Inactivation of microorganisms isolated from infected lower limb arthroplasty using High-Intensity Narrow-Spectrum (HINS) light

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Declaration of Authenticity and Author's Rights

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

Signed:

Date:

Acknowledgements

In no particular order I would like to thank several people who have helped make this thesis possible. I sincerely apologise to anyone who I have missed out; I hope you can forgive my weary head.

Jim Guthrie and his team at the Southern General Hospital microbiology lab supplied me with the isolates. James Eyland from the medical photography department captured the action shots in theatre. Aslam Mohammed and Bilal Jamal kindly modelled along with nursing staff. David Large and Dominic Meek encouraged the research in the first place, and the former agreed to help fund a portion of the degree. Lynne Horey provided literature searching expertise and found those articles that I could not access.

At the university Helen Grant and Scott MacGregor were my academic supervisors. Dave Currie provided constant entertainment and taught me basic microbiological techniques. John Anderson offered constructive commentary and discussion at various points during the degree. Michelle Maclean must be given special mention and praise for her tireless role as an advisor at all stages of the work.

Last but not least, my family and fiancé must be thanked for their patience and support. At times it must have felt like a longer road for them than I….

Abstract

Infection rates following arthroplasty surgery are reported between 1-2%, with considerably higher rates in revision surgery. The associated costs of treating infected arthroplasty cases are over four times the cost of the primary procedures, with significantly worse functional and satisfaction outcomes. In addition, multiple antibiotic resistant bacteria are developing, so to reduce the infection rates and costs associated with arthroplasty surgery, new preventative methods are required. HINS-light is a novel blue light inactivation technology which kills bacteria through a photodynamic process, and is proven to have bactericidal activity against a wide range of species. The aim of this study was to investigate the efficacy of HINS-light for the inactivation of microorganisms isolated from infected arthroplasty cases.

Specimens from hip and knee arthroplasty infections are routinely collected in order to identify possible causative organisms and susceptibility patterns. This study tested a range of these isolates for sensitivity to HINS-light. During testing, suspensions of the pathogens were exposed to increasing doses of HINS-light (of 123mW/cm^2 irradiance). Non-light exposed control samples were also set-up. The samples were then plated onto agar plates and incubated at 37°C for 24 hours before enumeration.

Complete inactivation (greater than 4-log reduction) was achieved for all of the clinical isolates from infected arthroplasty cases. The typical inactivation curve showed a slow initial reaction followed by a period of rapid inactivation. The doses of HINS-light exposure required ranged from 118 – 2214 J/cm^2 respectively. Gram-positive bacteria were generally found to be more susceptible than Gram-negative. A similar trend was also found when isolates were exposed whilst seeded on agar surfaces.

As HINS-light utilises visible-light wavelengths it can be safely used in the presence of patients and staff. This unique feature could lead to possible applications such as use as an infection prevention tool during surgery and post-operative dressing changes.

Contents

Chapter 1

Introduction

Total hip and knee replacement surgery is safe and cost effective (Ethgen et al, 2004). In Scotland alone there were over 14,000 such procedures performed in 2010 (Scottish Arthroplasty Project, 2010), with a further 164,000 hip and knee arthroplasty operations in England and Wales (National Joint Registry England & Wales, 2010). Prosthetic infection is a major but infrequent complication of the surgery, with an incidence between 0.6% and 2% per joint per year (Phillips et al, 2006; Jamsen et al, 2009). Revision of infected implants is associated with substantial morbidity and has significant economic implications.

Any new developments in reducing the burden of prosthetic joint infection are welcome. High-Intensity Narrow-Spectrum (HINS) light is an antimicrobial visible-light based technology that has been developed at the Robertson Trust Laboratory for Electronic Sterilisation Technologies (ROLEST) at the University of Strathclyde. The aim of this project is to assess the efficacy of HINS-light against positive microbial specimens obtained from infected lower limb arthroplasty cases. The following is a breakdown of the chapters and a brief description of the content.

Chapter 2 is the background and literature review. The rationale for joint replacement surgery is explained along with a discussion of the common complications that may occur. Strategies to reduce infection are reviewed, along with diagnosis and treatment of the infected joint. The last section of this chapter investigates the role of light technology in fighting infection, assessing available technologies and their uses. This incorporates a review of 405-nm HINS-light for microbial inactivation.

Chapter 3 discusses the epidemiology of arthroplasty infection in the Southern General Hospital over a period of one year. A microbiological review of the infected arthroplasty pathogens is also performed.

Chapter 4 is titled Microbiological Methodology. This provides information on the microorganisms, culture media, equipment and microbiological methods used throughout the investigations. The origin of each specimen and the site of infection are also described.

Chapter 5 describes the 405nm HINS-light LED systems used for exposure of infected arthroplasty isolates. The basic workings of the LED arrays are explained. Routine experiments assessing the behaviour of the systems in a laboratory setting are described, and the results presented.

Chapter 6 reports on the use of the 405 nm HINS-light LED systems for the inactivation of the clinical infected arthroplasty isolates. This chapter describes the experimental arrangements for exposure of the microorganisms to the 405 nm light. Inactivation results for bacterial suspensions and bacteria seeded onto surfaces are presented. A comparison of the efficacy of 405 nm HINS-light for inactivation of the clinical isolates is made with the published literature.

Chapter 7 is the general conclusions and recommendations for further work. This provides a summary of the key findings and limitations of the work. The potential future directions of the research are covered along with potential clinical applications.

Chapter 2

Background and literature review

2.0 General

This literature review can essentially be divided into six sections. Firstly is the analysis of the indication and clinical role of arthroplasty surgery. This section will also cover some of the complications that may occur, including infection. The second section will focus on the pathogenesis of prosthetic joint infections. The third section reviews the measures that are already in place to prevent infection. These prophylactic steps can be further subdivided by their timing in relation to the surgery: before, during and after. The fourth section will cover how the diagnosis of infection is actually made. The fifth section discusses in broad terms the principles of treatment and the options currently available. The last of the six sections examines the use of light-based technologies in the fight against infection. This will highlight both ultraviolet and visible light technologies, but will focus mainly on high-intensity narrow-spectrum 405 nm light (HINS-light).

2.1 The indications, aims and clinical role of arthroplasty surgery

Arthroplasty surgery is performed for failure of the respective joint. There are several possible mechanisms of this failure. Osteoarthritis is the main indication in the UK, be it primary or secondary to trauma. Inflammatory arthritis such as rheumatoid arthritis is the next most common. Other indications include metabolic disorders, tumours and congenital conditions, but the list is not exhaustive. The most commonly replaced joints are the hip and the knee, but technology is continuously evolving for high quality prostheses to be used in other parts of the human body.

The clinical decision to offer a joint replacement operation is based on symptoms in combination with radiological findings. The patient frequently complains of pain and an associated reduction in function. The X-ray changes noted include a reduction in

the joint space, bone sclerosis or thickening, the formation of bone cysts and bony outgrowths known as osteophytes. The radiological appearances of normal hip and knee joints are shown alongside arthritic joints in Figures 2.1 and 2.2 respectively.

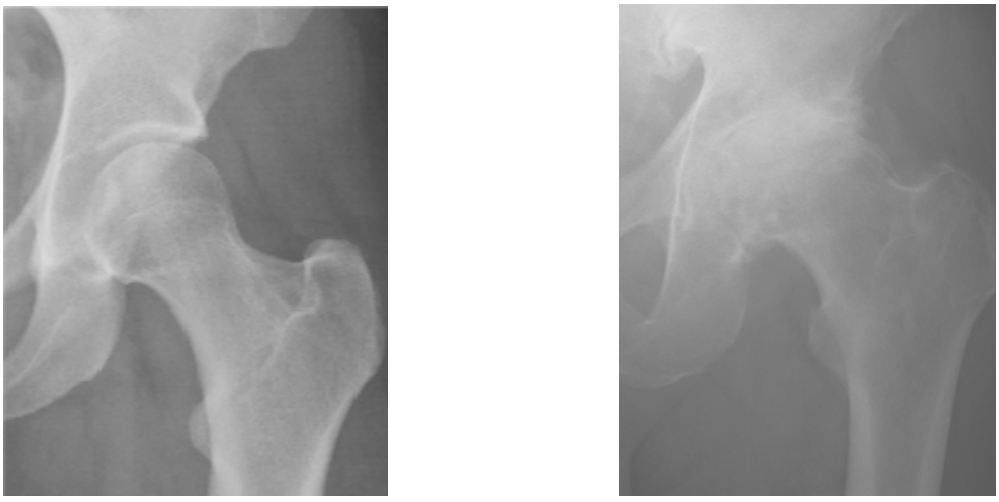


Figure 2.1 *A normal hip X-ray is shown on the left. A hip affected by osteoarthritis is shown on the right for comparison*

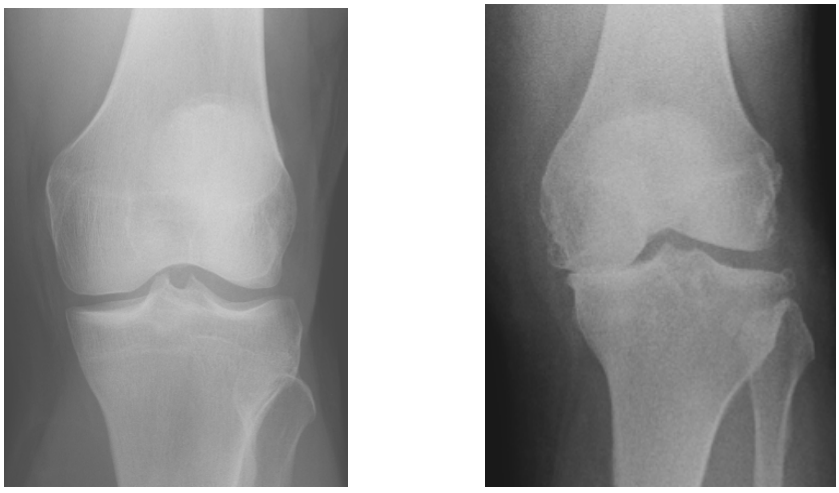


Figure 2.2 *A normal knee X-ray is shown on the left. A knee affected by osteoarthritis is shown on the right for comparison*

The aim of the operation is to give the patient a stable pain-free joint with a good range of movement. This is achieved by removing the worn cartilage and bone

surfaces, and replacing them with an articulating prosthesis. Examples of the post-operative X-ray appearances of a hip and knee are shown in Figure 2.3.

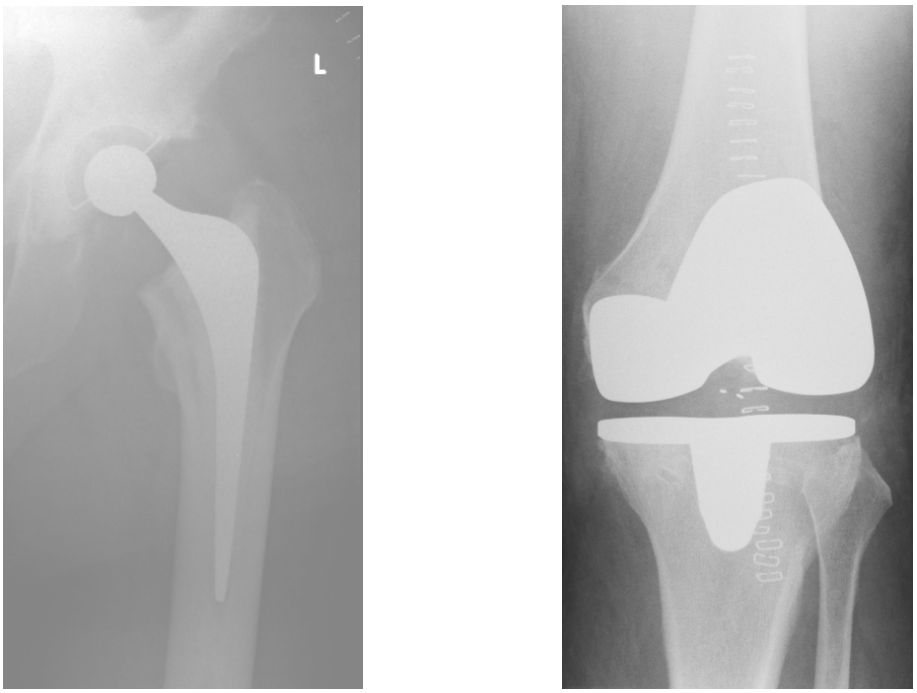


Figure 2.3 *A total hip replacement X-ray is shown on the left. A total knee replacement X-ray is shown on the right*

The hip is described as a ball and socket joint. The socket or pelvis side of the hip is anatomically referred to as the acetabulum, while the ball is known as the head of the femur or thigh bone. In order to replace the femoral head a stem is inserted into the femur and a head placed on top, as can be seen in Figure 2.4.

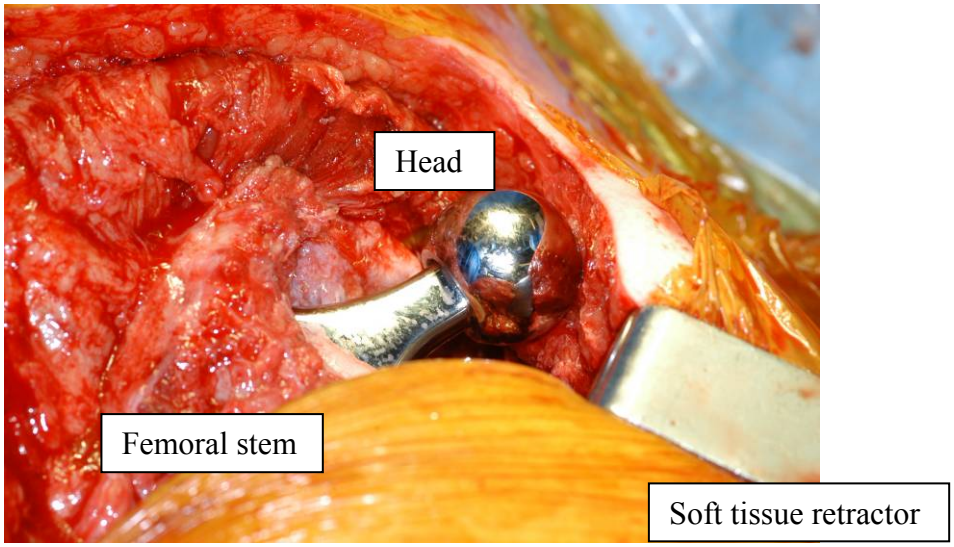


Figure 2.4 *An example of the femoral stem and articulating head of a total hip replacement in situ. The acetabular cup or socket is deep and is not shown*

The knee joint is commonly referred to as a hinge joint. Though this description is not strictly speaking true, it is useful for explaining the key components of a total knee replacement. On one side of the hinge is the lower end of the femur and on the other the upper part of the tibia or leg bone. The two metallic components articulate via a high density polyethylene tray, as can be seen in Figure 2.5.

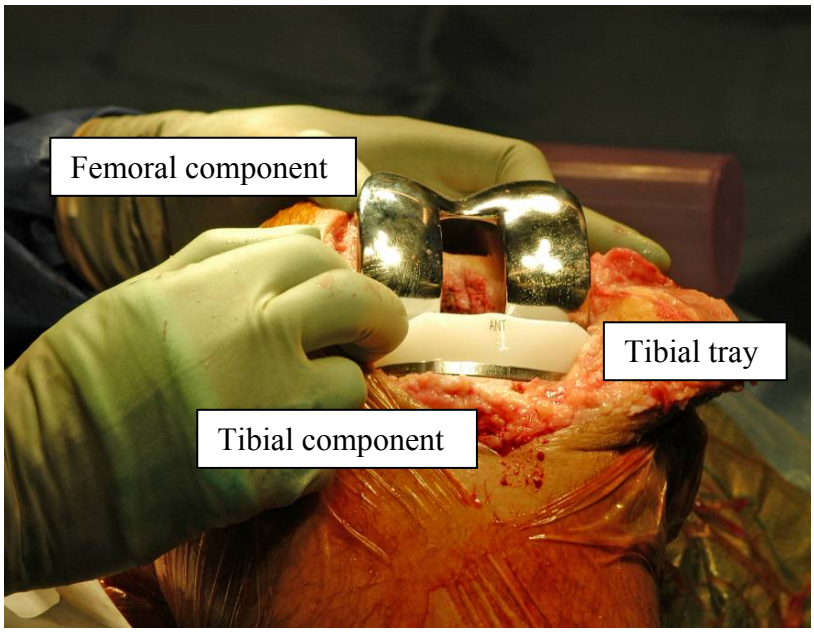


Figure 2.5 *An example of a total knee replacement in situ*

Soon after arthroplasty surgery the patient is encouraged to mobilise. Once safe and independent on their feet, the patient can return home to gradually increase their level of activity. Over the subsequent weeks to months they will continue with their rehabilitation and return to a good level of function free of their previous symptoms.

In the 2010 annual report by the National Joint Registry (NJR) for England and Wales it was noted that 79,413 total hip replacements (THR) and 84,527 total knee replacements (TKR) had been performed in 2009. In Scotland, the trend was slightly different with a greater number of THRs performed than TKRs. The Scottish Arthroplasty Project 2010 report noted that there were 7,168 THRs in 2009, and 6,884 TKRs. In the Southern General Hospital (SGH) alone, there were 566 joints replaced (305 THR, 261 TKR) in 2009 (Southern General Arthroplasty Database). With such large volumes of joint replacements being performed each year, things can go wrong. Some potential problems of arthroplasty surgery are discussed in the next section.

2.1.1 Complications of arthroplasty

There are numerous potential complications of joint replacement surgery, most of which are outwith the scope of this brief overview. Some of the frequent or major medical problems include blood clots (thromboembolism), heart attack (myocardial infarction), low blood count (anaemia) and infection (chest or urinary).

The surgical complications are usually more local to the site operated upon. The joint may feel unstable, which can lead to dislocation in THR. Alternatively, there may be wear of the components, frequently a polyethylene related problem. The implant may also simply loosen from the bone with time. The most challenging problem that arthroplasty surgeons face, however, is infection. A summary of the NJR 2010 annual report showed that it was the second most common reason for TKR revision surgery. The list of the other causes is summarised in Table 2.1. In the same report for THR, infection was again noted to be one of the leading causes for revision surgery (10%).

Table 2.1 *Reasons for revising a TKR, modified from the NJR 2009*

Reason for revision	Percentage
Aseptic loosening	24%
Infection	**18%**
Pain	14%
Instability	11%
Wear of polyethylene component	9%
Malalignment	6%
Stiffness	4%
Periprosthetic fracture	2%
Other	12%

The precise costs in terms of human life and financial expense are difficult to estimate. The patient inevitably requires a prolonged hospital stay, antibiotic therapies, and further operations in the form of washouts and of course potentially a revision procedure. The disruption to the patient's personal life is frequently

devastating and their return to work unpredictable. Evans et al (2009) report that the Centers for Disease Control and Prevention estimate that there are approximately 290,000 surgical site infections per year in the USA. They have estimated that this costs between $1-10 billion in direct and indirect medical costs, irrespective of the societal financial burden. Furthermore, they report that approximately 8000 patient deaths were linked to these infections, with *Staphylococcus aureus* being the lead pathogen.

2.2 Pathogenesis of prosthetic joint infections

The pathogenesis of implant related infections differs from other post-surgical infections because of the presence of biomaterials. The mechanism of infection is thought to involve a complex interaction between the microorganisms, the implant and the host.

The initial surgical incision creates an 'immuno-competent fibro-inflammatory zone' which is susceptible to colonisation by microbes. As the critical dose of microorganisms required for a clinically relevant infection is significantly reduced in the presence of foreign material, the field is at risk (Campoccia et al 2006). In addition, as orthopaedic implants are devoid of a microcirculation, a crucial host defence mechanism is not present (Zimmerli et al, 2004). The prosthesis also experiences micromovements which lead to wear and in turn local inflammatory responses which may further deplete the immune defence. Furthermore, the chemical nature of the implant along with its surface topography will influence microbial adhesion and subsequent colonisation (Campoccia et al 2006).

Bacteria responsible for prosthetic joint infections are often opportunistic. They take advantage of the weakened implant-tissue interface for adsorption. The microorganisms may then interact with the biomaterial surface by joining host extracellular matrix proteins or indeed forming their own adherent biofilm. Existence within a biofilm is a survival mechanism by which the bacteria live clustered and resist environmental factors such as antibiotics and the host immune system, making them very resistant to killing (Trampuz et al, 2006). For these reasons prosthetic

joints are particularly susceptible, and strategies are sought to minimise the chances of any infection developing.

2.3 Preventing infection

There are many steps taken to reduce the risk of a patient developing an infected joint replacement. Some measures have been in place for many years, while some are recent advancements in technology. They may be classified according to their timing in relation to the surgery, as can be seen in the following three sub-sections.

2.3.1 Preoperative

This is the time period prior to the patient having their operation, and really begins once the patient is deemed a potential candidate for a THR or TKR. Patients at high risk of an infection must be identified and optimised prior to surgery. Table 2.2 lists some key modifiable risk factors and some potential solutions.

Table 2.2 *Modifiable risk factors for prosthetic joint infection with potential solutions. Adapted from Evans et al 2009*

Modifiable risk factor	**Potential solution prior to THR/TKR**
Local or remote infection	Course of antibiotics
Diabetes	Blood sugar optimisation
Smoking	Cessation
Malnutrition/obesity	Dietetics advice/weight loss as appropriate
Anaemia	Cause identified and treated +/- transfusion
Rheumatoid/Inflammatory arthritis	Optimise medical therapy, minimise steroid use
MRSA	Topical decolonisation regime

The table is clearly not exhaustive, but covers the common groups of problem patients encountered in the clinical setting. Most hospitals have a preoperative assessment clinic where these issues are identified and addressed as best possible.

2.3.2 Intraoperative

This is the period of time when the patient is in the operating room environment. Prophylactic antibiotics are regarded as one of the most effective methods in the battle against infection. There are many controversies over the choice of antibiotic, with no universal agreement. It is thought that the antibiotic should ideally penetrate tissue well, have a long half-life to cover the procedure, be non-toxic and cheap (Hanssen et al, 1997). The optimal time to administer the prophylactic antibiotics is prior to skin incision (Burke, 1961).

The operating theatre itself may be a source of infection with many pieces of equipment (Figure 2.6). A deep clean is performed at the end of every day, with a surface clean after each operative case to reduce the potential surface contaminants.

Figure 2.6 *An example of an orthopaedic theatre at the SGH*

Lidwell et al (1982) demonstrated that ultra clean laminar airflow systems are effective in reducing the incidence of sepsis in total hip and knee replacement surgery from 1.5% to 0.6%. This is now routine practice for all orthopaedic arthroplasty theatres, and is shown in Figure 2.7. Holten et al (1990) provided specific guidelines

suggesting that the bacterial concentration should be less than 180 CFU/m^3 during an operation in a conventional theatre and less than 10 CFU/m^3 in an ultraclean theatre. These remain the accepted benchmarks to reduce surgical infection.

Figure 2.7 *An example of a laminar airflow system*

As well as the theatre environmental factors, there are many precautions that the surgical team must undertake. Prior to any operation the surgeons and nursing staff perform a hand scrub. The use of a nail cleaner and scrub brush (Figure 2.8) followed by rigorous cleaning for a total of ten minutes has been mandated (Khan et al 2003).

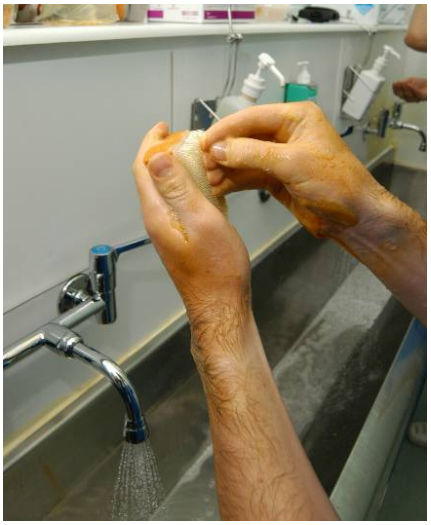


Figure 2.8 *Surgical hand scrub*

After scrubbing a body exhaust suit is typically worn for arthroplasty surgery. The evidence for this is controversial, but is based on work by Sir John Charnley (1979) that suggested that this provides the patient with additional protection from bacterial shedding from the hair, skin and mouth of the surgical team. In addition, double gloves are worn due to the large number of perforations during orthopaedic surgery, and the theoretical protection that an extra layer of gloves gives to both the patient and surgeon (Hanssen et al, 1997). A picture of the full attire is shown (Figure 2.9).

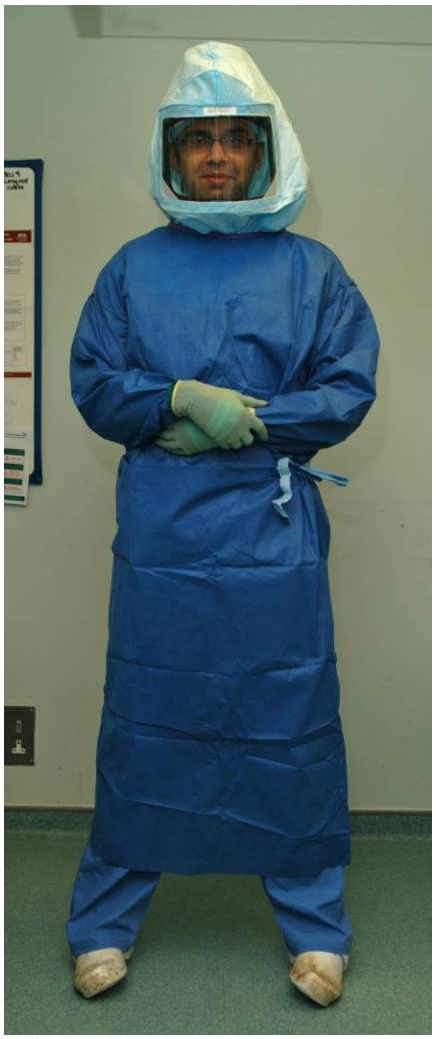


Figure 2.9 *Body exhaust suit with surgical gown, gloves and shoes*

The surgical team are then ready to prepare or clean the relevant limb of the patient. There is much debate in the literature as to the ideal antiseptic agent, with the two most commonly used being either povidone-iodine or chlorhexidine gluconate based. Most data suggest equal efficacy in decreasing the initial bacterial skin contamination

(Fletcher et al, 2007). The agent of choice at the SGH is iodine based, and the prepping of a limb can be seen in progress in Figure 2.10.

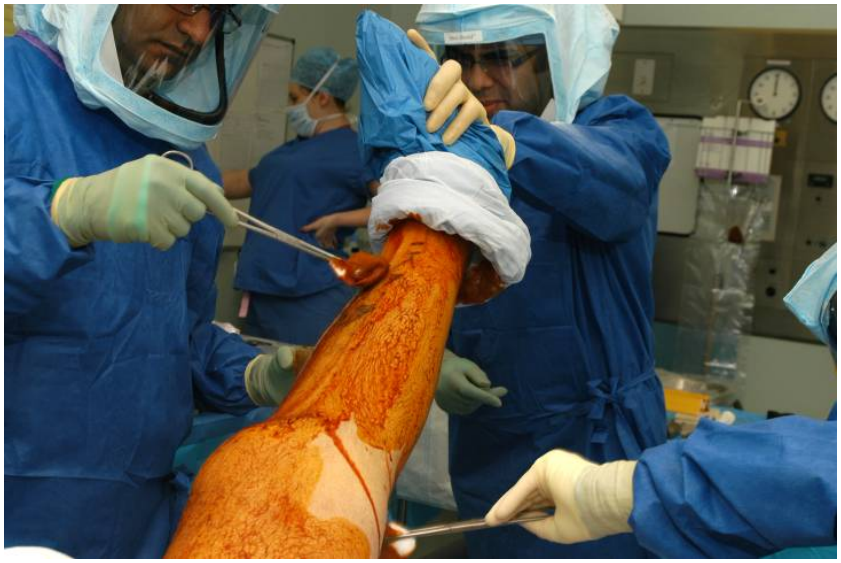


Figure 2.10 *A leg is cleaned with a povidone-iodine based antiseptic prior to total knee arthroplasty surgery as indicated by the TKA marking above the ankle*

The next intraoperative step to minimise the risk of infection is the application of surgical drapes. These effectively seal off the groin and perineum, areas that are known to harbour bacteria. An occlusive iodophor-impregnated plastic drape is then applied over the surgical site. This has been shown to improve wound contamination rates, but no associated reduced rates of infection have been reported (Fletcher et al, 2007). Nonetheless, for theoretical reasons these are widely used. Fully prepared and draped limbs ready for hip and knee replacement surgery can be seen in Figure 2.11.

