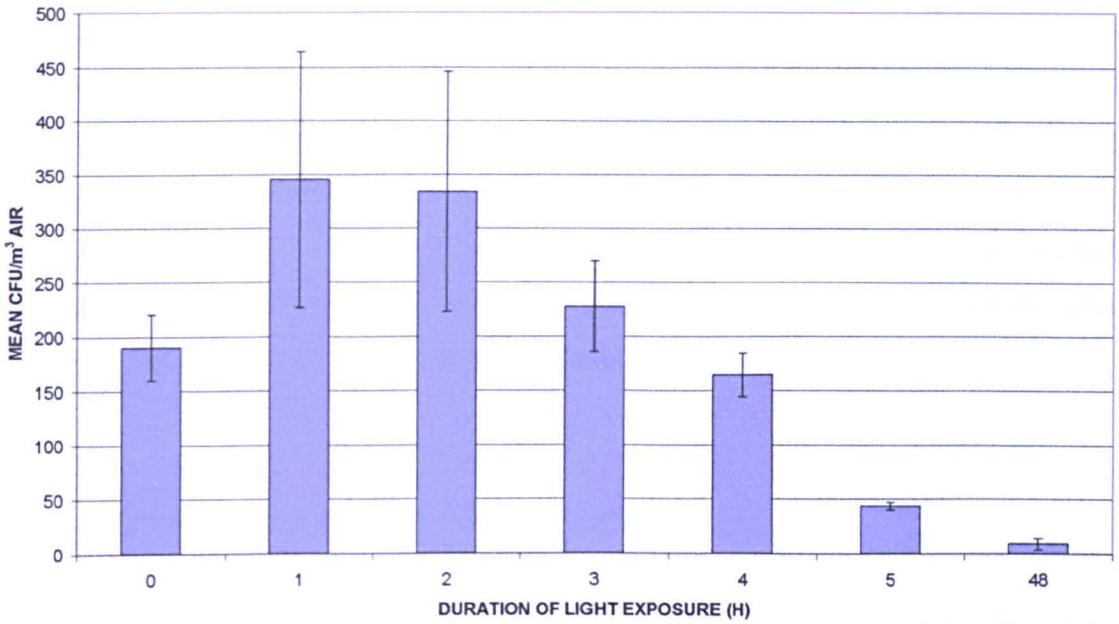

FIGURE 5.5 *Effect of 1, 2, 3, 4, 5 and 48-hour light exposure on the growth of bacteria in air samples obtained from within a microbiology laboratory.*

Figure 5.5 shows the results for the light exposure of air samples collected using the SAS Super-180 air sampler. As with the previous experiment (Figure 5.4), light exposures of 1, 2, 3, 4, 5 and 48 hours were applied. This experiment almost exactly replicated the results found with the light exposure of PUV-treated and non-treated air samples: 1-hour exposure induced photorepair of the organisms, with the count again approximately doubling; the bacterial counts after 2 and 3-hour exposures were increased compared to the control samples but were again decreasing in value from the count obtained after 1-hour exposure; and 4 and 5-hour exposures induced counts lower than the control samples with the mean CFU count/m^3 air reducing as the duration of light exposure increases, until near total reduction was observed after 48-hour exposure.

In summary, the initial photoreactivation experiments which were carried out using PUV-treated and non-treated samples brought to light a valuable discovery – that prolonged exposure to light outwith the germicidal UV-C wavelengths had a bactericidal effect on the isolated airborne bacteria. Since this effect was observed for both the PUV-treated and non-treated isolates it was obvious that it was not related to UV-C-mediated damage incurred during treatment with the PUV air disinfection system. In addition, this lethal effect could not be due to germicidal wavelengths, which induce UV-mediated DNA damage, since UV-C wavelengths were absent from the emission spectrum of the light cabinet.

5.3 LIGHT TREATMENT OF SURFACE BACTERIA

With the discovery of the possible biocidal properties of non-UVC light, the study progressed from the use of air samples to bacterial cultures of known species and population size, thus allowing more accurate and reproducible results. Initial experiments focused on the effect of light exposure on surface-inoculated bacteria.

5.3.1 Preparation of Bacteria

Bacteria were cultured for experimental use as described in Section 3.1.2. After an 18-hour incubation period, the broths were centrifuged at 3400 rpm for 10 minutes and the resultant pellet re-suspended in PBS. This suspension was then diluted to the required concentration.

5.3.2 Qualitative Data: Initial Experiments

As an initial experiment, 10^4 CFU/ml populations of both *Staphylococcus aureus* NCTC 4135 and *Escherichia coli* NCTC 9001 were spiral plated onto NA plates. The plates were then either incubated in light or dark conditions, for periods of 24 and 48 hours. As with the air-sample experiments, dark incubation conditions were established by wrapping the plates in foil, thus ensuring all plates were incubated in exactly the same environmental conditions. This was carried out in duplicate.

TABLE 5.1 *Effect of dark and light incubation on the growth of E. coli NCTC 9001 and S. aureus NCTC 4135 (+ Growth; - No Growth).*

GROWTH CONDITION	INCUBATION CONDITION	*E.coli* GROWTH	*S. aureus* GROWTH
DARK	37°C for 24 H	+	+
DARK	37°C for 48 H	+	+
LIGHT	37°C for 24 H	+	-
LIGHT	37°C for 48 H	+	-
LIGHT → DARK	24 H Light → 24 H Dark	n/a	-

S. aureus and *E. coli* were selected for use in this experiment so as to test one Gram-positive and one Gram-negative species (*S. aureus* and *E. coli*, respectively).

The results shown in Table 5.1 show that, when incubated in dark conditions, both *S. aureus* and *E. coli* grew as normal. Both 24 and 48-hour light-incubated *E. coli* showed a lawn of growth similar to the dark-incubated control samples. On the other hand, when *S. aureus* was incubated in the light for periods of both 24 and 48 hours, no growth was observed on the plate surface. An additional test was performed where a 24-hour light-incubated *S. aureus* plate was transferred to dark-incubation conditions for a further 24 hours in order to determine if the light was merely suspending growth that would resume on subsequent dark incubation. No growth was found after the total incubation period.

In conclusion, these results showed that the light was having some form of lethal effect on the *S. aureus* cells, but not on the *E. coli* cells.

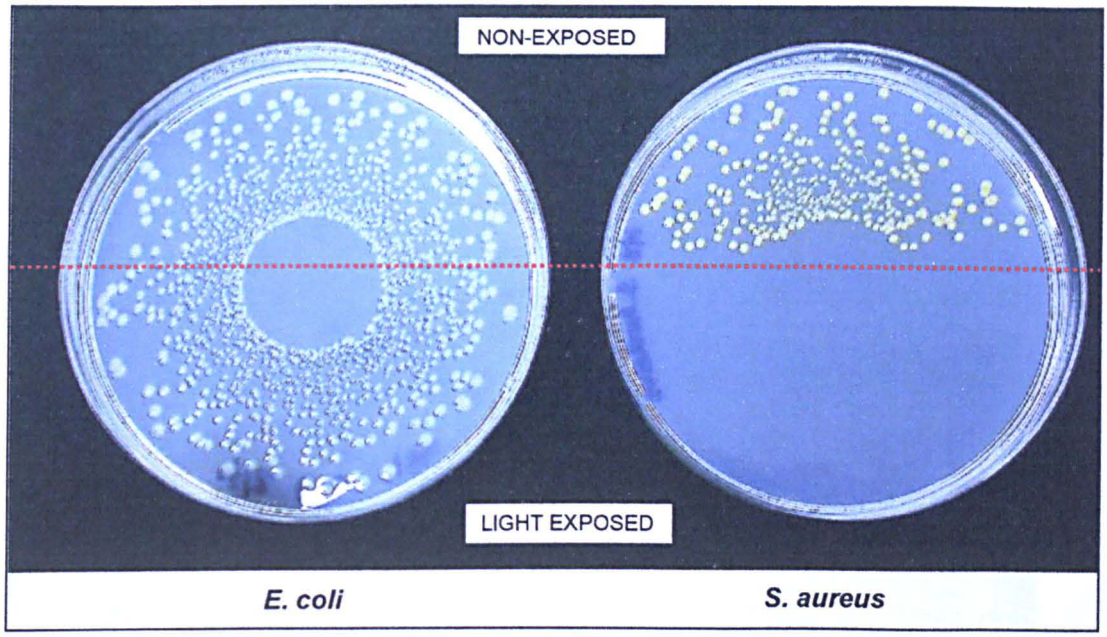

FIGURE 5.6 *Visual comparison of the effect of light exposure on S. aureus NCTC 4135 and E. coli NCTC 9001. Both nutrient agar spread plates were sealed so that only half of each plate was exposed to the light. Incubation/exposure was at 37 °C for 24 hours.*

In order to ensure that these results were not due to other factors such as differences in agar plates, temperature, relative humidity, etc., a comparison of light and dark exposure was made using one NA plate. This was done by spiral plating a 10^4 CFU/ml population of *S. aureus* onto a NA plate. Half the plate was then covered with black tape – creating dark incubation conditions – and the other half left uncovered to allow light exposure. This procedure was repeated using *E. coli*. Plates were incubated at 37°C for 24 hours and Figure 5.6 shows the results. It can be clearly seen that the light exposure had a bactericidal effect on the growth of *S. aureus* but no effect on *E. coli*. The slight inhibition along the edge of the dark/light interface for *S. aureus* was due to stray light. This comparison rules out environmental parameters as being the cause of the lethal effect.

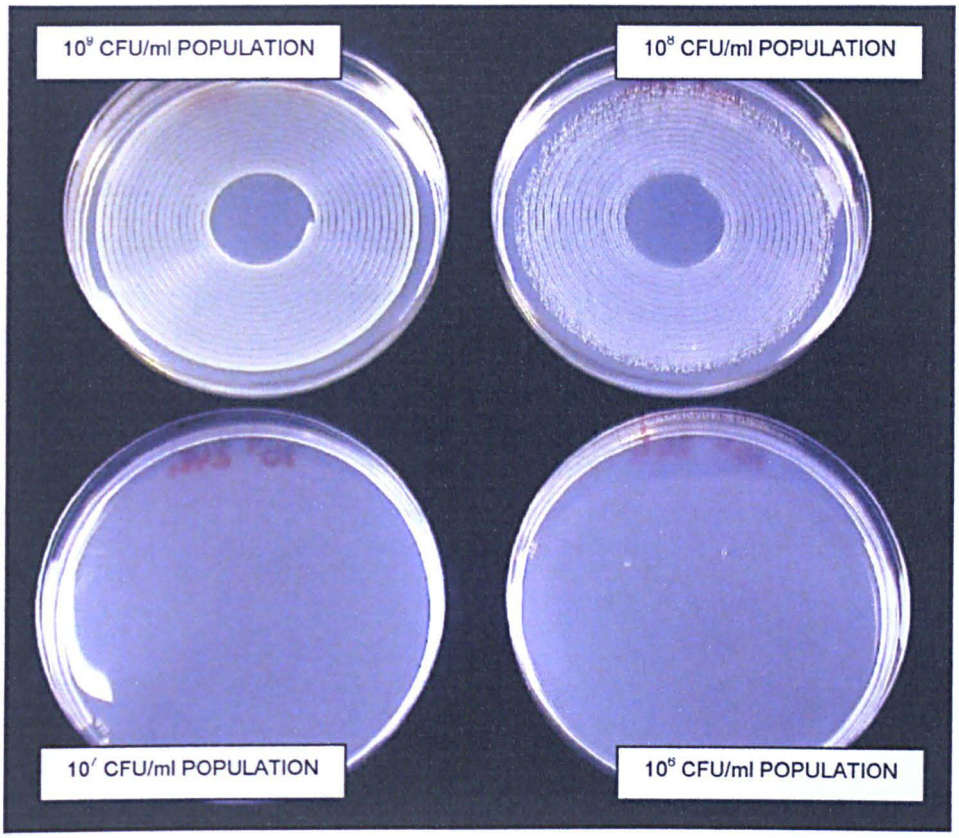

FIGURE 5.7 *Effect of light exposure on differing populations of S. aureus NCTC 4135 on nutrient agar spread plates. The photo shows 10^9, 10^8, 10^7 and 10^6 CFU/ml starting populations following exposure at 37°C for 24 hours.*

With the observation that light in the light incubation cabinet had a lethal effect on *S. aureus* plated onto NA, an investigation followed to examine the effect of light exposure on different populations of *S. aureus*. For this, populations of 10^9, 10^8, 10^7 and 10^6 CFU/ml were prepared in PBS and spread plated onto NA plates. These plates were then sealed and incubated at 37°C for 24 hours in the light cabinet.

Figure 5.7 shows a visual comparison of the results. It is evident that light exposure of 10^6 and 10^7 CFU/ml populations induced complete inactivation of the 100 µl sample spread plated onto the nutrient agar surface. For populations of 10^8 and 10^9 CFU/ml however, inactivation was less obvious. This was likely due to the high starting populations, and shading which reduces exposure of the cells to the light treatment [Lamont, 2005].

5.3.3 Quantitative Data

With the effect now confirmed qualitatively, work progressed in order to obtain quantitative results. Experiments were performed using 5 different microorganisms: *S. aureus* strains LMG 8064 and NCTC 4135, clinical isolate MRSA 16a, *E. coli* NCTC 9001 and, a *Micrococcus* strain isolated during air sampling and pulsed UV inactivation (pulsed isolate No.1 in Table 4.3). In order to investigate any role of the agar medium, several such media were used.

5.3.3.1 Treatment Method

Bacterial suspensions were prepared and diluted giving a starting population of 10^2 – 10^3 CFU/ml. These were then spread plated onto agar plates and sealed prior to incubation within the light cabinet. The plates were light exposed for durations of 1, 2, 3, 4, 5 and 24 hours, and subsequently wrapped in foil for the remainder of the 24-hour incubation period. Control samples were prepared which were foil-covered for the entire 24-hour period.

Low starting populations were used to obtain countable results on the agar surfaces. Also, as 100 µl sample volumes were being light exposed on the agar surfaces, bacterial counts were recorded as mean CFU per 100 µl rather than per millilitre.

5.3.3.2 Results

Figure 5.8 shows the effect of light exposure on *S. aureus* LMG 8064. It can be seen that after 3 hours exposure, the light had an obvious lethal effect on this bacterium, with the population/100 µl being reduced by almost half after a 5-hour exposure and near total inactivation occurring after 24 hours.

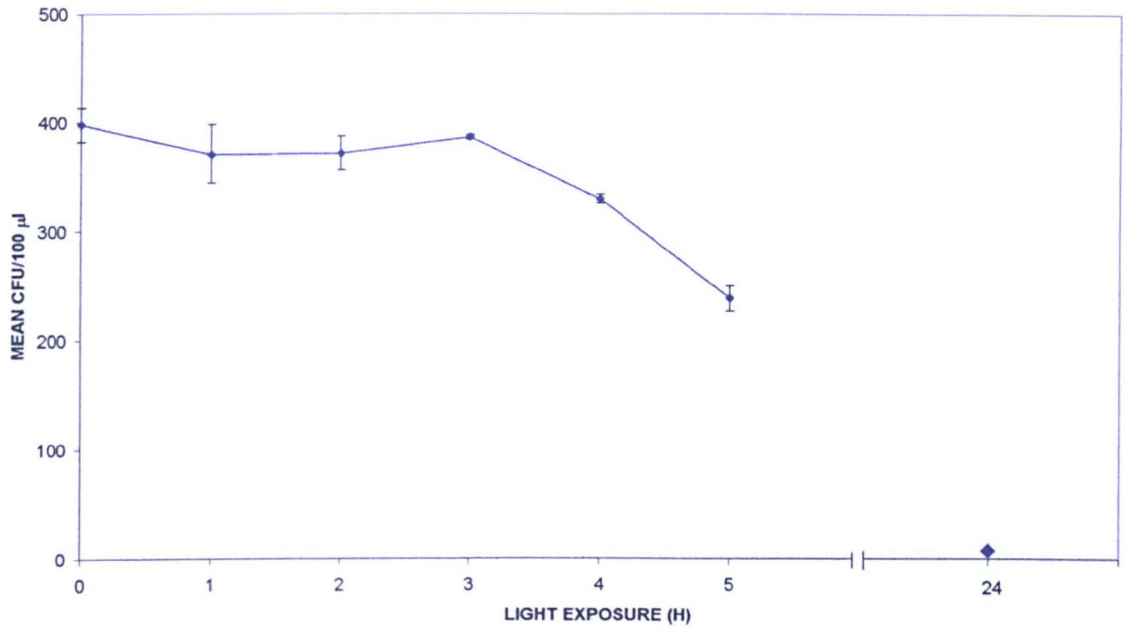

FIGURE 5.8 *Effect of light exposure on Staphylococcus aureus LMG 8064 survival when exposed on Nutrient agar*

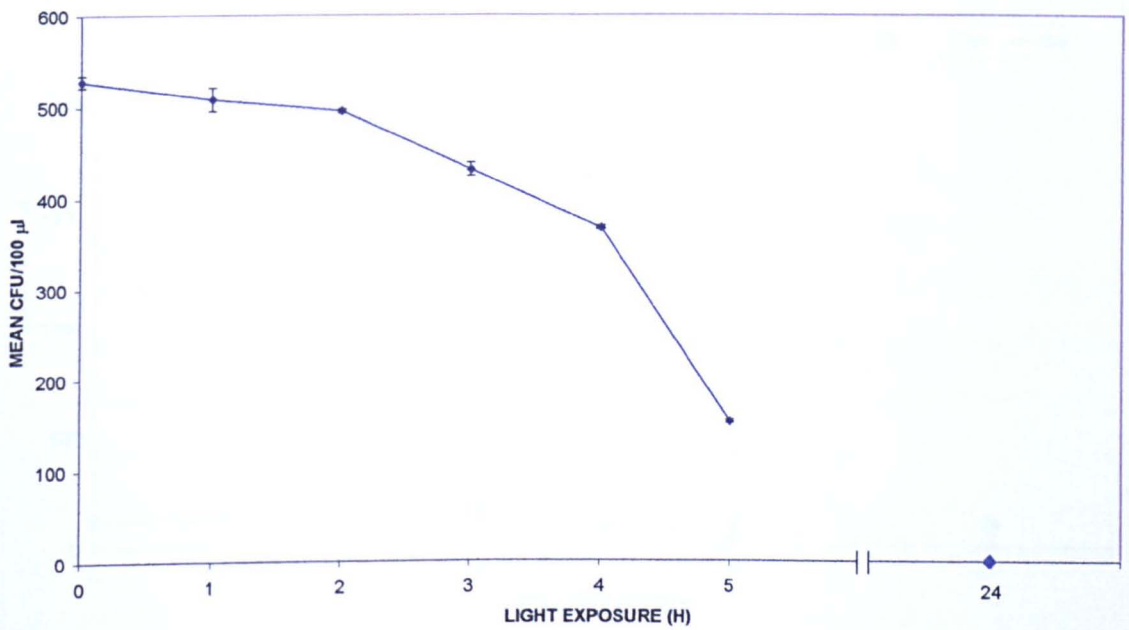

FIGURE 5.9 *Effect of light exposure on Micrococcus sp. survival when exposed on Nutrient agar*

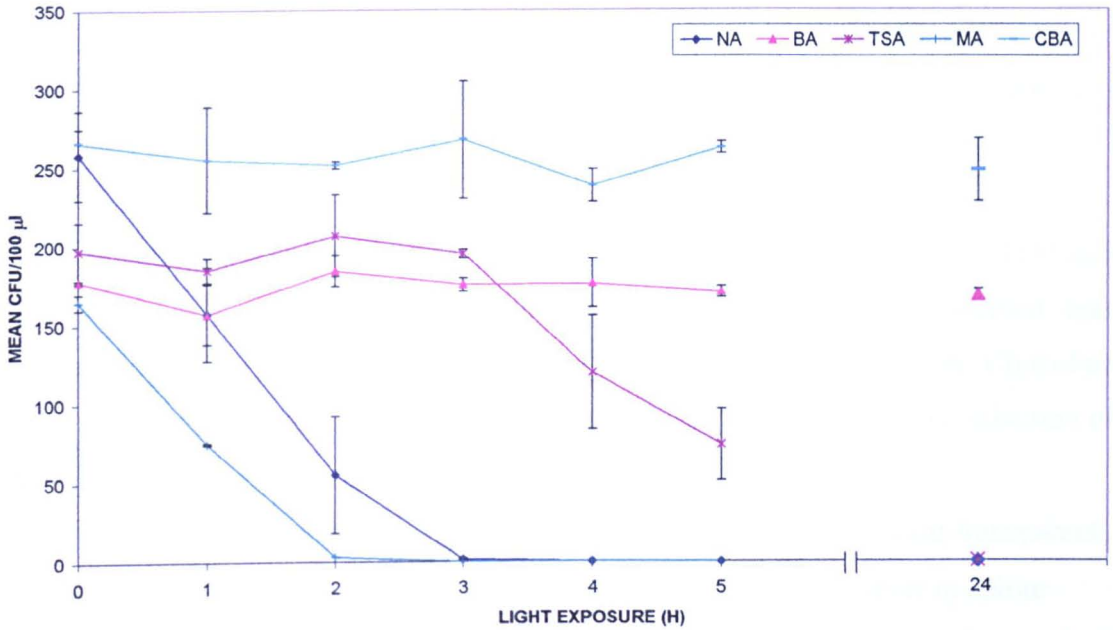

FIGURE 5.10 *Effect of light exposure on Staphylococcus aureus NCTC 4135 survival on a selection of media at 37°C.*

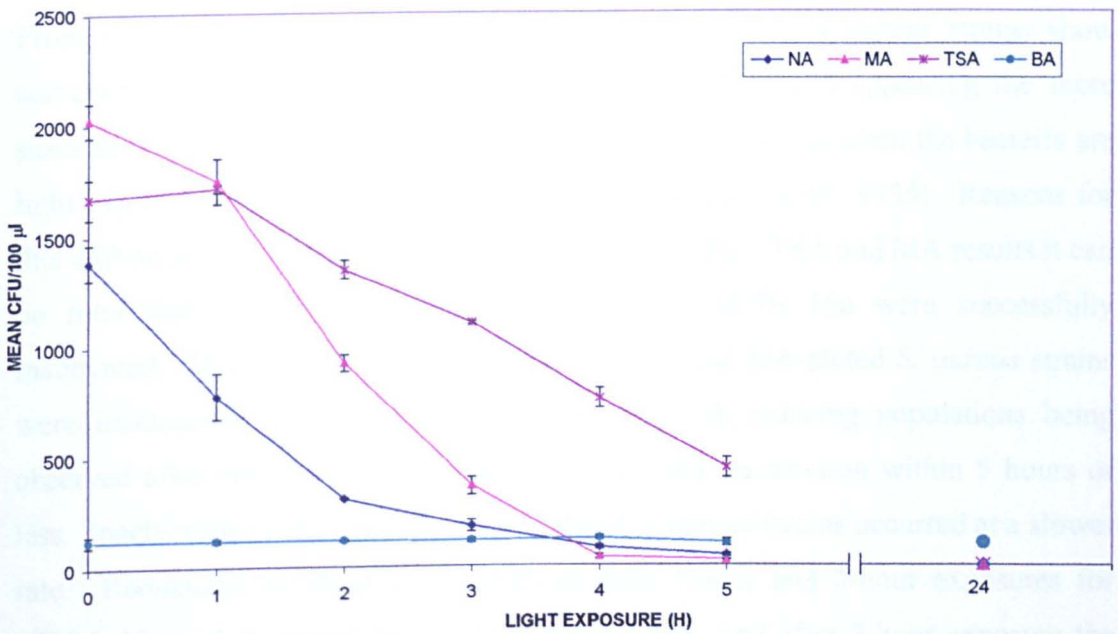

FIGURE 5.11 *Effect of light exposure on MRSA 16a survival when exposed on a selection of media at 37°C.*

The effect of light exposure on the *Micrococcus* isolate can be seen in Figure 5.9. A similar lethal effect to that seen with *S. aureus* LMG 8064 is shown, although here the effect appears to be more gradual with a reduction in count after each hour of exposure. Also, the micrococci appear to be slightly more sensitive to the effects of the light exposure, with an almost 5-fold reduction after a 5-hour exposure.

Figures 5.10 and 5.11 show the effect of light exposure on *S. aureus* NCTC 4135 and MRSA 16a, respectively, plated onto a selection of agar surfaces: Nutrient agar (NA), Tryptone Soya agar (TSA), Milk agar (MA), Blood agar (BA) and Chocolate Blood agar (CBA) – the latter used only for *S. aureus* NCTC 4135. The selection of this media was based on a number of factors;

- NA is a general-purpose medium and is straw-coloured and semi-transparent.
- TSA has similar nutritional content to NA but is slightly darker in colour
- MA again has a similar colour to NA, and growth on MA is thought to induce pigment production within pigment-containing bacteria such as *S. aureus*
- BA contains red blood cells and is red in colour
- CBA is red-brown in colour due the presence of lysed red blood cells

From these graphs it can be seen that both *Staphylococcus aureus* strains show susceptibility to light exposure, with *S. aureus* NCTC 4135 appearing the more susceptible of the two. The exception to this, in both cases, is when the bacteria are light exposed on BA (and CBA in the case of *S. aureus* NCTC 4135). Reasons for this will be proposed in Section 5.6. Focusing on the NA, TSA and MA results it can be seen that both *S. aureus* NCTC 4135 and MRSA 16a were successfully inactivated, albeit at differing rates. Both the NA and MA-plated *S. aureus* strains were inactivated with relatively similar trends, with reducing populations being observed after only 1-hour light exposure, and total inactivation within 5 hours or less. Inactivation in the case of the TSA-plated *S. aureus* strains occurred at a slower rate. Reductions in count were observed after 1-hour and 3-hour exposures for MRSA 16a and *S. aureus* NCTC 4135 respectively, and after 5-hour exposure the populations had reduced to less than half the original, with total inactivation following 24-hour exposure.

Figure 5.12 shows the effect of light exposure on NA and BA-plated *E. coli*. During the 1 to 5-hour exposure times, fluctuations in counts are observed – in particular with the NA-plated samples – but after 24-hour exposure the count per 100 µl remained similar to the control counts (zero-hour light exposure). This agrees with the negative qualitative results obtained previously.

It is not known why there is such large scatter for the inactivation results for *S. aureus* exposed on NA (Figure 5.12). Although this required further investigation, it was not followed up in the present study due to the preferential progression of the work to the use of more intense light sources (Chapter 6).

Fluctuations are likely to occur throughout the total 24-hour exposure period with all the exposed bacterial samples but due to hourly sampling only being performed during the first 5 hours, the degree of fluctuation between 5 and 24 hours is undetermined. Also, due to the final sample being taken after 24 hours the exact time of complete inactivation for all the tested samples (with the exception of *E. coli* which was unaffected) is undetermined – all that is known is the level of inactivation after a 24-hour exposure.

5.3.4 Investigation of Temperature Effect

A possible explanation for this bacterial inactivation is a lethal increase in the temperature that the microorganisms may be subjected to upon light exposure. It was found however that when *S. aureus* NCTC 4135 was again exposed to light treatment using the same experimental procedure as before (section 5.3.3.1), but at a cabinet temperature of 30°C rather than 37°C, similar results were obtained (Figure 5.13). This indicates that inactivation is not a result of over-heating of the organism.

The fact that a lethal effect occurs for *S. aureus* but not for *E. coli*, both of which can be grown at temperatures up to and above 40°C, is further evidence that the inactivation process is not a thermal one.

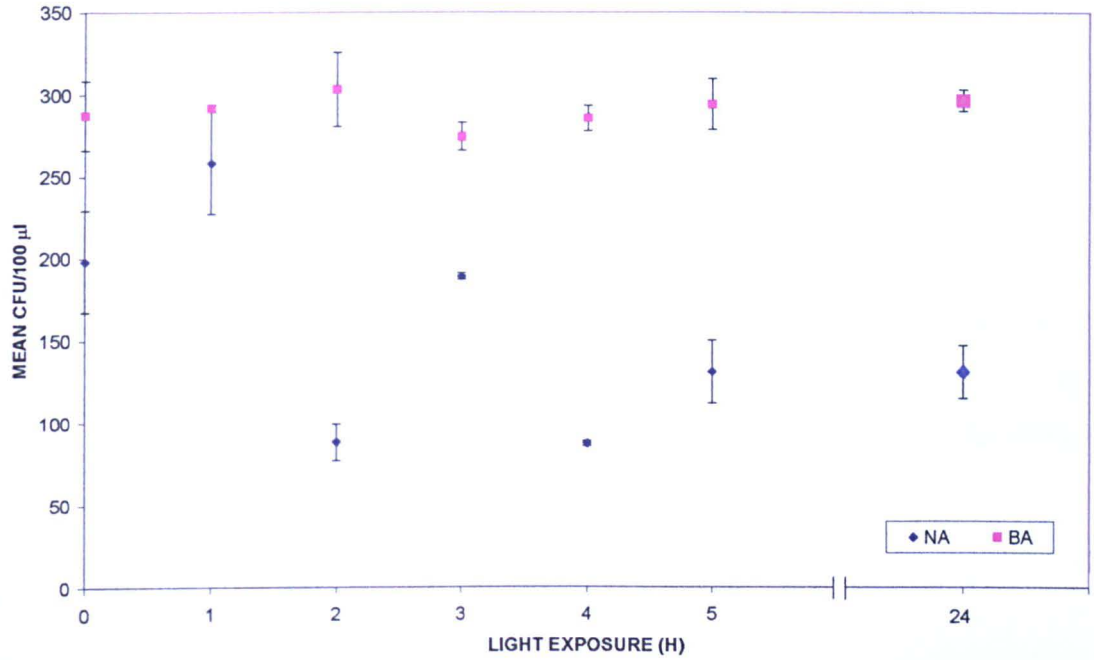

FIGURE 5.12 *Effect of light exposure on E. coli survival when exposed on Nutrient and Blood agar*

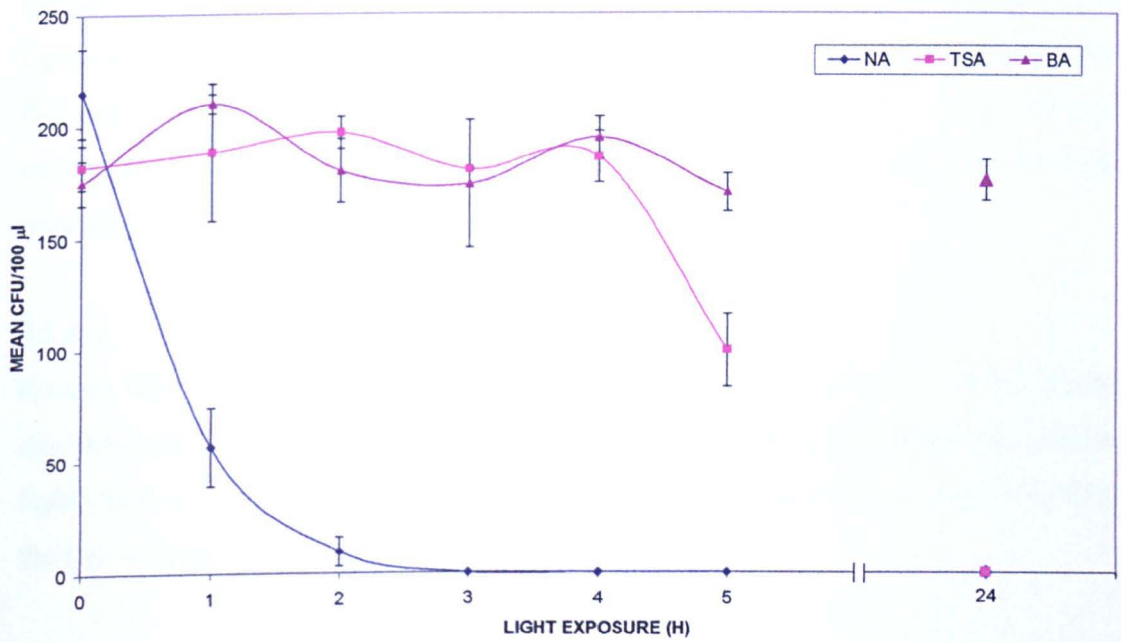

FIGURE 5.13 *Effect of light exposure on S. aureus NCTC 4135 survival when exposed on a selection of media at 30 ℃.*

5.3.5 Further Investigation of Different Media

As described in Section 5.3.3, Milk agar is believed to induce the production of pigments – a carotenoid pigment in the case of *S. aureus*. A study of possible medium protection was therefore made through culturing *S. aureus* in two different ways **prior to light exposure on the surface of Nutrient agar**:

(i) on Milk agar in an attempt to induce pigment production, which may ultimately provide protection against the light exposure, and

(ii) on Blood agar in order to investigate if 24-hour culture on this agar prior to visible-light exposure imparts some form of protective effect to the bacteria or, if protection is only provided when light-exposed while plated on the surface of Blood agar.

5.3.5.1 Bacterial Preparation and Treatment Method

Staphylococcus aureus NCTC 4135 was spread plated onto MA and incubated in the dark at 37°C for 24 hours. The plate was then maintained at room temperature for a further 24 hours as this is believed to further induce pigment development [Brown and Foster, 1970]. The colonies were then washed off the plate (using PBS) and resuspended in 9 ml PBS. Dilutions were then prepared, spread plated onto NA and light-exposed as before. For the BA experiment, *S. aureus* was spread plated onto BA and one plate dark incubated and another light incubated. After 24-hour incubation at 37°C, the bacteria were suspended and diluted in preparation for light exposure as in the MA experiment.

5.3.5.2 Results

Results from these experiments are shown in Figures 5.14 and 5.15. It was found that prior growth on either BA or MA does not confer any survival properties against light exposure. Also, light or dark incubation of the BA spread plates does not affect the rate of inactivation.

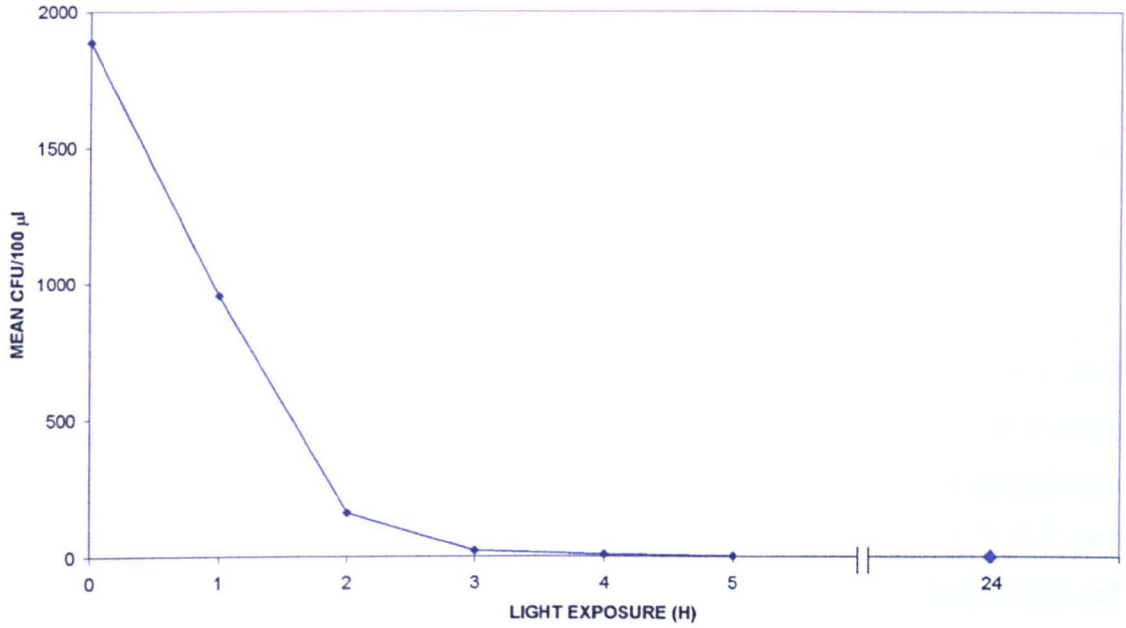

FIGURE 5.14 *Effect of light on S. aureus cultured on MA prior to exposure on NA*

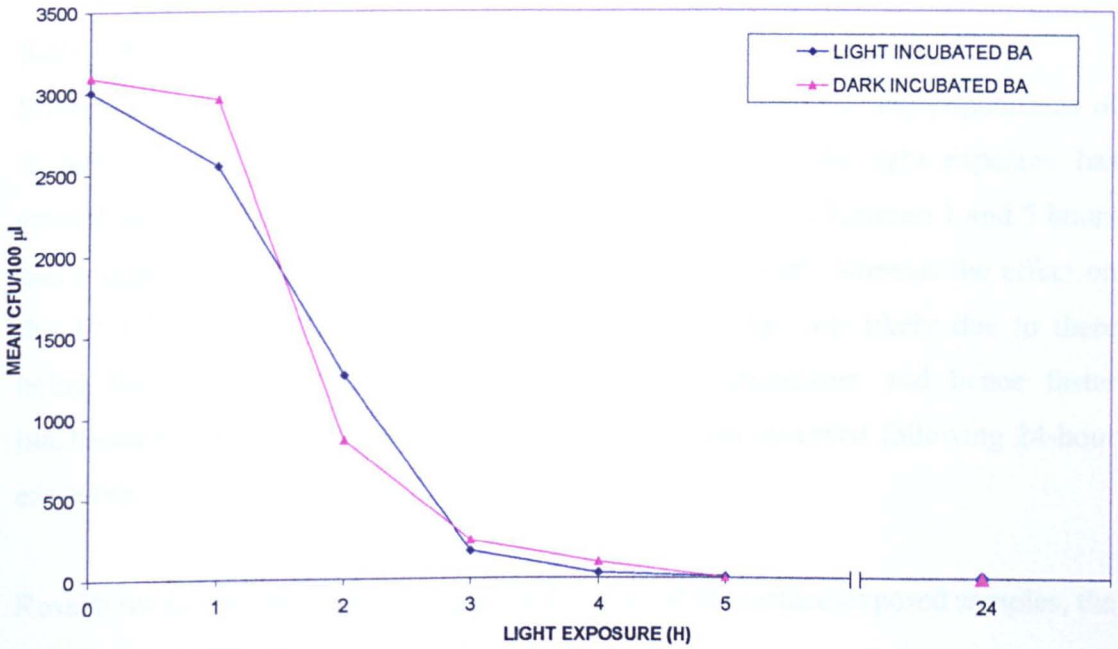

FIGURE 5.15 *Effect of light on S. aureus cultured on BA prior to exposure on NA*

5.4 LIGHT TREATMENT OF BACTERIAL SUSPENSIONS

With the biocidal effects of non-UVC light now established for surface-inoculated bacteria, the study was further advanced by investigating the treatment of bacterial suspensions. The use of suspensions also allowed the exposure of higher bacterial starting populations.

5.4.1 Bacterial Preparation and Treatment Method

Staphylococcus aureus and *E. coli* were prepared for experimental use as described in Section 3.1.2. Bacterial suspensions were diluted to give a range of starting populations. Four millilitre volumes of the selected dilution were then dispensed into sterile Petri dishes, sealed and placed in the light cabinet. As before, 1, 2, 3, 4, 5, and 24-hour light exposures were used, after which the samples were wrapped in foil and left in the cabinet for the remainder of the 24-hour incubation period. Control suspensions were covered in foil and placed in the cabinet for the entire 24-hour period. After exposure/incubation the suspensions were plated onto NA and incubated at 37°C for 24 hours.

5.4.2 Results

Figure 5.16 shows the effect of light exposure on different starting populations of *S. aureus* NCTC 4135. Results clearly demonstrate that the light exposure has caused inactivation of the suspended cells. Light exposure of between 1 and 5 hours had a gradual effect on the 10^4 and 10^5 CFU/ml populations, whereas the effect on the 10^3 CFU/ml population occurred more rapidly. This was likely due to there being less shading of the organisms within the suspension and hence faster inactivation. For all populations, total inactivation was observed following 24-hour exposure.

Results for *E. coli* are shown in Figure 5.17. As for the surface-exposed samples, the light had no inactivating effect on *E. coli*.

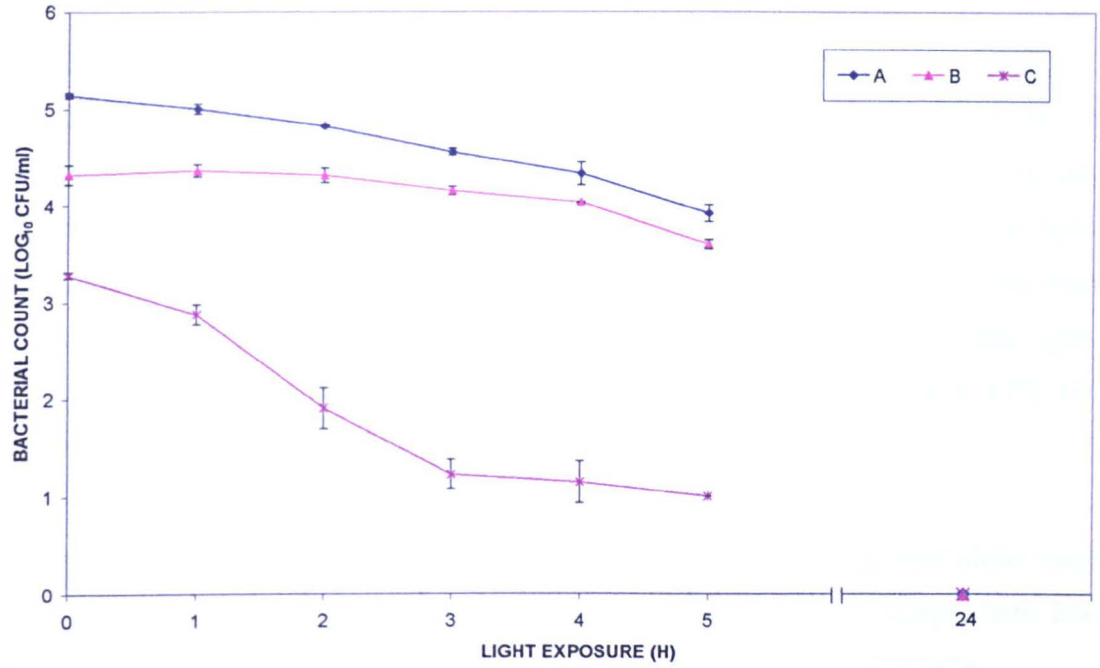

FIGURE 5.16 *Effect of light exposure on the growth of Staphylococcus aureus NCTC 4135 suspensions of differing starting population (A: 10^5 population; B: 10^4 population; C: 10^3 population)*

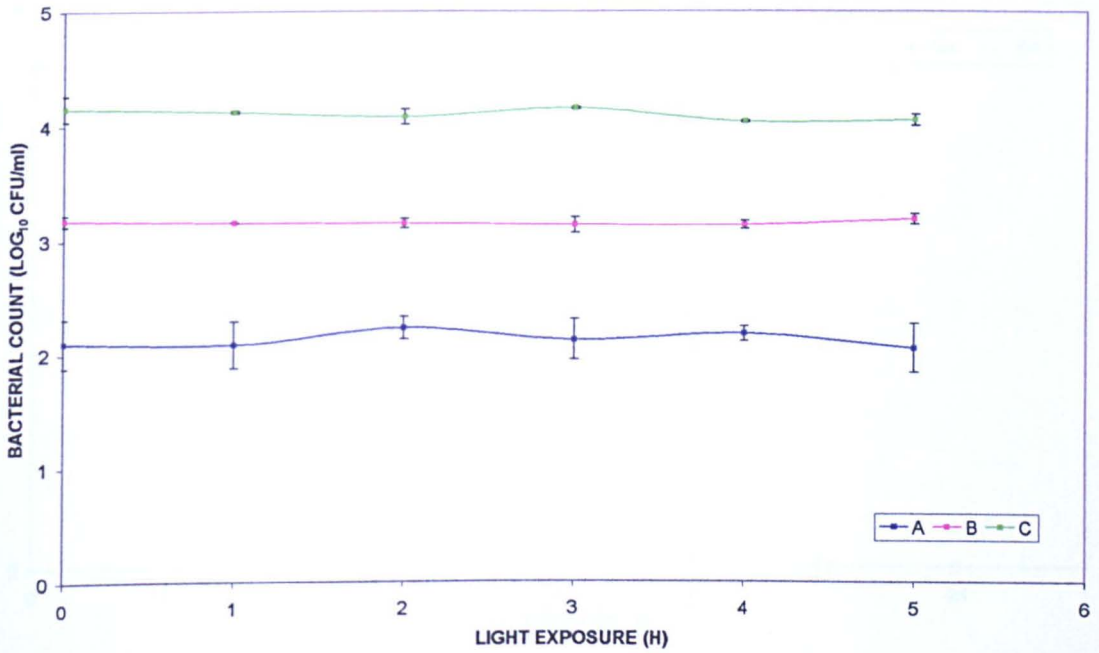

FIGURE 5.17 *Effect of light exposure on the growth of Escherichia coli NCTC 9001 suspensions of differing starting population (A: 10^2 population; B: 10^3 population; C: 10^4 population)*

5.4.3 Bacterial Growth on Blood Agar after Exposure

Previous results showed, firstly, that *S. aureus* NCTC 4135 plated onto a BA surface is not inactivated following 24-hour light exposure in the light cabinet (Figure 5.10), and secondly, that growth on BA prior to light exposure on NA does not impart protection against light inactivation (Figure 5.15). A further medium experiment was carried out to examine whether plating a *S. aureus* **suspension** onto BA after light exposure would induce any survival response within the cells. This experiment followed the exact method described in Section 5.4.1 with the exception that light-exposed samples were plated onto BA rather than NA before incubation at 37°C for 24 hours.

The results are shown in Figure 5.18. The NA and BA-plated samples show very similar counts, leading to the conclusion that plating a suspension sample onto BA after light exposure does not induce any survival effect in the *S. aureus* cells.

