DEPARTMENT OF ELECTRONIC & ELECTRICAL ENGINEERING

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Susceptibility of *Clostridium difficile* to 405 nm Light and Possible Mechanisms to Enhance Sporicidal Activity

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ABSTRACT

Clostridium difficile is currently one of the most significant causative agents of healthcare-associated infection. The ability of *C. difficile* to form highly resilient spores has limited the competence of current strategies for environmental decontamination, and advances to reduce environmental contamination and patient infection are urgently sought.

The results of this study demonstrate the sporicidal efficacy of 405 nm violet-blue light to achieve up to 5 $\log_{10}$ reductions in *C. difficile* spores. In comparison, vegetative cells demonstrated an increased susceptibility, requiring a 10-fold lower dose to achieve a comparable level of inactivation.

This study progressed to demonstrate enhanced sporicidal efficacy upon combination of chlorinated disinfectants with 405 nm light at both high (up to 225 $mWcm^{-2}$) and low (0.4 $mWcm^{-2}$) irradiances. For decontamination of spores in suspension, a 50% increased spore susceptibility was observed upon exposure to a 33% lower light dose when in combination disinfectants. On clinically-relevant surfaces, up to 100% increased spore reductions were observed upon combination of low irradiance 405 nm light with selected disinfectants.

Further significant findings of this study include the enhanced susceptibility of spores upon triggering germination, with inactivation achieved using up to 77% less dose. Also established was the critical requirement of oxygen for photo-inactivation of this anaerobic pathogen, thus supporting the mechanism being a result of photoexcitation of naturally-occurring porphyrins inducing ROS production, oxidative damage and ultimately cell death.

This study has confirmed the fundamental sporicidal efficacy of 405 nm light and further highlights possible mechanisms to enhance sporicidal activity. 405 nm light has several advantages over current in-house cleaning procedures and novel sporicidal technologies, including its safety for human exposure permitting continuous decontamination of the patient environment, and the work of this thesis supports the potential for sporicidal efficacy to be achieved if utilised as a complementary strategy with standard cleaning regimes.

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CHAPTER 1

Introduction

Clostridium difficile is a Gram-positive, anaerobic bacillus that is a major cause of antibiotic-associated diarrhoea in the developed world, with the spectrum of illness ranging from asymptomatic colonization, to life-threatening toxic megacolon and pseudomembranous colitis (in 0.4-3% of cases), with mortality rates of approximately 5% (De Sordi et al., 2015; Sayedy, 2010; Johnson & Gerding, 2011; Dubberke & Wertheimer, 2009; Burns et al., 2010a). *C. difficile* is responsible for approximately 15-25% cases of antibiotic-associated diarrhoea, and virtually all pseudomembranous colitis in adults (Barbut et al., 2009; Landelle et al., 2014). In the past decade *C. difficile* has increased to endemic proportions, with recent data suggesting that it has replaced methicillin-resistant *Staphylococcus aureus* as the most common cause of healthcare-associated infection (Nelson et al., 2016; Miller et al., 2011). *C. difficile* is associated with an increased length of hospital stay and cost, as well as substantial morbidity and mortality.

C. difficile is spread via the faecal-oral route and is commonly transmitted throughout healthcare settings via the hands of healthcare workers (Hung et al., 2013). In recent years the environment has been shown to play an important role in the spread of disease as patients excrete large volumes of spores and vegetative cells in their faeces into the environment (Doan et al., 2012; Landelle et al., 2014; Thompson, 2012). *C. difficile* can cause major contamination problems due to its ability to form highly infectious and resilient spores, which are 10-15 times more resistant to various chemical and physical agents than non-sporulating bacteria, enabling *C. difficile* to withstand exposure to heat, oxygen, alcohol and certain disinfectants, therefore making these spores difficult to eradicate and facilitating environmental persistence and transmission (Davies et al., 2011; He et al., 2013). This resilience enables spores to survive on contaminated surfaces for up to five months, in comparison to their vegetative counterparts which can survive for only 15 minutes on dry surfaces, or up to 6 hours on moist surfaces (Vohra & Poxton., 2011). Minimising environmental contamination by *C. difficile* spores could greatly reduce the further propagation of disease within the clinical environment, and thus minimise

prolonged hospital stays, increased costs associated with treatment, and death as a result of disease.

Despite the development of a range of novel disinfection and sterilisation technologies, *C. difficile* remains a significant problem within the healthcare environment. Current in-house cleaning recommendations include disinfection using chlorinated disinfectants; however these have several drawbacks including their corrosive nature and the release of irritating vapours affecting healthcare workers (Rupnik et al., 2009). Furthermore, these chlorinated disinfectants have been shown to be inadequate for complete disinfection of the hospital rooms, with *C. difficile* remaining in the environment upon admission of subsequent patients (Barbut et al., 2009; Otter et al., 2013). Also, due to the persistence of spores within the clinical environment, 'no touch' systems utilising ultraviolet (UV) light or gaseous disinfectants such as hydrogen peroxide are becoming more common methods of decontamination within the hospital (Otter et al., 2013; Weber et al., 2016). However, there are several drawbacks associated with these technologies including potential material degradation issues, low penetrability, and carcinogenic and toxic effects meaning that the devices can only be employed in vacant, sealed rooms (Elmnasser et al., 2007; Fu et al., 2012; Irving et al., 2016; Murdoch et al., 2013; Nicholson et al., 2005).

A recent technology that has been proposed for environmental decontamination applications is the use of 405 nm violet-blue light. Although less germicidal than UV light, violet-blue light has the advantage that it can be used at levels which provide an antimicrobial effect whilst being non-detrimental to exposed individuals. For implementation of this technology within the hospital environment, a ceiling-mounted lighting system that incorporates 405 nm violet-blue light with a blend of white light has been designed. The high-intensity narrow-spectrum light environmental decontamination system (HINS-light EDS) is installed alongside hospital lighting allowing for safe continuous decontamination of the environment (Bache et al., 2012). Recent clinical studies have shown the efficacy of the HINS-light EDS for reducing levels of environmental contaminants of common causes of nosocomial infections including staphylococci, and reducing overall total viable counts. However, there is no data available with regards to the efficacy of this novel optical decontamination system for the inactivation of *C. difficile* spores and the

potential reduction of environmental contamination in patient environments (Bache et al., 2012; Maclean et al., 2010; Murdoch et al., 2010).

The focus of this study is to determine the efficacy of 405 nm light for the inactivation of *C. difficile* spores, and to establish the potential for it to be utilised in clinically-relevant scenarios. Work will establish the sensitivity of both the vegetative cells and spores to 405nm light under different clinically-relevant situations including on a range of surfaces and in the presence of current recommended chlorine-based disinfectants. Work will further investigate some of the more fundamental aspects of spore sensitivity including environmental oxygen conditions and the effects of inducing spore germination. An overview of each chapter is provided below:

Chapter 2 - Background and Literature review provides a background of the significant healthcare-associated pathogen, *Clostridium difficile*, and also the significance of the sporulation and germination stages of its life cycle. A review is provided of the current problems associated with *C. difficile* in the clinical setting, including environmental contamination and drawbacks in the current infection prevention and control measures. New and developing technologies for the control of *C. difficile* are discussed followed by the introduction of the antimicrobial 405 nm light technology, which will be the focus of this study.

Chapter 3 - Materials and Methods provides a detailed description of the microbiological methodology and optical experimental test systems used throughout the study.

Chapter 4 - Susceptibility of Spore-Forming Bacteria to antimicrobial 405 nm light investigates the use of high and low irradiance 405 nm light on a range of *C. difficile* strains. Inactivation kinetics are established for the exposure of both vegetative cells and spores, in suspension, seeded onto surfaces, and when present in organically-rich faecal contamination. Comparison of the susceptibility of *C. difficile* is also made with the aerobic, spore-forming organism, *Bacillus cereus*.

Chapter 5 - Synergistic Efficacy of 405 nm Light and Chlorinated Disinfectants investigates the potential for a synergistic sporicidal effect to be achieved through the combined use of 405 nm light with low-concentration chlorinated disinfectants, which are used for clinical environmental decontamination. The susceptibility of *C. difficile* spores exposed to light treatment alone, disinfectant alone, and combinational treatments were compared to identify potential synergistic oxidative effects.

Chapter 6 - Investigation of other factors influencing the susceptibility of C. difficile to 405 nm light presents a range of experiments which investigate factors that may influence the efficacy of 405 nm light for *C. difficile* inactivation. This included investigating the role of oxygen in the photodynamic inactivation process, and also the potential for increased susceptibility upon stimulation of spore germination. In addition to this, the susceptibility of *C. difficile* spores to UV-C light was investigated, enabling a comparison of the susceptibility of this organism to these differing regions of germicidal light.

Chapter 7 - Conclusions and Future work discusses the significance of the results obtained throughout this study and proposes potential applications for this disinfection system within the clinical environment. Recommendations for further investigations into the fundamental understanding of the interactions of violet-blue light with this key healthcare pathogen, and also developments towards the potential practical application of this antimicrobial technology are also discussed.

CHAPTER 2

Background and Literature Review

GENERAL

This literature review will discuss the current problems associated with the spore forming bacterium *Clostridium difficile*, including infection, spread and decontamination problems alongside the current precautions and decontamination procedures currently in place to minimise the spread of *C. difficile* within healthcare facilities. Furthermore, this review will discuss the drawbacks with current infection prevention and control procedures and the need for novel decontamination technologies, with a particular focus on the use of antimicrobial violet-blue 405 nm light.

2.1. *CLOSTRIDIUM DIFFICILE* DISEASE

Clostridium difficile infection (CDI) is defined as "someone in whose stool *C. difficile* toxin has been identified at the same time as they have experienced diarrhoea not attributable to any other cause, or from cases of whose stool *C. difficile* has been cultured at the same time as they have been diagnosed with PMC" (pseudomembranous colitis) (Wiuff et al., 2011). Clinical features include diarrhoea, lower abdominal pain and systemic symptoms including fever, anorexia and nausea (Hung et al., 2013). *C. difficile* infection results from a lack of equilibrium within the microbiota, leading to colonisation followed by the secretion of two toxins, enterotoxin TcdA and the cytotoxin TcdB which cause damage to the intestinal epithelium resulting in inflammation and subsequent clinical symptoms (Paredes-Sabja et al., 2014). Hospitalisation and antibiotic treatment are the most significant risk factors for *C. difficile* colonisation, with an emphasis on broad-spectrum antibiotics which can disrupt a wide variety of anaerobic microflora. In particular, third generation antibiotics have been associated with *C. difficile* associated diarrhoea (CDAD), such as cephalosporins, clindamycin or flouroquinolines. Other factors that may lead to enhanced susceptibility to CDI include old age, therapies that can alter the balance of the gut microbiota, immunodeficiency, proton pump inhibitor consumption, feeding tubes and prior hospitalization (Dawson et al., 2009; Lin et al., 2013; McFarland, 2005).

2.1.1. RATES OF INCIDENCE

In the past 15 years the incidence of *C. difficile* has dramatically increased in the USA and Europe; this increase has been associated with hyper virulent strains which are resistant to antibiotics, produce more toxin, form spores more readily and adhere to the intestinal epithelium better than wild type strains (Ivarsson et al., 2015). In 2007-2008, in patients aged over 65 alone, there were 32,628 *C. difficile* infections costing the UK National Health Service (NHS) at least £75 million (Davies et al., 2011). In Scotland, the total number of reported cases increased from 214 to 2,130 during the period from 1988 to 2000 (Lee et al., 2001). In 2012 there were 1,646 deaths involving *C. difficile* infection in England and Wales (Deaths Involving *C. difficile*, England and Wales, 2012). In the US there are 453 000 cases of CDI/year, and 29,500 deaths, and in Europe, there are 172,000 cases of CDI a year with a 9% mortality rate (direct or indirect) (Frädrich et al., 2016; Lessa et al., 2015; Barbut, 2015). Due to the lengthened stay, additional antibiotics and laboratory testing, it is estimated that the additional cost associated with a patient with CDI is approximately £5,640 (Wilcox et al., 1996; Vonberg et al., 2008). Recent data has suggested that *C. difficile* has replaced Methicillin-resistant *Staphylococcus aureus* (MRSA) as the most common cause of healthcare associated infection (Miller et al., 2011).

2.1.2. DEVELOPMENT OF DISEASE

Following the acquisition of *C. difficile*, the patient can then develop disease or remain asymptomatic; although the exact incubation period for *C. difficile* is unknown, it is relatively short and is thought to be about 1 to 2 days after exposure, and no longer than 7 days (Shim et al., 1998). The infectious dose of *C. difficile* is unknown, however it is very low with the minimum infectious dose in mice reported as 7 $CFUcm^{-2}$ cage space (Lawley et al., 2009). Patients with CDI excrete in excess of 10^3 - 10^7 *C. difficile* spores and cells per gram of faeces (Wheeldon et al., 2008; Wilcox, 2003; Best et al., 2010a; Mulligan et al., 1984). Figure 0.1 demonstrates the steps following the acquisition of *C. difficile* leading to disease. Although the immune response does not confer protection from *C. difficile* colonisation, the systemic response influences the outcome of disease following colonisation. An increased systemic response, resulting in greater serum levels of IgG antibodies against Toxin A is associated with asymptomatic colonisation, whilst lower increases in serum levels of antibodies in response to Toxin A is associated with the development of diarrhoeal disease (Kyne et al., 2000).

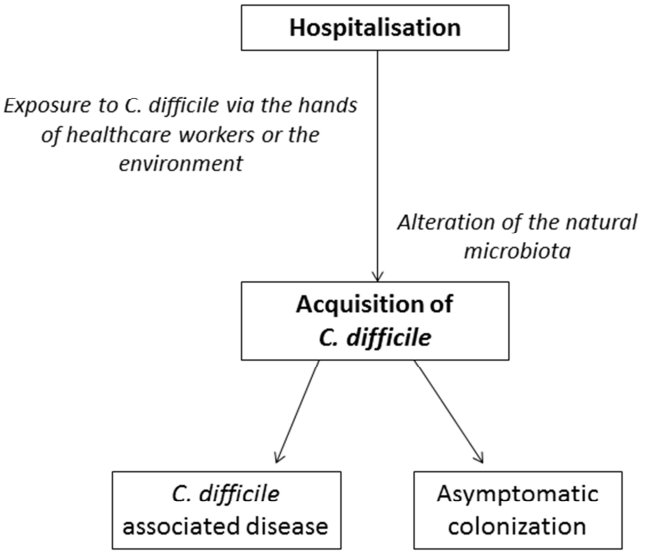

Figure 0.1 Typical 3 step process of *C. difficile* infection following admission of a patient with no *C. difficile* infection to the acquisition of *C. difficile* leading to CDI or asymptomatic colonization.

2.1.3. *C. DIFFICILE* EPIDEMIC

In recent years large outbreaks of *C. difficile* associated disease in the UK, North America, Australia and continental Europe, have been attributed to the emergence of hyper virulent strains such as an epidemic strain North American PFGE type 1 (NAP1) (also characterized as PCR-ribotype 027), which has increased resistance to fluoroquinolone antibiotics (Riggs et al., 2007). During 2004-2006 there were severe outbreaks of *C. difficile* PCR-ribotype 027, with this strain being associated with more than 40% of CDI cases in subsequent years. Evidence indicates that the emergence of this strain was driven by fluoroquinolone prescribing (Brazier et al., 2008; He et al., 2013). The estimated mutation rate of *C. difficile* BI/NAP1/027 is 1.47×10^{-7} – 5.33×10^{-7} (95% confidence intervals) substitutions per site per year, equivalent to 1-2 mutations per genome per year; approximately 10 times slower than that of *Streptococcus pneumoniae* and *S. aureus* (He et al., 2013). The 027 strain has been found to produce 16-fold higher concentrations of toxin A and 23-fold higher concentrations of toxin B *in vitro* (Gould & McDonald, 2008; Warny et al., 2005). In 2005, a study in Canada reported a severe outcome in 12.5% of infections caused by the BI/NAP1/027 strain, in comparison to 5.9% when infected with an alternative strain (Miller et al., 2010). The BI/NAP1/027 strain further produces a binary toxin, which is thought to

act as an additional virulence factor and has been associated with more severe diarrhoea, higher case fatality and is thought to be associated with a higher rate of recurrence of disease (Eckert et al., 2014; Stewart et al., 2013).

Independent acquisition of fluoroquinolone resistance by ribotype 027 strains has been reported on at least two occasions, with studies further demonstrating the association of the epidemic spread of ribotype 027 with the emergence of fluoroquinolone resistance (Mcdonald et al., 2005a; He et al., 2013). Additionally, the prescribing of fluoroquinolones has been highlighted as a major risk factor in the development of CDI (Pepin et al., 2005). As a result, reduced prescribing of fluoroquinolones has been introduced, and has been temporally associated with reductions in both 027 associated disease and overall CDI incidence (Ashiru-oredope et al., 2012; Valiquette et al., 2007).

2.2. *C. DIFFICILE* CARRIAGE AND ACQUISITION

2.2.1. CARRIAGE RATES WITHIN THE HOSPITAL

CDI is referred to as hospital-acquired if the onset of symptoms occurs more than 48 hours following admission to hospital or less than 4 weeks after discharge from a health care facility (Gupta & Khanna, 2014). *C. difficile* is transmitted mainly via the faecal-oral route, however in recent years the importance of the environment in the role of transmission of the disease has been highlighted. Johnson and Gerding (2011) stated that asymptomatic carriers usually outnumber symptomatic patients by several fold within the hospital, with the rate of colonization among hospitalised patients often as high as over 20% for those hospitalised for more than one week. For patients who are initially negative for *C. difficile*, the risk of acquiring the organism increases in proportion to the length of hospital stay (Johnson et al., 1990; Johnson & Gerding, 2011). In a study by Johnson et al. 60 of 282 patients with long-term hospital stays across three wards were culture positive for *C. difficile* during their stay, and of these 51 were symptom-free throughout the duration of their stay, even following treatment with antibiotics, demonstrating the high rate of asymptomatic patients (Johnson et al., 1990). It has been reported that *C. difficile* does not pose a threat for healthy individuals and this carriage may be transient or persistent (Friedman et al., 2013; Ozaki et al., 2004).

Only about 4-8% of healthy adults in the community are colonized with *C. difficile*, compared to 20-40% of hospitalized patients (Gould & McDonald, 2008; McFarland et al.,

1989; Viscidi et al., 1981). In older generations the carriage rate has been reported to rise, with an approximate carriage rate of 50% in nursing homes and long term care facilities (Binion, 2016). As mentioned, not all of these individuals have an active infection, but it is important to note that carriage rates increase with age and exposure to the health system. Hung et al. reported that *C. difficile* colonization (CdC) in the stools of patients is also a factor contributing to CDI, with Hung Y-P et al. demonstrating that patients with toxic CdC (tCdC) were 10-fold more likely to develop CDI than those without tCdC (Hung et al., 2014). This supported previous findings of Hung Y-P et al., in which it was reported that 51.3% of patients with tCdC in stools developed CDI whereas only 12% of patients without tCdC present in their stools developed CDI (Hung et al., 2013). In contrast to this, Johnson and Gerding carried out a study in which the results did not indicate that asymptomatic carriers were at increased risk of developing CDI; this may be due to the fact that colonization with non-toxigenic strains may prevent colonisation with toxigenic strains which may lead to disease (Friedman et al., 2013; Johnson et al., 1990; Johnson & Gerding, 2011). However, it is important to note that these colonized individuals may be a source of nosocomial contamination and infection. It is thought that colonization with non-toxic *C. difficile* may confer protection from toxigenic *C. difficile* strains, with a study by Boriello and Barclay demonstrating that prior colonisation of hamsters with non-toxigenic strains protected them from subsequent infection with toxigenic strains (Borrielo and Barclay, 1986). An explanation for this may be that asymptomatic colonization of *C. difficile* was associated with an immune response to *C. difficile* toxins resulting in high serum levels of IgG against toxin A, explaining the decreased risk of diarrhoea associated with asymptomatic colonisation with *C. difficile* (Kyne et al., 2000; Shim et al., 1998).

2.2.2. OCCUPATIONAL RISK FOR HEALTHCARE WORKERS

Many studies have focused on the frequency of *C. difficile* infection and colonisation amongst inpatients, however Friedman et al. (2013) carried out a study reporting the prevalence of *C. difficile* amongst healthcare workers (HCW). It has been reported that *C. difficile* is an underestimated occupational risk for HCW; however there are many contrasting studies with regards to this. Studies carried out by Carmeli et al. (1998) and Hell et al. (2012) reported that *C. difficile* stool colonization was not identified in 55 and 112 HCW, respectively, whereas other studies have indicated that colonization of HCW may occur at a similar frequency as that amongst healthy adults with carriage detected in 13% HCW by van Nood et al. (2009). The study by Friedman et al. investigated the colonization

rates of 214 HCW, with a mean age of 43 (ranging from 21-65) with results reporting that colonization with *C. difficile* amongst HCW is rare. The difference in colonization rates across different hospital settings may be due to a range of success in infection prevention strategies such as hand washing and the use of gloves.

2.2.3. *C. DIFFICILE* CARRIAGE IN THE COMMUNITY

CDI is defined as community-acquired if the onset of symptoms occurs in the community, within 48 hours of admission to a hospital or more than 12 weeks following hospital discharge.

Although other reservoirs of *C. difficile* are likely to exist outside hospitals the incidence of community acquired CDI is very low at 7.7 cases per 100,000 person-years (Hirschhorn et al., 1994; Johnson & Gerding, 2011). It has been reported that *C. difficile* does not pose a threat for healthy individuals and carriage may be transient or persistent (Friedman et al., 2013; Ozaki et al., 2004). In the community, it is estimated that only approximately 4-8% of healthy adults are colonized with *C. difficile*, in comparison to 20-40% of hospitalized patients, however the epidemiology of community acquired CDI is very poorly recorded (Gould & McDonald, 2007; McFarland et al., 1989; Viscidi et al., 1981). Hirschhorn et al. (1994) reported that, following a retrospective cohort study using records of a health maintenance organisation in order to determine CDI rates following the use of specific antibiotics, the overall risk of community acquired CDI was less than 1 per 10,000 antibiotic prescriptions; as was expected a higher risk is associated with specific antibiotics. However, in recent years the incidence of community-associated CDI has been seen to increase, with Health Protection Scotland (HPS) reporting that 26% cases of CDI were community associated, 68% being hospital associated cases and the remaining 6% unknown (Banks, 2015). 37% of the CA-CDI cases were not prescribed antibiotics prior to diagnosis. In this retrospective study, both community associated and hospital associated cases appear to have the same common ribotypes (Banks, 2015). In order to get a better understanding of the possible spread of disease, further studies could consider having personal contact with CA-CDI patients to determine if there is any links between CA-CDI and the hospital environment or if they have been in contact with individuals with HA-CDI.

2.2.4. CLINICAL CONTAMINATION

Contamination of the hospital environment with *C. difficile* was first reported in 1979, and alongside colonized humans and contaminated equipment, the environment is one of the

main reservoirs for *C. difficile* within hospitals (George et al., 1979). Further investigations into the contamination of the hospital environment has reported that the frequency of positive samples in rooms occupied by asymptomatic patients was 29%, and 49% in rooms occupied by patients with CDI (McFarland et al., 1989) with a particularly high percentage of positive samples in commodes, followed by bathroom floors (Wilcox et al., 2003). Due to the ability of spores to survive in the environment for up to five months, adequate decontamination is of the upmost importance for the rooms of patients with CDI (Kim et al., 1981; Vohra & Poxton, 2011).

Surfaces most frequently contaminated with spores are the floors, bathroom areas and the toilet (Rupnik et al., 2009; McFarland et al., 1989; Barbut et al., 2009). A study carried out by Barbut et al. reported that 74% of rooms (23 of 31) that had housed patients with CDI were contaminated with *C. difficile* spores. Furthermore, a 12% increase in risk of nosocomial CDI was reported when patients were in the immediate vicinity, or later occupants, of a room occupied by a patient with CDI (Bartlett & Gerding, 2008; Shaughnessy et al., 2011).

Wilcox et al. (2003) reported that contamination of some environmental sites is significantly more persistent, including commodes, toilet floors and bed frames which were found to be *C. difficile* positive on more than 50% of occasions. Samples for this study were collected from two elderly medicine wards using a sterile cotton wool swab, moistened with 0.25% ringers solution and cultured on cycloserine cefoxitin supplemented agar (CCA) containing lysozyme. Enrichment for *C. difficile* was performed by placing environmental swabs in Robertsons cooked meat broth followed inoculation on to lysozyme CCA (Wilcox et al., 2003). Contamination of radiators, non-toilet floors and curtain rails was relatively infrequent (~30%). Earlier studies, carried out by Kaatz et al. (1988) reported that the areas with the highest contamination rates were floors and bathroom surfaces in a study in which samples were collected from floors walls, windows, the bathroom environment, bedframes and doors in an outbreak ward. Kaatz et al. used rodac plates containing 0.1% sodium taurocholate supplemented with cefoxitin-cycloserine-fructose agar (CCFA). Best et al. (2012) reported that toilet flushing may be a major source of environmental contamination in the bathrooms of patients due to the production of aerosols following flushing, with a 10-fold reduction in *C. difficile* recovery at seat level when the toilet lid was closed upon flushing. It is estimated that patients excrete approximately $1 \times 10^3 – 1 \times 10^7$ of *C. difficile* per 1g of faeces, therefore the reduction of aerosol production could significantly minimise

C. difficile contamination within the bathroom environment (Best et al., 2010b; Dawson et al., 2011).

At present, there is a lack of standardisation of methods currently used to assess the environmental contamination of the clinical environment with regards to CDI spores. As a result, this may be a confounder when comparing studies.

2.3. *C. DIFFICILE* SPORES, VEGETATIVE CELLS AND GERMINATION

A major problem associated with the control of *C. difficile* contamination is the ability of the organism to form highly resilient spores which can survive in the environment for prolonged periods of time and withstand extreme conditions (Landelle et al., 2014; Wheeldon et al., 2008). Following the ingestion of these spores, *C. difficile* then undergoes germination under optimal conditions to produce vegetative cells which are responsible for causing disease.

2.3.1. SPORES

C. difficile spores are primarily responsible for the high rates of *C. difficile* disease and recurrence within the healthcare environment, as spores are the major transmissive form, and are highly resilient to various environmental and chemical stressors. *C. difficile* sporulation is triggered under conditions limiting survival, for example in the aerobic environment (as *C. difficile* is a strict anaerobe), and allows for metabolic dormancy until favourable conditions return. It has previously been demonstrated that patients with CDI can excrete up to approximately 10-fold more metabolically active vegetative cells in their faeces in comparison to spores, which then undergo sporulation in the aerobic environment; however it has been suggested that these vegetative cells can remain viable on moist surfaces for up to 6 hours (Jump et al., 2007; Worthington & Hilton, 2016). These endospores, shed in the faeces or subsequently formed from vegetative cells in the clinical environment, possess the ability to withstand a range of environmental stresses, including various cleaning detergents, and can persist within hospital environments for months (Landelle et al., 2014; Wheeldon et al., 2008; Awad et al., 2014; Wilcox & Fawley, 2000).

Spores have various properties which contribute to their resistance, including the low permeability of the spore coat, a thick outer proteinaceous coat, a low water content and a high level of dipicolonic acid (DPA) and divalent cations in the spore core (Setlow et al., 2000; Setlow et al., 2002). The physiological differences between spores and vegetative cells are shown below in Figure 0.2. The spore coat and cortex restrict the access of

potentially damaging chemicals into the spores, thus protecting it from oxidising agents and chemicals, such as disinfectants, and high temperatures (Pol et al., 2001). The spore coat is important due to the presence of enzymes, such as superoxide dismutase and catalase, enabling the detoxification of chemicals preventing reactions within the spore's interior. The inner membrane, found underneath the spores germ cell wall, plays a significant role in the protection against chemicals due to its low permeability; increases in permeability are associated with an increase of sensitivity to chemicals (Setlow, 2000; Cortezzo et al., 2004). Furthermore, saturation of DNA by α/β small acid soluble proteins (SASP) protects the DNA. Setlow (2000) reported that the loss of α/β – type SASP renders *B. subtilis* much more susceptible to damage by heat, UV radiation, desiccation, oxidizing agents and other chemicals. Although the dormancy of spores promotes protection under unfavourable conditions, their dormancy also means that they cannot repair damage to their DNA, proteins and other components which may result in spore death. Recently, the ability of *C. difficile* spores to survive the temperatures and disinfectant treatment of typical hospital laundering was demonstrated, leading to the cross-contamination of bed linen during a wash cycle (Hellickson & Owens, 2007; Hamblin et al., 2005).

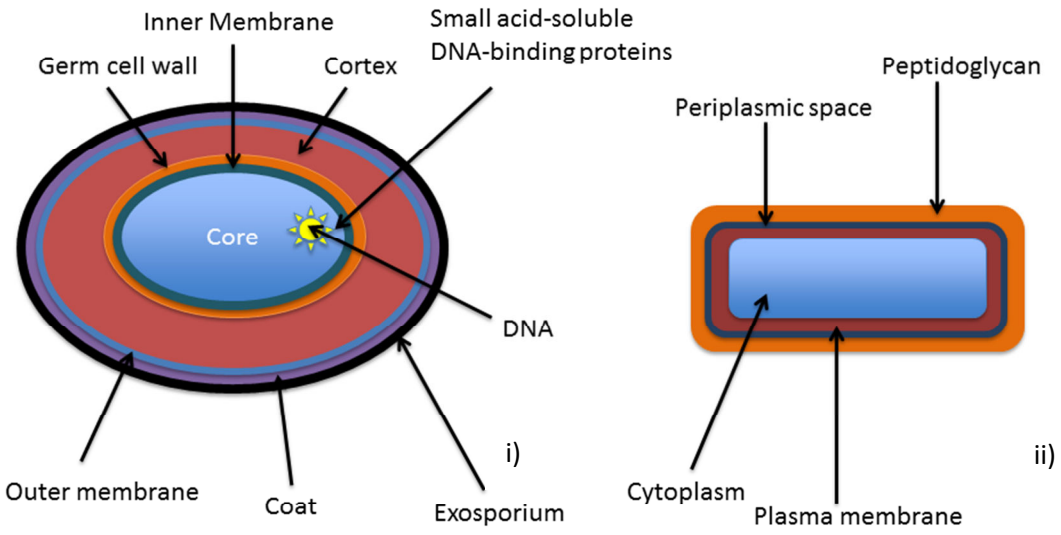

Figure 0.2 The physiological properties of the *C. difficile* i) spore ii) vegetative cell (Not to scale)

2.3.2. SPORULATION

Sporulation is the transformation of vegetative, viable forms of bacteria to dormant, resting spores in response to exposure to harsh environmental conditions that would normally

result in bacterial inactivation, allowing for the prolonged survival of the bacteria until suitable conditions are restored and the cell can again become viable following germination. Whilst vegetative forms of *C. difficile* are responsible for disease, they cannot survive in the environment. The initiation of sporulation of *C. difficile* vegetative cells results in the production of dormant spores, enabling the pathogen to persist and disseminate within the healthcare environment. Despite the importance of spores for the pathogenesis of *C. difficile*, very little is known with regards to the processes involved in sporulation (Fimlaid et al., 2013).

There are very limited studies with regards to *C. difficile* sporulation, with current knowledge based on comparisons with other spore-forming bacteria with well-known sporulation cycles. For example, *Bacillus cereus*, a Gram positive, facultative anaerobic endospore forming bacteria that can cause food poisoning and food spoilage and *Bacillus subtilis* are commonly used for comparison (Abee et al., 2011; Edwards & Mcbride, 2014). Sporulation results following an asymmetric cell division. In both *Bacillus* and *C. difficile* a regulatory protein (Spo0A) is responsible for regulating commitment to sporulation, acting as both a repressor and activator for transcription ensuring sporulation does not occur unless necessary for survival (Edwards & Mcbride, 2014). In *Bacillus*, under environmental stresses, a protein kinase activates the response regulator by phosphorylation. This phosphate is then transferred from the kinase to Spo0F, from Spo0F to Spo0B, and then from Spo0B to Spo0A; this phosphorelay tightly regulates the initiation of sporulation and is important to ensure that it is necessary to shut down cell division and commit to sporulation. Once Spo0A is phosphorylated, the cell becomes committed to sporulation resulting in the transcription of genes required for sporulation (Burbulys et al., 1991). AbrB, CodY and SinE inhibit the initiation of functions that are only needed for sporulation to occur (Saujet et al., 2011). Spo0A has been shown to be essential in *C. difficile* for sporulation, and is important for signal transmission (Fimlaid et al., 2013; Underwood et al., 2009).

In comparison to the *Bacillus* family, *C. difficile* appears to lack the phosphorelay with the sporulation initiation pathway comprising of a two-component system with Spo0A and associated kinases, which phosphorylate Spo0A directly, in the absence of Spo0F and Spo0B. It is hypothesised that this is regulated by 3 orphan kinases, CD2492, CD1492 and CD1579, and potentially other unidentified factors (Edwards & Mcbride, 2014; Pettit et al., 2014; Saujet et al., 2011; Underwood et al., 2009). *C. difficile* Spo0A proteins recognise a

consensus sequence and bind to it with a high affinity and specificity to its promoter regions (*Spo0A, Sig^{H}* and early sporulation genes that control σ^{F} and σ^{E} activation) (Rosenbusch et al., 2012). In *C. difficile* a SinR-like repressor and CodY are present - both negative regulators of early sporulation. However, *C. difficile* does not possess the AbrB repressor (Saujet et al., 2011). As many of the factors that activate or inactivate Spo0A in other spore forming bacteria are not contained within the sequenced *C. difficile* genome, there is still much to be discovered with regards to the regulatory components and signals that feed into Spo0A activation within *C. difficile*.