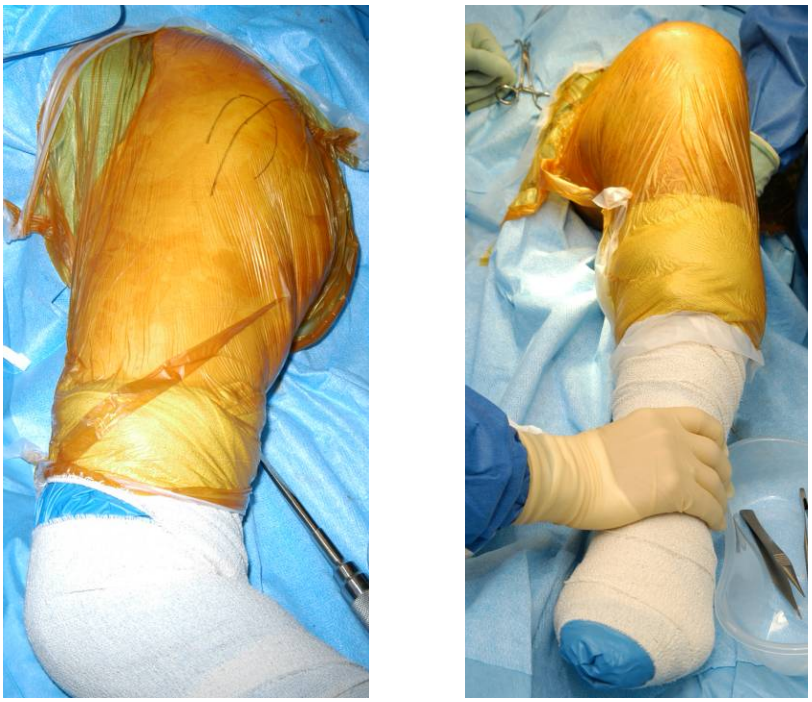
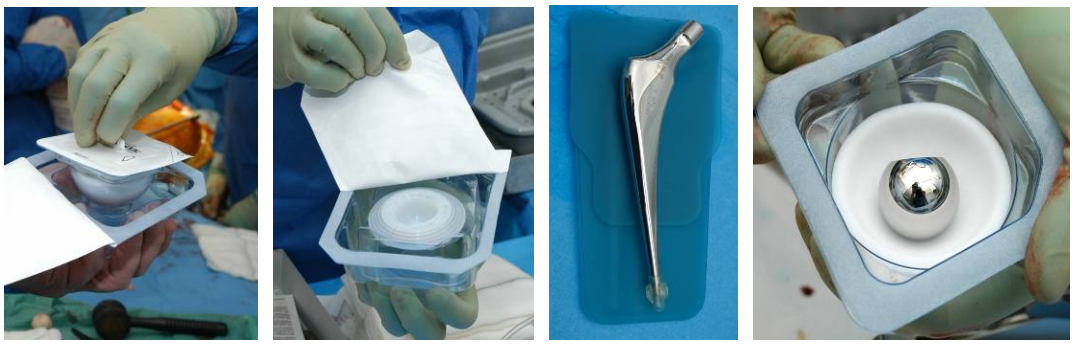


Figure 2.11 *A fully prepared and draped hip (left) and knee (right)*

All of the equipment used during the operation is sterile. In particular, all of the surgical instruments are autoclaved and checked prior to use; while the prosthetic implants are kept in individually sealed sterile packets. Some examples of the packaging precautions for a total hip replacement (Figure 2.12) and a total knee replacement are shown below (Figure 2.13).

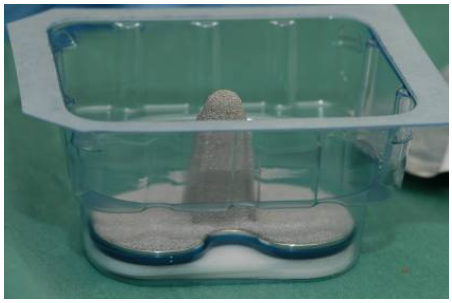


(a) (b) (c) (d)

Figure 2.12 *Implants for a total hip replacement. a) The outer packaging followed by a further protective and sealed internal layer over the b) acetabular cup. Similar measures are in place for the c) femoral stem and the d) head*

(a)

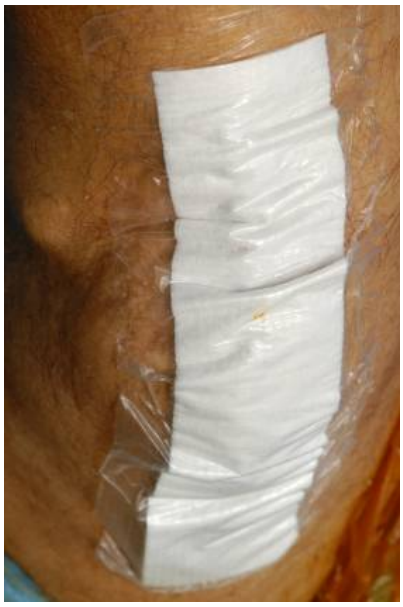
(b)



(c)

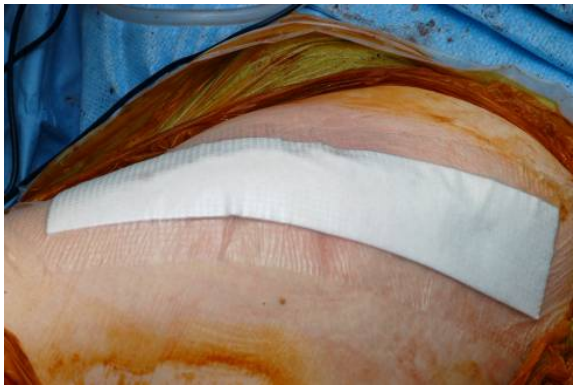
Figure 2.13 *Implants for a total knee replacement. The individually packed and sealed a) femoral component b) tibial insert and c) tibial tray*

Once the operation is complete, the wound is closed with a combination of sutures and/or surgical clips, and a dressing is applied. This is usually in the form of a non-adhesive but absorbant pad, with a peripheral seal. The occlusive seal of the dressing is thought to reduce post-operative rates of infection (Fletcher et al 2007). After a knee replacement this is supplemented with a sterile bulky dressing, which provides some pressure on the wound to reduce bleeding, and acts as a splint to prevent excessive movement in the immediate postoperative period. The hip is a more difficult area to which to apply a bulky dressing, and for that reason is not routinely applied. Examples of the dressings used to minimise exposure to pathogens is shown in Figure 2.14.



(a)

(b)



(c)

Figure 2.14 *Surgical dressings for a) a knee b) bulky dressing applied on the same knee and a c) lightly padded dressing following a hip replacement*

2.3.3 Postoperative

This is the period of time after the patient has had their arthroplasty surgery. Although this is indefinite, it is a term which is usually applied until the surgical site has healed. Wound management is therefore the main focus in preventing infection. It is recommended that the surgical dressing should be maintained for between 24-48 hours (Mangram et al 1999). Though some surgeons may choose to leave the original surgical dressing on for longer, there is little evidence for this. The key concept relates to keeping the wound clean and dry, and that any dressing changes should be performed using an aseptic technique (Bosco et al 2010). In addition, antimicrobial dressings may be used, but are not routinely available in the UK.

The overall hospital environment is the other major area where many precautions are in place to minimise the risk of joint infection following surgery. Though routine cleaning measures are in place, Dancer et al (2008) demonstrated in a ward based study that by simply employing additional cleaning staff there was a 32.5% reduction in environmental contamination. Similarly, Boyce et al (2009, 2008) advocate the role of hydrogen peroxide vapour (HPV) for cleaning and reducing microbial contamination. Unfortunately both techniques are limited by their cost, while the use of HPV may not be considered practical i.e. staff and patients need to vacate rooms and the rooms need to be sealed. Simple steps, such as hand hygiene procedures, which include the easy availability of hand sanitisers in between each patient contact episode, have successfully been put in place by most hospital trusts. However, there is limited evidence to show that this has actually reduced nosocomial infection rates.

2.4 Diagnosis of an arthroplasty infection

Diagnosis of infection is most commonly based on a combination of the patient's symptoms and the results of the subsequent clinical investigations. Arthroplasty infections in broad terms can be classified (Coventry 1975) as early (less than 3 months after surgery), delayed (3-24 months after surgery) and those that present late (greater than 24 months after surgery).

In those patients who present early, the clinical symptoms are frequently wound associated. These include constant pain, erythema and warmth and frequently have a history of a leaking or discharging wound. The delayed and late presentation group of patients usually have more subtle signs that will include aching from the joint, but often other symptoms are non-specific leading to diagnostic dilemmas. Both the early and delayed group of infections are thought to be acquired at the time of prosthesis implantation, with the late infections occurring secondary to haematogenous spread (Zimmerli et al 2004).

The clinical investigations performed to assess whether a prosthetic joint is infected can be further subdivided into those that are blood based, radiological or require fluid/tissue samples. The investigations act as a diagnostic aid, but no single test has perfect sensitivity and specificity and should ideally not be used as screening tests.

The routine peripheral blood parameters assessed in the potentially infected arthroplasty patient include a white cell count (WCC), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). More recently interleukin-6 (IL-6) has also been suggested (Bottner et al 2007). Berbari et al (2010) report in their review of the literature that IL-6 is best for diagnostic accuracy, followed by CRP, ESR and WCC. IL-6 is not routinely measured in NHS practice due to cost and lack of availability, along with the reported high diagnostic yields from CRP and ESR results when they are combined (Austin et al 2008, Greidanus et al 2007).

The radiological analysis of any prosthetic joint includes plain X-rays taken in perpendicular planes. This allows assessment of some non-specific changes that may be suggestive of infection. The changes typically noted are periosteal reaction,

osteolysis and bone resorption around the implant, an example of which can be seen in Figure 2.15.

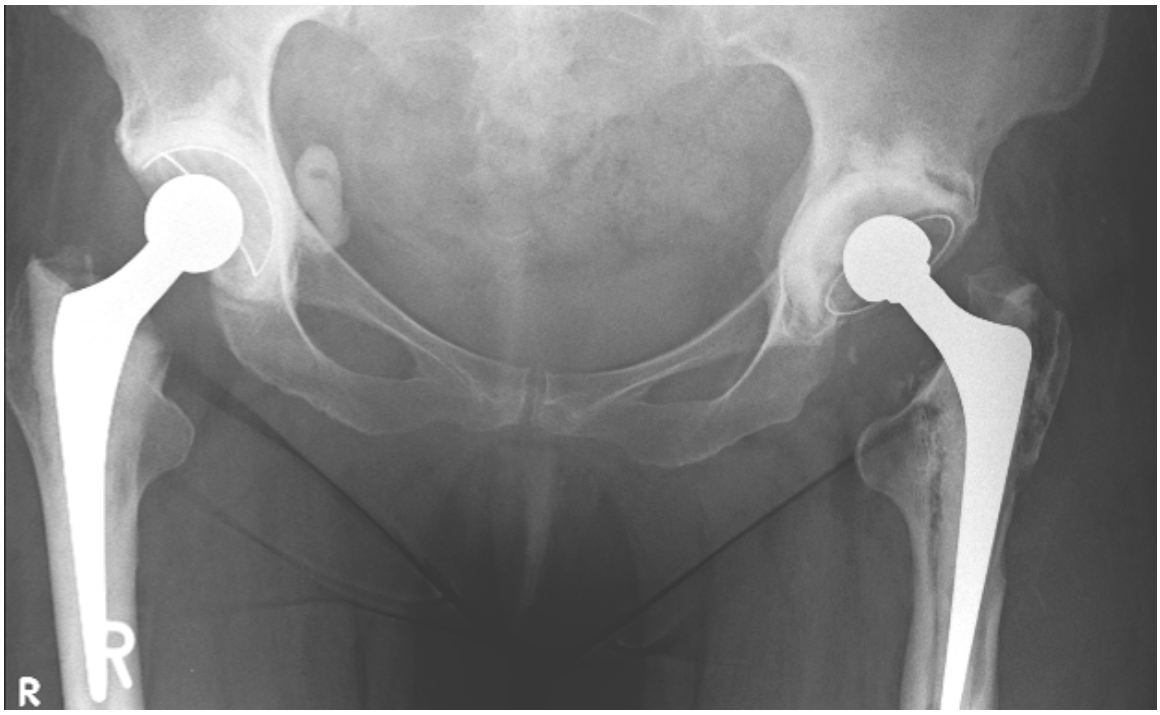


Figure 2.15 *A pelvis X-ray showing bilateral THRs. The left THR has marked osteolysis and bone resorption around the femoral component, with a normal right THR for comparison*

These changes describe a local inflammatory response with resultant bone destruction over time. Another imaging modality commonly employed in the infection setting is a radionucleotide scan. There are various radioisotope markers including technetium-99m, indium-111 and gallium-67 that can be used to give an indication of bone turnover, with the area affected by infection lighting-up (Bauer et al, 2006). The appearance of the previously noted infected left THR (Figure 2.15) can be seen on radionucleotide scan in Figure 2.16.

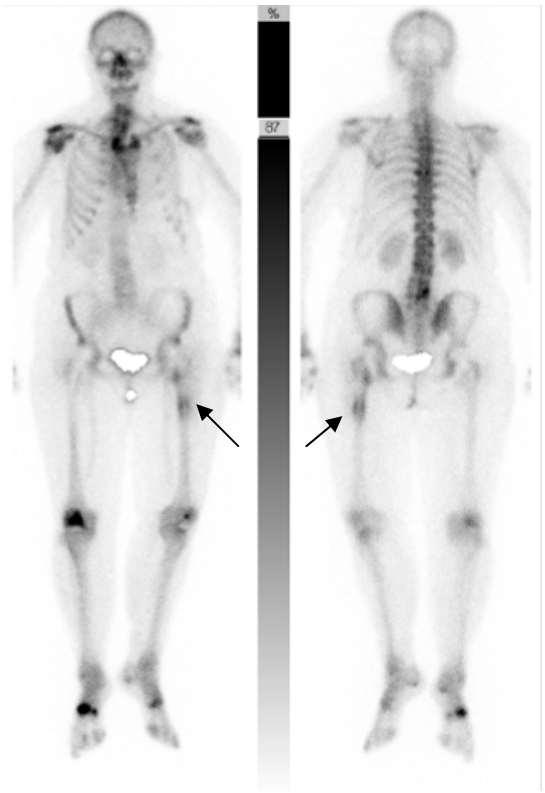


Figure 2.16 *A radionucleotide scan showing increased uptake around the left hip (shown by the arrows) compared to the right. There are other incidental lit-up areas*

A microbiological diagnosis of fluid or tissue samples taken from and around the suspected area of infection is presumed to be the gold standard. However, getting microbiological confirmation is often challenging and a subject of much debate, with new tests and theories continuously proposed. Earlier papers hypothesised that in the setting of a radiologically loose implant requiring revision surgery, a simple Gram stain may provide a quick and cheap answer as to whether the joint is infected, or has naturally loosened. However, this idea has firmly been refuted on the basis of a lack of sensitivity (Atkins et al 1998, Spangehl et al 1999). The fluid surrounding an infected joint has been an area of further exploration. A synovial or joint fluid cell count and differential has been reported as being a useful adjunct. Ghanem et al (2010) found that a leukocyte count of >1100 cells/$10^{-3}$$cm^3$ and neutrophil percentage of >64% could be used to diagnose infection. Though the studies in this area are promising, no consensus has been reached as to the exact cut-off values or units that should be used. Frozen sections of intraoperative samples have also been successfully used for the diagnosis of infection. Nunez et al (2007) believe that immediate

laboratory analysis in equivocal cases provides a rapid and accurate answer. Though this histological examination seems ideal, it is limited in certain settings by pathologist availability and the potential for confounding factors such as implant membranes that may confuse the picture (Bauer et al 2006).

Clearly the diagnosis of infection can be difficult, and any measures that can be taken to prevent it from ever occurring are welcome. For those with an established joint infection however, appropriate treatment must be tailored to the patient. Some of the key factors in managing such cases are covered in the following chapter.

2.5 Principles of treating a patient with an infected prosthesis

The management of a patient with an infected arthroplasty is difficult, and is dealt with on an individual case basis. However, in broad terms the management can be divided into medical and surgical treatments, with a combination of both modalities usually employed. This section provides a brief overview of both sets of therapies, and discusses how the timing (early versus delayed/late) of the infection may influence management.

2.5.1 Medical management of joint infection

The mainstay of medical or non-operative management is antibiotic therapy. This should ideally be given after the isolation of an organism with known sensitivities, but this is often not possible due to the diagnostic problems highlighted in the previous section. In such cases empirical 'best guess' antibiotics are prescribed based on the patient's general health. The method of delivering antibiotics, and the duration of therapy remain a contentious issue in the literature. Bernard et al (2010) advocate a regime of intravenous medication for one week, followed by oral for a further five weeks; a total of six weeks therapy. They report that this is preferred over traditional longer courses, which conferred no additional benefit in their study.

An alternative approach must be adopted in patients for whom surgery is contraindicated, or those who simply refuse. Lifelong suppressive oral antibiotics may be sufficient to control the clinical manifestations of infection, but will almost certainly not eradicate it (Trampuz et al, 2006).

2.5.2 Surgical management of joint infection

There are many potential surgical options dependent on the timing of the infection in relation to arthroplasty surgery, the pathogen responsible and the medical condition of the patient. Simple wound debridement with or without exchange of liners is a long established first line treatment in the group of early infections (Tsukayama et al 1986). For more deep seated and chronic infections, the prosthesis is often removed. Whether this should be immediately exchanged for a new prosthesis following thorough lavage or after a delayed time period (usually six weeks) is a matter of much debate. Oussedik et al (2010) feel that in selective revision surgery cases immediate exchange is better. In cases where the repeat surgery has failed on multiple attempts, joint fusion in the form of an arthrodesis, joint excision or amputation may be necessary (Bengston et al, 1989).

To avoid these catastrophic surgical complications alternative treatments are sought. Light technology is an area which is continuously explored, some of the details of which will be covered in the next section.

2.6 The role of light technology in the fight against infection

The potential of antimicrobial light as a treatment adjunct has long been considered. In this section some of the available technologies are described, along with their respective roles. In addition, a review of HINS-light technology and some of its uses thus far are discussed.

2.6.1 Ultraviolet light

Ultraviolet (UV) light is a region of the electromagnetic spectrum with wavelengths shorter than that of visible radiation, but longer than X-rays. UV wavelengths are 100-400 nm. The spectrum of UV light can be further subdivided into long-wave (UV-A), medium-wave (UV-B) and short-wave (UV-C). These subdivisions are based on their effects on tissue, commonly human skin (Bolton 2001). Photobiochemical reactions are induced by each subdivision of UV light that leads to distinct but overlapping damage to nucleic acids, which is a direct result of absorbed radiation energy or photons (Block, 1991). The damage induced corresponds to the DNA absorbance spectrum, which is maximal for UV-C (100-290 nm). This falls significantly for UV-B (290-320 nm) and is almost negligible for UV-A (320-400 nm). Therefore, UV-C has the greatest potential for DNA damage. However, for a natural UV source i.e. the sun, it is largely screened out by the stratospheric ozone layer before reaching the earth's surface, and consequently UV-B is more often implicated for adverse effects such as skin cancer (Miller et al, 1999).

Wavelengths less than 200 nm are strongly absorbed by oxygen within air, and are named vacuum UV; as such wavelengths are indeed only active in a vacuum. Therefore, the biologically active portion of the electromagnetic spectrum lies in the range of 200nm-320 nm. This is also known as the germicidal irradiation range because of its ability to inactivate microorganisms efficiently (Block, 1991). Absorption of UV-B and UV-C photons induces damage to genomic DNA, with a single photon having the potential to induce a carcinogenic or even a lethal effect (Sinha and Häder, 2002). In turn, it is these wavelengths which are exploited by UV light sources for a broad spectrum of disinfection technologies. Ultraviolet

Germicidal Irradiation (UVGI) is increasingly used for indoor air quality improvement, disease control and has biodefence applications (Bahnfleth et al, 2005).

Microorganisms have varying responses to UVGI. Chang et al (1985) used a collimated beam for exposure at 254 nm and found that the most susceptible organisms were Gram-negative bacteria followed by Gram-positive bacteria, viruses, spores and protozoa. In addition, the extent of UV damage to the pathogen is influenced by the dose applied. Microbial inactivation is actually accomplished at relatively low doses of UV radiation, and therefore can be applied inexpensively (Block 1991).

More recently interest has developed in the use of pulsed ultraviolet (PUV) light as a rapid sterilisation technology with a variety of commercial and industrial applications ranging from medical manufacturing to processing water, air or foods. PUV, as the name suggests, delivers a high peak power and high dose of broad-spectrum UV light in very short time span. The process is much more effective than conventional UV sources (Block 1991, Elmnasser et al 2007). The role of PUV light has been investigated in various settings by the ROLEST team. Wang et al (2005) demonstrated the wavelength-dependent germicidal efficacy of PUV for *E.coli* and Lamont et al (2007) showed inactivity of both poliovirus and adenovirus in response to PUV. Anderson et al (2000) reported the inactivation of both food-borne enteropathogenic bacteria and fungi using PUV, while Maclean et al (2004) described how the technology could be used as an air disinfectant for university lecture rooms.

UV light unfortunately has adverse side effects for humans, particularly noted on the skin and the eyes. Erythema or redness (as in sunburn), accelerated skin aging and neoplasms of the skin are the main dermatological complaints. Cataracts and painful photokeratitis are the principal ophthalmological problems (Bolashikov et al 2007). As a result of these safety issues, individuals cannot be directly exposed to UV light disinfection technologies. Another factor limiting the application of UV light is the potential for degradation of some plastic materials (Zhao et al 2006).

The efficacy of UV light for air decontamination in the operating theatre has also been questioned. Most air decontamination systems will have their UV light source

contained within housing. However, in some cases where UV is used for whole-room decontamination, the room must be vacated and sealed off during light use, which has practical implications. Furthermore, as discussed in section 2.3.2, there are multiple changes of air in the operating theatre, and UV light is not as effective in this setting. This prompted the authors of a recent review to conclude that UV-light is not a stand-alone technology (Memarzadeh et al, 2010). The combination of the significant side effects and unclear benefits in the theatre setting of UV light have necessitated further research in this field.

2.6.2 Blue light

In addition to UV light, visible light has been shown to have antimicrobial properties, with wavelengths in the blue region having the highest antimicrobial activity. Previously, the main field of antimicrobial work with blue light was in relation to dermatological conditions, primarily acne, where *Propionibacterium acnes* is the causative bacteria (Tzung et al, 2004). Though it is part of the normal microflora of the skin, it may cause inflammation and acne if trapped in a hair follicle (Todar 2011). *P. acnes* naturally produce lots of intracellular metal-free porphyrins which absorb light energy at the blue spectrum without the need for trigger molecules. These intracellular porphyrins can therefore act as endogenous photosensitisers. The use of blue light (typically 400-420 nm) for acne therapy leads to excitement of these intracellular porphyrins, singlet-oxygen production and eventually *P.acnes* destruction (Papageorgiou et al, 2000). Blue and red light combination treatments have also been trialled by Papageorgiou et al (2000). The authors report that the red light (660 nm) has anti-inflammatory properties that have proven to be superior to solely blue light exposure.

Work by Guffey et al in 2006 suggests further bactericidal uses of blue light. The authors report efficacy of both 405 nm and 470 nm blue light against both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The same wavelengths of blue light were applied to methicillin-resistant *Staphylococcus aureus* (MRSA) in two separate *in vitro* studies by Enwemeka et al (2008, 2009). In both papers the authors found that blue light was efficacious in killing bacteria in a dose-dependent manner.

2.6.3 405 nm High-Intensity Narrow-Spectrum light (HINS-light)

HINS-light is a narrow band of visible violet/blue light, with peak wavelength of 405 (±5) nm that induces inactivation of a range of bacterial pathogens. The technology works via a photodynamic inactivation effect which is triggered by absorption of the light. This in turn leads to photosensitisation of intracellular molecules and the production of reactive oxygen species, primarily singlet oxygen. The subsequent result of this is bacterial cell death (Maclean et al 2010). Use of 405 nm HINS-light for inactivation of pathogenic microorganisms has been developed by researchers in the ROLEST at the University of Strathclyde. In this section the evolution of the technology, research and current applications will be discussed.

Early work was based on the reported efficacy and bactericidal properties of both visible light and PUV discussed in the previous sections (2.6.1 and 2.6.2). This led the ROLEST team (Maclean et al 2008a) to specifically investigate and characterise the germicidal effect of high-intensity visible light on *S.aureus*. They found that the visible wavelength range of 400-500 nm induced staphylococcal inactivation. By using a combination of filters they were able to identify that bandwidths of 430-500 nm did not cause significant inactivation, with the most bactericidal activity noted at 405 ± 5 nm. This narrow-spectrum light was reported to also be effective against MRSA, but less so against *E.coli*.

Maclean et al (2008b) followed the previous study by testing the hypothesis that staphylococcal inactivation through visible-light exposure was a result of photo-excitation of endogenous porphyrins. Oxygen is essential for photodynamic inactivation, and hence a series of oxygen enhancement and depletion experiments using scavengers were conducted. The presence of oxygen for visible light inactivation of *S.aureus* was important, and supported the hypothesised mechanism of inactivation.

The ROLEST team then assessed the susceptibility of a variety of medically important bacteria to inactivation by the 405 nm light (Maclean et al 2009). The pathogens chosen were those commonly associated with hospital-acquired infections,

and both gram-positive and gram-negative species were successfully inactivated. For a given reduction in population, the authors noted that gram-negative species required longer exposure times than gram-positive species. Murdoch et al (2010) studied the effect of high-intensity 405 nm visible light on selected gram-negative enteric pathogens. Light exposure led to effective inactivation of these food-related bacterial pathogens, with *Campylobacter jejuni* identified as the most sensitive organism. The authors of both papers acknowledge that visible-inactivation is much less efficient than UV-inactivation, but highlight the greater operational safety it offers, and in turn the potential for widespread applications.

The laboratory success of the HINS-light technology led to the development of a large scale decontamination system. This system was introduced into a clinical setting by Maclean et al (2010) in conjunction with clinical collaborators at the Glasgow Royal Infirmary. The authors aimed to evaluate the role of this novel decontamination technology they called 'High-Intensity Narrow-Spectrum light Environmental Decontamination System' (HINS-light EDS). This is a ceiling-mounted device emitting high intensity 405 nm light that provides continuous disinfection of the air and contact surfaces in occupied environments. The HINS-light EDS was tested in hospital isolation rooms used to treat burns patients. Use of this system led to an almost 90% reduction in surface bacteria levels when the room was unoccupied, and between 56-86% when an infected burns patient was present. The results suggest that the HINS-light EDS can result in bacterial decontamination in a clinical environment. Bache et al (2012) have recently expanded the use of HINS-light EDS to both the outpatient and inpatient setting with similarly positive results.

The risk of wound related infections with all operations, and in particular arthroplasty surgery, has been highlighted throughout this chapter. McDonald et al (2011) have recently investigated what effects HINS-light has on an *in vitro* model of wound healing. The authors used a fibroblast-populated collagen lattice (FPCL) model, and exposed it to various doses of HINS-light. They then analysed the response of the lattice at a cellular level. The FPCL contraction and cell numbers were found to be unaffected by the light at therapeutic doses up to 18 Jcm^{-2}. In combination with previous work, this suggests potential roles of HINS-light in maintaining wound sterility or as wound disinfection tool.