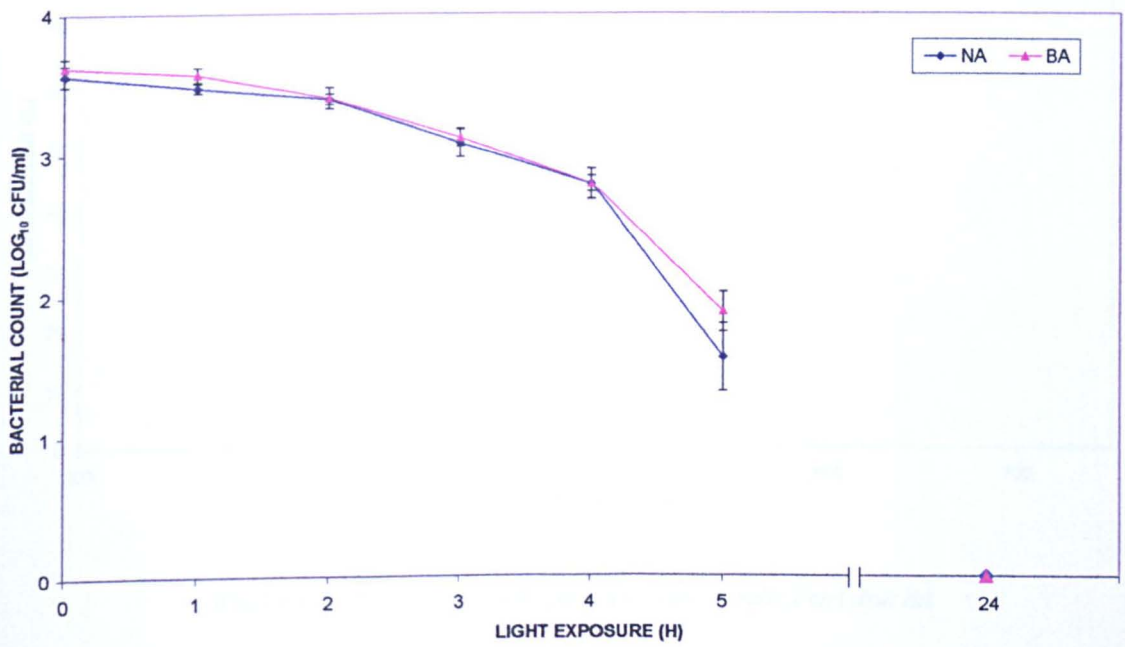

FIGURE 5.18 *Comparison between the surviving counts of exposed suspensions of S. aureus NCTC 4135 plated on to NA and BA plates*

5.5 INVESTIGATION INTO CAUSATIVE WAVELENGTHS

The aim of this section of the study was to establish which region of the electromagnetic spectrum was responsible for the lethal effect on the *Staphylococcus aureus* (and *Micrococcus*) strains. As ascertained in Section 5.3, inactivation cannot be a result of UV-C (240 – 280 nm) – or even UV-B wavelengths (280 – 320 nm) – because they are not emitted by the cabinet light source, as seen from the spectrum in Figure 5.3.

Since all experiments were conducted with the Petri dish lids in place, the transmission spectrum of the acrylic lid was obtained, as shown in Figure 5.19, in order to examine any possible light absorption. Comparison with the emission spectrum of the light cabinet source (Figures 5.2 and 5.3) shows that good transmission does take place for all source wavelengths.

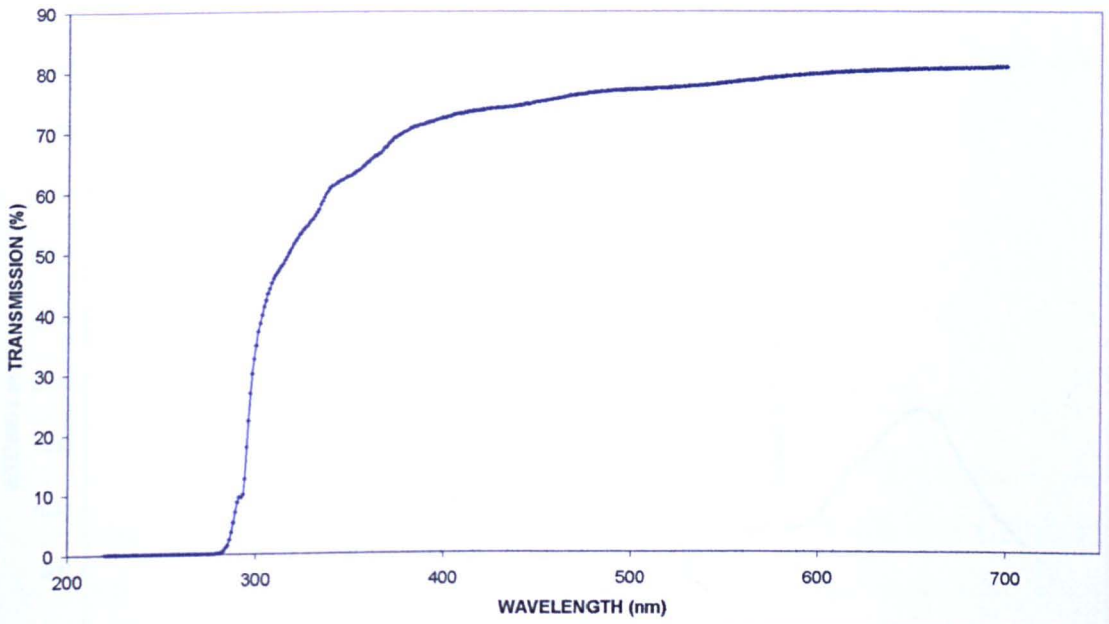

FIGURE 5.19 *Transmission spectrum of the acrylic Petri dish lid*

The next step in the investigation was to determine whether the effect was caused by either the UV-A (320- 400 nm), which is known to be capable of damaging bacteria

via the production of intra-cellular active species such as singlet oxygen and hydroxyl radicals [Oguma *et al*, 2002], or the visible region (400 – 700 nm). The easiest way of doing this was to focus on the effects of the visible wavelengths by filtering out wavelengths of <400 nm. This was done using a commercially available 400 nm longwave pass filter (which only allowed transmission of >400 nm). The emission spectrum of the light cabinet through this filter is shown in Figure 5.20.

A 10^3 CFU/ml population of *S. aureus* NCTC 4135 was spread plated onto NA and the 400 nm longwave pass filter was placed on top of the Petri lid. The remaining uncovered area was covered with foil to ensure all light striking the plate passed through the filter. The plates were then incubated in the light cabinet at 37°C for 24 hours.

Results showed that the transmitted wavelengths caused the complete inactivation of *S. aureus*. This confirmed the causative wavelengths were above 400 nm: **within the visible region**.

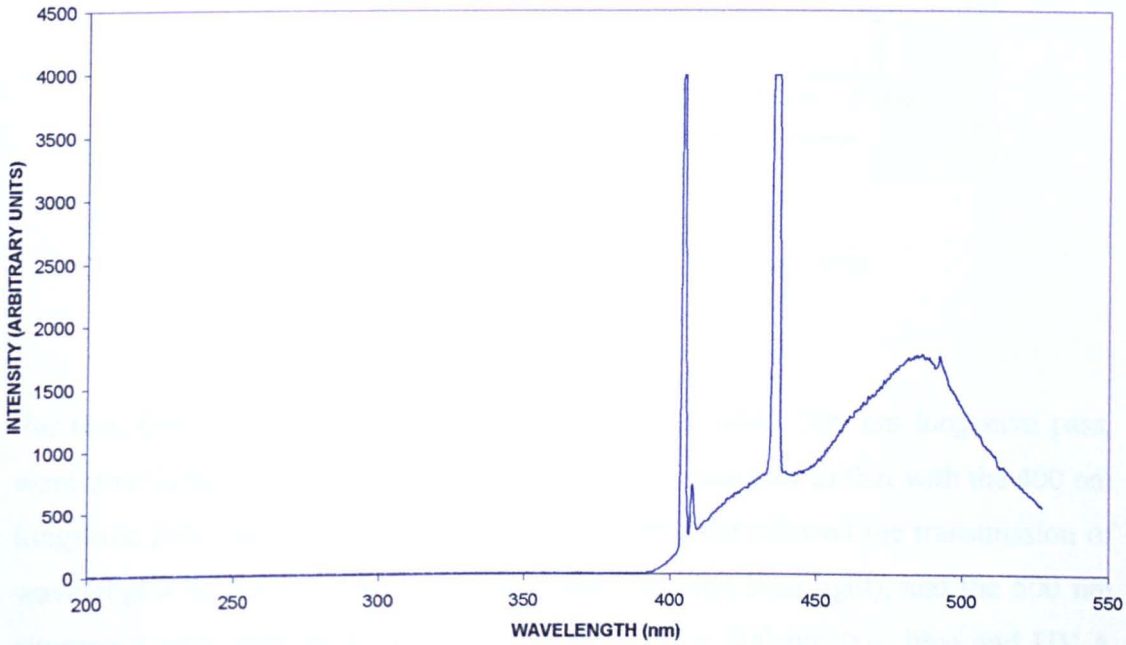

FIGURE 5.20 *Emission spectrum of light cabinet (200 – 500 nm) through a 400 nm longwave pass filter*

With the causative wavelengths now confirmed as being >400 nm, investigations continued with an aim to identify the part of the visible spectrum responsible for inactivation.

Visible light, shown in Figure 5.21, is part of the electromagnetic spectrum and is of wavelengths longer (and less energetic) than UV but shorter (and more energetic) than infrared, and is from approximately 380 to 750 nm. Violet/Blue is the first colour in the spectrum with a wavelength of 400 – 500 nm and was therefore the first wavelength region investigated.

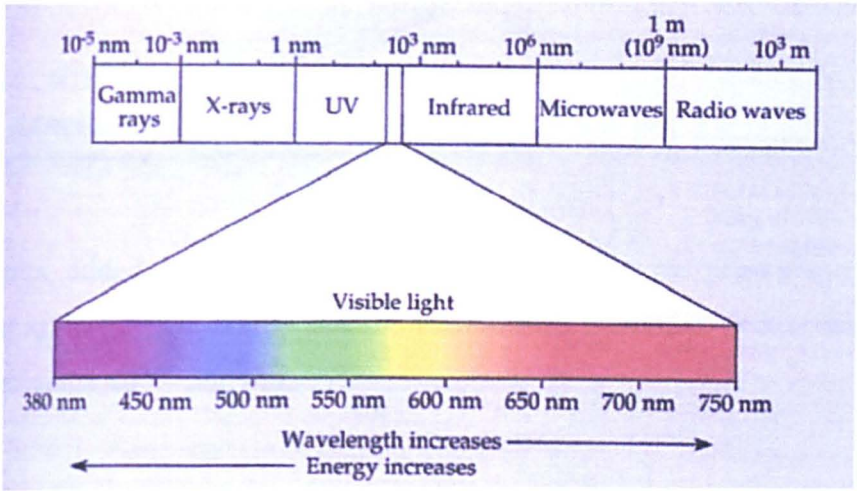

FIGURE 5.21 *Visible light spectrum [Campbell, 1996]*

For this, two other filters, a 500 nm shortwave pass and a 500 nm longwave pass, were used in turn and testing was performed in the same way as that with the 400 nm longwave pass filter. The 500 nm longwave pass filter allowed the transmission of wavelengths of 500 nm and above (therefore blocking blue light), and the 500 nm shortwave pass filter transmitted wavelengths below 500 nm (i.e. blue and UV-A light). The transmission spectra of these are shown in Appendix B.

Results showed that wavelengths below 500 nm caused the complete inactivation of *S. aureus*, whereas exposure of *S. aureus* to wavelengths of 500 nm and above caused no bactericidal effect. This confirmed the causative wavelengths to be below 500 nm.

TABLE 5.2 *Summary of Filter Results. Results are recorded as bacterial kill (+) or no kill (-) as determined by the presence absence of a zone of inhibition at the exposed area on the agar surface.*

FILTER	BACTERIAL KILL AFTER 24 H EXPOSURE
400 nm LONGWAVE PASS	+
500 nm SHORTWAVE PASS	+
500 nm LONGWAVE PASS	-
DARK CONTROL (NO FILTER)	-
LIGHT CONTROL (NO FILTER)	+

These results, added to the fact that the causative wavelengths were above 400 nm, lead to the important conclusion that the wavelengths causing *S. aureus* inactivation were in the range **400 – 500 nm**.

5.6 SUMMARY AND DISCUSSION

The main findings of the work with the light incubation cabinet described in this chapter are:

- Photoreactivity does occur for pulsed-UV-treated air samples but following sufficient visible-light exposure, the photoreactivity is outweighed by inactivation.
- Visible-light inactivation occurs for a range of Gram-positive bacteria – both surface-inoculated and in liquid suspension – but not for *E. coli*, the only Gram-negative bacteria tested.
- Temperature is not a factor in the visible-light inactivation process.
- The active wavelengths are in the range 400 nm to 500 nm.

Also investigated was the role of the medium used for surface exposure of the bacteria. Exposure on NA, TSA or MA resulted in complete inactivation, albeit at slightly differing rates. Exposure on BA however, showed complete survival of the organisms. The first thought was that this medium conferred some type of survival properties to the bacteria. This was subsequently ruled out and exposure on CBA was then performed. The method of preparation for this medium causes lysis of the red blood cells (RBC) and subsequent denaturing of the enzyme catalase released by the RBCs upon lysis. The possibility that catalase within the BA was detoxifying the reactive oxygen species produced by the bacterial cells upon exposure to UV-A, therefore preventing inactivation, was ruled out by this experiment as inactivation was again absent when exposed on the CBA.

From these media results and the fact that the causative wavelengths were found to be in the visible region of the electromagnetic spectrum between 400 nm and 500 nm, it was concluded that the most likely reason for bacterial survival while exposed on BA and CBA was light absorption by these red/red-brown coloured media. Medium light absorption is also the most likely reason for the slightly decreased inactivation rate when the bacteria were exposed on TSA compared to NA and MA.

The absence of susceptibility for *E. coli* may relate to the fact that this organism is Gram-negative. It is as yet undetermined whether *E. coli* is resistant to the bactericidal effects of the light exposure or if the inactivation effect was merely not being induced by the light intensity and exposure times being implemented in this study. Exposure of *E. coli* to a more intense light source will aid in this investigation, and will be further investigated in Chapter 6.

The observation that visible light has bactericidal properties has been previously documented with other bacteria – most notably, the acne-associated bacterium *Propionibacterium acnes*. Research into *Propionibacterium acnes* has found that irradiation of this organism with blue light leads to photosensitisation of intracellular

molecules called porphyrins, which in turn generate singlet oxygen and eventually cell death [Ashkenazi *et al*, 2003; Papageorgiou *et al*, 2000].

Other bacteria that have been found to be susceptible to visible-light exposure include *Helicobacter pylori* and some oral black-pigmented bacteria [Ganz *et al*, 2005; Soukos *et al*, 2005; Ashkenazi *et al*, 2003], and these bacteria, and the mechanism of their inactivation, will be discussed in the following chapters.

This chapter used a light incubation cabinet for the exposure of bacteria and bacterial suspensions to visible-light. For progression of the experimental work it was decided that the use of a more intense light source, which would potentially decrease exposure times, would be the ideal next step. This forms the basis for the experimental work in the following chapters.

Chapter 6

INACTIVATION OF STAPHYLOCOCCAL SPECIES THROUGH VISIBLE LIGHT EXPOSURE

6.0 BACKGROUND

Although there is a wide variety of both pathogenic and opportunistic microorganisms that are problematic within the hospital environment, none has been under the spotlight more intensely than methicillin-resistant *Staphylococcus aureus* – the so-called '*superbug*'.

Staphylococcus aureus is a Gram-positive cocci, ~1 μm in diameter, commonly found colonising skin (favouring moist skin folds), as well as in the oropharynx, gastrointestinal and urogenital tracts. *S. aureus* causes disease through the production of toxins or via the direct invasion and destruction of tissue [Murray *et al*, 1998] and the diseases are summarised in Table 6.6. Methicillin-resistant *S. aureus* (MRSA) causes the same diseases as methicillin-sensitive *S. aureus* (MSSA) but because of the difficulties in treating MRSA infections they can have more serious, often fatal results.

Staphylococci are commonly found in the nasopharynx and on the skin, with approximately 30% of healthy humans being asymptomatic carriers, rising to 50% in healthcare workers and hospital inpatients [Cooper *et al*, 2003]. As a result, shedding of the organism is common and is responsible for many hospital-acquired infections. This organism can also survive on dry surfaces for long periods, with a study by Neely and Maley [2000] indicating that staphylococci (and enterococci) can survive for days to months after drying on common hospital fabrics and plastics.

TABLE 6.6 *The wide-spectrum of diseases caused by Staphylococcus aureus infection [Murray et al, 1998; Sleigh and Timbury, 1998]*

DISEASE TYPE	DISEASE MANIFESTATION
Cutaneous	Pustules; Carbuncles; Abscesses; Impetigo; Folliculitis; Sycosis barbae; Conjunctivitis; Furuncles; Wound infections (including postoperative sepsis)
Toxin-mediated	Scalded skin syndrome; Toxic shock syndrome; Food poisoning
Deep infections	Bacteraemia; Septicaemia; Endocarditis; Pneumonia; Empyema; Osteomyelitis; Septic arthritis; Pyaemia

S. aureus transmission, particularly within the health-care environment, is mainly through person-to-person via direct contact or contact with fomites such as contaminated clothing or bed linens [Murray *et al*, 1998]. Airborne transmission has also been documented, with movement in the wards, and actions such as bed-making, being responsible for elevated airborne levels [Shiomori *et al*, 2002; Shiomori *et al*, 2001].

Hospital-acquired *Staphylococcus* infection usually manifests itself as a blood-stream infection, a surgical site infection or pneumonia [Block, 1991]. Endogenous infection can also occur. The majority of infections are thought to be initiated during occupation of hospital wards. Here, the risk of infection from MSSA and MRSA – as well as other hospital-acquired infections – is high due to the likelihood of cross-contamination from a variety of sources such as medical staff, contaminated fomites and the air environment.

Due to the problems caused by MRSA infection, the UK Department of Health introduced compulsory reporting in April 2001. Since then there has been an 8% increase (17,933 to 19,311) in the number of reported *S. aureus* bacteraemias, and a

5% increase (7,250 to 7,647) in the number that are methicillin-resistant [National Audit Office, 2004]. The overall proportion of *S. aureus* infections that are methicillin-resistant in the UK is 45% and the European Antimicrobial Resistance Surveillance System data for 2002 highlighted that the UK has among the worst rates in Europe, along with Italy, Greece and Spain [Tiemersma *et al*, 2004]. Statistics such as these reinforce the fact that current hospital cleaning and sterilisation measures are not efficient enough – therefore there is an urgent need for the development of an effective method for the prevention and control of MRSA infection.

Numerous reasons, both medical and financial, have made hospital-acquired infections a worldwide issue of growing concern. Although much of the media and political focus is on MRSA, there are other very significant infection-causing bacteria present within the hospital environment. Patients, due to pre-existing conditions and/or immunocompromisation, are predisposed to infection by organisms such as coagulase-negative *Staphylococcus* (CONS), *Streptococcus* and multiple-drug resistant *Enterococcus*, either through direct contact or airborne transfer. The acquisition of infection from MSSA, MRSA and these other organisms results in increased hospital stays, often serious healthcare complications, and in some cases, death. The effect of visible-light exposure on a selection of these organisms was investigated in this study.

Coagulase negative staphylococci (CONS) are Gram-positive cocci (0.5 – 1.0 μm). They are the major coloniser of human skin but, as with *S. aureus*, they are also found in the oropharynx, gastrointestinal and urogenital tracts [Murray *et al*, 1998]. Coagulase-negative staphylococci is a collective name, and this group includes all species of staphylococci found in humans with the exception of *S. aureus* (which produces the coagulase enzyme). Species include *S. epidermidis*, *S. saprophyticus*, and *S. haemolyticus*, and as with *S. aureus*, multiple-antibiotic-resistance is becoming a problem. These organisms are more commonly associated with opportunistic infections such as bacteraemia, endocarditis, catheter and shunt infections and prosthetic joint infections [Murray *et al*, 1998], and are usually

introduced into a host via their adherence to catheters and other synthetic material such as prosthetic valves and joints, grafts, and shunts. Here they may cause localised infection or progress to a systemic, potentially fatal, bacteraemia [Murray *et al*, 1998]. Data from the National Nosocomial Infections Surveillance System from January 1990 to May 1999 indicate that CONS are isolated from bloodstream infections in patients in intensive care units more often than *Staphylococcus aureus* (37.3% versus 12.6%) [NNIS System, 1999].

Streptococcus pyogenes is a Gram-positive coccus (0.5 – 1.0 μm) that produces a large number of powerful toxins and enzymes [Sleigh and Timbury, 1998]. It is commonly found colonising the oropharynx of healthy children and adults, and is associated with infections such as pharyngitis, pyoderma, scarlet fever, erysipelas, cellulitis, streptococcal toxic shock syndrome, rheumatic fever, glomerulonephritis, bacteraemia and necrotizing fasciitis –hence often referred to as 'flesh-eating bacteria' [Murray *et al*, 1998]. It has been reported that pharyngeal carriage or skin infection among hospital personnel is a common source of *S. pyogenes* nosocomial transmission, either via direct or droplet contact, with infection most often occurring in surgical and obstetric patients [Takahashi *et al*, 1998].

Enterococcus faecalis is another Gram-positive coccus commonly found colonising the large intestine. Over the last decade these bacteria have become recognised as the leading cause of nosocomial bacteraemia, urinary tract infection and surgical wound infection [Huycke *et al*, 1998]. In addition to these, they can also induce endocarditis and meningitis in severely ill hospitalised patients [AMM, 1997]. Antibiotic resistance among enterococci is also a noted problem – vancomycin-resistant enterococci (VRE) was first reported in 1986 and now many strains are showing complete resistance to all conventional antibiotics [Huycke *et al*, 1998; Murray *et al*, 1998]. Resistance of these strains to vancomycin and aminoglycosides is of particular concern due to the resistance being mediated by plasmids which can be transferred to other bacteria [Murray *et al*, 1998].

6.1 OUTLINE

As concluded in the previous chapter, visible light possesses bactericidal properties against some bacteria including *Staphylococcus aureus* and MRSA. Because of the long exposure times required when using the light cabinet, it was decided that for progression of this investigation, the use of a more intense light source, rich in the visible light region of interest, would be beneficial. Mercury-Xenon and Xenon spotlight sources, used in conjunction with a 400 nm longwave pass filter, were deemed as suitable up-grades from the fluorescent tubes of the light cabinet.

Initial experiments involved the treatment of surface contamination using the Mercury-Xenon lamp, with the work then progressing to the treatment of bacterial suspensions using the Xenon lamp.

This chapter details the effects of visible-light exposure on a range of Staphylococcal strains including *Staphylococcus aureus* NCTC 4135, MRSA LMG 15975, MRSA 16a, and *Staphylococcus epidermidis*, in addition to strains of *Enterococcus faecalis*, *Streptococcus pyogenes* and *Escherichia coli*.

The final section of the chapter goes on to investigate the effects of differing experimental parameters on the rate of bacteria inactivation. These parameters include temperature, sample volume, lamp output intensity and dose.

6.2 EXPERIMENTAL ARRANGEMENT

This section details the light sources and the experimental arrangements used for this section of the study.

6.2.1 Experimental Light Sources

As the research progressed, a more intense light source was required and a spotlight source (Lightningcure LC5, Hamamatsu Photonics UK Ltd.) with a 200 W Mercury-Xenon lamp was selected.

The emission spectrum of this lamp from 200 to 500 nm is shown in Figure 6.1. The Mercury-Xenon lamp has output in the visible region but also a high emission of UV wavelengths (200 – 380 nm).

At a later stage, the lamp was replaced with a 150 W Xenon lamp (Hamamatsu). Figure 6.2 shows the emission spectrum of the Xenon lamp from 200 to 500 nm. The reduced UV and increased visible wavelength emission of this lamp made it a more suitable light source.

Since the emission spectra of the two lamps, in particular the Mercury-Xenon lamp, contained UV wavelengths, a 400 nm long-wave pass filter was used in conjunction with the lamps in order to ensure the transmission of only visible wavelengths (>400 nm) and omission of all UV-light. This guarantees that inactivation is through the action of visible-light wavelengths. The emission spectra of the Mercury-Xenon and Xenon lamps through this 400 nm long-wave pass filter are also shown in Figures 6.1 and 6.2, respectively.

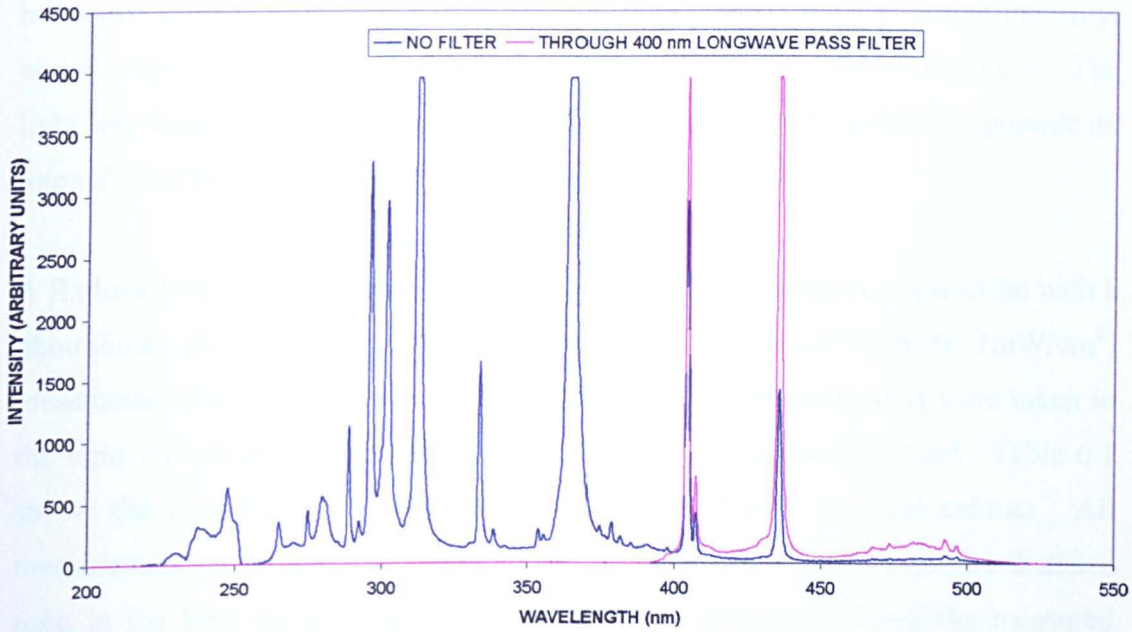

FIGURE 6.1 *Emission spectrum of Mercury-Xenon lamp from 200 to 500 nm. Also shown is the emission spectrum when passed through a 400 nm long-wave pass filter.*

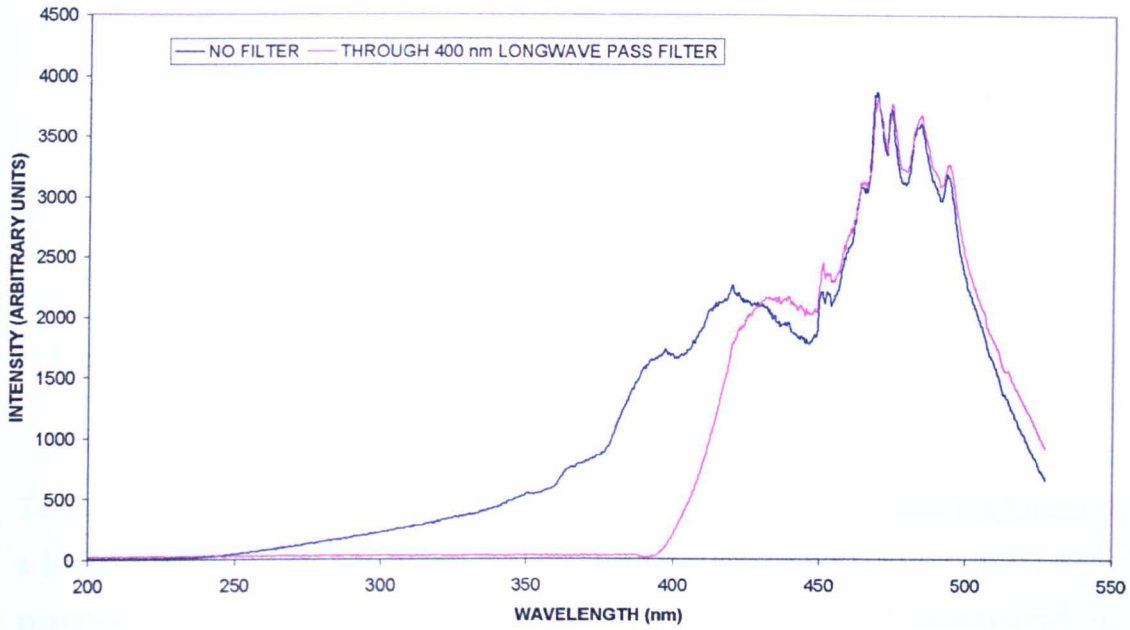

FIGURE 6.2 *Emission spectrum of Xenon lamp from 200 to 500 nm. Also shown is the emission spectrum when passed through a 400 nm long-wave pass filter.*

Each of the Mercury-Xenon and Xenon lamps was contained within a Lightningcure housing – shown in Figure 6.3 – and control of the lamp (ON/OFF, output intensity, etc) was by a digital display and touch pad located on the front panel of the unit. The light was transmitted along a light guide made up of a bundle of fibres to provide an intense source of light with a divergence angle of 12.4°.

A Radiant Power Meter (model 70260, L.O.T.-Oriel Ltd.) used in conjunction with a photodiode detector was used to obtain absolute power density (mW/cm^2) measurements of the Xenon lamp. In addition to this, measurements were taken in the light cabinet in order to compare the effective power densities used. Table 6.1 shows the power density values of the Xenon lamp and the light cabinet. All measurements were taken at a distance of 25 cm, as this was the exposure distance used in the light cabinet experiments detailed in Chapter 5. From the measured values it can be seen that the Xenon lamp has a much higher power density, with values for the total and >400 nm emission approximately six times those for the fluorescent tubes of the light cabinet.

TABLE 6.1 *Comparison of the power density values of the light cabinet and the Xenon lamp. All measurements were taken at a 25 cm distance from the light source.*

PARAMETER		LIGHT SOURCE	
		LIGHT CABINET*	XENON LAMP
POWER	TOTAL	3.48 mW	20.6 mW
	> 400 nm	3.07 mW	17.2 mW
	<400 nm	0.41 mW	3.4 mW

* 12 FLUORESCENT TUBES

To investigate the radial profile of the Xenon lamp the light-guide was positioned at a height of 25 cm from the bench-top and set at an output intensity of 100%. The power meter detector, positioned on the bench-top, was then moved from one side of the beam to the other, in increments of 1 cm, and the intensity measured at each point. From Figure 6.4, it can be seen that the intensity varies across the radius of the light beam, and two peaks of intensity occur.

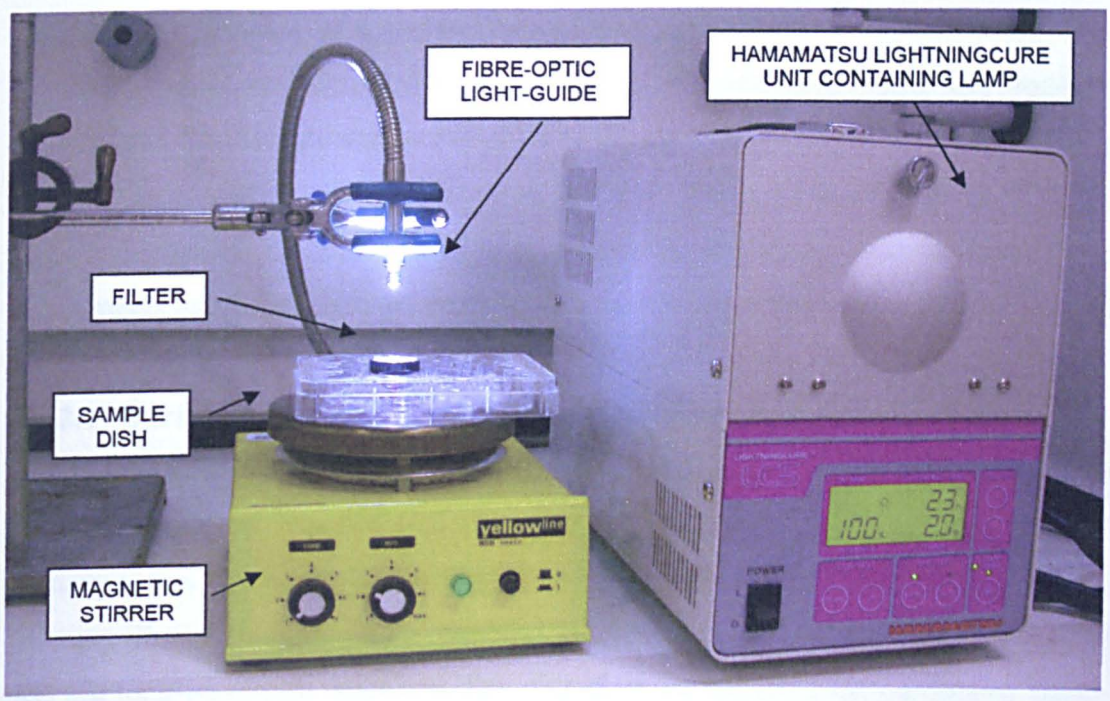

FIGURE 6.3 *Components of the Visible-Light Exposure System*

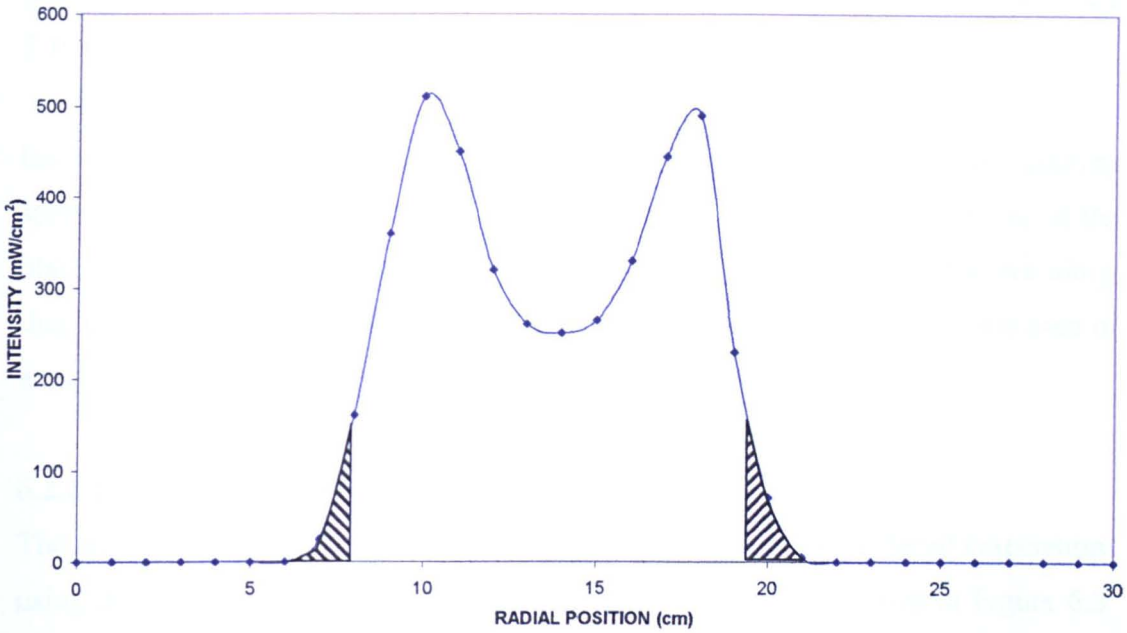

FIGURE 6.4 *Radial profile of the Xenon lamp with the light-guide at a distance of 25 cm from the detector. Highlighted are the less intense tails of the light beam which are not transmitted into the sample well when exposed at a 5 cm distance (discussed in Section 6.2.2.2).*

6.2.2 Experimental Arrangement

For this study, there were two experimental arrangements. The first was for the light treatment of surface-inoculated bacteria and used the Mercury-Xenon lamp, and the second was for the light treatment of bacterial suspensions, and this used the more efficient Xenon lamp. The set-ups were similar in principle, but due to the different aims of inactivating bacteria (1) on surfaces and (2) in suspensions, slight adjustments had to be made between the two in order to optimise the systems.

6.2.2.1 Surface-inoculated Bacteria Treatment System

To investigate the effectiveness of >400 nm light for inactivation of *S. aureus* on agar surfaces, the experimental arrangement was as follows: a suspension of *S. aureus* was spread plated onto NA and the fibre-optic light source (from the Mercury-Xenon lamp) was positioned 5 cm above the inoculated NA plate. The 400 nm long-wave pass filter was then placed on top off the plate lid directly below the lamp. The fact the Petri lid was kept on the plate during exposure was not a

problem as it does not absorb any wavelengths above 400 nm – as shown in Figure 5.19.

Because of the geometry of the deposition pattern of the spiral plater when preparing spread/spiral plates (Figure 3.2), the light source was not aimed at the centre of the plate but midway between the centre and edge. Successful bacterial inactivation is then characterised by a zone of clearing on the agar plate, directly below the area of exposure.

6.2.2.2 Bacterial-Suspension Treatment System

The experimental arrangement for the visible-light exposure of bacterial suspensions using the Hamamatsu Spot Light Source with Xenon lamp is shown in Figure 6.3. The bacterial sample was held within one well of a 12-well multidish (Nunc, Denmark), shown in Figure 6.5, and this sample well also contained a 7 mm x 2 mm magnetic follower (Fisher Scientific). A light shield (black PVC cylinder of height 16 mm and diameter 25 mm) was placed around the sample well to minimise the amount of stray light entering the well. The 400 nm longwave pass filter was placed on top of the well. The dish was then positioned directly under the light source on a magnetic stirrer (Yellowline MSH Basic). This, in conjunction with the magnetic follower, permitted continuous mechanical agitation of the sample during light exposure.

For the experiments detailed in this chapter all parameters were kept constant. The volume of bacterial suspension used in each experiment was 2 ml and the output intensity of the lamp was maintained at 100%. In each experiment control samples were used. These were subjected to identical conditions but were not exposed to light.

A significant parameter, also maintained fixed, was the distance of 5 cm between the light source and the surface of the bacterial sample. As illustrated in Figure 6.6, at this distance the whole well is light-exposed using the beam width shown in Figure 6.4 less the shaded-edge sections.

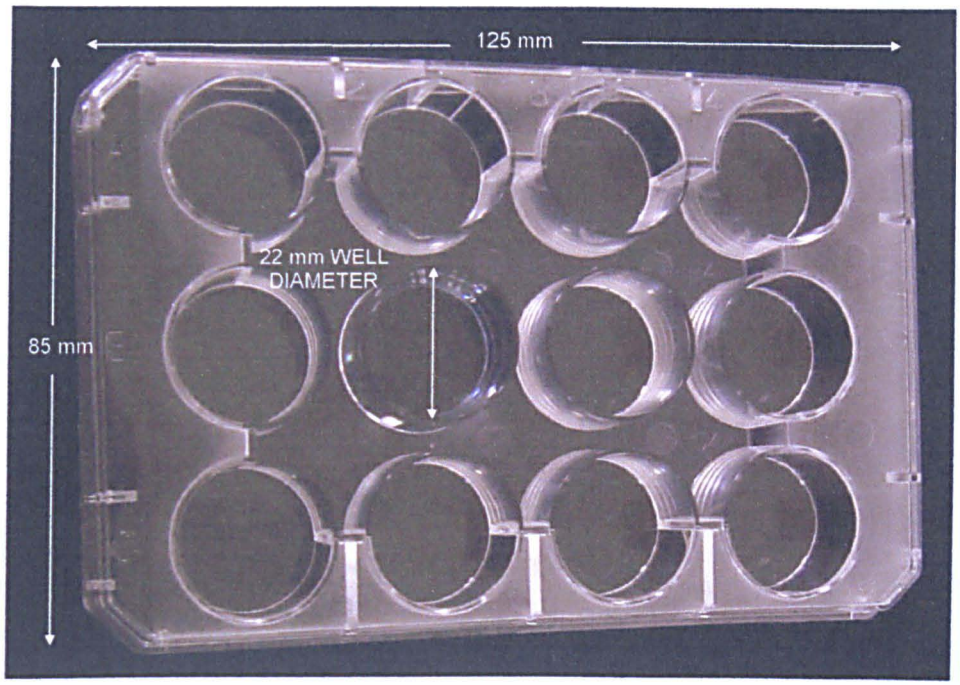

FIGURE 6.5 *12-well multidish (Nunc, Denmark) used as the sample dish for these experiments. In standard experimental procedure, the bacterial suspension is dispensed in one of the 12 wells, each with a diameter of 22 mm, and exposed to the xenon lamp through a 400 nm longwave pass filter.*

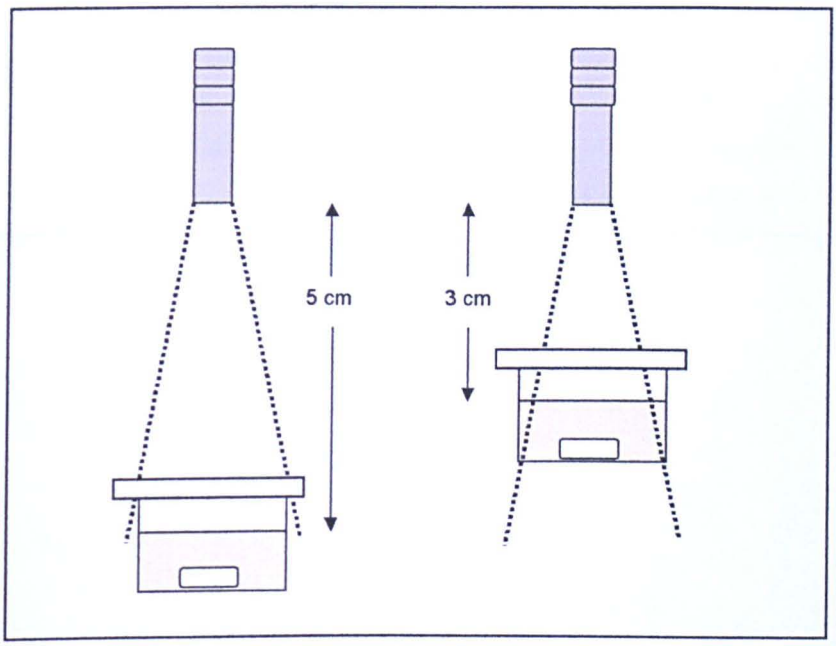

FIGURE 6.6 *Comparison of the use of 5 cm and 3 cm distances for bacterial-suspension exposure.*

With the confirmation of the use of a 5 cm exposure distance in these experiments, the next parameter to confirm was the power of the >400 nm light to which the bacteria were exposed when treated at a 5 cm distance.

Measurements were taken using the radiant power meter with the detector positioned 5 cm from the light source. Because the intensity of the original Xenon lamp declined with lamp operating time, during the course of the study the lamp was replaced. The power densities for the original and replacement lamps were 0.35 ± 0.05 W and 0.40 ± 0.05 W, respectively.

Table 6.2 is a summary of the experimental parameters, maintained constant throughout the investigation. The only changing parameters were the bacterial strains being exposed and the durations of light exposure.

TABLE 6.2 *Summary of the experimental parameters kept constant throughout the investigation*

EXPERIMENTAL PARAMETER	VALUE
SAMPLE VOLUME	2 ml
EXPOSURE DISTANCE	5 cm
LAMP OUTPUT	100%
POWER DENSITY	0.35 ± 0.05 W/cm^2 *
	0.40 ± 0.05 W/cm^2 #

* LAMP 1 VALUE # LAMP 2 VALUE

6.3 VISIBLE-LIGHT TREATMENT OF SURFACE BACTERIA

Populations of 10^5 and 10^4 CFU/ml of *S. aureus* NCTC 4135 were prepared and spread plated (100 µl) onto NA. The inoculated plates were treated as described in Section 6.2.2.1. Exposure times of 2, 4, 6, 8, 10, 15 and 20 minutes were used, with the lamp output intensity set at 100%. Plates were then incubated at 37°C for 24 hours.

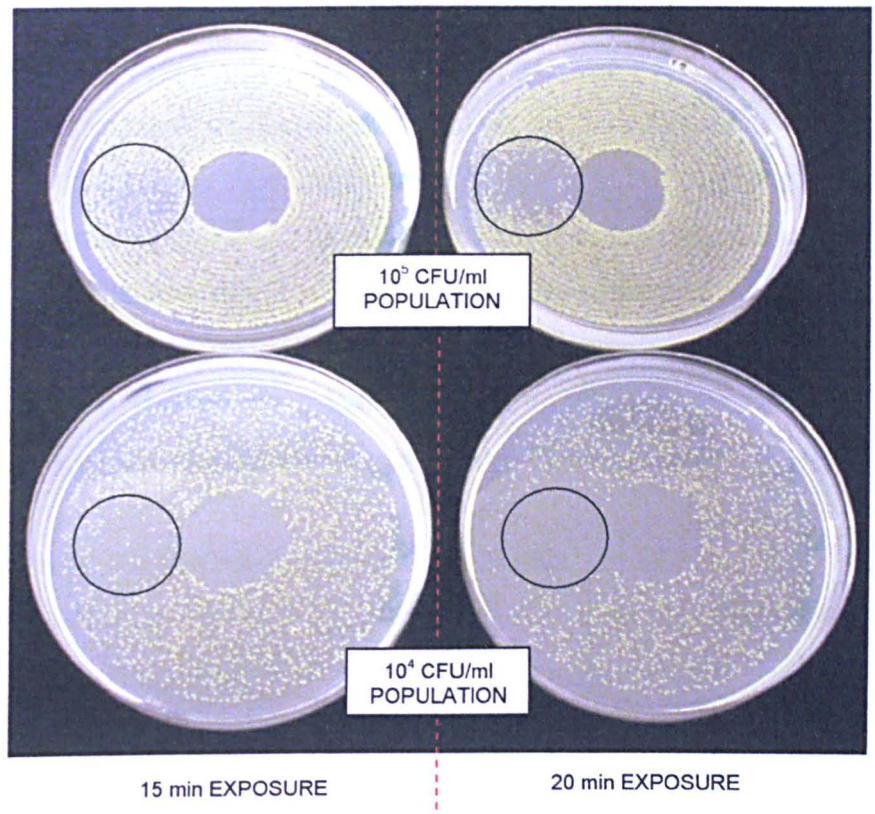

FIGURE 6.7 *Visual comparison of the effects of 15 and 20-minute light exposure on populations of 10^4 and 10^5 CFU/ml.*

Figure 6.7 shows the results for a selection of light-treated plates. It can be seen that inhibition of bacterial growth was evident after 15 minutes of light exposure and after 20 minutes, this was much more pronounced. Light exposures of 2, 4, 6, 8 and 10 minutes showed no growth inhibition. The results show that bacteria on agar surfaces can indeed be successfully inactivated through exposure to light of wavelengths >400 nm.