In addition to Spo0A σ^{H} is essential for sporulation in *Bacillus subtilis* and *C. difficile*. It is a key regulator for the transition from exponential growth to stationary phase growth, via the upregulation of the expression of genes associated with sporulation and mediating transcriptional changes when switching (Edwards & Mcbride, 2014). *Sig^{H}* appears to upregulate the expression of the early sporulation genes of *spo0A* and *CD2492* genes. Similarly, Spo0A appears to upregulate σ^{H} expression resulting in a positive feed forward loop (Edwards et al., 2016; Saujet et al., 2011). Furthermore, σ^{H} also positively controls the *spo0J, spoIIP* and *soj* gene products that are included in chromosomal segregation during asymmetric division (Saujet et al., 2011). σ^{H} is also associated with the genes encoding sigma factors of sporulation, and genes involved later in sporulation. It has a negative effect on the expression of the *app* operon encoding a predicted oligopeptide permease.

CCPA and CodY influence the sporulation in both *Bacillus* and *Clostridium* species (Edwards et al., 2016). CCPA represses *C. difficile* sporulation via *sigF* and Spo0A during the early stages of sporulation and results in indirect repression of CD1579 (Antunes et al., 2012; Antunes et al., 2011). CodY represses both sporulation and toxin production. CodY regulates *opp* and *sinR* which are both associated with the inhibition of the initiation of sporulation (Dineen et al., 2010; Nawrocki et al., 2016). There are still many questions with regards to how CodY regulates *C. difficile* sporulation, with various sporulation initiation factors in *C. difficile* still unknown (Nawrocki et al., 2016). The oligopeptide permeases, Opp and App, prevent sporulation by importing peptides into the cell.

The factors discussed that play a role in the regulation of *C. difficile* sporulation are shown in Figure 0.3, however there is much yet to be learnt to fully understand all the factors involved in *C. difficile* sporulation and their specific roles.

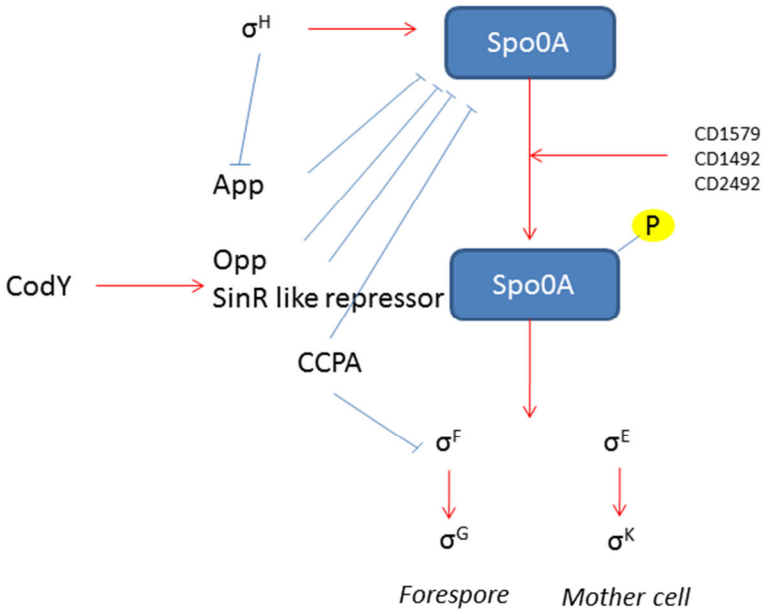

Figure 0.3 Schematic representation of the known factors resulting in the activation and repression of *C. difficile* sporulation leading to the formation of the forespore and mother cell. This is a complicated pathway, with current knowledge lacking information with regards to all genes and regulators involved.

2.3.3. GERMINATION

Germination is the irreversible loss of spore specific properties in response to the restoration of suitable conditions for bacterial growth and survival. Following germination the bacteria returns to its viable vegetative state which, in the case of *C. difficile*, allows for the release of toxins resulting in disease.

In healthy individuals, *C. difficile* cannot colonise due to competition with the naturally occurring microbiota. However, the use of antimicrobials disrupts the natural gut flora destroying the indigenous bacteria within the small intestine enabling *C. difficile*, which is resistant to a wide range of antibiotics, to colonise within the epithelial lining of the gastrointestinal tract and grow in a non-competitive environment.

In the healthy gut bile salt hydrolase enzymes, produced by bacteria in the natural gut microbiome, are deconjugated from amino acids in the intestinal lumen. In addition to this, the bacteria mediate the transformation of primary bile salt acids to secondary bile salt acids via the enzyme 7-alpha-dehydrolase which converts the bile acids cholate and chenodeoxycholate to deoxycholate and lithocholate. In the altered microbiota, the ability of bacteria to deconjugate the amino acids and the presence of 7-alpha-dehydrolase is reduced and as a result the presence of primary bile salts (for example taurocholate) and

conjugated bile salts is increased, and secondary bile salts are reduced (Blanchi et al., 2016). *C. difficile* favours an environment rich in primary bile salts, and has been shown to be inhibited in the presence of secondary bile salts, and thus can colonise the gut (Theriot et al., 2015). Bacterial fermentation is further altered, leading to a decrease in the presence of fatty acids and resulting in excess fermentation substrates, such as carbohydrates and amino acids, which can be used as metabolites by *C. difficile* for both germination and sporulation (Blanchi et al., 2016). As a result of this altered gut microbiome, this provides favourable conditions for the germination and outgrowth of *C. difficile* spores to vegetative cells and as such leads to colonisation which can result in disease.

Following the induction of these favourable conditions spores undergo germination. This is initiated when germination receptors come into contact with germinants (often amino acids and bile salts) resulting in the spores returning to their vegetative state; this is essential for disease (Dawson et al., 2009; Paredes-Sabja et al., 2008). Dembek et al. (2013) reported that bile salts (cholate, taurocholate, glycocholate and deoxycholate) stimulate *C. difficile* spore germination. There are two main stages in the germination of *C. difficile*, however very little is known about *C. difficile* germination and outgrowth (Dembek et al., 2013). Germination of the *Bacillus* species has been well studied, and has been reported to occur in two stages; Figure 0.4 demonstrates the main events occurring in each stage in germination in *Bacillus*.

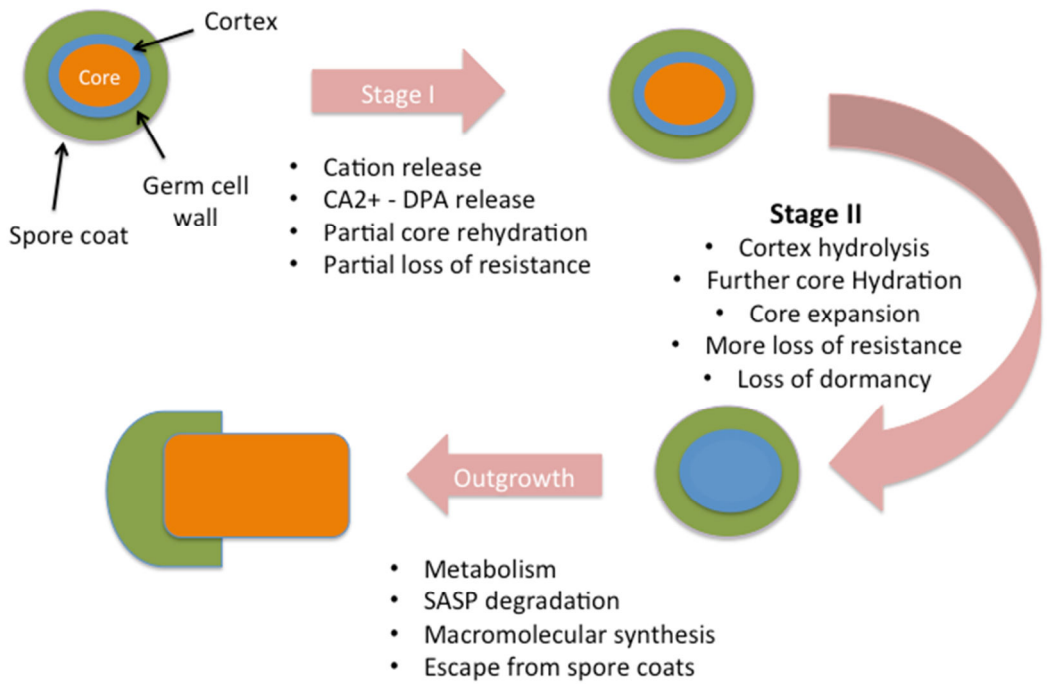

Figure 0.4 The step by step events that occur during germination of *Bacillus subtilis* under favourable conditions for the spore to return to exponential growth (Adapted from Setlow 2003)

The events in both stages of germination in *Bacillus subtilis* occur in the following order.

- In stage 1, following the binding of the germinant, the spore is committed to germination. In this stage monovalent cations, H+, Na+ and K+ are released, alongside calcium dipicolinic acid (Ca-DPA), which makes up approximately 25% of the spore dry weight. This loss in impermeability to water leads to water influx (Burns et al., 2010b; Setlow, 2003).
- In the second stage of germination, the spore peptidoglycan cortex is enzymatically digested and the water content of the spore returns to that of the vegetative cell, resulting in the loss of spore specific resistance (Burns et al., 2010b; Nguyen Thi Minh et al., 2010). At the end of stage two, small acid-soluble spore proteins (SASP) are hydrolysed to amino acids which are used in protein synthesis by the growing cell (Nguyen Thi Minh et al., 2010; Setlow, 2003).

C. difficile spore germination will be discussed in further detail in Chapter 6.

2.3.4. TOXIGENICITY OF *C. DIFFICILE* AS A RESULT OF GERMINATION

Whilst *C. difficile* spores are responsible for the transmission of disease, vegetative cells produce toxin A and toxin B (TcdA and TcdB), large enterotoxins encoded within a pathogenicity locus (PaLoc), and are associated with virulence. Under favourable conditions these are produced in copious amounts (Kazanowski et al., 2014; He et al., 2013; Warny et al. 2005; Gerding et al. 2014). TcdA and TcdB are thought to be the main virulence factors associated with disease symptoms, with the generation of at least one of these toxins being essential for the pathogenesis of this organism (Faulds-Pain et al., 2014; Kuehne et al., 2010; Lyras et al., 2009). The release of toxins A and B results in disruption to the normal intestinal mucosa by inactivating the transformation pathway, mediated by Rho family proteins, responsible for the proper construction of the actin cytoskeleton and signal transduction by GTP; this results in damage to enterocytes, leading to cytoskeletal changes resulting in the release of fluids and inflammatory products due to apoptosis (Burns et al., 2010b; Kazanowski et al., 2014; McFarland, 2005). The release of these toxins leads to an acute inflammatory response and subsequent damage to the intestinal epithelium and disease can range from mild diarrhoea to toxic megacolon and sepsis syndrome which can lead to morbidity and mortality (Denève et al., 2009; Solomon, 2013). Toxin B is associated with the major problems related to infection, whereas toxin A is associated with the secretion of fluid within the digestive tract, mucosal inflammation and structural damage (Kazanowski et al., 2014). The recent epidemic strain NAP1 (or ribotype 027) has been found to produce a further toxin, *C. difficile* transferase (CDT), alongside toxin A and toxin B; CDT is a binary toxin. This discovery of the presence of the binary toxin in epidemic strains has indicated a possible role it may play in the pathogenesis. Furthermore the strains found to possess this third toxin also possess further changes including increased resistance to fluoroquinolones and the presence of an 18 bp deletion and a stop codon in the tcdC gene encoding an anti-sigma factor involved in down regulation of toxin A and B production (Dupuy et al., 2008).

Although the production of toxins is essential for the pathogenesis of *C. difficile*, several other properties also contribute to the disease including bacteria surface associated proteins which are often involved in host colonization and immune evasion. An example of this is the S-layer protein, a paracrystalline structure that envelopes the vegetative cell (Fagan et al., 2009). The S-layer is composed of two post-translational cleavage products of SlpA which spontaneously form a heterodimeric complex bound to the cell wall (Fagan et

al., 2009). The S-layer proteins play a role in host colonisation, including adhesion and invasion, in addition to conferring protection from the host immune system (Calabi et al., 2002; Fagan et al., 2009; Merrigan et al., 2013; Spigaglia et al., 2011). S-layer mutants have been found to have increased sensitivity to killing by innate immune effectors alongside producing fewer spores than the wild-type (Kirk et al., 2015). Additionally S-layer mutants have been shown to be non-virulent.

2.4. *C. DIFFICILE* CONTROL METHODS

Overall, following the discussions in Sections 2.2 and 2.3, it is evident that *C. difficile* poses a great problem due to various factors including its virulence and ability to survive in the environment for prolonged periods for time. As such, a range of control factors need to be in place within the healthcare environment to minimise the spread of disease. Current strategies for the prevention of *C. difficile* disease and transmission will now be discussed.

2.4.1. CONVENTIONAL *C. DIFFICILE* CONTROL METHODS

2.4.1.1. Treatment

The recommended therapy for CDI is metronidazole (500 mg three times per day for 10–14 days), with vancomycin therapy (125 mg four times per day for 10–14 days) indicated for severe disease (Cohen et al., 2010; Moore et al., 2013). Approximately 20% of patients treated initially with metronidazole or vancomycin experience recurrent disease, which can be treated using the same antibiotic, and approximately 20-50% of these recurrences are the result of reinfections as opposed to relapses of the same strain. Relapses tend to occur in the first two weeks following treatment (Public Health England, 2013). If the patient has had recurrent CDI, the chances of further relapses increases to 50-65% (McFarland, 2005; Public health England, 2013). In 2012, oral fidaxomicin was approved for the treatment of CDI in Europe (Johnson & Wilcox, 2012). Studies have shown that fidaxomicin was not inferior to vancomycin in the initial cure of CDI, however it was superior in reducing recurrence and sustained clinical cure (Jones et al., 2013). The main disadvantage of fidaxomicin is the cost, as the acquisition cost is considerably higher than that of vancomycin, which is more expensive than metronidazole. Patients with mild disease may not require antibiotic treatment, however if necessary oral metronidazole is recommended; oral metronidazole is also recommended for moderate disease (Wilcox et al., 2013). For patients with severe CDI oral vancomycin is recommended, however patients with a high

risk of recurrence, including elderly patients, should be considered for treatment with fidaxomicin (Kazanowski et al., 2013; Public Health England, 2013)

Antimicrobials are excreted into the gastrointestinal tract, where *C. difficile* colonises resulting in *C. difficile* being subjected to selective pressure, which is thought to be a key factor in the epidemiology of *C. difficile*; therefore, an increase in the use of particular antibiotics for the treatment of CDI leads to increased resistance (Ackermann, 2003). Furthermore, *C. difficile isolates* have been demonstrated to acquire resistance to agents not used for the treatment of CDI, giving some strains a selective advantage for their spread (Coia, 2009). Ackermann (2003) stated that 'transfer of resistance genes within a pool of multiple drug-resistant bacteria seems to be a likely process'. McDonald et al. (2005) reported the changing epidemiology of *C. difficile* strains, with the recent epidemic strain ribotype 027 (NAP1) demonstrating resistance to fluoroquinolone antibiotics gatifloxacin and moxifloxacin (Mcdonald et al., 2005b; Rupnik et al., 2009). This acquisition of multiple drug resistance mechanisms limits CDI treatment and management of severe infections. Since 2000, substantially higher failure rates have been reported for metronidazole therapy (18.2%) (Kelly & LaMont, 2008).

2.4.1.2. Chemical cleaning

Cleaning refers to the removal of dirt and soiling, removing microbes in the process, whilst disinfection refers to the killing of a high percentage of pathogens on surfaces.

Chemical cleaning is used for routine and terminal cleaning of rooms that currently, or have previously, housed patients with CDI. Current control methods for *C. difficile* typically focus on patients with CDI or those suspected to have CDI, in which patients are contact restricted and the patient rooms are cleaned with 10% bleach solutions using conventional cleaning methods such as mopping floors and the use of sponges on surfaces using recommended chemical agents. Adequate disinfection is of the upmost importance as the rooms of patients with CDI can reach contamination rates of up to 50%, whilst those of asymptomatic patients can reach approximately 25% contamination, with spores surviving in the environment for up to five months (Rupnik et al., 2009; McFarland et al., 1989). Therefore it is very important that asymptomatic carriers are taken into account within the hospital, as this may currently be a major problem contributing to the spread of *C. difficile* infections. Previous studies carried out (Riggs et al., 1992; 1998; 2007) demonstrated that approximately two thirds of patients colonized with *C. difficile* become asymptomatic faecal

carriers. In 1992, Clabots et al. reported that, within a study hospital ward, most cases of nosocomial acquired CDI were epidemiologically linked to disease transmission from new, asymptomatic patients admitted to the ward. In addition to this, Riggs et al., (2007) found that the frequency of contamination of skin and environmental surfaces among asymptomatic carriers was nearly as high as that among patients with CDI, and spores on the skin of asymptomatic patients were easily acquired on investigators' hands. These findings suggest that asymptomatic carriers have the potential to contribute significantly to the transmission of epidemic and non-epidemic *C. difficile* infection in long-term care facilities. Currently, infection prevention control guidelines do not recommend the isolation of asymptomatic patients or the introduction of universal screening to identify asymptomatic patients, as there is currently not enough evidence to support that this could reduce infection rates. The introduction of patient screening and isolation of asymptomatic patients could lower transmission of *C. difficile* within the hospital. A mathematical model investigating the potential benefit of introducing screening of all patients was examined, with results concluding that screening was cost effective in all scenarios, with a saving of US $16,071/ 1,000 admissions could be made, (assuming a colonisation rate of 10.3% and a 75% compliance rate) however this study has made many assumptions (Bartsch et al., 2012). However, this could also lead to increased costs associated with screening, including false positives, as well as issues with limited isolation rooms (Wiuff et al., 2014).

Currently, there are two main measures to control CDI within the hospital:-

- Measures to prevent cross infection
- To reduce the risk of CDI by improving antimicrobial prescribing

Barrier methods frequently used to prevent the spread of CDI include isolation of patients with CDI, the use of gowns and gloves by healthcare workers, and hand hygiene. In a study carried out by Landelle et al. (2014) it was confirmed that contact with ungloved hands is a risk factor for *C. difficil*e transmission and that alcohol rubs, that are effective against other organisms, do not remove *C. difficile* spores; washing with soap and water after contact with infected patients is more efficient at removing spores from hands than alcohol based products (Landelle et al., 2014; Rupnik et al., 2009). Another source of *C. difficile* contamination was the handles of electronic thermometers; the replacement of these thermometers with disposable thermometers has reduced the spread of pathogens between patients and has resulted in marked reductions in CDI rates (Rupnik et al., 2009).

Reducing environmental contamination by *C. difficile* spores is difficult as many traditional detergents are not sporicidal, and in some cases may promote sporulation. A sporicidal hypochlorite solution can significantly reduce *C. difficile* contamination rates, and therefore CDI rates; however these detergents are commonly chlorinated and have many drawbacks including their corrosive nature and may cause cutaneous and respiratory irritation to humans, therefore cleaning with bleach is often reserved for use during an epidemic or CDI outbreaks (Rupnik et al., 2009).

2.4.2. ENVIRONMENTAL CLEANING WITH SPORICIDAL DISINFECTANTS

For *C. difficile*, and many other bacterial spores, disinfection should be carried out in two steps:

(i) Surfaces must be thoroughly cleansed of all visible soil

(ii) A surface disinfectant approved by the Environmental Protection Agency (EPA) for control of *C. difficile* endospores must be applied according to the manufacturer's directions.

It is critical that the instructions on the label be followed exactly for the *C. difficile* endospores to be killed as ineffective manual cleaning and the use of products not containing chlorine may lead to increased sporulation and further dissemination of *C. difficile* spores (Fawley et al., 2007; Wilcox & Fawley, 2000). Quaternary ammonium compound-based solutions and 70% methylated spirits commonly used for the disinfection of surfaces are ineffective against the spores produced by *C. difficile* (Worthington & Hilton, 2016). Chlorine-based products are considered the cheapest and easiest environmental disinfection method but have limitations such as the release of irritating vapours, their corrosive nature, inhibition by organic matter and lack of staff compliance with adhering to the correct disinfection method (Doan et al., 2012).

2.4.2.1. Recommended sporicidal disinfectants

Cleaning using detergents is not efficient for the removal of spores, therefore cleaning with chlorine-releasing disinfectants is recommended for the cleaning of rooms housing patients with CDI. The use of disinfectants is vital for reducing the transmission of *C. difficile* spores from the environment and minimising the spread of *C. difficile*. There are several disinfectants recommended for cleaning of spores in the clinical environment. Some key examples are:

- **Sodium hypochlorite**

Sodium hypochlorite is a chlorine releasing agent that is widely used for hard-surface disinfection (household bleach) or as a disinfectant within the hospital. Hypochlorites are the primary choice for laboratory disinfectants due to their wide microbicidal spectrum, comparatively rapid action rate and the ability to inactivate organisms at low temperature and concentrations. Sodium hypochlorite is recommended for use at concentrations ranging from 1,000 ppm - 100,000 ppm available chlorine for environmental cleaning of areas contaminated with *C. difficile* (Barbut et al., 2009; Heling et al., 2001; Maillard, 2012). Although considered to be the cheapest and most effective disinfection method, there are several drawbacks associated with sodium hypochlorite including its corrosive nature, the release of irritating vapours, and inhibition by the presence of organic matter (Baines, 2005; Barbut et al., 2009).

When bacterial spores are treated with chlorine releasing agents, such as sodium hypochlorite, the spores lose refractivity and the spore coat separates from the cortex resulting in lysis (McDonnell & Russell, 1999). Chlorine releasing agents (CRA) are oxidising agents that destroy the cellular activity of protein; it is thought that the moiety responsible for bacterial inactivation is hypochlorous acid (HOCl) as CRA activity is greatest when the percentage of undissolved HOCl is highest. Following the formation of hypochlorous acid, this is further decomposed and it is the oxygen released from this reaction that is responsible for oxidation resulting in inactivation due to degradation and hydrolysis (Estrela et al., 2002).

Numerous studies have investigated the efficacy of detergents versus hypochlorite cleaning for management of cases of CDI. A two year ward-based study, which evaluated the effects of environmental cleaning with hypochlorite versus detergent on the incidence of cases of CDI, demonstrated a statistically significant decrease in incidence on the ward utilising hypochlorite (Wilcox et al., 2003). Furthermore, in a study comparing the efficacy of 5 commonly used cleaning agents (including neutral detergent and hydrogen peroxide) only hypochlorite was found to be effective at inactivating *C. difficile* spores (Fawley et al., 2007).

- **Actichlor plus**

Actichlor plus is a chlorine based disinfectant recommended by the NHS for cleaning and disinfection in the presence of patients with CDI, with surfaces and equipment cleaned

twice daily (Management of *Clostridium difficile* infection, 2013). Actichlor is recommended for use at a concentration of 5,000 ppm. Actichlor combines a chlorine compatible detergent with sodium dichloroisocyanurate (NaDCC) in a single tablet format. The microbicidal activity of sodium dichloroisocyanurate tablets may be greater than that of sodium hypochlorite solutions containing the same total available chlorine as only half of the total available chlorine is free (HOCl and OCl^-), whereas the remainder is combined (monochloroisocyanurate or dichloroisocyanurate). As free available chlorine is used up, the latter is released, restoring the equilibrium. In addition to this sodium hypochlorite solutions are alkaline, whilst sodium dichloroisocyanurate solutions are acidic; HOCL is believed to dominate under acidic conditions (Bloomfield & Miles, 1979; Rutala et al., 2008).

A study by Dawson et al. (2011) reported that Actichlor alongside another chlorine releasing agent, Haztab, and the peroxygen Perasafe were the most effective biocides for the inactivation of all three PCR-ribotypes of *C. difficile* tested (012, 017 and 027 PCR-ribotypes) in a study assessing the susceptibility of these PCR-ribotypes to numerous chlorine releasing agents (CRAs), peroxygens, quaternary ammonium compounds (QAC) and a chlorhexidine hand wash.

- **Chlorine dioxide**

Chlorine dioxide is an oxidizing biocide that has a broad spectrum of activity against bacteria, viruses, yeasts, mycobacteria and spores, with high-level sporicidal activity following contact times as short as 30 seconds. Chlorine dioxide is not an irritant. Chlorine dioxide results in the inhibition of protein synthesis (Benarde et al., 1967). This happens as when a bacterial cell comes into contact with chlorine dioxide, which is constantly searching for an additional electron, an electron is donated from the bacterial cell wall. This results in a breach in the cell wall, enabling contents to pass across the cell wall (Maris, 1995; Roller et al., 1980). This disruption to the transport of nutrients results in the inhibition of protein synthesis leading to cell lysis. Tristel is a chlorine dioxide based disinfectant that is recommended for use by the NHS for the inactivation of *Clostridium difficile* spores, however is not the first choice of disinfectant. It is recommended for use at a concentration of 100-120 ppm. An advantage of Tristel is, as it is a chlorine dioxide based product, it directly reacts with cysteine, methionine, tryptophan and thiamine; these four amino acids, especially cysteine, play a crucial role in all living systems, including microbes, therefore it is suggested that microorganisms cannot build up resistance against chlorine

dioxide (Noszticzius et al., 2013). A recent study has suggested that chlorine dioxide is non-toxic to humans due to the size difference between mammalian cells in comparison to microorganisms and furthermore due to the multicellular components of humans conferring protection in comparison to the single cell nature of bacteria, thus making bacteria more susceptible to inactivation (Noszticzius et al., 2013).

- **Hydrogen peroxide**

Hydrogen peroxide is known for its fungicidal, bactericidal, sporicidal and viricidal activity. It is recommended for the disinfection of rooms of patients with CDI in European guidelines (Dubberke et al., 2014; Vonberg et al., 2008). It is widely used for disinfection and sterilization and is a good alternative to chlorine based disinfectants. Similar to the previously discussed sporicidal disinfectants, hydrogen peroxide exerts oxidative damage on the spore, via the production of hydroxyl free radicals, which causes disruption of the spore coat, allowing for access to the cortex enabling the destruction of essential cell components including lipids proteins and DNA (Bloomfield & Arthur, 1994; Thompson, 2012). Virox STF is a commercially available disinfectant containing 7% H_20_2, and has been demonstrated to be sporicidal (Perez et al., 2005). A disadvantage associated with hydrogen peroxide, however, is the ability of some bacterial species to develop resistance and as a result as their catalase enzyme is able to decompose H_2O_2 rapidly (Harris et al., 2002; Noszticzius et al., 2013).

2.4.2.2. Drawbacks of current disinfection protocols

Despite the development of a range of novel disinfection and sterilisation technologies, *C. difficile* remains a significant problem within the environment of various healthcare settings. Current guidelines recommend the use of chlorinated disinfectants for the decontamination of surfaces (Wilcox et al., 2011). There are many drawbacks associated with the current decontamination methods used to disinfect the rooms of patients diagnosed with *C. difficile*, with samples collected from rooms of patients who are neither infected nor colonized with *C. difficile* reaching up to 8%, demonstrating the inefficiency of currently used disinfectants at eliminating *C. difficile* (Barbut et al., 2009). Furthermore, although chlorinated disinfectants currently used for the elimination of *C. difficile* spores have been demonstrated to significantly reduce *C. difficile* contamination rates, drawbacks include their corrosive nature and the release of irritating vapours affecting healthcare workers (Rupnik et al., 2009). Another problem associated with chlorinated disinfectants is

the lack of staff compliance with adhering to the correct disinfection method (Doan et al., 2012). Multiple studies have demonstrated that less than 50% of surfaces are properly cleaned using different methods of assessment, as summarised in Table 0.1 (Boyce et al., 2011; Huang et al., 2015; Goodman et al., 2008; Carling et al., 2008; Weber et al., 2016).

Due to the problems associated with current disinfection methods, and the failure to achieve consistent high rates of cleaning and disinfection of surfaces, recent advances have seen an interest in the use of novel no touch technologies for whole room disinfection as an additional infection control strategy.

2.5. DEVELOPMENTS IN TERMINAL CLEANING

As discussed, terminal cleans upon vacation of hospital rooms can be done using conventional cleaning methods. In addition to cleaning using conventional methods, a range of new technologies have become available. These will now be discussed.

2.5.1. HYDROGEN PEROXIDE VAPOUR

Hydrogen peroxide vapour is a broad spectrum oxidising agent that produces highly reactive hydroxyl radical ions that attack DNA, membrane lipids and other essential cell components leading to cell death (Maclean et al., 2015; Weber et al., 2016). Due to its broad spectrum of activity against a wide range of bacteria, spores and viruses, it is becoming increasingly popular for the decontamination of surfaces and objects within unoccupied hospital rooms and wards and for terminal cleaning. An advantage of hydrogen peroxide vapour is that it is non-toxic following decomposition to water and oxygen (Barbut et al., 2009; Davies et al., 2011). As breakdown is required for the vapour to become non-toxic, patient rooms must be sealed throughout treatment. Hydrogen peroxide is highly favourable as it has a higher material compatibility, it is non-toxic to humans following breakdown and less detrimental to the environment than hypochlorite based disinfectant (Barbut et al., 2009).

There are two types of hydrogen peroxide delivery systems, these are discussed below.

2.5.1.1. Aerosolized hydrogen peroxide (aHP)

ASP Glosair (formally known as Sterinis) and Oxypharm Nocospray are examples of aerosolized systems that make use of a pressure-generated dry mist aerosol of hydrogen peroxide (as shown in Figure 0.5)**.** These technologies require experienced operator supervision and training. The recommended dose for hospitals is 6 ml/m^3 per cycle. The

chemical composition is usually 5%–7% hydrogen peroxide, 50 ppm silver and 95% de-ionised water (Barbut et al., 2009; Weber et al., 2016). During this cycle electrically charged particles circulate in the air, adhere to microbial particles in the air and on surfaces resulting in inactivation (Barbut et al., 2009; Boyce, 2009; Maclean et al., 2015). As mentioned previously hydrogen peroxide naturally decomposes, therefore it does not need to be removed from the environment.

a) b)

Figure 0.5 Examples of aerosolized hydrogen peroxide systems a) Glosair^{TM} 400 ASP and b) Oxypharm Nocospray

Aerosolized hydrogen peroxide has been demonstrated to inactivate a wide range of bacteria, including *Clostridium difficile*, Vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* (Barbut et al., 2009; Boyce, 2009; Chan et al., 2011). A study carried out by Barbut et al. reported a 91% reduction of *C. difficile* in the environment was achieved following the use of hydrogen peroxide mist for terminal cleaning; this is in comparison to terminal cleaning with sodium hypochlorite (a 0.5% bleach solution applied using a soaked cloth for 10 minutes across 16 rooms) which demonstrated a 50% room contamination decrease (Barbut et al., 2009). A further investigation carried out by Barbut el al., (2009) in which 3 toxigenic strains of *C. difficile* were inoculated onto PVC or laminate and placed in a vacant room near the bed and in the bathroom, reported that following one cycle of aerosolized hydrogen peroxide decontamination, a ~4.18 $\log_{10}$ reduction in the initial contamination of spore carriers was observed.

The study carried out by Barbut et al. (2009) reported residual contamination on 3 sites following treatment with hydrogen peroxide vapour, however this may be due to the reported deterioration of the surfaces. This highlights the need for materials to be deterioration free to prevent the presence of residual spores following terminal cleaning, as lacerations in materials may act as a reservoir for pathogens. Laboratory tests on *Bacillus subtilis* spores found significant differences in the decontamination efficacy of hydrogen peroxide gas on porous and non-porous surfaces, with a 1.2 $\log_{10}$ reduction on carpet, and 2.2 $\log_{10}$ reduction on bare pine wood, compared with >7.5 $\log_{10}$ reduction on paper wallboard, formica laminate and glass, therefore the surfaces in the room to be decontaminated with hydrogen peroxide should be taken into consideration (Barbut et al., 2009).

2.5.1.2. Hydrogen peroxide (H_2O_2) vapour systems

The H_2O_2 vapour systems generate a 30-35% hydrogen peroxide, with a dose of 10 g/m^3 recommended per cycle (Boyce, 2009; Fu et al., 2012; Weber et al., 2016). Examples of H_2O_2 vapour systems include Steris VHP and Bioquell HPV, as shown in Figure 0.6. Both these technologies require experienced operator supervision and training. Unlike aHP, vaporized H_2O_2 systems have an aeration unit because active catalytic conversion is required to aid decomposition of the hydrogen peroxide into non-toxic by-products (Otter & French, 2009).

Figure 0.6 Examples of hydrogen peroxide vapour systems a) The Bioquell BQ-50 and b) The Steris VHP 100ED

Although aHP may be seen as an easier option in comparison to hydrogen peroxide vapour systems, hydrogen peroxide vapour systems have generally been found to have greater efficacy against a range of nosocomial pathogens including *S. aureus, A. baumannii*, *C. difficile and Klebsiella pneumoniae* (Otter & French, 2009). Fu et al. (2012) demonstrated that HPV had a greater efficacy than aHP for the inactivation of MRSA and *A. baumannii*, and furthermore also demonstrated that HPV had greater diffusion in treated rooms than the aHP system. This is supported by a study by Holmdahl et al. (2011) in which all *Geobacillus stearothermophilus* biological indicators on carriers were inactivated following 3 different exposures to HPV, in comparison with only 10% following exposure to the one aHP test, with 79% inactivation demonstrated in a further 2 aHP tests. A study by Boyce et al. (2008) documented the significant reduction in incidences of CDI due to terminal cleaning using HPV in a university affiliated hospital over two consecutive 10 month periods during which there was a *C. difficile* epidemic. However, it is important to note that no control wards were used in this study, therefore these contamination rates may be due to natural fluctuation.

A further study comparing the efficacy of the Bioquell Hydrogen peroxide vapour system to that of a UV-C decontamination technology, the TRU-D UV-C device (to be discussed further in Section 2.5.2.3), reported a >6 $\log_{10}$ *C. difficile* kill following treatment with the Bioquell Hydrogen peroxide vapour system, in comparison to a <2 $\log_{10}$ *C. difficile* kill observed following treatment with the UV-C device. This study reported that, although HPV was significantly more effective against spores and rendering surfaces culture negative than UV-C, UV-C is faster and easier to use than HPV with a mean cycle time of 73 minutes and 153 minutes (with a delivered dose of 96 Jcm^{-2} for UV-C), respectively (Havill et al., 2012).