2.7 Summary

Hip and knee replacement operations are commonly performed throughout the UK with a high patient satisfaction. Occasionally there are complications, and infection is among the most serious. Though many steps are taken to minimise this risk, it has not been completely eradicated. The prosthetic joint infection may be difficult to diagnose, and no treatment modality currently available is fully effective.

Bactericidal light technology has been available for some time, but there have been many limitations restricting its use. Recent advances in visible light technology now offer a theoretically safe treatment for a problem challenging the orthopaedic community worldwide. In this thesis I aim to assess if HINS-light can inactivate pathogens isolated from infected arthroplasty cultures.

Chapter 3

Epidemiology of arthroplasty infection

3.0 General

This chapter discusses the microorganisms isolated from infected arthroplasty cases in the Department of Microbiology at the SGH over a period of one year.

3.1 Microbial culture results

All of the positive cultures from lower limb arthroplasty at the SGH Department of Microbiology were recorded in a database by the laboratory technicians. Between the 1st May 2009 and 30th April 2010 there were a total of 51 clinical isolates noted. There were slightly less TKRs performed than THRs over the study period as discussed in Chapter 2, and accordingly there were fewer isolates from TKRs. More infections were observed in surgery on the right hand side of the patient than the left. No obvious explanation was apparent. These data are summarised in the Figure 3.1.

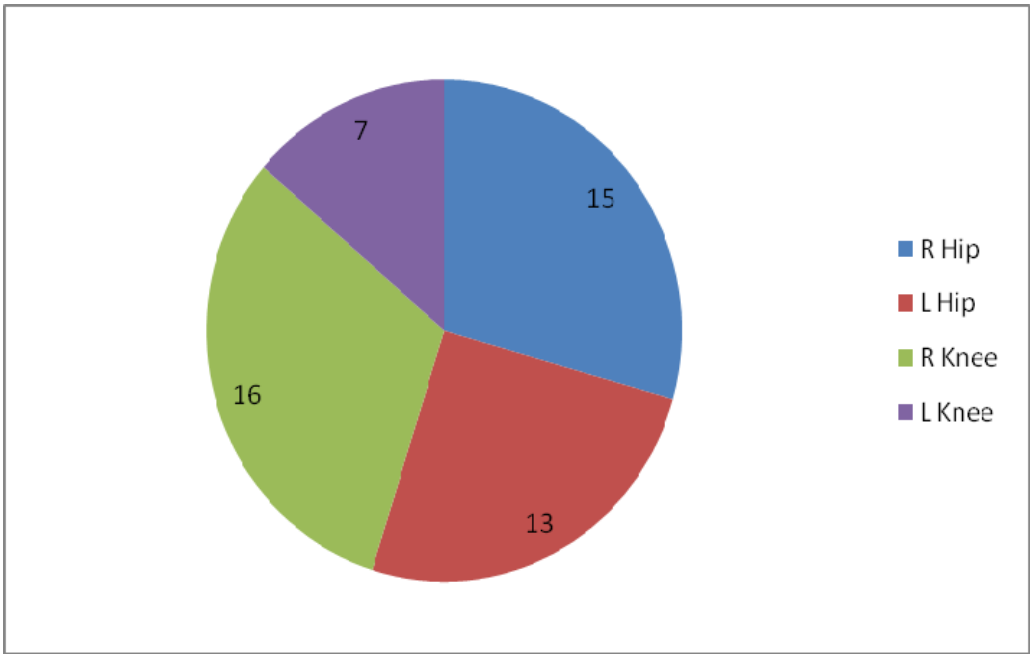


Figure 3.1 *Infected arthroplasty positive cultures by site and side*

There were various sampling methods used by the clinicians. Most commonly the positive cultures were obtained from swab (26/51) or tissue (23/51) specimens. The remaining two isolates were grown from pus aspirates.

The distribution of all positive specimens by microorganism can be seen in Table 3.1.

Table 3.1 *Positive cultures grown from infected lower limb arthroplasty over one year from the Southern General Hospital Department of Microbiology (* Gram stain not performed for Candida albicans as it is a yeast)*

Gram Stain Result	Microorganism	Positive cultures n = 51
+	*Staphylococcus aureus*	23
+	*Coagulase negative Staphylococcus*	6
+	*Staphylococcus epidermidis*	2
+	*Corynebacterium striatum*	2
+	*Enterococcus* species	1
+	*Enterococcus faecalis*	1
+	*Micrococcus* species	1
+	*Streptococcus* species	1
+	Group B *Streptococcus*	1
+	*Streptococcus pneumoniae*	1
-	*Escherichia coli*	4
-	*Klebsiella pneumoniae*	4
-	*Pseudomonas aeruginosa*	2
-	*Serratia marcescens*	1
N/A *	*Candida albicans*	1

It is apparent that the majority of arthroplasty infections were caused by gram positive bacteria, with *S. aureus* the most commonly isolated pathogen. These findings are in keeping with recent studies (Campoccia et al 2006, Sharma et al 2008, Trampuz et al 2006). None of the *S. aureus* samples identified by the hospital Department of Microbiology were methicillin-resistant.

3.2 Characteristics of microorganisms associated with arthroplasty infection at SGH

This section provides some background information on the genus and/or species of the isolates identified in Table 3.1.

3.2.1 Staphylococci

The genus *Staphylococcus* is a group of Gram-positive cocci that are catalase positive and facultatively anaerobic. They occur in microscopic clusters that classically resemble grapes. Bacteriologic culture of the nasopharynx and skin frequently yields staphylococci. Though numerous species of *Staphylococcus* are described, only *S. aureus* and *S. epidermidis* have significant interactions with humans [Block, 2001]. Both species led to clinically relevant arthroplasty infections.

3.2.1.1 *Staphylococcus aureus*

This was the single most common pathogen identified as noted in Table 3.1. *S. aureus* is approximately 1μm in diameter and is characterised by the presence of the coagulase enzyme. Other staphylococci are coagulase negative. *S. aureus* colonies have a typical golden-yellow appearance when grown on plate culture. Disease is caused through the production of toxins or via the direct invasion and destruction of tissue (Murray *et al,* 1998).

3.2.1.2 *Staphylococcus epidermidis* and other coagulase-negative staphylococci

S. epidermidis is the most common species of the coagulase negative staphylococci (CNS), though over 30 are recognised. *S. epidermidis* is a universal skin commensal associated with opportunistic infections, particularly of biomedical devices such as prostheses. The colonies are typically white in appearance, but morphologically similar to *S. aureus*. Few toxins are produced, with the pathogenicity based on the production of an exopolysaccharide that leads to a biofilm (Greenwood et al, 2007). CNS have previously been regarded as apathogenic. However, their important role as pathogens and increasing incidence are now recognised. The virulence factors are not as well established as the other staphylococci, though bacterial polysaccharide components are involved in their attachment to foreign materials such as prosthetic devices (Huebner et al, 1999).

3.2.2 Corynebacteria

This term refers to Gram-positive, aerobic, club-shaped rods that are non-sporing. The *Corynebacterium* genus has numerous medically important species, with the best known being *Corynebacterium diphtheriae*. This is the cause of diphtheria, a local infection of the respiratory tract that produces toxins leading to significant systemic effects. Some corynebacteria are, however, part of the normal flora, finding a suitable habitat all over the human body (Todar 2011).

3.2.2.1 *Corynebacterium striatum*

This species is most commonly a resident of the nose and skin. Though once thought of largely as part of the normal human flora, it is now recognised as a pathogen. Though bone and joint infections are rare, they can be severe, particularly in the immunocompromised host (Bhayani et al, 2009).

3.2.3 Enterococci

The genus *Enterococcus* is closely related to *Streptococcus*, and was formerly classified as faecal streptococci. These organisms are Gram-positive cocci that are

usually in pairs, not chains. As the name suggests, the natural habitat of the genus is the gut or intestines. The species most commonly associated with human disease are *E. faecalis* and *E. faecium* (Ward et al, 2009).

3.2.3.1 *Enterococcus faecalis*

In recent years this pathogen has become recognised as one of the leading causes of nosocomial or hospital-acquired infections. It is associated with urinary and biliary tract sepsis, peritonitis and surgical wound infection. The species can be particularly problematic due to antibiotic resistance. Vancomycin-resistant enterococci were first reported in 1986 and many strains are now showing complete resistance to all conventional antibiotics (Huycke et al, 1998).

3.2.4 Micrococci

The genera *Staphylococcus* and *Micrococcus* are distant members of the same family, *Micrococcaceae*. They are therefore Gram-positive, catalase-positive cocci that are 1-1.8μm in diameter. Though generally a harmless skin resident, it can cause opportunistic infections in the weakened host (Warren 2007).

3.2.5 Streptococci

The *Streptococcus* genus comprises a diverse and complex collection of gram positive cocci. They largely grow as chains or pairs. The genus is commonly classified by either the haemolytic reaction displayed on blood agar or the Lancefield grouping.

The former relates to the degree of red cell lysis surrounding a colony. Beta-haemolysis is complete haemolysis, alpha-haemolysis is partial haemolysis and a third group describes non-haemolytic colonies. The Lancefield grouping is a serological classification based on the major cell wall polysaccharide. There are approximately 20 Lancefield groups recognised (Todar 2011).

3.2.5.1 Group B *Streptococcus*

Group B streptococci may also be known as *Streptococcus agalactiae*, a gram-positive microorganism characterised by the presence of Group B Lancefield antigen. The main virulence factor is thought to be the polysaccharide antiphagocytic capsule, and this bacterium is recognised as a major cause of morbidity and mortality in neonates and pregnant women. In nonpregnant adults it is thought to largely impact upon the elderly or those who have chronic diseases (Farley et al, 1993).

3.2.5.2 *Streptococcus pneumoniae*

This species is alpha-haemolytic and aerotolerant anaerobic. Despite the name suggesting a predilection for the lungs, the microorganism causes infections throughout the body. The polysaccharide capsule is cited as the key virulence factor, with nasopharyngeal colonisation thought to occur in around 40% of the population (Todar 2011).

3.2.6 Enterobacteriaceae

This is a family of Gram-negative bacilli that are made up of over 100 species. As the name suggests, they normally reside in the intestines and may be referred to as coliforms. They are relatively small and non-spore forming, with variable motility. The pattern and type of substrate fermented is used differentiate them. They often cause pneumonia, urinary tract and wound infections and are often resistant to common antibiotics (Ward et al 2009). In the epidemiological study from SGH 3 separate isolates from this family were identified, namely: *Escherichia coli, Klebsiella pneumoniae* and *Serratia marcesans*.

3.2.6.1 *Escherichia coli*

This species is the predominant facultative organism of the gastrointestinal tract. It may grow over a wide temperature range, with some strains surviving temperatures as high as 60°C. *Escherichia coli* may be differentiated from other members of the Enterobacteriaceae family by its ability to ferment lactose amongst other

characteristic biochemical reactions. The various strains possess an array of pathogenic mechanisms. In brief, some of the virulence determinants include adhesins, invasins, toxins and the ability to withstand host defences (Todar 2011).

3.2.6.2 *Klebsiella pneumoniae*

This is a capsulate Gram-negative bacilli approximately 1-2 μm long. Though non-motile, most strains express fimbriae. Similar to *E. coli*, they may grow over wide-ranging temperatures, but optimal growth is at core body temperature (37°C). *K. pneumonia* most commonly causes serious infections in the hospital setting. The principal pathogenic mechanisms are thought to relate to the polysaccharide capsule which protect against phagocytosis, and the long-chain lipopolysaccharide that protects from serum complement (Greenwood et al 2007).

3.2.6.3 *Serratia marcescens*

S. marcescens is an anaerobic, motile, Gram-negative bacillus that was previously thought of as a harmless saprophyte, but is now recognised as an opportunistic pathogen that may cause outbreaks of infection (Yu 1979). It can also grow over a range of temperatures (5-40°C) and in pH levels ranging from 5-9.

3.2.7 Pseudomonads

This term covers a large group of Gram-negative, aerobic bacilli that are mainly found in soil or water. The majority are saprophytes and previously belonged to the genus *Pseudomonas*. Traditionally the genus *Pseudomonas* was applied to wide-ranging new species, but reclassification has led to many new genera including *Burkholderia* and *Stenotrophomonas* (Greenwood et al, 2007). The species most commonly associated with human infections is *Pseudomonas aeruginosa*.

3.2.7.1 *Pseudomonas aeruginosa*

P. aeruginosa is a non-sporing and non-capsulate rod-shaped organism. It is usually motile via 1-2 flagella. It is a strict aerobe that grows on a wide variety of culture

media. *P. aeruginosa* is an opportunistic pathogen causing both community and nosocomial infections with the main virulence factors being exotoxins. The species is notoriously resistant to antibiotics, with the following few still effective in combating it: aminoglycosides, some β-lactams and fluoroquinolones (Ward et al 2009).

3.2.8 *Candida*

Candida is a genus of yeasts that may be found in the commensal flora of about 20% of the normal population. The carriage rate is more prevalent in hospital patients and tends to increase with age. They commonly lead to superficial infections of the skin, nails and mucous membranes. The infection is usually associated with the patient's own endogenous reservoir, and can be particularly serious in the immunocompromised host.

In the laboratory setting two principal forms are recognised in culture. They appear as large, circular white colonies that may be joined end to end (pseudohyphae), or as buds (blastospores) [Greenwood et al 2007].

3.2.8.1 *Candida albicans*

This species accounts for 80-90% of all *Candida* related infections. It may be differentiated from other species by the pattern of fermentation and assimilation of carbohydrates. Superficial infections are usually managed with topical therapy, but deeper infections usually require intravenous anti-fungal agents (Ward et al 2009). It is not frequently noted in the orthopaedic literature as a cause of arthroplasty infection.

Chapter 4

Microbiological Methodology

4.0 General

This chapter details the microorganisms, culture media and equipment used throughout the research. The origin of each specimen and the site of infection are described. Microscopic images of the clinical isolates used in the experimental work are also shown.

4.1 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.

4.1.1 Clinical Arthroplasty Isolates to be tested for Sensitivity to HINS-light

All of the microbial isolates used in this study were obtained from the microbiology laboratory at the Southern General Hospital, Glasgow. This is the sole microbiology laboratory for the south side of the Greater Glasgow and Clyde NHS Trust. This includes the Southern General Hospital, Victoria Hospital and all of the surrounding GP surgeries. The isolates all produced clinically relevant THR or TKR infections requiring treatment.

Isolates from arthroplasty infections were selected for use in experimental studies to assess their sensitivity to HINS-light. This study aimed to test an organism from each of the different groups highlighted in Table 3.1. The bacterial and yeast strains selected for testing are listed in Table 4.1, along with the respective non-identifiable patient background data.

Table 4.1 *Details of the bacterial and yeast isolates from infected THR/TKR selected for experimental use*

Microbial Isolate	Collection date	Patient Age (Years)	Gender	Source
Staphylococcus aureus	27/05/2009	67	Male	Right knee tissue
Staphylococcus epidermidis	26/05/2009	68	Male	Right knee swab
Corynebacterium striatum	27/11/2009	75	Female	Right hip tissue
Enterococcus faecalis	14/09/2009	68	Male	Right knee tissue
Micrococcus species	13/07/2009	80	Female	Left hip tissue
Streptococcus species	22/07/2009	57	Female	Left knee tissue
Escherichia coli	25/07/2009	71	Male	Right knee swab
Klebsiella pneumoniae	11/06/2009	81	Male	Left hip swab
Serratia marcescens	27/01/2010	64	Male	Right hip pus aspiration
Pseudomonas aeruginosa	12/08/2009	62	Male	Right knee swab
Candida albicans	29/07/2009	79	Female	Left hip swab

4.1.2 Culturing and maintaining microbial cultures

At the SGH microbiology lab, when a positive culture was identified, the isolate was transferred onto Microbank™ beads (ProLab Diagnostics) containing cryopreservative solution for storage at -70°C in the hospital freezer. The isolates for testing for susceptibility to HINS-light were then transferred to ROLEST.

For recovery of the microbial isolate, an inoculated Microbank bead was removed under aseptic conditions and streaked onto an agar plate. The agar medium of choice was selected dependent on the organism being cultured. This streaked plate was then incubated at an appropriate temperature and duration (Table 4.2).

Table 4.2 *The bacterial and yeast strains selected for experimental use listed along with the appropriate culture medium and incubation settings*

Microorganism	Laboratory number	Growth Media	Temperature(°C)/ Incubation period
Staphylococcus aureus	R5630	Nutrient Broth & Agar	37°C/18 hours
Staphylococcus epidermidis	R5536	Tryptone soya Broth & Agar	37°C/18 hours
Corynebacterium striatum	R12808	Brain Heart Infusion Broth & Blood Agar	37°C/18 hours
Enterococcus faecalis	R10012	Nutrient Broth & Agar	37°C/18 hours
Micrococcus species	R7574	Nutrient Broth & Agar	37°C/18 hours
Streptococcus species	R7875	Brain Heart Infusion Broth & Blood Agar	37°C/18 hours
Escherichia coli	R8016	Nutrient Broth & Agar	37°C/18 hours
Klebsiella pneumoniae	R6260	Nutrient Broth & Agar	37°C/18 hours
Serratia marcescens	R00943	Nutrient Broth & Agar	37°C/18 hours
Pseudomonas aeruginosa	R8655	Nutrient Broth & Agar	37°C/18 hours
Candida albicans	R8177	Malt extract Broth & Agar	37°C/18 hours

After incubation, the isolate was sub-cultured and incubated on an agar slope, with the slope being stored in the laboratory fridge at 4°C for use as a regular source of inoculum. The purity of the bacterial isolates was checked by Gram staining (section 4.5.1) and visual identification under the microscope. Microscopy photographs of the clinical isolates are shown in Figures 4.1-4.10. All of the bacteria are shown after Gram staining and at a magnification of x1000; while the yeast is at a magnification of x400 and a Gram stain has not been performed.

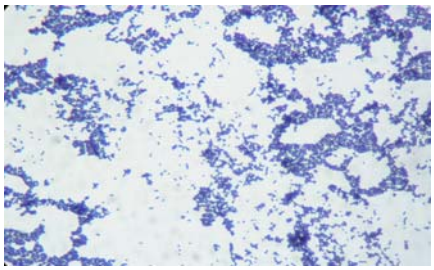


Figure 4.1 *Staphylococcus aureus*

Figure 4.2 *Staphylococcus epidermidis*

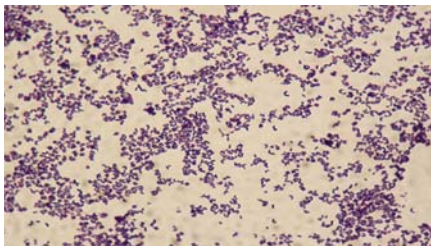


Figure 4.3 *Corynebacterium striatum*

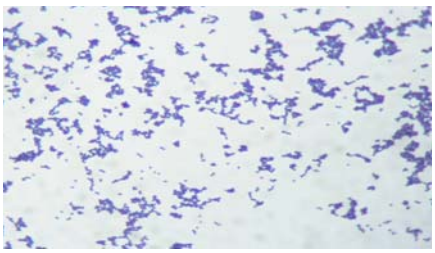


Figure 4.4 *Enterococcus faecalis*

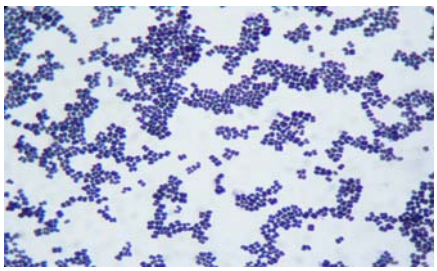


Figure 4.5 *Micrococcus species*

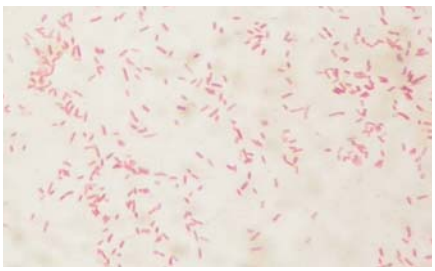


Figure 4.6 *Escherichia coli*

Figure 4.7 *Klebsiella pneumoniae*

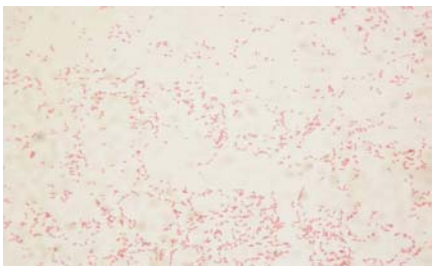


Figure 4.8 *Serratia marcescens*

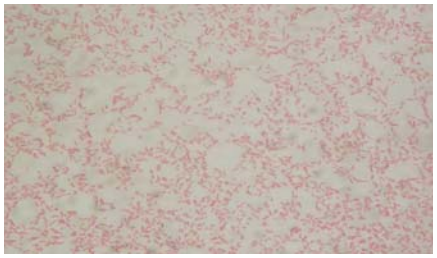


Figure 4.9 *Pseudomonas aeruginosa*

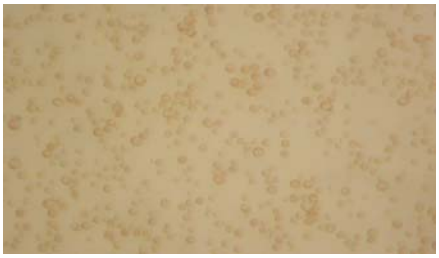


Figure 4.10 *Candida albicans*

In order to culture a microbial strain for experimental use, a loopful of organism from the agar slope (stored at 4°C) was aseptically extracted and inoculated into 100ml broth using a sterile wire loop. The broth was then incubated at the desired temperature and duration (Table 4.2) to provide a population of approximately 10^9 colony-forming units per millilitre (CFU/ml) for the bacteria, and 10^8 CFU/ml for the yeast. Under the specific growth conditions, 10^8 CFU/ml was the maximum that yeast could be grown to. The inoculated broth was then centrifuged and diluted to the population density required for testing. The method used for serial dilution of the microbial population is detailed in section 4.3.1.

All clinical isolates were successfully cultured with the exception of the *Streptococcus* isolate. Several attempts to culture this organism for experimental use unfortunately proved unsuccessful, therefore the effect of HINS-light on streptococcal inactivation could not be observed in this study. In addition, other CNS were not tested for their sensitivity to HINS-light, as they had only been identified as CNS, and the exact species were not defined.

4.2 Media

Culture media were prepared by dissolving the appropriate weight into distilled water as instructed by the manufacturer. The exact weights were measured using the Ohaus Navigator™ (Scientific Laboratory Supplies) digital scales system. The media were then sterilised at 121°C for 15 minutes in the bench top autoclave (Dixons Surgical Instruments Ltd).

After autoclaving, the molten agar was allowed to cool to 48°C. It was kept at this temperature in a water bath until poured into either 90mm Petri dishes to form agar plates, or 30 ml Universal bottles to prepare agar slopes for bacterial storage.

4.2.1 Broths and agars

These were made up according to the manufacturer specifications. The instructions for each media are shown as follows:

Broths

- NUTRIENT BROTH (Oxoid) 13g/L
- TRYPTONE SOYA BROTH (Oxoid) 30g/L
- MALT EXTRACT BROTH (Oxoid) 20g/L
- BRAIN HEART INFUSION BROTH (Oxoid) 37g/L

Agars

- NUTRIENT AGAR (Oxoid) 28g/L
- TRYPTONE SOYA AGAR (Oxoid) 40g/L
- MALT EXTRACT AGAR (Oxoid) 50g/L
- BLOOD AGAR BASE (Oxoid) 40g/L

For preparation of blood agar, after cooling the blood agar base in the water bath, defibrinated horse blood (Oxoid) was added to form a 7% v/v concentration blood agar (35 ml blood: 500ml agar).

4.2.2 Diluents and reagents

- PHOSPHATE BUFFERED SALINE (Oxoid) 1 tablet/100ml
  This was dispensed into either (1) 100ml volumes for bacterial pellet re-suspension or (2) 9ml volumes for use in the preparation of serial dilutions using a Brand Dispensette II Dispenser. These were then autoclaved at 115°C for 10 minutes in the Kestrel automatic autoclave (LTE Scientific).
- GRAM STAIN REAGENTS
  Crystal violet, Lugols iodine, Ethanol, Safronin and Distilled Water were used to stain the organisms. Technical details are described in section 4.5.1.
- HYDROGEN PEROXIDE (Fisher Scientific)
  6% hydrogen peroxide was used for the catalase test. The purpose of this test is discussed in section 4.5.2.
- OXIDASE STRIPS (Oxoid)
  Used for the oxidase test. This test is discussed in section 4.5.3.
- API strips (BioMérieux)
  The use of this identification test is highlighted in section 4.5.4.

4.3 Bacterial Enumeration

Microbial enumeration is the standard method used for the assessment of any inactivation technology. During the present study into the effect of HINS-light on clinical arthroplasty isolates, accurate enumeration of microbial samples pre- and post- treatment must be carried out in order to establish the effect of the HINS-light on the microbial population size.

For enumeration, control and test samples must be plated onto agar plates and incubated at a suitable temperature and duration (see Table 4.2). After incubation, the colony forming units (CFU) that had grown on the agar plates could be counted, and this could then be used to calculate the number of CFU per millilitre of sample (CFU/ml). The dilution and plating methods for enumeration of microorganisms that were used in this study are detailed in this section.

4.3.1 Serial dilutions

As mentioned in the previous section, serial dilution of the microbial population allowed the desired microbial concentration to be exposed to HINS-light and subsequently enumerated. After incubation, cultures were centrifuged for 10 min at 4300 rpm and the cell pellet re-suspended in 100 ml PBS, giving a suspension with a population density of approximately 10^9 CFU/ml for the bacterial isolates and 10^8 CFU/ml for the yeast. This microbial suspension was then serially diluted in PBS to provide the appropriate population densities for experimental use.

The process involved the addition of 1 ml of undiluted microbial suspension to 9ml phosphate buffered saline (PBS). This was thoroughly mixed using a Whirly mixer (FisherBrand). This created an evenly distributed cell suspension with a dilution factor of ten (10^{-1}). 1 ml of this newly formed 10^{-1} suspension was then added to a further 9 ml volume of PBS, resulting in a 10^{-2} dilution. These steps were then repeated until the desired dilution factor was achieved.

4.3.2 Plating techniques

Before and after a microbial suspension had been treated, a known volume was plated. This volume was dependent on the expected number of surviving colonies. Two principal plating methods were used: spiral plating and spread plating. The method chosen was dictated by the likely surviving population.

4.3.2.1 Spiral plate method

The WASP 2 spiral plater (Don Whitley Scientific) was used for this plating technique. A photograph of the device can be seen in Figure 4.11 below.

Figure 4.11 *WASP 2 Spiral plater*

The spiral plater has a vacuum suction component, on the right hand side of the picture above. This enables it to draw up the microbial suspension. The device then dispenses a 50µl volume of this suspension onto the surface of a rotating agar plate. The sample is deposited in a logarithmic fashion, with high concentrations centrally, reducing towards the periphery. This so-called Archimedes spiral shape can be visualised post incubation, as shown in Figure 4.12.