6.4 VISIBLE LIGHT TREATMENT OF BACTERIAL SUSPENSIONS

Following the successful inactivation of *S. aureus* on agar using the Mercury-Xenon lamp, work progressed to using the Xenon lamp for the inactivation of bacterial suspensions. This section investigates the effect of >400 nm light for the inactivation of *Staphylococcus aureus* strains including MRSA, as well as its effect against other medically important Gram-positive bacteria such as *Staphylococcus epidermidis*, *Streptococcus pyogenes* and *Enterococcus faecalis.*

6.4.1 Bacterial Preparation and Treatment Method

Bacteria were cultured as described in Section 3.1.2. After an 18-hour incubation period, the broths were centrifuged at 3400 rpm for 10 minutes and the resultant pellets re-suspended in PBS. These suspensions were then diluted to give a starting population of approximately 2 x 10^5 CFU/ml (unless otherwise specified). Using the treatment method described in Section 6.2.2.2, 2 ml samples of this suspension were then exposed to visible-light treatment for different times. Following exposure, samples were plated onto NA and incubated at 37°C for 24 hours.

6.4.2 Results: *Staphylococcus aureus* and MRSA

Figure 6.8 shows the results for *S. aureus* NCTC 4135 exposure to >400 nm light. Total inactivation (5-$\log_{10}$ reduction) of the bacteria took place after 30 minutes exposure. For a bacterial starting population of 2 x 10^7 CFU/ml, total inactivation was again observed but for an increased exposure time of 120 minutes, as shown in Figure 6.9.

When suspensions of MRSA LMG 15975 and MRSA 16a were exposed to visible-light treatment, successful inactivation was again observed. In Figures 6.10 and 6.11, it can be seen that a 5-$\log_{10}$ reduction in MRSA LMG 15975 population takes place after a 60-minute exposure, and for MRSA 16a, a 5-$\log_{10}$ reduction requires only a 45-minute exposure.

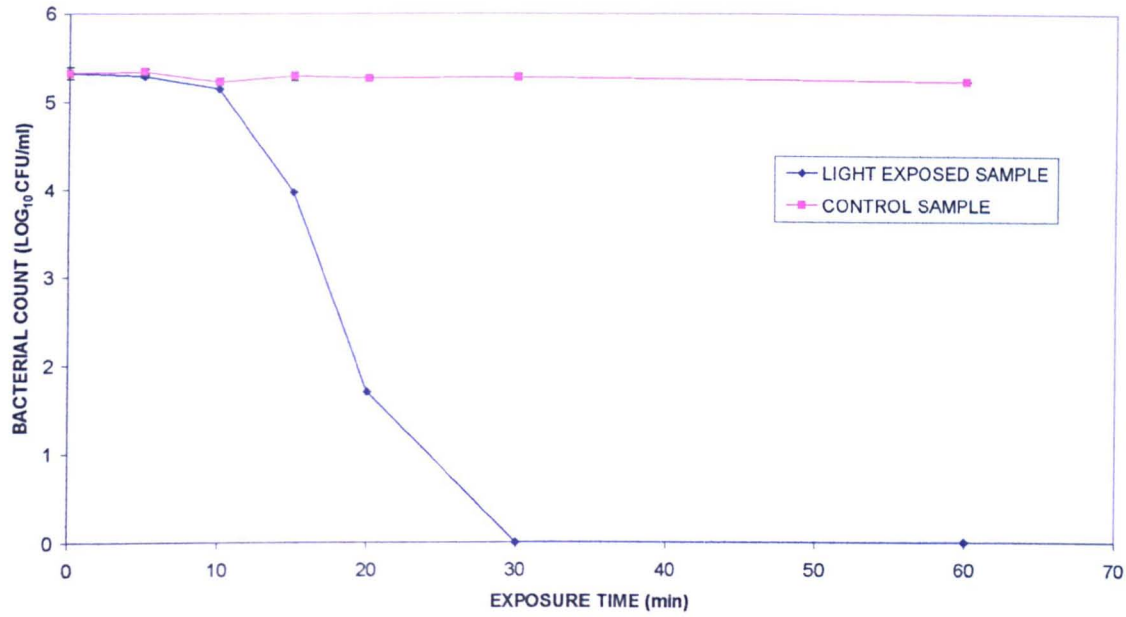

FIGURE 6.8 *The effect of >400nm light exposure on a Staphylococcus aureus 4135 suspension with a starting population of 2 x 10^5 CFU/ml.*

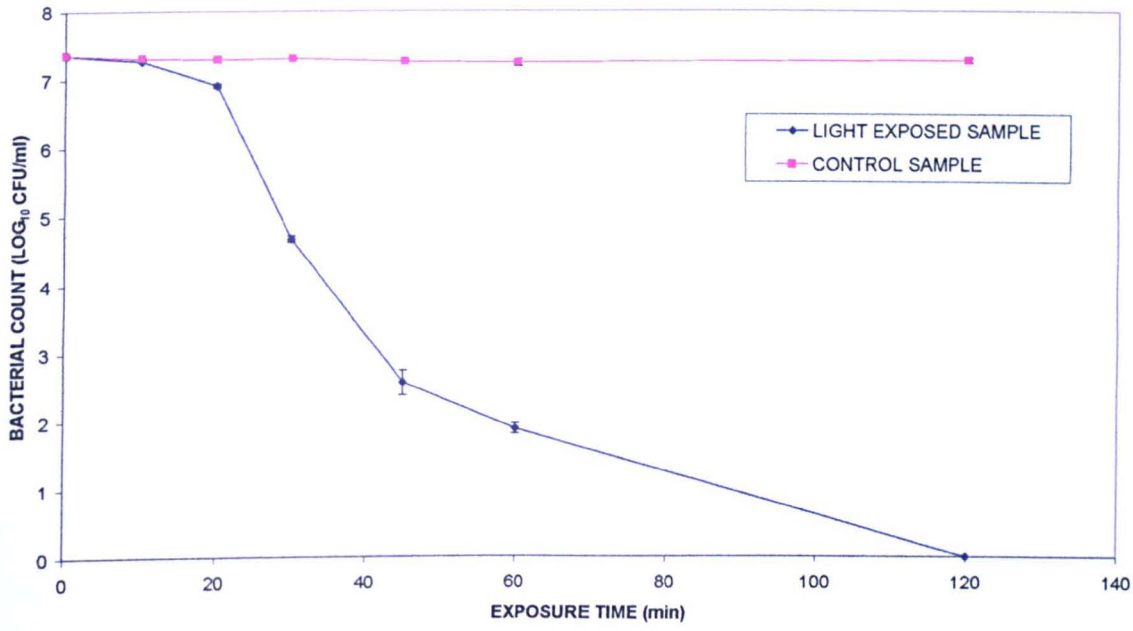

FIGURE 6.9 *The effect of >400nm light exposure on a Staphylococcus aureus NCTC 4135 suspension with a starting population of 2 x 10^7 CFU/ml.*

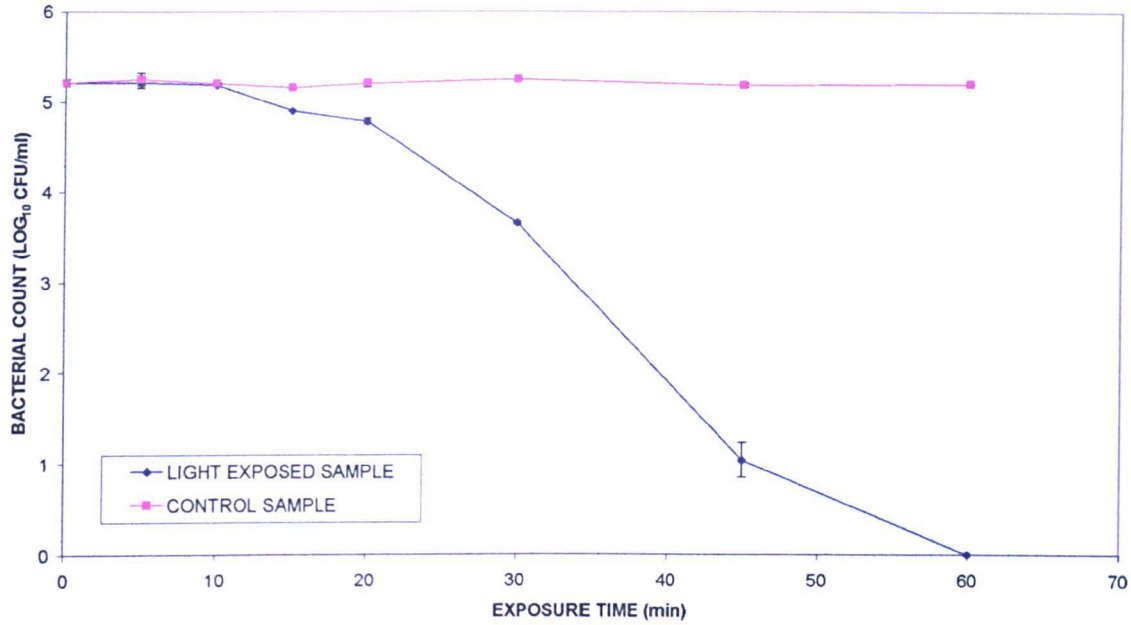

FIGURE 6.10 *The effect of >400nm light exposure on a MRSA LMG 15975 suspension.*

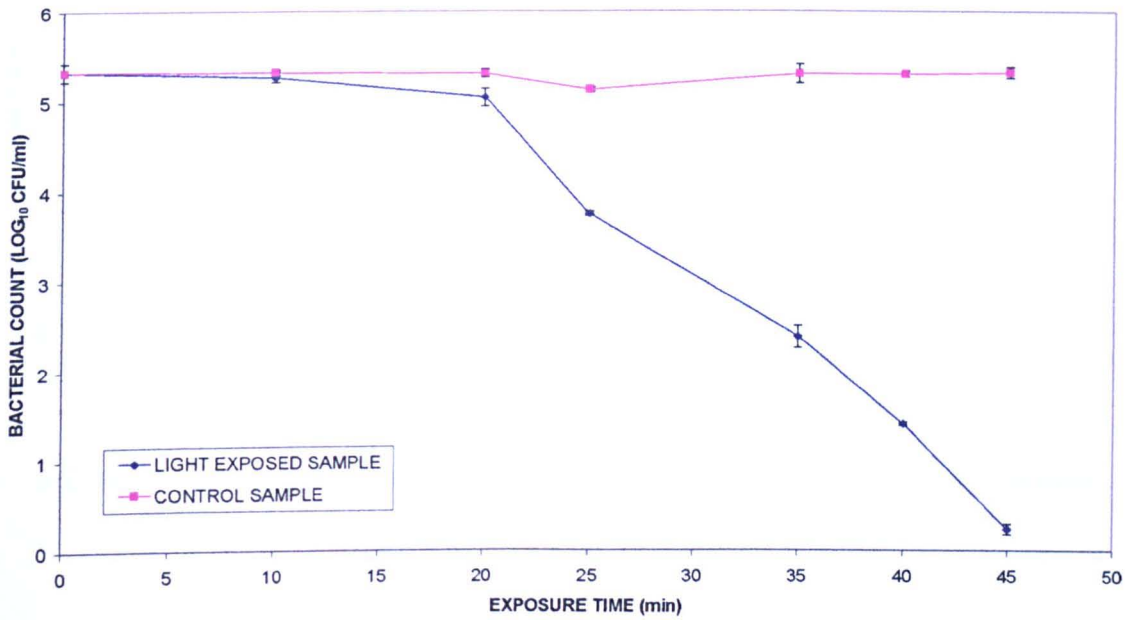

FIGURE 6.11 *The effect of >400nm light exposure on a MRSA 16a suspension.*

6.4.3 Visible Light Treatment of Other Medically Important Bacteria

With the successful application of visible light for the inactivation of *Staphylococcus aureus* strains including MRSA, the investigation broadened to investigate whether this visible-light treatment would be effective against other common hospital-acquired infection-causing bacteria. The preparation and treatment of all bacterial strains followed the same procedure as used for *S. aureus* and MRSA (Section 6.4.1).

6.4.3.1 *Staphylococcus epidermidis*

From Figure 6.12 it can be seen that the visible-light treatment was successful in the inactivation of *S. epidermidis* NCTC 7944, with complete inactivation (5-$\log_{10}$ reduction) being achieved after a 35-minute exposure.

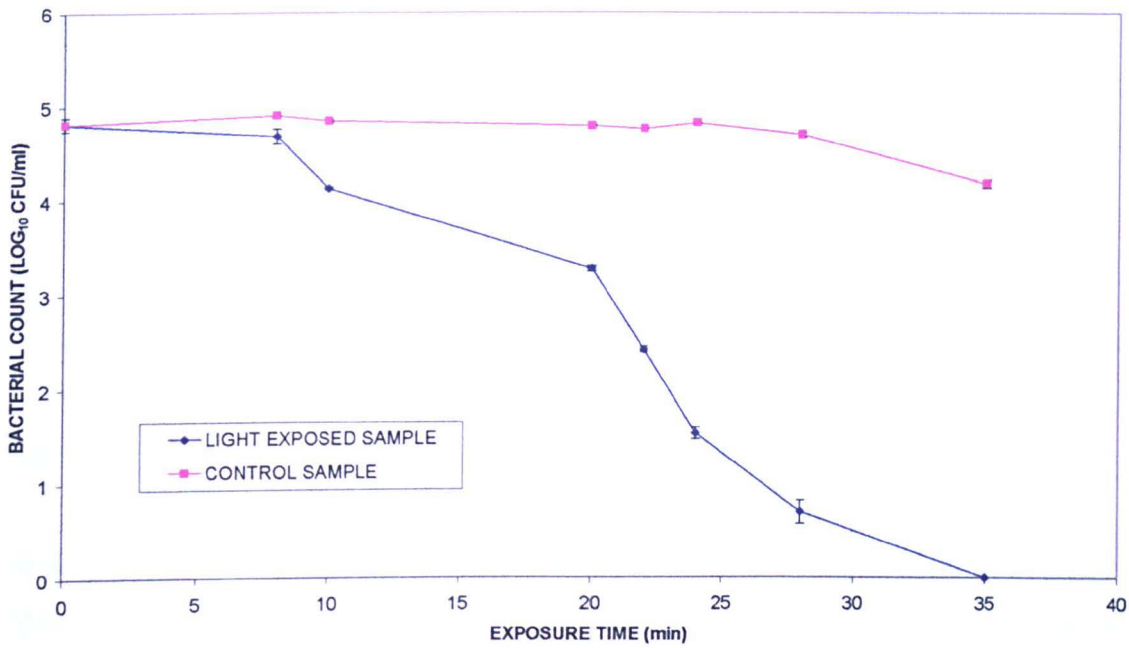

FIGURE 6.12 *The effect of >400nm light exposure on a Staphylococcus epidermidis suspension.*

6.4.3.2 *Streptococcus pyogenes*

Figure 6.13 shows the effect of visible-light exposure on a suspension of *S. pyogenes* NCTC 8198, with total inactivation of a 2 x 10^5 CFU/ml population being achieved after a 60-minute exposure.

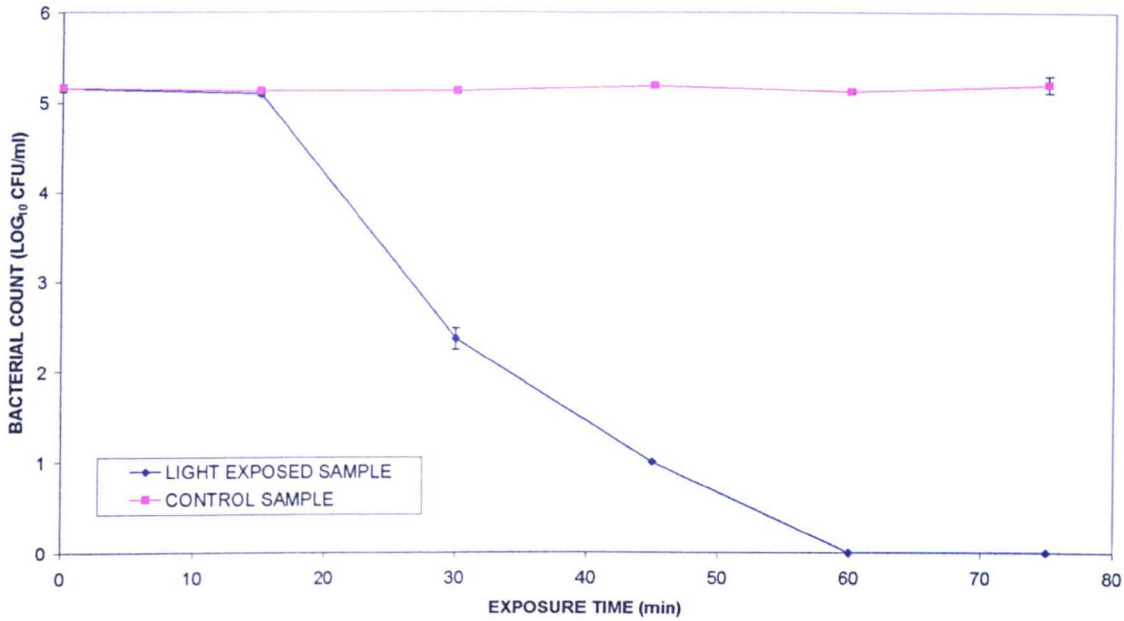

FIGURE 6.13 *The effect of >400nm light exposure on a Streptococcus pyogenes suspension.*

6.4.3.3 *Enterococcus faecalis*

The results in Figure 6.14 show that, although not as susceptible to visible-light treatment as *Staphylococcus aureus,* 120 minutes of light exposure provided a 3-$\log_{10}$ reduction in the bacterial population of a sample of *E. faecalis.*

6.4.3.4 *Escherichia coli*

As described in Section 5.3.2, *E. coli* survived within the light cabinet, either because visible light has no detrimental effect on it, or the light intensity in the light cabinet was not sufficient to induce inactivation. The results of Figure 6.15 demonstrate that visible-light exposure does have the ability to inactivate *E. coli,* although this inactivation requires a much longer exposure period than for other bacteria examined, with a 2-$\log_{10}$ reduction requiring 120 minutes of exposure. Due to the extended exposure period, the temperature was also measured post-exposure and was found to be 39°C – again ruling out temperature as the cause of this inactivation.

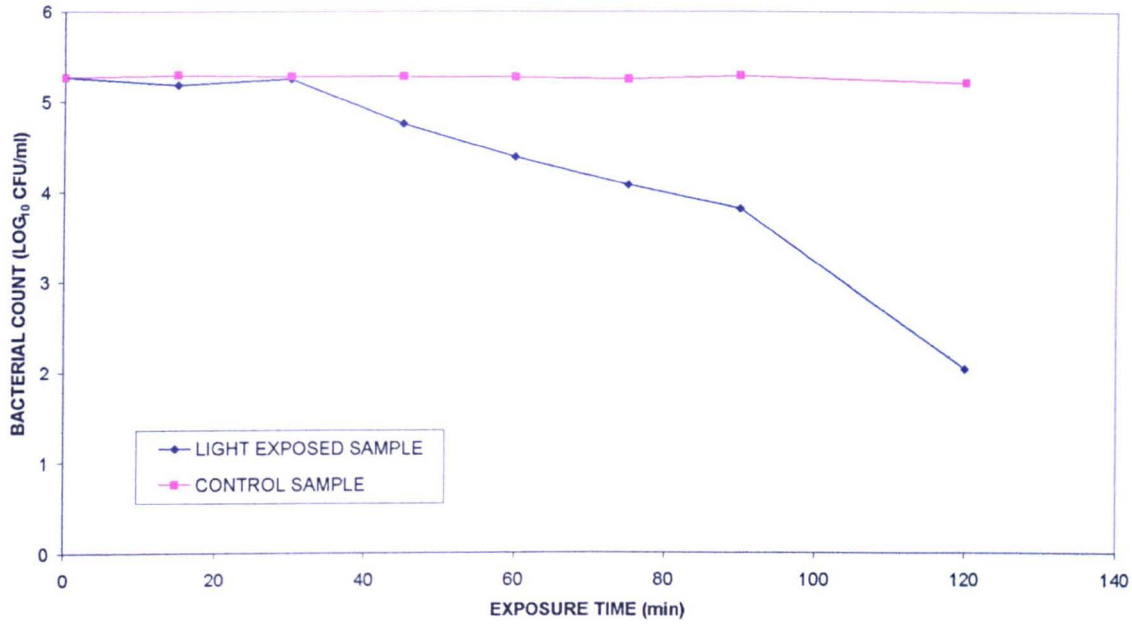

FIGURE 6.14 *The effect of >400nm light exposure on an Enterococcus faecalis suspension.*

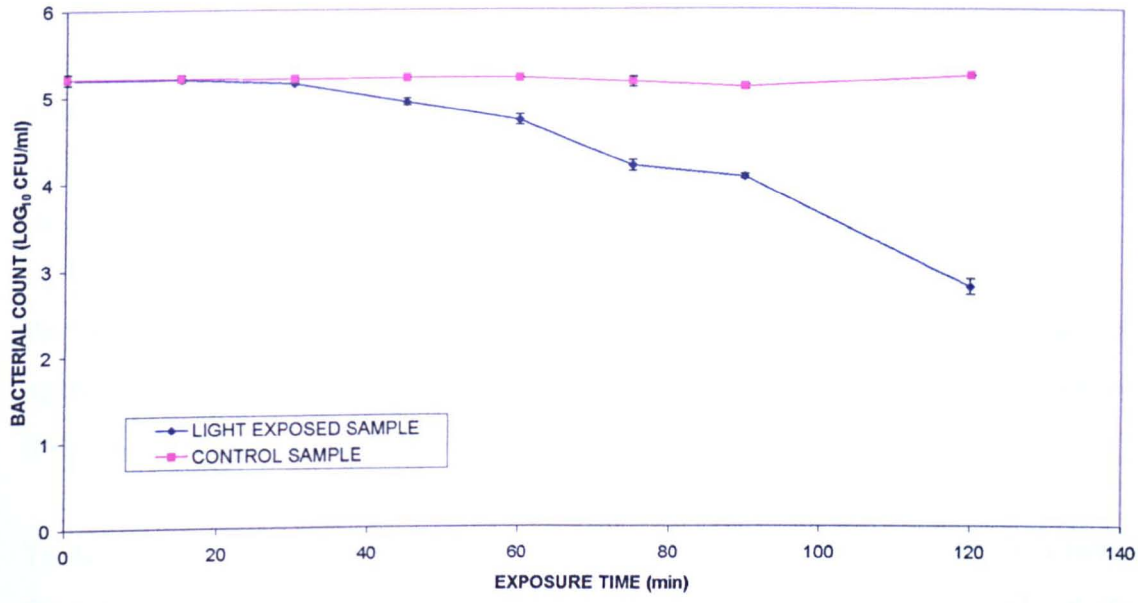

FIGURE 6.15 *The effect of >400nm light exposure on an Escherichia coli NCTC 9001 suspension.*

6.5 INVESTIGATION OF FACTORS AFFECTING INACTIVATION

Following on from the confirmation that visible light can inactivate *Staphylococcus aureus* strains including MRSA, the remainder of the chapter investigates the factors that may have an effect on this inactivation. Experimental parameters such as sample temperature and volume, and light source characteristics, including output intensity, power and energy, were examined.

6.5.1 Investigation of Temperature Effect

As with the light-cabinet experiments, the effect of temperature was investigated to ensure that bacterial kill was the result of light exposure and not excessive heating. The possibility of creating a lethal rise in temperature was greater in this case due to the significantly higher power outputs of the Mercury-Xenon and Xenon lamps compared to that of the fluorescent tubes in the light cabinet.

To investigate the temperature rise on the agar surface a thermocouple (Kane May, UK) was used. The thermocouple was placed on the agar surface of a NA plate and allowed continuous measurement of any temperature rise while the plate was being exposed to the Mercury-Xenon lamp through the 400 nm long-wave pass filter. The fibre optic light-guide was positioned at a 5 cm distance from the agar plate and exposure times of up to 20 minutes were monitored in order to keep the parameters the same as those used in the actual experiments (Section 6.3).

For the exposure of bacterial suspensions to the Xenon lamp, temperature gain was again monitored using the thermocouple. In this case, the thermocouple was used to take measurements before and after exposures of different durations.

Table 6.3 shows the results of these investigations into temperature gain. For both the surface and suspension experiments, exposure to the Mercury-Xenon and Xenon lamps did induce a temperature rise, but the rise is not lethal to *S. aureus* since it survives temperatures up to 45°C [Holt *et al*, 1994]. The results of a further temperature investigation are detailed in Section 6.5.3.

TABLE 6.3 *Temperature experienced by solid and liquid media on exposure to >400 nm light emitted from the Xenon lamp at a 5 cm distance*

	TEMPERATURE (°C)	
TIME (min)	**AGAR SURFACE**	**SUSPENSION (2 ml)**
0	19	22
5	22	-
10	24	-
15	25	-
20	26	-
30	-	34
60	-	38
150	-	39

6.5.2 Effect of Sample Volume

Due to the possibility of light absorption by the suspension, and therefore a reduced rate of inactivation, it was necessary to investigate the effect of variations in sample volume.

Using the method described in Section 6.2.2.2, 1 ml, 2 ml, and 3 ml volumes of an MRSA LMG 15975 suspension, with corresponding depths of 4 mm, 7 mm and 10 mm, were exposed to increasing durations of >400 nm light treatment with a power density of 350 mW/cm^2. The initial population density (2.0 x 10^5 CFU/ml), exposure distance (5 cm) and output intensity (100%) of the lamp were kept constant throughout the experiments. After exposure all samples were plated onto NA and incubated at 37°C for 24 hours.

Figure 6.16 shows that >400 nm light-exposure of suspension volumes of 1 ml, 2 ml and 3 ml results in approximately the same inactivation rate for all volumes.

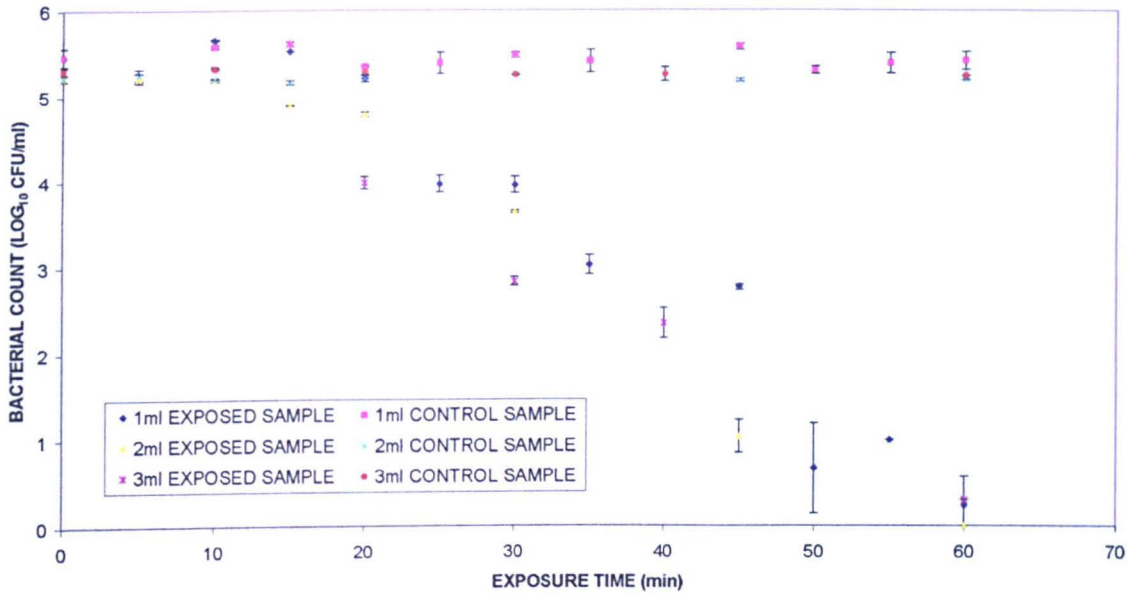

FIGURE 6.16 *The effect of different sample volumes on the inactivation rate of S. aureus LMG 15975 using >400nm light*

6.5.3 Effect of Lamp Output Intensity

The aim of this investigation was to confirm whether, as is found with UV studies, the degree of visible-light bacterial inactivation is governed only by the total dose; that is, the product of intensity (mW/cm^2) and exposure time (s), and not on their separate values.

Using the method described in Section 6.2.2.2, 2 ml volumes of the 2 x 10^5 CFU/ml *S. aureus* NCTC 4135 suspension were exposed to increasing durations of >400 nm light at output intensities of 50%, 75% and 100%. After exposure, all samples were plated onto NA and incubated at 37°C for 24 hours.

From the results plotted in Figure 6.17 and the inactivation energy values shown in Table 6.4, it can be seen that, as expected, lowering the intensity of the lamp

decreases the inactivation rate, but the total doses required for a 4.5-5.0 log_{10} reduction are similar.

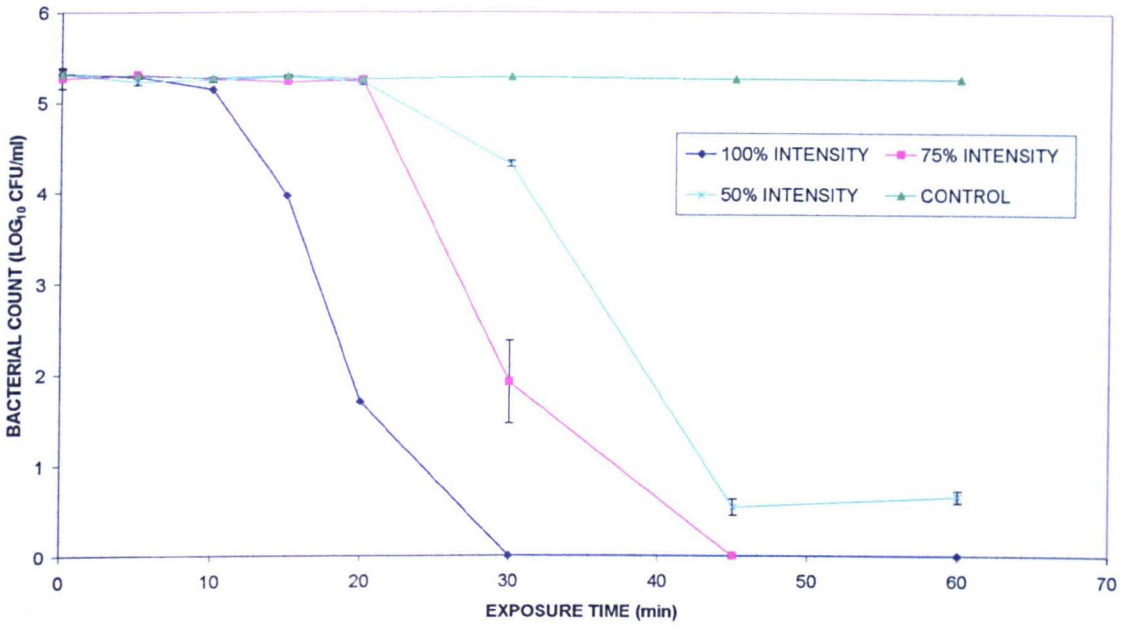

FIGURE 6.17 *The effect of different Xenon lamp output intensities on the inactivation rate of S. aureus NCTC 4135 using >400nm light at a 5cm distance*

TABLE 6.4 *Parameters for the inactivation of a 10^5 CFU/ml S. aureus NCTC 4135 suspension when using differing lamp output intensities*

EXPERIMENTAL PARAMETERS	LAMP OUTPUT INTENSITY		
	100%	**75%**	**50%**
EXPOSURE TIME (sec)	1800	2700	3600
POWER DENSITY (mW/cm^2)	350	270	180
LOG REDUCTION	5	5	4.5
DOSE (J/cm^2)	630	729	648
J/cm^2/LOG REDUCTION	126	145.8	144

TABLE 6.5 *Effect of exposure to differing lamp intensities on sample temperature*

LAMP INTENSITY	EXPOSURE DURATION	TEMP. RANGE (°C)	TEMP. RISE (°C)
100%	30 min	26 – 33	+ 11
75%	45 min	27 – 41	+ 14
50%	60 min	26 – 41	+ 15

During the investigation of the rate of different light intensities, sample temperatures were monitored before and after each exposure. Results, tabulated in Table 6.5, show that an increase in temperature is evident after all exposures. The temperature rises were different in each case, with an increasing rise in sample temperature being experienced on exposure to increasing durations of light treatment even though the lamp intensity was decreasing. The lowest temperature rise (11°C) was found with a 30-minute exposure to 100% intensity light and this was the exposure condition which produced the fastest rate of inactivation. This and the fact that these temperature rises are different, but the doses result in similar $\log_{10}$ reductions, further helps to eliminate the role of temperature in this form of bacterial inactivation.

6.5 DISCUSSION AND CONCLUSIONS

The use of the high intensity Mercury-Xenon and Xenon lamps significantly increased the rates of bacterial inactivation compared to those achieved while using the light incubation cabinet (Chapter 5), where inactivation of the same *S. aureus* strain was not observed until exposures of the order of hours.

Results from this study have shown that each of *Staphylococcus epidermidis* (CONS), *Streptococcus pyogenes* and *Enterococcus faecalis* is susceptible to visible-light treatment of wavelengths >400 nm. *S. epidermidis* and *S. pyogenes* were found to have inactivation rates similar to the tested *S. aureus* strains. *Enterococcus faecalis* was found to be less susceptible to the visible-light treatment.

The visible-light exposure of *E. coli* using the Xenon lamp produced interesting results. It appeared that exposure using this much more intense light source (compared to that of the fluorescent tubes in the light cabinet) induced inactivation – albeit at a decreased rate when compared to those of the other test species. For an exposure time of 120 minutes, a 99% reduction in *E. coli* population was achieved. With the negative results achieved on exposure in the light cabinet, this is a significant result as it establishes that *E. coli* can be inactivated by exposure to visible-light of wavelengths >400 nm, using a much more intense light source – a result of decreased susceptibility. Again any thermal-inactivation process was ruled out.

Through observation of the inactivation curves for all the tested bacteria, a common factor is observed – inactivation appears to begin after an initial period of inactivity. Microbial inactivation by continuous UV light produces a similar sigmoidal-shaped curve. In the case of continuous UV, the initial plateau is believed to be due to an injury phase (i.e. DNA damage) in response to the UV exposure. After this plateau, the maximum amount of injury is surpassed and minimal additional UV exposure is lethal for microorganisms and the population rapidly declines. Tailing at the end of the curve is due to factors such as UV resistance and/or experimental factors such as shading of the microorganisms [US FDA, 2000]. Pulsed-UV inactivation does not follow the same curve shape. Inactivation curves tend to follow a first-order model with a linear relationship between log concentration and UV dose, but if the bacterial concentration is high, deviation from this relationship can occur due to shadowing and cluster effects [Lani *et al*, 2006]. The immediate effect of pulsed-UV exposure – rather than the initial period of inactivity observed with continuous-UV and visible-light exposure described here – may reflect the fact that inactivation is due to cell disintegration (a result of rapid, instantaneous heating) in addition to DNA-damage [Wekhof, 2003].

Although the visible-light inactivation reported here displays the same sigmoidal-shaped inactivation curve as that caused by continuous UV light, it is suggested that differing mechanisms of inactivation must be occurring within the exposed bacteria.

The reasoning for this is that DNA damage resulting from continuous UV exposure has been shown to be a result of maximum UV absorbance by the DNA bases at 240-280 nm [Block, 1991]. The period of inactivity in the case of visible-light inactivation is also likely due to an 'injury phase', but rather than being caused by DNA-damage, it is likely the result of a build-up of energy or reactive molecules which must occur before induction of bacterial inactivation. As briefly mentioned in Section 5.6, visible-light inactivation of other bacteria such as *Propionibacterium acnes*, *Helicobacter pylori* and some black-pigmented bacteria has been documented [Ganz *et al*, 2005; Soukos *et al*, 2005; Ashkenazi *et al*, 2003]. The mechanism of inactivation in the case of each of these bacteria has been accredited to the photo-stimulation of endogenous intracellular molecules known as porphyrins using visible-light in the 400 nm to 500 nm region – more specifically 400 nm to 420 nm in the cases of *P. acnes* and *H. pylori* [Ganz *et al*, 2005; Ashkenazi *et al*, 2003]. Stimulation of these porphyrins leads to the production of reactive species, predominantly singlet delta oxygen ($^1O_2$) – and consequently, cell death [Hamblin and Hasan, 2004]. It is likely to be a similar inactivation mechanism which is occurring within the *S. aureus* and other species investigated in this study and this will be discussed in depth in Chapter 8.

Among bacterial genera, it is calculated that different doses are required before the induction of visible-light inactivation (see Table 6.7). Analysis within the one genera– more specifically the genus *Staphylococcus* – however, suggests that the initial period of inactivity always persists until the dose reaches the same value. Within the three tested *S. aureus* strains, a dose of 210 J/cm^2 was consistently required before inactivation of a 10^5 CFU/ml population could be induced within the cells. This was also true of the 10^7 CFU/ml population – even though the total inactivation time was much longer, thus suggesting that even at this higher population density, the visible wavelengths were able to sufficiently penetrate through the suspension to initiate the inactivation process. When observing the inactivation curve of *S. epidermidis* – also a member of the *Staphylococcus* genus – it was evident that this species required a dose of more than 192 J/cm^2 to initiate the inactivation process, similar to that for the *S. aureus* strains.

TABLE 6.7 *Doses required by different bacteria for initiation of inactivation by visible-light*

	INACTIVITY PERIOD (sec)	POWER DENSITY (mW/cm^2)	REQUIRED DOSE (J/cm^2)
Staphylococcus aureus NCTC 4135	600	350	210
Staphylococcus aureus NCTC 4135*	600	350	210
Staphylococcus aureus LMG 15975	600	350	210
MRSA 16a	600	350	210
Staphylococcus epidermidis	480	400	192
Streptococcus pyogenes	900	400	360
Enterococcus faecalis	1800	400	720
Escherichia coli	1800	400	720

* 2×10^7 CFU/ml starting population

Inactivation Energies for Different Bacteria

Although the inactivation energy data are useful for comparison of data for a particular bacterium – as in Section 6.4.3 – there are difficulties when making dose comparisons between different bacteria. The power meter measures light over the whole wavelength range above 400 nm but, if the inactivation method demonstrated in this study is the same as that shown for *P. acnes*, only a small portion of this wavelength range (between 405 nm and 420 nm) is responsible for inducing bacterial inactivation [Ashkenazi *et al*, 2003; Elman *et al*, 2003]. This would not present a problem if the same narrow wavelength range caused the inactivation for every bacterium examined, but different bacteria may involve different wavelengths for inactivation. Since the intensity of the lamp varies with wavelength (Figure 6.2), this means that the inactivating intensity may be different for different bacteria, even when the sensor is measuring the same intensity.

Consequently, absolute dose measurements are not meaningful since the sensor is recording intensities over the whole spectrum of the lamp, and not just the inactivating wavelengths, but for comparative purposes it is useful to consider the energy requirements for inactivation for this selection of bacteria. These data are shown in Table 6.8.

The values show that the organism most readily inactivated was *S. aureus* NCTC 4135, with a 5-$\log_{10}$ reduction requiring a dose (energy density) of 630 J/cm^2.

When comparing values of dose per $\log_{10}$ reduction it can be seen that relatively similar values are required for the inactivation of *S. aureus* NCTC 4135, MRSA 16a and *Staphylococcus epidermidis*; 126, 189 and 168 J/cm^2/$\log_{10}$ reduction respectively. Higher values of 252 and 288 J/cm^2/$\log_{10}$ reduction occur for MRSA LMG 15975 and *Streptococcus pyogenes*, and the more resistant of the organisms, *Enterococcus faecalis*, requires the higher value of 960 J/cm^2/log reduction.

The high value of dose per $\log_{10}$ reduction required for the inactivation of *E. coli* accounts for inactivation not being observed within the light cabinet; the value for *E. coli* being 11.5 times that for *S. aureus* NCTC 4135.

The slower inactivation observed with *E. coli*, and even *E. faecalis*, could be an example of different wavelengths within the visible light spectrum being required for their inactivation; their slower inactivation being a result of the lamp intensity being lower at the required inactivating wavelengths.

Further work will involve the use of narrow-band filters for the identification of the causative wavelengths, and with narrow-band data, absolute dose values will become more meaningful.

This chapter has described the use of an intense light source for the visible-light inactivation of a number of medically important bacteria including *Staphylococcus aureus*, MRSA, CONS, *Streptococcus pyogenes* and *Enterococcus faecalis*. The selection of the Xenon lamp allowed the rates of bacterial inactivation to be greatly increased compared to those achieved with exposure in the light incubation cabinet.

A significant development is the fact that this treatment is not only effective in inactivating *S. aureus* strains including MRSA, but has proved effective for use against a range of Gram-positive bacteria all of which are commonly associated with hospital-acquired infection.

TABLE 6.8 *Energy values required for the inactivation of a selection of bacterial species using broad-spectrum visible light (>400 nm light)*

ORGANISM	EXPOSURE TIME (sec)	POWER DENSITY (mW/cm^2)	LOG_{10} REDUCTION	DOSE (J/cm^2)	$J/cm^2/LOG_{10}$ REDUCTION
Staphylococcus aureus NCTC 4135	1800	350	5	630	126
Staphylococcus aureus NCTC 4135	7200	350	7	2520	360
MRSA LMG 15975	3600	350	5	1260	252
MRSA 16a	2700	350	5	945	189
Staphylococcus epidermidis NCTC 7944	2100	400	5	840	168
Streptococcus pyogenes NCTC 8198	3600	400	5	1440	288
Enterococcus faecalis	7200	400	3	2880	960
Escherichia coli NCTC 9001	7200	400	2	2880	1440

This method of bacterial inactivation using visible-light exposure – and the demonstrated susceptibilities of a range of major hospital-acquired pathogens – has revealed a potential disinfection method which may have implications for the health-care industry. Although the inactivation rates of the bacteria tested are significantly less than those achieved with continuous or pulsed-UV light illumination, this disadvantage is outweighed by the fact that human exposure to UV light has serious detrimental health effects. Dangers of UV exposure include eye damage and skin cancer [WHO, 2006], but the less energetic wavelengths used here are outside the UV wavelength range and so markedly reduce the risk of adverse health effects.

Work in subsequent chapters focuses on the further identification of the causative wavelengths, thus enabling the design of an efficient optical system for the inactivation of *Staphylococcus aureus*.

Chapter 7

INVESTIGATION INTO THE VISIBLE LIGHT WAVELENGTH SENSITIVITY OF *STAPHYLOCOCCUS AUREUS*

7.0 GENERAL

Preliminary research in Chapter 5 and inactivation data in Chapter 6 have led to the conclusion that prolonged exposure to light of visible wavelengths (>400 nm), and more specifically 400 – 500 nm light, can inactivate *Staphylococcus aureus*, MRSA and other medically important Gram-positive bacteria such as coagulase-negative *Staphylococcus*, *Streptococcus* and *Enterococcus*. This chapter focuses on the confirmation of these causative wavelengths.

Investigations using the high intensity Xenon lamp, in conjunction with a selection of commercially-available long-wave pass, short-wave pass and bandpass filters enabled firstly, the confirmation of the region of interest being between 400 nm and 500 nm (as demonstrated in Chapter 5) and secondly – and most significantly – the pinpointing of the causative wavelengths to within a 10 nm bandwidth.

Identification of the causative wavelength(s) precedes a discussion on the possible mechanism of bacterial inactivation.

7.1 OPTICAL FILTERS

A range of optical filters was used throughout the visible-light experiments and Table 7.1 shows their specifications. Three types of filter were used;

- Bandpass filters: permit transmission of a well-defined bandwidth of light. In this study, narrow-band filters, which have a transmission curve with a full width at half maximum (FWHM or bandwidth) of less than 20 nm, were used.
- Long-wave pass filters: permit transmission of wavelengths above a specified cut-off wavelength*.
- Short-wave pass filters: permit transmission of wavelengths below a specified cut-off wavelength*.

* The wavelength at which transmission is reduced to 50% of its peak value.

The filters had a diameter of 25 mm, with the exception of the 400 nm long-wave pass and 435 nm bandpass filters, which were square with an area of 50 mm^2. The transmission spectra for the filters were supplied by the manufacturers, and these are shown in Appendix B.

7.2 INVESTIGATION INTO THE CAUSATIVE WAVELENGTH RANGE

The discovery in Chapter 5, which established that 400 – 500 nm light can inactivate *Staphylococcus aureus*, was found through a series of experiments using 400 nm long-wave pass, 500 nm long-wave pass and 500 nm short-wave pass filters. For further investigation and a more accurate analysis, a combination of commercially available long-wave pass and short-wave pass filters was used to confirm that these causative wavelengths are in the region of 400 – 500 nm.

7.2.1 Power Density Measurements

The power density of the Xenon lamp through each of the long-wave and short-wave pass filters and some filter combinations was measured using the radiant power meter and detector and the results are shown in Table 7.2. All measurements were made at a distance of 5 cm from the lamp, with the Xenon light source operating at 100% output intensity.

TABLE 7.1 *Filter Specifications*

FILTER (nm)	TYPE	CW (nm)	FWHM (nm)	PEAK TRANSMISSION (%)	MANUFACTURER
320	Bandpass	321.79	10 ± 2	36.05	L.O.T.-Oriel Ltd.
330	Bandpass	330.89	10 ± 2	38.05	L.O.T.-Oriel Ltd.
340	Bandpass	341.29	10 ± 2	25.14	L.O.T.-Oriel Ltd.
350	Bandpass	351.83	10 ± 2	29.22	L.O.T.-Oriel Ltd.
360	Bandpass	361.64	10 ± 2	26.49	L.O.T.-Oriel Ltd.
370	Bandpass	370.75	10 ± 2	32.41	L.O.T.-Oriel Ltd.
380	Bandpass	380.14	10 ± 2	32.41	L.O.T.-Oriel Ltd.
390	Bandpass	392.91	10 ± 2	48.06	L.O.T.-Oriel Ltd.
400	Bandpass	402.86	10 ± 2	50.34	L.O.T.-Oriel Ltd.
400	Long-wave pass	n/a	n/a	- *	- *
405	Bandpass	405.2	10.2	45.1	Ealing Catalog. Inc.
410	Bandpass	411.93	10 ± 2	50.02	L.O.T.-Oriel Ltd.
415	Bandpass	416.61	10 ± 2	51.40	L.O.T.-Oriel Ltd.
420	Bandpass	420.17	10 ± 2	52.77	L.O.T.-Oriel Ltd.
430	Bandpass	430.87	10 ± 2	47.28	L.O.T.-Oriel Ltd.
435	Bandpass	436.8	8 ± 2	50.75	Ealing Catalog. Inc.
440	Bandpass	441.27	10 ± 2	58.23	L.O.T.-Oriel Ltd.
450	Bandpass	451.50	10 ± 2	54.97	L.O.T.-Oriel Ltd.
450	Long-wave pass	n/a	n/a	93.38	L.O.T.-Oriel Ltd.
460	Bandpass	462.58	10 ± 2	62.34	L.O.T.-Oriel Ltd.
470	Bandpass	471.38	10 ± 2	62.68	L.O.T.-Oriel Ltd.
480	Bandpass	481.60	10 ± 2	71.48	L.O.T.-Oriel Ltd.
490	Bandpass	492.52	10 ± 2	61.32	L.O.T.-Oriel Ltd.
500	Bandpass	501.97	20 ± 4	65.58	L.O.T.-Oriel Ltd.
500	Long-wave pass	n/a	n/a	93.40	L.O.T.-Oriel Ltd.
500	Short-wave pass	n/a	n/a	93.66	L.O.T.-Oriel Ltd.
550	Long-wave pass	n/a	n/a	90.39	L.O.T.-Oriel Ltd.