A study by Manian et al. (2013) questioned the need for hydrogen peroxide as results from this study indicated that improved cleaning practice may be just as beneficial as the inclusion of HPV for terminal cleaning. The study investigated the use of HPV for terminal cleaning of an entire community hospital and found that the rate of CDI was reduced by 37% when rooms where terminally cleaned using bleach followed by HPV. However, during this time nearly 50% of rooms tested were terminally cleaned 4 times with bleach alone due to lack of HPV availability, suggesting that HPV is not essential for the reduction of CDI, if this particular terminal cleaning method is used (Manian et al., 2013).

Although hydrogen peroxide has shown to be very efficient technology for terminal cleaning within the healthcare environment there are several limitations associated with the use of hydrogen peroxide.

- Removal of patients from room prior to performing decontamination
- Longer turnaround times compared to a typical bleach clean
- Staff training required
- Rooms need to be sealed to allow for breakdown to non-toxic components (Fu et al. (2012) found the levels of H_2O_2 peroxide were above exposure limits when measured outside un-sealed rooms)
- Skin irritant
- Involves the use of toxic chemical
- Short term effect as high contamination levels return after several days
- The presence of organic matter can reduce efficiency of hydrogen peroxide

(Davies et al., 2011; Fu et al., 2012; Maclean et al., 2015)

2.5.2. Ultraviolet Light

Ultraviolet light (UV) is another technology gaining evidence and support for effective terminal cleaning and environmental decontamination in hospitals. This section will give an overview of these UV based technologies and their applications. Due to the focus of the work of this thesis on light-based decontamination, I will provide a detailed review of UV light and it's applications within the hospital environment for terminal cleaning, with a focus on the terminal decontamination of CDI.

UV light has been used extensively for the decontamination of air alongside its applications for surface and water sterilization, and is able to effectively kill a range of bacteria, fungi and viruses (Guerrero & Barbosa, 2004; Menetrez et al., 2010). In the electromagnetic spectrum, UV light has a lower energy than x-rays and higher energy than visible light, with a wavelength range of approximately 200 nm to 400 nm. The UV region can be further subdivided into long-wave (UV-A), medium wave (UV-B), short-wave (UV-C) and vacuum-UV radiation. The damage caused by UV radiation corresponds to its photon energy, which is directly proportional to its frequency; as the wavelength of radiation increases, the frequency and energy of the photon decreases (Henderson, 1997; Tabrah, 2010; Maverakis et al., 2011; Wypych, 2015).

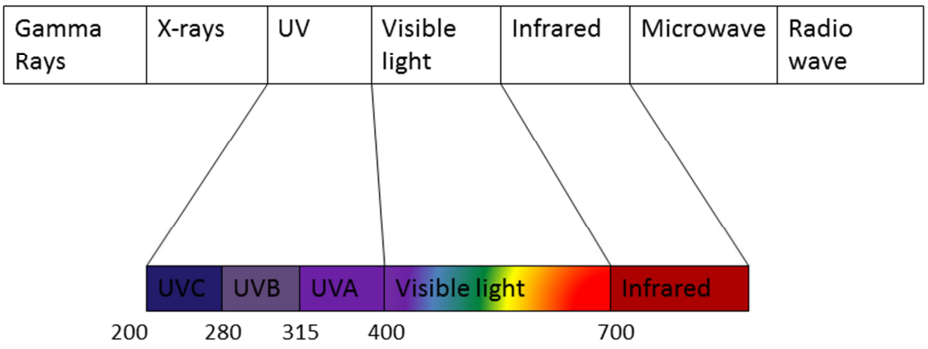

Figure 0.7 The Electromagnetic spectrum

2.5.2.1. Germicidal UV-C

Wavelengths between 200-280 nm (UV-C), are considered the most effective for germicidal activity as this corresponds with the bond energies of typical bonds found within biomolecules, therefore the photons have sufficient energy to induce reactions (UV Disinfection of Secondary Effluent, 2003). The germicidal activity of UV-C is attributed to its ability to cause damage to links in DNA as a result of pairing between thymine and cytosine bases. The formation of the spore photoproduct (SP), primarily cyclobutane-type dimers between adjacent pyrimidines on the same strand of DNA, interferes with replication, thus preventing growth and/or reproduction (Armstrong et al., 2006; Nicholson et al., 2005; Setlow, 2006). Once dimer formation reaches a specific level, irreversible damage occurs within the cell and microbial cell death occurs (Eischeid & Linden, 2007).

Until recently, there had not been much development in UV-C as an anti-infective approach, however since the realisation that the possible adverse effects to host tissue were relatively minor compared to its high activity in killing pathogens there has been a surge seen in the development of this technology (Yin et al., 2013). However, there are various limitations that are associated with prolonged and continuous exposure to UV-C, which can damage host cells and can be carcinogenic (Murdoch, 2012). UV-C has been shown to be effective for the inactivation of *C. difficile* spores; this will be discussed alongside a range of available UV-C technologies in Section 2.5.2.3.

In addition to UV-C, germicidal activity is also seen with UV-B and UV-A light. UV-A (320-400 nm) is the most penetrating but least damaging form of UV radiation, and is responsible for sun tanning. Nonetheless, prolonged exposure to UV-A can induce optical damage and skin damage including the reddening of the skin (erythema) and the development of skin

cancers (WHO, 2006). Unlike UV-C, UV-A and UV-B induce germicidal activity via indirect oxidative damage. Cytotoxic effects are mediated via sensitizer molecules which absorb energy which is then transferred to generate reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals and singlet oxygen. These ROS then react with DNA to damage bases and break strands. Protein-DNA cross-linking can also occur as a result.

2.5.2.2. UV repair mechanisms

Due to exposure to UV radiation from natural sunlight, several repair mechanisms have evolved in microorganisms to repair sub-lethal damage caused by exposure to UV-light; these mechanisms are ineffectual for bacteria that have been killed outright by UV exposure. The repair mechanisms are classified into dark-repair and light-dependant repair – also termed photoreactivation or photorepair.

Photoreactivation requires light in the near UV/blue light region, between approximately 300-500 nm and is catalysed by photolyase, an enzyme that directly reverses DNA damage. It is initiated by the absorption of photons by the chromophores of the photolyase, which are non-covalently bound to lesions. This results in the excitation and subsequent electron donation of a co-factor ($FADH^-$), which splits the adjacent pyrimidine dimers, resulting in the original pyrimidine monomers. This is a highly efficient process, with one dimer split for every blue light photon absorbed (Goosen & Moolenaar, 2008).

There are a number of dark-repair mechanisms, including base exclusion repair (BER) and nucleotide excision repair (NER). BER is the removal of a damaged base by a DNA glycosylase and is a frequently used DNA repair mode (Goosen & Moolenaar, 2008). NER, first observed in *E. coli*, involves the recognition of lesions followed by the removal of the DNA segment containing the lesion; this 'seek and cut' process is carried out by uvr-genes (Bugay et al., 2014). This is subsequently replaced with new, undamaged nucleotides using the undamaged single-stranded DNA remaining as a template for DNA polymerase and ligase to synthesize a short complementary sequence and seal the complementary strands; this process requires numerous proteins and enzymes.

2.5.2.3. Applications for Infection Control

Due to the high efficacy of UV for the inactivation of a wide range of bacteria and viruses, UV-C is a favourable choice for decontamination. There are several commercially available UV-C devices undergoing clinical trials for hospital room decontamination, many of which

are automatic non-touch disinfection systems, ensuring operator safety. Two of the main commercially available UV systems will now be discussed.

- **Tru-D device**

The Tru-D device manufactured by Lumalier Corporation, as shown in Figure 0.8, uses ultraviolet light (254 nm range) to eradicate pathogens. The device is placed in the centre of the room, and operators control the technology from an external location using a wireless remote control with motion sensing technology. The device, using low pressure mercury vapour lamps, requires an exposure time of ~20 minutes for the inactivation of vegetative cells and ~45 minutes for sporicidal effects, delivering a dose of 12,000 µWs/cm^2 and 22,000-36,000 µWs/cm^2, respectively (Nerandzic et al., 2010; Otter et al., 2013). Sensors on the device detect how much UV light bounces back from the surrounding surfaces and the device terminates the cycle when all sensors have determined that the desired dose has been delivered to all surfaces in the room (Boyce et al., 2011) . Tru-D automatically determines the room's darkest area based upon a reflected dose of UV-C and calculates the required duration of a given intensity to obtain a 3-6 $\log_{10}$ kill even in shadowed areas of a room such as dark corners, behind beds, underneath tables, etc.

Figure 0.8 The Tru-D UV-C emitting device used for decontamination of various nosocomial bacteria in laboratory and clinical environments.

Several studies have investigated the efficacy of the Tru-D UV-C emitting device for disinfection of various nosocomial bacteria, including *Vancomycin-resistant enterococci* (VRE), *Methicillin resistant Staphylococcus aureus* (MRSA) and *C. difficile* within the laboratory and clinical environment. Boyce et al. (2011) reported a 1.7-2.9 $\log_{10}$ reduction in *C. difficile* spores when a population of 10^5 $CFUml^{-1}$ was inoculated onto stainless steel carrier disks and placed in several areas within a hospital room, both in the direct and indirect line of UV-C. Nerandzic et al. (2010) further demonstrated a dose dependant 2-4 $\log_{10}$ reduction of MRSA, *C. difficile* and VRE. Following discharge of patients and conventional cleaning in 20 patient rooms' culture collections reported that over 90% of samples yielded positive culture results. Following a delivered dose of UV-C at 22,000 $\mu ws/cm^2$ for a median time of 67.8 minutes, the frequency of positive culture samples dropped to 47% (Boyce et al. 2011).

It is important to maintain a high level of terminal cleaning alongside the use of the Tru-D device to ensure all areas of the room are cleaned efficiently, as although the pathogen load does significantly have an impact on the efficacy of the Tru-D device, the presence of organic matter results in a lowered efficacy (Nerandzic et al., 2014). Furthermore, terminal cleaning is important as a study carried out by Sitzlar et al. (2013) reported that in the direct line of UV-C a 2-4 $\log_{10}$ reduction in *C. difficile* is achieved, however with indirect exposure a 1-2.4 $\log_{10}$ reduction was reported (Weber et al., 2016). Furthermore, a study carried out by Nerandzic et al. reported that when plastic carriers were placed 3 metres away from the device, a 1 $\log_{10}$ reduction in *C. difficile* spores was recorded, in comparison with a 2.6 $\log_{10}$ reduction achieved in direct line of site (Maclean et al., 2015; Nerandzic et al., 2010; Weber et al., 2016). For maximum efficiency of the Tru-D device, manufacturers recommend multiple cycles of UV-C devices from different locations (Boyce et al., 2011).

- **Pulsed xenon UV light**

Pulsed xenon UV (PX-UV) emits a broad range of UV (200-320 nm) in short pulses and uses a xenon gas flashlamp as the source (Nerandzic et al., 2015). An example of a PX-UV system is shown in Figure 0.9. The device is operated by a single user using a remote from outside the room. It possesses motion sensors that turn off the device if the door is opened. This device has a sporicidal setting of 5 minutes in each position, with a typical patient room requiring 3 positions, providing rapid decontamination (much faster than that achieved with continuous UV light). The entire disinfection process takes 15 to 20 minutes, including the setup of the device, radiation cycles and repositioning. The PX-UV system produces a

pulsed flash at a frequency of 1.5 Hz with an approximate output of 505 J per pulse and a pulse duration of less than 360 µs (Levin et al., 2013).

Figure 0.9 An example of a PX-UV device, Xenex, a PX-UV emitting device for terminal cleaning.

When combined with terminal cleaning, the ability of the PX-UV disinfection system has demonstrated the ability to achieve a 100% reduction in positive cultures of VRE (Stibich et al., 2011). Simmons et al. (2013) reported a 56% decrease in the mean plate count of MRSA following a change from standard terminal cleaning to a modified turnover clean and PX-UV disinfection cycle. Although the bacterial contamination levels were significantly lower in the rooms that received a turnaround clean and PX-UV, it is important to note that this report did not state what the 'modified' turnover clean comprised of and whether this is what contributed to the increased decontamination rates, or whether this is due to the PX-UV alone. Levin et al. (2013) reported that three 7 minute exposures of PX-UV following terminal cleaning contributed to a 53% reduction in the hospital associated CDI (HA-CDI) rate per 10,000 patient days; during the study period 73% of patients diagnosed with HA-CDI were placed in rooms that had not been treated with the PX-UV device due to time/equipment limitations. A significant reduction in *C. difficile* rates was demonstrated in both ICU and non-ICU areas (~40%) following discharge when PX-UV was employed (Vianna et al., 2016).

The advantages and disadvantages of UV light for environmental decontamination are summarised in Table 0.1.

Table 0.1 A summary of the advantages and disadvantages of the environmental disinfectants and technologies for the decontamination of rooms housing patients with CDI

Technology	Advantanges	Disadvantages
Conventional cleaning	• Can be carried out daily • Can be carried out in the presence of staff and patients • Required for the removal of soiling and standard cleaning of surfaces	• Corrosive nature of majority of cleaning agents • Several agents release irritating vapours (with the exception of chlorine dioxide) • User dependent • Only suitable and accessible surfaces treated • Demonstrated to not efficiently remove contaminants with contamination remaining on the admission of subsequent patients
Hydrogen peroxide	• Non-toxic following decomposition to water and oxygen • High material compatibility • Less toxic than hypochlorite • Whole room is treated • Good for terminal cleaning	• Organic matter, such as soiling, reduces efficacy • Experienced operator supervision and training required • Toxic before the breakdown of chemical • Rooms need to be vacated and require sealing • Longer turnaround times in comparison to conventional cleaning
UV	• Relatively short cycle time (15 minutes for vegetative cells, 50 minutes for spores with continuous UV-C and 15-20 minutes for PX-UV) • Does not produce by-products • No consumables are required, only capital costs of equipment and staff time • Does not require sealing of doors/vents	• As many items are out of the direct line of site, multiple cycles must be used. Potential for human error, as the operator must choose appropriate locations and this requires more operator time • Many UV-C systems rely on the measurement of reflected dose to determine the cycle; many factors can increase cycle times • Rooms must be vacated and doors closed • UV light is a mutagen • The long-term effect of UV-C on hospital materials has not been reported

2.5.3. VIOLET-BLUE LIGHT

There are many novel decontamination methods developed for clinical use, however several of these have various drawbacks, including the requirement of patient removal from the room, time consumption and only being adequate for terminal disinfection purposes, not daily cleaning. Furthermore, several of these methods are only adequate for temporary disinfection with the number or micro-organisms returning to pre-decontamination levels within hours (Bache et al., 2012).

Violet-blue light is light of 400 – 470 nm wavelength from within the visible spectrum. Blue light has been gaining interest due to its intrinsic microbial effect, using naturally occurring endogenous porphyrin molecules as photosensitisers, without the need for the addition of exogenous photosensitisers. Previous studies have demonstrated that visible light is the most effective for the excitation of these photosensitive molecules due to a high absorbance of light at ~400 nm (Maclean et al., 2008; Endarko et al., 2012; Maclean et al., 2013). Many microorganisms have exhibited sensitivity to killing using violet-blue light including, pathogenic bacteria, fungi, yeasts, and under certain circumstances, viruses (Maclean et al., 2009; McKenzie et al., 2014; Murdoch et al., 2013; Tomb et al., 2014; Yin et al., 2013). Furthermore violet-blue light has been shown to be safe for human exposure at bactericidal doses, and has been developed for continuous decontamination of the clinical environment (Maclean et al., 2010; McDonald, et al., 2011; Yin et al., 2013). The mechanism of inactivation will be discussed further in Section 2.5.3.1.

This study focuses on the use of violet-blue light with a wavelength of 405 nm which has been developed to allow for continuous cleaning of the hospital environment and is complementary to both the conventional cleaning and terminal cleaning techniques, previously discussed in Sections 2.4.2 and 2.5. This section will discuss the fundamental antimicrobial activity of 405 nm light and the clinical studies that have been performed demonstrating the efficacy of 405 nm violet-blue light for environmental decontamination.

Porphyrins are naturally occurring, pigmented light harvesting organic compounds. They are favourable for use for therapy as they are eco-friendly and human/animal safe for use. They are heterocyclic aromatic compounds, comprising four subunits of tetrapyrrole subunits linked by methane bridges – a schematic diagram is shown in Figure 0.10. Porphyrins are involved in various important biological functions including respiration (heme group) and photosynthesis (chlorophyll and bacteriochlorophyll) (Alves et al., 2015; Nitzan et al., 1994; Costa et al., 2012).

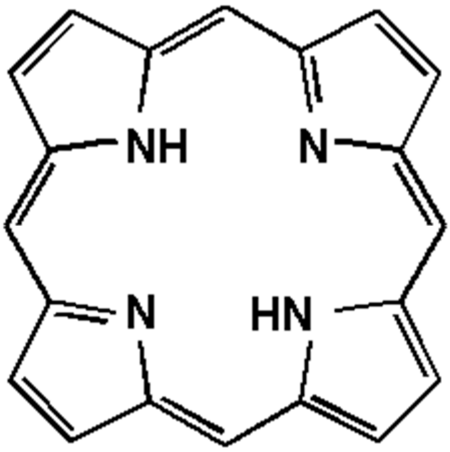

Figure 0.10 Schematic diagram of Porphine, the simplest porphyrin molecule (Josefsen & Boyle, 2008).

Porphyrins have demonstrated optimal absorption of photons of light at the peak wavelength of around 400 nm, with a bandwidth of ~10 nm, this is termed the soret band (Maclean et al., 2008). The soret band arises from a strong electronic transition from the ground state to the excited singlet state (Josefsen & Boyle, 2008). This is followed by four weaker absorptions, namely the Q bands, at higher wavelengths between 450-700 nm. There are two distinct groups of porphyrins, metal binding and non-metal binding. For photodynamic inactivation it is the non-metal binding porphyrin molecules which are involved because singlet oxygen, which is produced following photo-excitation of the porphyrin, is rapidly quenched by the metal moiety in metal-bound porphyrin molecules.

2.5.3.1. Mechanism of inactivation

Photodynamic inactivation (PDI) involves the use of visible light, oxygen and a photosensitiser. PDI involving exogenous photosensitizers such as dyes including methylene blue, rose bengal, or exogenous porphyrin molecules, is well-established and the mechanism of microbial damage is well understood (Huang et al., 2010; Baltazar et al., 2013; Costa et al., 2012; De Sordi et al., 2015; Kariminezhad & Amani, 2017). In the case of violet-blue light, it is the endogenous porphyrin molecules within the microbial cells which are relied upon to act as the photosensitizer in the inactivation process (Maclean et al., 2008; Maclean et al., 2009).

Following excitation of the naturally occurring intracellular porphyrins by photons of light (optimally ~405 nm), endogenous porphyrins in the singlet ground state, absorb a photon from light and become photo-excited. This excitation results in the production of an unstable, excited singlet state porphyrin which then transfers this energy by returning to

the singlet ground state, resulting in light emission, or through an intersystem crossing system where it is converted to the long-lived triplet state (Costa et al., 2012). The triplet state porphyrin then proceeds to react with oxygen or substrate resulting in the formation of highly cytotoxic species including singlet oxygen, hydrogen peroxide, superoxide and other free radicals resulting in oxidative damage of targets and ultimately cell death (Alves et al., 2015). Alternatively the triplet state porphyrin can decay to a ground state by phosphorescence emission. There are two main reaction pathways that can take place following excitation, shown in Figure 0.11 (Costa et al., 2012).

Type I reactions occur as a result of electron or hydrogen transfer from a substrate to the excited porphryin (reactions 1 and 2) to form a radical ion which then reacts with ground state oxygen leading to the production of reactive oxygen species (ROS) such as the superoxide radical ion (reaction 3); this relies on nearby proximity of the porphryin to the target (Alves et al., 2015). The reaction of superoxide with water leads to the production of hydroxyl radicals ($HO^{\cdot}$) (reaction 4) which can further react with biomolecules or combine resulting in the formation of hydrogen peroxide (reaction 5) (Wainwright, 1998). Type I reactions typically occur at the bacterial cytoplasmic membrane and further include the abstraction of allylic hydrogens from unsaturated molecules, such as phospholipids, with the radical species formed further reacting with oxygen to produce lipid hydroperoxide. Lipid peroxidation results in damage to the membrane, resulting in increased ion permeability. Further cell wall/membrane targets include aminolipids and peptides (Wainwright, 1998).

Reaction 1: $PS^{*} + Substrate \rightarrow PS^{\cdot +/-} + Substrate^{\cdot +/-}$

Reaction 2: $PS^{*} + Substrate\ H_2 \rightarrow PSH^{*} + Substrate\ H^{*}$

Reaction 3: $PS^{\cdot -} + {}^{3}0_2 \rightarrow PS + 0_2^{\cdot -}$

Reaction 4: $0_2^{\cdot -} + H^{+} \leftrightharpoons H00^{\cdot}$

Reaction 5: $2H00^{\cdot} \rightarrow H_20_2 + 0_2$

Type II reactions occur following the transfer of energy from the excited triplet state porphryin to molecular oxygen (${}^{3}0_2$), forming singlet oxygen (${}^{1}0_2$) (reaction 6) which reacts with the cell wall and enzymes leading to the inhibition of protein synthesis, nucleic acids,

peptides and anything in its environment (Costa et al., 2012; Wainwright, 1998). Singlet oxygen has a short half-life, ensuring a localised response. Type II pathways are thought to be the predominant pathway in PDT (Milgrom, 1997).

Reaction 6: $PS^* + {}^3O_2 \rightarrow PS + {}^1O_2$

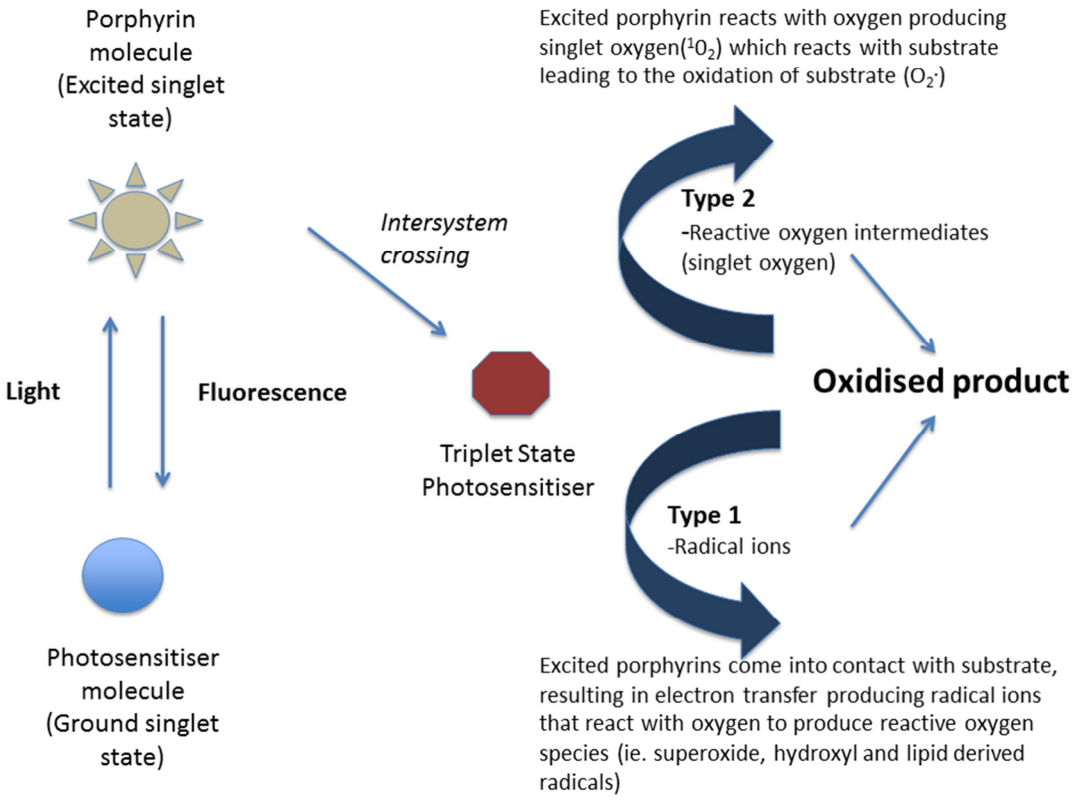

Figure 0.11 Diagram demonstrating the potential transfer of energy upon illumination of natural occurring porphyrins with 405 nm violet-blue light.

Recent studies have observed disruption of the cytoplasmic contents and disruption and breakage of the bacterial cell wall alongside the leakage of debris following treatment of *S. aureus* with blue light at a wavelength of 425 nm, demonstrating the induction of oxidative damage to the cell membrane as a result of exposure to blue light (Dai et al., 2013; Mckenzie et al., 2016). In studies comparing the inactivation of bacteria and mammalian cells following exposure to 405 nm light, bacteria were much more susceptable to inactivation than mammalian keratinocytes and osteoblasts. This is likely to be because mammalian cells have much more advanced mechanisms for protection and coping with

oxidative damage in comparison with bacterial cells (Dai et al., 2013; McDonald et al., 2013). Additionally, in relation to human exposure within the clinical environment, body tissues will be exposed, not individual cells and as such there are many layers of cells providing additional protection against violet-blue light.

2.6. POTENTIAL APPLICATIONS OF BLUE LIGHT

A range of studies have been carried out to investigate the antimicrobial efficacy of violet-blue light, and also to investigate the potential application of this antimicrobial light for a range of applications including wound treatment and environmental decontamination, and these will now be discussed.

2.6.1. ANTIMICROBIAL EFFICACY OF VIOLET-BLUE LIGHT

Violet-blue light treatment has been shown to be successful for the inactivation of a broad range of microorganisms. In 2008 Maclean et al. carried out a study using a broad-spectrum xenon white-light source in combination with a range of optical filters, demonstrating the efficacy of visible light at wavelengths of 400-420 nm against both methicillin sensitive and methicillin resistant *S. aureus* with a peak inactivation achieved following exposure of *S. aureus* to a wavelength of 405 nm (± 5 nm) (Maclean et al., 2008). The bactericidal effect of 405 nm blue light against *S. aureus* is further supported in a study by Guffey et al., and the sensitivity of an additional Gram negative bacilli *Pseudomonas aeruginosa* to 405 nm light was further demonstrated (Guffey & Wilborn, 2006). The potential for blue light at a wavelength of 405 nm for the inactivation of a range of Gram-positive and Gram-negative bacteria including *Staphylococcus epidermidis, Streptococcus pyogenes, Enterococcus faecalis, Clostridium perfringens, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris* and *Klebsiella pneumoniae* has been demonstrated, with Gram-positive bacteria demonstrating an increased susceptibility to inactivation than Gram-negative bacteria. An exception to this is *E. faecalis*, which required exposure to a higher dose than all other bacterial suspensions in the study for significant inactivation to be achieved (Maclean et al., 2009). Further studies have demonstrated the efficacy for 405 nm light for the inactivation of *Salmonella enterica*, *Shigella sonnei*, *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni* and *Mycobacterium terrae* (Endarko et al., 2012; Murdoch et al., 2012; Murdoch et al., 2010). *Mycobacterium terrae,* a Gram-positive organism, showed similar inactivation kinetics to that of Gram-negative organisms – an explanation for this may be the difference in conformation of the cell envelope of

mycobacteria, which may confer resistance to 405 nm light or resistance to reactive oxygen species as mycobacteria species can evade phagocytic oxidative burst damage. Violet-blue 405 nm light has also been shown to be effective against yeasts and moulds, such as *Saccharomyces cerevisiae, Candida albica*ns and both dormant and germinating spores of *Aspergillus niger* (Murdoch et al., 2013). As would be expected, the dormant spores of *A. niger* were the most resilient, likely due to the presence of a multi-layered pigmented spore coat containing aspergillin, a black coloured melanin-like compound making the spores particularly difficult to inactivate when exposed to visible and UV light. Upon germination of the dormant spores, enhanced susceptibility to 405 nm light was observed; this is likely due to a change in morphology upon germination with loss of the spore coat and other such changes.

With regards to bacterial spores, the sporicidal efficacy of blue light has been demonstrated against *B. subtilis* spores using visible light at wavelengths of 405 and 436 nm; the sensitivity was further demonstrated to increase upon germination of the spores (Abad-Lozano and Rodriguez-Velera, 1984). The addition of exogenous photosensitisers, o-toluidine blue and acridine orange did not result in enhanced sensitivity. This is most likely due to the impermeable nature of the spore coat. Further research has established the susceptibility of *Bacillus* spores, including *B. cereus*, *B. thuringiensis*, *B. subtilis* and *B. atrophaeus* in the presence of phenothiazinium dyes upon exposure to low doses of red light; *B. megaterium* was not susceptible (Demidova & Hamblin, 2005).

De sordi et al. (2015) demonstrated the efficacy of blue light, at a wavelength of 410 nm, to kill *C. difficile* in the absence of PS. Unfortunately this paper does not detail whether vegetative cells, spores or biofilms were used - it can however be assumed that these were vegetative cells due to the extremely low doses required to achieve inactivation with a dose of 0.54 Jcm^{-2} reported to achieve a 4 $\log_{10}$ reduction (De Sordi et al., 2015).

A study carried out by Maclean et al. investigated the effect of high intensity 405 nm light on both the vegetative cells and spores of the endospore forming bacterium *B. subtilis, B. megaterium* and *C. difficile* (Maclean et al., 2013). This research indicated that *C. difficile* was the most susceptible of the three organisms, with a 3.7 $\log_{10}$ $CFUml^{-1}$ reduction of vegetative cells following exposure to a dose of 48 Jcm^{-2}. In comparison to the vegetative cells, *C. difficile* spores required a dose of 1.15 $kJcm^{-2}$ to achieve a 2.7 $\log_{10}$ $CFUml^{-1}$ reduction. Maclean stated that 'during the spore germination process it is likely that there will be a point at which the spores lose their high resistance to 405 nm light'. Although

these initial studies have been carried out, more work is required to investigate the effects of 405 nm light on *C. difficile* and whether there is potential to develop this antimicrobial technology for application as a sporicidal treatment in clinical situations.

2.6.2. Potential of violet-blue light as an Antimicrobial Therapy

The successful demonstration of the efficacy of blue light against a range of microorganisms has led to clinical testing and the development of a range of applications targeted towards specific microorganisms for blue light therapy. These clinical applications include treatment for:

- **Acne vulgaris**

Blue light has been shown to be successful for the treatment of *Propionibacterium acnes*, the causative agent of acne vulgaris. This is of particular interest as *P. acnes* affects more than 80% of young adults, whilst more than 40% of *P. acnes* exhibit resistance to commonly used antibiotics, topical treatments are slow and other therapies are limited due to their side effects (Ashkenazi et al., 2003). Recurrence is also a problem associated with *P. acnes*. *P. acnes* naturally produce high amounts of porphyrins, and as such are susceptible to PDI using blue light without the need for exogenous photosensitizers. The naturally occurring metabolite, δ-aminolevulinic acid (ALA), can further enhance photosensitisation by enhancing the production of endogenous porphyrins (Ashkenazi et al., 2003). Ashkenazi et al. demonstrated the efficacy of violet blue light at a wavelength of 407-425 nm on *P. acnes* following exposure for 60 minutes, with enhanced inactivation observed when exposed more than once with 24 hour intervals (Ashkenazi et al., 2003). This is supported by the findings of Guffey and Wilborn, with results demonstrating a 95% kill of *P. acnes* following exposure to 405 nm light whilst 470 nm light was shown to achieve a 96% reduction in *P. acnes* populations (Guffey & Wilborn, 2006).

Clinical trials investigating the efficacy of violet blue light for the treatment of *P. acnes* have proved to be successful, with the Clearlight therapy system at an irradiance of 407-420 nm achieving a 77% reduction in lesions following treatment with blue light after treatment twice a week for up to 5 weeks (Kawada et al., 2002). In support of this, a further study using the Clearlight system reported achieving a significant decrease in inflammatory acne lesions following 8 treatments, spaced apart once weekly, with an 80% response to treatment observed in patients and a significant 59-68% decrease in the lesion size observed following treatment, with no adverse effects reported by patients (Elman et al.,

2003). In the initial study however, 10% of patients condition was reported to have worsened, whilst 10% of patients conditions were reported to have remained unchanged (Kawada et al., 2002).

- **Peptic ulcers**

H. pylori is the cause of peptic ulcers, and has been declared a carcinogen leading to the development of gastric cancer. *H. pylori* is found in more than 20-50% of humans in Western countries, with up to 90% of the population infected in certain countries (Ganz et al., 2005; Hamblin et al., 2005). Current treatments include the use of antibiotics; however these have side effects associated with them alongside the development of antibiotic resistance. Hamblin et al. (2005) demonstrated that 405 nm light at a dose of 20 Jcm^{-2} resulted in a 99.9% kill of 7 different strains of *H. pylori*; 405 nm violet blue light was shown to be the most effective wavelength of light for the inactivation of *H. pylori*. *Helicobacter Pylori* has been demonstrated to contain both the porphryins coproporphyrin (610 nm) and Protoporphryin IX (632 nm), explaining its high sensitivity to 405 nm light. In support of this, a further study reported that a 99.999% reduction in *H. pylori* was achieved following exposure to a dose of 32 Jcm^{-2}, totalling 5 minutes exposure (Ganz et al., 2005).

Following on from their previous *in vitro* studies, Ganz et al. then proceeded to deliver 405 nm light at a dose of 40 Jcm^{-2} to the gastric antrum of patients with *H. pylori* infections using an optical fibre passed through an endoscope for light delivery. The average reduction per gram of tissue reported was 91% in comparison to control tissue sections, with reductions in some patients reaching up to 99% with no tissue abnormalities reported following exposure to blue light (Ganz et al., 2005). A further study by Lembo et al. using a light source emitting 408 nm light to treat the entire stomach – the antrum of the stomach, the body of the stomach and the fundus – was carried out on 18 adults (Lembo et al., 2010). Reduction in *H. pylori* was observed in all parts of the stomach, with the highest reduction reported in the antrum with the mean reduction reported as 97.7%. In the body of the stomach and the fundus an 88% and 81% reduction, respectively, in *H. pylori* was reported. This is most likely due to the shape of the stomach, with light more likely to be able to access the antrum of the stomach (Lembo et al., 2010).