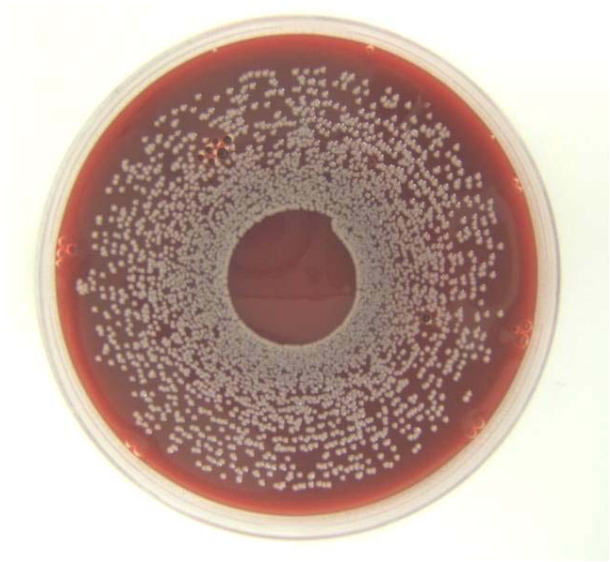


Figure 4.12 *Example of a WASP 2 spiral plate with logarithmic sample distribution*

After plate incubation the bacterial colonies were enumerated. This was performed either manually using the Stuart Scientific colony counter, or on the laboratory PC using the ACOLYTE software package (Figure 4.13). The key principle of both methods is that a centred counting grid is placed over the plate, physically or superimposed electronically. The grid segments correspond to known volumes of deposited sample. In turn, the CFU/ml of the plate is calculated by reference to the charts supplied with the manual counter, or electronically by the ACOLYTE software.

Figure 4.13 *ACOLYTE computer package with example counting grid on screen*

4.3.2.2 Spread plate method

Two separate techniques for the preparation of spread plates were used during this study. Similar to the spiral plated samples, an automatic setting is available on the WASP-2 plating machine for the preparation of spread plated samples. For this, the spiral plater settings were adjusted to allow 100µl of the bacterial sample to be deposited onto the agar plate surface in a linear (non-logarithmic) fashion.

The alternative method involves manually preparing the spread plates. 100µl of the microbial suspension was pipetted onto an agar plate. A sterile L-shaped spreader was then immediately used to evenly distribute the organism over the agar surface.

Once the spread plate samples were fully dry, they were incubated and then enumerated. In both spread plating techniques 100µl of bacterial suspension was used. Therefore the colony count of the entire plate was multiplied by a factor of ten to obtain the number of CFU/ml in the sample. If the sample was expected to contain few CFU due to HINS-light inactivation, larger volumes of the bacterial suspension, such as 200µl and 500µl, were used for improved sampling accuracy. In turn, the relevant multiplication factor of five or two, respectively, was applied to calculate the CFU/ml.

4.4 Other equipment

This section provides an overview of the other equipment used for microbiological analysis.

- Lec Medical refrigerators were used for storage at 4°C. Primarily this was for the retention of bacterial cultures on agar slopes.
- An OHAUS Navigator (Scientific Laboratory Supplies) digital balance was used for accurate measurement of media quantities.
- A Merit W4000 Distil provided water distilled of impurities for use in broth, agar and diluents preparation.
- 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 laboratory experimental work. These autoclaves were also used for the sterilisation of all contaminated waste prior to disposal.
- A Grant Water-bath (Scientific Laboratory Supplies) was maintained at 48°C to cool and preserve the agar in a molten state for pouring.
- A Bassaire Laminar Flow workstation was used for pouring the molten agar into sterile single vent Petri dishes or Universal bottles. This minimised the risk of air contaminants settling on the agar (Figure 4.14)

Figure 4.14 *Bassaire Laminar flow workstation with freshly poured agar plates*

- A rotary shaker incubator (New Brunswick Scientific) was maintained at 37°C for the culture of inoculated broths overnight. The shaking motion of the incubator ensured continual mixing and aeration, optimising conditions.
- Gilson Pipettes (100µl and 1ml) and sterile pipette tips were used to prepare dilutions and transfer the liquid bacterial suspensions with volumetric accuracy.
- Fifty millilitre centrifuge tubes (Nunc) containing the incubated broth were placed in the Heraeus Labofuge 400R Centrifuge (Kendo Laboratory Products). This was used to centrifuge the incubated broths at 4300 rpm for a period of 10 minutes. This resulted in a distinct bacterial pellet at the bottom of the tube along with a supernatant. The latter was discarded and the pellet re-suspended in PBS.
- A Nikon Eclipse E400 Microscope was used to examine the cell morphology, and confirm the absence of contaminants in the bacterial culture (Figure 4.15). There was a facility to attach a Nikon Coolpix 4500 digital camera to the microscope to permit microbial photographs.

Figure 4.15 *Nikon Eclipse E400 microscope in use for bacteria identification*

- Incubators (LTE Scientific) set at 37°C were used for the overnight incubation of agar plates. This incubation period allowed sufficient growth of the microorganism so that colony-forming units could be easily counted.

4.5 Microbiological and biochemical tests

A combination of microbiological and biochemical tests were used for the identification of bacterial isolates.

4.5.1 Gram stain

This was one of the first stages of bacterial identification, differentiating whether an organism was Gram-positive or Gram-negative. The Gram reaction is based on the structure of the bacterial cell wall. The steps involved in performing the test were as follows:

1. A drop of distilled water was placed onto a microscopic slide.
2. A wire loop was used to lift some bacterial colonies from the agar plate and they were emulsified into the drop of water.
3. This was air dried, and then fixed to the glass slide by passing it through the Bunsen burner flame three times.
4. The slide then has a bacterial smear and was placed onto a staining rack over a sink.
5. The slide was then stained with crystal violet for 1 minute (Figure 4.16).
6. The stain was then poured off and rinsed with Lugol's iodine.
7. The iodine was then poured off and decolourised by washing with ethanol briefly.
8. The slide was then counter stained with Safranin and left for approximately 1 minute.
9. The slide was rinsed with water and blotted dry
10. The stained bacteria could then be viewed under the oil immersion lens of the microscope (Nikon Eclipse E400).

Figure 4.16 *Crystal violet staining of slide on a rack over the sink*

In Gram-positive bacteria the dark purple crystal violet is retained by the thick layer of peptidoglycan forming the outer cell wall. The stain is held within its pores, which shut after dehydration by the ethanol washing stage. Gram-negative cells, however, have a thin peptidoglycan layer in the periplasm allowing solvent passage and subsequent easy removal of the crystal violet stain. The pink safranin counter stain affects the peptidoglycan layer, causing Gram-negative cells to stain pink.

4.5.2 Catalase test

This was a test used to determine the presence or absence of the enzyme catalase in the microorganism. The function of catalase is to catalyse the decomposition of hydrogen peroxide to water and oxygen. The test involved a small volume (3-4 drops) of 6% hydrogen peroxide to be dispensed onto a bacterial colony grown on an agar plate or slope. A positive response was demonstrated by vigorous bubbling occurring within seconds on the agar surface.

4.5.3 Oxidase test

The oxidase test was used to determine if an organism possesses the cytochrome oxidase enzyme. Oxidase strips which have a reactive end impregnated with N-N-dimethyl-p-phenylenediamine oxalate, ascorbic acid and α-napthol were used. The reagents turned a dark blue colour when oxidised, and remained colourless when reduced. The reactive end of the strip was dipped into the bacterial colony on an agar plate and the response noted. A blue colour change was regarded as positive, while no colour change was regarded as negative (Figure 4.17).

Figure 4.17 *Example of a positive oxidase test on the left, and negative on the right*

All 3 tests (Gram stain, catalase and oxidase) in combination with the colony morphology were used for the preliminary identification of bacterial cultures.

4.5.4 API identification test

API Strips (bioMerieux) enable identification of unknown bacterial isolates. Preliminary tests (Gram stain, oxidase test) must be first performed on the isolates in order to identify the specific type of API strip to be used. Though all of the isolates had been identified by the Southern General Microbiology Laboratory, the API strips were used for verification.

Each API strip has 20 microtubules containing dehydrated substrates. Bacterial cell suspension was added to each microtubule, and then the strip was incubated. The biochemical reactions (based on a colour change) noted in each tubule was entered into a computer database, and the identification software determined the likely identity of the bacterium tested. All of the results corresponded with the Southern General Microbiology Laboratory findings.

Chapter 5

405 nm HINS-light LED sources

5.0 General

As discussed in section 2.6.3, 405 nm high-intensity narrow-spectrum (HINS) light technology is effective for the photodynamic inactivation of microorganisms. For the purposes of the experiments in this study, two different types of 405 nm HINS-light light emitting diode (LED) were used: a 99-DIE LED array and a high intensity LED array. This chapter describes these light sources and how they behave in the laboratory setting.

5.1 405 nm 99-DIE LED array

LEDs provide a much higher intensity light emission when used as arrays rather than single LED units. A 99-DIE LED array (Opto Diode Corp., California, USA) was used in this study. This is composed of a closely packed array of 99 LEDs (9 x 11 rectangle), and can be seen in Figure 5.1.

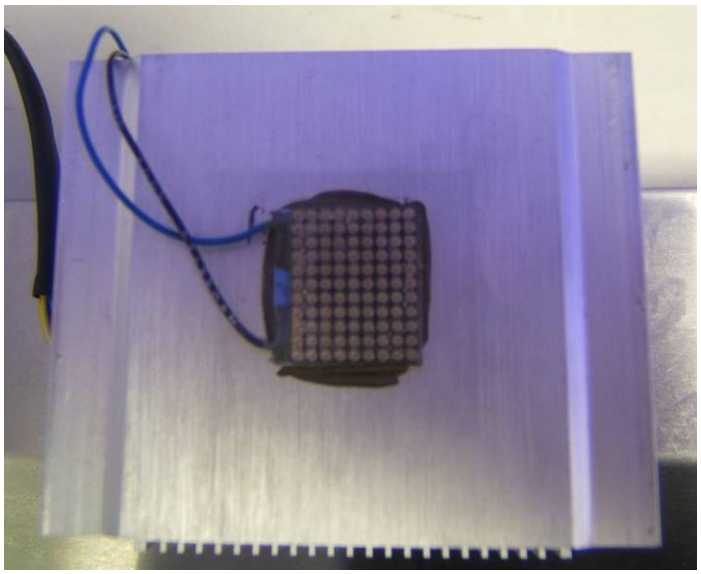


Figure 5.1 *The 99-DIE LED array (9 x 11 rectangle) attached to a heatsink*

This LED array has an area of 3.2cm^2 (2 x 1.6cm) and is powered by a DC supply (HQ POWER) with a controllable output in the range 0 – 3 A and 0 – 15 V. It emits violet light across a narrow spectral region and is made from indium-gallium-nitride. The 405 nm LED array has a centre wavelength (maximum emission) close to 405 nm, and the bandwidth is ~10 nm at full-width half-maximum as highlighted in Figure 5.2.

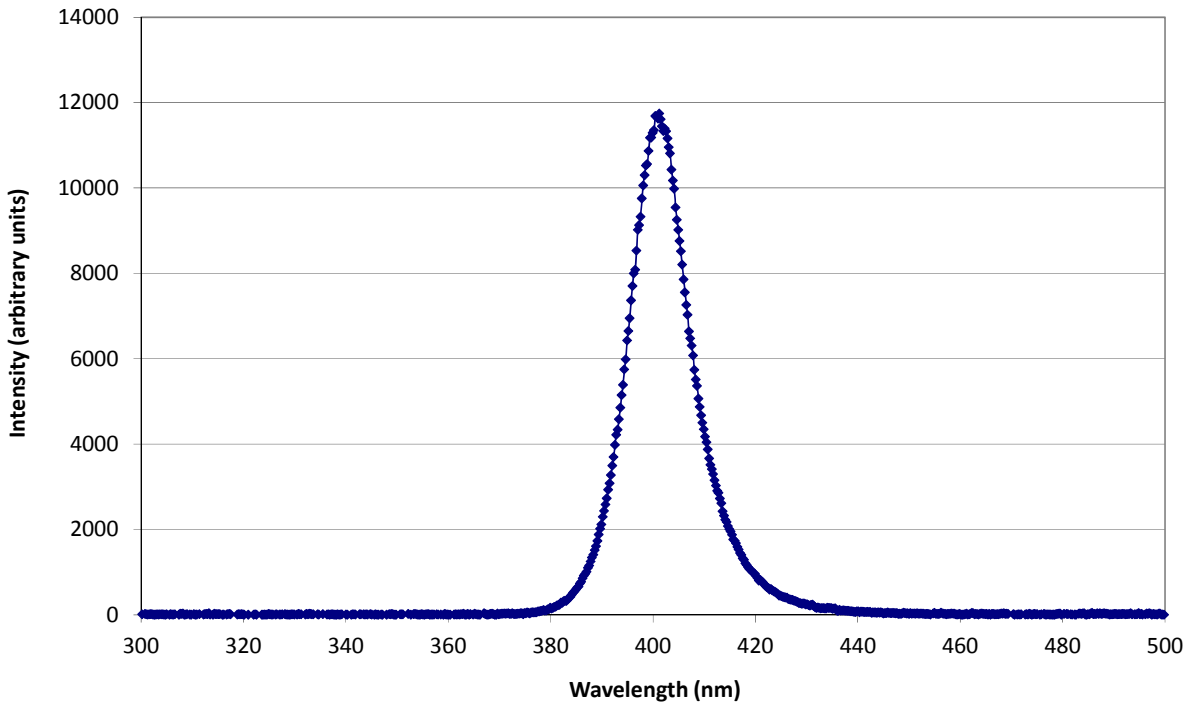


Figure 5.2 *Emission spectrum of the 99-DIE 405 nm LED array*

Though LEDs are known to have minimal heat dissipation, a heatsink and cooling fan were attached to the LED array as a precautionary measure. This ensured that a stable temperature around the LED array was maintained throughout testing. The LED array unit was mounted in a PVC housing, and this unit was used for exposure of all microbial **suspension** experiments as described in Chapter 6 (Part I).

5.2 405 nm ENFIS QUATTRO Light Engine

The light source used throughout for exposure of **surface-seeded** bacteria experiments was the ENFIS QUATTRO Mini Air Cooled Light Engine (Enfis Ltd). This is a larger 405 nm HINS-light source consisting of an array of 144 violet LEDs (12 × 12 square) with an area of 4cm^2 (Figure 5.3), powered using a 48V power supply.

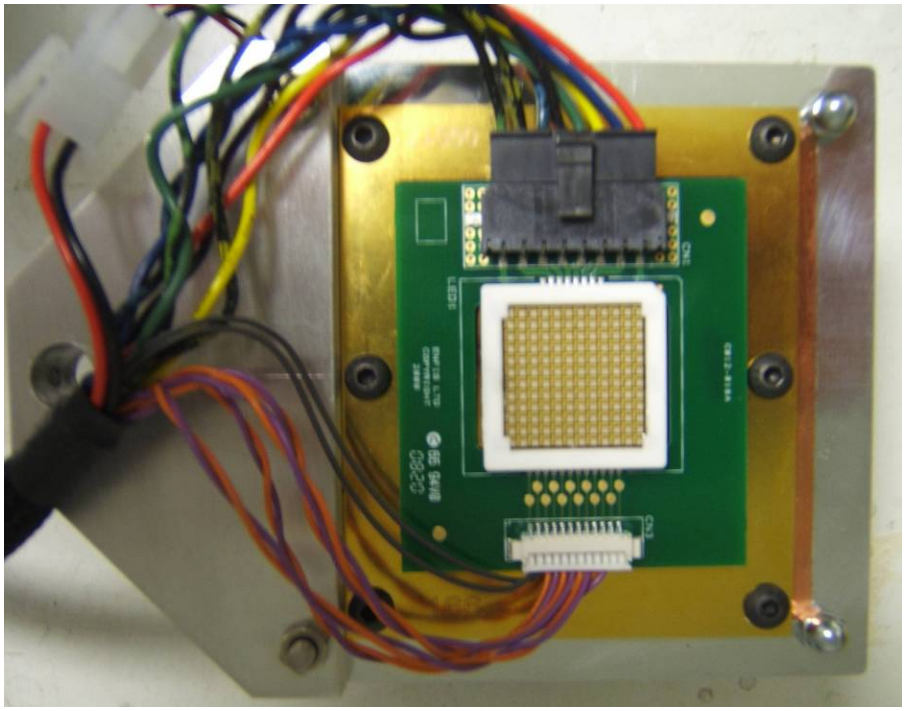


Figure 5.3 *The ENFIS QUATTRO LED array (12 x 12 square)*

This LED array system also emits 405 nm (± 5 nm) light, with a similar emission spectrum and peak wavelength to the 99-DIE LED array (Figure 5.4). The combination of a larger array surface area, increased number of packed LEDs, and the higher output power (irradiance) are the principal reasons why the ENFIS QUATTRO light engine was used for experiments investigating the exposure of agar surfaces seeded with microorganisms, described in Chapter 6 (Part II).

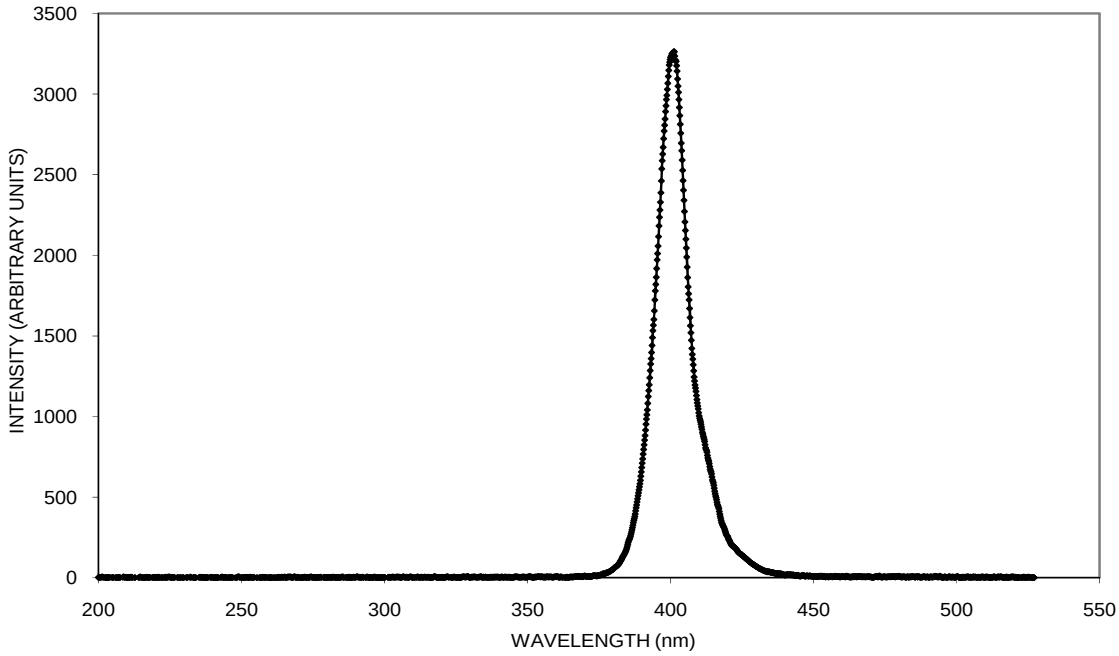


Figure 5.4 *Emission spectrum of the 405 nm ENFIS QUATTRO LED array*

As with the 99-DIE array, heat is produced from this LED array. To minimise this transfer of energy, the light engine has a metal heatsink, which absorbs and disperses heat, and a fan is also fitted to allow cooling, ventilation and the maintenance of a stable temperature around the array.

5.3 405 nm 99-DIE LED array: the effect of input current on output irradiance

As previously described in section 5.1, the 99-DIE 405 nm LED array was used for exposure of microbial suspensions. The experimental arrangement was such that the distance between the sample and the LED array was approximately 2 cm. The DC power supply for the LED array has a variable control. A radiant power meter in conjunction with a photodiode detector (model 70260, L.O.T. - Oriel Ltd) was used to measure the output power density (also known as 'irradiance') from the LED array when supplied with increasing input current (measured in Amperes). The photodiode detector head had a surface area of 1 cm^2, therefore the power meter measured irradiance in values of mW/cm^2. The detector can measure irradiances of up to 3 mW/cm^2. When the output irradiance was higher than this, a neutral density filter was attached to the detector head and the reading on the meter was multiplied by a factor of 100 in order to get the irradiance value. The power meter, detector head and neutral density filter can be seen in Figure 5.5.

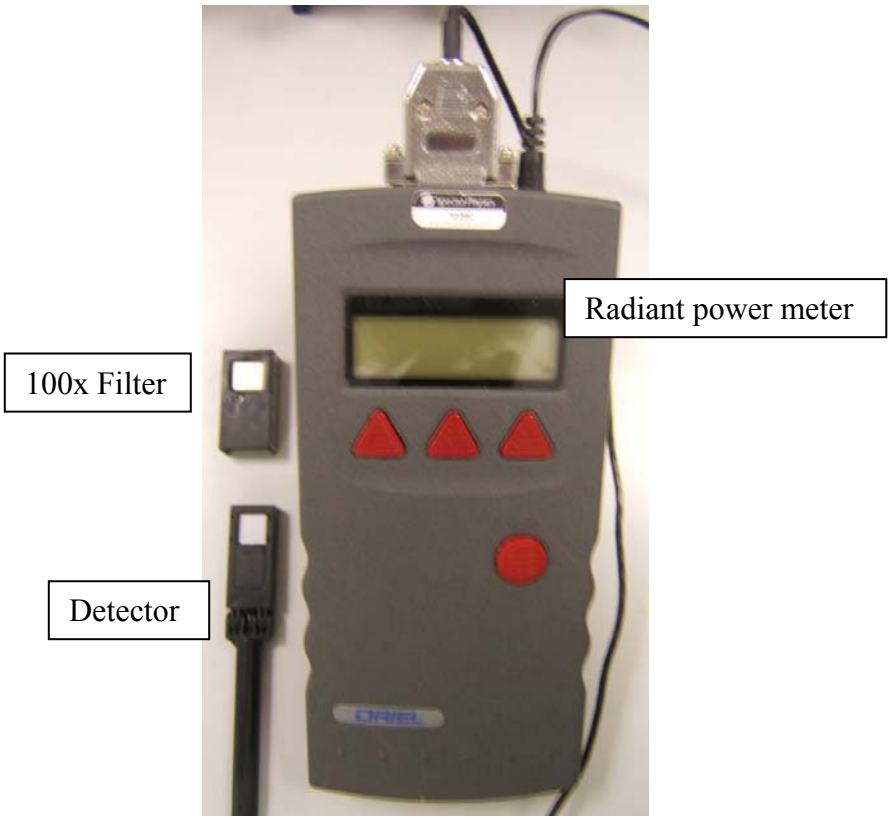


Figure 5.5 *Components of the power density measurement system*

The radiant power meter was used to quantify the irradiance emitted by the LED array upon varying the input current. The measurements were made from a distance of 2 cm, in keeping with the suspension experiments detailed in Chapter 6 (Part I).

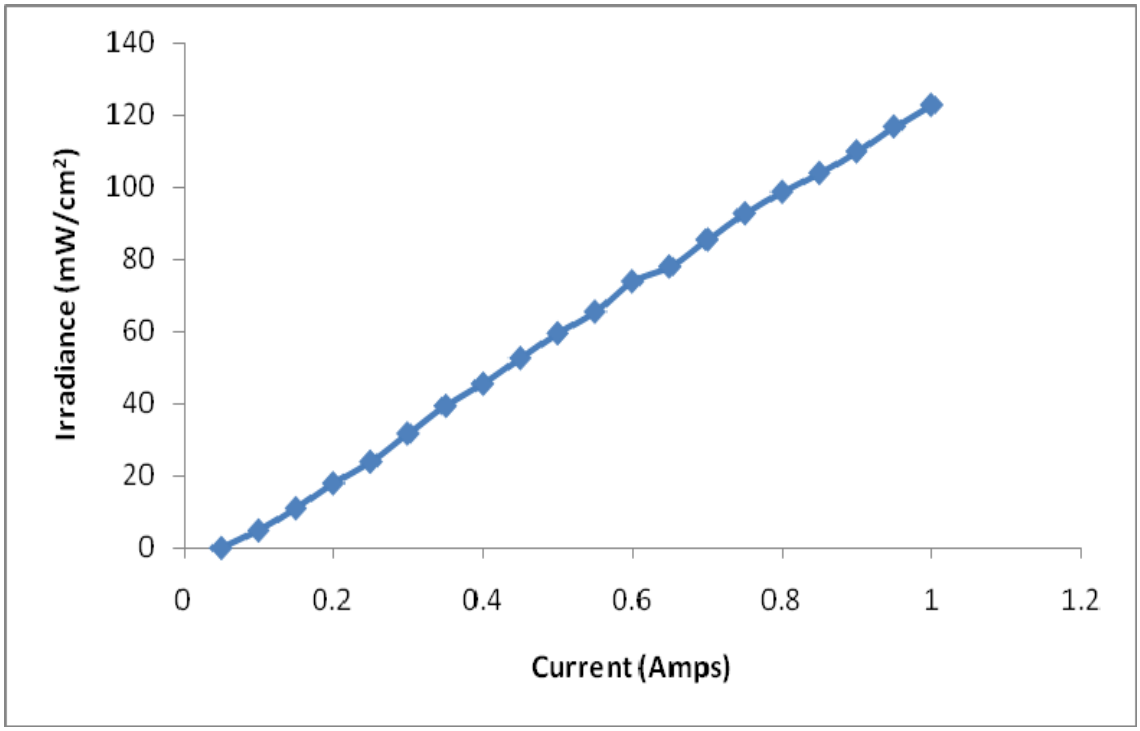


Figure 5.6 *The effect of input current on the output irradiance of the 405 nm 99-DIE LED array*

In Figure 5.6 a linear relationship was observed between input current and output irradiance.

In order to minimise the length of the experiments, for experimental use, the maximum measured irradiance of 123 mW/cm^2 was selected to be used. For all bacterial suspension exposure experiments the current was therefore set at 1 A, with an input voltage of 11.5V. This provided an output irradiance of 123 mW/cm^2 at 2 cm from the light source.

5.4 405 nm ENFIS QUATTRO light engine: the effect of exposure distance on output irradiance

As discussed in section 5.2, the ENFIS QUATTRO Mini Air Cooled Light Engine has a fixed 48V DC power supply that in turn provides steady output irradiance. However, the irradiance of the LED array can be varied by changing the distance between the source and the exposed surface. A radiant power meter was again used to measure the irradiance at various heights from the light source (Figure 5.7).