* Information not available

CW = centre wavelength

FWHM = full width half maximum

TABLE 7.2 *Power density values of the Xenon lamp (at 100% output intensity) through the long-wave and short-wave pass filters and filter combinations.*

FILTER(S)	TRANSMITTED WAVELENGTHS (nm)	POWER DENSITY (mW/cm^2)
400 nm long-wave pass	> 400	350
450 nm long-wave pass	> 450	304
500 nm long-wave pass	> 500	290
500 nm short-wave pass	< 500	160
550 nm long-wave pass	> 550	240
400 nm long-wave pass + 500 nm short-wave pass	400 – 500	132
450 nm long-wave pass + 500 nm short-wave pass	450 – 500	97

7.2.2 Treatment Method and Results

S. aureus NCTC 4135 was cultured as described in Section 3.1.2. After an 18-hour incubation period, the broths were centrifuged at 3400 rpm for 10 minutes and the resultant pellets re-suspended in PBS. The suspension was then diluted to give a population density of ~2.0 x 10^5 CFU/ml. Using the method described in Section 6.2.2.2, 2 ml volumes of the bacterial suspension were exposed to increasing durations of light treatment through each of the long-wave and short-wave pass filters. The exposure distance (5 cm) and output intensity (100%) of the lamp were maintained constant throughout. After exposure, all samples were plated onto NA and incubated at 37°C for 24 hours.

Figure 7.1 and Table 7.3 show the effects of the different wavelength ranges on the inactivation rate of *S. aureus* NCTC 4135 suspensions. Exposure to wavelengths of 500 nm and below induced the most rapid inactivation rate, with a dose per $\log_{10}$ reduction of 38.4 $J/cm^2/\log_{10}$, and this was thought to be the result of the inclusion of UV wavelengths, which are well known to have a germicidal effect. Wavelengths >400 nm and between 400 and 500 nm also caused total inactivation, at dose per $\log_{10}$ reductions of 126 and 58.6 $J/cm^2/\log_{10}$ reduction, respectively. Conversely,

when longer wavelengths of 500 nm and above were investigated, little to no inactivation was observed, even though the illuminating doses were much higher.

This confirms that the visible wavelengths inducing staphylococcal inactivation are in the region of 400 to 500 nm.

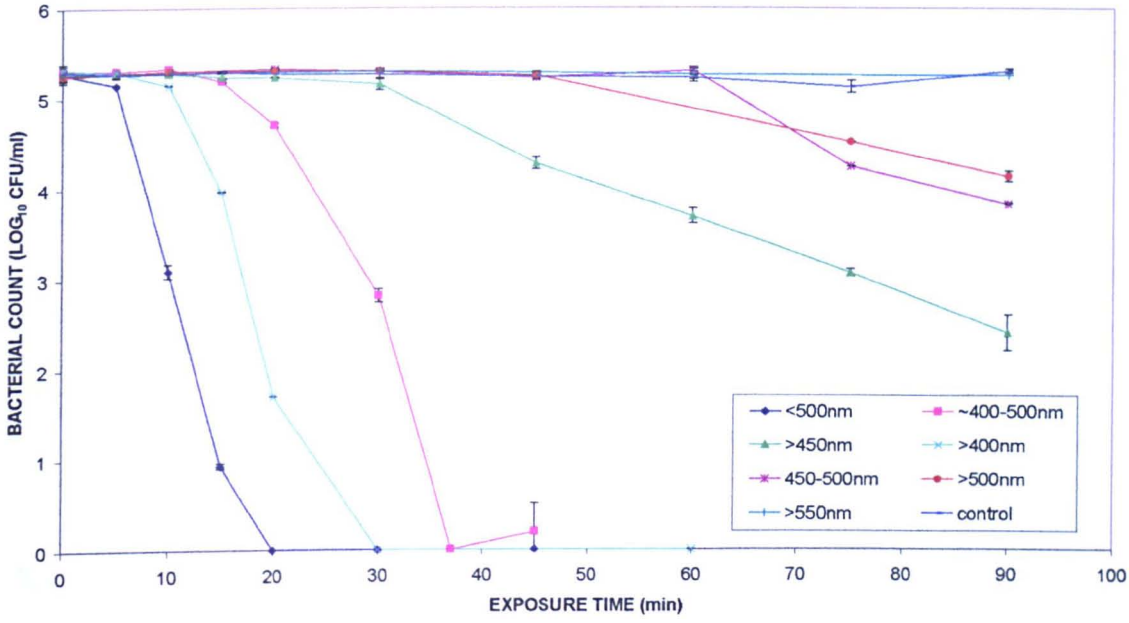

FIGURE 7.1 *Effect of different wavelength ranges on the inactivation rate of S. aureus NCTC 4135 suspensions.*

TABLE 7.3 *Inactivation energies required for treatment of a 10^5 CFU/ml S. aureus NCTC 4135 suspension using different wavelength ranges*

WAVELENGTH RANGE (nm)	EXPOSURE TIME (sec)	POWER DENSITY (mW/cm^2)	LOG_{10} REDUCTION	DOSE (J/cm^2)	$J/cm^2/LOG_{10}$ REDUCTION
<500	1200	160	5	192	38.4
>400	1800	350	5	630	126
400–500	2220	132	5	293	58.6
>450	5400	304	2.9	1641.6	566.1
450-500	5400	97	1.4	523.8	374.1
>500	5400	290	1	1566	1566
>550	5400	240	0	1296	-

Sample temperature was also monitored with the <500 nm, >400 nm and 400-500 nm exposures and the results are shown in Table 7.4. In all cases, the temperature rise is relatively small and is easily survived by *S. aureus*.

TABLE 7.4 *Effect of exposure to filtered wavelength ranges from the Xenon lamp on sample temperature*

WAVELENGTH RANGE (nm)	EXPOSURE DURATION (min)	TEMPERATURE RANGE (°C)	TEMPERATURE RISE (°C)
<500	20 (1200 sec)	23 – 29	+ 6
>400	30 (1800 sec)	26 – 34	+ 8
400-500	37 (2220 sec)	25 – 32	+ 7

7.3 INVESTIGATION INTO THE CAUSATIVE BANDWIDTH

Following on from confirmation that the causative wavelengths are between 400 nm and 500 nm, research advanced with an aim to further isolate the causative wavelengths to within a 10 nm bandwidth. To enable this, work progressed from the use of long-wave and short-wave pass filters to using a selection of bandpass filters isolating wavelengths between 320 nm and 500 nm at bandwidths of 10 nm (with the exception of the 500 nm filter: FWHM = 20 nm).

7.3.1 Power measurements

The power density of the light from the Xenon lamp transmitted through each of the bandpass filters was measured using the radiant power meter and detector. These are listed in Table 7.5 and plotted in Figure 7.2. As with the long-wave and short-wave pass filters, all measurements were made at a distance from the lamp of 5 cm, with the Xenon light source operating at 100% output intensity.

TABLE 7.5 *Power density of the Xenon lamp through each of the bandpass filters*

BANDPASS FILTER (nm)	POWER DENSITY (mW/cm^2)	BANDPASS FILTER (nm)	POWER DENSITY (mW/cm^2)
VISIBLE REGION		UV-A REGION	
400	4.3	320	0.33
405	3.27	330	0.42
410	4.6	340	0.40
415	5.1	350	0.49
420	5.4	360	0.53
430	5.3	370	0.82
435	5.1	380	1.61
440	6.1	390	4.0
450	6.5		
460	9.7		
470	10.3		
480	10.8		
490	9.7		
500	20.8		

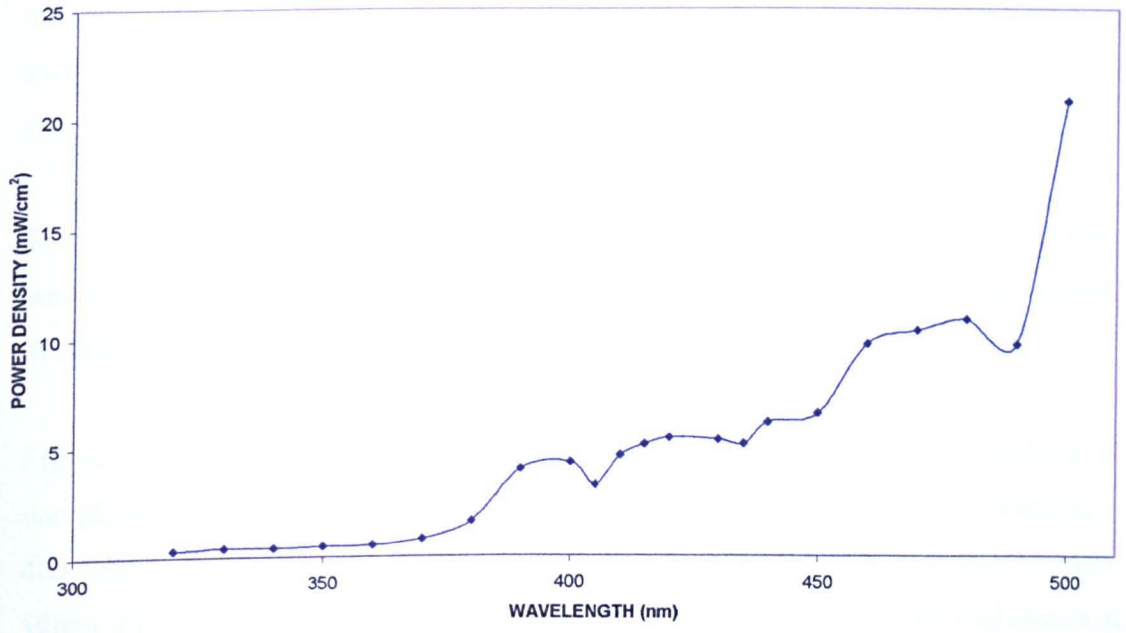

FIGURE 7.2 *Power density of the Xenon lamp through bandpass filters as a function of wavelength*

From Figure 7.2 it can be seen that a different power density of light transmits through each filter. This is due to a combination of the intensity variations within the emission spectrum of the Xenon lamp (Figure 6.2) and the differing percentages of transmission through the bandpass filters.

With the region of inactivation for visible light between 400 nm and 500 nm, the initial investigation focused on the visible bandpass filters. For comparison of the inactivation efficiencies for each narrow bandwidth, the output intensity of the lamp was amended for each filter so that the same power density was transmitted onto the bacterial sample for each filter. Since between 400 and 500 nm, the 405 nm bandpass filter transmitted light with the lowest power density (3.27 mW/cm^2), the output intensity of the lamp was adjusted so that this same value of power density was transmitted through all of the filters.

7.3.2 Treatment method and results

For light exposure of *S. aureus* NCTC 4135 using the bandpass filters, the bacterial culture and preparation procedures stated in Section 7.2.2 were repeated to prepare a suspension with a population density of ~2.0 x 10^5 CFU/ml. For each of the bandpass filters, a 2 ml aliquot of this suspension was light-exposed through the specified filter for a 2-hour duration. A black PVC cylinder was used to surround the sample well during exposure to prevent penetration of stray light. After exposure, all samples were plated onto NA and incubated at 37°C for 24 hours.

Since the power density through each filter was 3.27 mW/cm^2, and the exposure time was maintained constant at 2 hours, the dose to which each suspension was exposed at each bandwidth was 23.5 J/cm^2.

Figure 7.3 plots the $\log_{10}$ reduction of *S. aureus* NCTC 4135 as a function of wavelength. This graph highlights that the most effective bactericidal wavelength is 405 nm, with reduced effects observed at 400 nm, 410 nm, 415 nm and 420 nm. When the *S. aureus* suspension was exposed to wavelengths of 430 nm and above at an energy density of 23.5 J/cm^2, no inactivation was observed to occur.

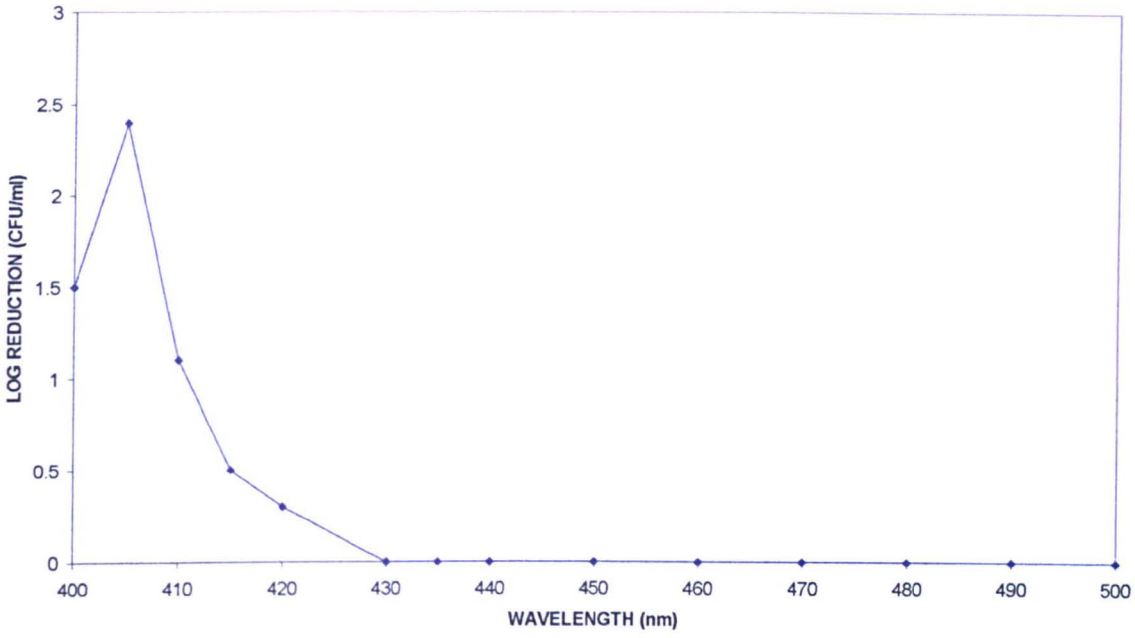

FIGURE 7.3 *Log reduction for S. aureus NCTC 4135 as a function of wavelength (400 – 500 nm) when exposed to a dose of 23.5 J/cm^2 at each of the bandwidths.*

Numerical data for the log reductions achieved through light exposure to 10 nm bandwidths of light from 400 nm to 420 nm (with CW increments of 5 nm) are listed in Table 7.6.

TABLE 7.6 *Log reduction values for the inactivation of S. aureus NCTC 4135 following exposure to 10 nm bandwidths of light from 400 nm to 420 nm, each with a dose of 23.5 J/cm^2.*

FILTER (nm)	INITIAL POPULATION, N_0 (CFU/ml)	FINAL POPULATION, N (CFU/ml)	$LOG_{10}(N/N_0)$ REDUCTION
400	2.30×10^5	6.80×10^3	1.5
405	$\mathbf{1.95 \times 10^5}$	$\mathbf{7.80 \times 10^2}$	**2.4**
410	2.05×10^5	1.54×10^4	1.1
415	1.6×10^5	4.55×10^4	0.5
420	1.80×10^5	9.80×10^4	0.3

7.4 GERMICIDAL EFFICIENCY

The inactivation capability at each wavelength can be quantified as the germicidal efficiency, defined as the $\log_{10}$ reduction of a bacterial population by inactivation per unit of light energy density in J/cm^2, [Wang *et al*, 2005].

Germicidal Efficiency, $\eta = \log_{10}(N/N_0)$ per J/cm^2

Figure 7.4 shows the germicidal efficiency for *S. aureus* NCTC 4135 as a function of wavelength for the wavelength range of 400 nm to 500 nm. The shape of this graph is the same as that of Figure 7.3 with the $\log_{10}$ reduction values converted to germicidal efficiency – a value that takes into account not only the $\log_{10}$ reduction but also the dose to which the bacteria has been exposed.

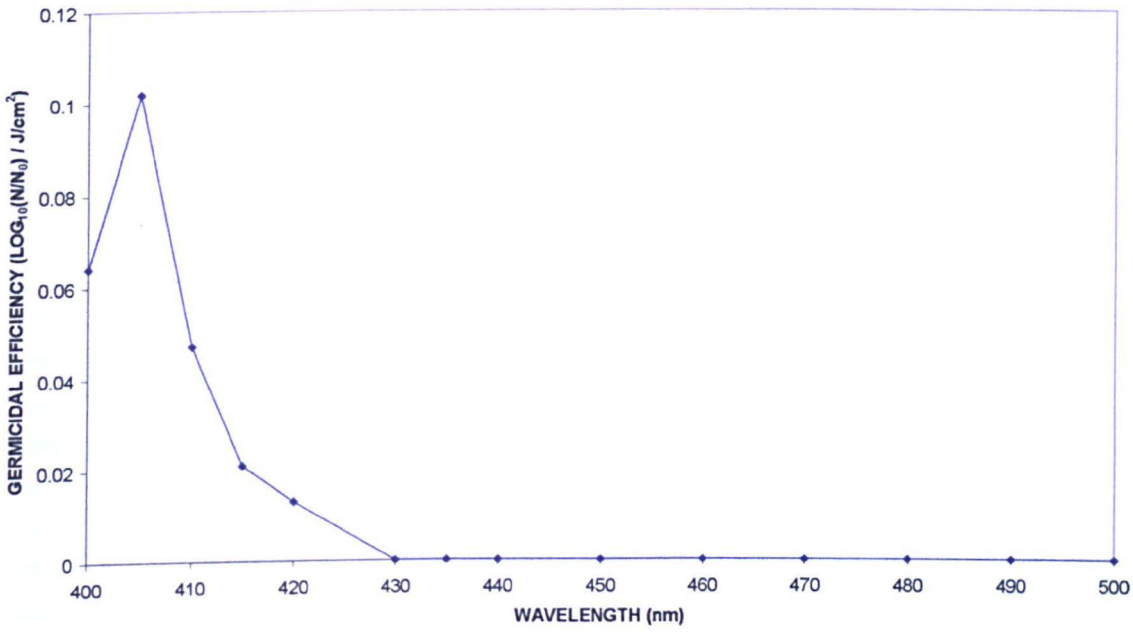

FIGURE 7.4 *Germicidal efficiency η ($\log_{10}(N/N_0)$ per J/cm^2) for S. aureus NCTC 4135 as a function of wavelength (400 – 500 nm)*

Although the germicidal efficiency peak is at 405 nm, 400 nm light also demonstrated good germicidal activity against the *S. aureus*, with a value of

0.064 $\log_{10}$ per J/cm^2. In order to investigate this lower wavelength range further, wavelengths down to 320 nm were examined using bandpass filters.

Because of the lower percentage transmission for many of the bandpass filters in the range 320 nm to 390 nm, power densities of 3.27 mW/cm^2 – as used for the 400 nm to 500 nm filters – could not be achieved. Therefore for inactivation measurements using the 320 nm to 390 nm filters, a range of power densities and exposure times was used and from these the doses, and ultimately the germicidal efficiencies, were calculated. These values, in addition to the corresponding values for the 400 nm to 500 nm bandpass filters are listed in full in Table 7.6.

TABLE 7.7 *Germicidal efficiencies for light inactivation of S. aureus NCTC 4135 from 320 to 500 nm in 10 nm bandwidths.*

FILTER (nm)	EXPOSURE TIME (s)	POWER DENSITY (mW/cm^2)	DOSE (J/cm^2)	LOG REDUCTION	GERMICIDAL EFFICIENCY ($\log_{10}(N/N_0)$/J/cm^2)
320	5400	0.84	4.5	4.3	0.955
330	5400	0.78	4.2	3.4	0.809
340	5400	0.53	2.8	1.8	0.643
350	7200	0.95	6.8	4	0.558
360	7200	0.70	5.1	1.6	0.314
370	7200	1.22	8.8	2.1	0.239
380	7200	1.76	12.6	1.9	0.151
390	7200	3.27	23.5	3.1	0.132
400	7200	3.27	23.5	1.5	0.064
405	**7200**	**3.27**	**23.5**	**2.4**	**0.102**
410	7200	3.27	23.5	1.1	0.047
415	7200	3.27	23.5	0.5	0.021
420	7200	3.27	23.5	0.3	0.013
430	7200	3.27	23.5	0	0
435	7200	3.27	23.5	0	0
440	7200	3.27	23.5	0	0
450	7200	3.27	23.5	0	0
460	7200	3.27	23.5	0	0
470	7200	3.27	23.5	0	0
480	7200	3.27	23.5	0	0
490	7200	3.27	23.5	0	0
500	7200	3.27	23.5	0	0

From Figure 7.5 (and the values in Table 7.7) it can be seen that after peaking at 405 nm, the germicidal efficiency then increases as the illuminating wavelength decreases.

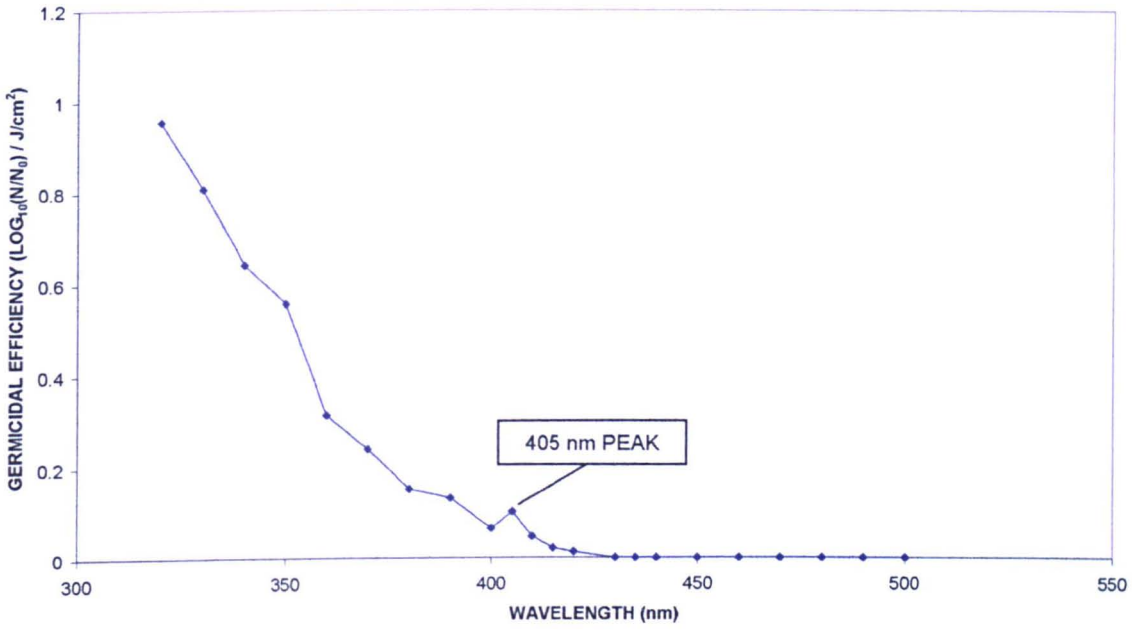

FIGURE 7.5 *Germicidal efficiency η ($log_{10}(N/N_0)$ per J/cm^2) for S. aureus NCTC 4135 as a function of wavelength (320 – 500 nm). Highlighted is the inactivation peak at 405 nm.*

7.5 DISCUSSION AND CONCLUSIONS

The results obtained in previous chapters have accumulated from the discovery of the bactericidal properties of visible-light between 400 nm and 500 nm wavelength to the successful application of these visible-wavelengths for the inactivation of a range of Gram-positive bacteria, (*S. aureus*, MRSA, coagulase-negative *Staphylococcus*, *Streptococcus* and *Enterococcus*) all of which are significant causes of hospital-acquired infections – particularly MRSA. The work in this chapter has focused on the identification of the causative wavelengths.

The results have highlighted that inactivation is evident using 400 nm to 420 nm wavelengths, with the most effective bactericidal activity at 405 nm ± 5 nm.

Wavelengths of longer than 430 nm induce no effect within the *S. aureus* cells. The occurrence of the peak at 405 nm suggests that some additional inactivation process may be initiated within the *S. aureus* cells at this wavelength.

In the wavelength-resolved investigations of UV inactivation of *E. coli* by Wang *et al* [2005; 2004], no measurable inactivation was found at wavelengths of 300 nm or above. The mechanism of UV inactivation for *S. aureus* is similar to that for *E. coli* [Gates, 1930], with damage being caused through energy absorption by their DNA bases, as illustrated in Figure 7.6.

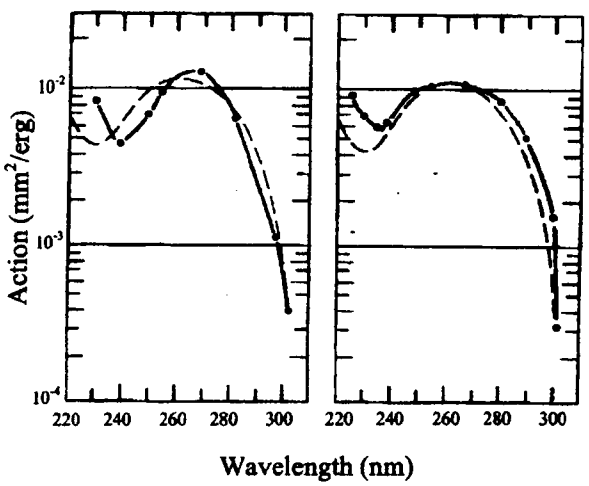

FIGURE 7.6 *Action spectra for UV killing of Escherichia coli (left) and Staphylococcus aureus (right). The broken line shows the relative absorption of DNA. [Gates, 1930]*

The reason for the lack of inactivation observed in *E. coli* for wavelengths above 300 nm may be due to the large differences in energies used between the studies. In the present study visible-light inactivation of *S. aureus* used a dose of 630 J/cm^2 for >400 nm wavelengths (Section 6.4.2), and for the study between 320 nm and 420 nm in 10 nm bandwidths, the doses used ranged from 4.5 to 23.5 J/cm^2. The studies of Wang *et al* [2005; 2004], used much lower doses of around 5 mJ/cm^2 to achieve similar $\log_{10}$ reductions, and this is a possible reason for the non-detection of germicidal activity above 300 nm.

It is possible that in the present study the non-effect of visible-light exposure on *E. coli* in the light incubation cabinet was due to photoreactivity by visible-light balancing inactivation. This might have been the case had the *E. coli* been UV-exposed prior to visible-light exposure, but since the bacteria were untreated – and there was no UV-C or UV-B wavelengths emitted by the fluorescent tubes – this is unlikely. Further evidence against this balance effect is that *S. aureus* cells have been found to show an enhanced rate of visible-light photoreactivation compared to *E. coli* [Adkins and Allen, 1982].

The inactivation of *S. aureus*, observed after prolonged exposure in the light incubation cabinet, is likely to have been a result of its sensitivity to wavelengths around 405 nm. As shown in Figure 5.3, the continuum of the fluorescent tube light sources within the cabinet contains 3 major peaks – one of which is at 405 nm. These peaks are part of the line spectra produced when an electrical discharge is passed through the mercury vapour within the tubes [Daish, 1971].

The important result obtained from this wavelength analysis of visible-light inactivation of *S. aureus* is that, although, as expected, the efficiency of inactivation falls off at the long-wavelength edge of the UV region, it remains finite on moving into the visible-wavelength region above 400 nm, with a peak occurring around 405 nm. It is evident that some additional inactivation process is initiated at this wavelength. Wavelengths in this same region have been found to induce the inactivation of some bacterial species – most notably *Propionibacterium acnes*, *Helicobacter pylori* and oral black-pigmented bacteria – through the photo-stimulation of endogenous porphyrins, which in turn results in singlet oxygen production and cell death [Ganz *et al*, 2005; Soukos *et al*, 2005; Ashkenazi *et al*, 2003]. The same mechanism of inactivation is likely to be occurring within the light-exposed *S. aureus* cells.

Papageorgiou *et al* [2000] investigated the effect of blue light on *P. acnes* and their findings further corroborate those established in this study. They found that "The sensitivity (of *P. acnes*) was highest for the lowest wavelength used (320 nm), decreasing continuously towards longer wavelengths but had a secondary maximum

in the blue region at 415 nm". This statement correlates almost exactly with the findings of this study shown in Figure 7.5, with the exception of a slight discrepancy concerning the wavelength of the "secondary maximum", but this can be explained. They state that 415 nm corresponds to the absorption maximum of the specific porphyrin molecules produced by *P. acnes*. The maximum of 405 nm determined in this study may indicate that other porphyrin molecules which have different absorption maxima are present within and/or produced by *S. aureus*.

A study by Griego and Spence [1978] also demonstrated the effectiveness of visible-light for inactivation, with results similar to those of the present study. On investigation into the effect of 290nm to 700 nm wavelengths on *Bacillus thuringiensis* spores they found that the highest rate of light-activated killing occurred with 400 nm visible-light. They stated that since the spores readily absorb 400-420 nm wavelengths, a photoproduct that prevents further replication is produced when a target molecule absorbs the energy.

Clearly the germicidal efficiency at this visible wavelength is much lower than for the UV wavelengths and this will be investigated further in Chapter 9, but this disadvantage may be more than outweighed by the big advantage of greater safety, thus allowing longer periods of continuous application and hence the application of higher doses.

In Chapter 8, this porphyrin/singlet oxygen mechanism will be examined in a series of experiments involving oxygen depletion and oxygen enhancement during visible-light inactivation of *S. aureus*. Chapter 8 will also include a detailed discussion of the mechanism of inactivation.

The identification of 405 nm (± 5 nm) as the most effective wavelength for the visible-light inactivation of *S. aureus* permits the selection of a more suitable light source compared to the broad-spectrum Xenon lamp used here. This will be investigated further in Chapter 9, where a practical 405 nm light source will be utilised for the inactivation of *S. aureus* and other medically important bacterial species.

Chapter 8

THE PROCESS OF VISIBLE-LIGHT INACTIVATION FOR *STAPHYLOCOCCUS AUREUS*

8.0 GENERAL

The work of the previous chapters has demonstrated that *Staphylococcus aureus*, MRSA and other medically important Gram-positive cocci can be optically inactivated using light of visible wavelengths. Further to this, filter studies using *S. aureus* enabled the identification of the causative visible wavelengths as being 400 nm to 420 nm, with optimal inactivation at 405 nm.

From the literature it is known that exposure to visible light, more specifically blue wavelengths in the 400 – 420 nm region, causes the inactivation of some bacterial species through the photo-stimulation of endogenous intracellular porphyrins [Ganz *et al*, 2005; Soukos *et al*, 2005; Ashkenazi *et al*, 2003]. This, viewed in conjunction with the wavelength sensitivity results obtained in Chapter 7, suggests that the inactivation of *S. aureus* (and other Gram-positive medically important bacteria) achieved here was also the result of the light stimulation of naturally occurring endogenous porphyrins.

It has been established that the exposure of bacterial porphyrins to visible-light, in particular 400-420 nm wavelengths, leads to porphyrin excitation, energy transfer and ultimately, the production of highly cytotoxic, oxygen-derived species – most notably singlet oxygen [Hamblin and Hasan, 2004]. The mechanism of this photo-toxic effect is oxygen-dependent [Wainwright, 1998].

This chapter examines and discusses the visible-light inactivation process, and consists essentially of two parts. In Part I, experiments examining the role of oxygen

are described. These help to determine the nature of the mechanism occurring within the light-exposed staphylococci. Experiments with altered oxygen concentrations were performed to investigate whether oxygen depletion and oxygen enhancement had any effect on the staphylococcal inactivation rate. This included an examination of the role of cell pigmentation in the inactivation process.

The results of the oxygen depletion/enhancement experiments provide verification of the important role played by oxygen in the visible-light inactivation process. In Part II, the process itself – referred to as photodynamic inactivation – is described in terms of interactions involving oxygen molecules and porphyrins to produce the reactive oxygen species, singlet oxygen ($^1O_2$).

PART I

8.1 OXYGEN DEPLETION

To investigate the significance of oxygen depletion in the inactivation process, the bacteria were light-exposed while suspended in oxygen-depleted environments. This was achieved by using, in turn, three scavengers of reactive oxygen species.

The oxygen scavengers used in the oxygen-depletion experiment were:

- Ascorbic acid, a strong reductant and radical scavenger [Niki, 1991]
- Catalase, a hydrogen peroxide scavenger [Henderson and Miller, 1986]
- Dimethylthiourea (DMTU), a hydroxyl radical scavenger [Feuerstein *et al*, 2005]

8.1.1 Treatment Method

Concentrations of 30 mM ascorbic acid, 20 U/ml catalase and 100 mM DMTU – as utilised in the study by Feuerstein *et al* [2005] – were used.

Staphylococcus aureus NCTC 4135 was again cultured as described in Section 3.1.2. After an 18-hour incubation period, the broths were centrifuged at 3400 rpm for

10 minutes and the resultant pellets re-suspended in PBS. The suspension was then diluted in PBS to give a population density of 2 x 10^6 CFU/ml, 1 ml of which was added to 9 ml of a scavenger solution. This produced a population of approximately 2 x 10^5 CFU/ml in the appropriate scavenger concentration. Two millilitre aliquots of these suspensions were then dispensed into the well of a multidish and, using the method described in Section 6.2.2.2, the bacteria-scavenger mixtures were exposed to 20 and 30-minute durations of visible-light treatment.

As in the previous experiments, visible-light exposure was performed using the Xenon lamp in conjunction with a 400 nm longwave pass filter to prevent UV-transmission, and continuous agitation of the sample was implemented throughout exposure. The exposure distance (5 cm) and output intensity (100%) of the lamp were again kept constant throughout the experiments. All samples were then plated onto NA and incubated at 37°C for 24 hours. Three sets of control samples were also prepared to provide reference data on (1) scavengers present with no light exposure, (2) scavengers absent with light exposure, and (3) scavengers absent with no light exposure.

8.1.2 Depletion Results

Results for the light exposure of scavenger-containing *S. aureus* suspensions are shown in Table 8.1 and Figure 8.1. From the table it can be seen that a 20-minute exposure in the presence of ascorbic acid and catalase results in only 0.7 $\log_{10}$ and 0.4 $\log_{10}$ reduction, respectively, with no significant reduction in the presence of dimethylthiourea (DMTU). After a 30-minute exposure, a reduction in *S. aureus* population of only approximately a single $\log_{10}$ was observed with all three of the scavengers. A 30-minute exposure without the addition of scavengers (control) resulted in close to total destruction of the initial population of 2.2 x 10^5 CFU/ml.

Figure 8.1 illustrates the effect of 30-minute light exposure on scavenger-containing *S. aureus* suspensions. It shows that the addition of scavengers significantly reduces inactivation, indicating that the presence of oxygen is important for the inactivation process.

TABLE 8.1 *Effect of oxygen scavengers on the population densities of S. aureus NCTC 4135 suspensions after 20 and 30-minute exposures to >400 nm light*

SCAVENGER	LIGHT EXPOSURE	BACTERIAL COUNT AFTER EXPOSURE TIME (CFU/ml)		
		0 min	**20 min**	**30 min**
CONTROL	-	2.2×10^5	1.9×10^5	2.2×10^5
(NO SCAVENGER)	+	**2.2×10^5**	**5.2×10^3**	**Between 0 and 10**
ASCORBIC ACID	-	2.0×10^5	1.6×10^5	1.7×10^5
(30 mM)	+	**2.0×10^5**	**4.7×10^4**	**2.5×10^4**
CATALASE	-	2.2×10^5	2.4×10^5	1.9×10^5
(20 U/ml)	+	**2.2×10^5**	**8.6×10^4**	**1.8×10^4**
DMTU	-	2.1×10^5	2.0×10^5	2.1×10^5
(100 mM)	+	**2.1×10^5**	**1.8×10^5**	**1.7×10^4**

(+, light exposed; -, non-light exposed)

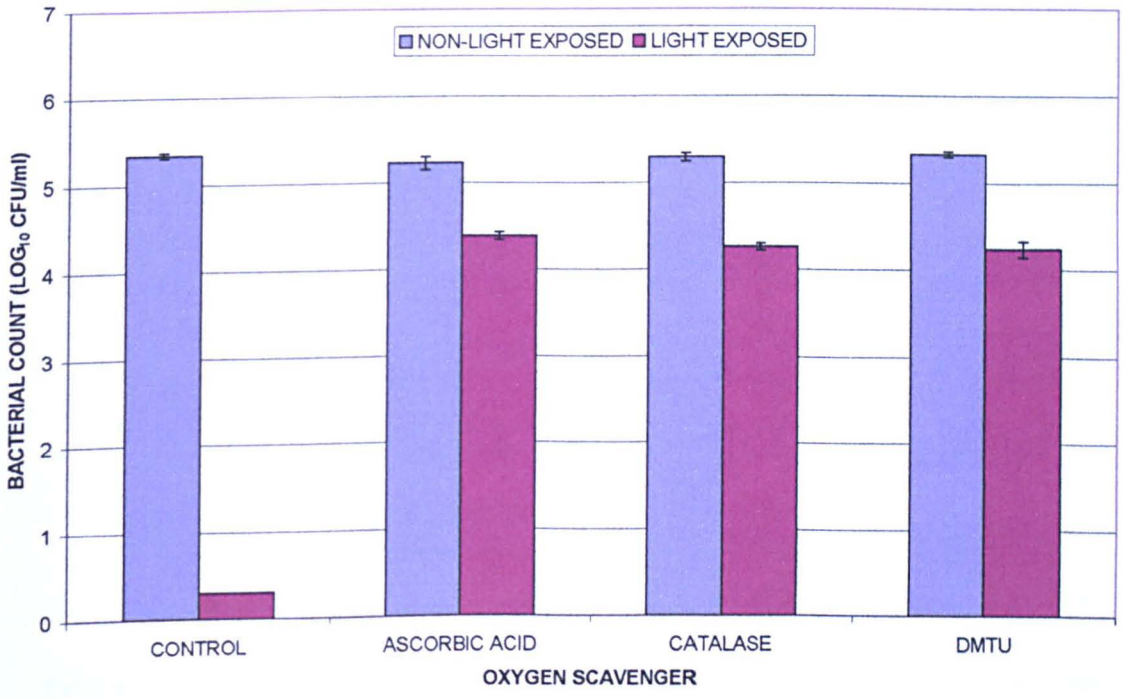

FIGURE 8.1 *Effect of the addition of reactive oxygen species scavengers to S. aureus NCTC 4135 suspensions, exposed for 30 minutes to >400nm light*

8.2 OXYGEN ENHANCEMENT

With the negative effect of oxygen scavengers on the inactivation process confirmed, in order to further strengthen the indication that this inactivation process is oxygen-dependent, an investigation into oxygen enhancement was carried out.

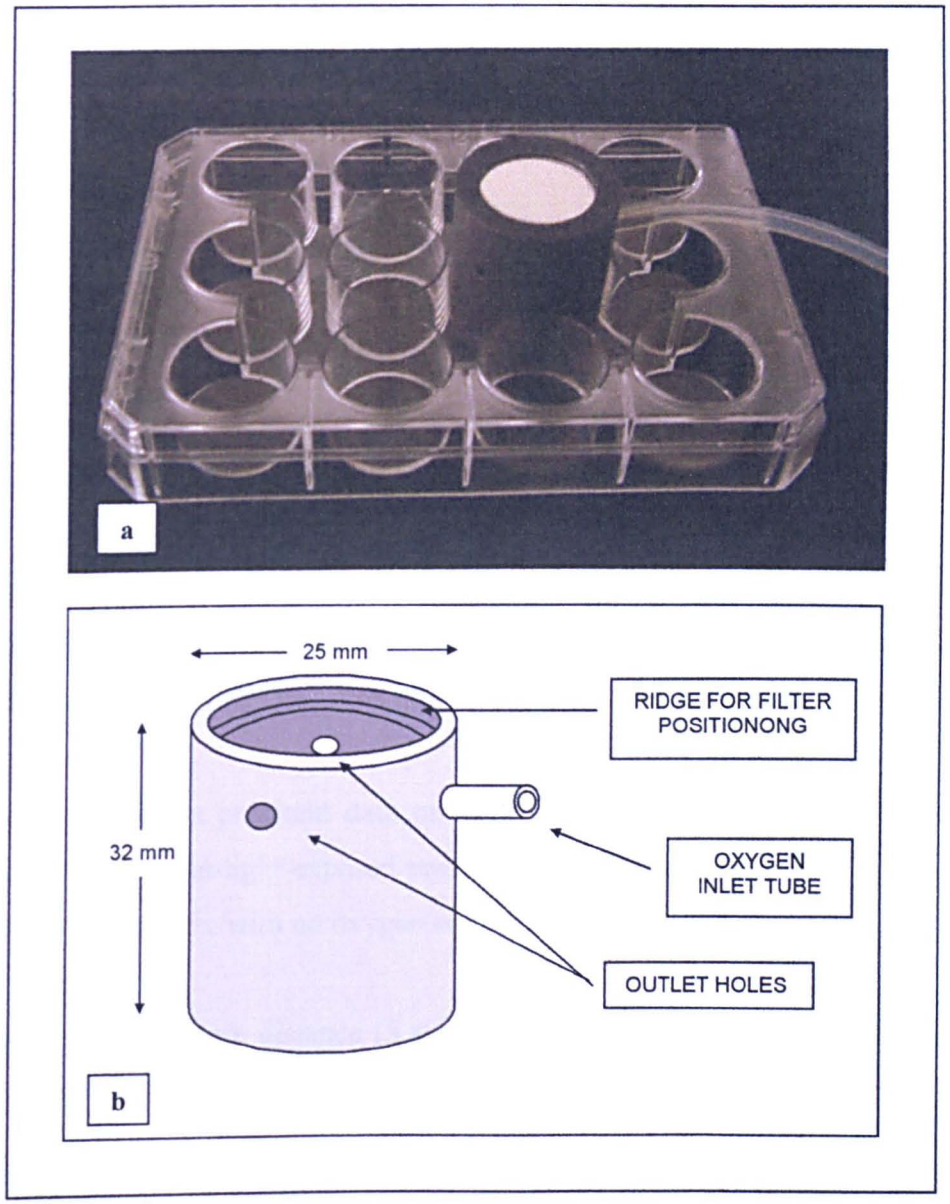

FIGURE 8.2 *Oxygen-enhancement delivery system. Photograph (a) shows the filter holder with oxygen inlet tube positioned around the sample well on the multidish; and diagram (b) highlights the main features and dimensions of the filter holder.*

8.2.1 Experimental Arrangement

To allow for the addition of gaseous oxygen, the original experimental arrangement (Section 6.2.2.2) was modified to allow a continuous flow of oxygen to be generated over the bacterial sample. The modification, depicted in Figure 8.2, was carried out as follows: A cylindrical PVC filter holder, of height 32 mm and diameter 25 mm, was constructed. An oxygen inlet tube was connected near the top of the filter holder. The filter holder fitted around the sample well and the filter slotted into the top of the holder. Two oxygen outlet holes (2 mm diameter) were placed around the perimeter near the top of the holder.

8.2.2 Treatment Method

Staphylococcus aureus NCTC 4135 was cultured as described in Section 3.1.2. After an 18-hour incubation period, the broths were centrifuged at 3400 rpm for 10 minutes and the resultant pellets re-suspended in PBS. The suspension was then diluted to give a population density of 2.0×10^5 CFU/ml. A 2 ml volume of the suspension was dispensed into the well of a multidish and covered with the filter holder and the 400 nm long-wave pass filter. The oxygen flow rate was set at 200 ml/min and, using the method described in Section 6.2.2.2, the oxygen-enhanced sample was exposed to increasing durations of visible-light treatment. All samples were then plated onto NA and incubated at 37°C for 24 hours.

Control experiments provided data on (1) light-exposed samples with no oxygen enhancement, (2) non-light-exposed samples with oxygen enhancement, and (3) non-light-exposed samples with no oxygen enhancement.

As before, the exposure distance (5 cm) and output intensity (100%) of the Xenon lamp were maintained constant throughout.

8.2.3 Enhancement Results

Figure 8.3 shows log-decay plots of bacterial population with and without oxygen-enhanced inactivation. Although both oxygen-enhanced and non-enhanced light exposures result in approximately a 5-$\log_{10}$ reduction after 30 minutes (540 J/cm^2

dose), the oxygen-enhanced inactivation occurs at a faster rate, with a 4.4-$\log_{10}$ reduction after 25 minutes (450 J/cm^2 dose) compared to a 1.2-$\log_{10}$ reduction with no oxygen-enhancement after the same time. Table 8.2 displays numerical data for comparison of the values for energy density per $\log_{10}$ reduction after a dose of 450 J/cm^2 for oxygen enhanced and non-enhanced treatment. From here it can be seen that for non-enhanced inactivation of a 2.0 x 10^5 CFU/ml *S. aureus* suspension the dose required is 375 J/cm^2 per $\log_{10}$ reduction, with around 3.5 times less specific dose being required for oxygen-enhanced inactivation.

The non-light-exposed/oxygen-enhanced and non-light-exposed/non-oxygen-enhanced control samples both experienced no inactivation. Clearly, oxygen enhancement does indeed accelerate the rate of visible-light inactivation of *S. aureus*.