- **Wound decontamination**

Burns are one of the most common, devastating forms of trauma and with a state of immunosuppression following severe burns, predisposing patients to potentially serious

infectious complications. Frequently these infections are caused by multidrug resistant organisms and thus are very difficult to treat. In addition to immunosuppression, burn wounds are particularly susceptible to colonisation resulting in infection due to the damage of cutaneous barriers. There is potential for the use of blue light for the treatment of nosocomial wound infections, with studies demonstrating the significant reduction of bacteria such as *P. aeruginosa*, MRSA *and A. baumannii* in potentially lethal mouse burn wounds, with no significant or irreversible damage caused to the skin (Dai et al., 2013; Zhang et al., 2014). Dai further demonstrated that blue light selectively inactivated *P. aeruginosa* over keratinocytes, with only a 0.16 $\log_{10}$ reduction in keratinocytes observed in comparison to a 7.64 $\log_{10}$ reduction achieved for *P. aeruginosa* following exposure of both populations to a dose of 109.9 Jcm^{-2} blue light, with an irradiance of 19.5 $mWcm^{-2}$ (Dai et al., 2013).

2.6.3. ENVIRONMENTAL DECONTAMINATION

As mentioned, violet-blue light has also been developed for environmental decontamination applications, with a number of studies demonstrating its efficacy within the hospital environment. The HINS-light environmental decontamination system (EDS) is a ceiling mounted lighting unit, installed alongside standard hospital lighting, which allows for continuous decontamination of the illuminated environment (Bache et al., 2012). The HINS-light EDS is designed to treat an area of 10 m^2, providing continuous disinfection of the air and all illuminated surfaces, inactivating any exposed bacteria (Maclean et al., 2013). This technology is not designed to replace current cleaning procedures, but to augment current infection control measures. The HINS-light EDS requires minimal staff training, causes no disruption to the normal hospital routine and the irradiances required to generate a bactericidal effect have been shown to be safe for humans.

A study carried out by Bache et al. (2012) investigated the effect of the HINS-light EDS on the levels of bacterial contamination in two burn unit environments: in single-bed isolation rooms housing burns patients and in a burns outpatient clinic room. For the inpatient study environmental sampling was conducted before installation of the HINS-light EDS, following use of the HINS-light for 2 days, and after the HINS-light EDS had been switched off for 2 days. Environmental samples were collected using contact plates at 8 am from a range of environmental sampling points around the room. The outpatient study samples were taken using the same methodology, but were collected 30 minutes prior to and following 8-hour clinics, with and without the use of HINS-light EDS. The results of this study demonstrated a

27%-75% reduction in the average number of bacterial colonies sampled in the rooms housing burn patients when the HINS-light EDS was in use, and up to an 80% increase in bacterial contamination following the HINS-light EDS being switched off. In the outpatient burns clinic in the absence of HINS-light EDS, a 174% increase in bacterial contamination was observed whilst during use of the HINS-light EDS a significantly reduced increase of 85% was observed. This increase in bacterial contamination was to be expected due to undressing of bandages resulting in the release of bacteria into the environment, paired with increased activity within the room during clinic hours. Thus, technologies with the potential to maintain a low bacterial burden within the environment, and in turn result in lowered transmission of bacteria to subsequent areas of the hospital, is of great interest and could result in lowered transmission of bacteria into open wounds (Bache et al., 2012).

This supports a previous study investigating the efficacy of the HINS-light EDS in an ICU isolation room, in which a 67% reduction in contamination across all sampled sites was demonstrated. Furthermore, this study demonstrated the return of bacterial contamination to normal levels following the removal of HINS-light EDS, similar to that observed in the study by (Maclean et al., 2010). This restoration of bacterial contamination levels demonstrates the efficacy of the HINS-light EDS for reducing bacterial contamination alongside maintaining low levels of contamination throughout periods of use (Maclean et al., 2010). Furthermore, this study investigated the decrease in contamination levels in a burns ward in a room occupied by an MRSA-positive burns patient over a 2 day period and a further 3 day extended period. The results of this study highlight the importance for the continuous use of the HINS-light EDS system, as a 56% reduction in MRSA contamination levels was observed following 2 days use of the HINS-light EDS system, in comparison to an 86% reduction following 3 days use (Maclean et al., 2010). A further study proceeded to investigate if surfaces the distance of the surfaces from the light source affected the decontamination efficacy, and found that, whilst a difference in the level of decontamination was observed between surfaces in closer proximity to the light, bacterial contamination was reduced on all surfaces. This is likely due to the potential of the HINS-light EDS for decontamination of airborne bacterial contamination.

2.7. AIMS OF THE CURRENT STUDY

In summary, this chapter has discussed the problems associated with *C. difficile* infection and contamination of the healthcare environment. This chapter has further discussed a range of current and new technologies and methodologies for the control of *C. difficile*, alongside the drawbacks associated with these methods.

Given the broad efficacy of blue light and its potential for infection control and potential clinical therapeutic use, a range of possible applications are being researched for blue light as previously discussed. However, as of yet, the literature survey showed that only one preliminary study with regards to the susceptibility of *C. difficile* to blue light had been carried out, and no other studies had expanded these observations (Maclean et al., 2013).

This project aimed to establish the bactericidal effect of 405 nm violet-blue light on *C. difficile* vegetative cells and spores under a range of conditions and environmental stresses, with the ultimate focus being towards investigating the potential of this antimicrobial technology to be developed for sporicidal environmental decontamination. In order to achieve this, work was undertaken with regards to determining the efficacy of 405 nm violet-blue light against spores, and possible mechanisms that would facilitate the enhanced decontamination of *C. difficile* spores in the clinical environment. As such, this research was split into 3 main research areas:

(i) *C. difficile vegetative cell and spore inactivation kinetics.*

(ii) *Investigation of a synergistic antimicrobial effect between 405 nm light and hospital disinfectants.*

(iii) *Factors influencing the susceptibility of C. difficile spores to 405 nm light.*

CHAPTER 3

Microbiological Methodology

3. OVERVIEW

This chapter details the microorganisms used in this study, alongside the various media, chemicals, diluents, reagents and equipment used throughout the research. Details of the standard microbiological techniques used for cultivation and enumeration, and also the light sources used in the inactivation studies are provided.

3.1. MEDIA

Media was prepared according to the manufacturer's instructions, with exact quantities weighed out and dissolved in distilled water. All media was then sterilised by autoclaving at 121°C for 15 minutes. Following sterilisation, agar was cooled in a 48°C water bath, and then dispensed into 90 mm single vent Petri dishes.

Media was pre-reduced by storage in an anaerobic environment for a minimum of 18 hours to remove any residual oxygen.

3.1.1. AGARS AND BROTHS

Agars

- Blood Agar [CM055B] (Oxoid Ltd, UK) 40 g/L
  After cooling, defibrinated horse blood [SRC0050C] (Oxoid Ltd.) was added to make a 7% blood concentration.
- Nutrient agar [CM0003] (Oxoid Ltd, UK) 28 g/L
- *Clostridium difficile* agar base [CM0601] (Oxoid Ltd, UK) 34.5 g/ 500 ml supplemented with *Clostridium difficile* Moxalactam Norfloxacin (CDMN) selective supplement [SR0173E] (Oxoid Ltd.) using freeze dried vials according to manufacturer's instructions.

Broths

- Brain heart infusion broth [CM225B] (Oxoid ltd, UK) 37 g/L
- Nutrient broth [CM0001] (Oxoid Ltd, UK) 13 g/L
- Columbia broth [294420] (SLS, UK) 35 g/L

- Clospore sporulation medium was prepared as follows (Perez et al., 2011):
  To a 500 ml Duran bottle add 300 ml of distilled water and then:

Special peptone mix [LP0072] (Oxoid Ltd, UK)	5.00 g
KH_2PO_4 [221309] (Sigma Aldrich, UK)	1.30 g
$(NH4)_2SO_4$	0.30 g
$CaCl_2.2H_20$ [C3306] (Sigma Aldrich, UK)	0.04 g
Yeast extract [LP0021] (Oxoid Ltd, UK)	5.00 g
K_2CO_3 [367877](Sigma Aldrich, UK)	1.74 g
$MgSO_4$	0.06 g
ddH_2O	up to 500 ml

3.1.2. Diluents and Reagents

- Phosphate buffered saline (PBS) [BR0014G] (Oxoid Ltd, UK) [BR0014G] – 1 tablet / 100 ml Distilled water
- Gram stain reagents : Crystal violet, Lugol's iodine, Ethanol and Safranin (Sigma Aldrich, UK)
- L-Ascorbic acid 99% [A92902] (Sigma Aldrich, UK)
- Bile salts [LP0055] (Oxoid Ltd, UK) 0.5% w/v in BHI broth
- Sodium taurocholate 0.5% w/v in BHI broth [AC230370050] (Fisher Scientific, UK)
- L-cysteine [168149] (Fisher Scientific, UK)
- Lysozyme [L6876] (Sigma Aldrich, UK)
- Trypsin [T2600000] (Sigma Aldrich, UK)
- Na_2HPO_4 [255793] (Sigma Aldrich, UK)
- NaH_2PO_4 [331988] (Sigma Aldrich, UK)
- 0.1 M sodium phosphate buffer – Prepared by weighing 8.19 g anhydrous Na_2HPO_4 and 5.84 g $NaH_2PO_4.H_2O$ and dissolving in 800 ml distilled H_20.
- 1% sodium dodecyl sulphate (SDS) [71727] (Sigma Aldrich, UK)
- 0.1 M Sodium hydroxide (NaOH)

3.1.3. PREPARATION OF SIMULANT FAECES

Artificial faeces was used in an attempt to simulate an environment similar to that in which vegetative cells and spores would be found in the clinical environment (due to the shedding of vegetative cells and spores in patient faeces), and to establish the inactivation kinetics of *C. difficile* vegetative cells and spores in faeces upon exposure to 405 nm violet-blue light in such environments. This was adapted from the simulant faeces composition produced by Colon et al., 2015 (Colón et al., 2015).

- Bakers Yeast (Marigold)
- Cellulose Microcrystalline powder [435236] (Sigma-Aldrich, UK)
- Psyllium husk powder (Wholefoods, UK)
- Miso paste (Yutaka)
- Fluka oleic acid [01008] (Sigma Aldrich, UK)
- NaCl (Fisher Scientific, UK)
- KCl (99+%) [P9541] (Sigma Aldrich, UK)
- $CaCl_2$ [449709] (Sigma Aldrich, UK)

Due to the very viscous nature of the simulant faeces, a modified simulant faeces was produced, the weights of the ingredients used in both recipes are listed in Table 3.1. 1 M NaOH was used to adjust the pH to 7-7.5.

Table 3.1. The composition of the original simulant faeces (Colon et al., 2015), and the alternative modified composition used to create modified simulant faeces in the present study.

	Original composition	Modified composition
Compound	**Amount (g kg^{-1})**	
Water	800	200
Baker's yeast	60	15
Microcrystalline Cellulose	20	0.625
Psyllium	35	4.375
Miso paste	35	8.75
Oleic acid	40	2.5
NaCl	4	1
KCl	4	1
$CaCl_2$	2	0.5

The changes made were mostly to reduce the concentration of microcrystalline cellulose and oleic acid in the mixture in order to reduce the viscosity. Faeces typically consists of 10-30% dry weight (dw) carbohydrates. The original recipe contained 10% cellulose and 17.5% psyllium; modifications to this recipe changed this to 1.25% cellulose and 8.75% psyllium – 10% carbohydrates in total in the final produce. Faeces typically consist of 5-25% fats, with oleic acid used for fat in the original recipe (20% dw); this was reduced to 5% in the modified recipe. Normally stools contain approximately 60-90% water, however diarrhoea occurs when not enough water is removed from the stool with diarrhoea reported to contain approximately 80- 91% water (Wenzl et al., 1995; Goy et al., 1976). The simulant faeces suspension was then mixed in a 1:50 ratio with water to produce a more diarrhoea like suspension.

3.2. MICROORGANISMS

The bacterial strains used throughout this study were:

- *Clostridium difficile* NCTC 11204, ribotype 001
  (National Collection of Type Cultures, Collindale, UK)
- *Clostridium difficile* NCTC 11209, ribotype 001 (the reference strain for *C. difficile*)
  (National Collection of Type Cultures, Collindale, UK).
- *Clostridium difficile*, ribotype 027
  (Obtained from the *C. difficile* reference Laboratory, NHSGCC, Glasgow)
- *Bacillus cereus* NCTC 11143
  (National Collection of Type Cultures, Collindale, UK)

C. difficile NCTC 11204 was used for the majority of the experimental work, with NCTC 11209 and the clinical isolate used as comparative strains. *B. cereus* is an aerobic sporulating bacteria used for comparison of inactivation kinetics with the anaerobic *C. difficile* spores.

3.3. CULTURE AND MAINTENANCE OF ORGANISMS

Microorganisms obtained from culture collections/reference laboratories were checked for viability by inoculation onto appropriate agar followed by Gram staining. They were then transferred onto Microbank^{TM} beads (Prolab Diagnostics) for storage at -20°C. Prior to experimental use, an inoculated bead was streaked onto an agar plate of the appropriate growth medium and cultured under the growth conditions required for the organism.

3.3.1. *C. DIFFICILE* VEGETATIVE CELLS

For preparation of vegetative cells, *C. difficile* was streak-inoculated onto blood agar (BA) plates and incubated at 37°C for 18 hours under anaerobic conditions (miniMACS Anaerobic Workstation, Don Whitley Scientific UK). Occasionally, anaerobic jars (Oxoid Ltd.) and Anaerogen sachets (Oxoid Ltd.) were also used for sample incubation. Post-incubation, the cells were washed off agar plates with PBS by agitation with an L-shaped spreader. This was then diluted to the desired population for experimental use. The absence of bacterial endospores was confirmed by heat treatment at 80°C for 10 minutes prior to enumeration on BA plates.

3.3.2. *C. DIFFICILE* SPORES

3.3.2.1. 10^3 CFUml^{-1} population for inactivation kinetics

For preparation of spores, *C. difficile* was inoculated into 15 ml Brain Heart Infusion (BHI) broth supplemented with 0.1% L-cysteine and incubated under anaerobic conditions at 37°C for 7 days. Post-incubation, the spore suspension was centrifuged at 4300 rpm for 10 minutes (Heraeus Labofuge 400R [Kendro Laboratory Product, USA]) and resuspended in 9 ml PBS. This suspension was heat treated at 80°C for 10 minutes to ensure only spores were present, and then diluted in PBS to the desired population for experimental use. An improved Neubauer Haemocytometer (Hawksley Technology, England) was used for enumeration of spore suspensions. This was stored at 2-4°C.

3.3.2.2. 10^3 CFUml^{-1} populations for germination experiments

Spores were prepared as before, however following heat treatment, they were washed in sterile distilled water 3 times by centrifugation to remove debris. These suspensions were stored at 2-4°C.

3.3.2.3. 10^6 CFUml^{-1} population preparation for inactivation kinetics

This methodology was adapted from that of Perez et al. (2011).

- From the stock suspension, stored on frozen microbeads, a loop was taken and streaked onto a pre-reduced CDMN agar plate. This was incubated at 37°C for 48 hours under anaerobic conditions.
- A colony was then resuspended in 5 ml pre-reduced Columbia broth (CB), and incubated for 48 hours under anaerobic conditions at room temperature.
- Following incubation 50 µl of this suspension was inoculated into 20 ml pre-reduced CB.
- Following incubation, the 20 ml CB was transferred into 500 ml Clospore broth and returned to anaerobic conditions, without agitation, and stored at 37°C for 10 days.
- Following incubation, the spore suspension was centrifuged in 50 ml batches, at 4500 rpm for 10 minutes; this was repeated until the total volume of the culture was centrifuged.
- The sediment was then pooled in one centrifuge tube and the pellet was washed once with 25 ml sterile distilled water. The pellet was resuspended in 10 ml sterile distilled water and transferred to a previously weighed 50 ml centrifuge tube. The previous centrifuge tube was washed several times with sterile distilled water and

then the volume in the new centrifuge tube was made up to 20 ml. This was centrifuged at 4500 rpm for 10 minutes, and the supernatant discarded.

- The pellet and centrifuge tube were then weighed, and the weight of the pellet was used to prepare the enzymatic solution (20 µg lysozyme per mg wet weight spore and 15 µg trypsin per mg wet weight spore, dissolved in 25 ml 0.1 M sodium phosphate buffer, pH 7), which was then sterilized through a Minisart 0.2 µm sterile filter (Satorius, UK).
- The pellet was resuspended in 10 ml 0.1 M sodium phosphate buffer and mixed, and then the enzymatic solution was added by filtration.
- This was sonicated on ice for 60 seconds (Ultra BT Ultrasonic bath U100 [Ultrawave Limited, Cardiff]), cooled on ice for 30 seconds, and then repeated.
- The suspension was then incubated at 45°C in a water bath for 30 minutes, followed by repetition of the sonication step. This was centrifuged at 4500 rpm for 10 minutes and washed once with sterile distilled water, and resuspended in 30 ml sterile distilled water. This was then heat treated for 10 minutes at 70°C, followed by submerging in ice for 5 minutes. This was centrifuged at 4500 rpm for 10 minutes, and resuspended in 10 ml sterile distilled water. This was stored at 2-4°C.

Following preparation, spores were analysed using phase contrast microscopy to ensure that spore populations were >99%. Spores were identified under the phase contrast microscope due to their bright phase appearance, as seen in Figure 3.1. Pictures were captured using Zeiss Axioimager Z1 phase contrast microscope viewed under ×100 oil immersion lens and pictures were captured using the Carl Zeiss MicroToolBox 2004 (MTB2004) software. Prior to experimental use, the spores were sonicated for 5 minutes to ensure spores were not aggregated into clumps.

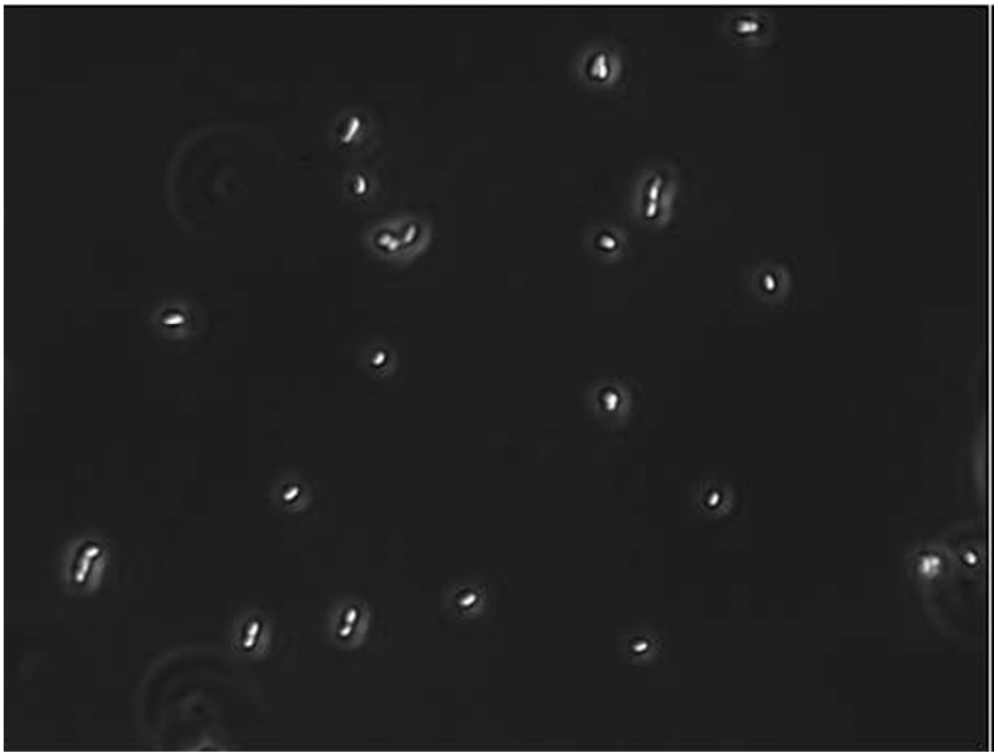

Figure 3.1 Phase contrast microscopy used to demonstrate that spore population was over 99%. Phase-bright spores are seen, with no debris/ dark vegetative cells observed.

Numerous other different attempts to cultivate a high titre of spores were unsuccessful. These methodologies are listed in Table 3.2.

Table 3.2 A summary of the methodologies used in an attempt to obtain a high titre of *C. difficile* spores.

Methodology	Population achieved
Spores were inoculated into 15 ml BHI and 0.1% L-cysteine, incubated at 37°C anaerobically for 7 days and centrifuged at 4500 rpm for 15 min, then resuspended in 1 ml PBS. (When centrifuged for a further 20 min and resuspended in 1 ml PBS, it gave 1 ml 10^5 $CFUml^{-1}$)	10^4 $CFUml^{-1}$
Spores were inoculated into 25 ml BHI supplemented with 0.1% L-cysteine, incubated anaerobically at 37°C for 7 days and centrifuged at 4300 rpm for 15 min then resuspended in 9 ml PBS.	10^4 $CFUml^{-1}$
A lawn of *C. difficile* was inoculated onto 10 blood agar plates for 7 days and incubated anaerobically at 37°C for 7 days. Spores were resuspended in 1 ml PBS and agitated using an L-shaped spreader. All the spore suspensions were pooled together, centrifuged at 4300 rpm for 10 min and then heat treated at 80°C for 10 min.	10^3 $CFUml^{-1}$
5 × 200 ml BHI broths were inoculated with *C. difficile* and incubated for 10 days anaerobically at 37°C. This was then centrifuged at 4500 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 2.5 ml sterile distilled water and 10 ml ethanol and incubated at room temperature for 1 hr. This was centrifuged as before and washed once with 2.5 ml chilled water then heat treated at 80°C for 10 min.	10^4 $CFUml^{-1}$
9 ml BHI supplemented with 0.1% w/v sodium taurocholate and 0.1% w/v L-cysteine was inoculated with *C. difficile* and incubated anaerobically at 37°C for 7 days. This was centrifuged at 4300 rpm for 10 min, resuspended in 9 ml PBS and heat treated 80°C for 10 min.	10^4 $CFUml^{-1}$
15 ml BHI supplemented with 0.1% w/v sodium taurocholate and 0.1% w/v L-cysteine was inoculated with *C. difficile* and incubated anaerobically at 37°C for 7 days. This was then centrifuged at 4300 rpm for 10 minutes and resuspended in 9 ml PBS and heat treated 80°C for 10 minutes.	10^4 $CFUml^{-1}$
A lawn of *C. difficile* was plated onto blood agar supplemented with 5% yeast and 0.1% L-cysteine and incubated 37°C 7 days. This was resuspended in 9 ml PBS and heat treated 80°C for 10 minutes. All 4 attempts became contaminated.	Contamination on each attempt
25 ml BHI supplemented with 0.1% w/v sodium taurocholate and 0.1% w/v L-cysteine was inoculated with *C. difficile* and incubated anaerobically at 37°C for 7 days. This was then centrifuged at 4300 rpm for 10 minutes and resuspended in 9 ml PBS and heat treated 80°C for 10 minutes.	10^5 $CFUml^{-1}$

3.3.3. *B. CEREUS* VEGETATIVE CELLS

For preparation of vegetative cells, *B. cereus* was inoculated into 100 ml nutrient broth and incubated aerobically at 37°C under rotary conditions (125 rpm) for 18 hours. Following incubation, the suspension was centrifuged at 4300 rpm for 10 minutes and then diluted in PBS to obtain a 10^3 $CFUml^{-1}$ population.

3.3.4. *B. CEREUS* SPORES

For preparation of spores, *B. cereus* was inoculated into 100 ml nutrient broth supplemented with 200 µl manganese sulphite at a concentration of 200 µl/100 ml broth and incubated aerobically at 37°C for 72 hours under rotary conditions (125 rpm). Following incubation, the spore suspension was centrifuged at 4300 rpm for 10 minutes. Serial dilutions in PBS were then prepared to obtain the desired 10^3 $CFUml^{-1}$ population. This was then heat treated at 80°C to ensure that only spores were present.

3.4. MICROBIOLOGICAL IDENTIFICATION TESTS

3.4.1. GRAM STAINING

Gram staining was used to confirm the purity of the stock cultures of the Gram positive bacteria used in this study. The methodology was carried out as follows:

- Using a sterile loop, a colony of the bacteria was transferred from an agar plate onto a microscope slide and mixed with a drop of water. This was fixed on the surface of the microscope slide by passing through a Bunsen flame.
- This was then flooded with crystal violet and left to sit for 30-60 seconds.
- This was rinsed with Lugol's Iodine and covered with fresh iodine and left to sit for 60 seconds.
- Ethanol was run over the surface to rinse until the colour ran clear.
- Safranin was then flooded onto the slide and left to sit for 1 minute before rinsing with water.
- The slide was blotted dry using the edge of a paper towel and then examined under the oil immersion microscope lens at ×1000 magnification. A Nikon Eclipse E400 Microscope was used to examine bacterial culture purity and cell morphology. A Nikon Coolpix 4500 digital camera was used to photograph the microorganisms.

Following Gram staining, Gram positive bacteria appear purple, whilst Gram negative bacteria are stained pink. This is due to the retention of the crystal violet stain by the

peptidoglycan of the cell wall of Gram positive bacteria. Gram negative bacteria, on the other hand, do not retain the crystal violet stain and are decolourised by the ethanol. Subsequent counterstaining using safranin results in the pink colour characteristic of Gram negative bacteria. A Gram stain of the *C. difficile* NCTC 11204 type culture strain used in this study is shown in Figure 3.2.

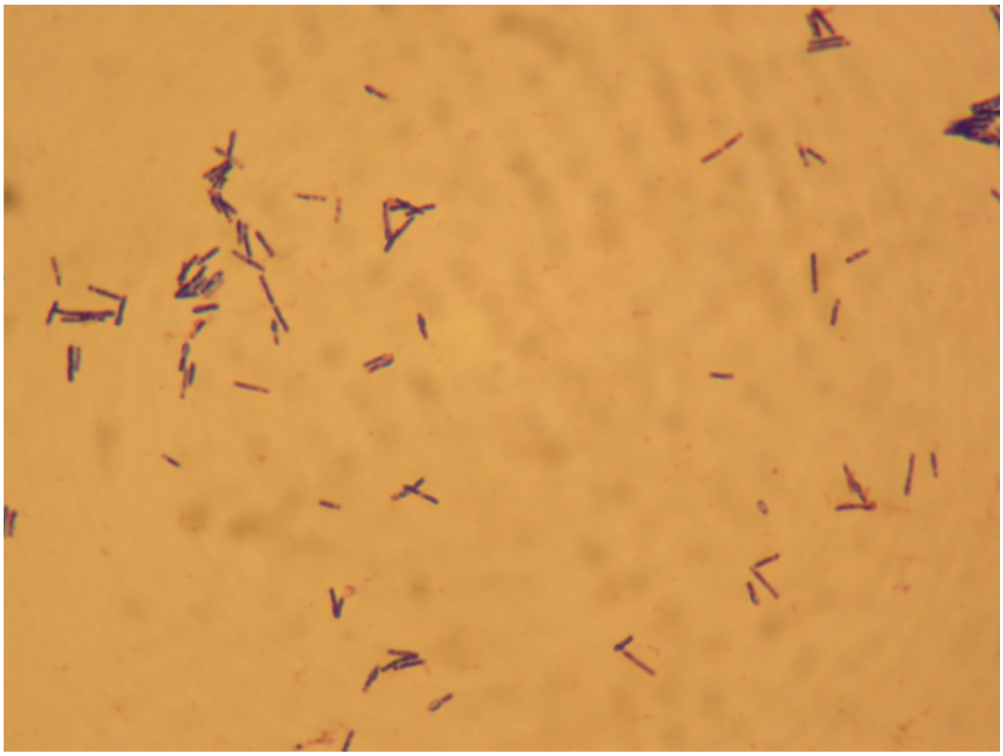

Figure 3.2 The morphology of Gram positive *C. difficile* observed under the ×1000 magnification oil immersion microscope lens (Nikon Eclipse E400 Microscope) following gram staining. Photographed using a Nikon Coolpix 4500 digital camera.

3.4.2. WIRTZ-CONKLIN STAIN

This was used to confirm spore suspensions were 99% spores for spore suspensions, and also to measure germination. Endospores (free endospores or those inside mother cells) retain the malachite green stain and appear under the microscope as a minty green colour, whereas vegetative cells and mother cells stain red. The methodology was carried out as follows:

- Using a sterile loop, a colony of bacteria was transferred from an agar plate onto a microscope slide and mixed with a drop of water. This was fixed on the surface of the microscope slide by passing through a Bunsen flame.

- A small piece of paper towel was then placed over the smear and saturated with 5% solution of malachite green and allowed to sit for 1 minute
- In a fume cupboard, the slide was heated using a Bunsen burner until steam was given off. More malachite green was applied as necessary. Just enough heat to allow continued steaming for 5 minutes was applied.
- The slide was allowed to cool, and then rinsed with water for 30 seconds.
- This was counterstained with safranin for 60-90 seconds and then rinsed with water and blotted dry.
- The slide was then examined under the oil immersion microscope lens (× 1000 magnification).

Figure 3.3 shows C*. difficile* NCTC 11204 spores stained using this methodology following preparation, as previously described in Section 3.4.2.

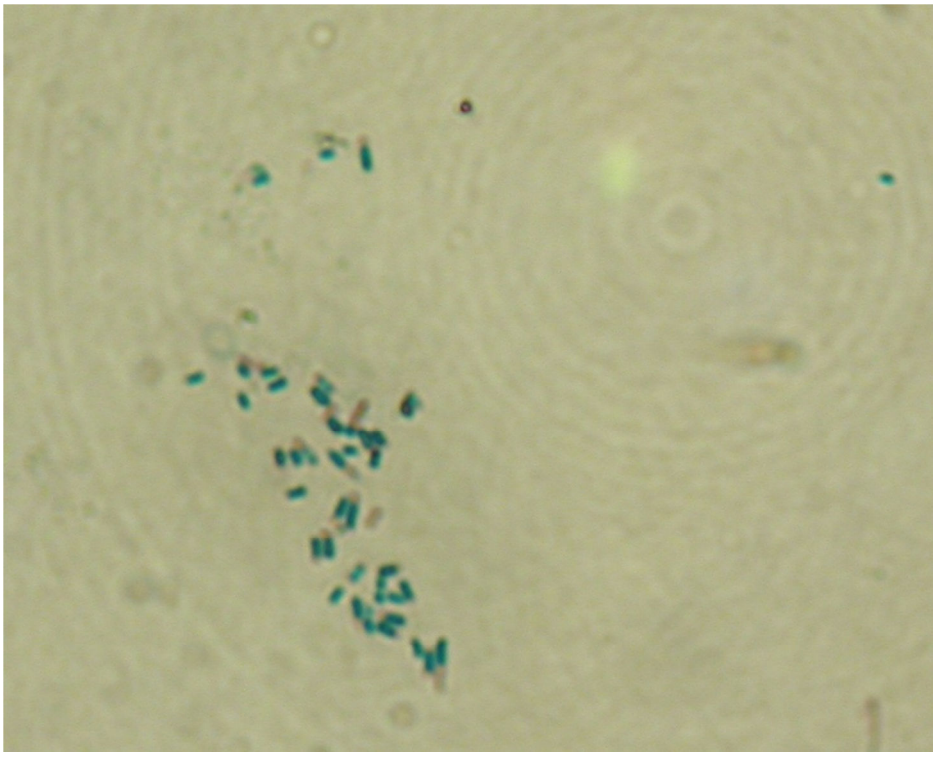

Figure 3.3 *C. difficile* spores stained using Wirtz-Conklin. Endospores are stained a mint green and vegetative cells red. Observed under the ×1000 magnification oil immersion microscope lens (Nikon Eclipse E400 Microscope). Photographed using a Nikon Coolpix 4500 digital camera.

3.5. 405 NM LIGHT EXPOSURE SYSTEMS

Light emitting diode (LED) arrays were the light sources used to investigate the sporicidal efficacy of 405 nm light. This section provides details of the 2 sources that were used: a

high irradiance source (used to provide light irradiances of 70 and 225 $mWcm^{-2}$) and a low irradiance source (used to provide an irradiance of 0.5 $mWcm^{-2}$).

3.5.1. ENFIS PHOTONSTAR INNOVATE UNO 24

The light source used for high irradiance exposure of bacteria to 405 nm light was an ENFIS PhotonStar Innovate UNO 24 405 nm light emitting diode (LED) array [PhotonStar Technologies, UK], which consisted of 24 LEDs with a peak output at approximately 405 nm. The light source and experimental set up for both suspension and surface exposures is pictured in Figure 3.4a and Figure 3.4b. The light source was held on a clamp stand and samples were placed directly below, at approximately 5 cm distance.

Optical analysis of the output confirmed a peak wavelength of 407 nm, and a bandwidth of 19 nm at FWHM (Figure 3.5). The spectral output was measured using a spectrometer [Ocean Optics HR4000] and SpectraSuite software. The LED array was powered by a DC power supply (40V Phillips Xitanium LED driver), and a cooling fan and heat sink were attached to the array allowing heat to dissipate from the source thereby minimizing heat transfer to the sample (Figure 3.4d). Irradiance was measured using a photodiode detector (Model-1Z02413; Ophir) and radiant optical power meter (Model-70260; Oriel Instruments) calibrated at 405 nm. This array further has a resistor to allow changes to be made to the output irradiance, as can be seen in Figure 3.4c. The outputs typically used for the exposure of suspensions and surfaces was 70 $mWcm^{-2}$ and 225 $mWcm^{-2}$, with samples being placed at a ~5 cm distance from the array. A Kane May KM340 thermocouple (UK) was used to measure the temperature of light exposed suspensions.