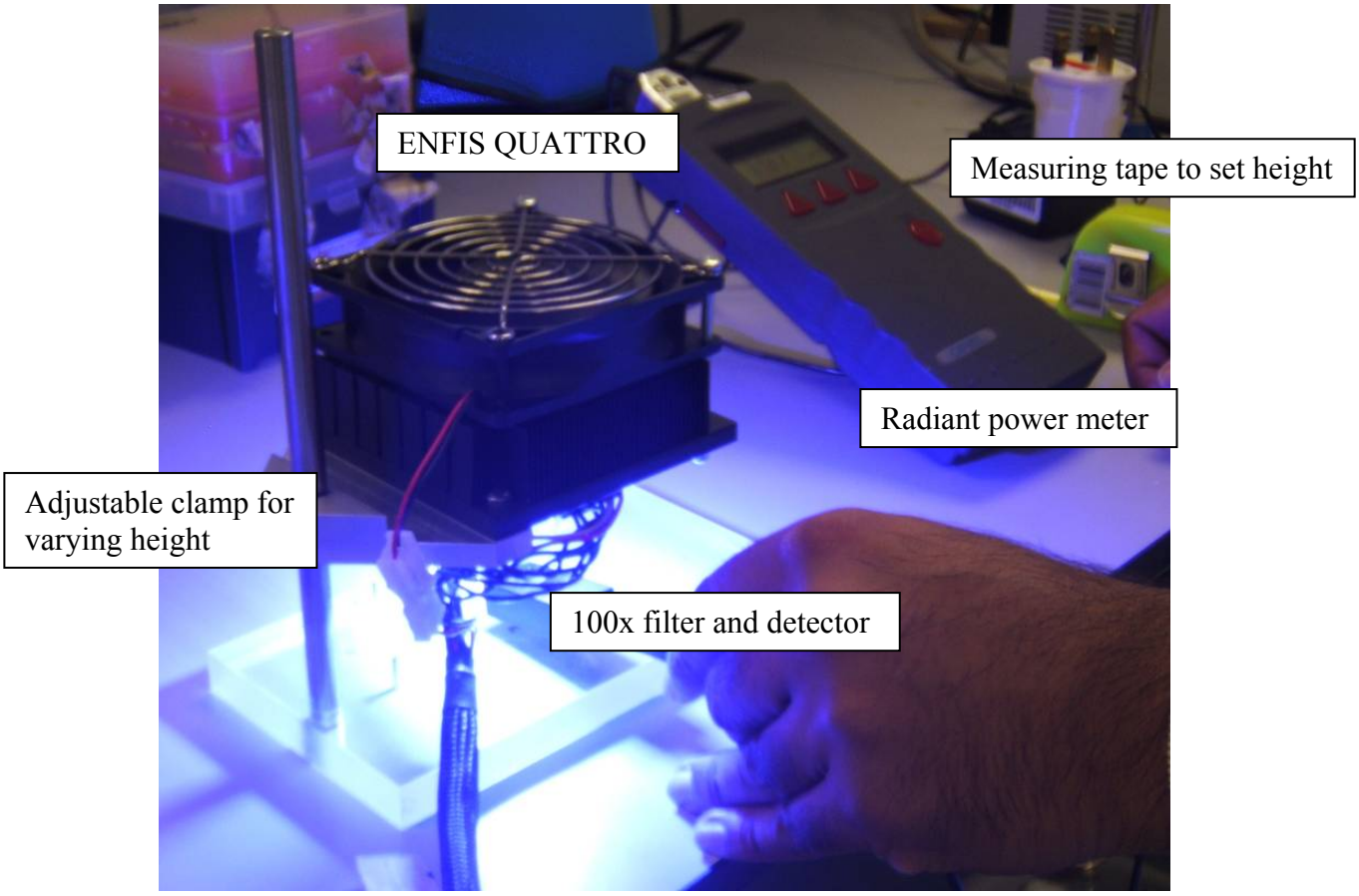


Figure 5.7 *Experimental set-up for measuring the effect of exposure distance on irradiance*

The effect of exposure distance on output irradiance is shown in Figure 5.8, and the results demonstrate that the further away the light is from the meter, the lower the irradiance.

Despite using the neutral density filter with the detector head, the irradiance readings at 30 mm from the source were too high to record, and hence values on Figure 5.8 start at a distance of 40 mm from the source. As this was the peak reading, and was marginally fluctuating, it was felt that a more consistent and reliable value may be obtained slightly further from the light source. At a height of 50 mm the irradiance reading directly under the light source was approximately 200 mW/cm^2. For all surface exposure experiments carried out during this study, the ENFIS QUATTRO Mini Air Cooled Light Engine was therefore maintained at a height of 50 mm from the seeded plate surface.

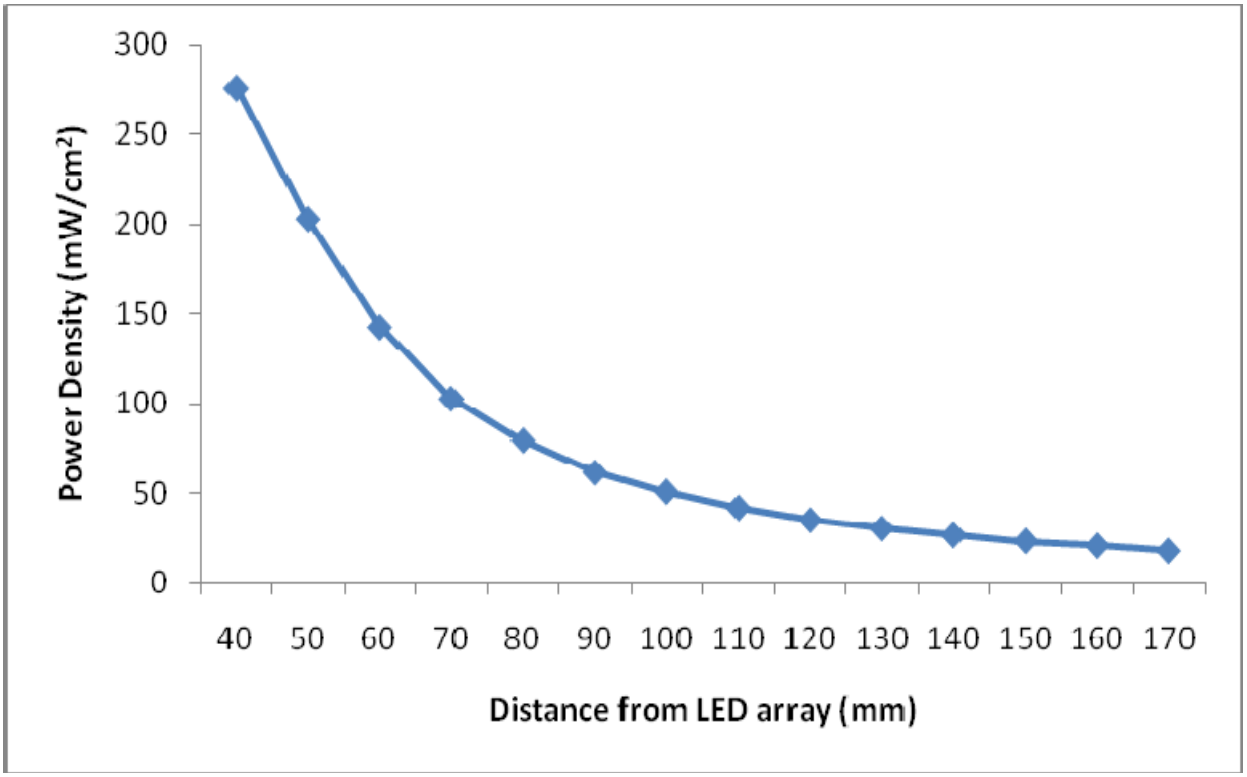


Figure 5.8 *The effect of exposure distance on output irradiance of the ENFIS QUATTRO 405 nm light engine*

5.5 405 nm ENFIS QUATTRO light engine: investigation of the radial spread of light on a surface

As stated in the previous section, all surface exposure experiments were carried out with the height of the lamp maintained at 50 mm from the surface. The surfaces to be exposed in these experiments were agar plates with microorganisms seeded on the agar surface. Each agar plate (Scientific Laboratory Supplies Ltd) used had a diameter of 90mm, and it was therefore hypothesised that when the plate was centralised underneath the light source, the highest irradiance reading would be at the centre of the plate (45 mm from the plate edge). As the ENFIS QUATTRO array beam spreads out, irradiance readings will be reduced towards the plate periphery.

The radiant power meter was used to measure the irradiance at 10mm intervals across a 90 mm distance (plate diameter) with the neutral density filter again being used on the detector head in order to measure the output irradiance. The thickness of the photodiode detector head was equivalent to that of the agar plate containing growth media and bacteria. This set-up can be seen in Figure 5.9.

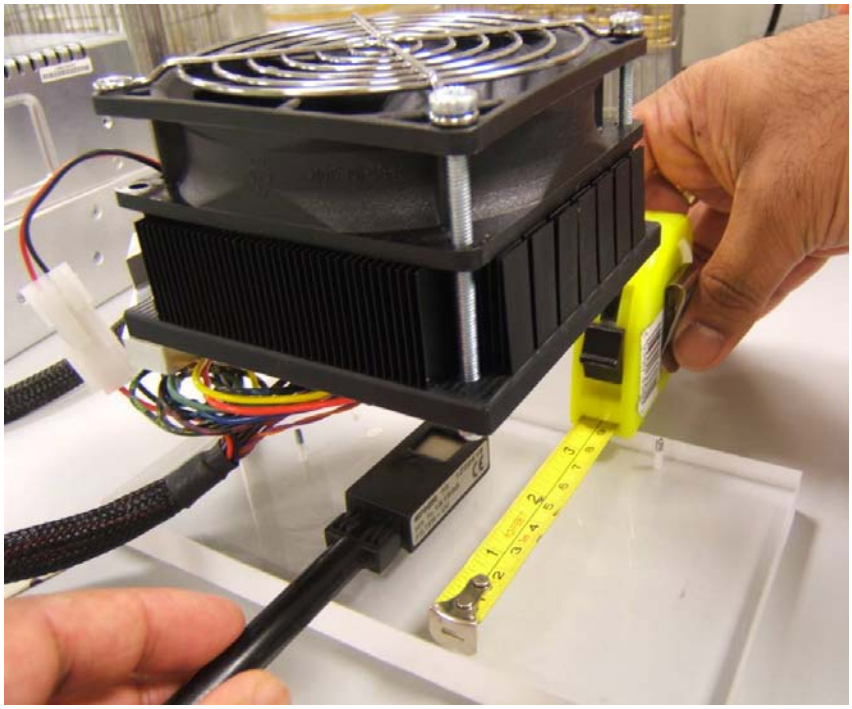


Figure 5.9 *Experimental set up for measuring the irradiance in relation to the radial spread of light*

Figure 5.10 shows that the irradiance varies across the radius of the light beam, with a bell-shaped curve observed. The highest irradiance was found in the central region (45mm from the periphery), directly underneath the light source. The irradiance at the 50 mm height was approximately 200mW/cm^2, as determined from the previous experiment. Irradiance measurements tailed off as the power meter moved away from the centre point to approximately 34mW/cm^2. It was therefore anticipated that there would be a higher kill rate at the centre of the plate with a reduced kill rate at the agar plate periphery due to the reduced irradiance.

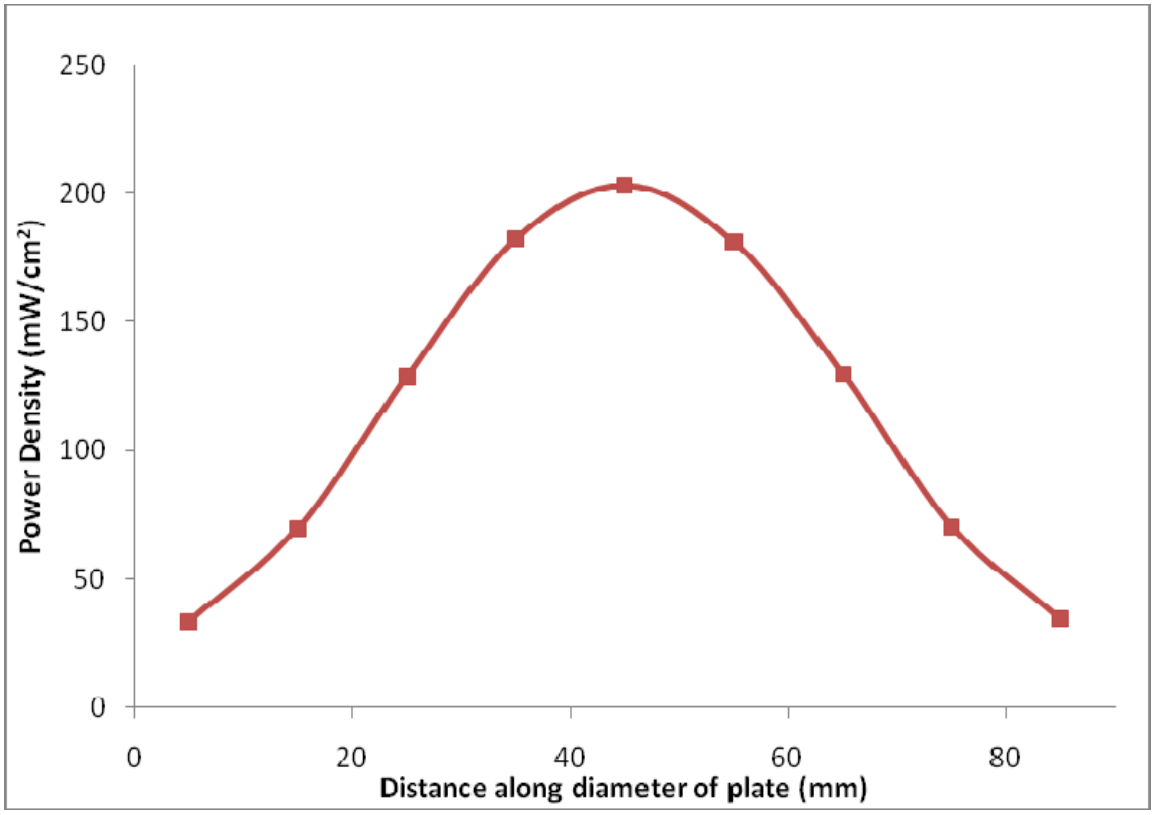


Figure 5.10 *Radial profile of the 405 nm ENFIS QUATTRO light engine at a height of 50mm from the detector, over a simulated plate diameter of 90mm*

Using this data, mathematical modelling was used to generate a 3D profile (Figure 5.11) and calculate the average irradiance across the agar plate surface. When the light source is at a distance of 5cm from the agar plate surface, the average irradiance across the plate surface was found to be 71 mW/cm^2 (data provided by Dr Endarko, ROLEST).

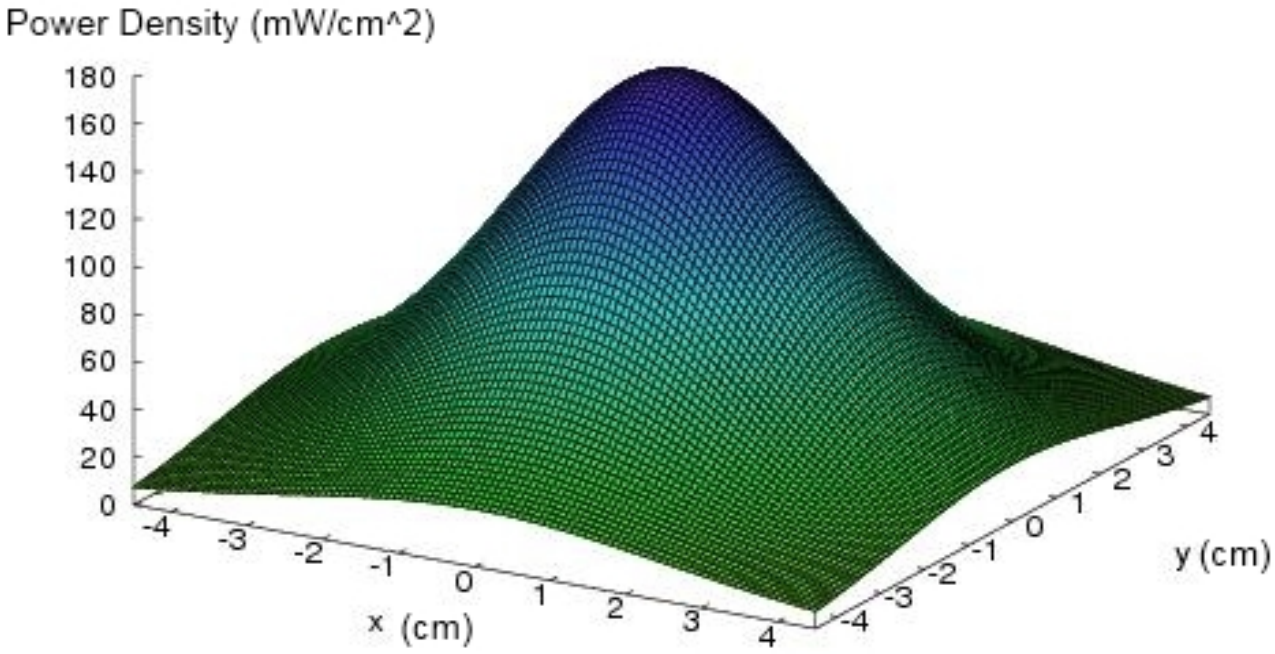


Figure 5.11 *3D model of irradiance across agar surface (5cm distance between agar & LED array). The average irradiance across the plate surface is 71 mW/cm^2.*

5.6 Summary of light source settings to be used in microbial exposure experiments

The standard parameters used for both of the 405 nm HINS-light LED arrays during microbial exposure experiments are summarised in Table 5.1. These settings were kept constant for all microbial exposure experiments.

Table 5.1 *Parameters for use of the 405nm HINS light LED arrays during experimentation*

Experimental parameter	**99-DIE LED array**	**ENFIS QUATTRO light engine**
Experimental arrangement	Exposure of microbial suspensions	Exposure of seeded agar plate surfaces
Exposure distance	2 cm	5 cm
Input voltage	11.5 V	48 V
Output irradiance from LED array	123 mW/cm^2	71 mW/cm^2

Chapter 6

HINS-light Inactivation of Clinical Arthroplasty Isolates

6.0 General

This chapter describes the experimental protocols for HINS-light exposure of the pathogens isolated from infected lower limb arthroplasty cases using the two HINS-light sources described in the previous chapter. The chapter is divided into two sections; Part I describes the HINS-light exposure of the clinical isolates in **suspension**, and Part II describes the HINS-light exposure of the clinical isolates seeded onto agar **surfaces**. The surface setting is believed to be most relevant to the clinical scenario. In both parts the various experimental arrangements are described, and the inactivation results presented.

Part I: HINS-light Exposure of Microbial Suspensions

6.1 Experimental arrangement for exposure of microbial suspensions

The bacterial isolates were stored and cultured for experimental use as described in section 4.1.2. After inoculating a 100 ml broth (the broth type dependent on the organism being tested – see section 4.1.2) with a loopful of organism from the agar slope, the broth was incubated at 37°C for 18 hours. This provided a population density of approximately 10^9 CFU/ml for the bacteria and 10^8 CFU/ml for yeast. This broth was then centrifuged at 4300 rpm for 10 minutes and the resultant pellet re-suspended in PBS. This suspension was then serially diluted as described in section 4.3.1 to provide a population density of approximately 10^5 CFU/ml. All of the diluted solutions produced were clear, with no turbidity.

A Gilson pipette and sterile tip was used to transfer 3ml of the liquid bacterial sample to the central well within a 12-well multidish (Nunc, Denmark). A 7mm x 2mm magnetic follower (Fisher Scientific) was added to the sample, as can be seen in

Figure 6.1. The multidish was then placed onto a magnetic stirrer (Yellowline MSH Basic), ensuring continuous mechanical agitation of the sample during light exposure.

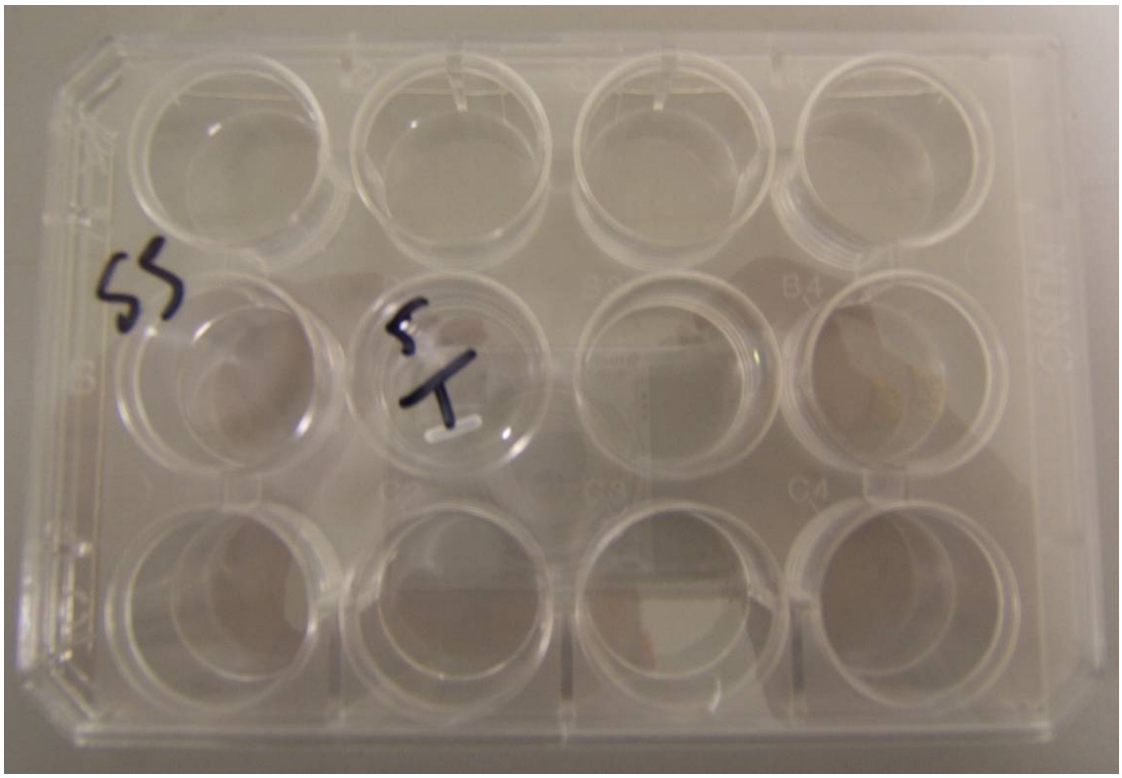


Figure 6.1 *12-well multidish (Nunc, Denmark) with bacterial suspension and magnetic follower (Fisher Scientific)*

The 99-DIE LED array, fan and PVC housing unit were then placed directly over the well containing the suspension to be exposed. The underside of the housing unit had an outer edge that fitted around the multidish, ensuring it was firmly held in place. The LED array was positioned to ensure that it is always directly above the sample well. The distance between the sample and the LED array was approximately 2cm, thus keeping the light intensity constant for all exposures.

The complete HINS-light exposure set-up with all components can be seen in Figure 6.2.

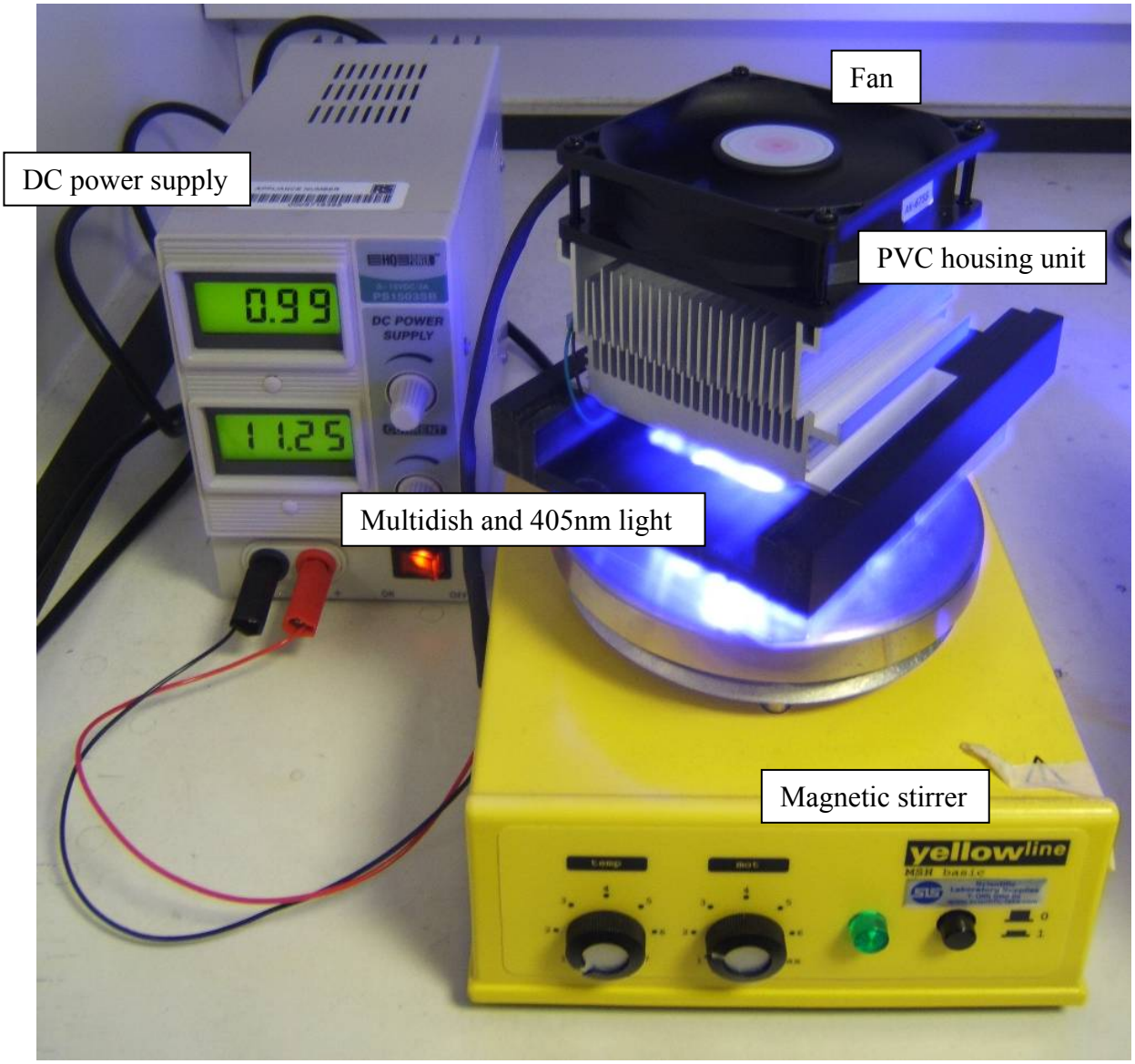


Figure 6.2 *HINS-light treatment system for exposure of bacterial suspensions*

A control sample was set-up for each test. This was a bacterial suspension held in the well of a multidish which was mechanically agitated for the same period of time as the test sample but not exposed to 405nm light. Following exposure, samples were plated onto agar plates (the type dependent on the organism being tested – see section 4.1.2) using a combination of spiral and spread techniques as described in sections 4.3.2.1 and 4.3.2.2. The plates were then incubated at 37°C for 24 hours before enumeration.

The key experimental parameters kept constant throughout testing on bacterial suspensions are summarised in Table 6.1.

Table 6.1 *Bacterial suspension treatment system: summary of key experimental parameters*

Experimental parameter	**Value**
Sample volume	3 ml
Exposure distance	2 cm
HINS light input current/voltage	1A (+/- 0.05) / 11.5V (+/- 0.25)
Irradiance from LED array	123 mW/cm^2

6.2 Results of HINS-light treatment of clinical isolates in suspension

The following sections report the efficacy of 405nm HINS-light for the inactivation of various microorganisms isolated from infected lower limb arthroplasty cases, using the experimental technique detailed in the previous section. The results of each of the individual microorganisms are presented, followed by a comparative summary of the results for Gram positive and Gram negative bacterial species. All of the charts in this section are X-Y scatter plots and include error bars for the standard deviation where applicable.

6.2.1 *Staphylococcus aureus*

The results of exposure of suspensions of *S. aureus* to 405nm HINS-light are summarised in Figure 6.3. From a mean starting population of 1.22 x10^5 CFU/ml, total inactivation (5-$\log_{10}$ reduction) of the bacteria took place after 20 minutes. The shape of the curve suggests a slow reaction over the first 5 minutes, followed by steady inactivation over the next 15 minutes until complete inactivation. The control line was relatively flat and unchanged throughout.