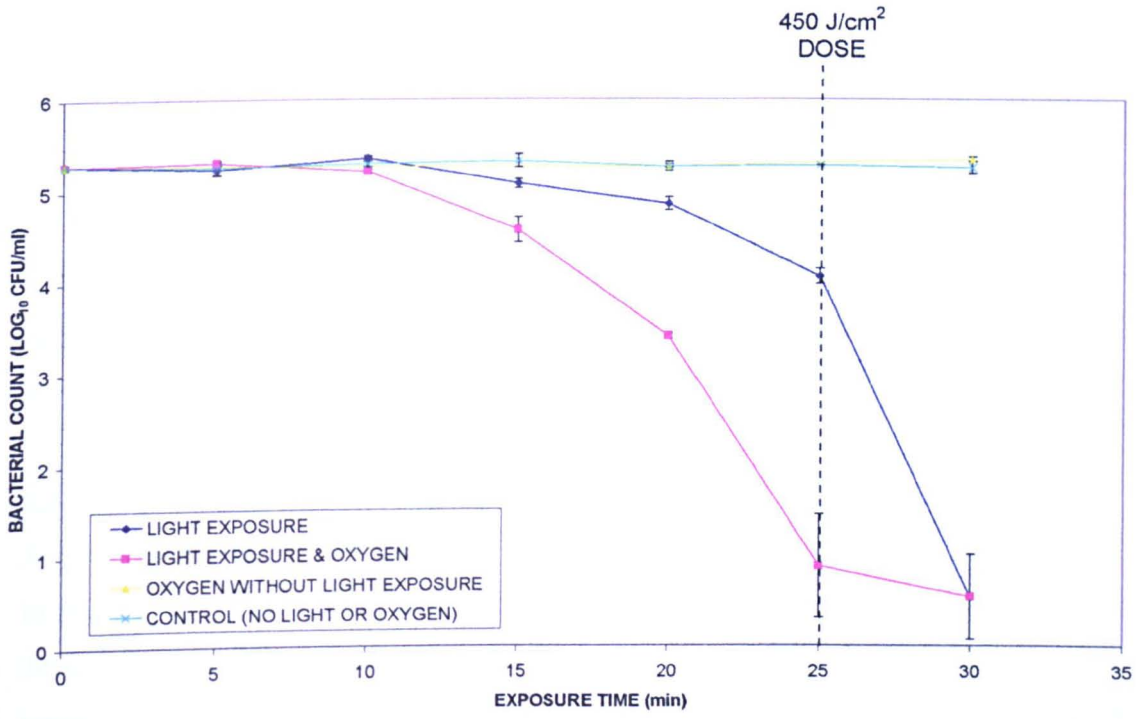

FIGURE 8.3 *The effect of oxygen enhancement on the inactivation rate of S. aureus NCTC 4135 by >400 nm light. Marked on the graph is the point at which a dose of 450 J/cm^2 is achieved.*

TABLE 8.2 *Effect of oxygen enhancement on the inactivation of a 2.0 x 10^5CFU/ml S. aureus suspension after a dose of 450 J/cm^2*

OXYGEN ENHANCEMENT	EXPOSURE TIME (sec)	POWER DENSITY (mW/cm^2)	DOSE (J/cm^2)	LOG_{10} REDUCTION	J/cm^2/LOG_{10} REDUCTION
YES	1500	300	450	4.4	**102.3**
NO	1500	300	450	1.2	**375**

8.3 EFFECT OF VISIBLE-LIGHT EXPOSURE ON *STAPHYLOCOCCUS AUREUS* PIGMENTATION

As indicated by their name, *Staphylococcus aureus* cells contain yellow pigmentation (*aureus* is Latin for golden) and these pigments have been identified as triterpenoid carotenoids, the predominant pigment being staphyloxanthin [Marshall and Wilmoth, 1981]. Aside from the important role of the carotenoid pigments in photosynthesis, they are known as efficient quenchers of $^1O_2$ and radicals, which they achieve through interception of the oxidative chain reactions [Polívka and Sundström, 2004].

The aim of this experiment was to investigate whether light exposure of *S. aureus* had any effect on its pigmentation, with a subsequent effect on the oxygen-quenching ability of the carotenoid.

8.3.1 Treatment Method

Staphylococcus aureus NCTC 4135 was cultured for experimental use as described in Section 3.1.2. After an 18-hour incubation period, the broths were centrifuged at 3400 rpm for 10 minutes and the resultant pellets re-suspended in PBS, giving a mean population of 1.99 x 10^9 CFU/ml. Using the treatment method described in Section 6.2.2.2, 3 ml of this suspension was then exposed to 3.5 hours of >400 nm light (at 100% intensity & a 5cm exposure distance) with continuous mechanical agitation. A control sample (non-light treated) was also used. After light treatment, the two samples were centrifuged (7800 rpm for 5 minutes) in order to obtain a pellet.

8.3.2 Treatment Results

Plating of the light-exposed and control samples showed that the light exposure resulted in an 8-$\log_{10}$ reduction in population, with a decrease from 1.99 x 10^9 CFU/ml to 1.5 x 10^1 CFU/ml. In addition to this inactivation, observation of the resultant pellets revealed that there was a distinct difference in pigmentation between the light-exposed and control samples. This can be seen in the Figure 8.4.

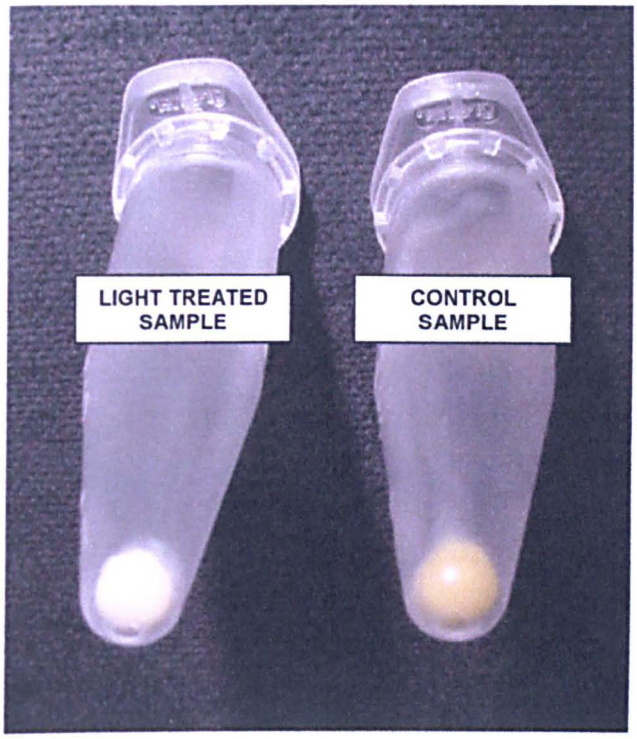

FIGURE 8.4 *Comparison of the pigmentation between light-exposed and non-exposed S. aureus NCTC 4135*

This result may have significance in the proposed theory that inactivation is mediated through the photo-excitation of intracellular porphyrins. As mentioned before, carotenoids serve as efficient quenchers of $^1O_2$ [Polívka and Sundström, 2004] and they are also proven to absorb light most strongly in the blue region of the electromagnetic spectrum [Campbell, 1996]. These factors suggest that the high intensity light may be photobleaching the carotenoid, resulting in its destruction. This would then eliminate any $^1O_2$ quenching effect, thus allowing the rapid progression of cellular destruction.

8.4 DISCUSSION OF EXPERIMENTAL RESULTS

The results of this study demonstrated that oxygen plays a strategic role in inactivation of *S. aureus* through exposure to visible light. Oxygen depletion had a negative effect on the occurrence of PDI of *S. aureus*. The addition of reactive oxygen scavengers significantly reduced the efficacy of the visible-light treatment, and these results correlate well with those of other similar studies.

Feuerstein *et al* [2005] investigated the effect of the same oxygen scavengers on the visible-light inactivation of the periopathogens, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Exposure using a range of blue-light-emitting sources photo-stimulated the naturally produced porphyrins to produce $^1O_2$ and radicals resulting in cell death. Upon addition of the scavengers they found a marked reduction in the percentage of bacteria growth [Feuerstein *et al*, 2005]. Similar results were also found in a study by Burns *et al* [1996], who used a Helium-Neon laser and toluidine blue O for the successful inactivation of *Streptococcus mutans*, but addition of methionine and sodium azide provided 98% protection to the normally lethal PDI effect.

As in the present study, Feuerstein *et al* [2005] found that the addition of oxygen scavengers did not completely protect the bacteria from inactivation and this was assumed to be due to their inability to effectively scavenge the fast-binding of the highly reactive $^1O_2$ (excited singlet states are very short-lived: in the region of 10^{-9} s [Fuchs and Thiele, 1998]), and/or their partially inefficient access to the reactive oxygen species generated within the cells [Feuerstein *et al*, 2005].

A number of studies have investigated the effect of oxygen-depletion through the exposure of bacterial samples in anaerobic environments. Feuerstein *et al* [2005] found that exposure of *P. gingivalis* and *F. nucleatum* to blue light under anaerobic conditions eliminated the phototoxic effect obtained under aerobic conditions. Similarly, a study by Henry *et al* [1996] concluded that the phototoxic effects of argon-laser irradiation against *Porphyromonas* and *Prevotella spp.* were dependent

on the presence of atmospheric oxygen after bacterial exposure while in an anaerobic pouch, yielded no inactivation, even using double the minimum inhibitory dose. Burns *et al* [1996] also documented no significant decrease in the viable count of *S. mutans* when irradiated under anaerobic conditions compared to a 60% decrease under aerobic conditions. These results suggest that the availability of oxygen is a rate-limiting factor for the production of $^1O_2$.

Oxygen-enhancement experiments in this study further established the significance of oxygen in the visible-light inactivation mechanism. The addition of gaseous molecular oxygen to the sample well during visible-light exposure successfully increased the rate of *S. aureus* inactivation, with a dose of 450 J/cm^2 resulting in approximately a 3-$\log_{10}$ increase in kill compared to non-enhanced inactivation. Burns *et al* [1996] also investigated the effect of oxygen enhancement on PDI but used deuterium oxide (D_2O) rather than O_2, as used in this study. The addition of D_2O resulted in a substantial 15-fold increase in the number of bacteria killed.

This study of the oxygen processes of depletion and enhancement is of a preliminary nature, designed only to verify the significance of oxygen. Future work to quantify the oxygen concentrations should involve systems where oxygen depletion and enhancement levels can be measured. For example, flow rate may be controlled and monitored, so that oxygen concentration can be determined. The monitoring and measurement of $^1O_2$ is another parameter which could be investigated to further elucidate that this is indeed the mechanism of action. A range of optical methods exists for this purpose and includes indirect spectroscopic detection using chemiluminescence and optical upconversion [Tromberg *et al*, 1991], and the piston source method [Lee *et al*, 1976; Duo *et al*, 2001].

PART II

8.5 PHOTODYNAMIC INACTIVATION PROCESS

Part I has investigated the role of oxygen in the visible-light inactivation of *S. aureus* through enhancement and depletion experiments. Results indicate that oxygen plays an important role in successful bacterial inactivation.

The dependence on oxygen for successful *S. aureus* inactivation, in addition to the wavelength sensitivity being in the region of 400-420 nm, provides further evidence that the mechanism of staphylococcal inactivation observed in this study is the photo-excitation of naturally-occurring intracellular porphyrins, and their function as endogenous photosensitisers. A discussion of the background and mechanism of this visible-light-mediated inactivation, known as photodynamic inactivation, now follows.

8.5.1 Photodynamic Inactivation

Photodynamic inactivation (PDI) – also known as photodynamic therapy, photoradiation or photochemotherapy – involves the use of a molecule called a photosensitiser, which is excited upon exposure to light of a particular wavelength, to destroy cells. The three components essential for PDI are a photosensitiser, light and oxygen [Moan and Peng, 2003].

The mechanism of PDI involves the administration of a photosensitiser molecule to a target area/liquid, followed by its illumination using visible-light of an appropriate wavelength. This excites the photosensitiser and, through a series of reactions, which will be discussed in Section 8.5.3, results in the production of reactive oxygen species – predominantly singlet oxygen ($^1O_2$). Singlet oxygen is an aggressive chemical species that reacts rapidly with surrounding biomolecules. The production of $^1O_2$ leads to the oxidation of biological molecules such as nucleic acids, proteins and lipids – and ultimately cell death [Hamblin and Hasan, 2004].

Photodynamic activity of chemical compounds towards microorganisms was discovered more than 100 years ago. Clinically, photodynamic inactivation (PDI) has been developed as a cancer treatment, followed by other medical applications such as the treatment of age-related macular degeneration, arthritis, psoriasis, Barretts oesophagus, atherosclerosis and restenosis in veins and arteries [Hamblin and Hasan, 2004]. The rapidly increasing emergence of microbial antibiotic resistance has ignited research interest into the use of PDI as an alternative antimicrobial treatment.

Clinical PDI therapy typically involves the application – either topically or intravenously – of a photosensitiser, of which hematoporphyrin derivative (HPD, also known as Photofrin) is the best-known example, [Hamblin and Hasan, 2004]. The interest of the present study however, is on the photo-stimulation of intracellular molecules called porphyrins – which essentially act as endogenous photosensitisers within the bacterial cells – without an applied photosensitiser.

8.5.2 Porphyrins

Chromophores are the part of a molecule responsible for its colour and their absorption of light is the initial step in all photochemical reactions [Campbell, 1996]. Prime examples of chromophores are the porphyrin molecules.

Porphyrins are a ubiquitous class of naturally occurring molecules involved in a wide variety of important biological processes including pigmentation changes, catalysis, oxygen transport and photosynthesis [Goldoni, 2002]. These molecules all have in common a substituted aromatic macrocyclic ring consisting of four pyrrole rings linked together by four methine bridges [Milgrom, 1997]. The pyrrole ring, shown in Figure 8.5, is a five-membered ring of four carbon atoms and one nitrogen atom, with a hydrogen atom bound to each. To derive the porphyrin macrocycle, four of the pyrrole units are connected together via four unsaturated =CH— groups, known as methine bridges [Milgrom, 1997]. This structure is shown in Figure 8.6. Addition of single side chains to this tetrapyrrole molecule determines the type and function of the porphyrin molecules, and in some cases, a metal ion or atom such as iron, zinc,

magnesium, nickel and cobalt, can be inserted in the centre of the macrocycle forming metalloporphyrins. For photodynamic inactivation, only metal-free porphyrins are useful. If a metal is present there is rapid quenching of any singlet oxygen produced and therefore no photodynamic action [Krasnovskii *et al*, 1982].

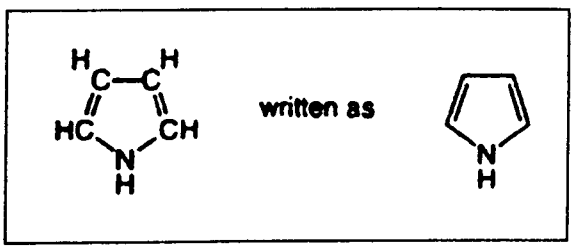

FIGURE 8.5 *Structure of the five-membered pyrrole molecule [Milgrom, 1997]*

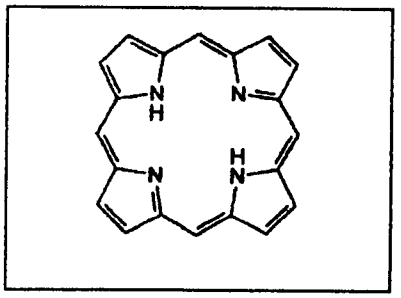

FIGURE 8.6 *Structure of the unsubstituted tetrapyrrole porphyrin macrocycle [Milgrom, 1997]*

The visible absorption spectrum of a porphyrin, shown in Figure 8.7, shows intense absorption (extinction coefficient >200,000) in the region of 400 nm, and this absorption maximum is referred to as the "Soret Band". The visible spectra of porphyrins also show several weaker absorptions at longer wavelengths of between 450 nm and 700 nm: these are termed "Q Bands" [Goldoni, 2002].

Due to this high absorption at approximately 400 nm, blue light is theoretically the most effective visible-wavelength for photosensitisation of porphyrins. The presence of the Q bands highlights that other wavelengths are capable of being absorbed and hence exciting porphyrins, but their low absorption means that very high intensity light would be required to induce the same effect as that for light around 400 nm.

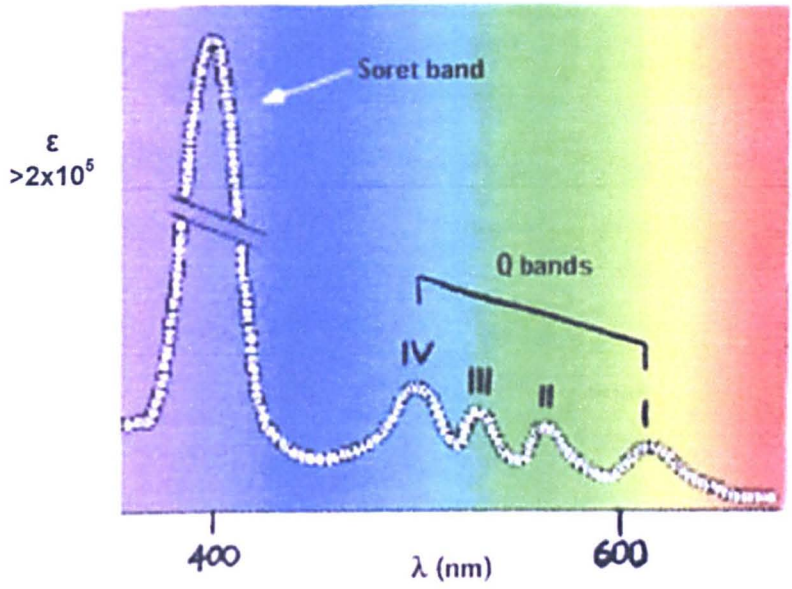

FIGURE 8.7 *Typical UV-visible absorption spectrum of porphyrins [Goldoni, 2002]*

8.5.3 Mechanism of Photodynamic Inactivation

Photodynamic inactivation of microorganisms involves the excitation of either exogenously applied or endogenous photosensitiser molecules using light of specific wavelengths. The photo-excitation of these molecules generates the production of reactive oxygen species – predominantly singlet oxygen ($^1O_2$) – which proceed to lethally damage cells. The exact mechanism of this excitation process will now be discussed with specific reference to porphyrins as the photosensitising molecule.

Upon illumination of the porphyrins (or other photosensitiser molecules) with light corresponding to their absorption maximum, the porphyrin undergoes excitation from a stable ground state to a singlet excited state. The singlet excited porphyrin can then either decay back to the ground state with the release of energy in the form of fluorescence (detectable at approximately 600 nm) or, as is the case with most porphyrins, this excited state may undergo intersystem crossing to the slightly lower energy but longer lived, triplet state [Wainwright, 1998; Hamblin and Hasan, 2004]. This triplet state then further reacts by one or both of two pathways, commonly referred to as Type I and Type II photoreactions – both of which are oxygen-dependent [Wainwright, 1998].

Type I photoreactions involve electron-transfer from the triplet-state porphyrin with the participation of a substrate, such as nearby cellular membranes or amino acids, to produce radical ions that can react with oxygen to produce cytotoxic species, such as hydroxyl, superoxide and lipid-derived radicals. Type II photoreactions involve energy transfer from the triplet-state porphyrin to ground-state molecular oxygen, producing the highly-oxidizing, excited-state $^1O_2$ [Hamblin and Hasan, 2004], and this is shown in diagrammatic form in Figure 8.8. This transfer of energy from triplet-state porphyrins to molecular oxygen is favourable since dioxygen, which is found in most cells, is one of the very few molecules with a triplet ground state [Smith and Forsyth, 1997].

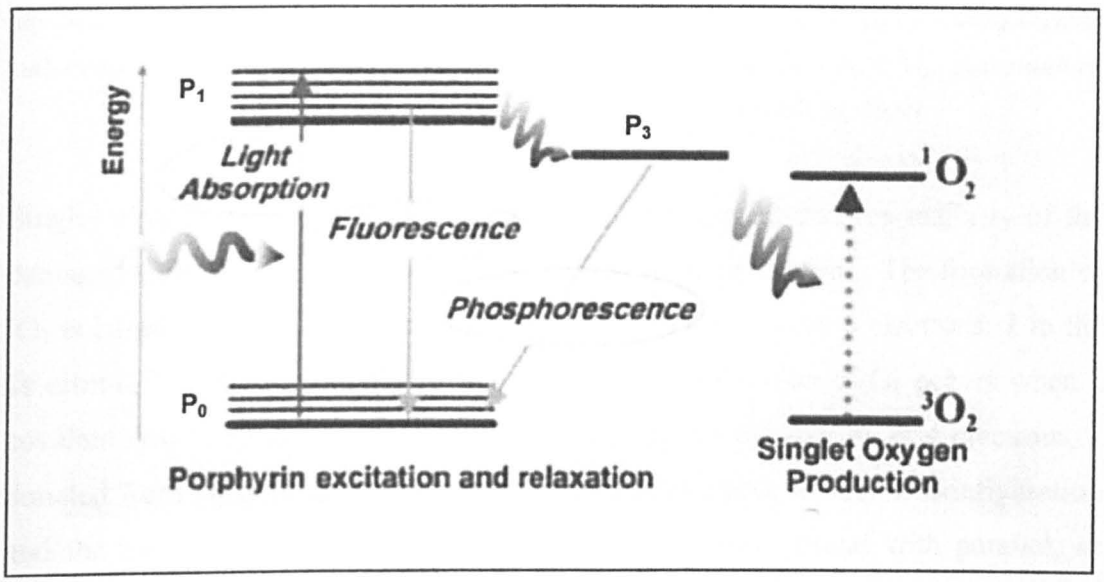

FIGURE 8.8 *Schematic diagram of the activation pathways during Type II photosensitization reactions [Lukšienė, 2005]*

Previous studies indicate that PDI processes utilising porphyrins or porphyrin-derivatives as the photosensitising molecules is mediated predominantly by the Type II photoreaction pathway [Fuchs and Thiele, 1998]. A summary of Type I and Type II photoreactions involved in photodynamic inactivation is shown in Figure 8.9.

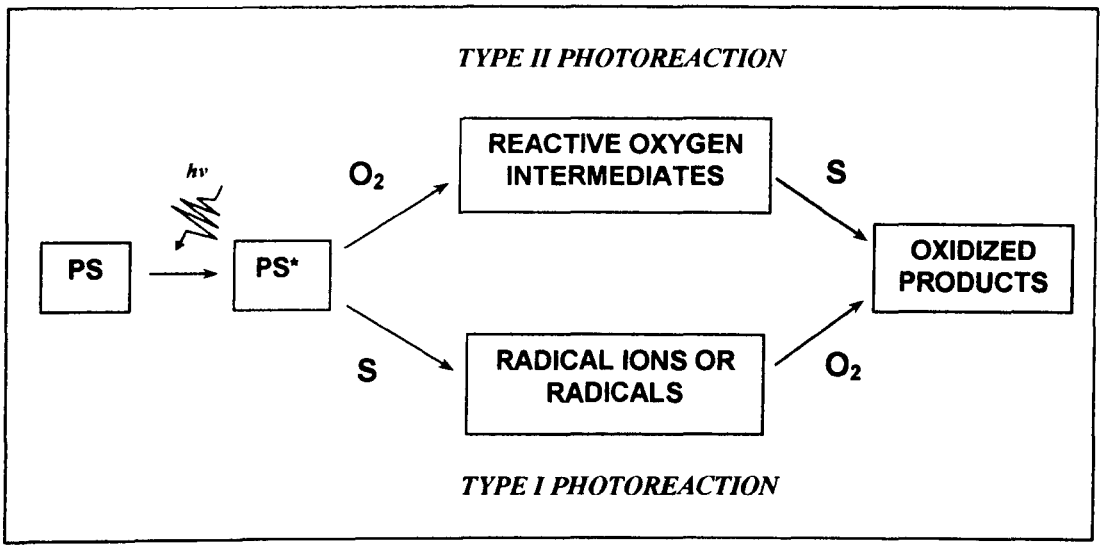

FIGURE 8.9 *Pathways of Type I and Type II photosensitization reactions. After light activation of the ground-state photosensitizer (PS), excited PS* can follow two pathways; Type I involving organic substrates (S) to produce free radicals or Type II which produces the highly toxic $^1O_2$. Both result in the oxidation and ultimate destruction of cells. [Maisch et al, 2004]*

Singlet oxygen itself is a highly reactive oxidising species and the majority of the damage resulting from PDI has been accredited to its generation. The formation of $^1O_2$ is based on its electron configuration. Oxygen atoms have 8 electrons, 2 in the 1s orbital, 2 in the 2s orbital and 4 in 2p orbitals. Formation of O_2 occurs when a covalent double bond is created between two atoms by the sharing of 4 electrons, 2 donated from each atom. Triplet oxygen is its most stable molecular configuration and the two outermost electrons each exist in their own orbital with parallel, or unpaired, spins. This makes molecular oxygen O_2 a diradical. The formation of $^1O_2$ occurs on addition of energy to triplet O_2 – for example by transfer from triplet-state porphyrins. This addition of energy flips the spin of an electron, causing it to pair with the other in a single outermost orbital, hence resulting in a vacant orbital [ShoreLaser and Esthetics, 2006].

Addition of another electron to this outermost orbital yields a superoxide anion, and addition of a second electron, a peroxide anion. A superoxide and a peroxide molecule can undergo further compositional change by acceptance of a proton,

thereby yielding water and hydrogen peroxide molecules, respectively [ShoreLaser and Esthetics, 2006].

Singlet oxygen has a very high reactivity with a variety of biomolecules and readily combines chemically with them [Fuchs and Thiele, 1998]. In addition to biomolecular damage, $^1O_2$ can also transfer its electronic energy to a donor molecule (thus exciting it) and return to the ground state – referred to as quenching [Fuchs and Thiele, 1998]. The half-life of $^1O_2$ in the cellular environment is in the region of 6 x 10^{-7} s, with its diffusion distance estimated to be approximately 0.1 μm [Fuchs and Thiele, 1998].

The primary target of porphyrin-mediated photoreactions is thought to be the cell membrane and this is supported by the findings of Ashkenazi *et al* [2003]. They documented that illumination of *P. acnes*, containing both naturally produced and ALA-induced porphyrins (predominantly coproporphyrin), resulted in an efflux of potassium ions (K^+) and significant phosphate loss – typical consequences of membrane damage, specifically of the ionic pumps. They argued that the damage was a result of membrane cross-linking alterations, and the free radical species produced may be responsible for diminishing K^+ and ATPase activity and cell death [Ashkenazi *et al*, 2003]. This is supported by the findings of Lambrechts *et al* [2005(I)] who identified the cytoplasmic membrane as being the target organelle in the PDI of *Candida albicans* using the exogenous photosensitiser TriP(4).

8.5.4 Microbial Photodynamic Inactivation using Endogenous Photosensitisers

Research into acne vulgaris has demonstrated that the photodynamic inactivation of *P. acnes* could be performed without the addition of photosensitisers. The reason for this, as confirmed in a study by Ashkenazi *et al* [2003], is that these bacteria naturally produce high amounts of intracellular metal-free porphyrins, mainly coproporphyrin III and protoporphyrin IX (PpIX) [Morton *et al*, 2005], without the need for any trigger molecules, such as δ-ALA. These intracellular porphyrins can therefore act as endogenous photosensitisers in the same way as exogenously applied or δ-ALA-induced porphyrins. The use of blue light, usually 407-420 nm, for acne

therapy therefore results in excitement of these intracellular porphyrins, singlet-oxygen production and eventually *P. acnes* inactivation [Papageorgiou *et al*, 2000]. Red light has also been used for acne treatment, but with minimal success compared to blue light, due to the high intensity necessary to initiate porphyrin excitation and bacterial destruction [Ashkenazi *et al*, 2003], as indicated by the absorption spectrum of Figure 8.7. Blue and red light combination treatments have also been utilised, and have proved superior to exposure to solely blue light, the red wavelengths being effective for their anti-inflammatory properties rather than their photosensitising capabilities [Papageorgiou *et al*, 2000].

This exploitation of high levels of intracellular porphyrins for endogenous photosensitisation is not limited to *P. acnes*. A number of studies have documented that visible-light exposure of other bacteria has an inactivation effect, with inactivation attributed to the photo-stimulation of endogenous porphyrins [Ganz *et al*, 2005; Soukos *et al*, 2005; Feurestein *et al*, 2004; Henry *et al*, 1996].

Photodynamic inactivation of *Helicobacter pylori* – colonisation of which in the human stomach is linked with peptic ulcers and possibly also with adenocarcinoma – has been documented. Ganz *et al* [2005] demonstrated successful use of 405 nm light for both the *in vitro* and *in vivo* eradication of *Helicobacter pylori*, thereby suggesting a new clinical treatment method for an organism which has been known to fail standard antibiotic treatment.

In addition to *P. acnes* and *H. pylori*, visible-light inactivation studies performed using a range of bacteria commonly associated with human periodontal disease have had successful results. Feurestein *et al* [2004] employed blue-light exposure for the inactivation of *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, Gram-negative periopathogens associated with periodontal bone loss and soft tissue inflammation, respectively. The Gram-positive oral bacterium *Streptococcus mutans* was also inactivated, without exogenous photosensitisers, but using significantly higher doses [Feuerstein *et al*, 2004]. Similarly, Henry *et al* [1996] inactivated a range of *Prevotella* and *Porphyromonas spp.*, without the use of exogenous photosensitisers.

A study by Soukos *et al* [2005] also successfully used a 380-520 nm light source for the photodynamic inactivation of *Prevotella* and *Porphyromonas* species. They postulated that endogenous photosensitisation of these bacteria was a consequence of their black pigmentation – the predominant form of which is protoporphyrin IX (PpIX) – and the fact that they accumulate various amounts of iron-free PpIX, which is photosensitive.

8.5.5 Microbial Photodynamic Inactivation using Exogenous Photosensitisers

Since most bacteria do not contain significant levels of porphyrins that enable rapid photosensitisation, as is the case with *P. acnes*, photosensitisation is typically enabled through the use of exogenous photosensitisers, such as halogenated xanthenes (Rose Bengal), acridines, phenothiazines (Toluidine Blue O) and perylenequinones (hypericin) [Hamblin and Hasan, 2004].

As an alternative to the use of exogenous photosensitisers, many *in vitro* experiments, and acne PDT treatments, have investigated enhancement of the production of endogenous porphyrins by pre-treating the bacterial cells with δ-aminolevulinic acid (ALA) [Ashkenazi *et al*, 2003; ShoreLaser and Esthetics, 2006], a naturally occurring metabolite that is the first committed intermediate in the biosynthesis of tetrapyrroles [Fukuda *et al*, 2005]. The addition of δ-ALA to bacterial cultures leads to the increased synthesis of uroporphyrin, coproporphyrin and protoporphyrin IX (PpIX) [Nitzan *et al*, 2004]. This build-up of 'endogenous' photosensitising molecules enables photodynamic inactivation upon illumination with visible-light.

Numerous *in vitro* studies have been performed using microorganisms, with successful results for bacteria, yeasts, viruses and parasites. Furthermore, results have consistently shown that photosensitisation of bacterial cells is independent of their antibiotic resistance spectrum [Nitzan *et al*, 2004]. Table 8.3 lists a representative selection of microorganisms that have been photo-dynamically irradiated.

TABLE 8.3 *Reports of photodynamic inactivation of microorganisms in vitro*

ORGANISM	PHOTOSENSITISER	LIGHT SOURCE	REFERENCE
BACTERIA			
Pseudomonas aeruginosa *Staphylococcus aureus*	TriP(4)	White light	Lambrechts et al, 2005[II]
MRSA	IgG-tin(IV)chlorine e6 conjugate	HeNe laser (632.8 nm)	Embleton et al, 2002
Escherichia coli	Cationic hydrophilic porphyrin	Blue, Green & Red light	Nitzan & Ashkenazi, 2001
Staphylococcus aureus *Staphylococcus epidermidis* *Streptococcus pyogenes* *Propionibacterium acnes*	Methylene blue	250 W OHP lamp (400-700 nm)	Zeina et al, 2001
Staphylococcus aureus	Hematoporphyrin	Not stated	Bertoloni et al, 2000
Deinococcus radiodurans	Rose Bengal	1000 W Xenon arc lamp (>320 nm)	Schafer et al, 2000
Escherichia coli	δ-ALA	150 W halogen lamp (400-800 nm)	Szocs et al, 1999
MRSA	Methylene blue & derivatives	Exal light box (350-800 nm)	Wainwright et al, 1998
Haemophilus parainfluenzae	δ-ALA	Argon dye laser (630 nm)	van der Meulen et al, 1997
Staphylococcus aureus	Toluidine blue O	HeNe laser (632.8 nm)	Wilson & Yianni, 1995
VIRUSES			
Herpes simplex virus-1	5,10,15,20-TSPP	Hg-medium pressure lamp (>650 nm)	Zecasin, 2000
HIV-1	Methylene blue	Visible light	Bachmann et al, 1995
FUNGI & YEASTS			
Candida albicans	TriP(4)	500 W halogen lamp (white light)	Lambrechts et al, 2005[I]
Aspergillus fumigatus	Green 2W	Light through diffusing fibre (630 nm)	Friedberg et al, 2001
PARASITES			
Acanthamoeba palestinensis	Tetracationic Zn(II)-phthalocyanine	Halogen lamp (600-700 nm)	Ferro et al, 2006

8.6 EVIDENCE FOR PORPHYRIN-MEDIATED PHOTODYNAMIC INACTIVATION OF *STAPHYLOCOCCUS AUREUS*

The importance of oxygen for the successful optical inactivation of *S. aureus*, added to the fact that the wavelengths required for optimal inactivation are in the region of 400-420 nm, provides further evidence for the mechanism of inactivation being the photo-excitation of naturally-occurring intracellular porphyrins.

The description of this inactivation method as the result of photo-stimulated porphyrins may also clarify some unexplained results in the previous chapter. Referring back to Section 7.2.2, if inactivation is indeed porphyrin-mediated, the Q bands present in the absorption spectrum of porphyrins may provide a solution as to why slight inactivation was witnessed when *S. aureus* suspensions were illuminated with light of wavelengths longer than 450 nm, between 450 and 500 nm and longer than 500 nm (Figure 7.1). The presence of these small absorption peaks, combined with the high intensity light used to illuminate the samples, raises the possibility that the lower rates of bacterial inactivation witnessed with these wavelengths was in fact also a result of porphyrin photosensitisation, and was only observed due to the high light intensities used.

Although endogenously-mediated PDI has been documented with bacterial species such as *P. acnes* [Ashkenazi *et al*, 2003], black pigmented bacteria [Soukos *et al*, 2005], and more recently, *H. pylori* [Ganz *et al*, 2005], this is the first documented evidence that *S. aureus* can be inactivated optically using 400-420 nm light without the use of exogenous photosensitisers or δ-ALA-induced porphyrins.

Much research has been done involving the photodynamic inactivation of *S. aureus* and MRSA with exogenous photosensitisers such as phenothiazinium dyes, including toluidine blue O [Wainwright *et al*, 1998; Wilson and Yianni, 1995] and methylene blue [Zeina *et al*, 2001; Wainwright *et al*, 1998], and porphyrin derivatives including hematoporphyrin [Bertoloni *et al*, 2000] and TriP(4) [Lambrechts *et al*, 2005(II)]. The induction of the intracellular porphyrin production through pre-treatment with δ-

ALA has also provided successful means for staphylococcal inactivation [Nitzan *et al*, 2004; Nitzan and Kauffman, 1999]. Throughout these studies, inactivation in the absence of exogenous photosensitisers or δ-ALA-induced porphyrins has either been dismissed or not discussed.

A study by Nitzan *et al* [2004] tested the light-inactivation of δ-ALA-induced *S. aureus* cultures, with controls of non-induced cultures also used. Exposure of these controls showed no inactivation and the reason for this is likely to be due to the use of a dose of 50 J/cm^2, very low in comparison to the 630 J/cm^2 used in this study to achieve a 5-log$_{10}$ reduction of a *S. aureus* population. Lambrechts *et al* [2005(II)] also investigated the effect of visible-light exposure on *S. aureus* in the absence of photosensitisers.

From the inactivation curves shown in Chapter 6, it was suggested that different bacterial genera require different doses before visible-light inactivation is initiated, but within a single genera, the pre-inactivation dose is always the same. Nitzan *et al* [2004] reported similar results with bacteria pre-treated with δ-ALA, and proposed that the diversity of the photo-inactivation reactions between the staphylococcal strains and the other five strains tested (*Streptococcus*, *Bacillus*, *Escherichia*, *Acinetobacter* and *Aeromonas*) was dependent on the diversity of the porphyrin(s) produced in the bacterial cell for each strain. They quantified the various porphyrins produced in a range of Gram-positive and Gram-negative bacteria upon induction with δ-ALA and found that the predominant porphyrin produced in both *S. aureus* and *S. epidermidis* was coproporphyrin, whereas there was no predominant porphyrin produced in the Gram-negative *E. coli*, *Acinetobacter* and *Aeromonas* strains. The amount of coproporphyrin produced by the staphylococcal strains was also five to six-fold higher than in these Gram-negative strains [Nitzan *et al*, 2004].

It appears that the cause for the high level of photo-inactivation of staphylococcal strains in studies involving δ-ALA pre-treatment [Nitzan *et al*, 2004], and the present study which utilises natural porphyrin levels, is due to the presence of high levels of coproporphyrin which is photosensitised and generates sufficient amounts of $^1O_2$

through blue-light illumination. This is further supported by the fact that *P. acnes*, which is readily inactivated by visible light through photo-stimulation of endogenous porphyrins, also contain high levels of coproporphyrin [Morton *et al*, 2005].

This study also found that the Gram-negative *E. coli* required much larger doses for its inactivation, with a specific dose of 1440 J/cm^2/log reduction compared to 126 J/cm^2/log reduction for *S. aureus*. Studies on the visible-light exposure of pre-treated δ-ALA *S. aureus* and *E. coli* suspensions, also found *S. aureus* to be much more susceptible to photosensitisation [Nitzan *et al*, 2004; Nitzan and Kauffman, 1999]. These findings are supported by other inactivation studies which used a range of light sources including blue light [Nitzan *et al*, 2004; Nitzan and Kauffman, 1999], 630 nm laser light [van der Meulen *et al*, 1997] and white light with a UV cut-off [Szocs *et al*, 1999], and found that Gram-negative species are less susceptible to photodynamic inactivation than Gram-positive species. In one study using pre-treatment with δ-ALA, which used a white-light source for illumination (as in this study), the inactivation of *E. coli* required at least 10 times higher doses to achieve similar activation levels to that for *S. aureus* [Nitzan and Kauffman, 1999]. The fact that similar ratios (approximately 10:1) are found for the eradication of *S. aureus* and *E. coli* using natural endogenous porphyrins, in this study, and using δ-ALA-induced porphyrins, in published studies, suggests that enhancement by pre-treatment with δ-ALA results in a direct amplification of the natural porphyrin content of the cells.

This study therefore provides the first evidence that *S. aureus*, in addition to the other medically important microorganisms tested in Chapter 6, can be inactivated optically using blue light of wavelengths 400-420 nm without the use of exogenous photosensitisers or ALA-induced porphyrins.

The rise in antibiotic-resistance is the major reason for the renewed interest in photodynamic inactivation (PDI) as an antimicrobial treatment method, rather than cancer therapy which the majority of PDI methods are aimed towards. The fact that this study has discovered a method which can inactivate a range of medically important microorganisms including MRSA without the use of any pre-treatments is

of great importance and could have a significant impact in a range of medical applications, and this will be discussed further in Chapter 10.

The finding that visible-light inactivation of *S. aureus* occurs more rapidly when exposure is performed in an oxygen-rich environment is significant for clinical applications. For example, in clinical application of visible-light inactivation in an operating theatre, oxygen enhancement could be used to optimise disinfection thus further reducing the risk of infection transmission during surgical procedures. Oxygen-enhancement adds to the benefit of this treatment being visible-light-based and non-detrimental to human health.

Chapter 9

405 nm LED LIGHT SOURCE

9.0 GENERAL

The work of the previous chapters has allowed progression to the conclusion that *Staphylococcus aureus* can be photo-dynamically inactivated using 400-420 nm visible-light, with optimal inactivation at 405 nm, through the photo-excitation of naturally-occurring endogenous porphyrins – most likely coproporphyrins. These porphyrins act as endogenous photosensitisers within the bacterial cells thus having the advantage over the more conventional Photodynamic Inactivation (PDI) mechanism, which requires the addition of exogenous photosensitisers for successful inactivation.

The identification of the causative wavelengths consequently allowed the selection of a more appropriate light source. The Xenon lamp utilised up till this point in the study was a broad-spectrum white-light source, using the complete visible spectrum (400-700 nm), with any UV wavelengths being filtered out. Identification of the causative wavelengths highlighted that the vast majority of the illuminating wavelengths were superfluous to the inactivation process. The use of a light source with emission primarily in the 400-420 nm region would be more efficient for inactivation, and as a consequence of using only a small part of the visible spectrum, should further minimise any thermal effects.

The desired light source should provide high intensity at 405 nm, and generate minimal heat dissipation. 405 nm light emitting diodes (LED) were considered the ideal next step. These provide a high-intensity narrow-spectrum (HINS) light beam, referred to as HINS-light.

This chapter investigates the use of HINS-light for the inactivation of *Staphylococcus aureus*, MRSA, *Streptococcus pyogenes* and also *Clostridium perfringens*, an emerging hospital pathogen which has recently been receiving significant media attention.

A comparison of the inactivation efficiencies of the LED and Xenon light systems used in this study, in relation to each other and to other published photodynamic inactivation systems is also discussed.

9.1 LIGHT EMITTING DIODES

405 nm LEDs are commercially available, and provide light emission of high intensity, when used as arrays rather than single LED units. A 99-LED array (Opto Diode Corp., California, USA), pictured in Figure 9.1, was chosen for this study.

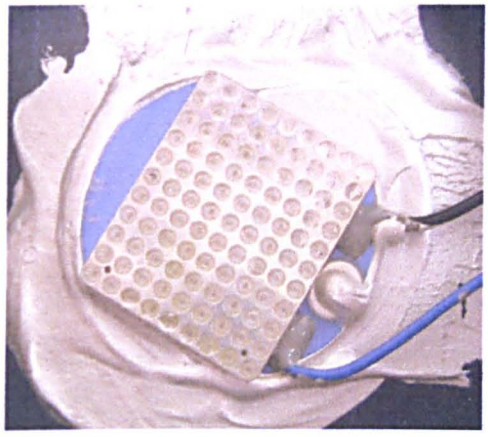

FIGURE 9.1 *99-LED array (20 mm x 16 mm)*

405 nm LEDs are made from indium-gallium-nitride (InGaN) and emit violet light across a narrow spectral region.

The emission spectrum of the 405 nm-LED array is shown in Figure 9.2. It can be seen that the centre wavelength (CW) for maximum emission is close to 405 nm, and the bandwidth is ~10 nm at FWHM. The angular distribution of the LED array is shown in Figure 9.3.

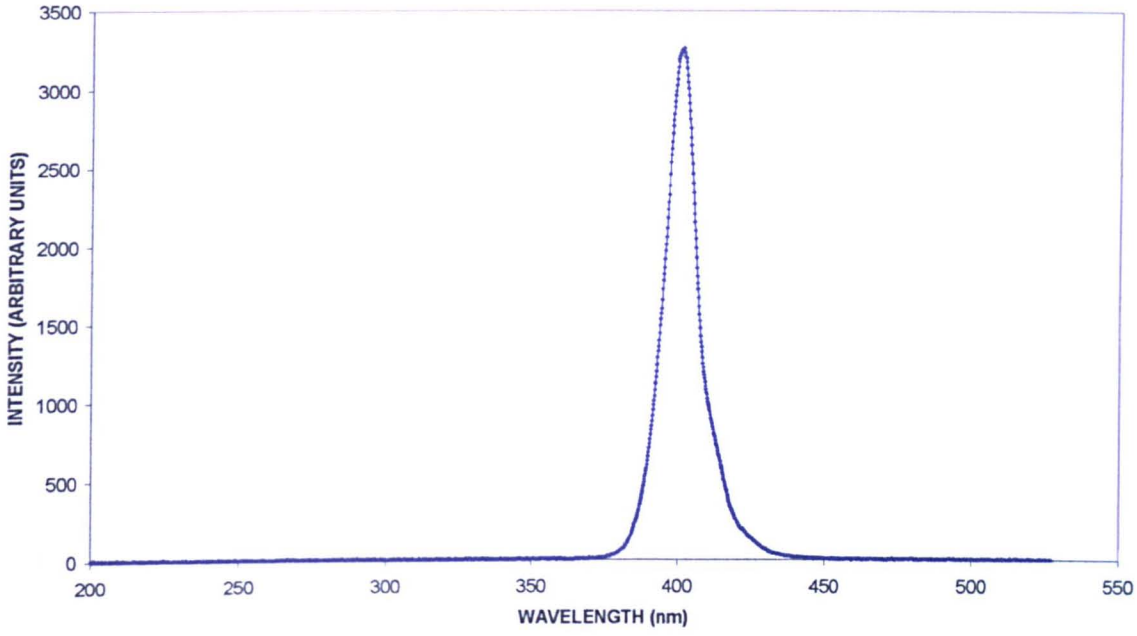

FIGURE 9.2 *Emission spectrum of 99-LED array*

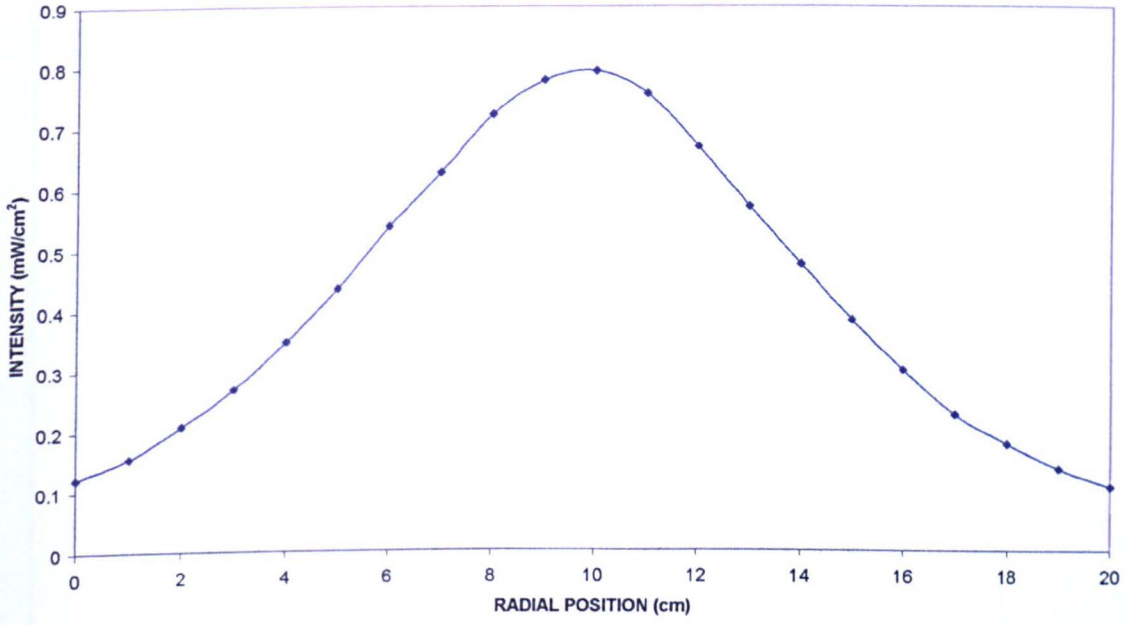

FIGURE 9.3 *Angular distribution of the 405 nm LED array at a distance of 10 cm from the detector*

9.2 EXPERIMENTAL ARRANGEMENT

Although LEDs have minimal heat dissipation, a cooling fan was attached to the LED array as a precautionary measure, to ensure temperature was maintained as stable as possible. The LED array and fan were mounted in a PVC housing. Photographs of the top and underside of this LED housing are shown in Figures 9.4a and b.