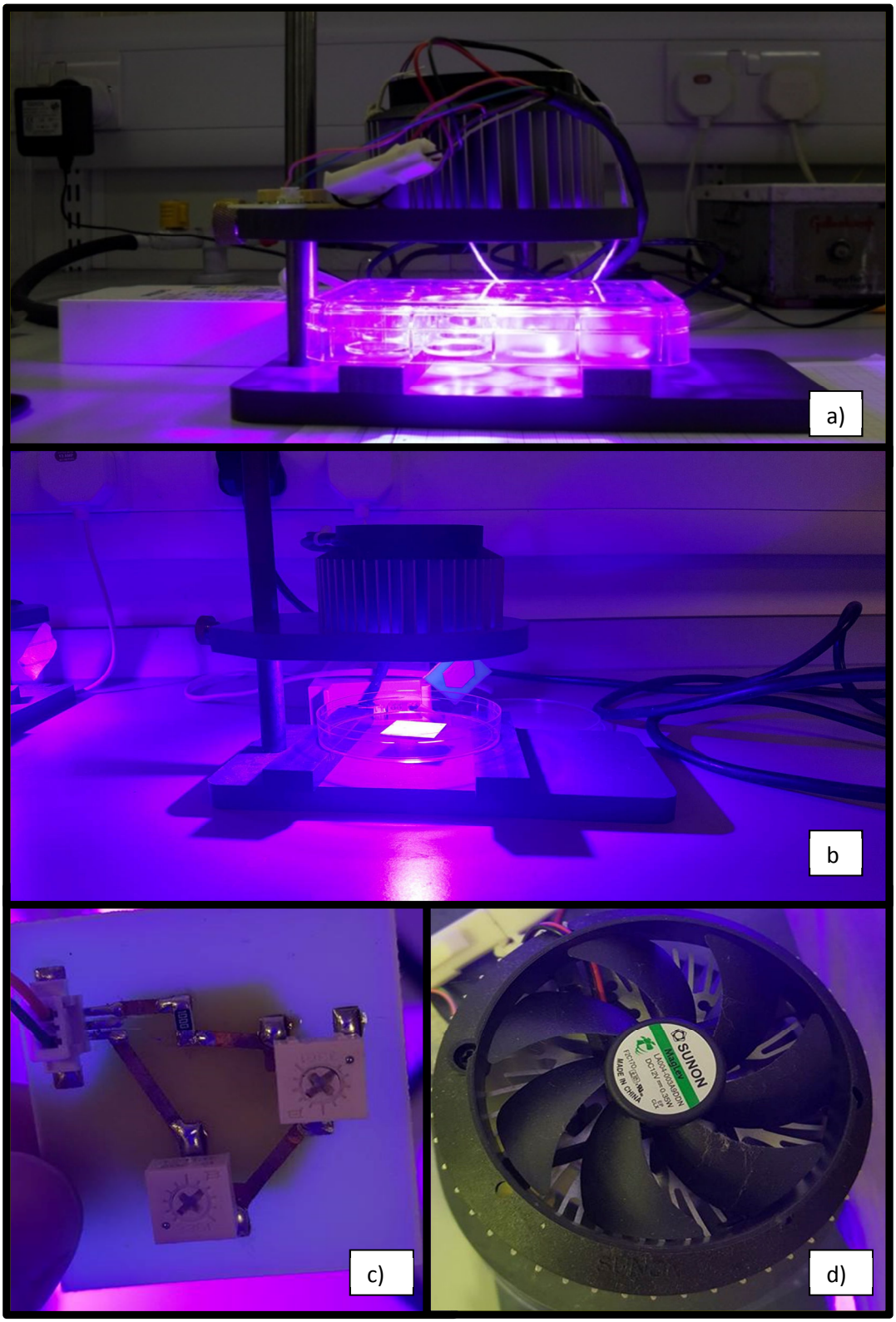

Figure 3.4 The experimental arrangement for the high irradiance 405 nm light exposure of vegetative cell and spore suspensions, and spores seeded on clinically relevant surfaces. The light source used was an Enfis PhotonStar Innovate UNO 24 light system. (a) Suspension experiments (b) Surface experiments (c) The resistor allowing control of the irradiance output from the light source (d) the cooling fan.

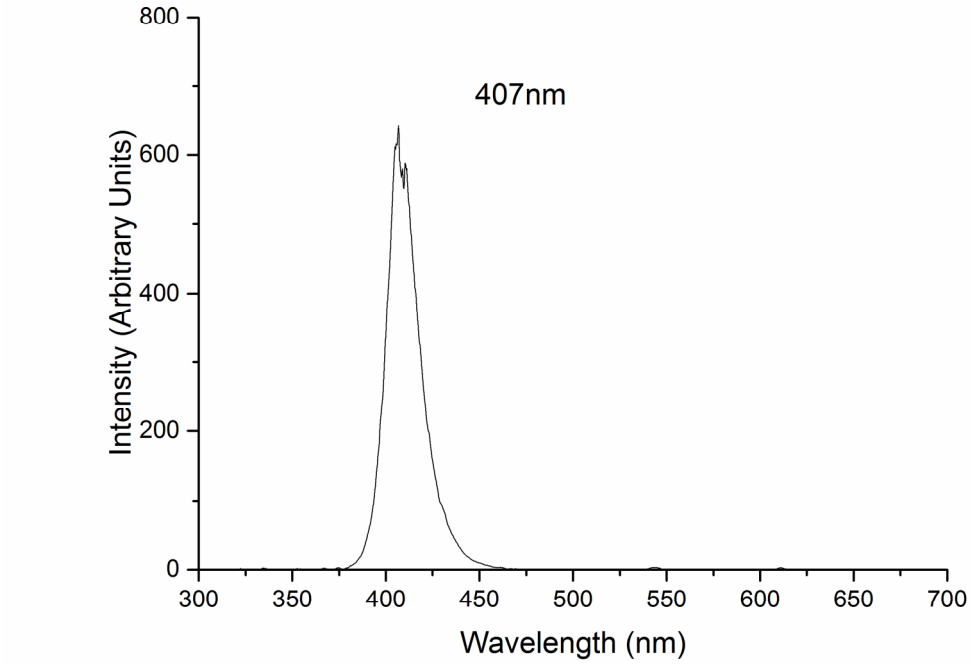

Figure 3.5 Optical emission spectra for the ENFIS PhotonStar Innovate UNO 24, with peak wavelength highlighted. The spectral output was measured using a spectrometer [Ocean Optics HR4000] and SpectraSuite software.

3.5.2. HINS-LIGHT ENVIRONMENTAL DECONTAMINATION SYSTEM

As detailed in Section 2.6.3, the High-Intensity Narrow-Spectrum Light Environmental Decontamination System (HINS-light EDS) is a ceiling mounted lighting system for microbial inactivation designed to illuminate an area of approximately 10 m^2. The lighting system consists of a matrix of LEDs which provide a continuous, low irradiance 405 nm illumination, with an irradiance of approximately 0.4 $mWcm^{-2}$, at a distance of 1.5 m. The system used in this study consisted of 16 violet 405 nm 99-DIE LED arrays (OptoDiode Corp, USA) and 5 broadband white LEDs; the integration of white LEDs produces lighting that is predominantly white and so blends well with standard room lighting. In order to minimise heat build-up, the blue and white LEDs were mounted to an aluminium heat sink (30 × 30 × 85 cm). A fresnel lens and diffuser were positioned in front of the LED matrix, to help blend and distribute the light output. The source was held in a 59 × 59 cm PVC housing unit and ceiling mounted. The emission spectra for the combined violet-blue light and broadband white light emitted from the HINS-light EDS is shown in Figure 3.6. This optical analysis of the output confirmed a peak wavelength of 401 nm and a bandwidth of 14 nm for the

violet blue LEDs. The light system is powered by two power supplies, one supplying to blue LED's (18.3 A and 15.9 V) and one to power the white LEDs (1.5 A and 10.3 V) allowing for independent control; these power supplies are kept in a lockable mobile cabinet.

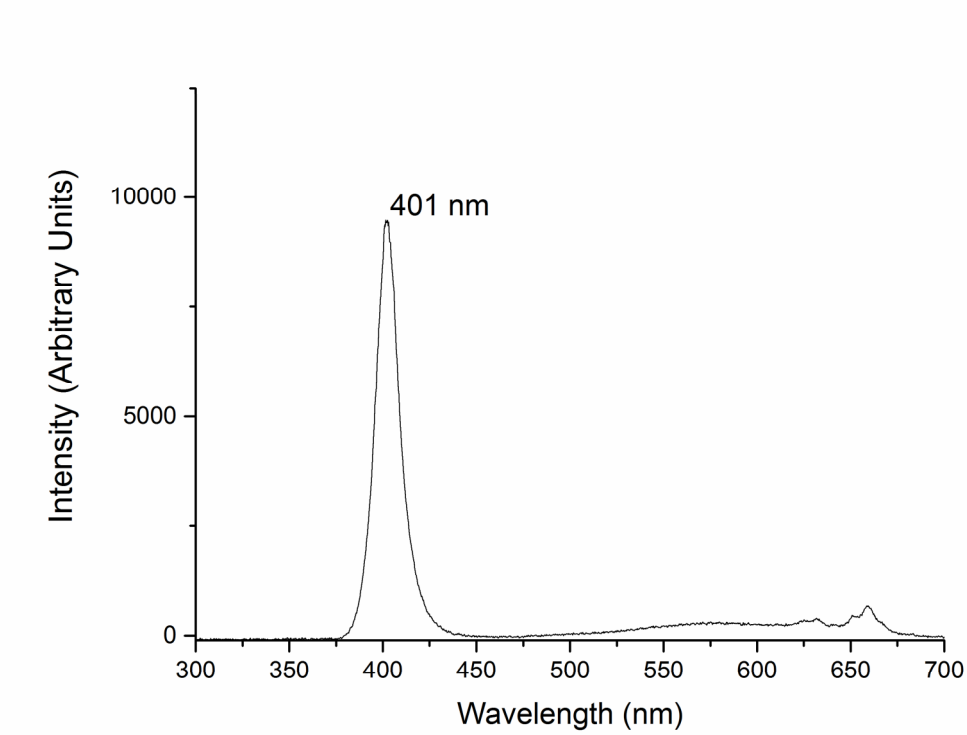

Figure 3.6 Optical emission spectra for the HINS-light EDS, with the peak wavelengths of the violet-blue LEDs highlighted. The spectral output was measured using a spectrometer [Ocean Optics HR4000] and SpectraSuite software.

The HINS-light EDS was mounted into the ceiling and surfaces inoculated for exposure were place on a gridded board, approximately 1.5 metres below the light source and exposed to 405 nm light for increasing time periods. The area of the board used for exposure experiments was 100 × 68 cm, permitting the simultaneous exposure of multiple samples. Prior to the exposure of bacteria, the irradiance profile of both the violet-blue light LEDs alone and the violet-blue and white LEDs together on the surface was measured using a photodiode detector (Model-1Z02413; Ophir) and radiant optical power meter (Model-70260; Oriel Instruments) and plotted, as shown in Figure 3.7. The irradiance for the blue LEDs averaged at approximately 0.4 $mWcm^{-2}$. Slight variation was measured along the grid, as shown in Figure 3.7. As such, following profiling of the irradiance onto the gridded board the samples were positioned in the central area (40 × 40) of the grid so that the exposure irradiance was uniform. This experimental arrangement is pictured in Figure 3.8.

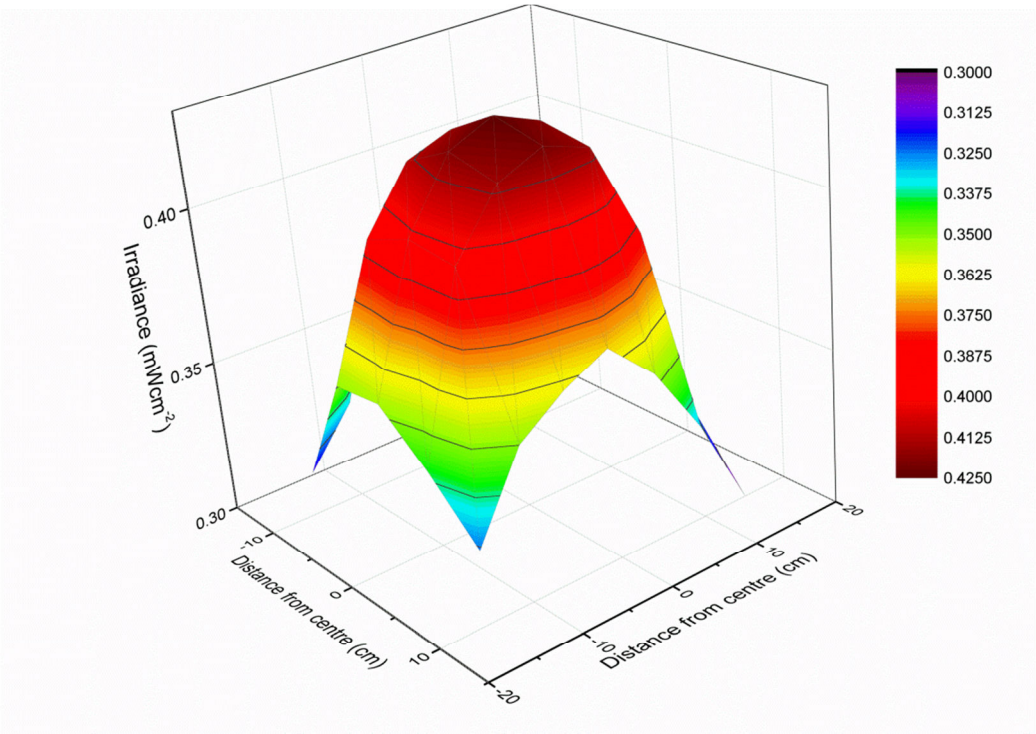

Figure 3.7 The distribution of HINS-light EDS irradiance across the gridded board, with the highest measured irradiance being the centre point directly below the source.

Figure 3.8 The experimental arrangement for the 405 nm light exposure of *C. difficile* spores inoculated onto surfaces. The irradiance was measured at each sample position on the board, with a 0.4 mWcm^{-2} irradiance average.

3.6. ULTRAVIOLET LIGHT EXPOSURE SYSTEMS

For a comparison of the inactivation kinetics of 405 nm light, *C. difficile* spores were exposed to UV-C light. The light source used was the UVP UVGL-25 UV lamp (Ultraviolet product, UK) (Figure 3.9). This low power device (4 Watts) has an output irradiance of ~0.79 mWcm^{-2} for UV-C at a distance of 1 cm. Figure 3.10 shows the emission spectrum of this UV-C light source.

Figure 3.9 UVP UVGL-25 UV lamp used for the exposure of bacterial suspensions and bacteria seeded on surfaces UV-C light.

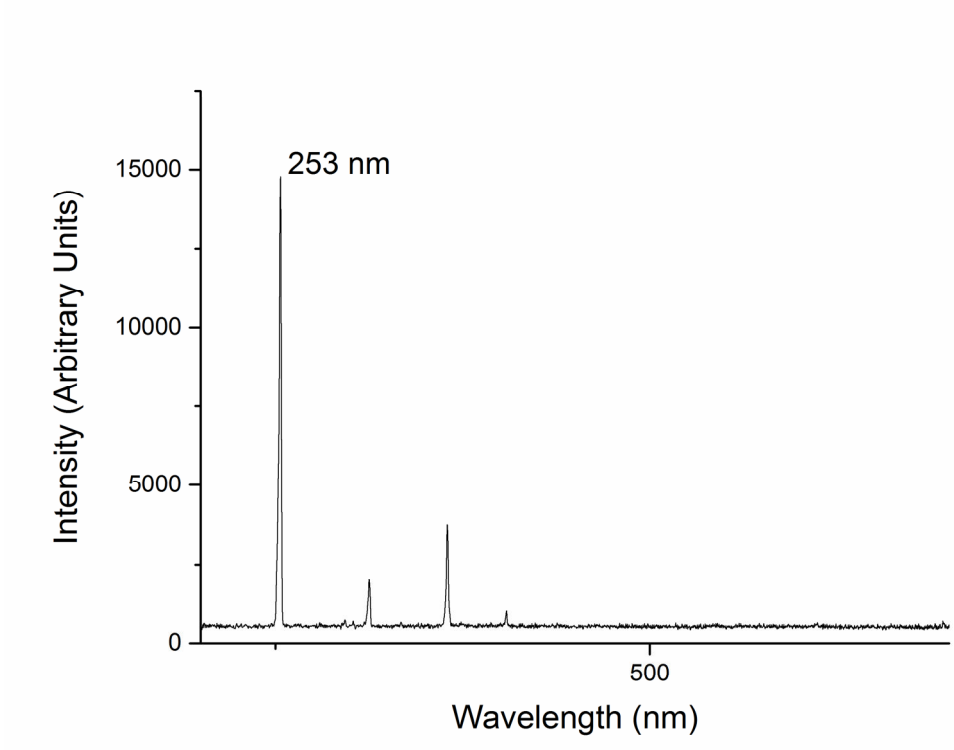

Figure 3.10 Emission spectrum of UV-C light from the UVP UVGL-25 UV lamp, showing a peak output at 253 nm.

3.7. BACTERIAL ENUMERATION

In bacterial inactivation studies, it is of great importance to accurately enumerate the surviving population pre- and post-light exposure to enable an accurate calculation of the decrease in the viable bacteria. To enumerate the population, the samples must be plated onto agar and incubated under optimal conditions: 37°C for 48 hours under anaerobic conditions for *C. difficile* and 24 hours under aerobic conditions for *B. cereus*. If the samples of interest are expected to be too numerous for counting, they must be diluted prior to plating to ensure quantifiable results. Following incubation, the population is enumerated and recorded as colony forming units per millilitre ($CFUml^{-1}$). This section describes the preparation of serial dilutions, plating and enumeration techniques used throughout this study.

3.7.1. SERIAL DILUTIONS

Serial dilutions were prepared by pipetting 1 ml of the neat bacterial suspension into 9 ml PBS, to give a 10^{-1} $CFUml^{-1}$ dilution. The diluted sample was vortexed using a whirly mixer (Fisherbrand, UK) to achieve a homologous suspension. 1 ml of this suspension was then pipetted into a further 9 ml volume of PBS, to produce a 10^{-2} $CFUml^{-1}$ and so on, until the desired population was achieved.

3.7.2. PLATING AND ENUMERATION

Following exposure, either neat or diluted samples of the exposed suspension and non-exposed controls were plated onto the agar of choice for the bacterial species: *C. difficile* was plated onto Blood agar (BA) plates and *B. cereus* was plated onto nutrient agar (NA) plates.

In general, post-exposure, manual spread plates were prepared, with 100 μl or 500 μl samples pipetted onto BA (or NA for *B. cereus*) and spread evenly using an L-shaped spreader. These were incubated at 37°C for 48 hours under anaerobic and aerobic conditions for *C. difficile* and *B. cereus*, respectively. Spread plates were enumerated manually by counting the total colonies present on the plate. The total colony count was classified as colony-forming units per millilitre ($CFUml^{-1}$). Specific details of the plating methodologies are provided in the relevant chapters.

3.8. DATA ANALYSIS

3.8.1. CALCULATING DOSE

To calculate the dose of light exposure (Jcm^{-2}) using the measured irradiance and exposure times, the following calculation was used:

$$\textit{Dose } (Jcm^{-2}) = \textit{Irradiance } (Wcm^{-2}) \times \textit{exposure time (seconds)}$$

3.8.2. STATISTICAL ANALYSIS

Experimental data are an average of a minimum of triplicate independent experimental results, measured in duplicate ($n \geq 6$), with error bars representing the standard deviation (SD). As samples were directly plated onto BA plates, some samples counted were below the detection limit (2 $CFUml^{-1}$ when using 500 μl samples and 10 $CFUml^{-1}$ when using 100 μl spread plates) however these have been included in graphs to demonstrate the near-to-complete inactivation effect achieved. Unless otherwise stated, data were analysed using one-way analysis of variance (ANOVA) using Minitab statistical software Version 15. In all statistical analysis in this thesis, actual P values will be provided with significant differences accepted at $P \leq 0.05$.

CHAPTER 4

Investigation into the Inactivation of Spore Forming Bacteria Using 405 nm Light

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4. GENERAL

Sections 2.5.3-2.6.2 discussed the broad antimicrobial effects of 405 nm light against a range of problematic pathogenic organisms. Currently there is very little research in the literature with regards to the susceptibility of *C. difficile* to 405 nm light, with only one research article currently available (Maclean et al., 2013). This current chapter will investigate the fundamental interactions of 405 nm light with both the vegetative cells and spores of *C. difficile* to obtain quantitative data to establish the efficacy of 405 nm light against vegetative cells and spores. This will be done using both small-scale suspension experiments and surface seeded experiments.

This chapter investigates:

- The inactivation kinetics of *C. difficile* spores and their vegetative cell counterparts in suspension.
- The inactivation kinetics of *C. difficile* spores and their vegetative cell counterparts on clinically relevant surfaces.
- The inactivation kinetics in the presence of artificial faeces upon illumination of 405 nm light, to mimic how bacterial contamination is likely to be found in the clinical environment following shedding of vegetative cells and spores into the environment as a result of bowel movements.
- If other *C. difficile* strains and/or spore forming bacteria exhibit sensitivity to 405 nm light, and in addition, to determine if they exhibited similar inactivation kinetics to that of the model *C. difficile* strain selected for the majority of this study.

The aim of the experiments in this chapter was to obtain initial inactivation kinetics of both vegetative cells and spores upon exposure to 405 nm light under a range of settings, and to establish if there was a variance in these inactivation kinetics in the presence of organic

matter and between different *C. difficile* strains and in comparison to that of other spore forming bacteria.

4.1. INACTIVATION KINETICS OF *C. DIFFICILE* VEGETATIVE CELLS AND SPORES UPON EXPOSURE TO 405 NM LIGHT WHEN IN SUSPENSION

The aim of these experiments was to determine if 405 nm light could be used for the inactivation of the spore forming bacteria *C. difficile*, and to compare the inactivation kinetics with their vegetative counterparts both in suspension and on clinically relevant surfaces.

4.1.1. BACTERIAL PREPARATION

Clostridium difficile NCTC 11204 (ribotype 001), which has been reported to be present in 33 of 58 UK hospitals and is also prevalent in multiple other countries, including Sweden, the United States and Japan, was used primarily for the purpose of this study (Stubbs et al., 1999; Kato et al., 2001; Wullt et al., 2003; Johnson et al., 1999). *C. difficile* bacterial vegetative cell or spore suspensions previously prepared - as detailed in Section 3.3.1 for vegetative cell suspensions and 3.3.2 for spore suspensions - were prepared for this study.

4.1.2. TREATMENT METHOD

A 2 ml volume of the bacterial vegetative cell or spore suspension (10^3 CFUml^{-1}) was transferred to a 12-well multi-dish. This was then placed 5 cm below the LED array on a stand to allow any heat to dissipate from under the multi-dish. The light array used was the ENFIS Photostar Innovate UNO 24 (for high irradiance exposures), as detailed in Section 3.5.1. This experimental set up is pictured in Figure 3.4. The suspensions were exposed to an irradiance of 70 mWcm^{-2} and 225 mWcm^{-2} for vegetative cell suspensions and spore suspensions, respectively, for increasing time durations. Control samples were subjected to identical laboratory conditions (atmospheric pressure, humidity and temperature), but exposed to ambient laboratory lighting levels for the same time intervals as samples exposed to 405 nm light. The ambient light in the laboratory was generated by standard fluorescent ceiling mounted lamps, the ambient light irradiance at control sample surface level was measured using a radiant optical power meter (Model-70260; Oriel Instruments) and found to be (0.2 mWcm^{-2}).

Following exposure, exposed samples and non-exposed controls were plated onto blood agar and incubated at 37°C for 48 hours under anaerobic conditions. Samples were then

enumerated and viable bacterial counts recorded as $\log_{10}$ CFUml^{-1}, as discussed in Section 3.7.2. Experiments were repeated in triplicate.

In order to ensure that 405 nm light was the only cause of inactivation in the suspensions, temperature changes were recorded at each time point following exposure of the samples to high irradiances of 225 mWcm^{-2}. This was done using a thermocouple (Kane May KM340), with a maximum increase of 9°C recorded.

4.1.3. Optical Analysis

A Biomate 5 UV-visible Spectrophotometer (Thermo Scientific, USA) was used to measure light transmission through *C. difficile* suspensions over a scan of wavelengths of 300-700 nm. PBS was used to zero the instrument.

A Shimadzu spectrofluorophotometer (RF-5301-PC, America) was used to measure the fluorescence of *C. difficile* suspensions.

4.1.4. Results: 405 nm light Inactivation of *C. difficile* vegetative cells and spores in suspension

Initial experiments exposed *C. difficile* vegetative cells and spores to increasing doses of 405 nm light, at an irradiance of 70 mWcm^{-2} and 225 mWcm^{-2} respectively, in order to determine the inactivation kinetics of the National Culture Collection strain NCTC 11204. The results are displayed graphically in Figure 4.1 and Figure 4.2.

The results in Figure 4.1 demonstrated that vegetative cells were susceptible to inactivation upon exposure to 405 nm light. As shown in Figure 4.1, the population remained fairly constant with doses of up to 105 Jcm^{-2}, and after which significant inactivation was observed, with a 1.45 $\log_{10}$ reduction after exposure to 189 Jcm^{-2} (P=0.006), and complete inactivation (99.9% reduction) of the 3.3 $\log_{10}$ population achieved after exposure to 252 Jcm^{-2} (P<0.001). Figure 4.2 shows the comparative results for the inactivation of spores. Inactivation was achieved, but as expected, the spores proved to be significantly more resilient to light treatment, with approximately ten times the dose required to achieve a similar 3.5 $\log_{10}$ reduction as that achieved with the vegetative cells. Inactivation kinetics showed a significant 0.5 $\log_{10}$ reduction after a dose of 810 Jcm^{-2} (P=0.001), and 3.3 $\log_{10}$ reduction after 2.43 kJcm^{-2} (P<0.001).

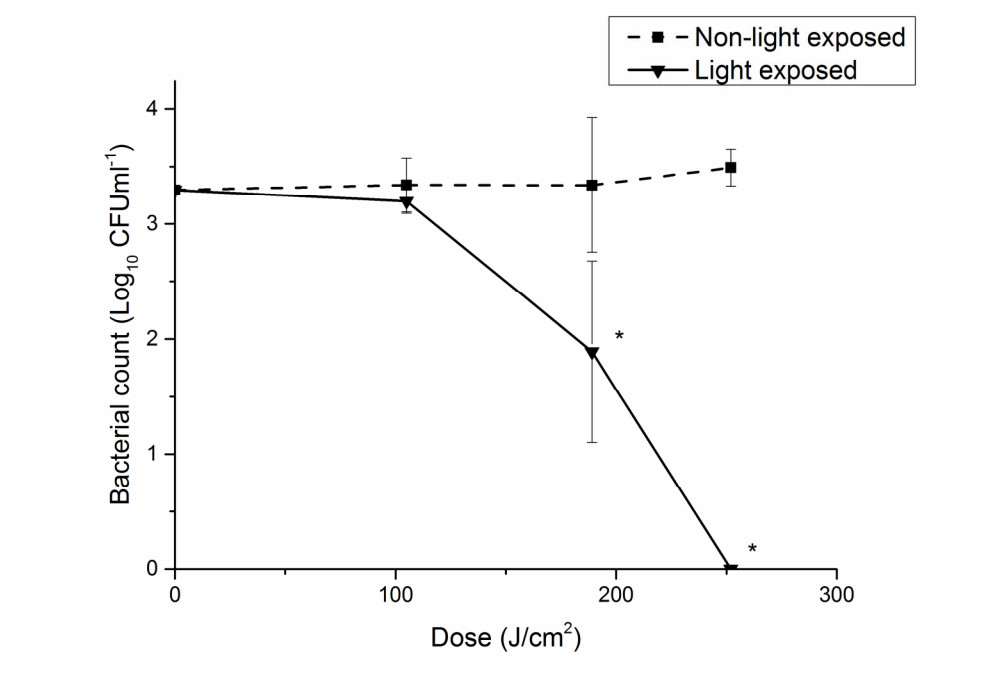

Figure 4.1 The susceptibility of *C. difficile* vegetative cells suspended in PBS to 405 nm light (70mWcm^{-2}). Control samples were non-light exposed for the equivalent time periods (0, 25, 45 and 60 minutes), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation (P≤0.05). Each data point is a mean value ± SD (n=6).

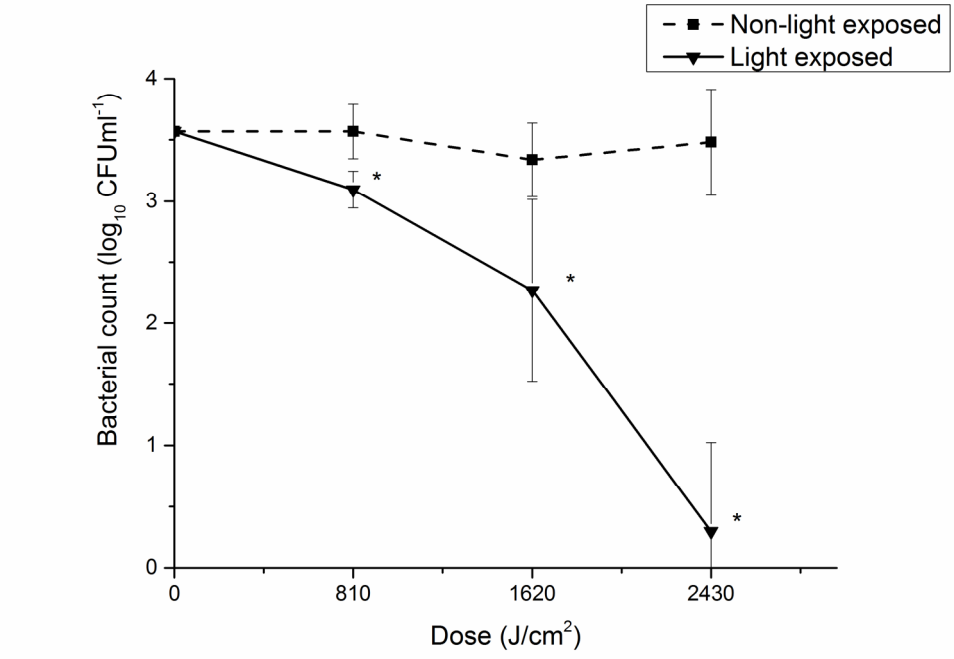

Figure 4.2 The susceptibility of *C. difficile* spores suspended in PBS to 405 nm light (225 mWcm^{-2}). Control samples were non-light exposed for the equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation (P≤0.05). Each data point is a mean value ± SD (n=6).

Figure 4.3 shows the differential optical transmission spectra, T, for spore and vegetative cell suspensions obtained using the Biomate 5 UV-visible Spectrophotometer (as discussed in Section 4.1.3), PBS with no spores was used as a control in these optical spectra measurements. At a wavelength of 405 nm, Figure 4.3 demonstrates that 405 nm light has a 99% transmission through vegetative cells, whilst only 78.5% light transmits through the spore suspension, as shown in Figure 4.3. The results demonstrating that T=99% for the vegetative cell suspension indicates that light transmission was the same as through PBS alone. This slightly lower transmission of light through the spore suspension may also play a role in lowering the spore susceptibility to 405 nm light, due to lower penetration of 405 nm light through the suspension.

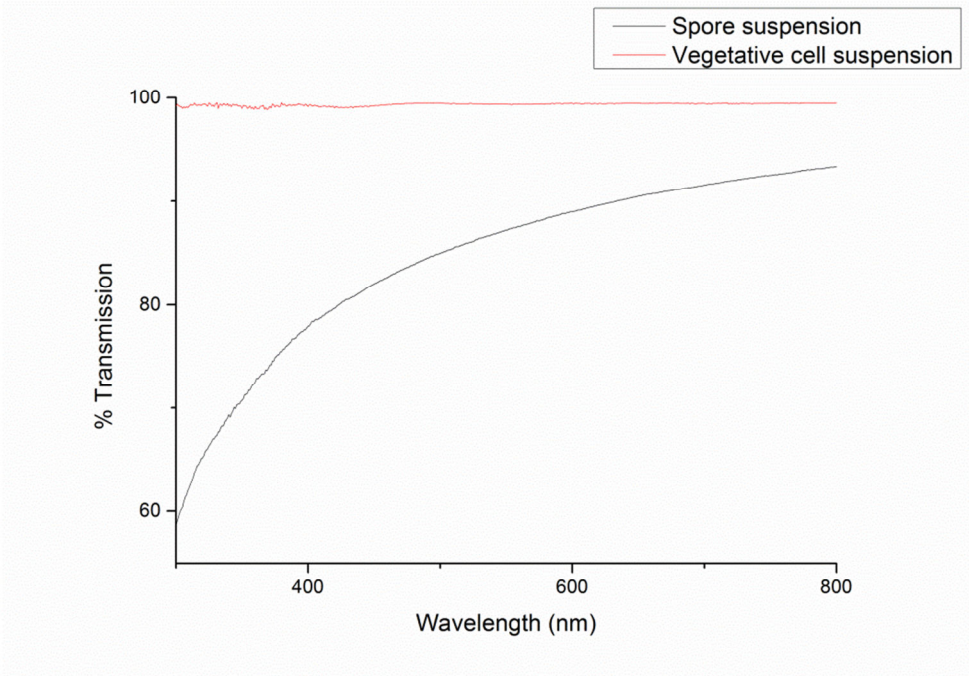

Figure 4.3 Comparison of the transmission of light through 10^3 CFUml^{-1} populations of vegetative cell and spores at wavelengths ranging from 300-800 nm.