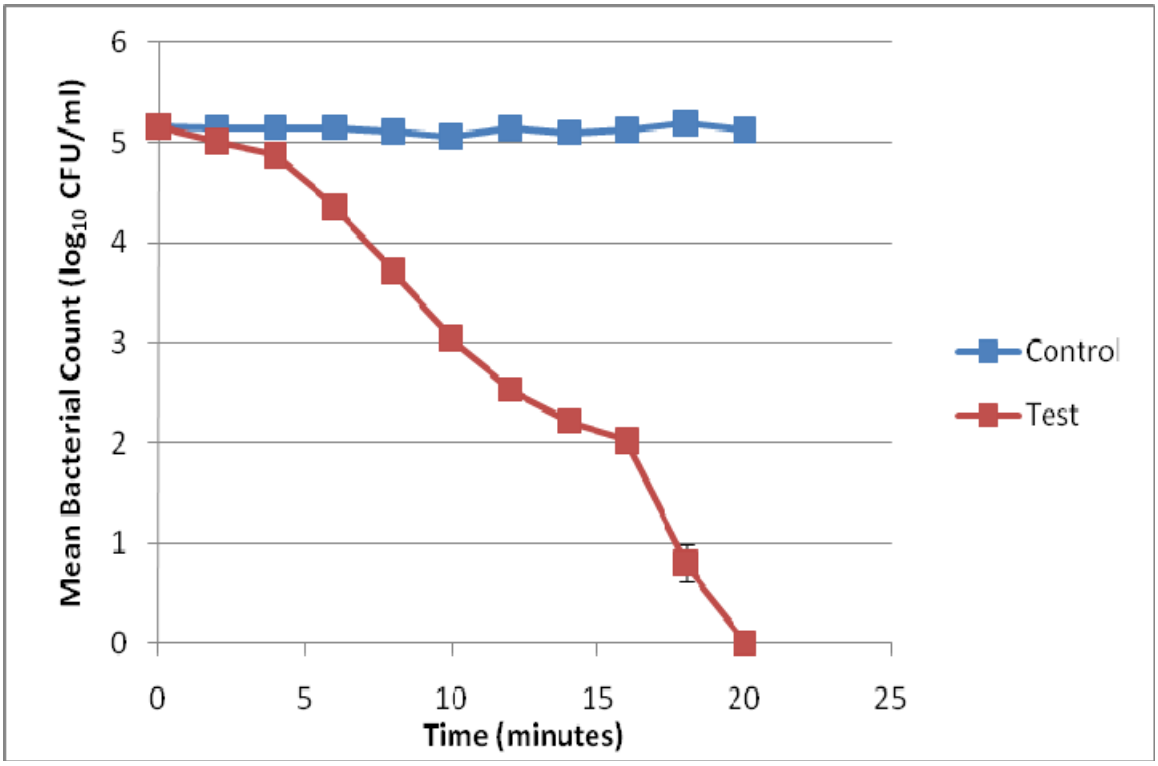


Figure 6.3 *Inactivation of Staphylococcus aureus in suspension by HINS-light exposure (123 mW/cm^2 irradiance). Mean ± S.D., n=4*

6.2.2 *Staphylococcus epidermidis*

From a bacterial starting population of approximately 1.30 x10^5 CFU/ml, complete inactivation was again observed but by a reduced exposure time of 16 minutes. The inactivation is charted in Figure 6.4. The curve shape again indicates slow inactivation initially, but after 6 minutes, inactivation occurred at a rapid rate. There was no significant change in the control sample.

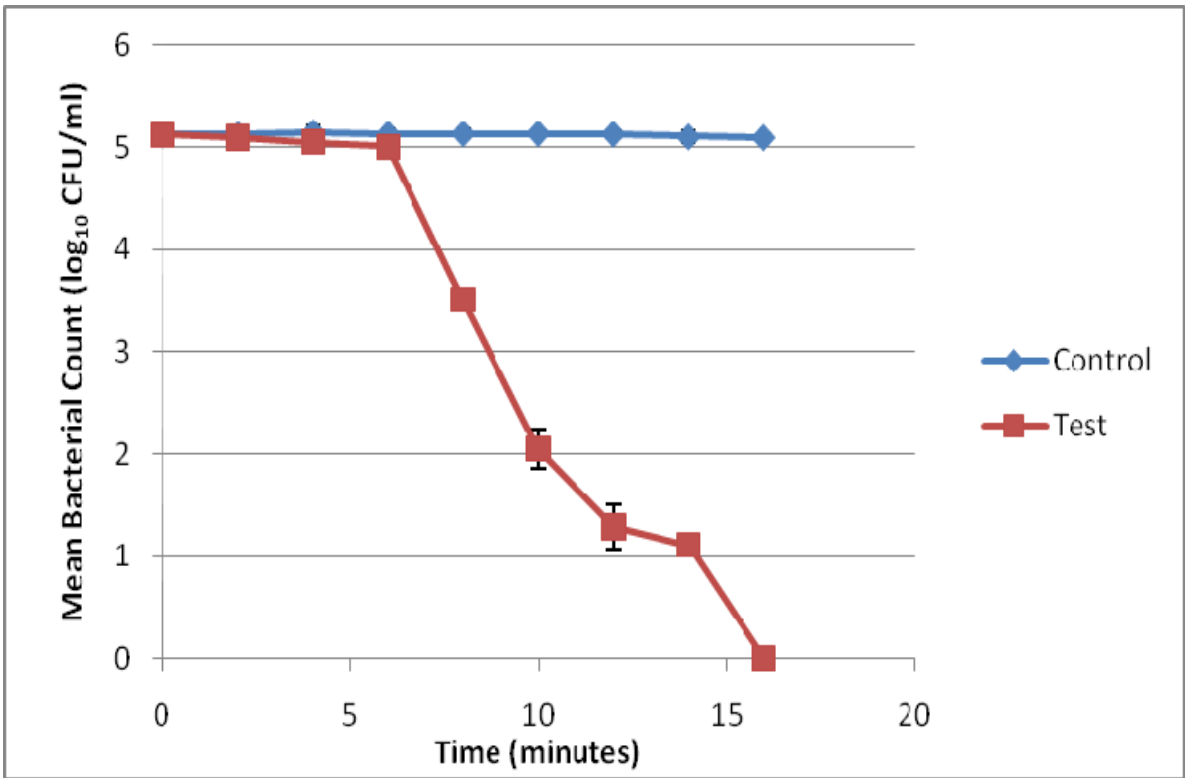


Figure 6.4 *Inactivation of Staphylococcus epidermidis in suspension by HINS-light exposure (123 mW/cm^2 irradiance). Mean ± S.D., n=4*

6.2.3 *Corynebacterium striatum*

The results of *C. striatum* exposure to HINS-light are summarised in Figure 6.5.

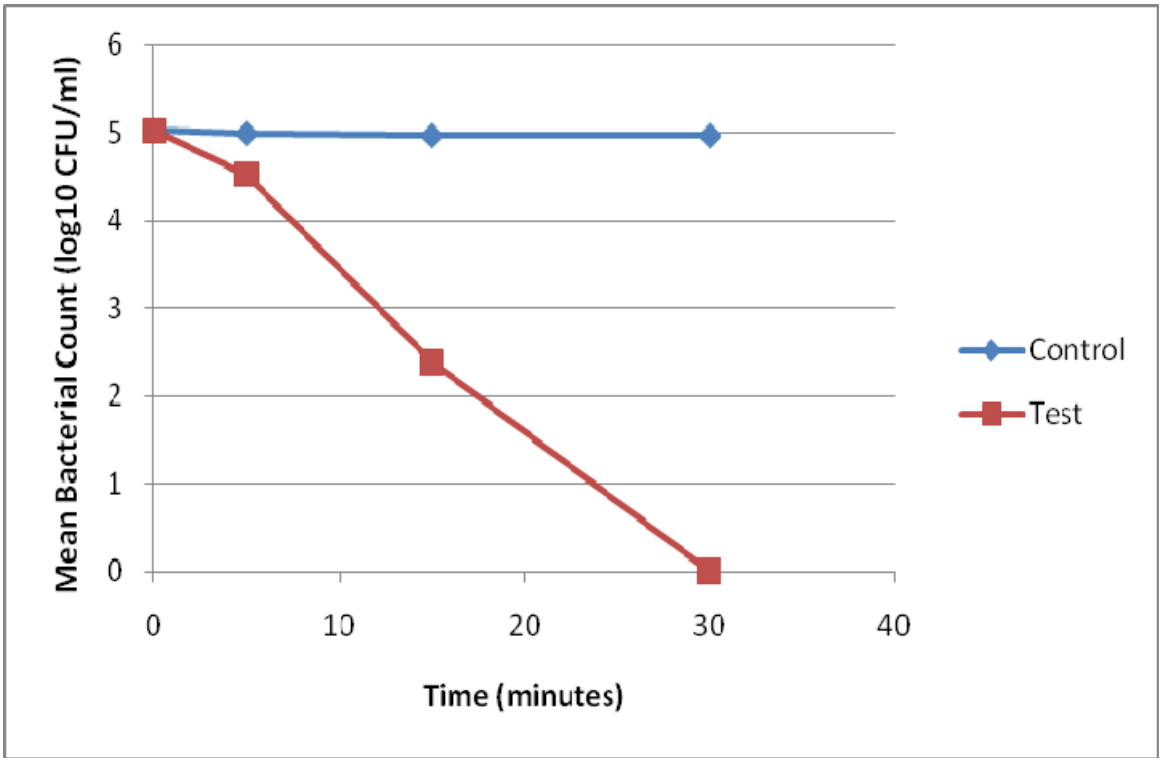


Figure 6.5 *Inactivation of Corynebacterium striatum in suspension by HINS-light exposure (123 mW/cm^2 irradiance). n=1*

The mean starting population was 1.06 x10^5 CFU/ml. The test curve became much steeper after 5 minutes, with steady inactivation noted thereafter. Based on the figures already seen, it is a possibility that complete inactivation of the bacteria may have occurred prior to 30 minutes. However, due to limited supplies of defibrinated horse blood for preparation of agar plates the sampling points were limited to 0, 15 and 30 minute exposures. More importantly, it was confirmed that the 405nm HINS-light was able to inactivate the bacteria over a relatively short period of time. The control line was again unchanged.

6.2.4 *Enterococcus faecalis*

This was a more resilient bacterium as can be seen in Figure 6.6. Despite a slightly lower average starting population of 5.27×10^4 CFU/ml, less than a 1-$\log_{10}$ reduction took place after 1 hour of HINS-light exposure. The slow initial phase of inactivation noted in previous bacteria tested was over a considerably longer period of time with *E. faecalis*. After 1 hour the curve was much steeper, indicating an increased rate of inactivation. Complete inactivation was observed after a 2 hour exposure period. The control line was flat throughout.

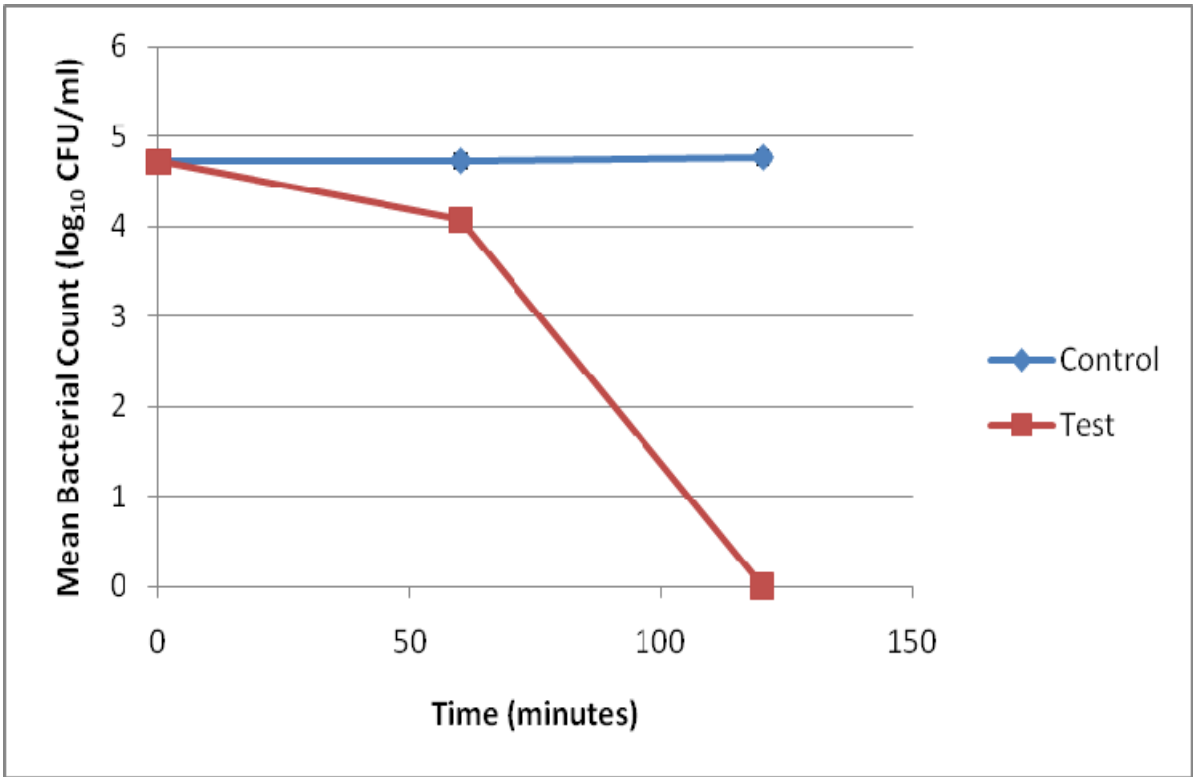


Figure 6.6 *Inactivation of Enterococcus faecalis in suspension by HINS-light exposure (123 mW/cm^2 irradiance). n=2*

6.2.5 *Micrococcus* species

Complete inactivation of the microorganism (~5-$\log_{10}$ reduction) occurred after 1 hour of HINS-light exposure. It was noted that after 15 minutes exposure to HINS-light, less than a 1-$\log_{10}$ reduction had occurred from a starting population mean of 7.15 x10^4 CFU/ml. Due to time restrictions, only two exposure times were investigated, but the successful inactivation demonstrated after a 1-hour exposure period confirmed that 405nm light was effective against the pathogen. This information is summarised in Figure 6.7 below. The inactivation curve displayed similar patterns of inactivity to previous experiments, with a slow initial period followed by a steeper, steadier portion. The control line was relatively flat again.

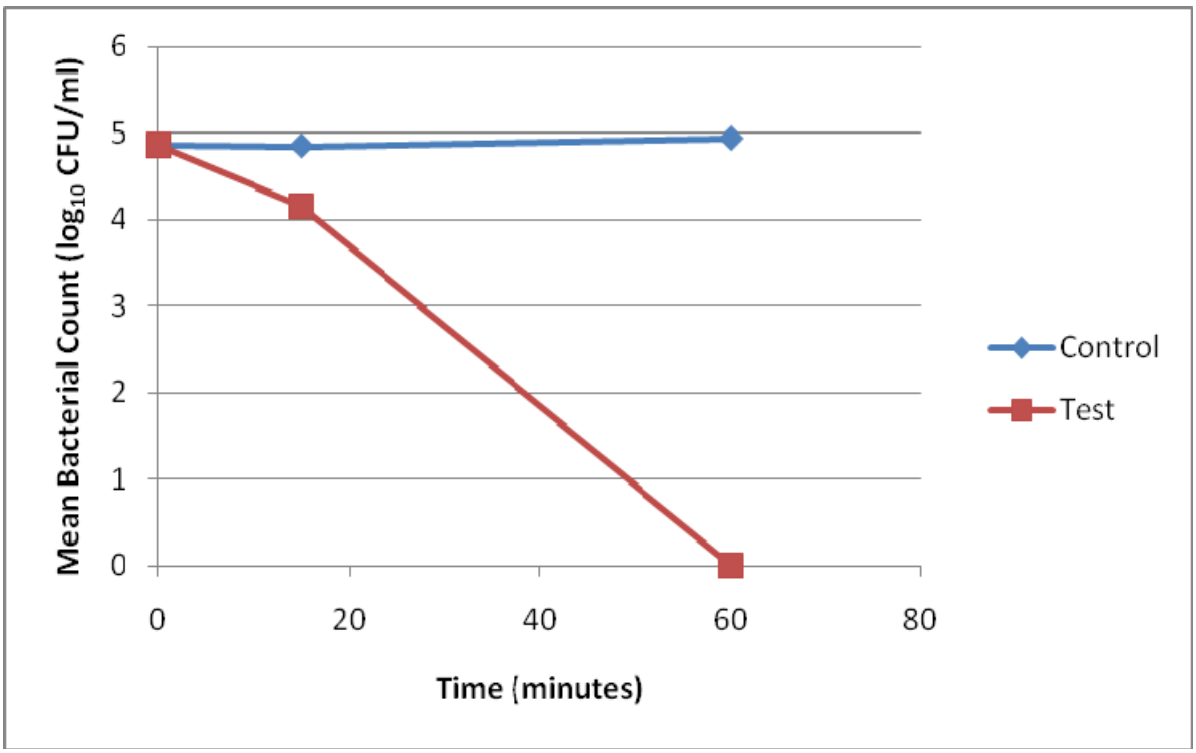


Figure 6.7 *Inactivation of Micrococcus species in suspension by HINS-light exposure (123 mW/cm^2 irradiance). n=1*

6.2.6 *Escherichia coli*

This was the first Gram-negative bacteria tested, and it required 5 hours of 405nm HINS-light exposure before complete inactivation of a mean starting population of 1.03 x10^5 CFU/ml was achieved. The test curve was fairly flat for the first 2 hours of exposure. Between 2-4 hours there was steady rate of inactivation. However, the graph was steepest after 4 hours, indicating a relative acceleration in terms of the rate of inactivation achieved. This was the longest exposure time required by any of the microorganisms tested in this study, demonstrating that this species was least susceptible to the bactericidal action of the 405 nm light. The inactivation curve can be seen in Figure 6.8. Of note, the control line behaved similar to previous experiments.

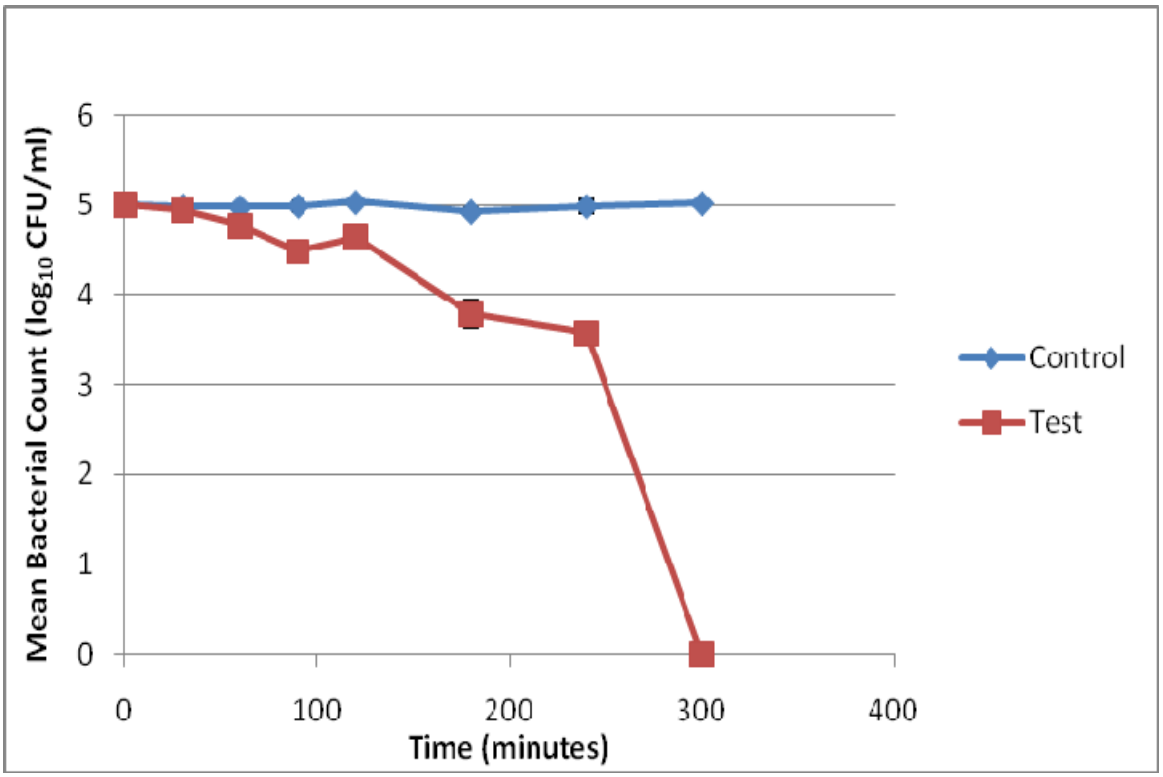


Figure 6.8 *Inactivation of Escherichia coli in suspension by HINS-light exposure (123 mW/cm^2 irradiance). n=1*

6.2.7 *Klebsiella pneumoniae*

From a mean starting population of 1.14 x10^5 CFU/ml complete inactivation of this pathogen was achieved after an exposure time of 3 hours. Figure 6.9 shows the inactivation curve. The test line shows gradual inactivation until 150 minutes, after which there is dramatic increase in the gradient. It is apparent that this bacterium continued the trend noted with the previous Gram-negative bacteria in terms of longer inactivation times being required. The control line was unremarkable and flat throughout.

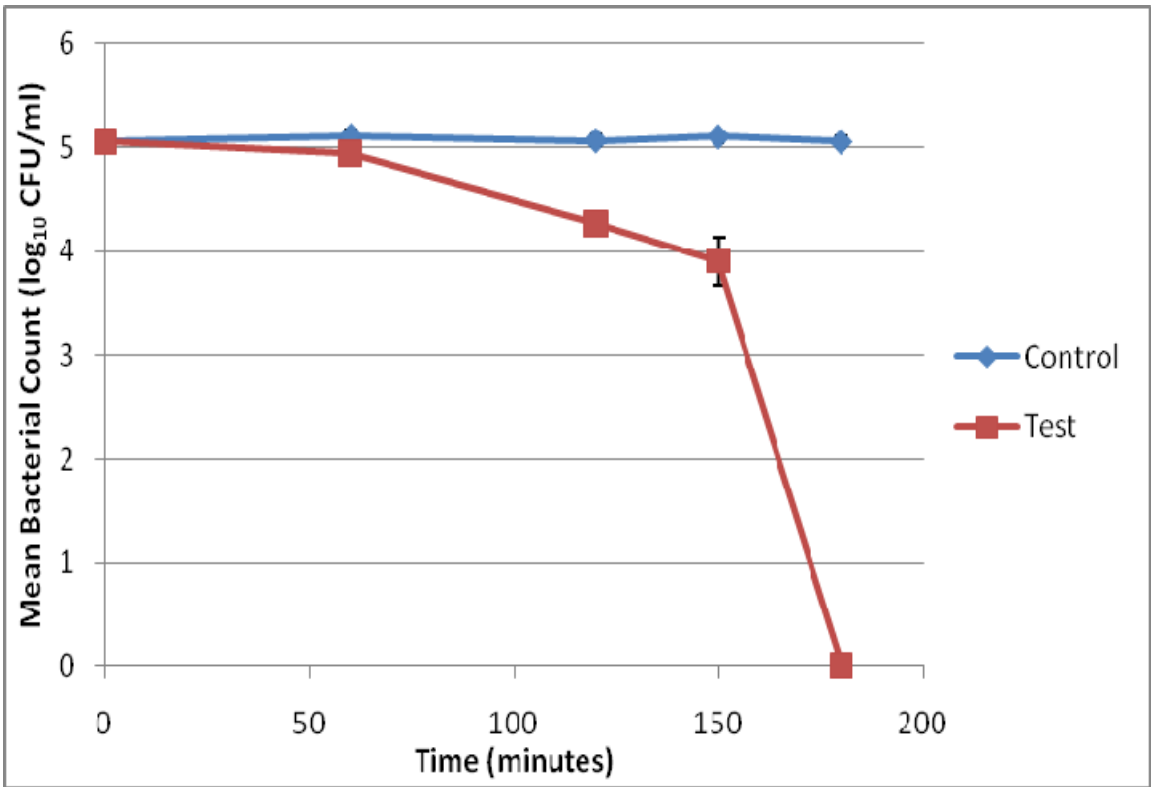


Figure 6.9 *Inactivation of Klebsiella pneumoniae in suspension by HINS-light exposure (123 mW/cm^2 irradiance). Mean ± S.D., n=3*

6.2.8 *Serratia marcescens*

From a starting population of approximately 1.5 x10^5 CFU/ml, complete inactivation took 4 hours. The inactivation curve for *S. marcescens* can be seen in Figure 6.10.

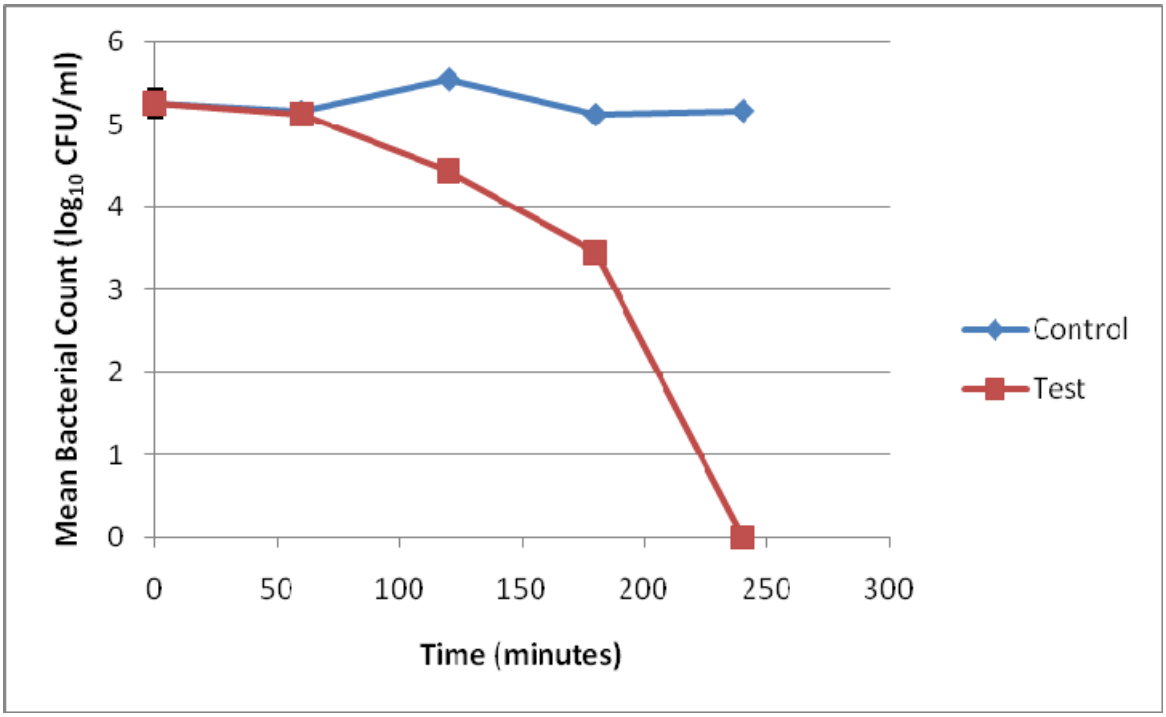


Figure 6.10 *Inactivation of Serratia marcescens in suspension by HINS-light exposure (123 mW/cm^2 irradiance). Mean ± S.D., n=3*

The test curve was similar to prior experiments. Between 0-3 hours there was a steady rate of bacterial inactivation observed. After 3 hours the test curve increased in terms of steepness, reflecting more rapid inactivation. The control line has an apparent slight peak at 120 minutes, but was largely flat for the experiments. The reason for this was unclear.

6.2.9 *Pseudomonas aeruginosa*

The results of *P. aeruginosa* inactivation are summarised in Figure 6.11. To achieve a 5-$\log_{10}$ reduction in population (mean starting population of 1.02 x10^5 CFU/ml), 90 minutes of 405nm light exposure was necessary.