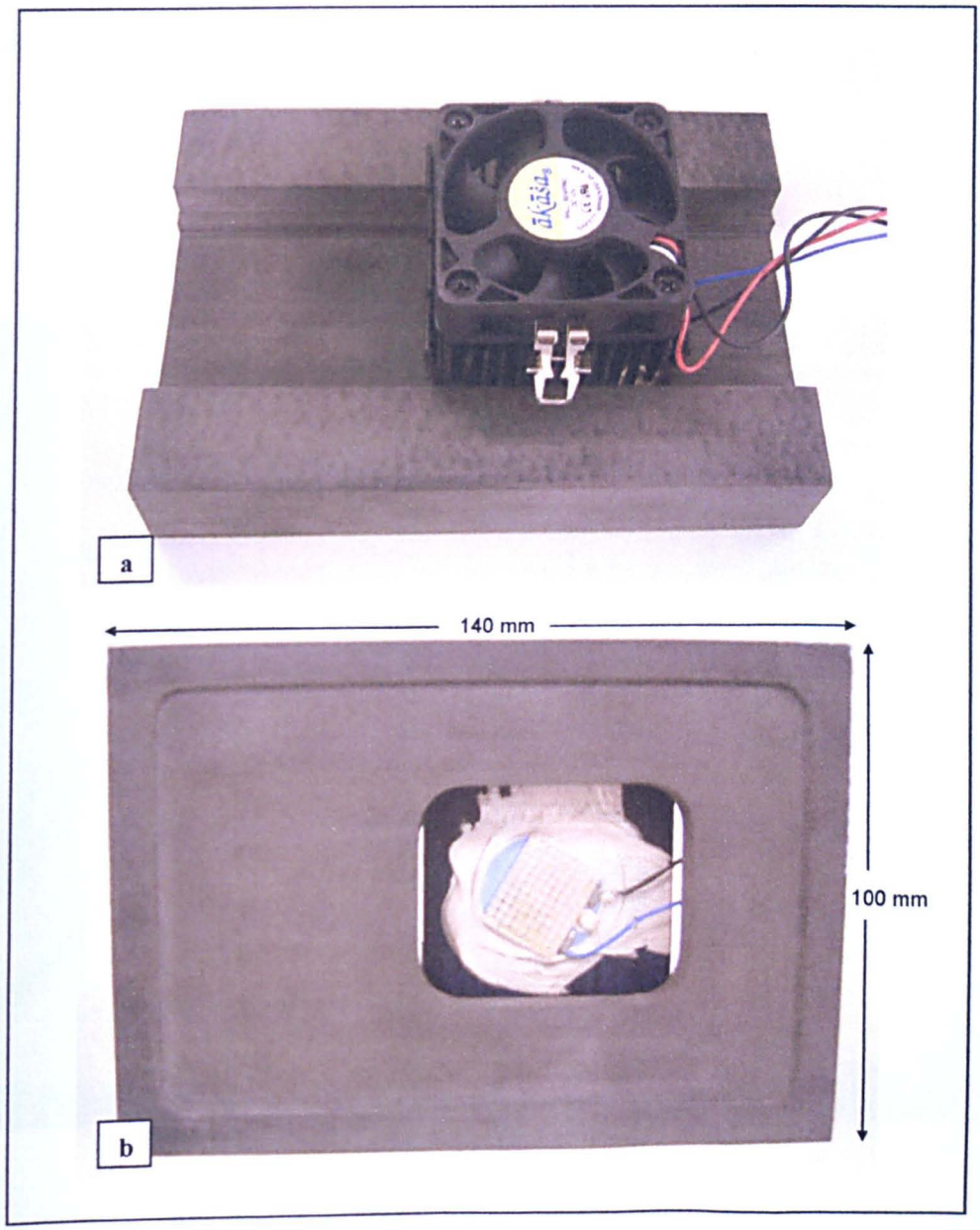

FIGURE 9.4 *LED housing and dimensions; The top view (a) shows the cooling fan which has been attached to the LED, and (b) shows the underside view of the LED housing*

It can be seen that the underside of the housing has an outer ridge that fits around the sample dish, holding it securely in place during exposure. The LED array is also fixed in position to ensure it is always directly above the sample well at exactly the same distance, thus keeping the light intensity constant for all exposures. Further details of these features are discussed later in the section.

The experimental arrangement for 405 nm light exposure of bacteria using the LED array light source is shown in Figure 9.5. This arrangement is similar to that used for the Xenon-lamp experiments (Chapters 6 and 7).

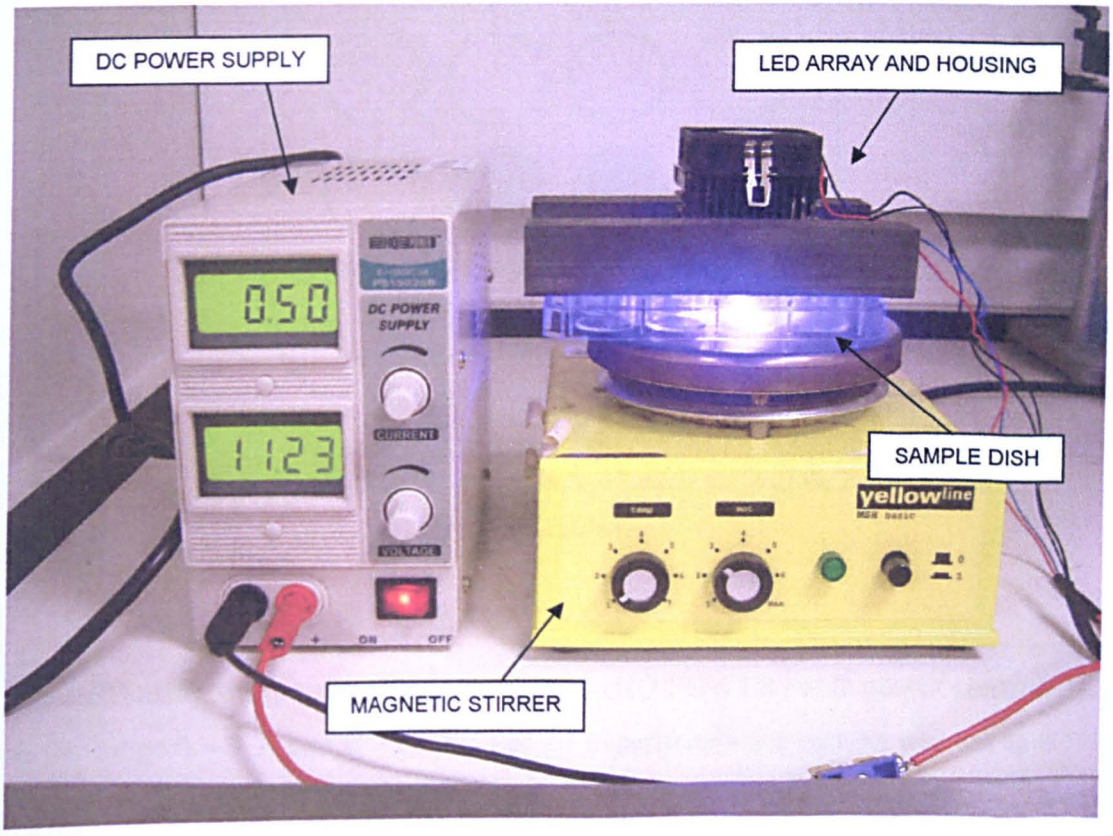

FIGURE 9.5 *Experimental arrangement*

The 2 ml bacterial sample, along with a small magnetic follower, was held within one of two wells of a 12-well micro-plate (the sample dish). The LED array was then positioned on top of the plate, with the overlapping edges of the housing ensuring it was held firmly in place. The two central wells were used for light exposure and these are highlighted in Figure 9.6. The LED housing held the LED array in position directly above one of these wells. To expose the other well, the plate was turned through 180°. The sample dish and LED was then positioned on a magnetic stirrer which, in conjunction with the magnetic follower, permitted continuous stirring of the sample during light exposure.

Control samples were established: these were continuously agitated but not exposed to 405 nm light.

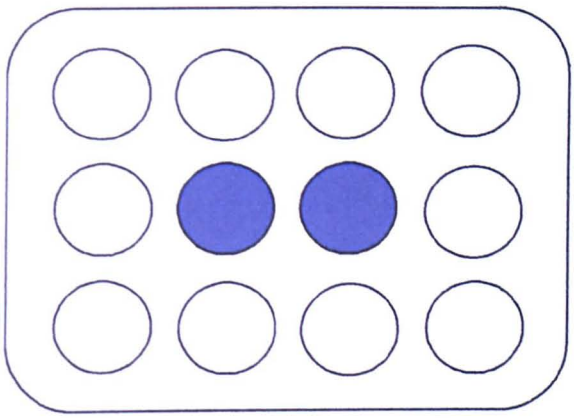

FIGURE 9.6 *Diagram of a 12-well sample dish highlighting (in blue) the 2 central wells used for light exposure*

The LED array was powered by a DC supply (HQ POWER) with output controllable in the range 0 – 3 A and 0 – 15 V. For all experiments the current was set to 0.5 ± 0.05 A at a voltage of 11.2 ± 0.1 V.

The distance between the sample and the LED array was approximately 2 cm, and at this distance, a power density of around 10 mW/cm^2 was recorded using the radiant power meter.

9.3 TREATMENT OF BACTERIAL SUSPENSIONS USING 405 nm LIGHT

This section investigates the effectiveness of the 405 nm LED array for the inactivation of a number of medically important Gram-positive bacteria, including *Staphylococcus aureus*, MRSA and *Streptococcus pyogenes*.

9.3.1 Bacterial Preparation and Treatment Method

Bacteria were cultured as described in Section 3.1.2. After an 18-hour incubation period, the broths were centrifuged at 3400 rpm for 10 minutes and the resultant pellets re-suspended in PBS. These suspensions were then diluted to give a starting population of approximately 2.0×10^5 CFU/ml.

As for the Xenon-lamp experiments, a 2 ml sample volume was dispensed into the well of a 12-well micro-plate. The plate was positioned under the housing ensuring the well to be exposed was situated below the LED array (as described in Section 9.2). Samples were exposed to varying durations of light treatment and then plated onto agar for incubation at 37°C for 24 hours. Control samples were also established: these were not light-exposed.

For the culture and plating of the bacteria, Nutrient broth/agar and Brain Heart Infusion broth/agar were used for the Staphylococcal and Streptococcal species, respectively.

9.3.2 Results

Figures 9.7 and 9.8 show the results of *Staphylococcus aureus* NCTC 4135 and MRSA 16a inactivation following treatment with the 405 nm LED array. For *S. aureus* it can be seen that a 5-$\log_{10}$ reduction in bacterial population was achieved after a 60-minute exposure, with total inactivation realised after a 75-minute exposure. Treatment of MRSA 16a with the 405 nm LED array resulted in a 5-$\log_{10}$ reduction in bacterial population after an exposure of 75 minutes.

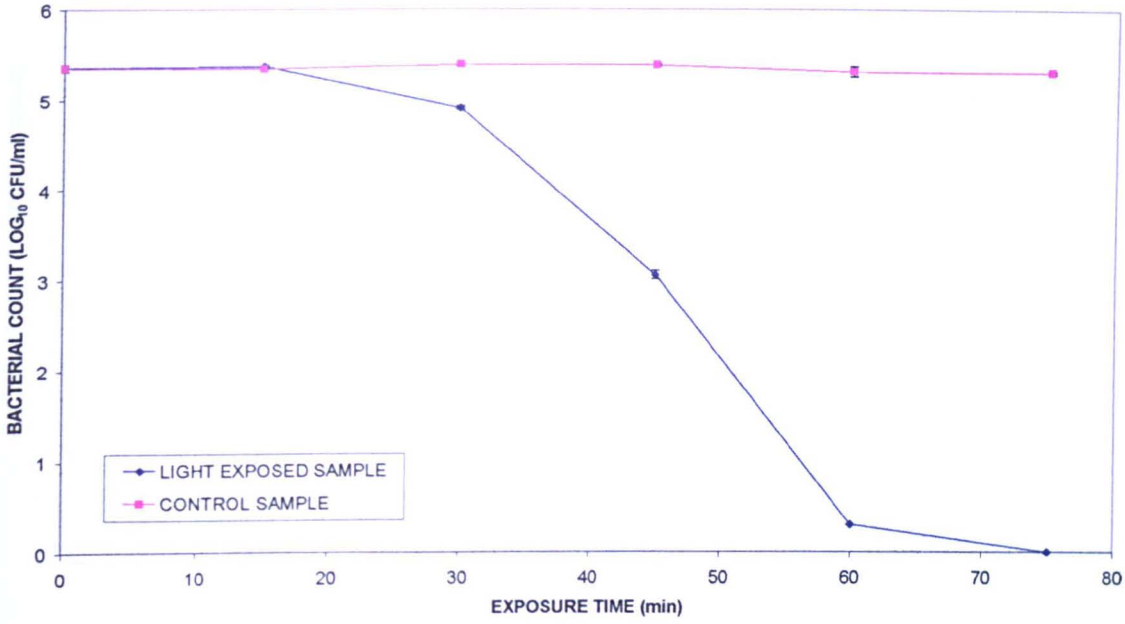

FIGURE 9.7 *Staphylococcus aureus 4135 inactivation with 405 nm LED array*

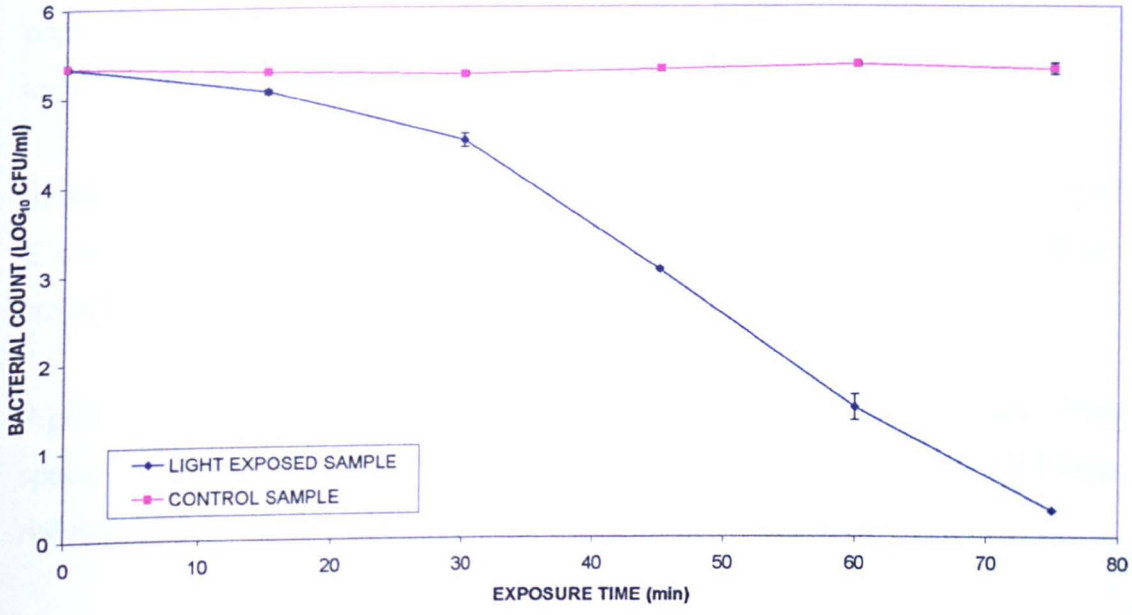

FIGURE 9.8 *MRSA 16a inactivation with 405 nm LED array*

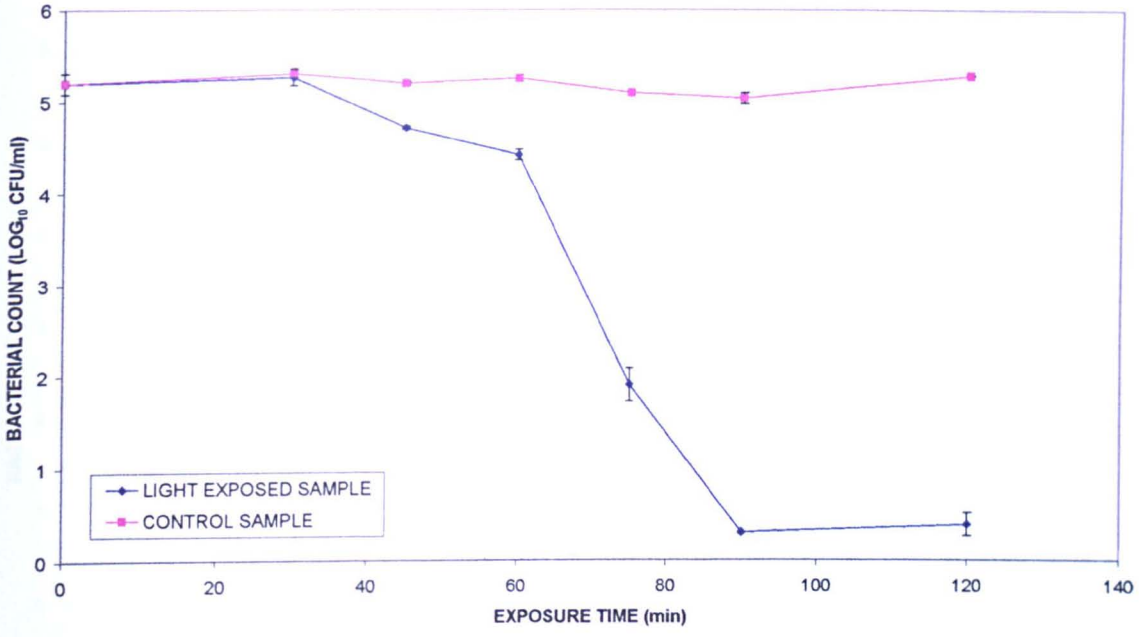

FIGURE 9.9 *Streptococcus pyogenes inactivation with 405 nm LED array*

Figure 9.9 is an inactivation curve for *S. pyogenes*, with the population showing a 5-$\log_{10}$ reduction after a 90-minute exposure. After 120 minutes of exposure, a population of less than 10 CFU/ml survived, out of a starting population of approximately 2 x 10^5 CFU/ml.

In addition to exposing the *Staphylococcus* and *Streptococcus* species, exposure of *E. coli* was also performed to investigate whether illumination using the LED array could induce its inactivation.

Figure 9.10 shows the results of *E. coli* exposure to the 405 nm LED array. This species again proved less susceptible to photodynamic inactivation, with a 2.5 $\log_{10}$ reduction achieved after 240 minutes of exposure.

Sample temperature was also monitored during exposure of these microorganisms, and in each case, had only risen to approximately 30°C after exposure – easily survived by each of the microbial species.

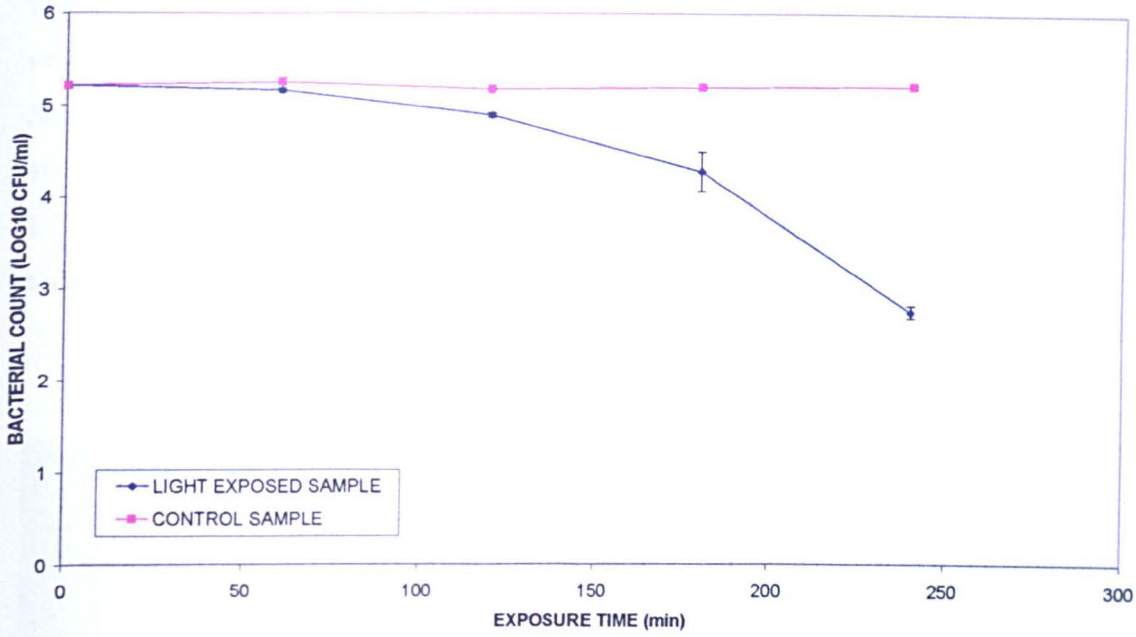

FIGURE 9.10 *Escherichia coli inactivation with 405 nm LED array*

9.4 INACTIVATION OF *CLOSTRIDIUM* SPECIES USING 405 nm LIGHT

Clostridium is another Gram-positive medically important organism. *Clostridium* species, in particular *Clostridium difficile*, have been associated with high mortality in elderly patients due to antibiotic-associated pseudomembraneous colitis. *Clostridium perfringens*, another medically relevant species, can cause a spectrum of diseases from a self-limited gastroenteritis to an overwhelming destruction of tissue in infections such as necrotizing fascitis which is associated with very high mortality (approaching 50%), even in patients who receive early medical intervention. Exogenous and endogenous *Clostridium difficile* infection can occur: exogenous infections transmit from person to person in hospital; and endogenous infections initiate with gastro-intestinal overgrowth of toxin-producing strains after treatment with antibiotics [Murray *et al*, 1998].

Since *Clostridium* is a significant hospital-acquired infection problem, it is desirable to investigate the susceptibility of this problematic organism to treatment with

405 nm light. *Clostridium* is rod-shaped and requires anaerobic growth conditions – rather than being coccal and aerobic, as were the previously successfully treated organisms (*Staphylococcus*, *Streptococcus* and *Enterococcus*). Gram stains of *Staphylococcus spp.* and *Clostridium spp.*, highlighting their different morphologies, are pictured in Figure 9.11. Because of the difficulty in culturing *Cl. difficile*, *Cl. perfringens* – another problematic *Clostridium* species frequently isolated in clinical specimens – was used in these experiments.

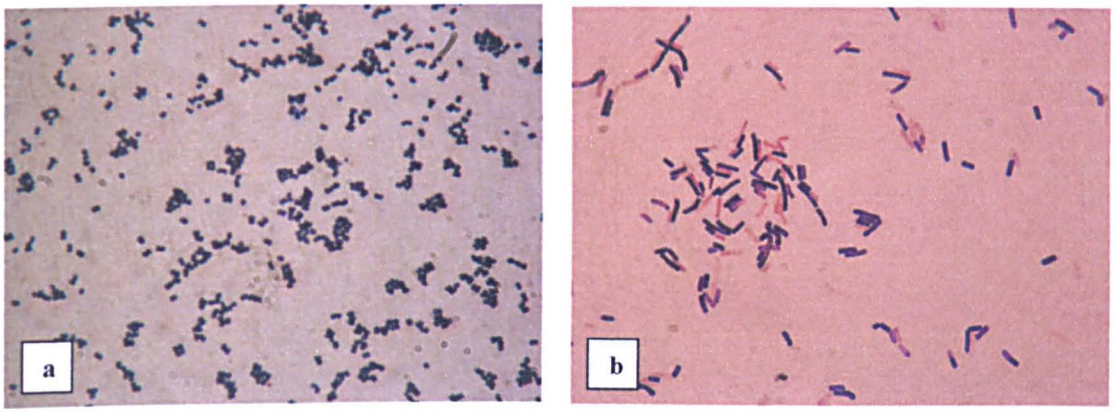

FIGURE 9.11 *Gram stains of (a) Staphylococcus aureus, a Gram-positive cocci, and (b) Clostridium perfringens, a Gram-positive rod.*

9.4.1 Bacterial Preparation and Treatment Method

Clostridium perfringens, being an anaerobic organism, must be cultured in a different manner to the organisms considered in Section 9.3. The procedure used for *Cl. perfringens* involved inoculation of a broth; namely the anaerobically-incubated Thioglycollate broth (TGB). Due to the need for minimal preparatory procedures to avoid exposing the organism to added oxidative stress, it was proposed that the sample be diluted direct from the TGB without centrifugation. On analysis of sterile TGB, however, it was found that its high absorbance (and low transmission) in the 405 nm wavelength region – as shown in Figure 9.12 – makes this method unsuitable.

An alternative method was used, as follows: *Cl. perfringens* 13124 was plated onto Thioglycollate agar (TGA) and placed within an anaerobic jar containing an AnaeroGen sachet. This generated anaerobic conditions within the sealed jar. The anaerobic jar was then placed into a 37°C incubator for 18 hours.

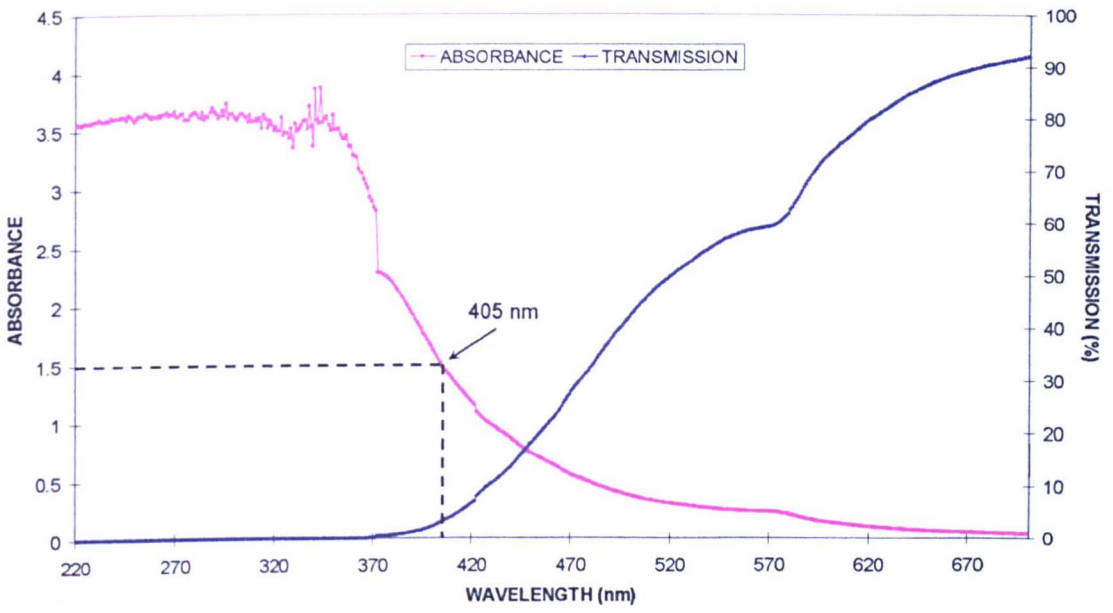

FIGURE 9.12 *Dependence on wavelength of absorbance and transmission of Thioglycollate broth*

After this incubation period, a loopful of bacteria was taken from the inoculated TGA plate and suspended in 9 ml PBS. This was then diluted to give a starting population of $10^4 - 10^5$ CFU/ml. Two millilitre volumes of the resultant suspension were then light-exposed using the system and method described in Sections 9.2 and 9.3.1, respectively.

8.4.2 Results

Figure 9.13 shows the results of *Cl. perfringens* inactivation by treatment with the 405 nm LED array. A single $\log_{10}$ reduction takes place after around 60 minutes and total inactivation (4.4 $\log_{10}$ reduction) after a 75-minute exposure.

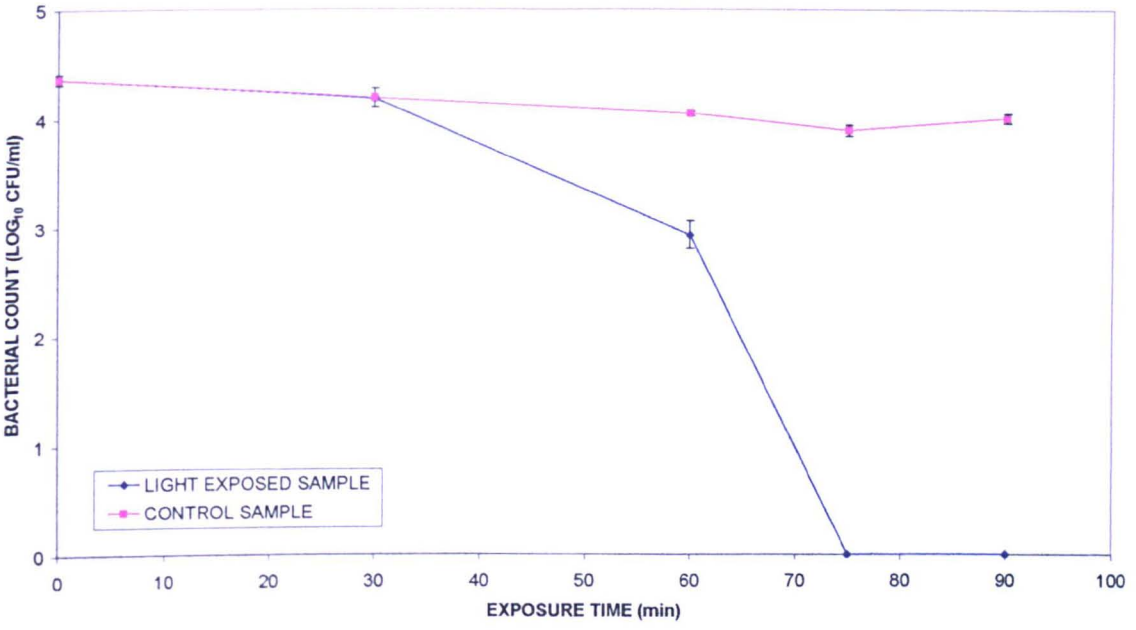

FIGURE 9.13 *Clostridium perfringens 13124 inactivation with the 405 nm LED array*

9.5 DISCUSSION OF INACTIVATION ENERGIES AT 405 nm

Table 9.1 allows comparison of inactivation parameters, including germicidal efficiencies, for *Staphylococcus aureus* NCTC 4135, MRSA 16a, *Streptococcus pyogenes* and *Clostridium perfringens* following exposure to the 405 nm LED array. The inactivation energies for *E. coli* have also been included for reference.

The responses of all four organisms to 405 nm inactivation are quite similar, with *Staphylococcus aureus* NCTC 4135 displaying the highest rate of inactivation, and hence the highest germicidal efficiency, with a value of 0.14 $\log_{10}$ CFU/ml per J/cm^2.

As with the Xenon-lamp exposures, *E. coli* could be inactivated but proved not as susceptible to visible-light inactivation as the Gram-positive organisms.

TABLE 9.1 *Energy values required for the inactivation of a range of bacterial species using narrow spectrum 405 nm light*

ORGANISM	EXPOSURE TIME (sec)	POWER DENSITY (mW/cm^2)	LOG_{10} REDUCTION	DOSE (J/cm^2)	$J/cm^2/Log_{10}$ Reduction	GERMICIDAL EFFICIENCY ($Log_{10}(N/N_0)$ per J/cm^2) *
Staphylococcus aureus NCTC 4135	3600	10	5	36	7.2	0.14 (± 0%)
MRSA 16a	4500	10	5	45	9	0.11 (± 0%)
Streptococcus pyogenes NCTC 8198	5400	10	5	54	10.8	0.09 (± 2%)
Clostridium perfringens 13124	4500	10	4.4	45	10.2	0.10 (± 1%)
Escherichia coli NCTC 9001	14400	10	2.5	144	57.6	0.02 (± 6%)

* Germicidal efficiency values including % uncertainty for the experimental results in this study (calculated using germicidal efficiency values to 3-decimal places). 0% uncertainty indicates no standard deviation from the mean for $\log_{10}N$ and $\log_{10}N_0$

TABLE 9.2 *Comparison of the energies required for bacterial inactivation using >400 nm and 405 nm filtered light from the Xenon lamp and a 405 nm LED array*

ORGANISM	DOSE (J/cm^2)			$J/cm^2/Log_{10}$ Reduction			GERMICIDAL EFFICIENCY ($Log_{10}(N/N_0)$ per J/cm^2)		
	>400 nm	405 nm (filter)	405 nm (LED)	>400 nm	405 nm (filter)	405 nm (LED)	>400 nm	405 nm (filter)	405 nm (LED)
Staphylococcus aureus NCTC 4135	630	23.5	36	126	9.8	7.2	-	0.102	0.14
MRSA 16a	945	-	45	189	-	9	-	-	0.11
Streptococcus pyogenes NCTC 8198	1440	-	54	288	-	10.8	-	-	0.09

As observed with the inactivation curves using Xenon lamp exposures (Section 6.3), a period of inactivity occurs until a specific dose is reached. Similarly, staphylococcal species require the least amount of energy (9 J/cm^2) before inactivation is observed, with *S. pyogenes* and *Cl. difficile* requiring double that with 18 J/cm^2 and, as expected, *E. coli* having the greatest energy requirement of 36 J/cm^2 – a dose equivalent to that required for total inactivation of a 10^5 CFU/ml population of *S. aureus* NCTC 4135.

It is useful to compare the 405 nm inactivation data obtained using the LED array with the data obtained using the Xenon lamp and both the 400 nm longwave pass filter (that provided light over a wide spectral range above 400 nm) and the 405 nm narrow bandpass filter (that provided 405 nm ± 5 nm light). Values are shown in Table 9.2.

It can be seen that the use of the 405 nm LED array as the light source has significantly reduced the doses required for inactivation of the three test organisms, *S. aureus*, MRSA and *S. pyogenes*, with respective doses of 17, 21 and 27 times lower than those required for inactivation using the Xenon lamp. Inactivation of *E. coli* was also achieved at a lower dose using the LED array, with a similar 2.0-2.5 $\log_{10}$ reduction being achieved with a dose 20 times less than with the Xenon lamp.

The reason for this large decrease in the required dose is the fact that the LED array emits only a narrow-band of wavelengths, the spectrum of which is very similar to the identified causative wavelengths of 400 – 420 nm which induce photo-excitation of the endogenous bacterial porphyrins. The use of this light source allows only high-intensity light of appropriate wavelength to illuminate the bacteria. With the Xenon lamp used in conjunction with a 400 nm longwave pass filter for UV cut-off, only a small fraction of the light energy contributes to inactivation –most is dissipated as heat within the sample.

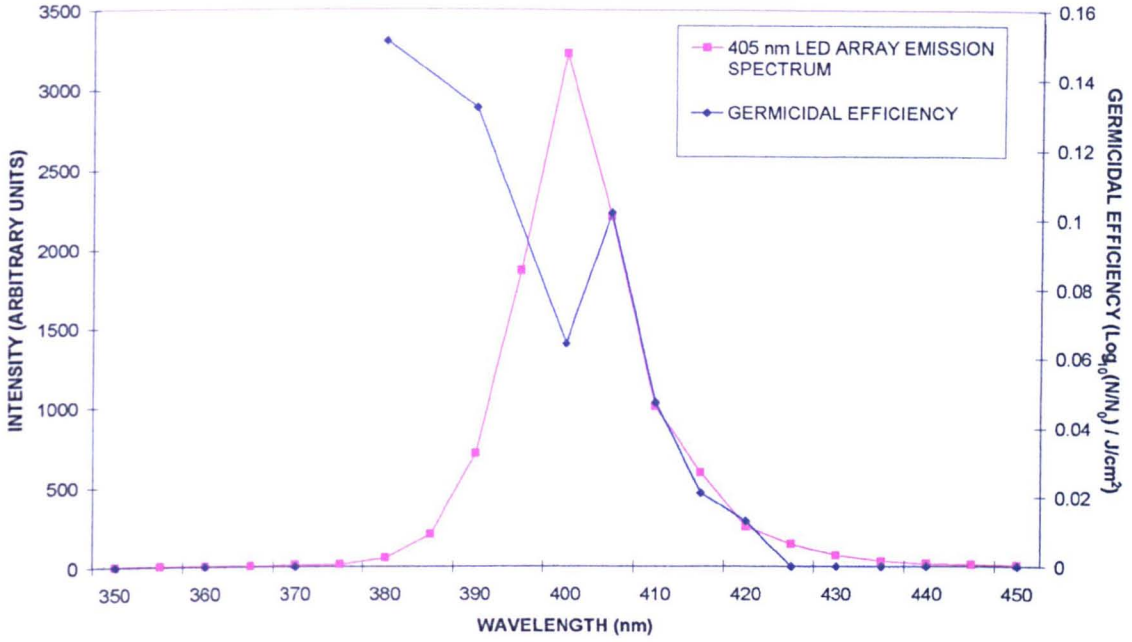

FIGURE 9.14 *Comparison of the emission spectrum of the 405 nm LED array and the germicidal efficiency of Staphylococcus aureus NCTC 4135*

Comparison of the inactivation data for *S. aureus* NCTC 4135 using the 405 nm LED array, with that using the Xenon lamp and 405 nm bandpass filter combination, shows that the LED array is about 40% more germicidally efficient. This can be accounted for by the difference in transmission profiles of the LED array (Figure 9.2) and the 405 nm bandpass filter (Appendix B, Figure B.14), the former having a closer match to the germicidal efficiency curve for *S. aureus* NCTC 4135.

A comparison of the LED array output emission and wavelength sensitivity of *S. aureus* is shown in Figure 9.14 and this demonstrates how essentially most of the light output from the LED array plays a role in inactivation.

Figure 9.15 displays three curves, each of which is a curve of best fit as a function of wavelength, for (i) the normalised LED output – green, (ii) the germicidal efficiency for *S. aureus* NCTC 4135 – blue, and (iii) the normalised product of (i) and (ii) – red. Curve (iii) illustrates the relative efficiency of the LED array as a function of wavelength for the inactivation of *S. aureus* NCTC 4135.

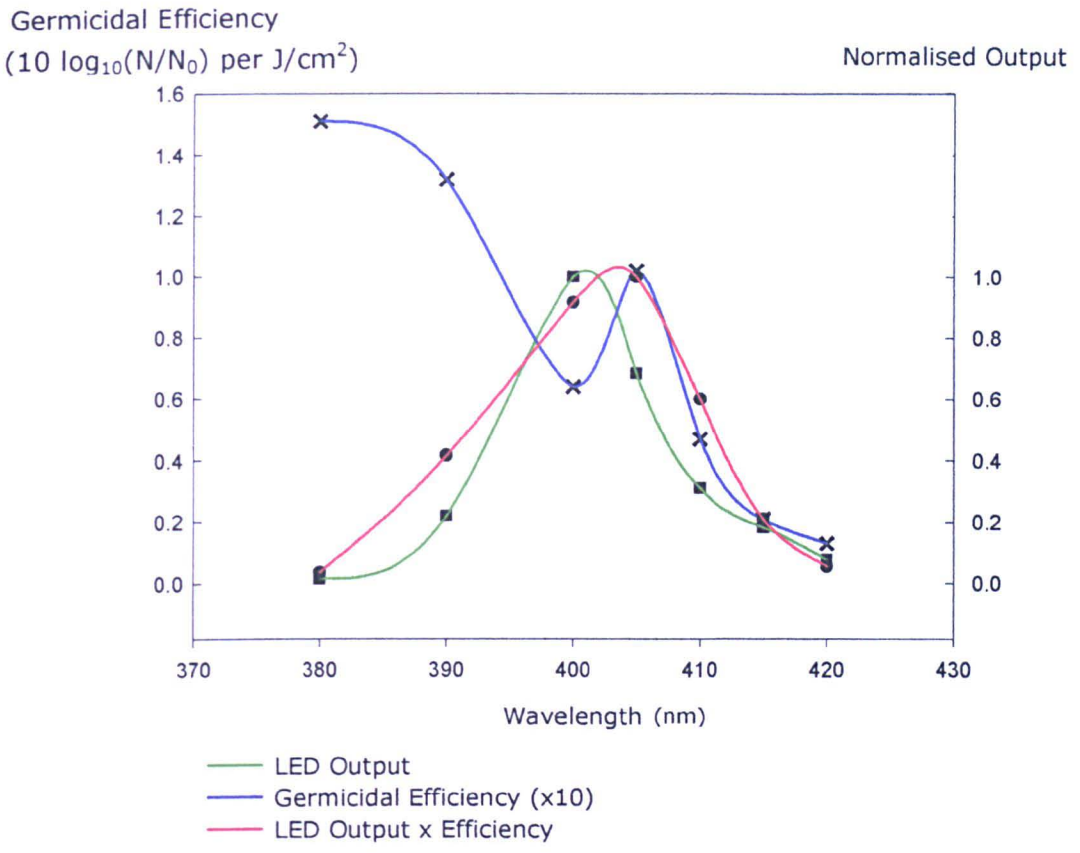

FIGURE 9.15 *Curves of LED output, germicidal efficiency, and the product of LED output and germicidal efficiency.*

Using the values of germicidal efficiency, comparisons can be made between the blue-light inactivation of the organisms in this study and those of other published studies.

As discussed earlier, detailed comparisons are not, however, possible even when source wavelengths are specified, because of variations with wavelength of both the source intensity and germicidal efficiency.

TABLE 9.3 *Germicidal efficiencies of 405 nm light treatment used in this study compared to values from published studies (Data in bold type are results from this study; * UV inactivated bacteria included for comparison purposes)*

BACTERIUM	PORPHYRINS	RADIATION (nm)	DOSE (J/cm^2)	$Log_{10}(N/N_0)$ reduction	EFFICIENCY ($Log_{10}(N/N_0)$ per J/cm^2)	REFERENCE
Propionibacterium acnes	Natural	407-420	75	2	0.03	Ashkenazi *et al*, 2003
Propionobacterium acnes	δ-ALA-induced	407-420	75	7	0.09	Ashkenazi *et al*, 2003
Helicobacter pylori	Natural	405	40	5	0.13	Ganz *et al*, 2005
Staphylococcus	δ-ALA-induced	407-420	50	5	0.1	Nitzan *et al*, 2004
Staphylococcus aureus	**Natural**	**405 (LED ARRAY)**	**36**	**5**	**0.14**	-
MRSA 16a	**Natural**	**405 (LED ARRAY)**	**45**	**5**	**0.11**	-
Streptococcus pyogenes	**Natural**	**405 (LED ARRAY)**	**54**	**5**	**0.09**	-
Clostridium perfringens	**Natural**	**405 (LED ARRAY)**	**45**	**4.5**	**0.10**	-
* *Escherichia coli*	NA	270	4×10^{-3}	1.6	430	Wang *et al*, 2005

Data in Table 9.3 demonstrate that the germicidal efficiency values obtained in this study for the inactivation of a range of medically important organisms are similar to those obtained in other published work for the PDI of *P. acnes*, *H. pylori* and *Staphylococcus*. Data for both natural and δ-ALA-induced endogenous porphyrins in *P. acnes* are shown and these highlight the improved germicidal action observed when porphyrin levels are amplified by δ-ALA pre-treatment. Also shown is the germicidal efficiency for *H. pylori* inactivated through blue-light photostimulation of its natural porphyrin levels. The 405 nm LED system is as efficient for the inactivation of MRSA and all the other medically important bacteria used in this study as are the established visible-light systems for *P. acnes* and *H. pylori*, which are now being used clinically.

It can be seen that the germicidal efficiency of *S. aureus* inactivation using the 405 nm LED array in this study was higher than that achieved in a study by Nitzan and Kauffman [1999], which exposed δ-ALA pre-treated *Staphylococcus*. This result demonstrates, for the first time, that *S. aureus* can be optically inactivated through exposure to blue light without the need for pre-treatment – and with efficiencies as high as that achieved with pre-treatment. By dispensing with the δ-ALA, the pre-treatment time of 4 hours is eliminated.

The germicidal efficiencies of *S. aureus* inactivation using exogenous photosensitisers are also compared to that found in this study and a selection of these is shown in Table 9.4. Studies which used dyes as the photosensitising molecules [Lambrechts *et al*, [II]; Zeina *et al*, 2001] have found only slightly higher germicidal efficiencies than those documented in the present work.

The most efficient photosensitisation was observed by in a study by Bertoloni *et al* [2000], with the use of the porphyrin derivative, Hematoporphyrin. This use of this photosensitizer and white, visible-light illumination, provided 10 times the germicidal efficiency of the 405 nm LED treatment used in the present work. The disadvantage that a pre-treatment period is required mitigates against the technique, particularly for large-surface-area inactivation.

TABLE 9.4 *Germicidal efficiencies for S. aureus using a selection of exogenous photosensitisers and δ-ALA-induced pre-treatment (Data in bold type is from the present work, using the 405 nm LED array for photo-excitation of S. aureus endogenous porphyrin)*

PHOTOSENSITIZER	IRRADIATING LIGHT	DOSE (J/cm^2)	LOG REDUCTION	GERMICIDAL EFFICIENCY ($Log_{10}(N/N_0)/J/cm^2$)	REF.
TriP[4] (5 μM)	White	27	5	0.18	Lambrechts *et al*, [II]
Methylene Blue (100 μg/ml)	White	25.2	5	0.19	Zeina *et al*, 2001
Hematoporphyrin (10μg/ml)	White	3.6	4.5	1.25	Bertoloni *et al*, 2000
δ-ALA induced porphyrins	White Blue	75 50	2.5 4	0.03 0.08	Nitzan & Kauffman, 1999
Endogenous porphyrins	**Blue**	**36**	**5**	**0.139**	-

9.6 CONCLUSIONS

In summary, this study has demonstrated several significant advantages of the HINS-light inactivation mechanism:

Inactivation of microorganisms is non-thermal.

When using the 405 nm LED array, sample temperatures only rise to approximately 30°C – easily survived by *S. aureus* – and the inactivation efficiency using the LED array is much improved compared to that for the Xenon lamp: these findings establish this inactivation mechanism as being non-thermal.

Treatment does not require the addition of photosensitisers.