To ensure that 405 nm light was also capable of completely inactivating a 10^5 CFUml^{-1} population of *C. difficile* spores in suspension, this population was exposed to 405 nm light at an irradiance of 225 mWcm^{-2}. The results of this experiment are shown in Figure 4.4, with a complete 5.16 $\log_{10}$ reduction observed ($P<0.001$) following a dose of 3.24 kJcm^{-2}, only a 25% increase in dose required than that for complete inactivation of the 10^3 CFUml^{-1} population. Unlike the 10^3 CFUml^{-1} previously discussed, following exposure of the 10^5

CFUml^{-1} population to a dose of 810 Jcm^{-2} no significant inactivation was observed. However significant inactivation is observed following exposure to a dose of 1.62 kJcm^{-2}, with a reduction of 0.76 log$_{10}$ CFUml^{-1} (P=0.001).

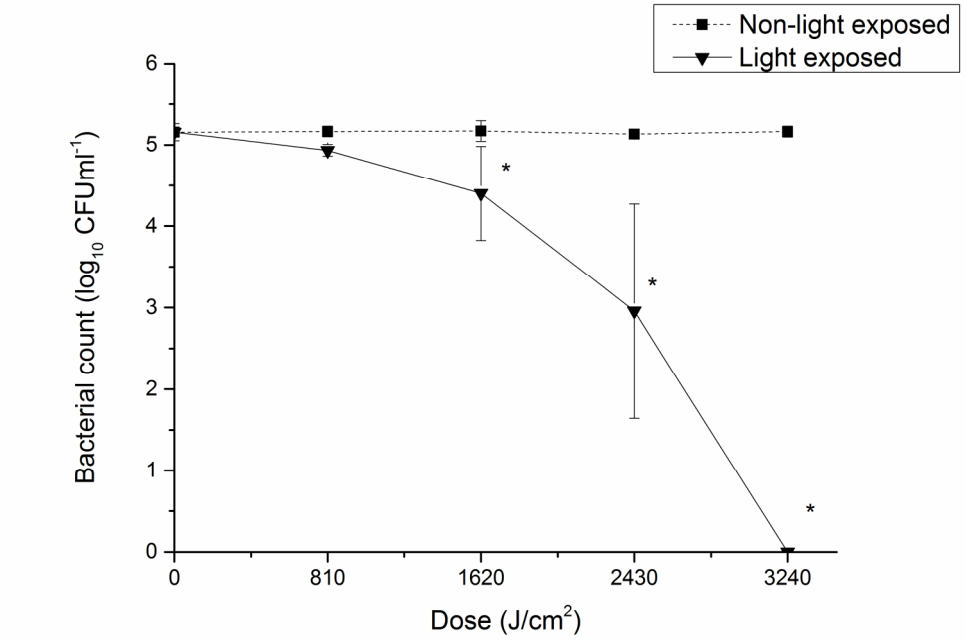

Figure 4.4 The susceptibility of *C. difficile* spores (10^5 CFUml^{-1}) suspended in PBS to 405 nm light (225 mWcm^{-2}).). Control samples were non-light exposed for the equivalent time periods (0, 1, 2, 3 and 4 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation (P≤0.05). Each data point is a mean value ± SD (n=6).

4.2. INACTIVATION OF *C. DIFFICILE* VEGETATIVE CELLS AND SPORES SEEDED ONTO CLINICALLY RELEVANT SURFACES

Experiments then proceeded to investigate the inactivation kinetics of *C. difficile* vegetative cells and spores seeded onto clinically relevant surfaces on which *C. difficile* could typically be found in the clinical environment to investigate:

- If differences were observed between suspension and surface inactivation kinetics
- If inactivation kinetics upon exposure to 405 nm light differed on a range of surfaces.

The surfaces chosen for this study were vinyl flooring, PVC, stainless steel and aluminium.

PVC is the most commonly used polymer in hospital plastics due to cost effectiveness, flexibility and optical properties. Examples of PVC within the clinical environment include bedpans, tubing and toilet seats (Block, 2004).

Stainless steel is a contact surface commonly found in healthcare settings (Block, 2004; Weaver et al., 2008) and it is often used as a reference surface in disinfection studies (Perez et al., 2005). Due to its easy to clean and non-oxidative nature, it is commonly used for hospital equipment such as trolleys, grab rails, wash basins and surgeon scrub units (Block, 2004; Weaver et al., 2008). Aluminium was used as a comparison metal as it has been recommended as an ideal choice for the manufacturing of medical instruments and equipment as it is recyclable, inexpensive and strong.

Vinyl flooring was also selected for inclusion due to the high levels of *C. difficile* contamination commonly found on the floors of bathrooms of patients with CDI following bowel movements, as a result of the high number of vegetative cells and spores shed in the faeces of symptomatic patients.

4.2.1. TREATMENT METHOD

Vegetative cell and spore suspensions were prepared as detailed in Section 3.3.1 and 3.3.2.1. 50 µl of the bacterial suspension, with a population density of 10^4 $CFUml^{-1}$, was seeded onto surfaces using a pipette. Seeded surface coupons (1 inch × 1 inch) were held in a petri dish, and, in the case of the spore tests, were dried at 50°C for 20 ± 5 minutes depending on the surface material. For vegetative cell experiments, the seeded coupons were not dried onto the surface as heating at 50°C would result in desiccation, and furthermore vegetative cells can only survive in the environment for over 15 minutes if wetted (Jump et al., 2007). Coupons were then placed 5 cm below the ENFIS Photostar Innovate UNO 24 (for high irradiance exposures), on a stand to permit airflow around the sample dish and allow any heat to dissipate from under the petri dish. This experimental set up is pictured in Figure 3.4b. The coupon was then exposed to 405 nm light, at an irradiance of 70 or 225 $mWcm^{-2}$ for vegetative cells and spores, respectively, for increasing time periods. Controls were subject to identical conditions, but exposed to normal laboratory lighting levels (0.2 $mWcm^{-2}$).

Post-exposure, vegetative cells and spores were resuspended in 5 ml PBS and agitated using an L-shaped spreader; samples were spread plated onto BA plates and incubated at 37°C for 48 hours under anaerobic conditions. Samples were then enumerated and viable bacterial counts recorded as $\log_{10}$ $CFUml^{-1}$, as discussed in Section 3.7.2. This was then used to calculate the population in $CFUml^{-1}$ that would be present in the spore suspension prior to seeding on surfaces. Experiments were repeated in triplicate.

4.2.2. RESULTS: INACTIVATION OF *C. DIFFICILE* VEGETATIVE CELLS AND SPORES SEEDED ONTO SURFACES

As shown in Figure 4.5, vegetative cells seeded onto surfaces indicates an enhanced sensitivity to 405 nm light in comparison to those in suspension upon exposure to an irradiance of 70 $mWcm^{-2}$ with a 3-3.5 log_{10} reduction observed on all surfaces following exposure to a dose of 63 Jcm^{-2}. On all surfaces near complete inactivation was observed following exposure to a dose of 189 Jcm^{-2}. On both stainless steel and aluminium surfaces the control population was found to dramatically reduce upon prolonged exposure to the aerobic environment, with a 1.17 log_{10} and 3.08 log_{10} reduction in the bacterial count on stainless steel surfaces, and a 3.25 log_{10} and 3.37 log_{10} reduction in bacterial count on aluminium surfaces, following 45 minute and 60 minutes exposure to the aerobic environment.

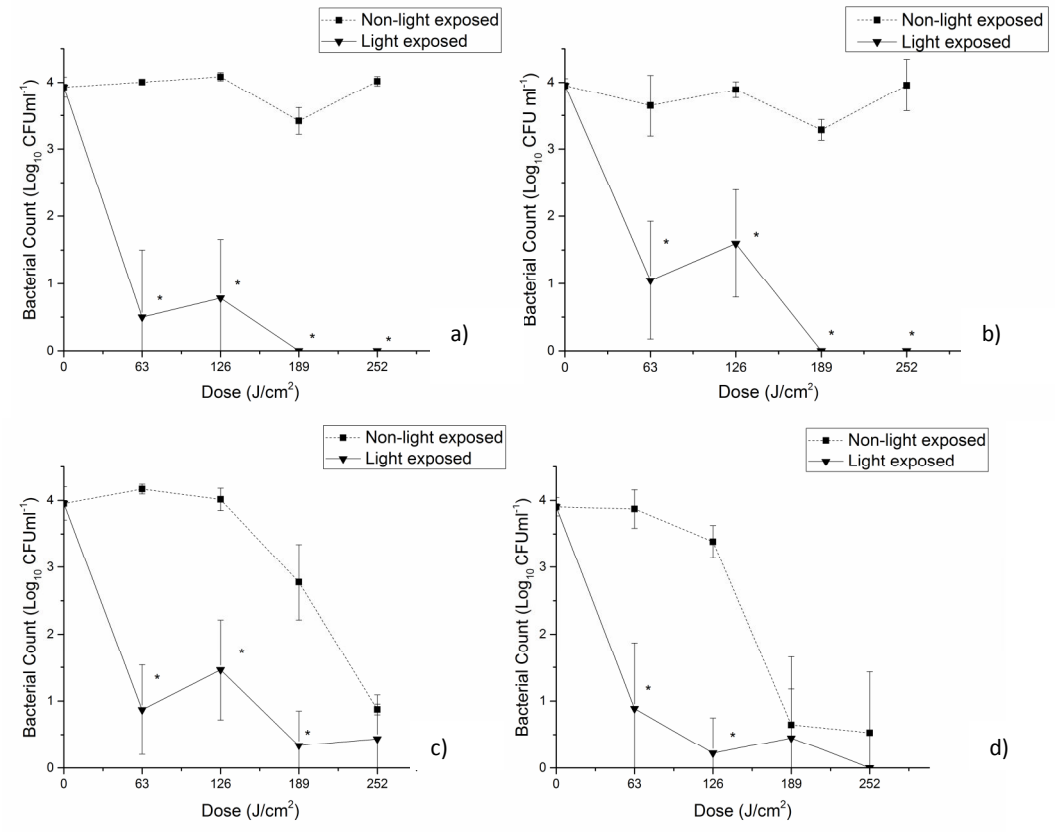

Figure 4.5 The susceptibility of *C. difficile* vegetative cells seeded onto clinically relevant surfaces to 405 nm light (70mWcm^{-2}) (a) PVC (b) Vinyl flooring (c) Stainless steel (d) Aluminium.). Control samples were non-light exposed for the equivalent time periods (0, 15, 30, 45 and 60 minutes), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

The sporicidal effects of 405 nm light at an irradiance of 225 mWcm^{-2} on *C. difficile* spores seeded onto a range of clinically relevant surfaces is shown in Figure 4.6. Spores demonstrated the greatest susceptibility to 405 nm light on PVC surfaces, as seen in Figure 4.6a, with a significant 1.38 $\log_{10}$ reduction observed following exposure to a dose of 810 Jcm^2 (P=0.006) and complete inactivation following exposure to a dose of 2.43 kJcm^{-2} (P=0.003). On vinyl flooring, following exposure to a dose of 810 Jcm^{-2} a significant 0.73 $\log_{10}$ reduction was observed (P=0.049). Following exposure of spores seeded onto vinyl surface coupons to a dose of 2.43 kJcm^{-2}, a significant 1.42 $\log_{10}$ reduction (P=0.003) in spores was observed, however this then seems to plateau, with a similar 1.46 $\log_{10}$ reduction (P=0.002) observed following exposure to a higher dose of 3.24 kJcm^{-2} (Figure 4.6b). Following exposure to a dose of 3.24 kJcm^{-2} similar inactivation kinetics were observed on stainless steel and aluminium, with a 1.66 $\log_{10}$ and 1.62 $\log_{10}$ reduction in bacterial spores observed, similar to that on vinyl flooring (P<0.001).

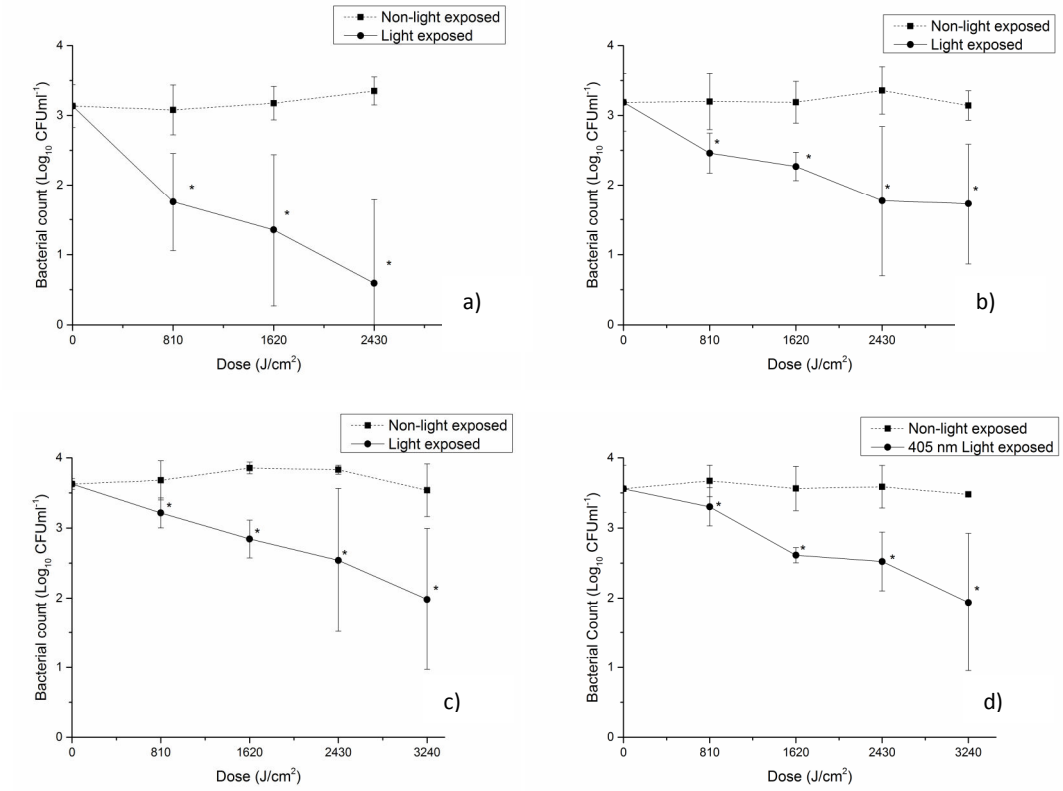

Figure 4.6 The susceptibility of *C. difficile* spores seeded onto clinically relevant surfaces to 405 nm light (225 mWcm^{-2}) (a) PVC (b) Vinyl flooring (c) Stainless steel (d) Aluminium.). Control samples were non-light exposed for the equivalent time periods (0, 1, 2, 3 and 4 hours) therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

4.3. INACTIVATION KINETICS OF *C. DIFFICILE* IN SIMULANT FAECES UPON EXPOSURE TO 405 NM LIGHT

Due to the shedding of high titres of vegetative cells and spores in the excreta following bowel movements of patients with CDI, this study investigated if the successful inactivation of *C. difficile* vegetative cells and spores upon exposure to 405 nm light could be inhibited or limited by the presence of organic matter that would occur alongside shedding.

4.3.1. TREATMENT METHOD

Bacterial suspensions were prepared as described previously in Section 3.3.2, but in the case of these experiments, the final serial dilution was inoculated into faecal suspension, which comprised of ¼ or 1/50 dilutions of the simulant human excreta in water. The recipe for the simulant human excreta is detailed in Section 3.1.3.

Exposure of spores suspended in simulant faeces was carried out using the same experimental protocol as carried out for the previous suspension experiments, as detailed in Section 4.1.2.

For light exposure of spores seeded onto surfaces, 100 µl of the spore suspension, prepared as described previously in Section 3.2.2.1, was mixed with 100 µl of faecal suspension (1/4 or 1/50 dilution in water) and then seeded onto PVC, vinyl flooring, stainless steel or aluminium. The exact same experimental protocol was carried out as before for the previous surface experiments (section 4.2.1).

4.3.2. RESULTS: INACTIVATION OF *C. DIFFICILE* IN SIMULANT FAECES UPON EXPOSURE TO 405 NM LIGHT

As patients with CDI shed vegetative cells and spores in their faeces it is highly possible that the bathroom environment of patients will have high contamination rates of *C. difficile* in the presence of faecal matter. As such, it is of importance to know if the presence of this organic matter will limit the inactivation of *C. difficile* spores upon exposure to 405 nm light in the clinical environment.

The results in Figure 4.7 show, that when *C. difficile* spores were suspended in the 1:4 simulant human excreta:water suspension and exposed to 405 nm light up to a dose of 6.48 $kJcm^{-2}$, very little inactivation was observed. The reduced levels of 405 nm transmission through this suspension is likely the explanation for the dramatically decreased susceptibility of spores to 405 nm light, with only 0.05% transmission of 405 nm light measured through the faecal suspension (Figure 4.8), however the simulant faecal matter

was very viscous and not representative of that of a patient with diarrhoea at this dilution. To address this, spores were resuspended in a 1:50 dilution of the simulant faeces. Transmission of 405 nm light in this case was still exceptionally low at 1%, but was an 80% increase to that of the 1:4 faecal preparation. When suspended in this 1:50 dilution, enhanced inactivation was observed upon exposure to 405 nm light at a dose of 225 $mWcm^{-2}$. Following exposure to a dose of 1.62 $kJcm^{-2}$, a complete 3.11 log_{10} inactivation was observed in the 1:50 simulant faecal suspension: a 1.68 log_{10} increased reduction in comparison to that achieved when spores suspended in PBS were exposed to the same dose of 405 nm light (P=1.03).

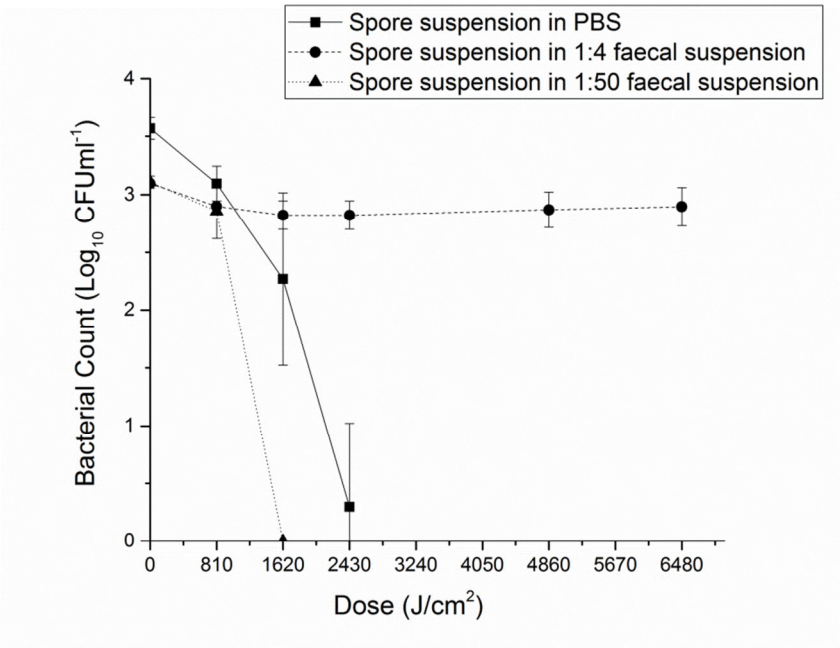

Figure 4.7 The effect of 405 nm light on *C. difficile* spores suspended in PBS, ¼ dilution of simulant faecal secretion and a 1/50 dilution of simulant faecal secretion (225mWcm^{-2}).). Control samples were non-light exposed for the equivalent time periods (0, 1, 2, 3, 6 and 8 hours) therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

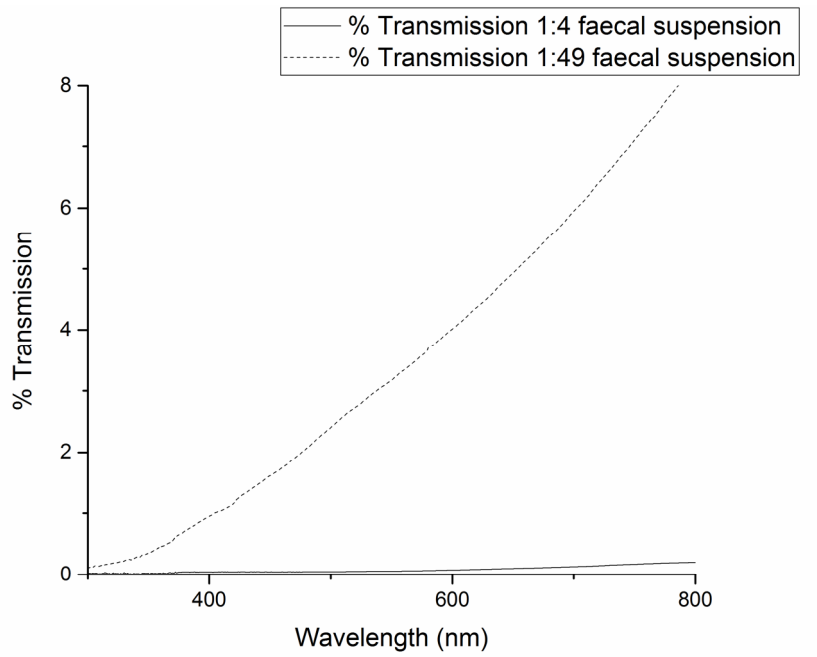

Figure 4.8 Comparison of the transmission of light through the 1:4 and 1:50 diluted simulant faecal suspensions.

Unlike the results in suspension (Figure 4.7), spores on surfaces did not exhibit enhanced susceptibility to 405 nm light when seeded whilst in the 1:50 simulant faeces, as shown in Figure 4.9a-d. A maximal 0.28 $\log_{10}$ reduction was observed on PVC surface coupons following exposure to a dose of 2.43 $kJcm^{-2}$ (Figure 4.9a): a 2.27 $\log_{10}$ decreased spore inactivation in comparison to the almost complete reduction observed when spores suspended in PBS were seeded onto surfaces (Figure 4.6a). In the presence of simulant faeces on all other surface coupons, a 0.94-1.06 $\log_{10}$ reduction in spore inactivation was achieved following exposure to a dose of 3.24 $kJcm^{-2}$ (Figure 4.9b-d), in comparison to that achieved previously upon exposure of spores suspended in PBS seeded onto surfaces, in Figure 4.6b-d.

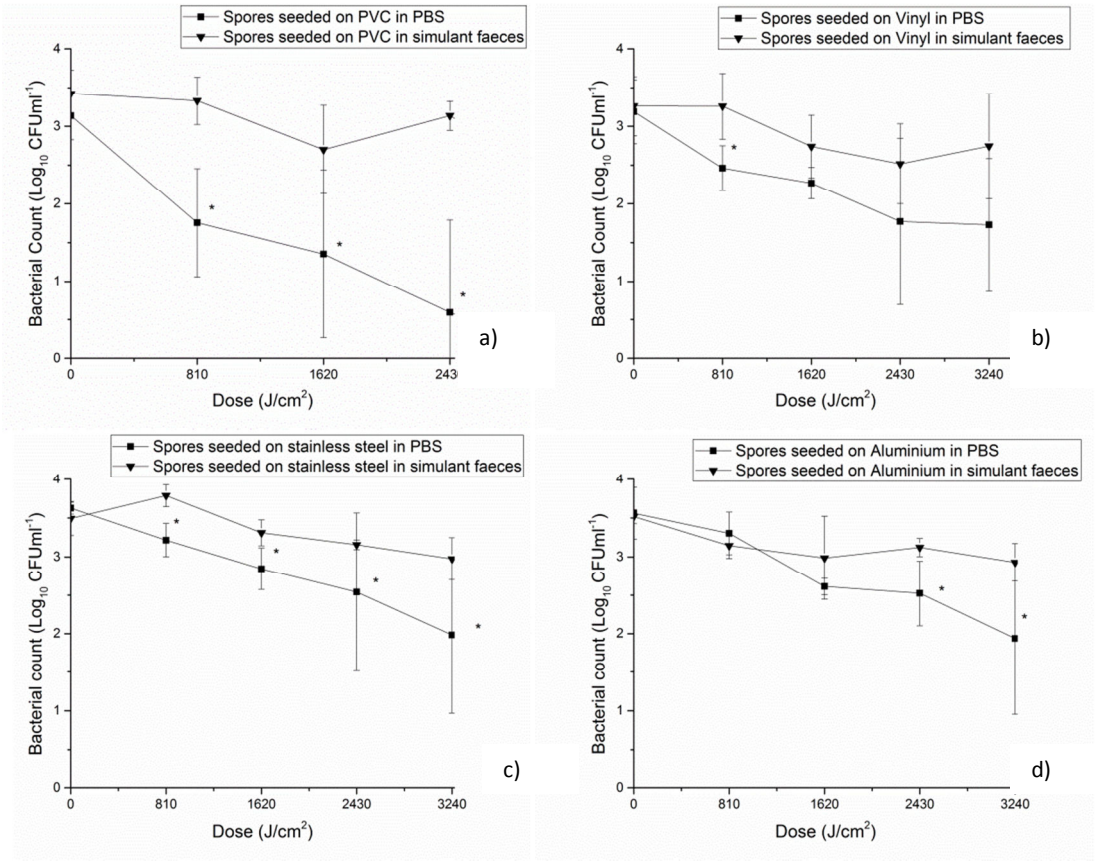

Figure 4.9 The effect of 405 nm light (225mWcm^{-2}) on *C. difficile* spores seeded onto a) PVC b) Vinyl flooring c) Stainless steel and d) Aluminium. Spores were suspended in PBS or a 1/50 dilution of simulant faecal secretion before seeding onto the surface coupons. The inactivation kinetics of spores suspended in PBS upon exposure to 405 nm light are those previously reported in Figure 4.6. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

To offer an explanation for the enhanced inactivation of spores by 405 nm light whilst in suspension with the simulant faecal matter, fluorescence emission spectroscopy was performed. This aimed to determine and identify the presence of porphyrins within any of the ingredients used for the simulant faeces by using fluorescence spectrophotometry, whereby specific fluorescence signals are associated with endogenous porphyrin molecules. The results in Figure 4.10 and Figure 4.11 highlight the emission spectra of individual suspension preparations of each of the components used to produce the simulant faecal matter. Excitation of the 1/10 dilution of the miso paste suspension and the 1/10 dilution of the yeast suspension at 405 nm displayed peak emissions between 453-489 nm and 504-535 nm, respectively. Excitation of the psyllium suspension, as seen in Figure 4.11, displayed a peak emission at 467 and 670 nm.

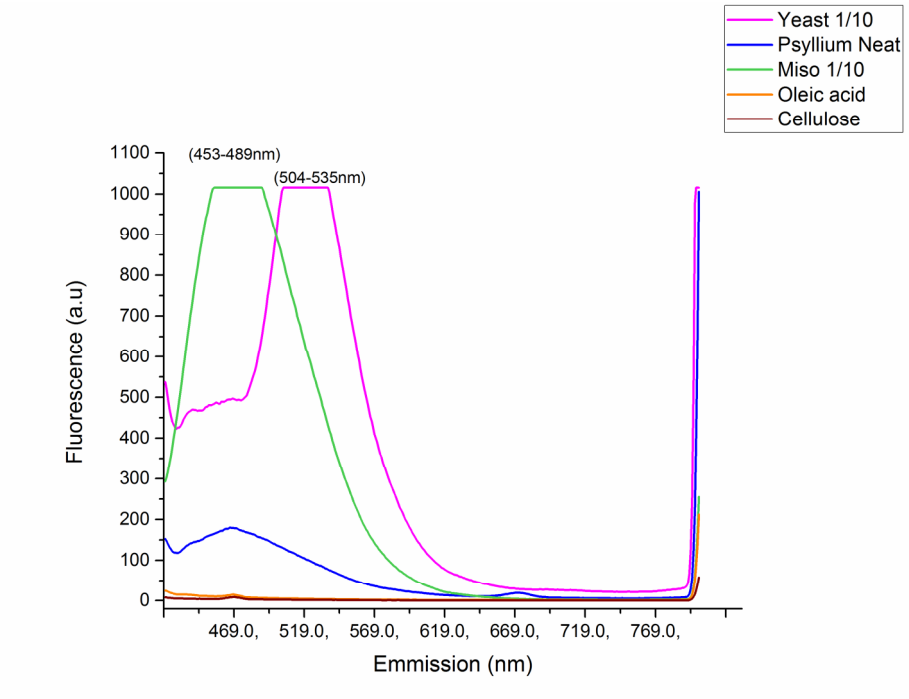

Figure 4.10 Fluorescence spectra of the separate components of the simulant faecal matter suspension. Fluorescence emission spectra were detected from suspension preparations using an excitation wavelength of 405 nm. Emission spectra are shown between 500-800 nm.

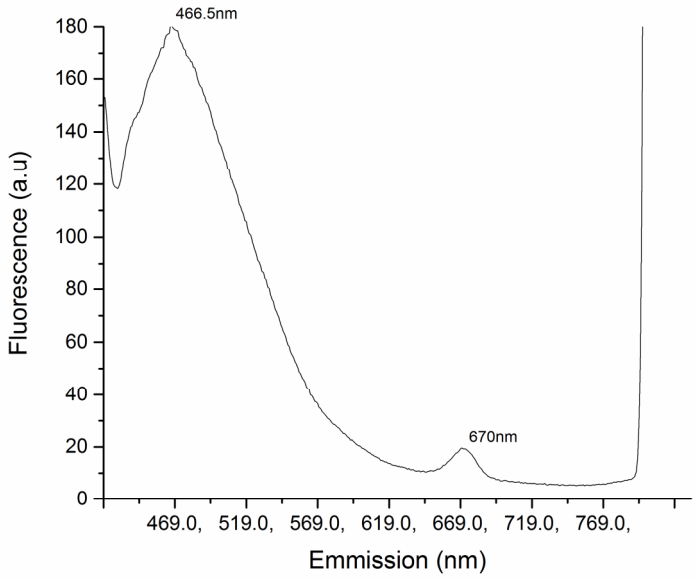

Figure 4.11 Fluorescence emission spectra of psyllium suspension. Fluorescence emission spectra were detected from spore suspension preparations using an excitation wavelength of 405 nm. Emission spectra are shown between 500-800 nm.

4.4. COMPARISON OF THE INACTIVATION KINETICS TO THAT OF OTHER STRAINS AND SPORE FORMING BACTERIA

Tests were conducted to compare the inactivation kinetics of the type-culture strain NCTC 11204, with those of one other type-culture strain (NCTC 11209) and a clinical isolate strain (ribotype 027), in order to establish strain to strain variation in susceptibility, and also use of the strain ribotype 027 would be more representative of *C. difficile* found within the clinical environment. This section also compares the inactivation kinetics to that of the aerobic, spore forming bacterium, *Bacillus cereus*.

4.4.1. BACTERIAL PREPARATION

The bacteria used for these experiments were *C. difficile* (NCTC 11204, NCTC 11209 and Ribotype 027) and *B. cereus* vegetative cells and spores. *C. difficile* strains NCTC 11209 and Ribotype 027, used as a comparative strains, were grown under identical conditions to NCTC 11204, as discussed in Section 3.3.2.1. *B. cereus* vegetative cells and spores were prepared as described in Section 3.3.3 and 3.3.4.

4.4.2. TREATMENT METHOD

Spore suspensions were exposed to the ENFIS Photostar Innovate UNO 24 (high irradiance 405 nm light) at an irradiance of 225 $mWcm^{-2}$ as detailed in Section 3.5.1.

4.4.3. RESULTS: COMPARISON OF THE INACTIVATION KINETICS TO THAT OF OTHER STRAINS AND SPORE FORMING BACTERIA

Several studies have reported varying susceptibilities of *C. difficile* strains to various different decontamination methods indicating that different *C. difficile* strains may exhibit differing characteristics in response to different stresses. To investigate if there is any difference in the inactivation kinetics between different *C. difficile* strains following exposure to high irradiance 405 nm light, comparative studies were carried out using type-collection strain NCTC 11209, as shown in Figure 4.12a, and a clinical isolate of ribotype 027, as shown in Figure 4.12b. Figure 4.13 shows the comparative inactivation kinetics of all 3 strains. The results demonstrate that the type-collection strain NCTC 11204 is the most susceptible to 405 nm light, with NCTC 11209 and Ribotype 027 exhibiting similar inactivation kinetics. *C. difficile* NCTC 11209 spores demonstrated an enhanced resilience to inactivation by 405 nm light, requiring a dose of 3.24 $kJcm^{-2}$ to achieve a complete 3.1 $\log_{10}$ reduction ($P<0.001$). Following exposure *of C. difficile* NCTC 11209 to 2.43 $kJcm^{-2}$, at which near complete reduction was observed with the NCTC 11204 strain, only a 1.23 $\log_{10}$

reduction was observed (P<0.001). Significant reduction was initially observed following a dose of 1.62 kJcm^{-2} for this strain, in comparison to a significant reduction achieved following exposure to a dose of 810 Jcm^{-2} for the type-collection NCTC 11204 strain.

The inactivation kinetics of the clinically obtained *C. difficile* strain, as seen in Figure 4.12b, were very similar to that of NCTC 11209, with a significant 0.41 $\log_{10}$ inactivation observed following exposure to a dose of 1.62 kJcm^{-2} and exposure to a dose of 3.24 kJcm^{-2} obtaining complete inactivation (P<0.001).

Upon comparison of all 3 strains, as seen in Figure 4.13, all 3 strains were significantly different to one another following exposure to a dose of 1.62 kJcm^{-2} (P<0.001), whilst following a dose of 2.43 kJcm^{-2} both NCTC 11209 and Ribotype 027 were not statistically different, with only NCTC 11204 exhibiting almost complete inactivation.