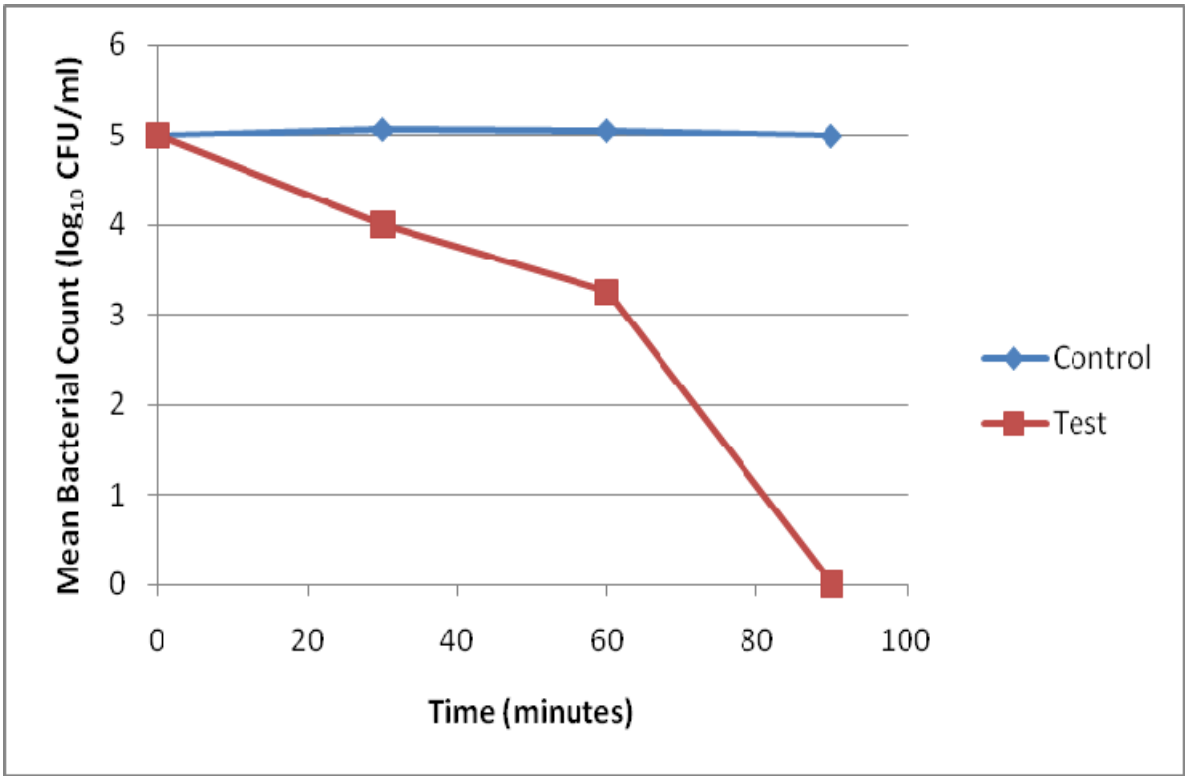


Figure 6.11 *Inactivation of Pseudomonas aeruginosa in suspension by HINS-light exposure (123 mW/cm^2 irradiance). Mean ± S.D., n=2*

The test curve demonstrated the recurring themes of steady inactivation, followed by a sharp increase in the rate of inactivation. In the case of *P. aeruginosa* this acceleration in bacterial inactivation took place after 60 minutes of HINS-light exposure. This was the quickest inactivation observed in any of the Gram-negative bacteria tested.

6.2.10 *Candida albicans*

This was the only yeast tested for its sensitivity to 405nm HINS-light. Experiments used a slightly lower starting population of 3.36 x 10^4 CFU/ml noted. The inactivation curve for *C. albicans* can be seen in Figure 6.12.

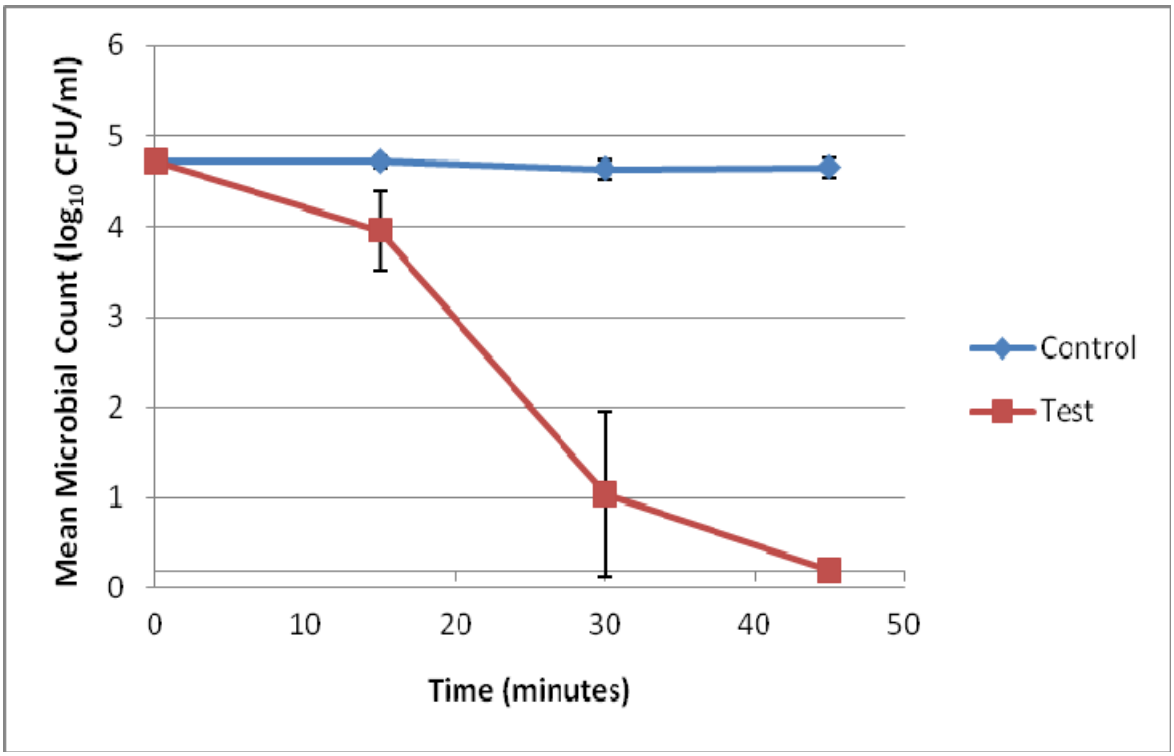


Figure 6.12 *Inactivation of Candida albicans in suspension by HINS-light exposure (123 mW/cm^2 irradiance). Mean ± S.D., n=3*

After 15 minutes exposure less than a 1-log_{10} reduction was observed. Over the course of a 30 minute exposure a 3-log_{10} reduction had taken place in one experiment, but when repeated complete inactivation was seen, hence the large standard deviation noted by the error bars. In all experiments complete inactivation was noted after 45 minutes. The test curve shape is similar to that observed for bacterial inactivation. There is gradual inactivation initially, followed by an increased gradient in the curve between 15-30 minutes. Unlike the bacterial experiments, however, this is followed by an apparent reduced rate of inactivation between 30-45 minutes. This may simply reflect skewed results from one of the three experiments, or indeed the fact that yeast respond differently from bacteria to HINS-light. The control line was flat.

6.3 Summary of bacterial and yeast suspension experiments

A graph comparing the inactivation kinetics for all the **Gram-positive** bacteria exposed to 405nm HINS-light can be seen below in Figure 6.13.

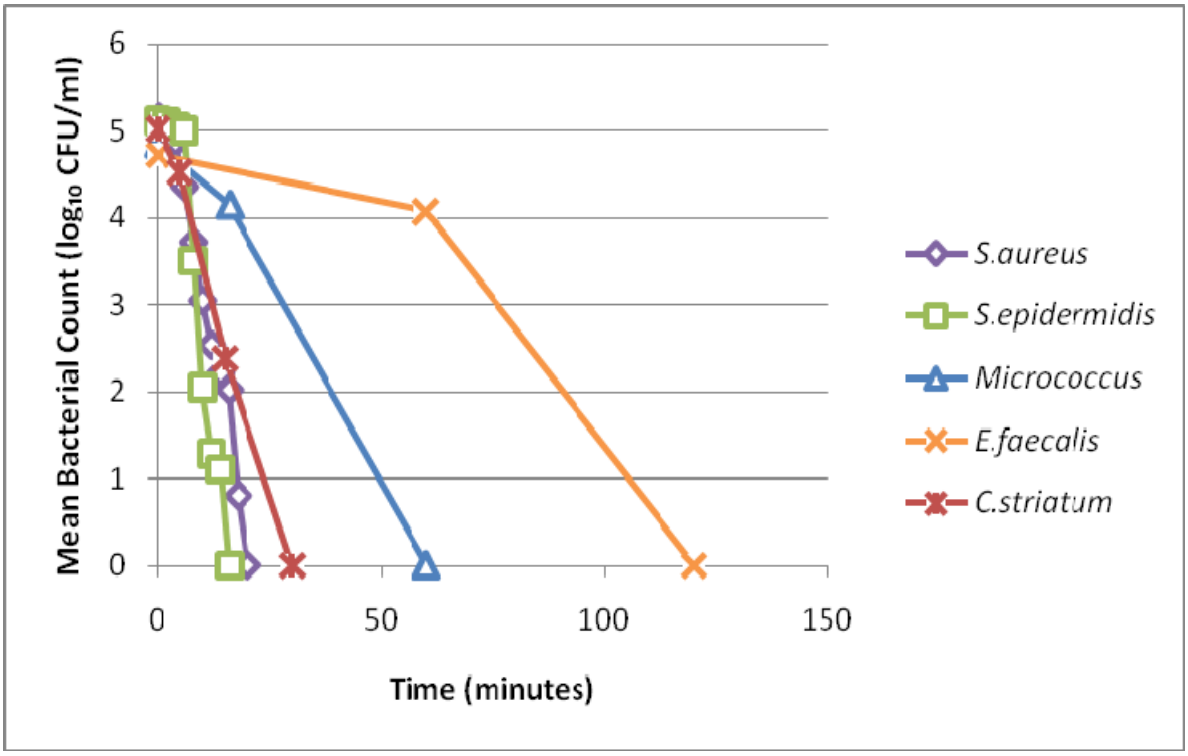


Figure 6.13 *Comparison of the kinetics for the HINS-light inactivation of the Gram-positive bacterial isolates in suspension (123 mW/cm^2 irradiance)*

With the exception of *E. faecalis,* all of the Gram positive bacteria were successfully inactivated in under an hour, with an approximate 5 $\log_{10}$ reduction achieved in each case. This graph demonstrates that all of the Gram-positive bacteria were inactivated fairly rapidly whilst in suspension. *S. epidermidis* was the quickest to be completely inactivated in 16 minutes, followed closely by *S. aureus* which took 20 minutes. *E. faecalis* was the slowest to be inactivated, taking around 120 minutes, but this may be a slight overestimation in view of the fewer time points that were used in the *E. faecalis* experiments.

A similar graph comparing the inactivation kinetics for all of the **Gram-negative** pathogens can be seen in Figure 6.14.

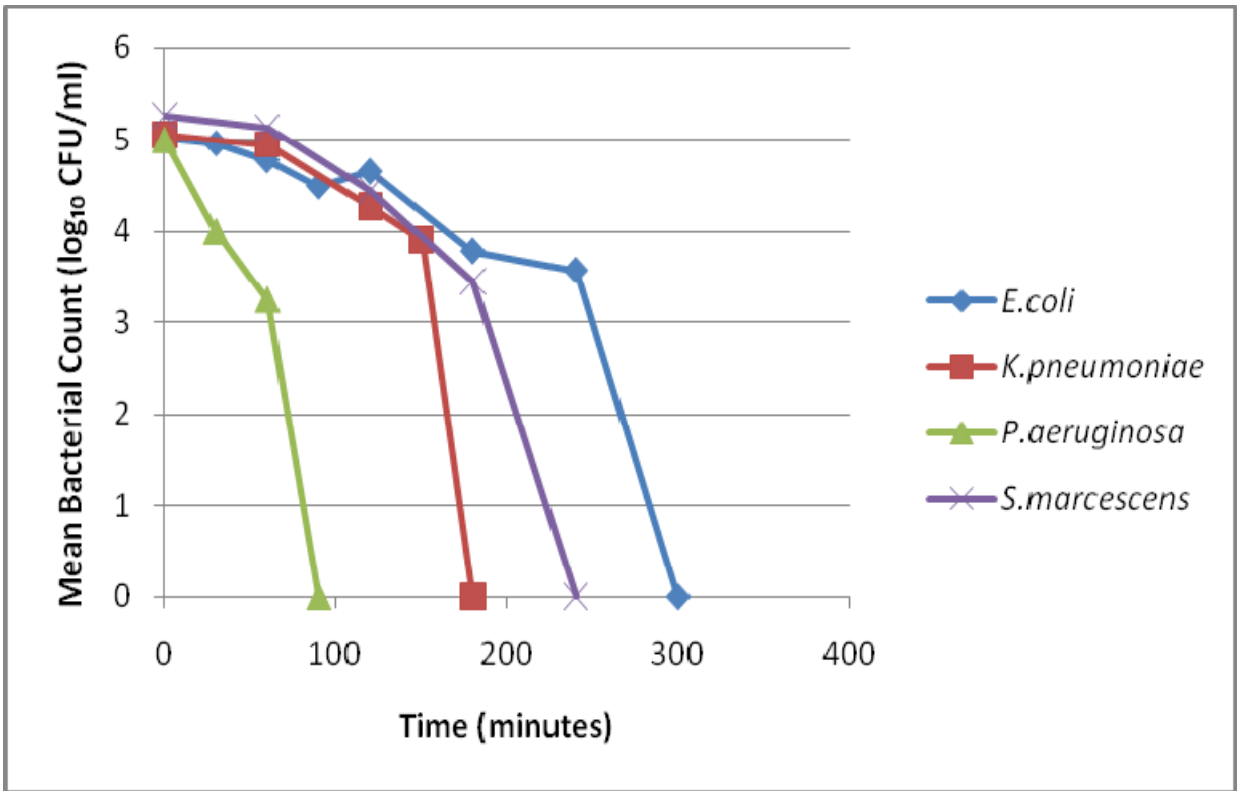


Figure 6.14 *Comparison of the kinetics for the HINS-light inactivation of the Gram-negative bacterial isolates in suspension (123 mW/cm^2 irradiance)*

As previously noted, the inactivation times for the Gram-negative organisms were clearly longer than those for the Gram-positive bacteria. The longest time required for inactivation was 5 hours of 405 nm light exposure; and this was for *E.coli*. The other enterobacteriaceae, namely *K. pneumoniae* and *S. marcescens*, required slightly shorter exposure times of 3 and 4 hours respectively for complete inactivation. *P. aeruginosa* was the last Gram-negative organism tested, and was found to be inactivated quickest, over the course of 90 minutes.

C. albicans was the sole yeast isolate exposed to HINS-light, and a clear 4 $\log_{10}$ reduction was apparent after 45 minutes. As the starting populations were different from the bacteria, it is not possible to be precise about how long a 5 $\log_{10}$ reduction would have taken. However, based on the curve shape observed in Figure 6.12, one would hypothesise complete inactivation by 60 minutes. Comparing this with Figures 6.13 and 6.14, this would suggest *C. albicans* behaved like slowly inactivated Gram-positive bacteria, or fairly fast inactivated Gram-negative bacteria.

6.4 Doses of HINS-light used in bacterial and yeast suspension experiments

When the irradiance and exposure time of the HINS-light applied to the microorganisms is known, the dose applied may be calculated based on the formula:

E (energy or dose) = **P** (power or irradiance) x **t** (time, in seconds)

A summary of the doses of HINS-light applied for each microorganism can be seen in Table 6.2. It should be noted that the exposure time detailed in the table reflects the time required for complete inactivation of the microbial populations, as per the inactivation curves in sections 6.2.1-6.2.10.

Table 6.2 *Doses of HINS-light required for complete inactivation of microorganism*

Microorganism	Exposure Time (s)	Power Density (mW/cm^2)	Dose (J/cm^2)
S. aureus	1200	123	147.6
S. epidermidis	960	123	118.1
C. striatum	1800	123	221.4
E. faecalis	7200	123	885.6
Micrococcus	3600	123	442.8
E. coli	18000	123	2214
K. pneumoniae	10800	123	1328.4
S. marcescens	14400	123	1771.2
P. aeruginosa	5400	123	664.2
C. albicans	2700	123	332.1

The highest dose of HINS-light required for inactivation was for *E. coli* at 2214 J/cm^2, while the lowest was for *S. epidermidis* at 118.1 J/cm^2. Generally higher doses were necessary to completely inactivate the Gram-negative bacteria in comparison to the Gram-positive. The dose of HINS-light required for *C. albicans* was 332.1 J/cm^2, which falls closer to the Gram-positive spectrum of inactivation.

6.5 Germicidal efficiency

Based on the experimental results for the HINS-light exposure of microbial suspensions, from section 6.2.1-6.2.10, the inactivation capability of the 405nm HINS-light can be quantified. This is known as the germicidal efficiency (GE). This can be defined as the $\log_{10}$ reduction of a given microbial population by inactivation per unit of light energy density in J/cm^2, also known as the dose (Wang *et al*, 2005). This calculation can be shown as:

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

Table 6.3 provides a summary of the GE of the 405 nm light emitted from the 99-DIE LED array for all the pathogens tested.

Table 6.3 *Germicidal efficiency of 99-DIE LED array against pathogens isolated*

Pathogen	Dose (J/cm^2)	Log_{10} Reduction	Germicidal Efficiency ($log_{10}(N/N_0) / J/cm^2$)
S. aureus	147.6	5.16	0.0350
S. epidermidis	118.1	5.12	0.0434
C. striatum	221.4	5.02	0.0227
E. faecalis	885.6	4.72	0.0053
Micrococcus	442.8	4.85	0.0110
E. coli	2214	5.01	0.0023
K. pneumoniae	1328.4	5.05	0.0038
S. marcescens	1771.2	5.26	0.0030
P. aeruginosa	664.2	5.00	0.0075
C. albicans	332.1	4.52	0.0136

The results show that microorganisms in suspension can be successfully inactivated through exposure to light of wavelengths of 405 nm. The highest GE was noted for *S. epidermidis*, with a value of 0.0434. The lowest GE was seen with *E. coli*, where a value of 0.0023 was observed. The Gram-positive bacteria generally had a higher GE than Gram-negative bacteria, with the one exception being *E. faecalis*. *P. aeruginosa* had a higher GE than it. The yeast isolate (*C. albicans*) had a GE of 0.0136, which was marginally better than *Micrococcus*, and was considerably higher than all of the Gram-negative bacteria.

6.6 Effect of temperature

As with all microbial inactivation technologies, it is important to establish that inactivation is a result of the decontamination treatment being applied and not any co-founding factors. In this case, it was important to establish that inactivation found in this study was a result of exposure to the microbicidal 405 nm light and not the effect of temperature causing excessive heating of the bacterial samples.

To investigate the temperature change in the bacterial suspensions during light exposure, the suspension temperature was measured at regular intervals during exposure using a thermocouple (model KM340, Kane-May). The longest period of light exposure used in the present study was 5 hours (for *E. coli*) and as such, the results for this 5-hour exposure period are presented in Figure 6.15. Even after 5 hours exposure, the temperature reached a maximum of only 39°C. *E. coli*, the bacteria subjected to this duration of light exposure, is known to grow at temperatures of over 40°C (Fotadar et al, 2005). All other microorganisms were inactivated in 4 hours or less, when the suspension had reached 37°C, core body temperature. All isolates were *in vivo* specimens, and hence any inactivation process was not thermal.

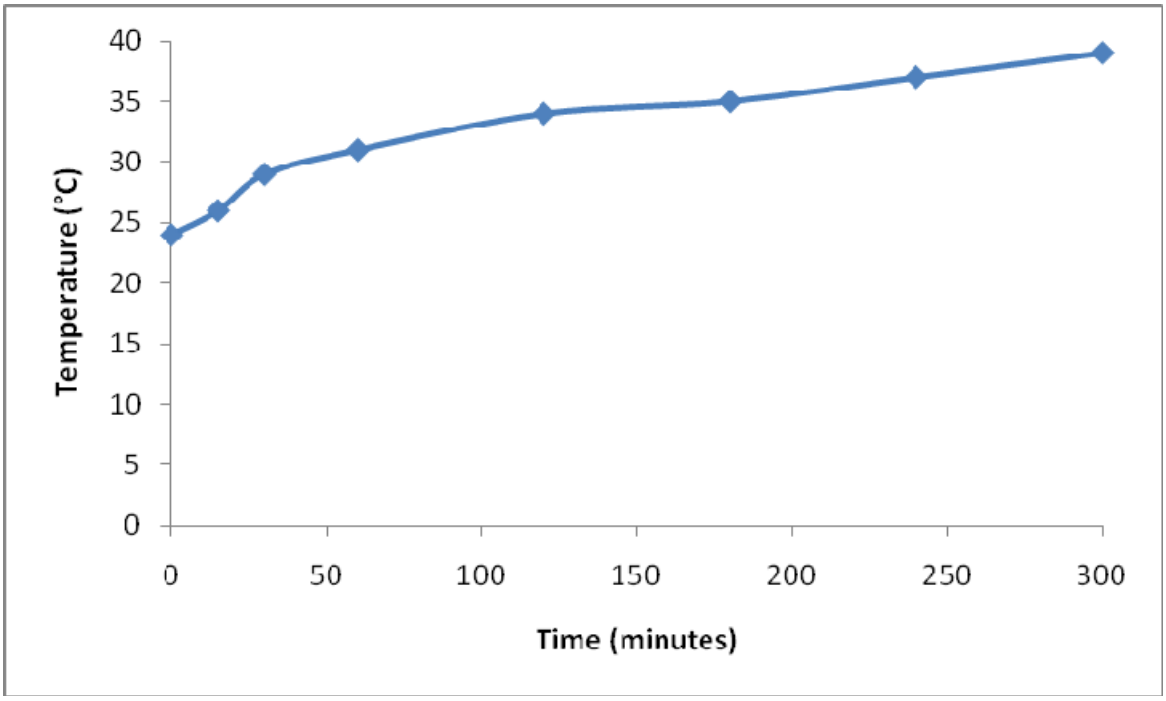


Figure 6.15 *Effect of light exposure on liquid suspension temperature*

Part II: HINS-light Exposure of Microorganisms Seeded onto Agar Surfaces

6.7 Surface-inoculated microbial treatment system

The aim of this series of experiments was to investigate the effectiveness of HINS-light for the inactivation of microorganisms seeded onto a solid surface – in this case, the surface of an agar plate. The bacteria were cultured and prepared in a similar manner to that for suspension experiments, as described in section 6.1. The principal difference with the surface experiments was the dilution process, as for these experiments samples were required to have a lower starting population than was used in the suspension tests. Once the microbial pellet was resuspended in PBS, it was serially diluted to approximately 10^3 CFU/ml. This was to allow accurate manual counting post-incubation.

A Gilson pipette and sterile tip was used to transfer 100µl of the 10^3 CFU/ml microbial suspension onto the agar surface (the type of agar being dependent on the microorganism being tested – see table 4.2). Using a sterile L-shaped spreader, the sample was evenly distributed over the surface of the agar plate. Once dry, this plate was then positioned under the ENFIS QUATTRO LED light engine at a fixed height of 5cm (Figure 6.16).

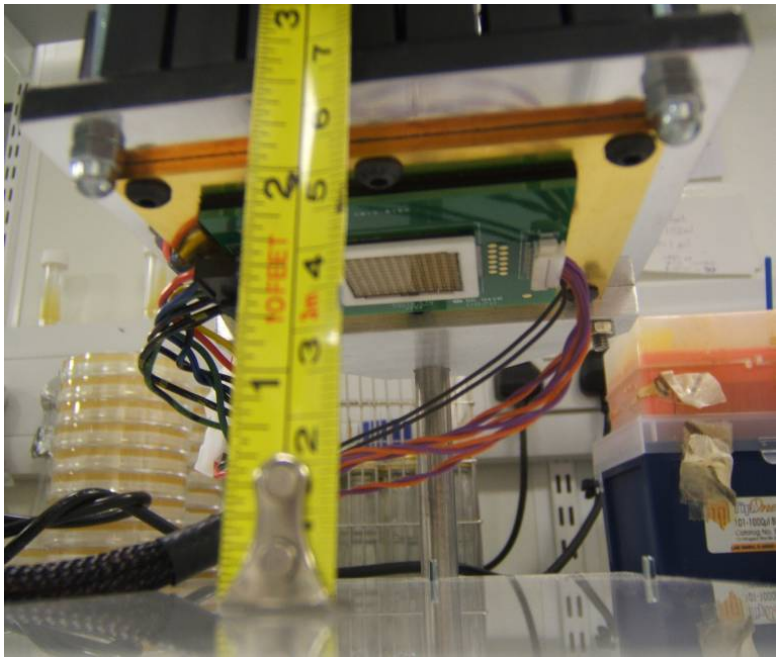


Figure 6.16 *ENFIS Quattro LED light engine maintained at a height of 5cm from the agar surface for all exposure experiments*

The light settings used are described in the earlier section 5.5. The final experimental arrangement can be seen in Figure 6.17 below.

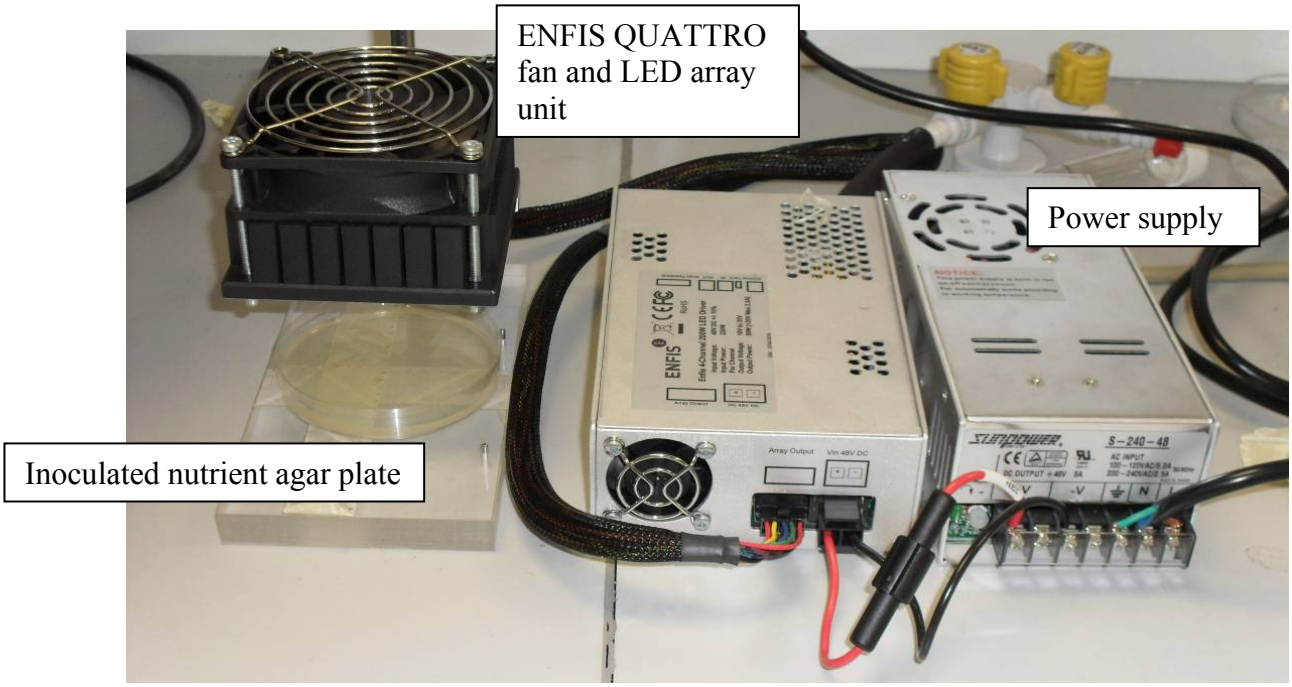


Figure 6.17 *HINS-light exposure system for surface inoculated bacteria*

A control sample was set-up for each test. This was a surface inoculated agar plate which was left on the laboratory bench and not exposed to high intensity 405nm light. Following exposure, test and control plates were incubated at 37°C for 24 hours before enumeration. The key experimental parameters kept constant throughout surface testing are summarised in Table 6.4.

Table 6.4 *Surface-inoculated bacteria treatment system: summary of key experimental parameters*

Experimental parameter	**Value**
Sample volume	100µl
Exposure distance	5cm
HINS light input voltage	48V
Irradiance from LED array	33.5-203.1mW/cm^2 (peripheral to central) Average across the plate = 71 mW/cm^2

6.8 Results of HINS-light treatment of surface-inoculated pathogens

This section reports the findings obtained when the clinical isolates were exposed to HINS-light whilst seeded on the surface of agar plates. The experimental technique used is as detailed in the previous section.