Comparison of the germicidal efficiency for 405 nm exposure of *S. aureus* in this study with published studies using exogenous photosensitizers found that the use of dyes and δ-ALA pre-treatment resulted in little difference to the efficiency obtained in this study. Conversely, the use of porphyrin-derivatives resulted in a significant increase in germicidal efficiency. The advantage of the blue HINS-light treatment developed in this study is that it can be performed without the requirement for exogenous photosensitizers or a δ-ALA pre-treatment period – and this is likely to be advantageous for many potential clinical applications such as environmental disinfection procedures.

Treatment is less efficient than with UV-light, but has significant safety advantages – particularly for large scale applications.

When the differences in germicidal efficiency between blue-light and UV-light are compared, it is obvious that UV-light exposure is by far the more efficient treatment method. But in spite of the much lower efficiency of blue-light inactivation, this process has clear advantages in terms of its ease of use and substantially greater safety – as already proved with respect to the use of light of similar wavelengths for the inactivation of *P. acnes* (407-420 nm) and *H. pylori* (405 nm) in clinical applications.

The comparison of germicidal efficiencies from different studies also highlighted that the 405 nm LED system is as efficient for the inactivation of MRSA, *Clostridium* and the other medically important Gram-positive bacteria tested here as are the established visible-light systems for *Propionibacterium acnes* and *Helicobacter pylori* – both now being used clinically. This suggests that the intense LED-array system at 405 nm has potential for clinical use in the suppression of MRSA and other bacteria including *Clostridium* and coagulase-negative *Staphylococcus* for both large-scale and small-scale applications.

Chapter 10

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK

10.0 GENERAL

This study investigated the light-inactivation of a range of medically important microorganisms and this chapter reviews the results of the development and application of firstly, a PUV-light air disinfection system, and secondly, a visible-light-based system, now termed HINS-light. Also discussed in this chapter are ideas for future work that would enable the further development and potential clinical application of these systems.

10.1 CONCLUSIONS

10.1.1 Microbial Air Sampling and the Development and Application of PUV-light Air Disinfection System

The air sampling performed in university lecture theatres demonstrated that airborne bacterial contamination levels rise significantly over the period of a lecture and that this increase in contamination is a result of the presence of personnel. With a recommended threshold level of 500 CFU/m^3 in lecture theatres [Cheong and Lau, 2003], it was evident that the majority of the levels reported in this study were significantly higher – in one case 5 times higher – than this recommended level. These high contamination levels made university lecture theatres ideal situations for the testing of a novel PUV-light air disinfection system.

The application of this PUV-light system to disinfect contaminated air samples was shown to cause a significant reduction in the levels of airborne bacterial population,

with a decrease of 80% observed in one case. Ideal employment of this pulsed system would be either within HVAC systems or as a stand-alone system.

From the successful results achieved in this study, it is obvious that, with further research and development, the use of a PUV-light air-disinfection would markedly reduce levels of airborne microbial populations, thereby reducing levels of infectious agents and potential allergens. Consequently, PUV-light may have significant potential applications in the control of indoor air quality for a variety of situations ranging from the workplace and domestic settings to more clinical environments such as healthcare establishments.

10.1.2 Discovery of Visible-Light Inactivation Potential

The major discovery of this study was the fact that prolonged exposure to visible light has bactericidal properties against *Staphylococcus aureus*. This finding was uncovered during an investigation into the photoreactivating capabilities of airborne bacterial isolates treated with PUV light. Exposures in the light incubation cabinet of up to three hours – using a bank of fluorescent lights – did induce photoreactivation within the illuminated organisms, but extended exposures resulted in inactivation in both the pulsed and non-pulsed control organisms.

Following these unexpected results, the photoreactivation study was set aside in favour of investigating further this discovery of the potential bactericidal effects of particular light wavelengths. The wavelength range responsible for this bactericidal effect was identified using a selection of optical filters and was found to be between 400 and 500 nm. This discovery has important implications due to these wavelengths being within the visible region rather than the UV region, the latter being typically associated with bacterial inactivation.

10.1.3 Visible-Light (>400 nm) Inactivation of MRSA and other Medically Important Bacteria

In order to increase the inactivation rates observed using exposures in the light incubation cabinet, a Xenon lamp, which was significantly more intense, was

employed, in combination with a 400 nm longwave pass filter (for UV cut-off) to examine visible-light inactivation of a number of medically important Gram-positive bacteria.

Exposure to >400 nm light from the Xenon lamp proved successful for the inactivation of *Staphylococcus aureus*, MRSA, coagulase-negative *Staphylococcus* (CONS), *Streptococcus pyogenes* and *Enterococcus faecalis*. The use of the Xenon lamp also significantly increased the rates of bacterial inactivation, with total inactivation of an *S. aureus* suspension of population 2 x 10^5 CFU/ml being achieved within a 30-minute exposure period.

10.1.4 Wavelength Sensitivity of *Staphylococcus aureus*

The wavelength sensitivity of *S. aureus* was investigated in the spectral range from 320 nm to 500 nm using narrow bandpass filters with a 10 nm bandwidth. The important result was that although, as expected, the efficiency of inactivation falls off at the long-wavelength edge of the UV region, it remains finite on moving into the visible-wavelength region above 400 nm, and a peak then occurs around 405 nm. This suggested that some additional inactivation process was being initiated at this wavelength. Analysis of the effect of the visible wavelengths found that an inactivation effect was observed between 400 nm and 420 nm, with optimal inactivation at 405 nm.

Other workers have found that wavelengths in this same region do cause inactivation of some bacterial species – most notably *Propionibacterium acnes* and *Helicobacter pylori* which are associated with acne and peptic ulcers, respectively. The inactivation process involves the photo-excitation of endogenous porphyrins, which ultimately results in singlet-oxygen production and cell death. Similarities between results in this study and other published studies strongly suggested that it was this same mechanism of inactivation that occurs within the visible-light exposed *S. aureus* cells.

10.1.5 Mechanism of Blue-Light Inactivation

In order to support the hypothesis that staphylococcal inactivation was a result of the photo-excitation of its endogenous porphyrins, the role of oxygen – which is essential for photodynamic inactivation – in the visible-light inactivation of *S. aureus* was investigated through oxygen enhancement and depletion experiments.

Results indicated that the presence of oxygen is important for successful staphylococcal inactivation. Significantly reduced inactivation was observed when *S. aureus* cells were light-exposed in an oxygen-depleted environment. Conversely, light-exposure of *S. aureus* in an oxygen-enhanced environment resulted in increased inactivation.

This dependence on oxygen for successful *S. aureus* inactivation, in addition to the wavelength sensitivity being in the region of 400-420 nm, provided further evidence for the mechanism of staphylococcal inactivation observed throughout this study being the result of photo-excitation of naturally-occurring intracellular porphyrins, most likely coproporphyrin, and their function as endogenous photosensitisers.

This study therefore provides the first evidence that *S. aureus*, and other medically important Gram-positive bacteria including coagulase-negative *Staphylococcus*, *Enterococcus* and *Clostridium*, can be inactivated optically using blue light of wavelengths 400-420 nm. Significantly, the mechanism for *S. aureus* does not require the use of exogenous photosensitisers or ALA-induced porphyrins – as used in previous studies concerning staphylococcal photodynamic inactivation.

10.1.6 Use of an Improved Light Source – HINS-Light

The identification of 405 nm as the wavelength inducing maximum inactivation led to the development of a High Intensity Narrow Band light source, now termed HINS-light. A source made up of a 405 nm LED array was assembled, and used to expose the bacteria to the most germicidally-efficient wavelength. Blue-light treatment successfully inactivated all the tested medically important Gram-positive species including MRSA and *Clostridium* species, with comparisons of the germicidal

efficiencies of the Xenon lamp and 405 nm LED array confirming the latter as a significantly more efficient light source for visible-light inactivation.

10.1.7 Potential Applications of HINS-light

This method of bacterial inactivation using visible/blue light exposure – and the demonstrated susceptibilities of a range of major hospital-acquired pathogens –has revealed a potential disinfection method that may have wide application in the health-care environment, most significantly in the losing battle against MRSA. Health-care statistics suggest that the existing control and prevention methods are failing to deal with the problem [National Audit Office, 2004; Tiemersma *et al*, 2004], and with MRSA developing resistance to even the most-recently developed antibiotic 'Linezolid' [Cooper *et al*, 2003], there is an urgent need for new and effective disinfection methods.

Exposure to blue-light (HINS-light) and white-light containing blue wavelengths, has proved successful for the inactivation of a range of significant Gram-positive bacteria including MRSA, methicillin-sensitive *S. aureus*, coagulase negative *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Clostridium* species. The advantage of the visible-light treatment developed in this study over other published photodynamic inactivation methods is that it can be performed without the requirement for exogenous photosensitisers or a δ-ALA pre-treatment period – a property which is likely to make it advantageous for many potential clinical applications such as environmental disinfection procedures.

Clinical systems using visible-light in the blue-violet spectral region have been established for *Propionibacterium acnes* and *Helicobacter pylori*, and the values of the germicidal efficiencies of inactivation are similar to those measured in the present work with the 405 nm LED-array system for MRSA, *Clostridium* and other medically important Gram-positive bacteria. This suggests that the intense LED-array system at 405 nm has potential for clinical use in the suppression of MRSA and other bacteria including *Clostridium* and coagulase-negative *Staphylococcus*.

Although the germicidal efficiency at this visible wavelength is much lower than for the UV wavelengths, this disadvantage is outweighed by the fact that human exposure to UV light has serious detrimental health effects. Dangers of UV exposure include eye damage and skin cancer [WHO, 2006], but the less energetic wavelengths used here are longer than 400 nm and within the visible region of the electromagnetic spectrum, therefore markedly reducing the risk of adverse health effects. Indeed, the safety of such systems have already been established through their clinical use for the inactivation of *P. acnes* (407-420 nm) and *H. pylori* (405 nm).

This attribute of the blue-light inactivation method allows longer periods of continuous application and hence the use of higher doses. Consequently, a possible application of HINS-light will be as a continuous source supplementary to the normal room lighting in a clinical environment. In this type of application, due to the natural pervasive nature of light, all exposed parts of the room would be bathed in HINS-light. This area of application has the potential to lead to the development of a room-decontamination system designed to disinfect the air and all surfaces and materials in the room, without causing discomfort or hurt to the occupants. The potential uses of such a system would be wide-ranging in hospital, health-care and exercise facilities.

HINS light can be viewed as a method for both the control and eradication of MRSA and other organisms. The developed system has the potential to maintain a room free from MRSA and other bacterial contamination (for example, in an operating theatre or any other room where contamination should be eliminated), as well as to disinfect a contaminated area, such as in an isolation ward where MRSA is being shed by an infected individual. The fact that visible-light inactivation of *S. aureus* occurs at a much greater rate when exposure is performed in an oxygen-rich environment may be of added benefit. For example, in some clinical applications of visible-light inactivation; such as in operating theatres, it may be possible to use oxygen enhancement to optimise disinfection, thus reducing the risk of infection transmission during surgical procedures.

Although applications within the healthcare industry are the most obvious, Community-Acquired MRSA (CA-MRSA) is a growing problem, particularly in areas such as nursing homes, prisons, army barracks and public gyms [Weber, 2005]. As a consequence of this, potential applications of HINS-light, again most likely as an adjunct to installed room-lighting fixtures, may be found in the wider public sector.

Because of the safety benefits of HINS-light, potential applications are not limited to the large-scale treatments described above. Localised applications – as have been used for the inactivation of *Propionibacterium acnes* in acne therapy – would also have beneficial uses within the clinical environment. For example, irradiation of wounds during and post-operation, and irradiation of surgical equipment and implants such as prosthetics, shunts and catheters, are all target areas in which HINS-light can potentially be applied for the control and prevention of hospital-acquired infection.

10.2 FURTHER WORK

10.2.1 Pulsed Ultraviolet-Light Air Disinfection

Although the general principle that pulsed UV-rich light can successfully be applied as an air disinfection method, further development of the system was not undertaken during this study, because of the change in direction of the research, following the discovery of the potential of visible-light as a bactericidal treatment. Further research and improvements to the pulsed-light treatment are needed in order to maximise its efficiency in reducing bacterial numbers. Parameters such as the air influx rate, pulse rate and pulse energy need to be investigated in order to optimise the system for particular applications.

This study focused only on the effectiveness of this system for inactivating bacterial airborne microorganisms. An important addition to this work would be an investigation into the effects of PUV-light on airborne fungal and viral populations.

Data of this kind would enable a realistic assessment of the potential of PUV-light as an air disinfection system for clinical use in healthcare and public situations.

10.2.2 HINS-Light Inactivation

This technology has exciting potential, and further work is required for attainment of optimal germicidal efficiency. This work should focus on both fundamental and applied studies.

At the fundamental level, the present study has proposed that the visible-light inactivation of *S. aureus* is the result of blue-light photo-excitation of the bacterial endogenous porphyrins, with the most efficient wavelengths being 400-420 nm, and optimal inactivation being achieved at 405 nm. Excitation with these wavelengths induces the generation of highly reactive $^1O_2$ and other radicals, ultimately resulting in damage and cell death.

Examination of the porphyrins involved in the inactivation of *S. aureus* can be made using High-Performance Liquid Chromatography (HPLC). The process involves analysis of light-exposed and non-light-exposed samples of *S. aureus*, with HPLC separating and identifying the levels of the different porphyrins within the bacteria. HPLC has been applied successfully in fundamental studies of *P. acnes* and δ-ALA pre-treated *S.aureus* and has found the predominant porphyrin in both to be coproporphyrin [Ashkenazi *et al*, 2003; Nitzan *et al*, 2004].

Investigations into any structural damage in the *S. aureus* cells following exposure to blue-light can be carried out using transmission electron microscopy. A study by Ashkenazi *et al* [2003] used this method to detect leakage in *P. acnes* cells which had been illuminated with 407-420 nm light. This leakage was deemed to be a direct result of membrane damage.

Preliminary results in Chapter 8 verified the significance of oxygen in the inactivation process and this again supports the role played by oxygen-dependent photodynamic inactivation in the mechanism of inactivation observed in *S. aureus*.

Quantitative analysis of oxygen concentrations can be performed for systems where depletion and enhancement levels can be measured – for example, flow rates may be controlled and monitored, so that oxygen concentrations can be determined. Singlet oxygen ($^1O_2$) is a significant parameter in the inactivation process. Singlet oxygen concentrations are normally measured using optical methods including indirect spectroscopic detection using chemiluminescence and optical upconversion [Tromberg *et al*, 1991], and the piston-source method [Duo *et al*, 2001; Lee *et al*, 1976].

Expansion of the present study to investigate the effectiveness and efficiency of HINS-light for the inactivation of other microbial species including bacterial spores (such as *Clostridium* spores), Gram-negative bacteria (such as *Pseudomonas aeruginosa*), fungi (such as *Aspergillus* spores) and viruses (such as influenza and SARS virus) will add to the value of HINS-light irradiation, and enable further potential applications to be identified.

For the control of the Gram-positive organisms investigated in this study, a combination of large-scale and localised HINS-light irradiation would be required due to the locations and typical routes of infection used by these organisms, many of which are antibiotic-resistant. The control and prevention of MRSA, for example, would be benefited by both large-scale and localised treatments. Large-scale treatment would control environmental contamination, arising from continuous shedding by dispersers and transfer from the hands of infected patients and medical staff, which can survive on dry surfaces for long periods [Murray *et al*, 1998]. Localised treatment for wound irradiation would prevent infection through what is thought to be the major route for bacteraemia.

Control of *Staphylococcus epidermidis* and other coagulase-negative staphylococci would be best applied through localised irradiation of prosthetics and surgical equipment such as catheters, shunts and artificial heart valves, although large-scale irradiation would aid in the control of these organisms which are the predominant contaminants of hospital floors [Ayliffe *et al*, 2001].

Enterococcus, which has also demonstrated susceptibility to HINS-light, is a hardy organism, many species of which demonstrate antibiotic resistance, and targeting this organism would be through predominantly localised treatments for wound irradiation.

Identification of the areas from which these organisms become transmitted and initiate infection, along with germicidal efficiencies, will allow development of HINS-light systems for both large-scale and localised applications for use in the healthcare industry for the control and prevention of hospital-acquired pathogens.

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Appendix A

AIR SAMPLING CORRECTION TABLE

6. CALCULATION OF RESULTS

6.1. Colony Forming Units per 1000 litres of air

The number of organisms counted on the surface of the "Contact Plate" must first be corrected for the statistical possibility of multiples particles passing through the same hole. The statistical formula is taken from work by J. Macher. Correction Tables are given below for both the 55 mm standard Contact head, the 84 mm Maxi-Contact head and the Petri head. The probable count (Pr) is then used to calculate the Colony Forming Unit (CFU) per cubic metre of air sampled.

CORRECTION TABLE TO ADJUST COLONY COUNTS FROM A 219-HOLE IMPACTOR USING STANDARD 55 mm CONTACT PLATES AND 90mm PETRI PLATES

Colonies r	MPN Pr	Colonies r	MPN Pr	Colonies r	MPN Pr	Colonies r	MPN Pr	Colonies r	MPN Pr	Colonies r	MPN Pr
1	1	41	48	81	101	121	178	161	269	201	642
2	2	42	46	82	102	122	178	162	263	202	654
3	3	43	48	83	104	123	180	163	297	203	667
4	4	44	49	84	106	124	182	164	301	204	680
5	5	45	50	85	107	125	185	165	305	205	695
6	6	46	51	86	109	126	187	166	309	206	611
7	7	47	53	87	110	127	189	167	313	207	627
8	8	48	54	88	112	128	192	168	317	208	646
9	9	49	55	89	114	129	194	169	322	209	666
10	10	50	57	90	116	130	196	170	326	210	687
11	11	51	58	91	117	131	199	171	331	211	712
12	12	52	59	92	119	132	201	172	335	212	739
13	13	53	60	93	121	133	204	173	340	213	770
14	14	54	62	94	122	134	206	174	344	214	807
15	15	55	63	95	124	135	209	175	349	215	851
16	17	56	64	96	126	136	212	176	354	216	906
17	18	57	66	97	128	137	214	177	359	217	975
18	19	58	67	98	130	138	217	178	365	218	1068
19	20	59	68	99	131	139	220	179	370	219	1367
20	21	60	70	100	133	140	222	180	375		
21	22	61	71	101	135	141	225	181	381		
22	23	62	73	102	137	142	228	182	387		
23	24	63	74	103	139	143	231	183	393		
24	25	64	76	104	141	144	234	184	399		
25	26	65	77	105	142	145	237	185	405		
26	28	66	78	106	144	146	240	186	412		
27	29	67	80	107	146	147	243	187	418		
28	30	68	81	108	148	148	246	188	425		
29	31	69	83	109	150	149	249	189	432		
30	32	70	84	110	152	150	252	190	439		
31	33	71	86	111	154	151	255	191	447		
32	34	72	87	112	156	152	258	192	455		
33	36	73	89	113	158	153	261	193	463		
34	37	74	90	114	160	154	264	194	471		
35	38	75	92	115	162	155	268	195	480		
36	39	76	93	116	165	156	271	196	489		
37	40	77	95	117	167	157	275	197	498		
38	42	78	96	118	169	158	278	198	508		
39	43	79	98	119	171	159	282	199	519		
40	44	80	99	120	173	160	285	200	530		

r = colony forming units counted
Pr = probable count

[Cherwell Laboratories Ltd., 2002]