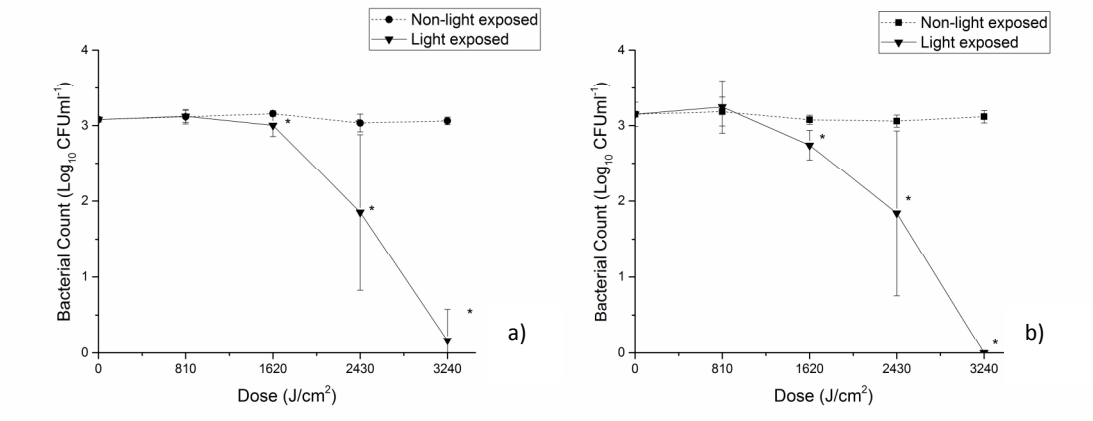

Figure 4.12 The effect of increasing doses of 405nm light (225mWcm^{-2}) on *C. difficile* spores suspended in PBS: (a) NCTC 11209 *C. difficile* spores and (b) Clinical ribotype 027 spores.). Control samples were non-light exposed equivalent time periods (0, 1, 2, 3 and 4 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

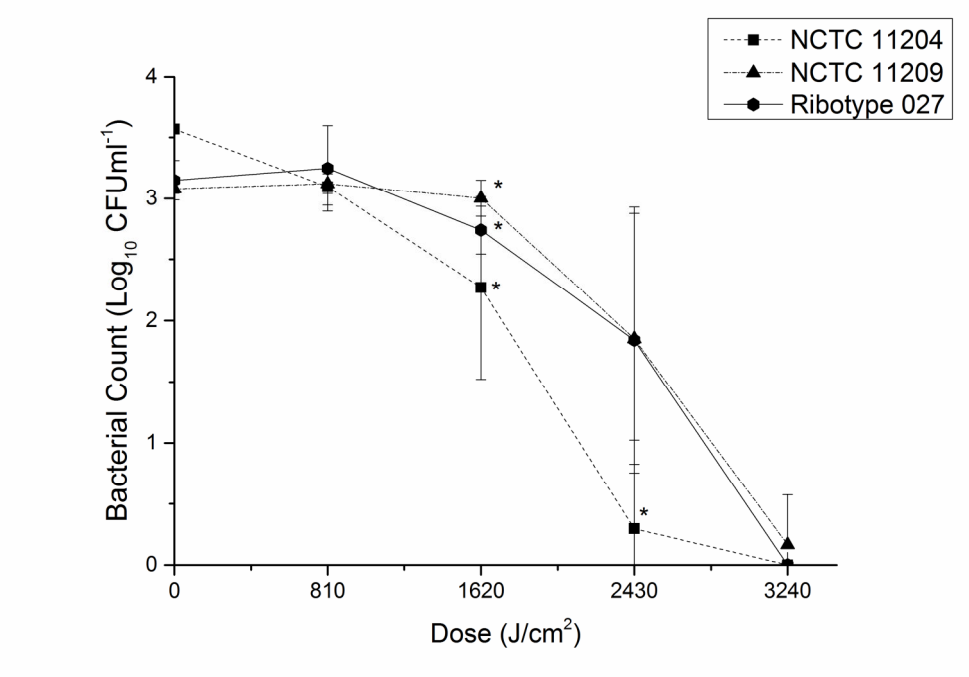

Figure 4.13 Comparison of the inactivation kinetics of the spores of all 3 *C. difficile* strains upon exposure to 405 nm light (225mWcm^{-2}). * represent significant bacterial inactivation compared to one another (P≤0.05). Each data point is a mean value ± SD (n=6).

This study then proceeded to compare the inactivation kinetics of the *C. difficile* vegetative cells and spores upon exposure to 405 nm light previously obtained in Section 4.1.4 to that of *B. cereus* vegetative cells and spores.

The enhanced resilience of *C. difficile* spores in comparison to its vegetative counterparts, previously demonstrated in Figure 4.1 and Figure 4.2 in Section 4.1.4, is similar to that of other spore forming bacteria, such as the spores of *Bacillus* species (Melly et al., 2002; Silva et al., 2013). *Bacillus* spores exhibit an increased resistance to various chemicals that routinely inactivate vegetative cells including hydrogen peroxide, alcohols, phenols, chlorhexidine and benzalkonium compounds (Demidova & Hamblin, 2005; Melly et al., 2002; Setlow & Setlow, 1993). Furthermore, the spores of both *Clostridium* and *Bacillus* species have been reported to be 5-50 times more resistant than their corresponding vegetative cells to 254 nm UV radiation (Nicholson et al., 2000; Setlow & Setlow, 1988; Setlow, 2001) .

For a direct comparison of the inactivation kinetics between both spore-forming bacterial species, the inactivation kinetics of *B. cereus* vegetative cells and *B. cereus* spores, shown in Figure 4.14 and Figure 4.15, were investigated. The results in Figure 4.14 and Figure 4.15 demonstrate that, similar to that observed for the *C. difficile* vegetative cells and spore

populations, *B. cereus* vegetative cells were much more susceptible to inactivation upon exposure to 405 nm light than spores, with *B. cereus* spores requiring 20× more dose than their vegetative counterparts to achieve a ~3.5 $\log_{10}$ reduction. The results in Figure 4.14 demonstrate a 3.63 $\log_{10}$ reduction in vegetative cells following exposure to 405 nm light at a dose of 126 Jcm^{-2} ($P<0.001$). In comparison to this, a 3 $\log_{10}$ reduction was achieved when *B. cereus* spores were exposed to 405 nm light at a dose of 2.43 $kJcm^{-2}$ ($P<0.001$).

These results demonstrate that *B. cereus* vegetative cell inactivation is initiated at a much lower dose than that of *C. difficile*, with ~3.5 $\log_{10}$ reduction achieved at a dose of 126 Jcm^{-2} ($P<0.001$) and 252 Jcm^{-2} ($P<0.001$) for *B. cereus* and *C. difficile,* respectively. Significant inactivation of *B. cereus* vegetative cells is observed following exposure to 84 Jcm^{-2} ($P<0.001$) (Figure 4.14).

B. cereus spores demonstrated a significant reduction in bacterial count of 0.88 $\log_{10}$ $CFUml^{-1}$ at a dose of 810 Jcm^{-2} ($P<0.001$). Spores of both *B. cereus* and *C. difficile* have a similar inactivation rate requiring a dose of 2.43 $kJcm^{-2}$ for complete inactivation; indicating that following sporulation, both *C. difficile* and *B. cereus* have similar protection mechanisms. The spores of both *C. difficile* and *B. cereus* require at least a 10× higher dose for inactivation compared to that of their vegetative counterparts.

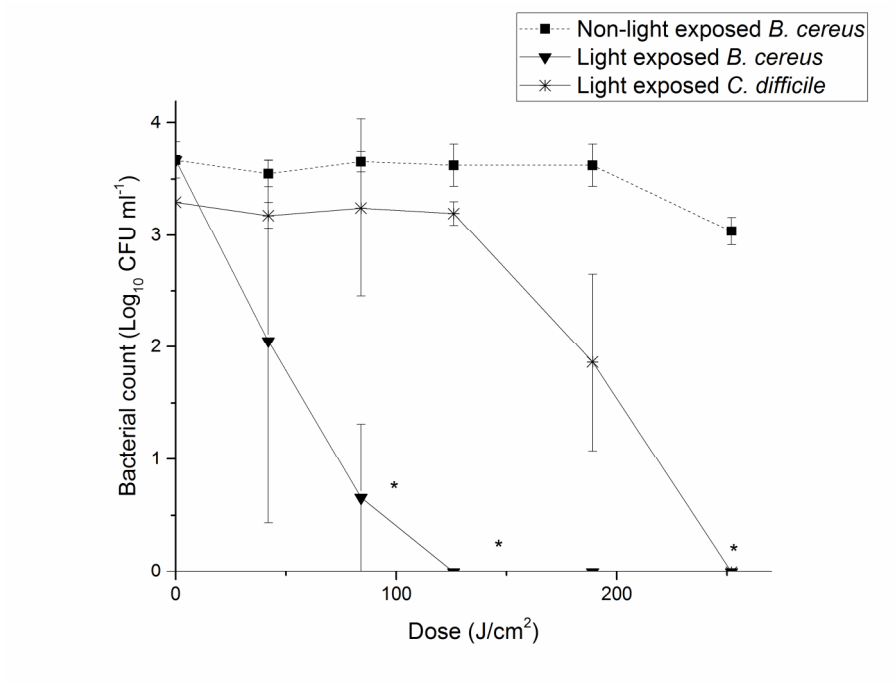

Figure 4.14 The susceptibility of *Bacillus cereus* vegetative cells suspended in PBS to 405 nm light (70 mWcm^{-2}). Control samples were non-light exposed for equivalent time periods (0, 10, 20, 30, 45 and 60 minutes), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. This graph displays the comparative inactivation kinetics of *C. difficile* vegetative cells suspended in PBS. * represent significant bacterial inactivation compared to the non-light exposed controls (P≤0.05). Each data point is a mean value ± SD (n=6).

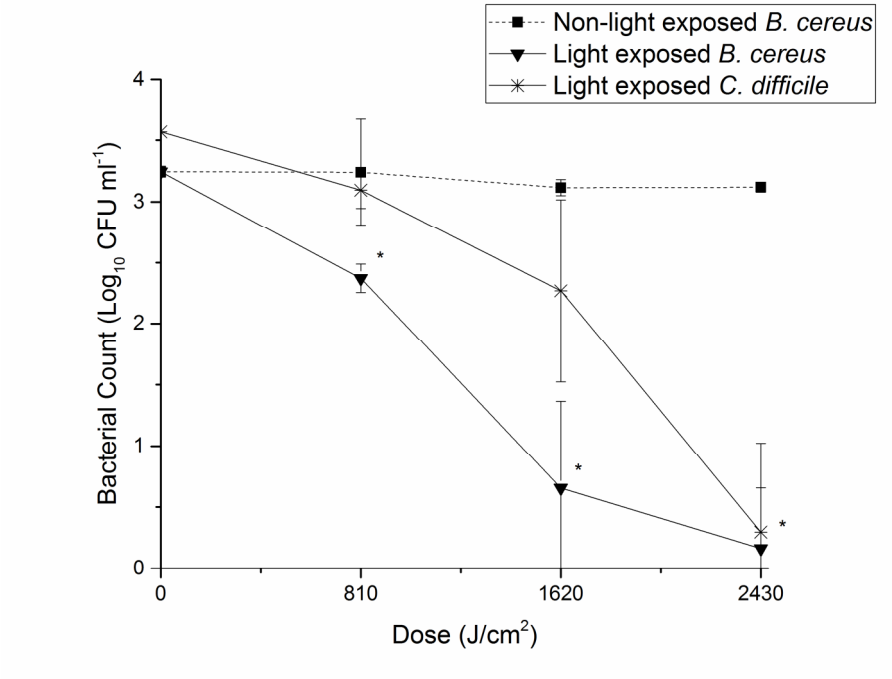

Figure 4.15 The susceptibility of *Bacillus cereus* spores suspended in PBS to 405 nm light (225 mWcm^{-2}). Control samples were non-light exposed for equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. This graph displays the comparative inactivation kinetics of *C. difficile* spores suspended in PBS. * represent significant bacterial inactivation (P≤0.05). Each data point is a mean value ± SD (n=6).

4.5. DISCUSSION

The results of this study have demonstrated that 405 nm light is capable of inactivating both the vegetative cells and spores of *C. difficile*, with the potential to achieve up to a 5 $\log_{10}$ reduction in *C. difficile* spores following a delivered dose of 3.24 $kJcm^{-2}$.

The results in Figure 4.1 and Figure 4.2 demonstrated that *C. difficile* spores are much more resilient to inactivation by 405 nm light than their vegetative counterparts, requiring ~10× the dose to achieve a 99.9% reduction in vegetative cell population, with a dose of 252 Jcm^{-2}, whilst a similar level of spore inactivation required an applied dose of 2.43 $kJcm^{-2}$. These findings support a previous study that investigated the effect of high intensity 405 nm visible light on both the vegetative cells and spores of the endospore forming bacteria *B. subtilis, B. megaterium* and *C. difficile* (Maclean et al., 2013). This paper indicated that *C. difficile* was the most susceptible of the three organisms, with a 3.7 $\log_{10}$ $CFUmL^{1}$ reduction of vegetative cells following exposure to a dose of 48 Jcm^{-2}. In comparison to the vegetative cells, *C. difficile* spores required a dose of 1.15 $kJcm^{-2}$ to achieve a 2.7 $\log_{10}$ $CFUml^{-1}$ reduction.

The significant difference in dose required for inactivation of spores (2.43 $kJcm^{-2}$) in comparison to that required for the inactivation of vegetative cells (252 Jcm^{-2}) was expected as spores are resilient, difficult to inactivate structures, developed under stressful environmental conditions, and formed to enable bacteria to survive until suitable conditions (such as transfer to the human body) return. The spore resistance is a result of various factors conferring protection including the thick proteinaceous spore coat, the low water content of the spore and the presence of small acid-soluble proteins (Nicholson et al., 2000; Rogers et al., 2005; Setlow & Setlow, 1993).

It is also important to note that, as shown in Figure 4.3, the transmission of light across the measured spectrum was much lower through the spore suspension than through the vegetative cells suspension at 405 nm. This decrease in light transmission may also play a role in the reduced inactivation, with a potential explanation for this lowered transmission being the reflection of light by the spore coat, preventing the transmission and absorbance of some of the light through the spore suspension.

In support of the results observed in Figure 4.1, a previous study investigating the capability of violet-blue light for the inactivation of *C. difficile* vegetative cells demonstrated that, at a wavelength of 410 nm, a 4 $\log_{10}$ reduction in *C. difficile* vegetative cells was achieved in the

absence of exogenous photosensitisers following an administered dose of 0.54 Jcm^{-2}, (De Sordi et al., 2015); although the dose delivered in the present study was much lower, this may due to the use of a different *C. difficile* strain – strain R20291 – and the different peak wavelength of the light source. Furthermore, this study spotted 20 µl of the vegetative suspensions onto BHI surfaces prior to exposure, this seeding onto nutritious surfaces may have further increased the susceptibility of the vegetative cells as nutrients may act as photosensitisers. The study by De Sordi et al. further supports the results shown in Figure 4.1 and Figure 4.2, with the results demonstrating enhanced resilience of *C. difficile* spores in comparison to vegetative cells (De Sordi et al., 2015).

This increased resilience of *C. difficile* spores compared to that of vegetative bacteria has also been demonstrated with other decontamination methods, such as UV-C. A study by Rutala et al. (2010) achieved a >99.9% reduction in vegetative bacterial counts on surfaces following a 15 minute exposure to UV-C radiation in comparison to a 99.8% reduction in *C. difficile* spores following a 50 minute exposure to UV-C. This increased resilience of spores in comparison to vegetative cells is further supported by a study demonstrating that, following induction of spore germination, *C. difficile* spores become much more susceptible to inactivation by UV-C radiation and heat, due to the restoration of vegetative cell properties and irreversible loss of spore properties (Nerandzic & Donskey, 2010).

Photodynamic therapy (PDI) uses a similar inactivation mechanism to that of violet-blue light, however it requires the use of exogenous photosensitizers, and has been demonstrated to be successful for the inactivation of several spore forming species. Photosensitizers may have different effects and targets depending on the microbial species. Following excitation of the exogenous photosensitizer by a specific wavelength of light, the mechanism of inactivation is similar to that of violet blue-light, occurring via the type I or type II pathway resulting in the production of ROS, such as singlet oxygen, and ultimately cell death.

There are very few studies investigating the effect of blue light on *C. difficile* to date. Rutala et al. (2016) presented their findings investigating the inactivation of a range of bacteria upon exposure to 405 nm light on test surfaces with a maximal 65% reduction in *C. difficile* spores observed following exposure to disinfecting blue light at an irradiance of ~29 – 38 Jcm^{-2}.

De sordi et al. (2015) demonstrated the efficacy of PDI for the inactivation of 6 clinically relevant *C. difficile* strains in biofilms, planktonic cells and germinating spores, using red light or infrared laser light, at wavelengths of 665, 652 or 784 nm, in the presence of 13 different photosensitizers whilst not damaging human HT-29 cells at effective antimicrobial dose. This study then progressed to demonstrate the efficacy of blue light, which at a wavelength of 410 nm, was able to kill *C. difficile* in the absence of PS; this paper, however, does not detail if these were vegetative cells, spores or biofilm - it can be assumed that these were vegetative cells due to the extremely low doses required to achieve inactivation with a dose of 0.54 Jcm^{-2} reported to achieve a 4 log_{10} reduction (De Sordi et al., 2015).

Photodynamic inactivation has previously been shown to be successful for the inactivation of *C. difficile* spores, with Wilson et al. (2006) demonstrating a 100% inactivation rate following exposure of *C. difficile* suspension (consisting predominantly of vegetative cells) to white light (400-700 nm) in combination with a cellulose acetate coating containing Toluidine blue O over a 4 hour period. Additionally, UV-A in combination with tetracycline and chitosan has been shown to be an effective method for killing with 100,000-fold decrease in viable cells following a combination of the three treatments; this study however does not state if vegetative cells or spores were used for this study (Choi et al., 2015).

As discussed in Section 2.5.3.1, the mechanism of inactivation upon exposure of pathogens to 405 nm light involves oxidative damage due to the production of reactive oxygen species, resulting in non-specific destruction of the cellular target, and ultimately cell death. It has been speculated that less oxygen-tolerant bacterial species may be particularly susceptible to the effects of ROS, as they are likely to possess fewer key oxidative regulators than most aerobes (Jean et al., 2004; Murdoch et al., 2012). However, reactive oxygen species have been shown to play a crucial role in *C. difficile* disease as a result of the toxins, TcdA and mostly TcdB, inducing a cytotoxic effect in the host by ROS (Frädrich et al., 2016; Qiu et al., 1999). As such, it is likely that *C. difficile* possesses ROS protective mechanisms. Glutamate dehydrogenase (GDH), an important metabolic enzyme, has been demonstrated to be important for the normal growth of *C. difficile*, with the presence of active extracellular GDH protecting *C. difficile* against H_20_2 – allowing *C. difficile* to protect itself from ROS produced during the host immune response (Girinathan et al., 2014). This protective mechanism may offer a further explanation as to why *C. difficile* spores require

such high doses of 405 nm light for inactivation despite their anaerobic nature, in addition to the protection conferred by the spore coat.

The nature of the surface also can also have an impact on the effects of photodynamic inactivation, as such this study then proceeded to compare the efficacy of 405 nm light on a range of clinically relevant surfaces in comparison to that achieved when vegetative cells and spores were exposed in suspension, to obtain comparative inactivation kinetics to determine if any changes in spore susceptibility were observed between those in suspension and seeded onto surfaces (Brovko et al., 2005). The results of this study demonstrated that vegetative cells were much more susceptible to 405 nm light when seeded onto clinically relevant surfaces in comparison to those in suspension, with a 3-3.5 log_{10} reduction observed on all surfaces investigated following exposure to a dose of 63 Jcm^{-2} in comparison to a 1.41 log_{10} reduction observed in suspension following exposure to 3× the dose, with the highest susceptibility observed on PVC surfaces. This enhanced susceptibility of vegetative cells to 405 nm light when seeded onto surfaces in comparison to those in suspension may be in part a result of desiccation, with vegetative cells shown to survive 15 minutes on dry surfaces, whilst moistened vegetative cells can survive up to 6 hours – as such the suspension may confer protection of vegetative cells against desiccation in the initial suspension experiments (Vohra & Poxton, 2011).

Conversely, *C. difficile* spores seeded onto clinically relevant surfaces appeared to be more resilient upon exposure to 405 nm light in comparison to those in suspension, with a dose of 2.43 $kJcm^{-2}$ achieving near complete inactivation only on PVC surfaces (P=0.003). On vinyl, stainless steel and aluminium surface coupons an approximately 50% lowered spore inactivation was observed following exposure to 25% increased dose in comparison to that observed with spores in suspension.

Luksiene et al. (2009) compared the inactivation kinetics of *B. cereus* vegetative cells and spores in suspension and on food related surfaces upon illumination with visible light, of wavelength 400 nm, in the presence of 7.5 $mmol^{-1}$ 5-aminolevulinic acid (ALA), to encourage the production of endogenous photosensitizers. The findings of this study reported that *B. cereus* vegetative cells and spores were more susceptible to inactivation upon illumination in suspension. Following exposure of vegetative cells in suspension to a dose of 18 Jcm^{-2}, a 6.3 log_{10} reduction was achieved, whilst on food related surfaces a 4

log_{10} reduction was observed. Similarly, upon exposure of spores to the same dose of visible light in suspension a 3.7 log_{10} reduction was observed, whilst on plastic packaging a 2.7 log_{10} reduction was observed (Luksiene et al., 2009).

Similarly, a study in which *B. cereus* spores were exposed to UV light at a wavelength of 254 nm both in suspension and on a range of surfaces (including aluminium foil and PVC), spores in suspension were more susceptible the those on surfaces (Blatchley III et al., 2005). Results of this study demonstrated that in suspension an approximate 4 log_{10} reduction in *B. cereus* spores was achieved following exposure to a dose of 30 $mJcm^{-2}$. Spores air-dried onto surfaces demonstrated a much higher resistance to UV_{254}, with much more varying results – presumably as a result of the differing surface characteristics. On aluminium foil a ~3 log_{10} reduction was observed with a dose of 80 $mJcm^{-2}$, whilst spores on plastic surfaces demonstrated a lower susceptibility to UV_{254}, with a ~3 log_{10} reduction observed following exposure to 100 $mJcm^{-2}$; however the lower reflectivity of PVC surfaces may contribute to these results (Blatchley III et al., 2005).

The ability to inactivate both *C. difficile* vegetative cells and spores in the presence of organic faecal matter is of importance as patients with CDI shed both anaerobic vegetative cells and spores in the faeces, with vegetative cells then undergoing sporulation in the aerobic environment to form spores (Rineh & Kelso, 2014). If the vegetative cells, demonstrating a higher susceptibility to 405 nm light than spores, could be inactivated in the faecal matter before undergoing sporulation this could allow for a much greater eradication of the bacteria within the clinical environment within smaller time frames. This could also lead to lower reinfection rates of patients, or infection of cohabiting patients, as a result of unintentional ingestion of *C. difficile* through the faecal-oral route.

In order to investigate the susceptibility of 405 nm light on *C. difficile* spores in the presence of faecal matter, simulant faecal excreta was produced and spores were exposed to 405 nm light both in suspension with the simulant faeces and seeded onto clinically relevant surfaces in the presence of simulant faces. Due to time constraints, only spores were used. As vegetative cells are much more susceptible to 405 nm light than spores it can be assumed that if the spores are successfully inactivated following exposure to a particular dose, then the vegetative cells would be inactivated at a dose lower than this.

Upon suspension of *C. difficile* spores in simulant faeces, diluted in a 1/50 dilution with water, enhanced inactivation of *C. difficile* spores was observed with a 33% reduced dose required for complete inactivation of spores suspended in simulant faeces in comparison to spores suspended in PBS. Furthermore, at a wavelength of 405 nm, 78.5% transmission levels were recorded through PBS suspensions, whereas through artificial faeces only 1% transmission levels were recorded, as shown in Figure 4.3 and Figure 4.8. It is of interest that despite the approximately 80× lower transmission through the faecal suspension compared to that of the PBS suspensions, enhanced sporicidal effects were still observed in the presence of simulant faecal matter. Although the artificial faeces is only a simulated version of faecal matter, and is not an exact representation of the consistency or opacity of faeces passed from patients with CDI, it is a good indicator that the naturally occurring molecules present in faecal matter may be sensitive to 405 nm light. These results indicate the potential for *C. difficile* susceptibility to 405 nm light to be enhanced when suspended in simulant faeces.

On clinically relevant surfaces, spore susceptibility to 405 nm light was not enhanced in the presence of simulant faecal matter. On PVC surfaces following a delivered dose of 2.43 $kJcm^{-2}$ a 90% lowered rate of spore inactivation was observed in the presence of simulant faecal matter in comparison to that of spores in PBS seeded onto surfaces. On all other clinically relevant surfaces a 64% reduction in bacterial spore inactivation was observed in the presence of simulant faeces. The reason for this enhanced resilience of spores on surfaces in the presence of simulant faeces may be due to the presence of organic load minimising the penetration of 405 nm light on the surfaces; as shown in Figure 4.8 only a 1% transmission of 405 nm light is observed in simulant faeces. A possible explanation for the enhanced inactivation of spores in suspension with faecal matter and not on clinically relevant surfaces is that, whilst in suspension 2 ml volumes of the spore–simulant faecal suspension were added to a 12 well-plate, whereas on surfaces 100 µl of the faecal suspension was seeded onto the surfaces and thus this reduction in the volume of the faecal components present may have reduced the presence of the photosensitive molecules and porphyrins assumed to have been present. The lowered presence of photosensitive molecules alongside the lowered transmission of light through the organic load may offer an explanation for the lowered inactivation of spores seeded onto clinically relevant surfaces in the presence of the simulant faecal matter. Equal volumes of simulant faeces and spore suspensions were added onto the surface coupons, whereas on the

surfaces in the vicinity of patients with CDI, it is likely that smaller volumes of faecal matter would be present which may also have a more watery consistency and thus allow for higher transmission of 405 nm light. It is also important to note that in Section 4.2.2 when spores in PBS suspension were seeded onto clinically relevant surfaces they became much more resilient to 405 nm light than those in suspension exposed to 405 nm light, in Section 4.1.4.

Results from fluorescent spectrophotometry were used to confirm the general presence of porphyrins within the individual components comprising the organic simulant faecal matter. In Figure 4.10 the relatively high broad peaks seen early in the spectrum following the excitation of the miso paste and inactivated yeast suspensions at 405 nm may be the result of the presence of a photosensitive molecule that can absorb light in this region demonstrated by the fluorescence peaks around 454-489 nm and 504-535 nm, respectively. It is unlikely that this is indicative of the presence of porphyrin molecules as porphyrin emission is typically further up the spectrum. The fluorescent peak at 670 nm following excitation of the psyllium suspension at 405 nm, Figure 4.11, is indicative of the presence of Uroporphyrin III (Dai et al., 2013). However, further analysis would be necessary in order to provide full identification.

Different interactions have been recorded upon exposure of different *C. difficile* strains to a range of disinfection techniques. For example, when using sporicidal wipes it was observed that different wipes interacted differently with spores from different PCR ribotypes, with some spores becoming loosely associated in wipes leading to the release of spores on different surfaces upon multiple uses, thus potentially leading to the unsafe disposal of wipes (Siani et al., 2011). In a study by Dawson et al. (2011) the susceptibility of 3 PCR ribotypes of *C. difficile* to 4 different disinfectants was investigated with the results indicating that there were differences in the susceptibility of each of the PCR ribotypes to the different disinfectants. This is consistent with results by Vohra & Poxton (2011) who reported that 5 different disinfectants demonstrated differing inactivation rates when used on both vegetative cells and spores of different *C. difficile* strains, with epidemic ribotypes in particular demonstrating higher resilience.

As the results of this study have demonstrated the susceptibility of *C. difficile* type-culture strain NCTC 11204 vegetative cells and spores to 405 nm light, it was of importance to also confirm that other strains of *C. difficile* were similarly susceptible to 405 nm light in order to

ensure that there was no major strain-to strain differences, which would be important if violet-blue light technology was to be deployed for *C. difficile* decontamination across the healthcare environment. Investigations were carried out to compare the inactivation kinetics of the culture collection strain (NCTC 11209) alongside that of a clinical strain (Ribotype 027). The results demonstrated the susceptibility of both NCTC 11209 and Ribotype 027 to 405 nm light, with a 25% increased dose required to achieve complete inactivation of both these strains in comparison to the *C. difficile* type culture strain NCTC 11204 selected for this study. The results have demonstrated that all 3 strains were susceptible to 405 nm light demonstrating its efficacy for the inactivation of spores and highlighting its potential for use within the hospital. Although some strains may require longer periods of exposure to achieve inactivation, this is feasible as the HINS-light EDS system is designed for continuous use.

This study then proceeded to compare the inactivation kinetics of both the vegetative cells and spores to that of another Gram positive spore forming bacteria – *B. cereus*. *B. cereus* is naturally found in soil environments and is known for its ability to cause severe foodborne illness (Banerjee et al., 2012; Luksiene et al., 2009; Nam et al., 2014). The results of this study demonstrate that that *B. cereus* vegetative cells were more sensitive to 405 nm light, requiring a 50% lowered dose than *C. difficile* vegetative cells for complete 3.3-3.7 $\log_{10}$ inactivation. The spores of both *C. difficile* and *B. cereus* demonstrated similar inactivation kinetics, requiring exposure to a dose of 2.43 $kJcm^{-2}$ 405nm for a near complete 3.3-3.6 $\log_{10}$ inactivation. This indicates that the vegetative structures of both bacteria may have different mechanisms of protection from environmental stress, however following the formation of spores, these bacteria both show a similarly high resilience to stress; this may be due to the development of similar structures and protection mechanisms upon sporulation.

In a similar study carried out by Maclean et al., (2013) *C. difficil*e spores were reported to be more susceptible to 405 nm light than that of *B. cereus* spores, with a dose of 1.15 $kJcm^{-2}$ achieving a 2.7 $\log_{10}$ and a 1.9 $\log_{10}$ reduction in *C. difficile* and *B. cereus* spores, respectively, however these results were not statistically significant (Maclean et al., 2013). Furthermore, this study reported that *C. difficile* vegetative cells were more susceptible to 405 nm light than *B. cereus* vegetative cells, with a dose of 48 Jcm^{-2} achieving a 3.7 $\log_{10}$ reduction in *C. difficile* vegetative cells, whilst only a 0.5 $\log_{10}$ reduction in *B. cereus* vegetative cells was observed at this dose (Maclean et al., 2013). A possible explanation for

the increased resilience of *B. cereus* vegetative cells when compared to that of *C. difficile* vegetative cells observed the study by Maclean et al., opposite to that observed in the current study, is that a lower irradiance of 405 nm light was used (40 $mWcm^{-2}$) in comparison to that used in the current study (70 $mWcm^{-2}$). As such, the threshold energy levels of *B. cereus* vegetative cells may be higher than that of *C. difficile* to initiate inactivation; however, once these thresholds are achieved the vegetative cells are much more susceptible to inactivation.

A previous study by Demidova et al. demonstrated the susceptibility of *B. cereus* spores to visible light at a wavelength of 630 nm in the presence of TBO (50 µM), with a 99.999% reduction achieved following exposure to a dose of 40 Jcm^{-2}, whilst a study by Abad-Lozano et al reported that the addition of photosensitizers did not increase the susceptibility of spores to visible light, most likely due to the impermeability of spores (Demidova & Hamblin, 2005; Abad-Lozano and Rodriguez-Velera, 1984). Furthermore, the study by Demidova et al. demonstrated the increased resilience of *B. cereus* and *B. subtilis* spores in comparison to their vegetative counterparts, with the spores requiring approximately 3-4× and 100× the concentration of TBO to achieve inactivation than their vegetative counterparts, respectively; this further suggests that different species from the same genus may have more enhanced protective mechanisms than others (Demidova & Hamblin, 2005).

The susceptibility of spores of different species have previously been shown to vary considerably upon photoinactivation. De silva et al. demonstrated that upon exposure of *Bacillus* spores to white light at 400-800 nm in the presence of tricationic porphyrin photosensitiser, a 3.5 log_{10} reduction in *B. cereus* were observed. In comparison, only 1 log_{10}, 0.5 log_{10} and 0.6 log_{10} reductions were observed for *B. subtilis*, *B. licheniformis* and *B. sphaericus*, respectively, even with increased concentrations of photosensitizer and exposure to a higher irradiance of light (De Silva et al., 2012).

4.6. CONCLUSIONS

In conclusion, this initial proof-of-concept research has demonstrated the efficacy of 405 nm light for the inactivation of *C. difficile* vegetative cells and spores, with up to 5 log_{10} $CFUml^{-1}$ reductions observed, requiring an applied dose of 3.24 $kJcm^{-2}$. Additionally, spores demonstrated an increased resilience to 405 nm light in comparison to their vegetative counterparts, requiring a 10-fold increase in dose to achieve a similar 3 log_{10} reduction.

Furthermore, this novel decontamination method has been demonstrated to be similarly effective for the inactivation of other *C. difficile* strains and sporulating bacteria, such as *B. cereus* which was used in this study. Enhanced sporicidal effects when spores are suspended in the simulant faecal matter have indicated that the presence of naturally occurring photo-reactive species in the faecal matter may enhance the oxidative effects of 405 nm light. However, on surfaces the organic load may have minimised the transmission of light and thus these experiments would need to be carried out under clinical conditions to investigate if this enhanced inactivation would occur in key problem areas, such as the bathrooms of patients with CDI.

This chapter utilised high light irradiances to establish the fundamental susceptibility of *C. difficile* to 405 nm light. Developing on from the establishment of this key data and the evidence of the high doses required for spore inactivation, it is of great interest to investigate whether the susceptibility of spores can be enhanced upon combination with other stressors or by inducing germination, thus enabling the use of lower doses of 405 nm light, and/or faster inactivation rates. These factors will be investigated in the following chapters.

CHAPTER 5

The Synergistic Sporicidal Activity of 405 nm Light and Disinfectants

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5. GENERAL

C. difficile is transmitted via the faecal-oral route, with the main route of transmission being the hands of healthcare workers. However, in recent years, the role of the environment has been highlighted as a major source for the transmission of *C. difficile*. The ability of the spores to survive for up to 5 months in the environment, alongside the high volume of spores shed in the faeces of infected patients, results in an alarmingly high rate of spores within the hospital environment. This can be further heightened by inadequate cleaning of the hospital surfaces, such as with sporicidal wipes, which have been demonstrated to transfer spores to other surfaces (Siani et al., 2011). Inefficient cleaning can result in enhanced transmission of spores which can subsequently be ingested or spread further following transmission from the environment to the hands of patients and healthcare workers (Leggett et al., 2016; Siani et al., 2011).