This study investigated the exposure of microbial isolates on agar surfaces, and as such, the calculation of the population densities surviving following HINS-light exposure differed slightly from that used for the suspension exposure experiments. Results for exposure of liquid suspensions were recorded as colony-forming units per millilitre (CFU/ml), but the results for exposure of bacteria seeded onto agar surfaces are recorded as CFU per agar plate (CFU/plate).

An example inactivation curve of results for the yeast *C. albicans* is presented. This is followed by summary charts of the results for Gram-positive and Gram-negative bacteria. All of the charts in this section are X-Y scatter plots. Unfortunately, due to time limitations, experiments could not be repeated and therefore there are no error bars present on the charts.

6.8.1 Exposure of surface-inoculated pathogens – an example inactivation curve

C.albicans was one of the first isolates exposed to the ENFIS QUATTRO LED 405nm light engine whilst seeded onto agar surfaces. As can be seen in Figure 6.18, complete inactivation was achieved after 40 minutes. The test curve shows steady inactivation until 20 minutes, and there is perhaps a slight acceleration following this between 20 and 30 minutes. After 30 minutes the curve becomes less acute, not dissimilar in pattern to what was observed in the suspension experiments. The control curve appears to undulate slightly with the time period tested, but is overall fairly flat throughout. Variations in the control line are likely to be more apparent in the surface experiments due to the use of low seeding populations and 'real' counts. High populations and log counts, as used in the suspension experiments, do not show fluctuations as readily due to the scales used.

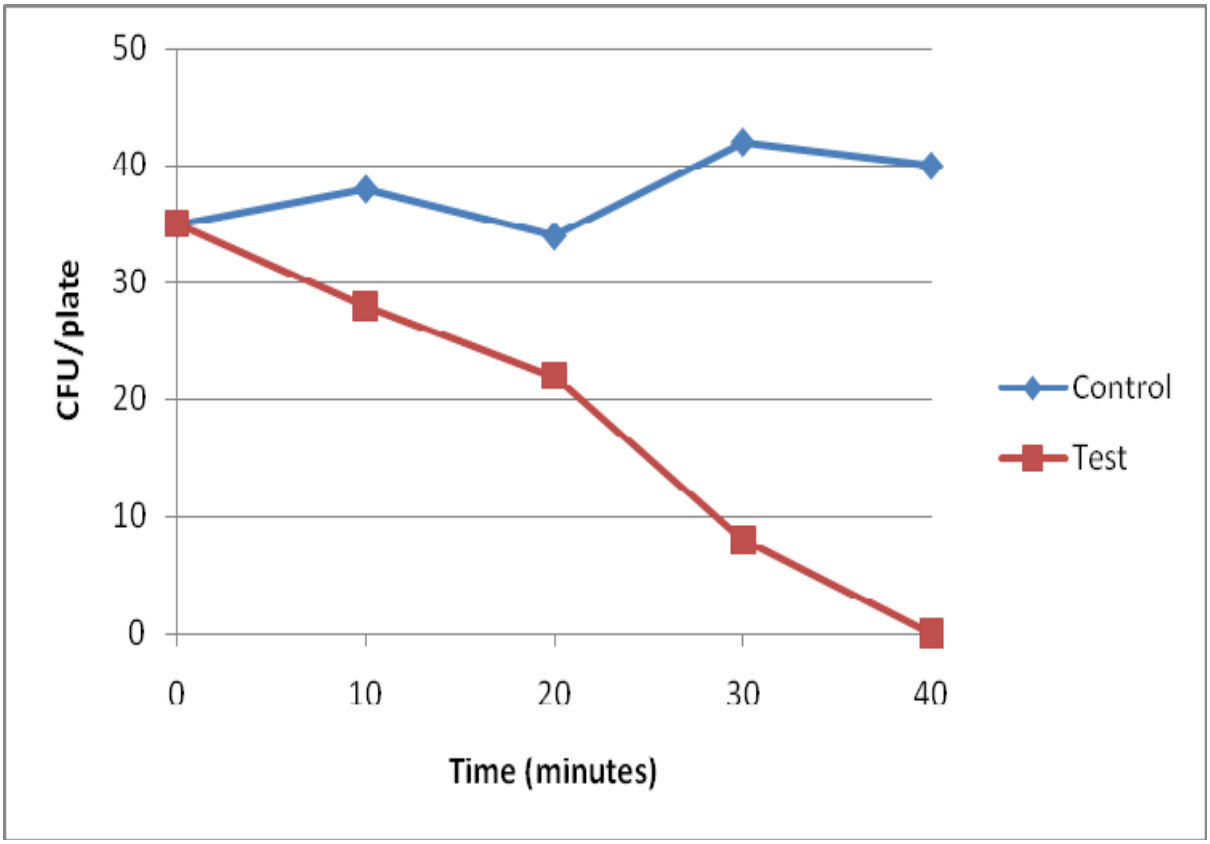


Figure 6.18 *C.albicans surface inactivation in response to HINS light (average 71 mW/cm^2 irradiance)*

6.8.2 Summary of Gram-positive surface experiments

As mentioned in section 6.8, the number of CFU/plate surviving post-exposure was recorded. Though similar graphs could be produced for all of the clinical isolates tested, it was apparent that the starting populations for each of the pathogens varied significantly, therefore conversion of the raw data to give percentage reduction allowed the efficacy of the HINS-light for inactivation of the isolates to be directly compared. Comparison of the inactivation kinetics for the Gram-positive organisms is shown in Figure 6.19.

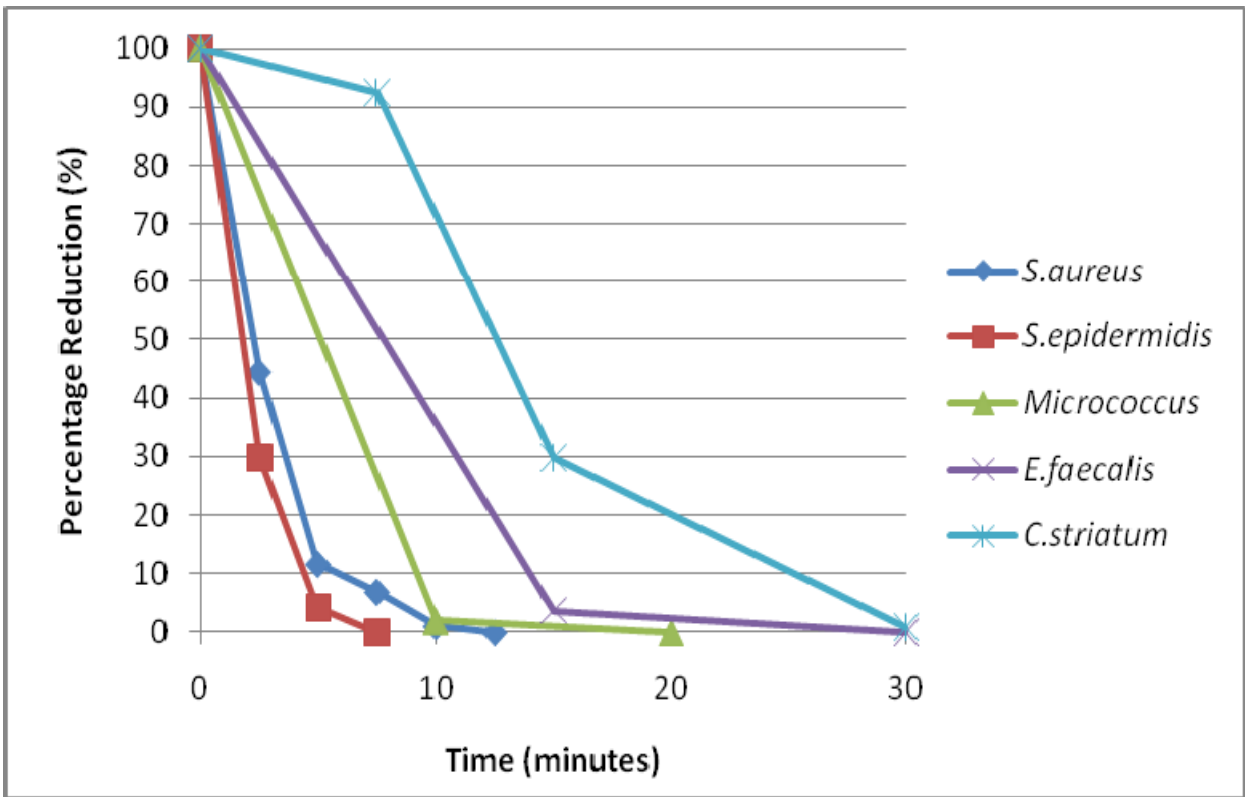


Figure 6.19 *Comparison of the kinetics for the HINS-light inactivation of the Gram-positive bacterial isolates seeded onto agar plate surfaces (average 71 mW/cm^2 irradiance)*

The graph shows that all of the gram-positive bacteria underwent complete inactivation within 30 minutes. *S. epidermidis* was the pathogen to be inactivated in the shortest period of time, in 7.5 minutes. This was closely followed by *S. aureus*, which had a similarly shaped curve. Both *Micrococcus* and *E. faecalis* exhibited

similarly shaped inactivation curves, with over 95% inactivation having taken place when the first time point was recorded for each respectively. With the exception of *C. striatum*, all of the organisms appeared to undergo rapid inactivation in what may be described as an exponential decay type pattern.

The inactivation curve of *C. striatum* showed a slow initial response to the 405 nm light, but this was followed by an accelerated period. The rate of inactivation then slowed again before complete inactivation. As this exact pattern was not observed for the other Gram-positive bacteria, it possibly suggests a difference when using the blood based agar as the seeded surface. Use of blood agar which is red in colour, compared to the pale yellow colour of nutrient agar and tryptone soya agar, may affect HINS-light absorption. This was explored further, and is discussed in section 6.8.4.

A feature that all of the curves shared, including *C. striatum,* was the apparent deceleration at the end of the trendlines. This may be referred to as 'tailing', and is likely to be as a result of the uneven light distribution across the plate surface shown in Figures 5.10 and 5.11. Bacteria in the centre of the plate, will therefore, be killed faster than those bacteria towards the periphery.

6.8.3 Summary of Gram-negative surface experiments

A similar inactivation kinetics graph can be seen for the Gram-negative pathogens in Figure 6.20. All of the isolates were fully inactivated within 60 minutes.

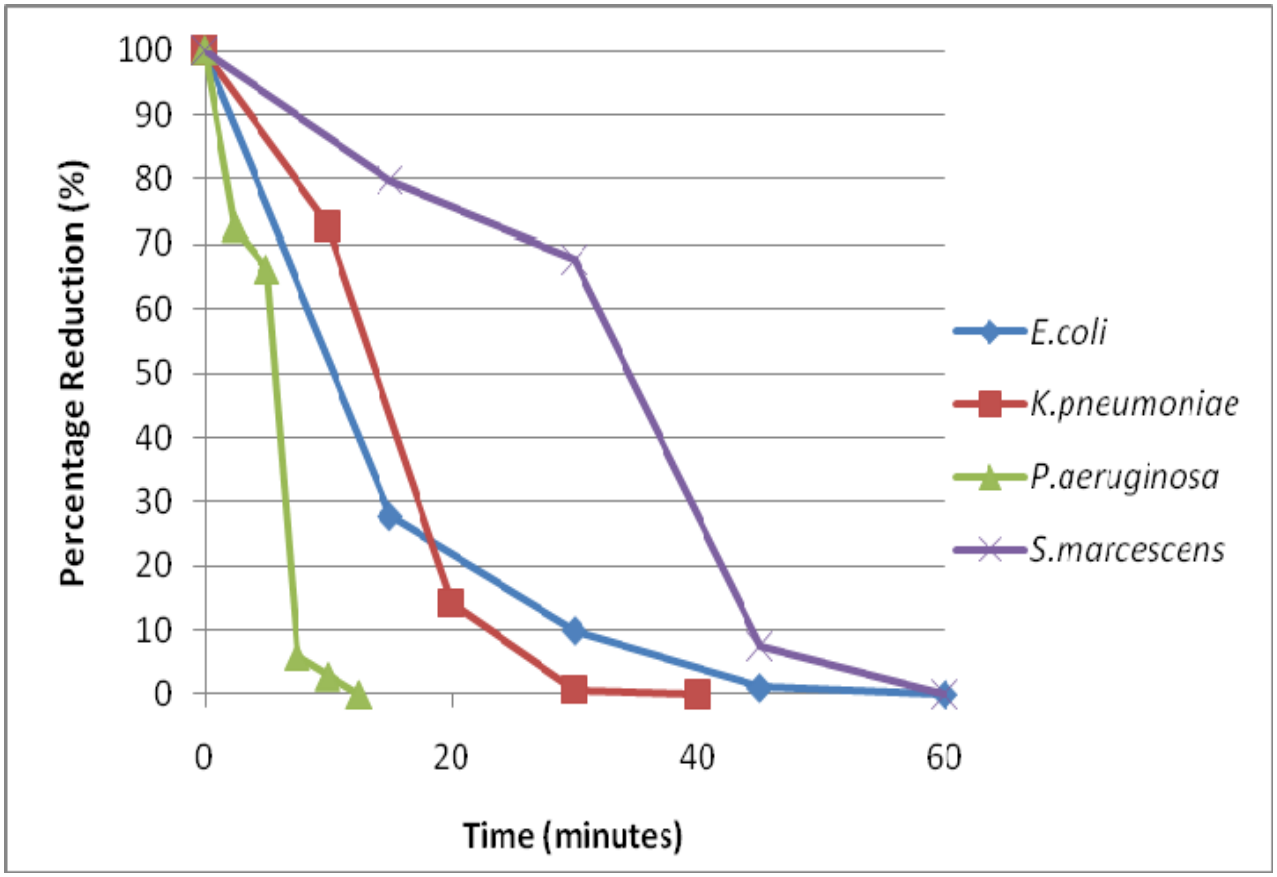


Figure 6.20 *Comparison of the kinetics for the HINS-light inactivation of the Gram-negative bacterial isolates seeded onto agar plate surfaces (average 71 mW/cm^2 irradiance)*

P. aeruginosa was the Gram-negative bacterium inactivated in the shortest period of time, taking 12.5 minutes. The gradient is fairly steep throughout, with a relative deceleration towards the end of the curve. The *K. pneumoniae* curve showed relatively slower progress initially, but a 60% reduction in the population was observed between 10 and 20 minutes of 405 nm light. This was again followed by a slower period of inactivation at the end of the curve.

The surface experiments using *E. coli* noted a steep decline in bacteria population after 15 minutes of HINS-light exposure. The line tailed off thereafter until full inactivation at 60 minutes. With *S. marcescens* there was slow initial progress. Between 30 and 45 minutes, however, rapid inactivation occurred. The deceleration pattern at the end of the curve in terms of bacterial inactivation was again observed. The 'tailing' pattern noted with all of the Gram-negative bacteria may be explained by the uneven light distribution over the plate surface, discussed at the end of the previous section 6.8.2.

6.8.4 Bacterial inactivation in the presence of blood

Arthroplasty surgery may lead to a bloody surgical field, and an experiment was set-up with the aim of potentially providing a crude indication as to the efficacy of 405nm light for inactivation of bacteria whilst on a blood-containing surface.

This experiment was carried out to investigate the effect of HINS-light exposure on bacteria seeded onto agar plates that contain blood. This experiment used plates containing blood agar, which is agar supplemented with defibrinated horse blood. This line of testing was of particular interest following the surface experiment results of *C. striatum* (section 6.8.2), the only isolate previously tested on blood based agar.

S. aureus was the test organism selected. The culture and preparation of the *S. aureus* was the same as that described in section 6.7, with the experimental parameters summarised in Table 6.4. The principal additions to the previous surface *S.aureus* experiments were that of surface-inoculated blood based agar control and test plates. The hypothesis was that due to the blood not being transparent, there would be increased absorption of the light by the blood, consequently potentially reducing the bacterial inactivation rate observed.

The results from the previous surface exposure experiments using *S. aureus* (Figure 6.19) identified that complete inactivation of the isolate was achieved after 12.5 minutes. On this basis we repeated the experiment for the same duration using both nutrient agar and blood agar plates, and counted the number of CFU per plate

surviving after HINS-light exposure. This was repeated for non-exposed control plates. The results are summarised in Table 6.5.

Table 6.5 *Experimental results of HINS light inactivation of S. aureus seeded on the surface of blood agar plates. Test plates were exposed to 12.5 min HINS-light treatment at an irradiance of 71 mW/cm^2; control plates were left for the same time period in laboratory lighting conditions.*

Setting	CFU/plate
Starting population	310
Nutrient agar control	320
Nutrient agar test	0
Blood based agar control	313
Blood based agar test	91

Table 6.5 shows that there was complete inactivation of *S. aureus* seeded on nutrient agar surfaces, similar to previous experiments. However, when seeded on blood agar plates, 29% of the bacteria remained when tested over an identical period of time. Although not tested, it is anticipated that complete inactivation of the organism would have been achieved with a longer exposure time (i.e. an increased dose).

The results confirmed our hypothesis that in the presence of blood there was reduced bacterial inactivation. Clinically, this very provisional experiment suggested that the microorganisms may be slightly less susceptible to 405nm light in a blood field.

6.9 Summary and Discussion

The primary aim of any arthroplasty surgery is to relieve pain. Over 178,000 hip and knee replacements are performed in Great Britain annually (Scottish Arthroplasty Project, 2010; National Joint Registry England & Wales, 2010). With such high volumes of surgery being performed, complications are inevitable, and infection remains one of the most challenging to deal with. Though there are many preventative measures in place, an infection rate of around 1% is still quoted to patients. The management of an infected prosthetic joint usually involves a combination of medical and surgical modalities, but outcomes are variable. Any new technologies to reduce the burden of arthroplasty infection are welcome.

HINS-light is a recently developed antimicrobial technology developed by the ROLEST team at the University of Strathclyde. It has a narrow band of visible blue light with a peak wavelength of approximately 405 nm. The inactivation of pathogens is achieved via a photodynamic inactivation effect, which is triggered by absorption of the light and is mediated by porphyrins (Maclean et al, 2010). Though much work has been done with HINS-light in the laboratory, the potential clinical role is still evolving (section 2.6.3). In this thesis, the sensitivity of clinical isolates to HINS-light was examined.

All of the pathogens isolated from infected lower limb arthroplasty that could be cultured and identified were exposed to HINS-light. In both the suspension and surface exposures, all of the microorganisms were inactivated using 405 nm light. Some specific trends or patterns were observed during testing, and are described along with a summary of the results in this section.

During suspension experiments an irradiance of 123 mW/cm^2 was applied, and *S. epidermidis* was the bacteria inactivated in the shortest period of time, 16 minutes. This Gram-positive bacterium required the lowest dose of all those tested, and as a result had the highest GE. *E. coli* took the longest period of time to achieve complete inactivation, 300 minutes. This Gram-negative bacterium therefore required the highest dose of all those tested, and in turn had the lowest GE. Murdoch et al (2010) found the GE of *E. coli* O157:H7 exposed to 405 nm light to be 0.02, ten times greater

efficiency than what we noted. There are many potential reasons for this, including differences in the exact nature of the light source used in each study. This may lead to differences in the emission from each source, and different peak wavelengths. Alternatively, this may simply be a reflection on the different bacterial strains examined in the separate studies.

The Gram-positive bacteria were generally noted to require lower doses of HINS-light exposure to achieve inactivation in comparison to the Gram-negative bacteria. This was similar to what has previously been published in 2009 by Maclean et al. The authors of that paper suggested that Gram-positive bacteria produce greater quantities of porphyrins, and specifically coproporphyrin, making them more readily inactivated by visible light than Gram-negative bacteria. The only yeast tested was *C. albicans*, and it was seen to behave in a manner more similar to the Gram-positive bacteria than Gram-negative, with respect to inactivation dosages and GE. The potential role of confounding factors such as temperature from the light source was disproven.

During surface experiments an irradiance of 33.5-203.1 mW/cm^2 (peripheral to central) was applied, giving an average across the plate of 71 mW/cm^2. *S. epidermidis* was the microorganism most susceptible to visible blue light treatment on an agar surface, with complete inactivation taking place in 7.5 minutes. *E. coli* and *S. marcescens* were the Gram-negative bacteria that required the longest period of light exposure prior to inactivation; 60 minutes. *C. albicans* was completely inactivated after 40 minutes of HINS-light surface exposure. This was longer than all of the Gram-positive bacteria and one of the Gram-negative bacteria - *P. aeruginosa*. This relative change in inactivation time with respect to the other microorganisms, may represent that *C. albicans* is more susceptible to HINS-light in a suspension form.

In the bacteria surface experiments Gram-negative pathogens generally continued to take longer to inactivate than Gram-positive. The final blood-based surface exposure experiment suggested that blood may hinder but not negate the inactivation properties of blue light.

The suspension inactivation curves for all of the bacteria followed a similar shape. There was an initial period of inactivity, followed by a rapid acceleration until

complete inactivation. The distinct inactivation times observed may be explained by the fact that different bacteria produce different porphyrins, the peak absorption wavelengths are therefore likely to vary, and in turn different wavelengths may be required for optimum photostimulation (Maclean et al, 2009). In contrast, the recurring pattern noted with the bacteria isolates in surface experiments was a steep initial gradient, followed by a slower phase prior to complete inactivation. This apparent deceleration or 'tailing' trend noted may be as a result of the uneven light distribution across the plate surface. Bacteria in the centre of the plate, will therefore, be killed faster than those bacteria towards the periphery.

When comparing the results of the suspension and surface experiments, there were clear similarities. The logarithmic reductions achieved in a suspension form for *S. epidermidis* and *S. aureus* were mirrored on seeded agar surfaces. *P. aeruginosa* remained the Gram-negative pathogen most susceptible to the effects of HINS-light in both sets of experiments. The enteric bacteria were noted to be the least responsive to HINS-light in all experiments. This may represent inherent resilience from their natural gut habitat.

Gram-negative bacteria remained generally less responsive to 405 nm light than Gram-positive, however, subtle changes between the individual bacteria were noted. For example, *E. faecalis* was the slowest pathogen to be inactivated in the suspension form, but on a surface was quicker than *C. striatum*. This may simply reflect blood interfering with blue-light absorption, or that *E. faecalis* is more sensitive on a surface. In both suspension and surface experiments *C. striatum* displayed unusual inactivation curves compared to the other Gram-positive bacteria. In the suspension exposures it did not have the typical slow start seen, but when surface exposed it did, the exact opposite of the other Gram-positives.

The overall inactivation times for all microorganisms were shorter in the surface experiments than the bacterial suspensions. There are some potential reasons for this. Firstly, the experiments were designed to have starting populations that were reasonable to manually count. Hence, lower volumes of less concentrated pathogens were used to yield a significantly lower starting population. Furthermore, there was higher irradiance from the LED array used in surface experiments, particularly over

the central portion of the plate. This may have contributed to the accelerated inactivation noted.

Although the visible-light inactivation on a surface reported here displays the same sigmoidal-shaped inactivation curve as that caused by continuous UV light, we know from sections 2.6.1 and 2.6.3 that differing mechanisms of inactivation are occurring. Blue light inactivation is mediated by porphyrins, whilst UV light inactivation is as a result of DNA damage. This point is reinforced when our work is compared to that of Chang et al (1985). The authors conducted a series of experiments using a collimated beam of UV light set at 254 nm and found that Gram-negative bacteria were more susceptible than Gram-positive bacteria. This is the opposite of the results reported with HINS-light thus far, and may reflect that Gram-negative bacteria are more susceptible to UV-based damage than porphyrin-related damage.

Chang et al (1985) also report that the dosage of UV light required for a 5-$\log_{10}$ reduction of both *S. aureus* and *E. coli* was approximately 10 mW-sec/cm^2. This is considerably less than any of the dosages required involving 405 nm blue light. The dosage of UV light required for inactivation was also seemingly the same for both bacteria. With HINS-light there is a marked difference in the inactivation dosage for the corresponding bacteria, with *E. coli* proving to be the most resistant. One of the few common features noted of both UV and HINS-light is the dose-mediated inactivation effect. Prolonged UV light exposure unfortunately has a significant human side effect profile, including a spectrum of dermatological and ophthalmological conditions. HINS-light does not have such sequelae, and therefore offers relative operational safety.

A practical advantage of the 405 nm blue light compared to other visible light systems is the lack of need for an exogenous photocatalyst or photosensitiser. Lambrechts et al (2005) describe photodynamic inactivation as a process that requires both visible light and a photosensitiser for cellular inactivation. Using white light and a cationic porphyrin they inactivated *S. aureus, P. aeruginosa* and *C. albicans* in both PBS and blood plasma settings in a dose dependent manner. From our results it is evident that a photosensitiser is not necessary, as we achieved complete inactivation of all these microorganisms in our study. However, the dosage of white light delivered in the

study by Lambrechts et al was 30 $mWcm^{-2}$, considerably less than what was used in the HINS-light experiments. Therefore, though the photosensitiser is not necessary, it may reduce the dosage of visible light required for microbial inactivation.

In brief, all clinical isolates from infected lower limb arthroplasty identified over the period of one year were successfully inactivated by HINS-light. The demonstration of their susceptibility means that there could be potential to develop HINS-light decontamination systems for use in applications relating to orthopaedic surgery and infections. The potential applications of HINS-lights with differing irradiance in various settings will be discussed in section 7.1.

Chapter 7

General conclusions and recommendations for further work

7.0 General conclusions

Hip and knee replacements are common, quality of life improving operations. Though all efforts are made to minimise the risk of infection, it is a recognised disastrous complication. The antimicrobial properties of UV light technology have long been recognised, but the role of visible light, and in particular blue light is a relatively recent innovation. This works by the process of photodynamic inactivation and does not require a photosensitiser.

All of the pathogens identified from infected lower limb arthroplasty over a 12 month period were exposed to 405 nm light and complete inactivation was achieved in a dose-related manner. Successful inactivation was observed in a suspension setting, and on surfaces. Gram-positive bacteria were more susceptible to HINS-light than Gram-negative. The treatment was noted to be less efficient than UV, but has significant safety advantages.

7.1 Recommendations for further work

In this section are ideas for future work that would enable the further development and potential clinical application of the HINS-light system. In this study 405 nm light has proven to be effective against microorganisms in both a suspension and surface setting. A limited experiment was carried out to assess inactivation properties in the presence of blood. This area requires detailed analysis, as arthroplasty surgery is often bloody. In infected cases there is often pus, and a biofilm may be present. The efficacy of the blue light here would help differentiate whether the light is useful for the treatment of infection, or only in prevention.

Ideally the HINS-light system that has been ceiling mounted for dressing clinics and burns patient isolation rooms, could continuously emit blue light in the operating theatre over the surgical field. However, from our experiments the irradiance of HINS-light was altered by the distance from the target, and whether it was directly centred over it. During surgery, this would suggest the need for either very high doses of light, or the need for a hand-held device to deliver the blue light locally. Clearly a device emitting high doses of blue light would interfere with the surgeon's tissue perception. This can be minimised by the development of glasses that absorb the blue light, or by mixing the blue light with white light, such that it is not seen. The latter strategy is currently employed with the environmental decontamination system. A hand-held device would not be practical unless mounted to surgical equipment, as this would add considerably to the operating time, a known independent risk factor for increasing infection rates in arthroplasty surgery (Småbrekke et al, 2004).

At present the technology is being used for environmental decontamination of isolation rooms in the Canniesburn Plastic Surgery Unit (Bache et al, 2012). Pending further work, HINS-light may help prevent infection in the Southern General Hospital orthopaedic operating theatres and beyond.

Appendix

Inactivation of microorganisms isolated from infected arthroplasty using High-Intensity Narrow-Spectrum light.

Podium presentations at:

1) Combined British and Irish Orthopaedic Associations Meeting, Dublin. September 2011
2) Scottish Committee for Orthopaedics and Trauma, Crieff.
   January 2012

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