Appendix B

FILTER TRANSMISSION SPECTRA

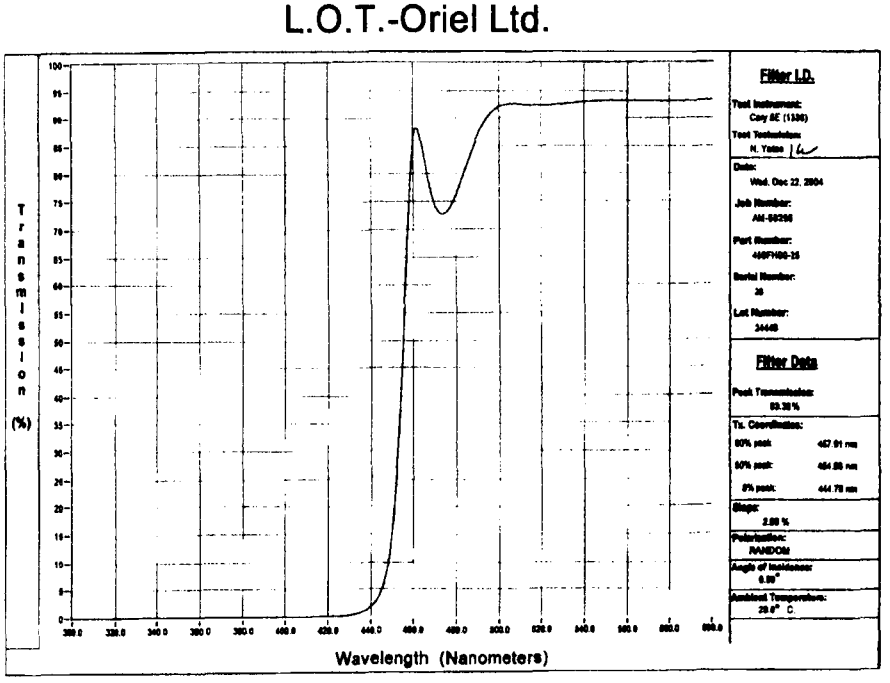

FIGURE B.1 *450 nm long-wave pass filter*

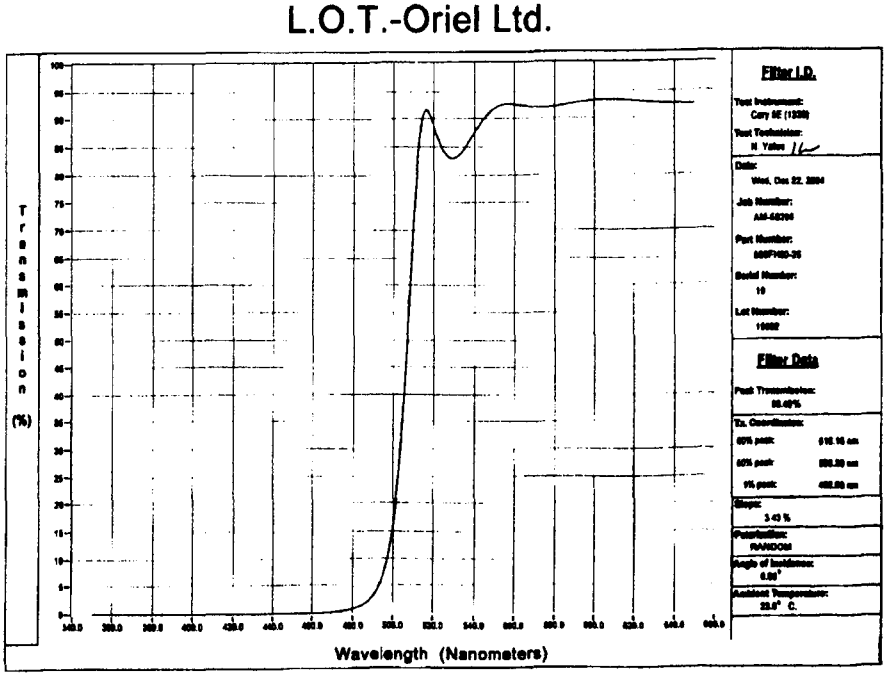

FIGURE B.2 *500 nm long-wave pass filter*

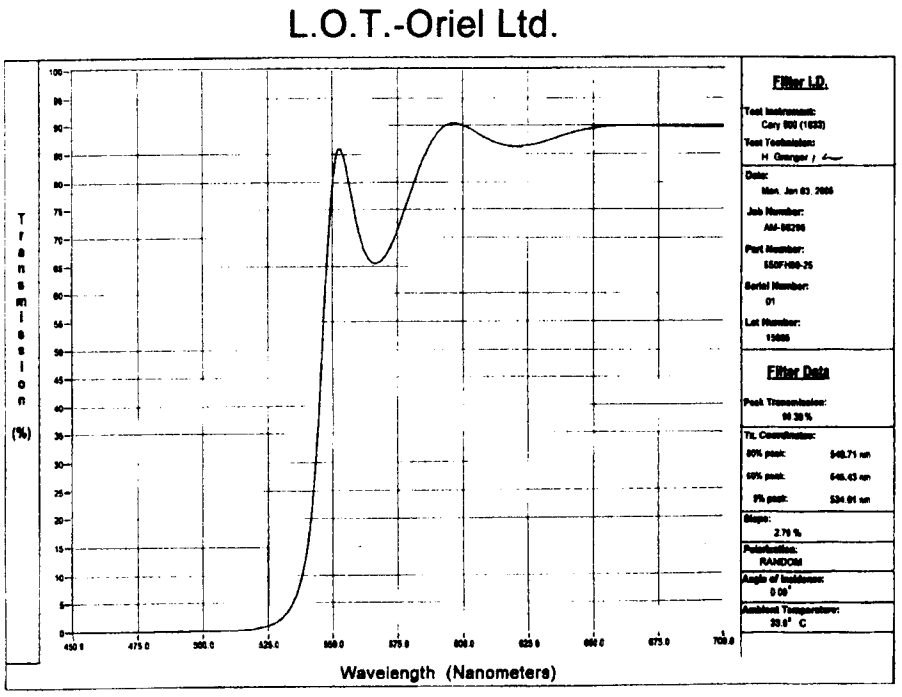

FIGURE B.3 *550 nm long-wave pass filter*

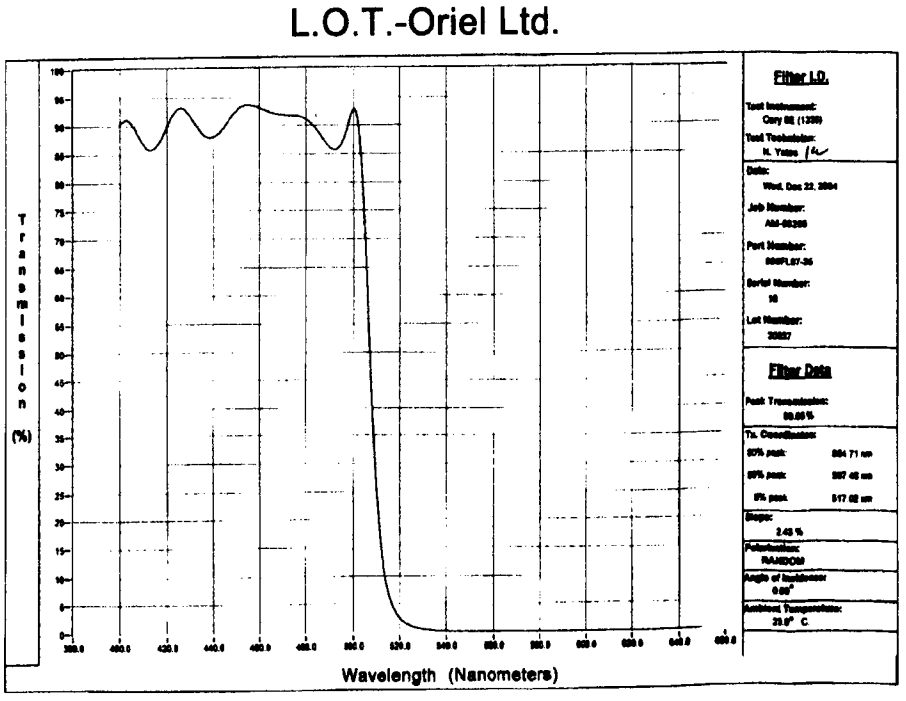

FIGURE B.4 *500 nm short-wave pass filter*

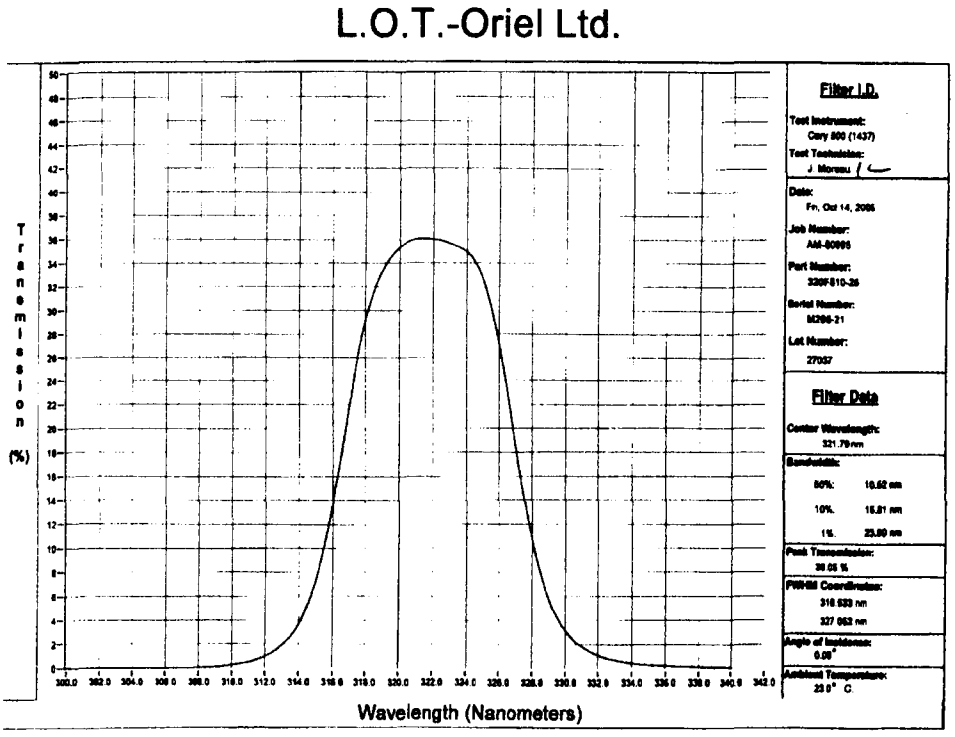

FIGURE B.5 *320 nm bandpass filter*

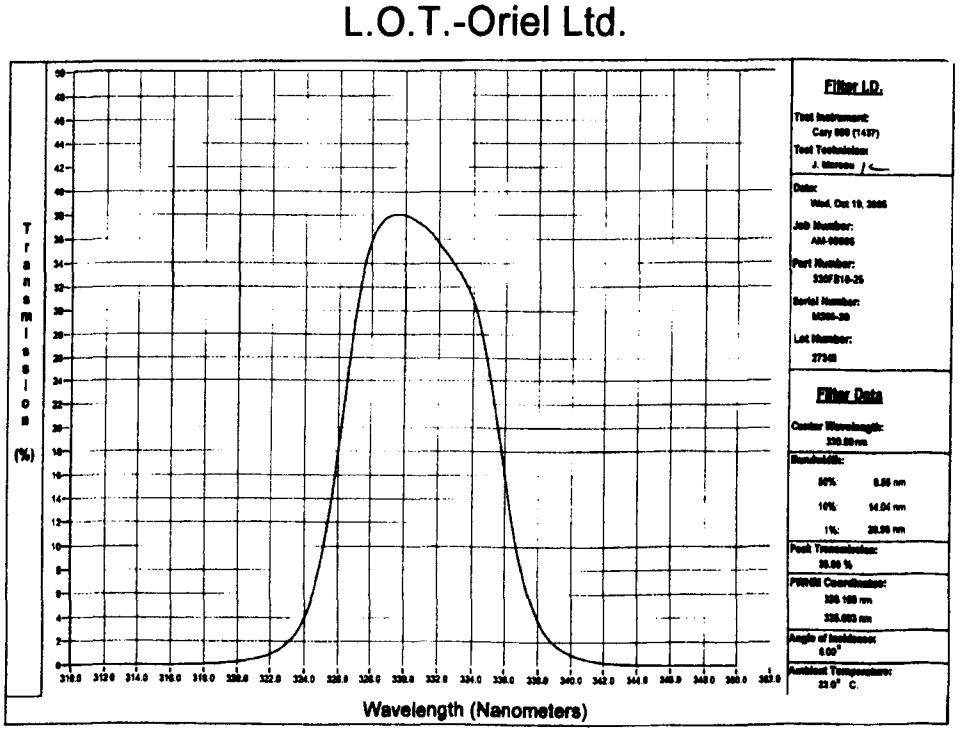

FIGURE B.6 *330 nm bandpass filter*

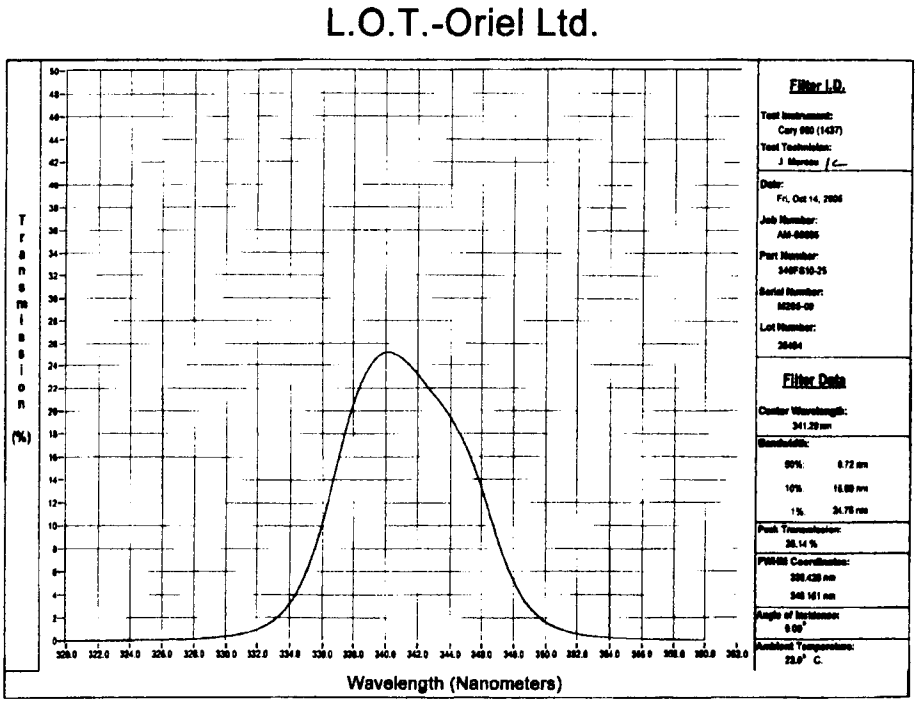

FIGURE B.7 *340 nm bandpass filter*

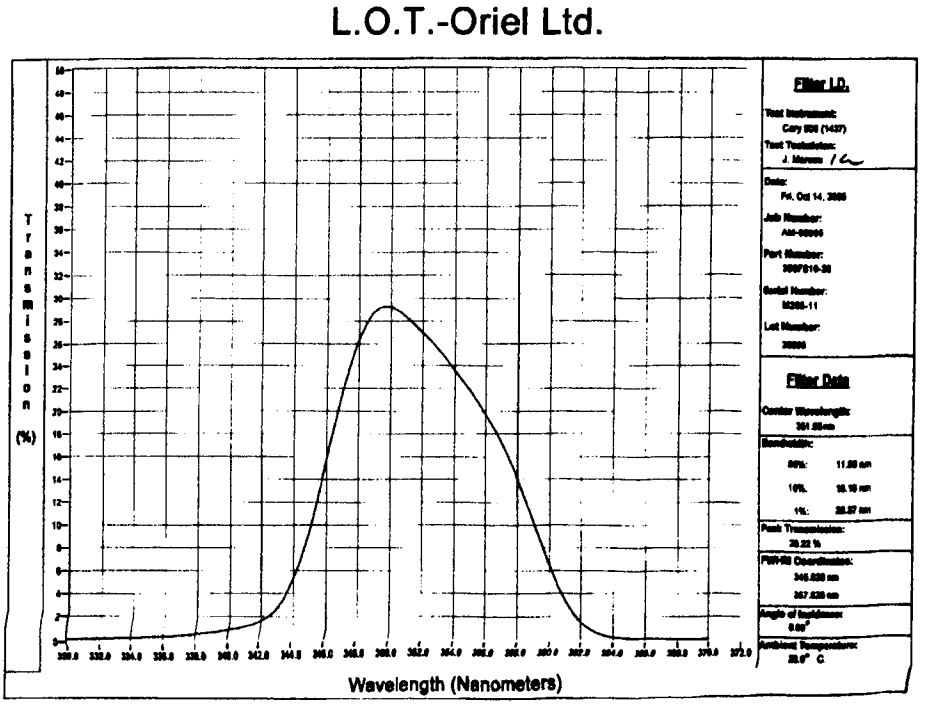

FIGURE B.8 *350 nm bandpass filter*

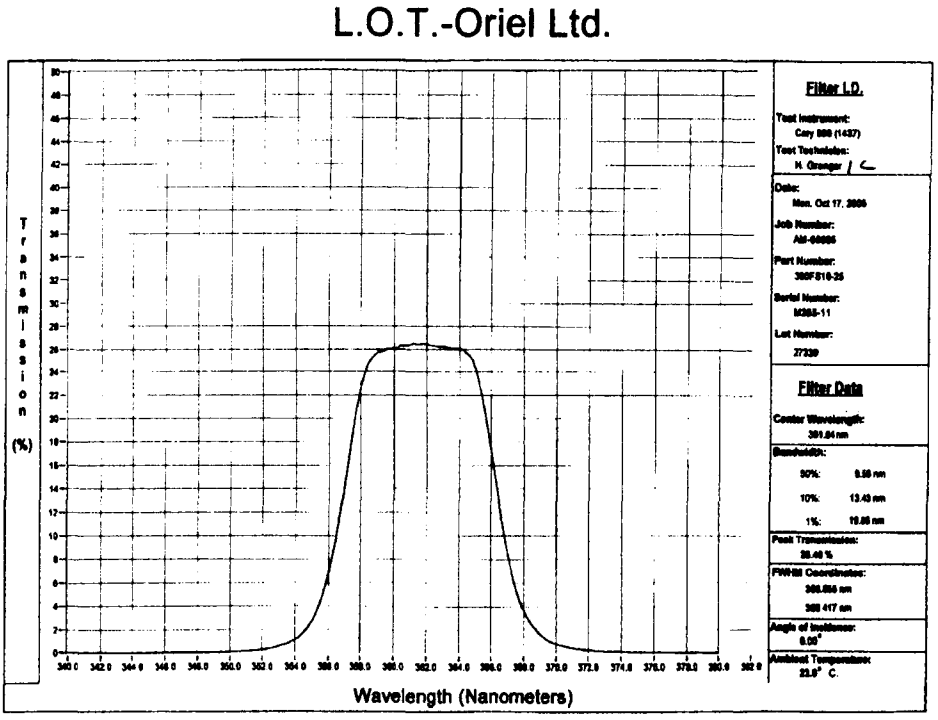

FIGURE B.9 *360 nm bandpass filter*

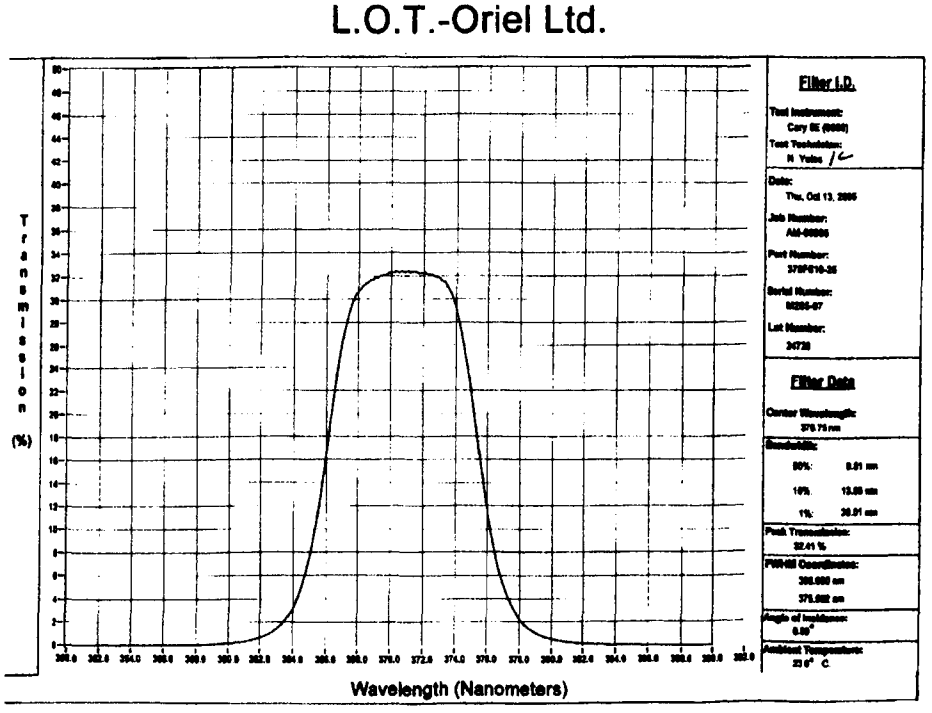

FIGURE B.10 *370 nm bandpass filter*

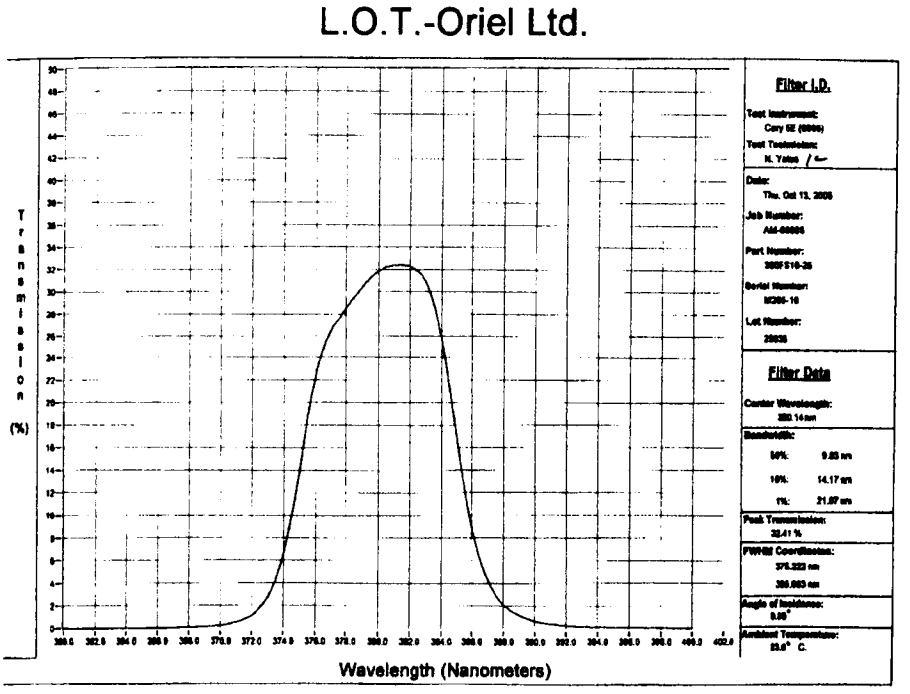

FIGURE B.11 *380 nm bandpass filter*

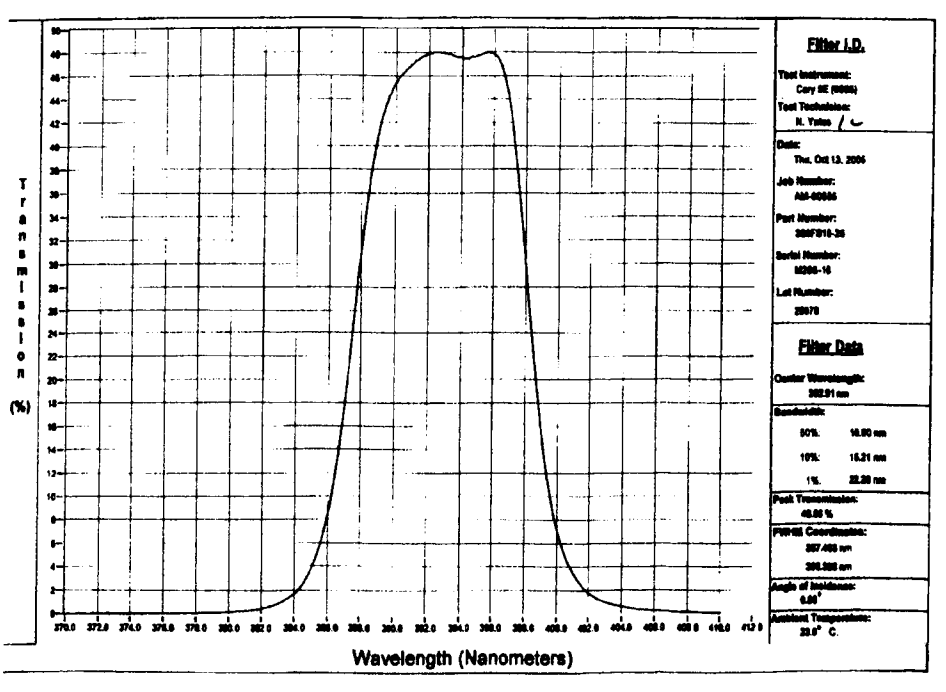

FIGURE B.12 *390 nm bandpass filter*

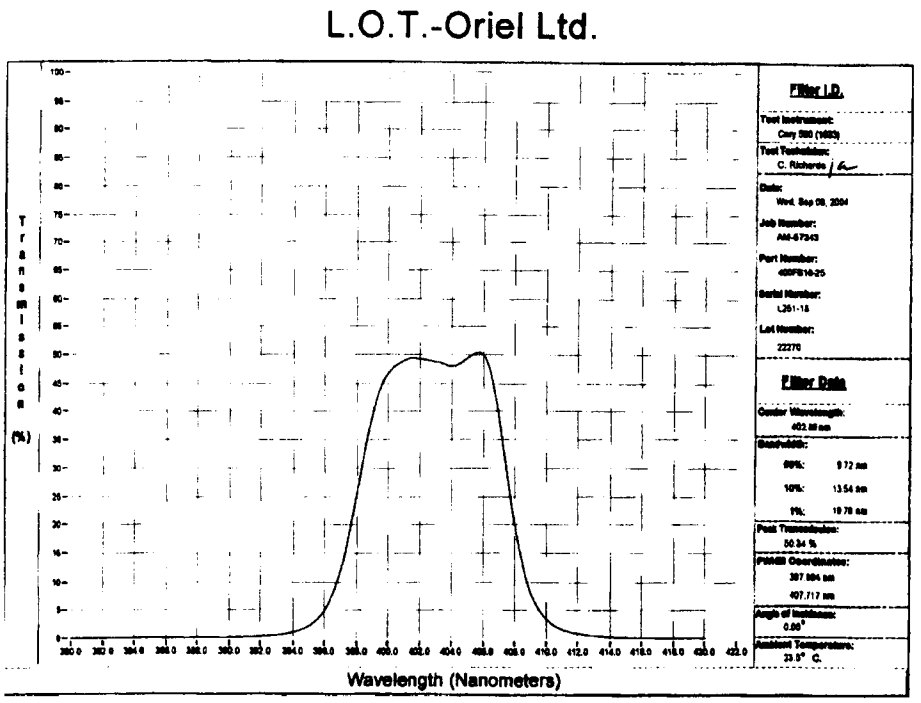

FIGURE B.13 *400 nm bandpass filter*

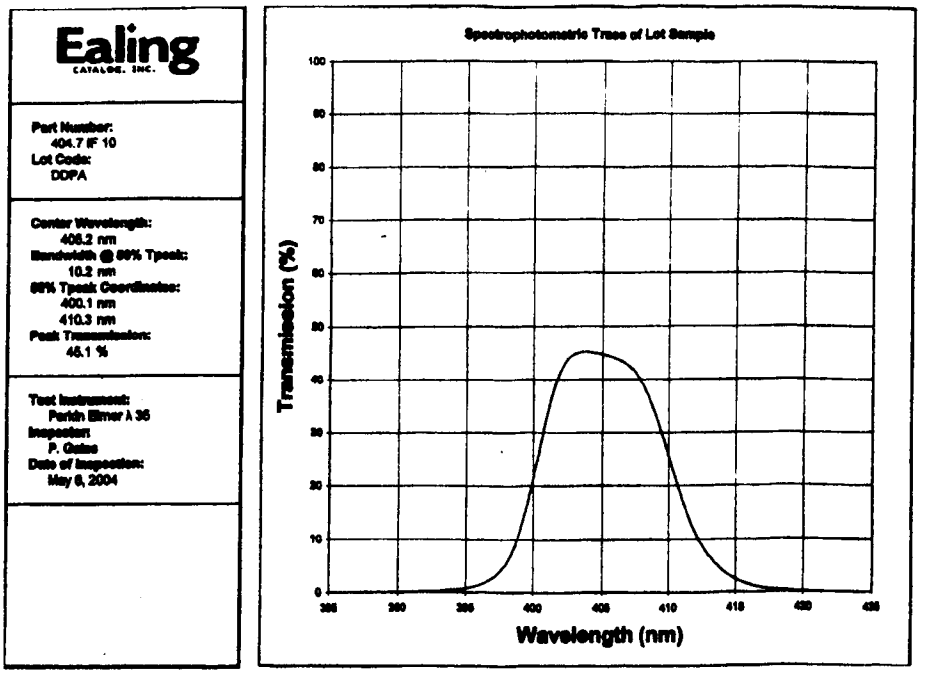

FIGURE B.14 *405 nm bandpass filter*

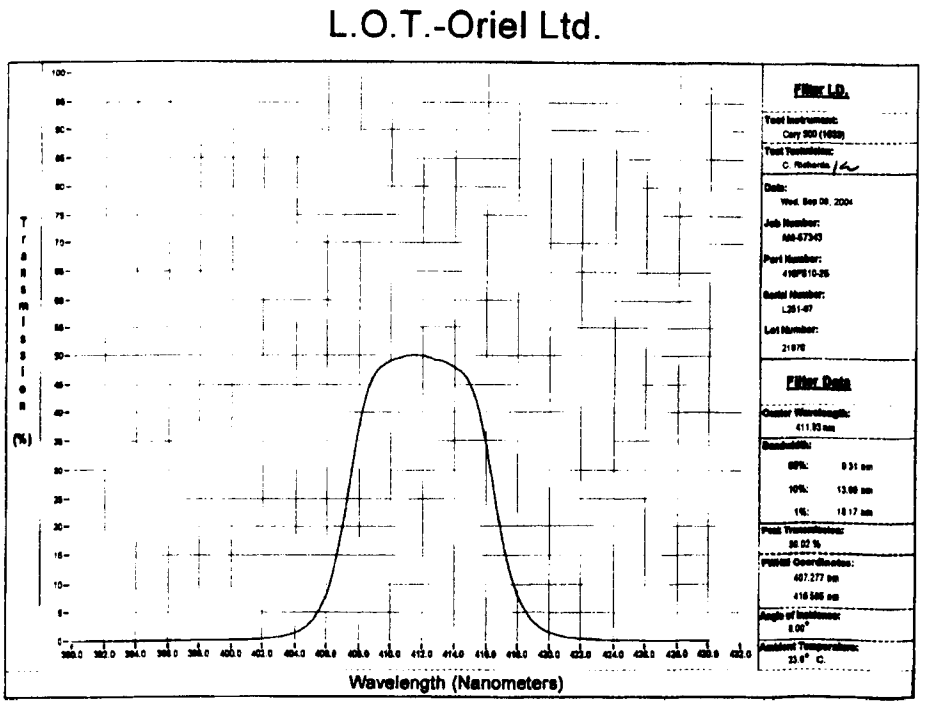

FIGURE B.15 *410 nm bandpass filter*

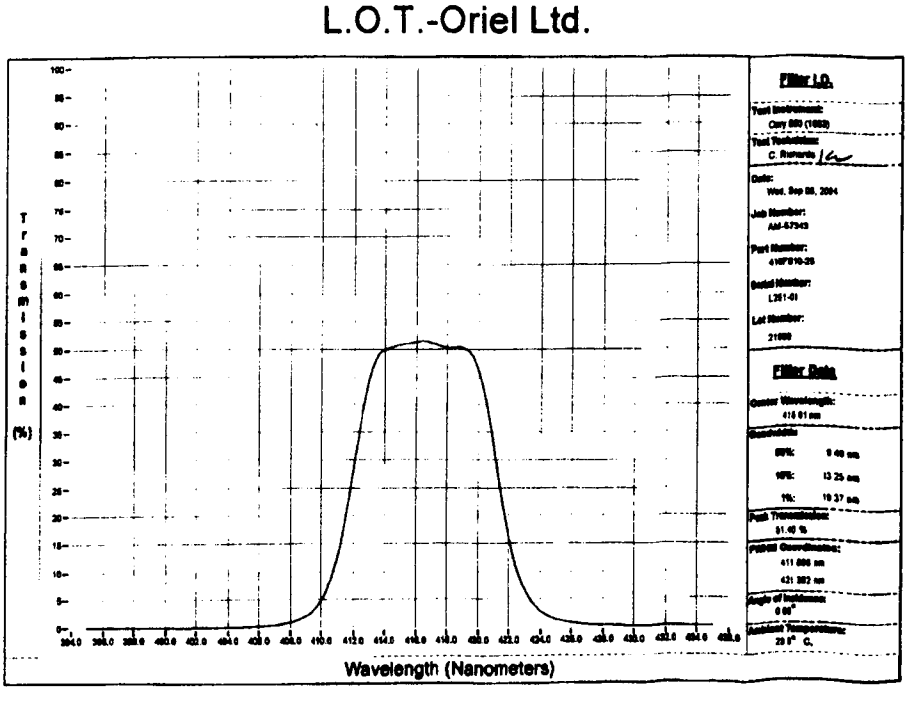

FIGURE B.16 *415 nm bandpass filter*

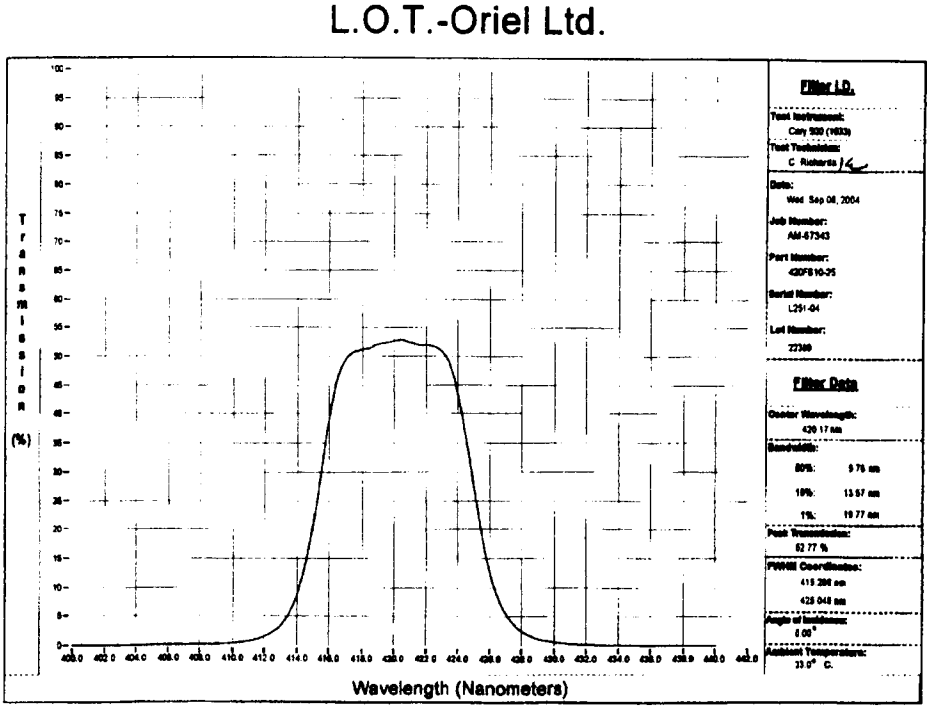

FIGURE B.17 *420 nm bandpass filter*

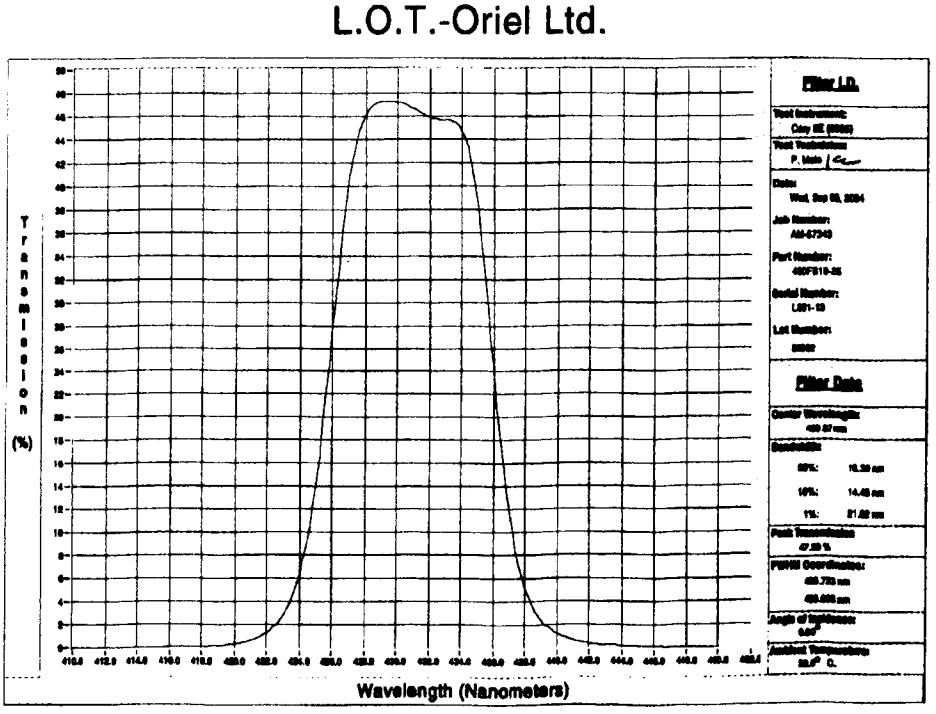

FIGURE B.18 *430 nm bandpass filter*

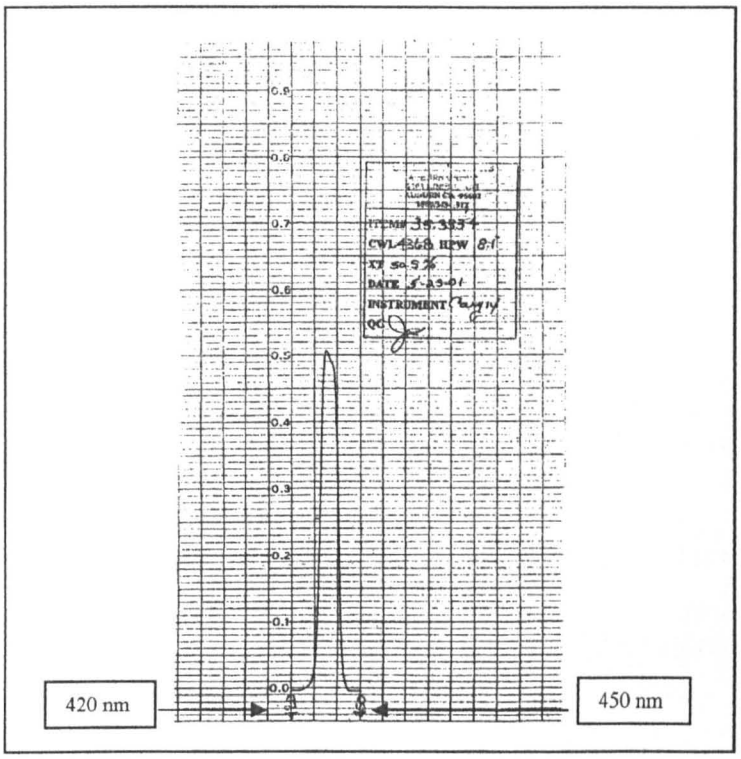

FIGURE B.19 *435 nm bandpass filter*

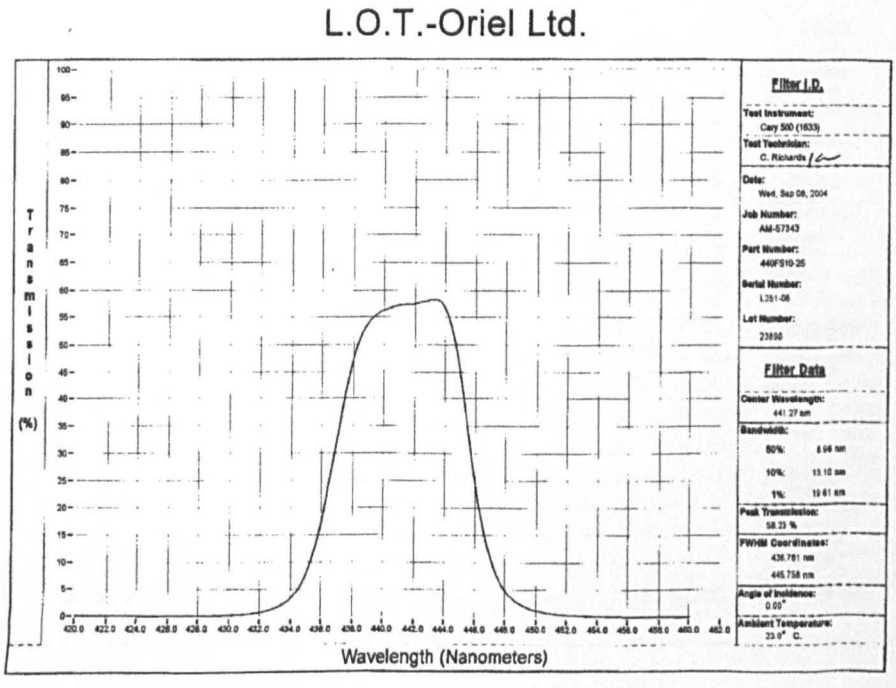

FIGURE B.20 *440 nm bandpass filter*

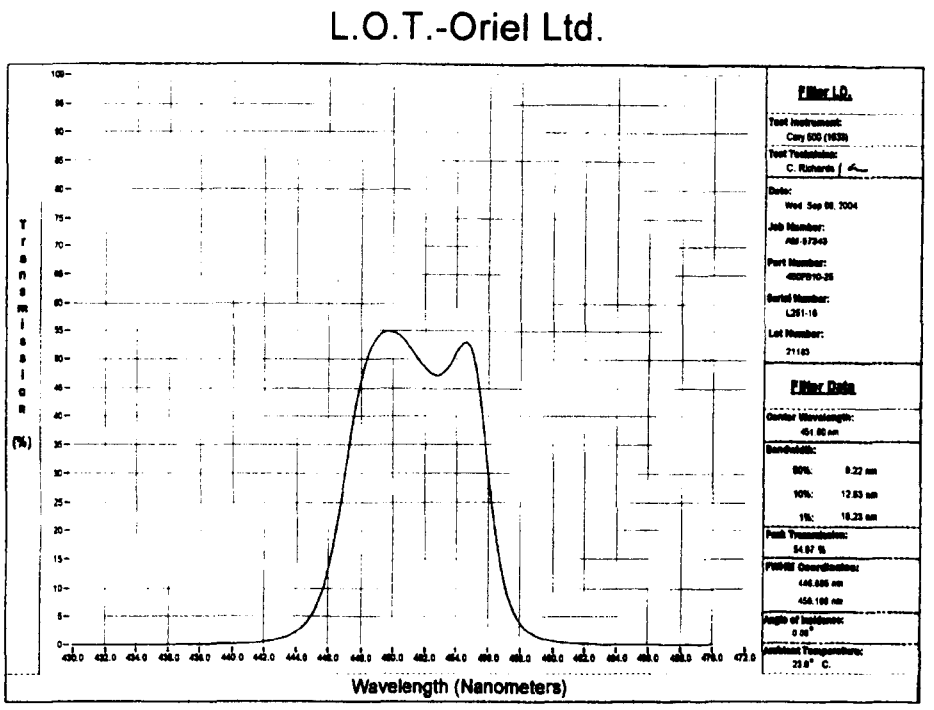

FIGURE B.21 *450 nm bandpass filter*

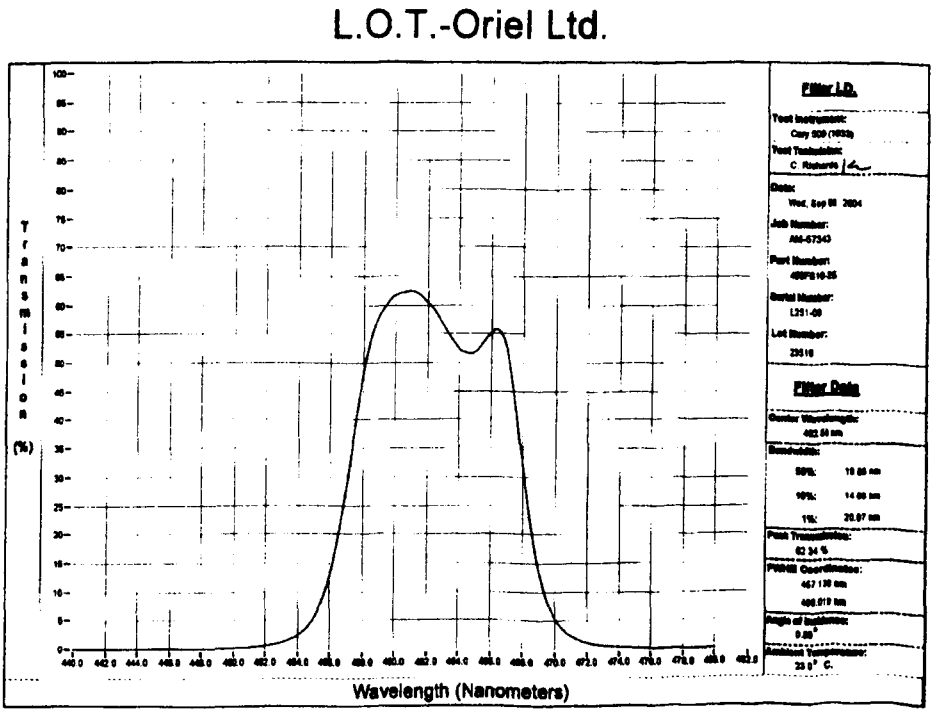

FIGURE B.22 *460 nm bandpass filter*

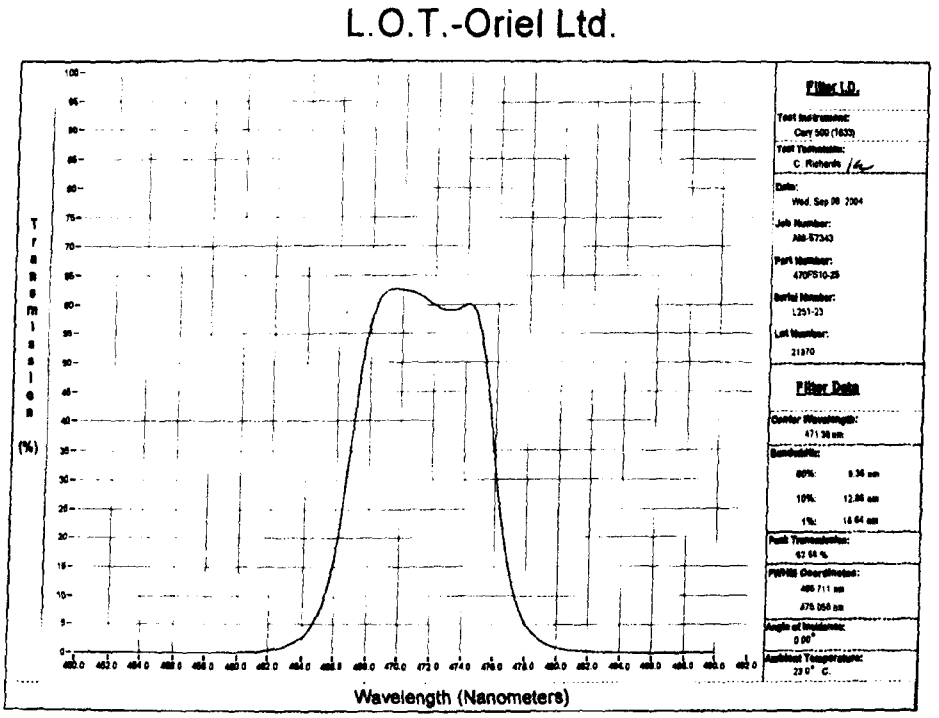

FIGURE B.23 *470 nm bandpass filter*

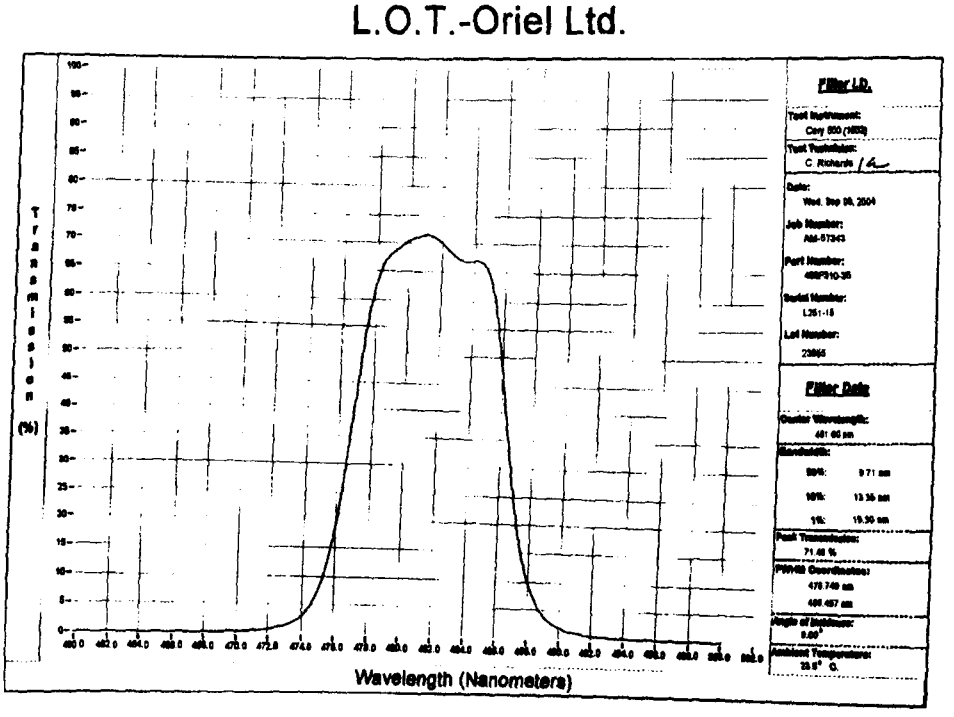

FIGURE B.24 *480 nm bandpass filter*

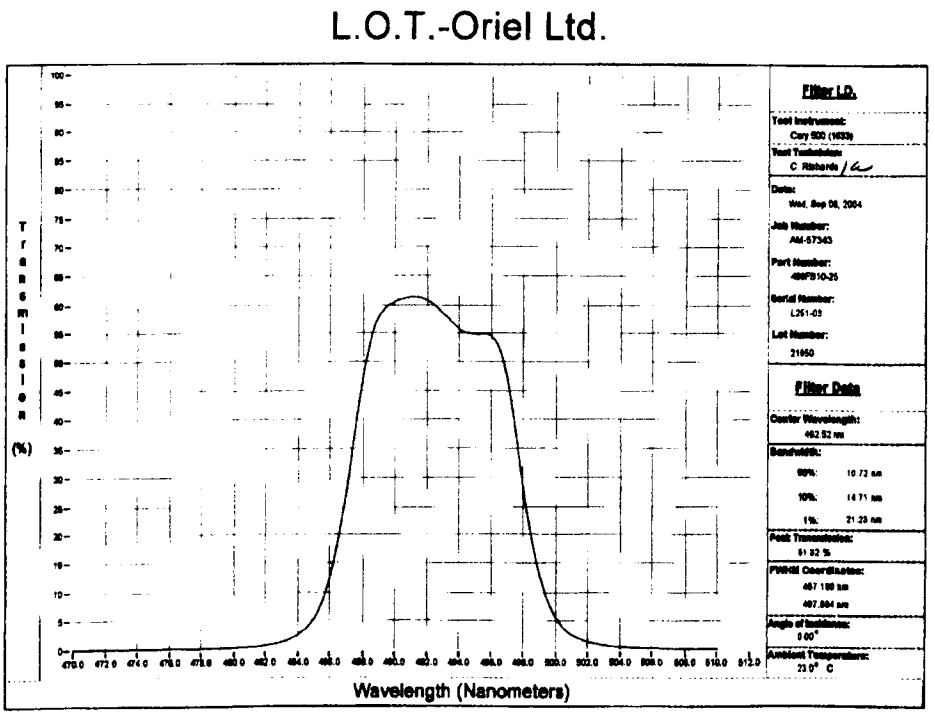

FIGURE B.25 *490 nm bandpass filter*

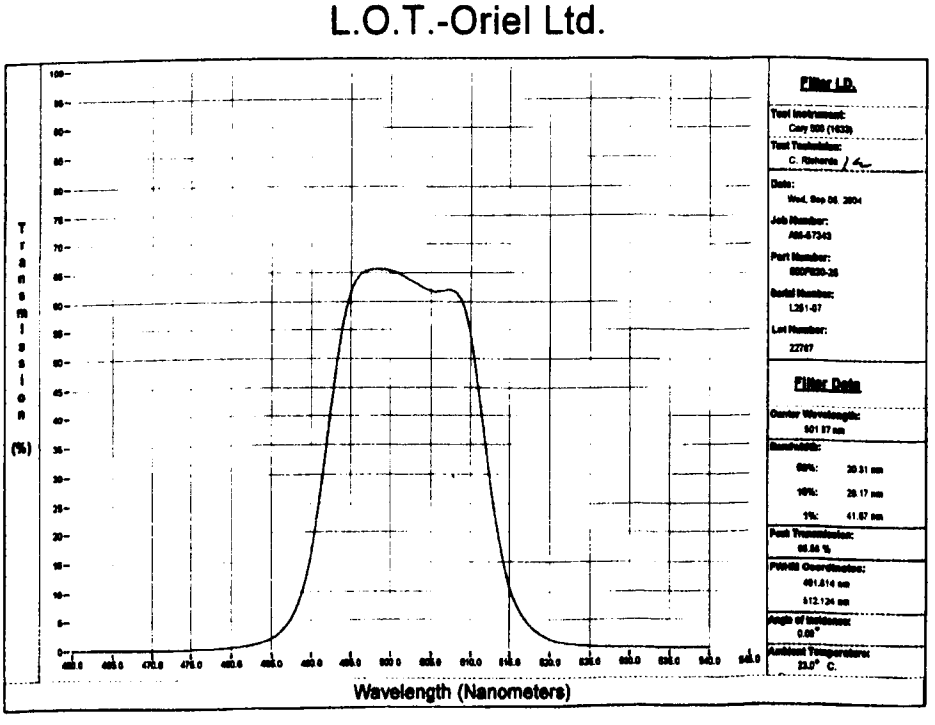

FIGURE B.26 *500 nm bandpass filter*

Appendix C

PUBLICATIONS

During the course of this study, work was submitted for publication and presented at national and international conferences. The publications and conferences at which they were presented are listed below, with the abstracts and papers subsequently included for reference.

1. M. Maclean, J.G. Anderson, S.J. MacGregor and N.J. Rowan. "Microbiological Quality of Air in University Lecture Theatres and the Potential Application of Pulsed Ultra Violet Light Disinfection", *Society for General Microbiology, 152nd Meeting, University of Edinburgh, Paper MI 14, 7-11 April 2003.*

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

In addition to the above publications on the developed pulsed UV air-disinfection technology, a patent regarding the work on the visible light inactivation of *Staphylococcus* species has been filed.

3. M. Maclean, S.J. MacGregor, J.G. Anderson and G.A. Woolsey, The Robertson Trust Laboratory for Sterilisation Technologies, University of Strathclyde.
   "Inactivation of Gram-positive Bacteria"
   New British Patent Application 0515550.2 (Filed: 29 July 2005)

Society for General Microbiology, 152nd Meeting,
University of Edinburgh, Paper MI 14, 7-11 April 2003.

MICROBIOLOGICAL QUALITY OF AIR IN UNIVERSITY LECTURE THEATRES AND THE POTENTIAL APPLICATION OF PULSED ULTRA VIOLET LIGHT DISINFECTION

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ABSTRACT

Airborne transmission of microorganisms is recognised as being a significant source of infection, with potentially fatal illnesses such as tuberculosis and meningitis spread via this route. Consequently, more research is required in this area to both establish the physical mechanisms of airborne transmission and develop effective means of achieving air disinfection.

The present study is concerned with an investigation of air quality in university lecture theatres. Due to high student numbers and absence of air quality control within lecture rooms, there is potential for the build-up of airborne contamination and therefore cross-infection.

In this study, a SAS Super-180 sampler was used to take microbial and fungal counts before, during and after a 1hour lecture in order to identify the variation in airborne contamination levels that occur, with results demonstrating a significant increase in airborne populations due to the presence of large congregations. Various environmental parameters were also monitored to examine their effect on the microbial air load. Also, a pulsed UV light system developed by the EPAST Group (which has had success in surface and liquid applications) was used to disinfect the air, and results highlight the effectiveness of the PUV system for the inactivation of airborne microorganisms.

THE DEVELOPMENT OF A PULSED UV-LIGHT AIR DISINFECTION SYSTEM AND ITS APPLICATION IN UNIVERSITY LECTURE THEATRES

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Abstract

Indoor air quality is an increasingly important issue in a wide variety of industrial, domestic and social settings, the most notable example being hospitals and healthcare facilities.

The present study is concerned with the application of pulsed UV-rich light as an effective means of air disinfection. For this, a system was developed which involved the passing of volumes of air through a path of pulsed UV-rich light. The treated samples were then collected using an SAS Super-180 air sampler and the effects of this pulsed light on airborne bacterial populations were then investigated.

Also, in an attempt to establish the extent of airborne bacterial contamination which can occur in specific situations, the microbiological air quality of university lecture theatres was monitored.

The results obtained demonstrate a significant increase in airborne bacterial populations due to the presence of large student congregations.

In addition, the application of UV-rich pulsed light was shown to be effective in reducing the levels of airborne bacteria within samples of air taken from the lecture theatres. Furthermore, treatment was found to be capable of eliminating the majority of the *Staphylococcus spp.* found in the air, and this is desirable since these bacteria are opportunistic pathogens compared to the saprophytic *Micrococcus spp.*, some of which survived treatment.

I. INTRODUCTION

Airborne transmission of microorganisms is recognised as being a significant source of infection, with many medically important bacteria, fungi and viruses capable of dispersal via this route. Although this has been a topic of longstanding importance, over recent years there has been a notable increase in interest in indoor air quality issues, both in an attempt to establish the physical mechanisms of airborne transmission and to develop effective means of achieving air disinfection [1, 2].

The majority of human-shed organisms are commensals and pose little threat to immunocompetent individuals. There is, however, the possibility that infectious organisms such as *Mycobacterium tuberculosis* and *Neisseria meningitidis* are being released through droplet nuclei ($\leq 5\mu m$ in diameter) which remain suspended in the air for long periods due to their small size [3]. These may subsequently cause infection in other individuals via the respiratory tract if the required infectious dose is inhaled.

Although the transmission of infectious aerosols is a major concern in the hospital environment, other less well-documented situations must also be taken into consideration. In areas where large numbers of people are brought together in close proximity, such as in universities/schools and on aeroplanes, there is the potential for the build-up of substantial airborne contamination and therefore cross-infection [4, 5].

It is because of this and other indoor air quality issues such as Sick Building Syndrome, that there is a need to develop effective air quality control methods that may be used in densely-populated indoor venues such as those mentioned above, and most importantly, in hospitals and other health-care facilities.

There are a variety of technologies for air quality control in use today including mechanical filtration (involving HEPA filters), mechanical filters with bactericides, ionising electrically enhanced filtration and UV radiation (2).

The main objective of this study was to develop an air disinfection system using pulsed UV-rich light. The authors have had previous success using pulsed UV light for liquid treatment applications (6). The microbial air quality of university lecture theatres was also investigated and reported. This situation was thought to be ideal for testing due to high student numbers and limited air quality control within lecture theatres. There therefore exists the potential for the build up of airborne contamination and therefore cross-infection. Investigations also progressed to consider whether these airborne bacterial populations could subsequently be reduced in number by the application of this pulsed UV-rich light disinfection system.

II. METHODS

A. Lecture Theatre Sampling

Measurements of air quality were made in a lecture theatre occupied by students for periods of 2 hr, 3 hr, 4 hr and 6 hr. Samples of air were taken at the beginning of each period, at the conclusion of each period, and 30 minutes after the conclusion of each period. Throughout each period, lecture theatre occupancy was over 75%, except during the 5-minute changeovers of personnel

which took place at hourly intervals. An SAS Super-180 sampler (Cherwell Laboratories Ltd., UK.) was used to collect the bacterial air samples onto nutrient agar (NA) plates. The sampler was programmed to collect volumes of 180 litres, and six replicate samples were taken at each sampling time. The NA plates were incubated at 37°C for 48 hours before enumeration. The incubation temperature of 37°C was applied, as this tends to select for a bacterial flora of human origin, and therefore, potential pathogens.

The results obtained were then used to calculate the mean cfu/1000l of air. Hourly samples were taken when the lecture room was unoccupied to establish typical background levels, and these were used as control data.

B. Pulsed Light System

Figure 1 shows the pulsed UV-rich light system used in these experiments. The system consists of a pulse power generator, flashlamp housing, airflow pipe and the SAS Super-180 air sampler. The air sampler is used to draw air into the system via the inlet holes. The air then passes along the pipe while being exposed to pulses of UV-rich light before impacting onto the sampling media contained within the head of the sampler. Also, the L-shaped pipe has an inner lining of aluminium foil in the vertical section to increase reflectance, and the bend section is coated with matt black paint to prevent UV rays reflecting onto the sampling media and thereby affecting the results.

The lamp used was a low pressure (450 torr), Heraeus Noblelight, xenon-filled flashlamp with a clear quartz envelope. The energy per pulse was 20J when the system was operating at 1kV and Figure 2 shows the emission spectrum of the flashlamp when operating at 1kV.

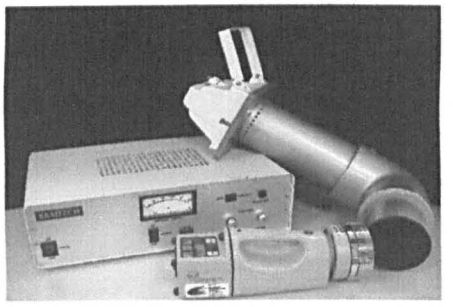

Figure 1. *Pulsed UV air disinfection system*

C. Pulsed Light Treatment of Lecture Theatre Air

Immediately after the conclusion of a 1-hour lecture period, 180 litres of air were passed through the system (Fig. 1) without samples being taken, allowing air representative of the surrounding environment to be drawn into the system.Triplicate air samples, each of 180l, were then collected on NA plates; these representing non-pulsed samples. The pulsed light system was then switched on and the pulsing frequency was set at approximately 5 pulses per second (PPS), operating at 1kV. 180 litres of air was again passed through the system before triplicate pulsed samples were taken. This procedure was then repeated after intervals of 10 and 20 minutes.

The university lecture theatre used for the sampling and pulsing experiments had a volume of 400m^3 and a maximum occupancy of 174. The estimated air change rate for this lecture room is 12.6 air changes/hr.

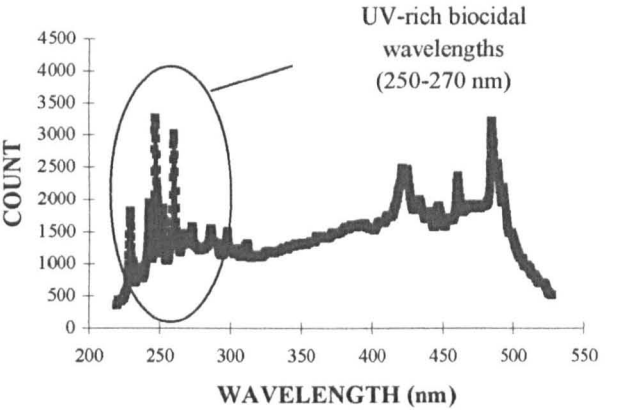

Figure 2. *Emission spectrum of flash lamp when operating at 1kV*

D. Identification of Bacteria

A number of isolates from the pulsing experiments were identified using microbiological and biochemical tests including Gram staining, and for selected isolates, full identification was found using API Staph kits (Biomerieux, USA). Identification was performed on pulsed and non-pulsed isolates to determine whether the biocidal effects of the treatment were uniform or selective against different components of the airborne microflora.

III. RESULTS

A. Lecture Theatre Sampling

Figure 3 shows the results recorded for bacterial levels in the sampled lecture theatre, with numbers in one case rising as high as 1173.3 cfu/1000l after a 4-hour period.

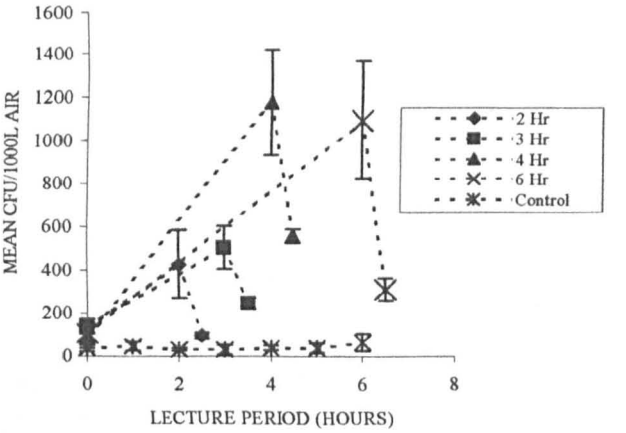

Figure 3. *Levels of airborne bacteria in a lecture theatre before (0 hrs) and after lecture periods of differing durations (2, 3, 4, 6 hrs), followed by levels monitored 30 minutes after the lecture theatre was vacated.*

A general trend can be seen which shows that as the duration of lecture period increases, so too does the concentration of airborne bacteria.

An exception to this is the airborne bacteria level after 6 hours, which was not significantly different from that obtained after the 4-hour period. The reason for this is not clear but could be due to changes in the environmental parameters or other interdependent stresses which the bacterial aerosols encounter during their launch, aerial transport and subsequent deposition.

Figure 3 also shows that during the 30-minute period after vacation of the room, the bacterial numbers have fallen to values similar to, or not far above, those detected prior to the start of the lecture period.

The control samples obtained from the unoccupied lecture theatre show a slight, but not a significant variation in the airborne bacteria level. Clearly, the presence of large congregations of students is the major contributor to airborne bacterial contamination in the lecture theatre.

B. Pulsed Light Treatment of Lecture Theatre Air

Immediately after a 1-hour lecture period, triplicate non-pulsed and light pulsed air samples were taken and the results of these are plotted in Figure 4. It can be seen that exposure to the pulsed light treatment caused a reduction in the airborne bacterial contamination, with the level decreasing from >540cfu/1000l to <105cfu/1000l.

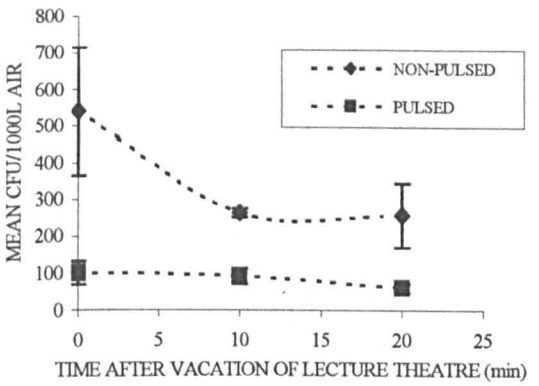

Figure 4. *Effect of pulsed light treatment on the airborne bacterial content of university lecture room air*

The cfu counts for the second and third non-pulsed samples, which were taken at approximately 10 minute intervals, were progressively lower. This is thought to be as a result of the airborne bacterial population beginning to settle – an effect that is also evident in Figure 3 by the low levels detected in samples taken 30 minutes after vacation of the lecture room. The mean cfu counts for the pulsed air samples remain comparatively similar, decreasing gradually from 102 to 63cfu/1000l of air.

The photographs of NA plates in Figures 5a and b provide a visual comparison of bacterial colony forming units present in non-pulsed and light-pulsed samples. It can be seen that pulsed light treatment has significantly reduced the number of cfu/180 litres of air sampled.

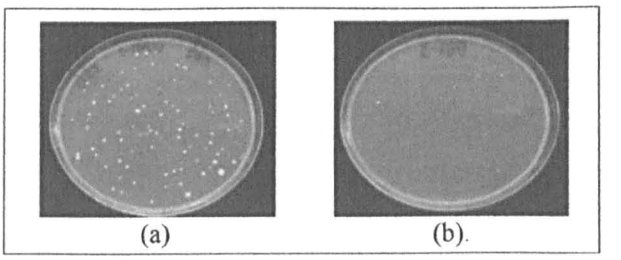

Figure 5. *Nutrient agar plates indicating the effectiveness of the pulsed light system for the inactivation of airborne bacteria. Figure (a) shows an air sample taken immediately after a 1-hour lecture period, and (b), a sample taken at the same time and subjected to pulsed light treatment.*

C. Identification of Bacteria

The characteristics of a range of bacterial isolates obtained from lecture theatre air are shown in Table 1. The colonies were selected on the basis of size and colour in an attempt to maintain the ratio as they appeared on the sampling plates. It can be seen from the results that the majority of isolates were Gram-positive cocci, with only two Gram-negative species being detected, and both being among the non-pulsed samples. It was also found that the majority of isolates surviving pulsed light exposure had a yellow pigmentation, whereas a larger population of cream isolates were found in the non-pulsed samples.

From the species identification tests it can be seen that a larger number of *Staphylococcus spp.* were found within the non-pulsed samples, in addition to some *Micrococcus spp.*, but in contrast, all of the identified pulsed isolates, with the exception of one, were *Micrococcus spp.*

Table 1. *Characteristics of bacterial isolates obtained from lecture room air. ([a] number out of 14 identified isolates; [b] number out of 9 identified isolates)*

		NON-PULSED	PULSED
GRAM STAIN	+ve	23	25
	-ve	2	0
SHAPE	Cocci	23	25
	Rods	2	0
COLOUR	Cream	15	4
	Yellow	10	21
ID	*Staphylococci*	10/14 [a]	1/9 [b]
	Micrococci	4/14 [a]	8/9 [b]

IV. DISCUSSION

These experiments have shown that airborne bacterial contamination levels in university lecture theatres can rise significantly over the period of a lecture. It is also evident that this increase in contamination is a result of the presence of personnel, either through human-shed organisms via skin scales and respiratory secretions, or through the re-suspension of dust containing microbes already deposited in the room, due to movement and air

currents. It is highly likely that all of these mechanisms contribute to this airborne contamination.

The limited air quality control measures available in situations such as the university lecture theatres investigated here are not usually a noteworthy problem since the vast majority of occupants are healthy and not susceptible to the largely non-pathogenic airborne bacterial flora. However, it is becoming increasingly evident that poor indoor air quality (IAQ) can have some effect on healthy occupants. Sick Building Syndrome is becoming more noted, particularly within the workplace. Given that individuals are spending the majority of their day indoors, the importance of efficient air quality control is high. Long-term exposure to allergens and pollutants has resulted in the development of hypersensitivity with symptoms including lethargy and headaches, as well as respiratory problems [7]. Studies of student performance indicate that poor IAQ may be having a detrimental effect, and recent data also suggests that mental tasks such as calculation, concentration and memory can be affected [8].

The application of UV-rich pulsed light was shown to be effective in reducing the levels of airborne bacteria within samples of air taken from the lecture theatres. Pulsed light treatment is believed to induce DNA-based damage within the bacteria as they pass through the system. The major type of lesions resulting from this exposure is cyclobutane pyrimidine dimers (CPD), with pyrimidine-pyrimidone [6-4] photoproducts also being produced. Other types of DNA damage also occur but to a much lesser extent [9].

Identification of the bacterial isolates from the pulsed and non-pulsed samples highlighted that the majority of bacteria capable of survival following pulsed light treatment were *Micrococcus spp.* containing yellow pigmentation. Consequently, there is the indication that pigmentation may be an influencing factor for the survival of airborne bacteria when exposed to UV light. *Micrococcus spp.* are found primarily on mammalian skin and in soil, but are also commonly isolated from air and food products [10]. Because of their prevalence in this range of environmental situations, they are generally robust organisms which can demonstrate varying degrees of UV resistance. Furthermore, treatment was found to be capable of eliminating the majority of the Staphylococcal species found in the air, and this is desirable since these bacteria are opportunistic pathogens compared to the saprophytic *Micrococcus spp.* which survived pulsed light illumination.

V. CONCLUSION

This study has demonstrated the extent of airborne bacterial contamination which may be encountered when large numbers of students congregate within close proximity of one another in university lecture theatres. The application of a pulsed UV-rich light system to disinfect contaminated air samples has been shown to cause a significant reduction in the levels of airborne bacterial population. Overall, it can be seen that the pulsed light disinfection system is effective in reducing airborne bacteria levels and with further research, may have future potential applications for the control of air quality.

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