Previous studies have demonstrated that, as the environmental burden of *C. difficile* increases, the prevalence of *C. difficile* hand carriage among health care workers also increases (Samore et al., 1996). Due to the frequent bowel movements of patients infected with *C. difficile*, the environment surrounding these patients is likely to be contaminated with *C. difficile* spores. It has been reported that the heaviest contamination is found on floors and bedrails. Other sites frequently found to be contaminated include windowsills, commodes, toilets, bedsheets and call buttons (Gerding et al., 2008). A recent study recovered *C. difficile* from the air at heights of 25 cm above the toilet seat after flushing (Thompson, 2012).

The ability of spores to adhere to surfaces commonly found within the healthcare environment, for example bed linen and stainless steel, may play a further role in the survival and transmission of *C. difficile* spores, with the attachment of bacterial spores to surfaces having previously been demonstrated to enhance their resilience to treatment using commercial liquid sterilants (Joshi, 2012; Sagripanti & Bonifacino, 2000). Factors

influencing the ability of *C. difficile* spores to adhere to surfaces include spore hydrophobicity and the presence of surface structures such as appendages and the exosporium (Joshi et al., 2012; Sorg & Sonenshein, 2008).

Currently, chlorinated disinfectants are recommended for environmental cleaning of rooms housing patients with *C. difficile* infections. However these have several drawbacks including their corrosive nature and the release of irritating vapours, which are harmful to healthcare workers (Rupnik et al., 2009). In addition to this, Carling et al. (2006) demonstrated that only 47% of intended surfaces are actually contacted by a disinfectant on a routine cleaning basis, with reports that positive *C. difficile* samples collected from rooms of patients who are neither infected nor colonized with *C. difficile* can reach up to 8%, demonstrating the inefficiency of currently used disinfectants at eliminating *C. difficile* (Barbut et al., 2009; Carling et al., 2006). It is also important to note that quaternary ammonium–based detergents and surfactants currently used for day to day cleaning within the hospital, whilst not exerting sporicidal effects, may actually promote sporulation (Gerding et al., 2008).

Generally, it is acknowledged that the use of chlorine releasing cleaning agents reduces the incidence of *C. difficile* contamination, however it is important to note that the effectiveness of these cleaning agents with regards to *C. difficile* inactivation has not been confirmed in all studies – with the effectiveness varying when tested *in vitro* on contaminated surfaces (Ungurs et al., 2011). For example, a 0.7 $\log_{10}$ reduction of spores was reported by Block et al., (2004) following 10 minutes exposure to 1,000 ppm sodium dichloroisocyanurate (NaDCC), in comparison to Wheeldon et al. (2008) who reported that 1,000 ppm achieved a 5.26 $\log_{10}$ reduction following 9 minutes exposure (Block, 2004; Wheeldon et al., 2008). Several studies have concluded that high levels of sporicidal activity require either prolonged exposure of the spores to disinfectants or use of a higher concentration of chlorine within cleaning detergents (Bloomfield & Arthur, 1994; Omidbakhsh, 2010; Rutala et al., 2008).

The previous chapter demonstrated the ability of 405 nm light, albeit at high levels, to inactivate the highly resilient *C. difficile* spores. Due to the mechanisms of action of both 405 nm light and chlorine based disinfectants being related to oxidative damage, it was of interest to establish whether enhanced sporicidal activity can be achieved by their combined use. By combining chlorinated detergents with 405 nm light, if a synergistic effect is observed upon the exposure of *C. difficile* spores to 405 nm light and current

recommended chlorine based cleaning products, this could lead to the enhanced inactivation of spores whilst enabling the use of lower concentration of chlorine and shorter exposure times.

Initial proof-of-concept experiments involved bacterial spore suspensions being exposed to high intensity 405 nm light alongside low concentration disinfectants to establish if a synergistic effect could be achieved. Following confirmation of a synergistic effect, experiments were progressed to then investigate the efficacy against spores on a range of surfaces commonly found within the clinical environment. Finally, the synergistic effect was investigated on a range of contaminated surfaces using low irradiance 405 nm light, at irradiances similar to what would be used in the clinical setting for continuous decontamination of the environment.

5.1. The Synergistic Activity of 405 nm Light and Disinfectants in Suspension

For initial proof of concept of the potential for a synergistic effect of the oxidative effects of 405 nm light and the chlorine based disinfectants, initial investigations were carried out in suspension using high irradiance 405 nm light (225 $mWcm^{-2}$).

5.1.1. Bacterial preparation

C. difficile spores were prepared as described in Section 3.3.2.1. *C. difficile* spores were cultivated in 15 ml BHI broth anaerobically at 37°C for 7 days. Following incubation, the suspension was centrifuged at 4300 rpm for 10 minutes then re-suspended in 9 ml PBS.

5.1.2. Disinfectants

The disinfectants used in this study were:

- Sodium hypochlorite (NaOCl; Fisher Scientific, UK)
- Actichlor (Ecolab Ltd, UK)
- Tristel (Tristel solutions Ltd, UK).

Disinfectants were prepared according to the manufacturer's instructions with subsequent dilutions made as required.

Before experiments investigating the synergistic activity of disinfectants in combination with 405 nm light could be carried out, it was important to firstly establish the disinfectant concentrations which would cause a negligible effect on the spores over the maximum exposure period required for inactivation of the spores using the 405 nm light alone (up to

4 hours), as shown in Figure 4.2. The spore suspension was resuspended in 9 ml of each disinfectant at a range of concentrations, to give a final 10^3 $CFUml^{-1}$ spore population, and exposed for up to 3 hours. Samples were plated onto BA and incubated anaerobically at 37°C for 48 hours. Samples were then enumerated and viable bacterial counts recorded as log_{10} $CFUml^{-1}$. The inactivation effects were then recorded as shown in Table 5.1. Based on these results, concentrations of 0.0001%, 0.001% and 0.1% were chosen for Tristel, Actichlor and sodium hypochlorite, respectively.

Table 5.1 Exposure of *C. difficile* spores to chlorinated disinfectants, at a range of concentrations, over a 3 hour period.

Disinfectant	Concentration	Initial Spore Count (log_{10} $CFUml^{-1}$ ± SD)	Surviving Spore Count (log_{10} $CFUml^{-1}$ ± SD)	% Inactivation
Sodium hypochlorite	0.1% *	3.91 ± 0.13	3.76 ± 0.19	3.8%
	0.2%	2.90 ± 0.33	1.02 ± 1.03	64.8%
Actichlor	0.001%*	3.18 ± 0.35	3.23 ± 0.26	0.0%
	0.01%	2.93 ± 0.38	0.00 ± 0.00	100.0%
Tristel	0.0001% *	3.36 ± 0.08	3.34 ± 0.18	0.59%
	0.001%	3.14 ± 0.21	2.67 ± 0.14	15.0%
	0.01%	2.28 ± 0.28	0.00 ± 0.00	100.0%

* Highlighted are the concentrations selected for use in later experiments

5.1.3. TREATMENT METHOD

For experiments investigating the synergistic effects of light and disinfectants, spores were light exposed whilst suspended in low concentration disinfectants.

The final spore dilution was suspended in the selected disinfectant concentrations to give a starting population of 10^3 $CFUml^{-1}$ spores. 2 ml sample volumes were then transferred to a 12-well multi-plate and exposed to increasing doses of 405 nm light, at an irradiance of 225 $mWcm^{-2}$, using the ENFIS Photostar Innovate UNO 24 (for high irradiance exposures) as detailed in Section 3.5.1. Controls were subject to identical conditions, but exposed to normal laboratory lighting levels (0.2 $mWcm^{-2}$).

Following exposure, both the exposed sample and the non-exposed control were plated onto blood agar and incubated at 37°C for 48 hours under anaerobic conditions. Samples

were then enumerated and viable bacterial counts recorded as $\log_{10}$ CFUml^{-1}, as discussed in Section 3.7.2. Experiments were repeated in triplicate.

5.1.4. Results: The Synergistic Activity of 405 nm light and Disinfectants in Suspension

Spores suspended in low, non-sporicidal concentrations of the disinfectants were exposed to 405 nm light at an irradiance of 225 mWcm^{-2} for increasing time periods. Results are shown in Figure 5.1, Figure 5.2 and Figure 5.3.

First looking at the control data, *C. difficile* spores suspended in 0.1% NaOCl, 0.001% Actichlor and 0.0001% Tristel alone over the 3-hr period showed no significant reduction in population (P=>0.05). In addition to this, the baseline sporicidal activity of 405 nm light when the spores were suspended in PBS demonstrated a requirement for doses of 0.81, 1.62 and 2.43 kJcm^{-2} to achieve reductions of 0.5, 1.3 and 3.5 $\log_{10}$ CFUml^{-1}, respectively.

Enhanced sporicidal activity was observed in all cases when the spores were exposed to 405 nm light whilst suspended in the three disinfectants. In the case of NaOCl (Figure 5.1), a 0.5 $\log_{10}$ increased reduction was achieved following combined exposure to a dose of 0.8 kJcm^{-2} (P=0.007) (Figure 5.1). Following combined exposure to a dose of 1.62 kJcm^{-2} and 0.1% NaOCl, an approximate 2.5× increase in spore inactivation was achieved, with a 3.2 $\log_{10}$ reduction, in comparison to a 1.3 $\log_{10}$ reduction achieved when exposed to 405 nm light alone (Figure 5.1).

When exposed to 405 nm light whilst suspended in Actichlor, a significant 1.1 $\log_{10}$ enhanced spore reduction was achieved following exposure to a dose of 0.8 kJcm^{-2} (P=0.001). Following combined exposure to a dose 1.62 kJcm^{-2} a 59% increase in spore inactivation was observed achieving near complete reduction. This is approximately 1.5 times lower a dose than that required to achieve a similar inactivation with 405 nm light alone (Figure 5.2).

Similar results observed following combined exposure with 0.1% NaOCl and 0.001% Actichlor were also seen upon combined exposure of 0.0001% Tristel with a dose of 1.62 kJcm^{-2}, with an increased 2 $\log_{10}$ reduction observed in comparison to that achieved with 405 nm light alone (Figure 5.3).

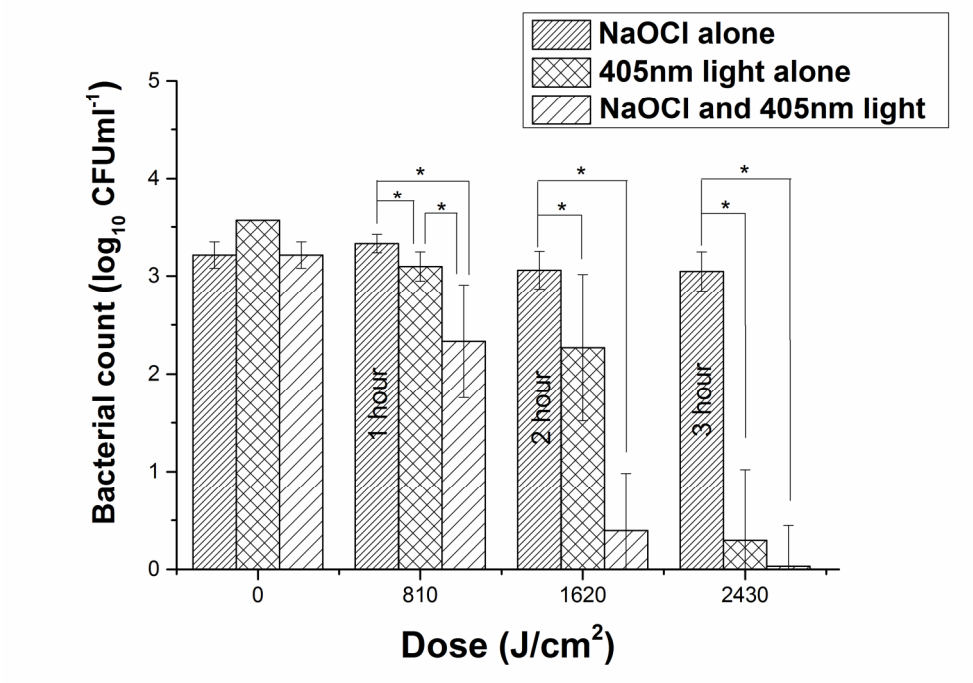

Figure 5.1 Comparison of the inactivation of *C. difficile* spores when exposed to (i) 0.1% sodium hypochlorite only (ii) 405 nm light alone (iii) 0.1% sodium hypochlorite combined with 405 nm light. Controls were suspended in negligible concentrations of disinfectant for the equivalent time periods (0, 1, 2 and 3 hours) and were non-light exposed. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

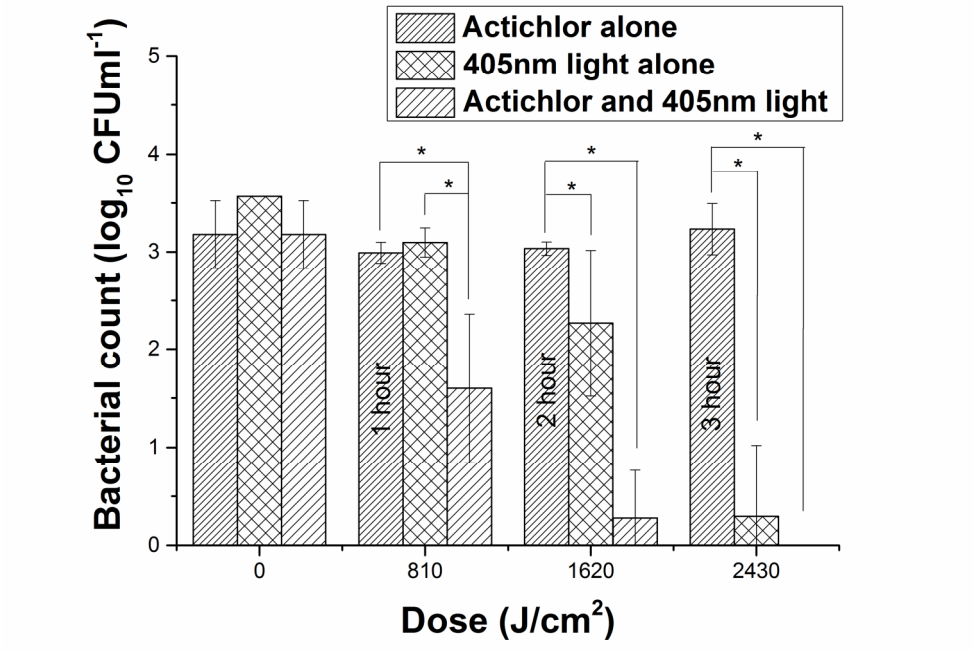

Figure 5.2 Comparison of the inactivation of *C. difficile* spores when exposed to (i) 0.001% Actichlor only (ii) 405 nm light alone (iii) 0.001% Actichlor combined with 405 nm light. Controls were suspended in negligible concentrations of disinfectant for the equivalent time periods (0, 1, 2 and 3 hours) and were non-light exposed. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

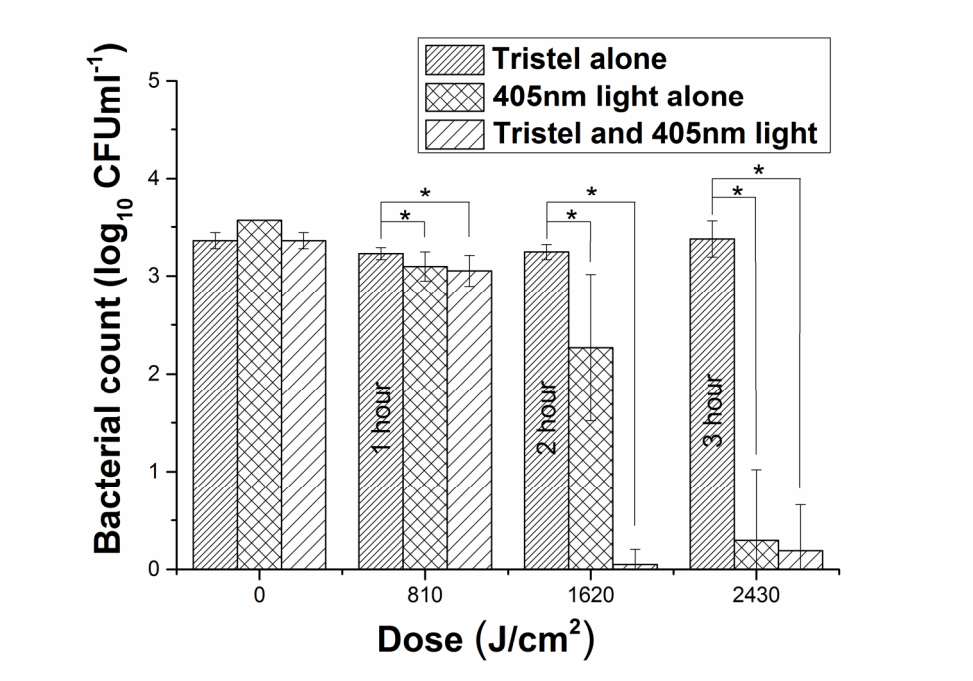

Figure 5.3 Comparison of the inactivation of *C. difficile* spores when exposed to (i) 0.0001% Tristel only (ii) 405 nm light alone (iii) 0.0001% Tristel combined with 405 nm light. Controls were suspended in negligible concentrations of disinfectant for the equivalent time periods (0, 1, 2 and 3 hours) and were non-light exposed. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

To allow for a direct comparison of the inactivation kinetics of the negligible concentrations of the disinfectants chosen for this study, and that of the recommended concentrations for sporicidal use, *C. difficile* spores were suspended in all 3 disinfectants prepared as described by the manufacturer's instructions without dilution – 1% NaOCl, 0.1% Actichlor and 0.01% Tristel. This was then repeated with simultaneous exposure to 405 nm light at an irradiance of 225 $mWcm^{-2}$. The results are shown in Table 5.2 and demonstrate that Actichlor is the most efficient agent for the disinfection of *C. difficile* spores, with a complete 3.13 log_{10} reduction achieved following 1 minute contact time at the manufacturers recommended concentration of 0.1%. This is followed by 1% NaOCl, which following which 3 minutes contact time, resulted in a complete 3.42 log_{10} reduction. When combined with 405 nm light, an enhanced 1.5× inactivation was observed following exposure for 1 minute. 0.01% Tristel was the least efficient sporicidal treatment, with only a 1.92 log_{10} reduction in spore count observed after 10 minutes exposure. Upon combination of this with 405 nm light, a complete 2.85 log_{10} reduction was observed after 10 minutes.

Table 5.2 Comparison of the efficacy of recommended concentrations of sporicidal disinfectants in the absence and presence of 405 nm light at an irradiance of 225 $mWcm^{-2}$ over a range of time periods. A starting population of 3.3-3.54 log_{10} $CFUml^{-1}$ was used and the surviving population enumerated (n=3).

Disinfectant	Time (minutes)	Exposure to disinfectant only ± SD ($CFUml^{-1}$)	Exposure to disinfectant and 405 nm light ± SD ($CFUml^{-1}$)
Actichlor	0	3.13 ± 0.9	3.24 ± 0.8
	1	0 ± 0	0 ± 0
NaOCl	0	3.42 ± 0.06	3.17 ± 0.20
	1	2.44 ± 0.25	1.65 ± 0.31
	2	0.33 ± 0.52	0.22 ± 0.53
	3	0 ± 0	0 ± 0
Tristel	0	2.9 ± 0.39	2.85 ± 0.34
	2.5	1.63 ± 0.80	1.79 ± 0.52
	5	1.29 ± 0.73	1.76 ± 0.23
	7.5	1.37± 0.71	0.91 ± 0.73
	10	0.98 ± 0.81	0 ± 0

5.2. THE SYNERGISTIC ACTIVITY OF 405 NM LIGHT AND DISINFECTANTS ON CLINICALLY RELEVANT SURFACES

Following on from the initial proof of concept validation that synergistic sporicidal activity can be achieved upon the combination of 405 nm light with low concentrations of chlorine based disinfectants that alone exhibit negligible sporicidal effects, it was of interest to determine if this was representative of what would be obtained on surfaces. This was done using both high intensity light, to provide proof of principle data, and also lower intensity light, similar to levels representative of those which has previously used for clinical environmental decontamination (Bache et al., 2012; Maclean et al., 2013). As such, this section is split into two parts which investigate:

- ***The synergistic decontamination of clinically relevant surfaces when low concentration disinfectants are used in conjunction with high irradiance 405 nm light***
- ***The synergistic decontamination of clinically relevant surfaces when low concentration disinfectants are used in conjunction with low irradiance 405 nm light***

5.2.1. BACTERIAL PREPARATION

C. difficile spores were prepared as described in Section 3.3.2.1. *C. difficile* spores were cultivated in 15 ml BHI broth anaerobically at 37°C for 7 days. Following incubation, the suspension was centrifuged at 4300 rpm for 10 minutes, then re-suspended in 9 ml PBS.

5.2.2. SURFACES

For this investigation, the surface materials selected, as detailed in Section 4.2, were:

- Stainless steel
- Vinyl flooring
- PVC
- Aluminium

The same concentrations of disinfectants with a negligible effect on the spores, as detailed in Section 5.1.2 were used for this study, unless otherwise stated. Prior to experiments, surfaces were cleaned using 70% ethanol to remove environmental soiling by spraying the surfaces with ethanol and drying. As surface coupons were used for subsequent experiments, surface coupons were soaked in virkon overnight post-exposure then washed in water and allowed to dry.

5.2.3. TREATMENT METHOD

50 µl of spore suspension was seeded onto surfaces using a pipette and dried at 50°C for 20 ± 5 minutes, depending on the surface material. Following drying, 100 µl of disinfectant was pipetted onto the spore inoculum.

5.2.3.1. Surface exposures

The surface coupons were exposed to increasing durations of 405 nm light. For high irradiance exposures the ENFIS Photostar Innovate UNO 24, at an irradiance of 225 $mWcm^{-2}$ (as detailed in Section 3.5.1) was used. For low irradiance exposures the HINS-light EDS (as detailed in Section 3.5.2) was used at an irradiance of ~0.4 $mWcm^{-2}$. Controls were exposed to (i) 405 nm light in the absence of disinfectants, and (ii) disinfectants in the absence of 405 nm light, to establish the sporicidal activity of each agent alone, and to demonstrate the synergistic effect when combined.

Post-exposure, spores were resuspended in 5 ml PBS and agitated using an L-shaped spreader. Samples were then spread plated onto BA plates and incubated at 37°C for 48 hours under anaerobic conditions. Samples were then enumerated and viable bacterial counts recorded as log_{10} $CFUml^{-1}$.

5.2.4. RESULTS: THE SYNERGISTIC ACTIVITY OF 405 NM LIGHT AND DISINFECTANTS ON CLINICALLY RELEVANT SURFACES

Initial experimental surface work investigating if the previously demonstrated synergistic effect could be achieved against spores seeded onto clinically relevant surfaces was carried out using high irradiance 405 nm light, at an irradiance of 225 $mWcm^{-2}$, and low irradiance 405 nm light more indicative of what would be used in the clinical setting, at an irradiance of ~0.4 $mWcm^{-2}$. The results of this study are now described.

5.2.4.1. High irradiance surface exposures

Figure 5.4 shows that, on PVC surface coupons, both 0.1% NaOCl and 0.001% Actichlor exhibited enhanced sporicidal activity in combination with 405 nm light at an irradiance of 225$mWcm^{-2}$. Upon exposure of *C. difficile* spores to 405 nm light alone on PVC surfaces, a dose of 2.43 $kJcm^{-2}$ achieved a 2.54 log_{10} $CFUml^{-1}$ reduction (P=0.003). Combined exposure with 0.1% NaOCl resulted in complete 3.4 log_{10} inactivation following exposure to a dose of 810 Jcm^{-2} (P=0.001), an increased 2 log_{10} reduction in comparison to 405 nm light alone at this dose (P=0.006).

Actichlor also achieved complete inactivation of spores in combination with 405 nm light at a dose of 1.62 $kJcm^{-2}$ ($P<0.001$), achieving an almost 2× increase in spore inactivation in comparison to 405 nm light alone.

Tristel, at a concentration of 0.0001%, showed very little synergy on PVC, with the inactivation kinetics showing similar results to that of the spores exposed to 405 nm light alone, with a 3 log_{10} reduction following exposure to 405 nm light in combination with 0.0001% Tristel ($P<0.001$).

On vinyl flooring, as demonstrated in Figure 5.5, 0.001% Actichlor demonstrated the greatest synergy with 405 nm light with an increased 2.23 log_{10} reduction following exposure to 2.43 $kJcm^{-2}$ ($P<0.001$).

0.1% NaOCl also demonstrated enhanced sporicidal activity, with a 2.25 log_{10} reduction achieved after exposure to a dose of 810 Jcm^{-2} ($P<0.001$), 3× that observed when exposed to 405 nm light only. Complete inactivation was not observed, with a maximum of 2.83 log_{10} reduction achieved following combined exposure to 2.43 $kJcm^{-2}$ ($P<0.001$). However, this was a 1.41 log_{10} greater reduction in spores than that achieved with 405 nm light alone.

No synergy was observed in the presence of 0.0001% Tristel on vinyl flooring, with a a 1.49 log_{10} reduction in *C. difficile* spores achieved following combined exposure with 2.43 $kJcm^{-2}$ 405 nm light.

Figure 5.6 compares the enhanced sporicidal effects of 405 nm light and all 3 disinfectants on stainless steel. A slightly enhanced sporicidal effect was observed on all 3 surfaces, with a maximal increase of 0.9 log_{10} spore inactivation upon combined exposure to 2.43 $kJcm^{-2}$ with 0.001% Actichlor.

On stainless steel 2.43 $kJcm^{-2}$ 405 nm light alone achieved a reduction in spore count of 1.09 log_{10} ($P<0.001$). Comparatively, in the presence all 3 disinfectants, synergy was observed with a 1.64-2 log_{10} reduction achieved on all surfaces. 0.001% Actichlor demonstrated the greatest enhanced sporicidal activity and 0.1% NaOCl the least, with 1.8× and 1.5× increased sporicidal activity upon exposure to 2.43 $kJcm^{-2}$, respectively, in comparison to exposure to this dose of 405 nm light alone. However, none of the results were significantly different ($P=>0.05$).

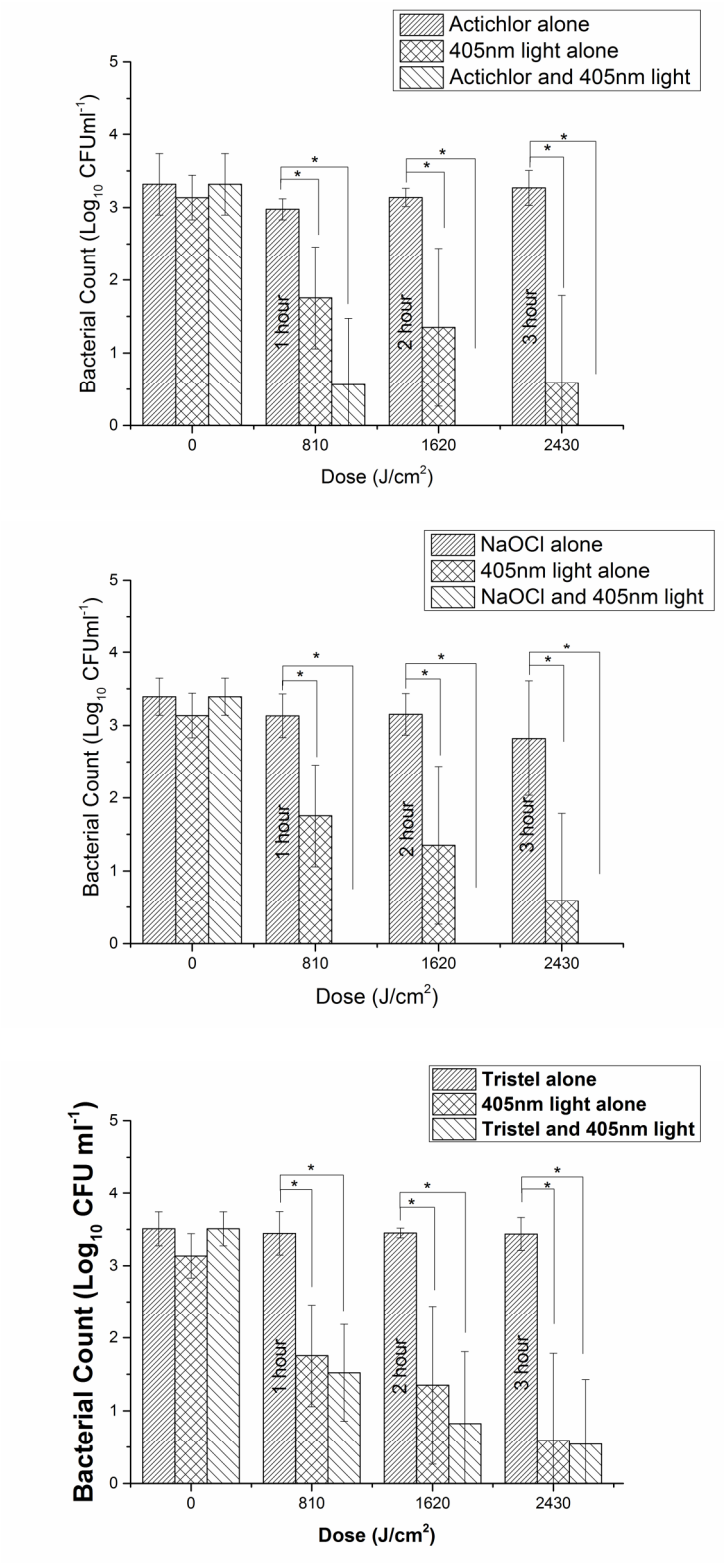

Figure 5.4 Spores on PVC surfaces exposed to disinfectants (0.0001% Tristel, 0.1% sodium hypochlorite (NaOCl) and 0.001% Actichlor) in combination with 405 nm light (225 mWcm^{-2}). Control samples were exposed to (i) 405nm light alone and (ii) disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

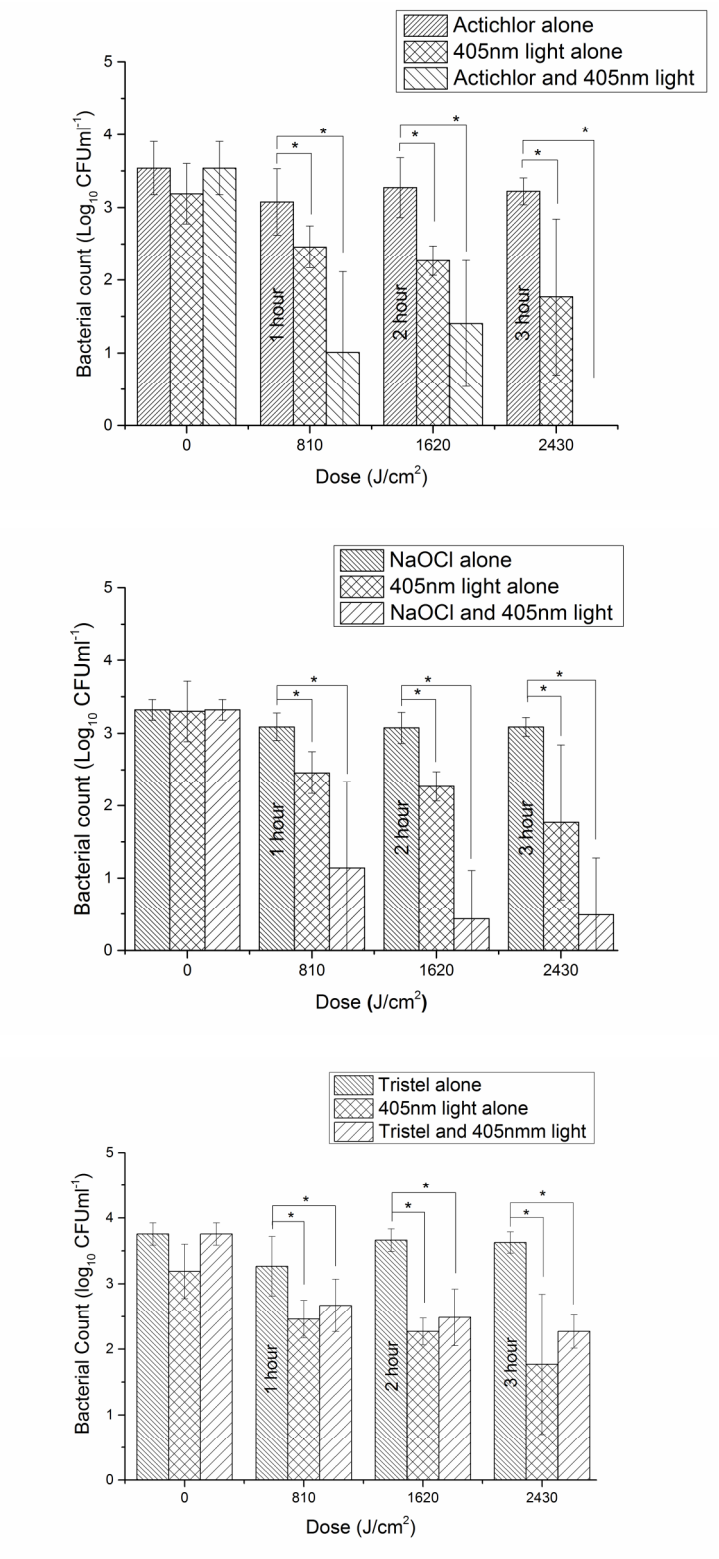

Figure 5.5 Spores on Vinyl flooring exposed to disinfectants (0.0001% Tristel, 0.1% sodium hypochlorite (NaOCl) and 0.001% Actichlor) in combination with 405nm light (225 mWcm^{-2}). Control samples were exposed to (i) 405 nm light alone and (ii) disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

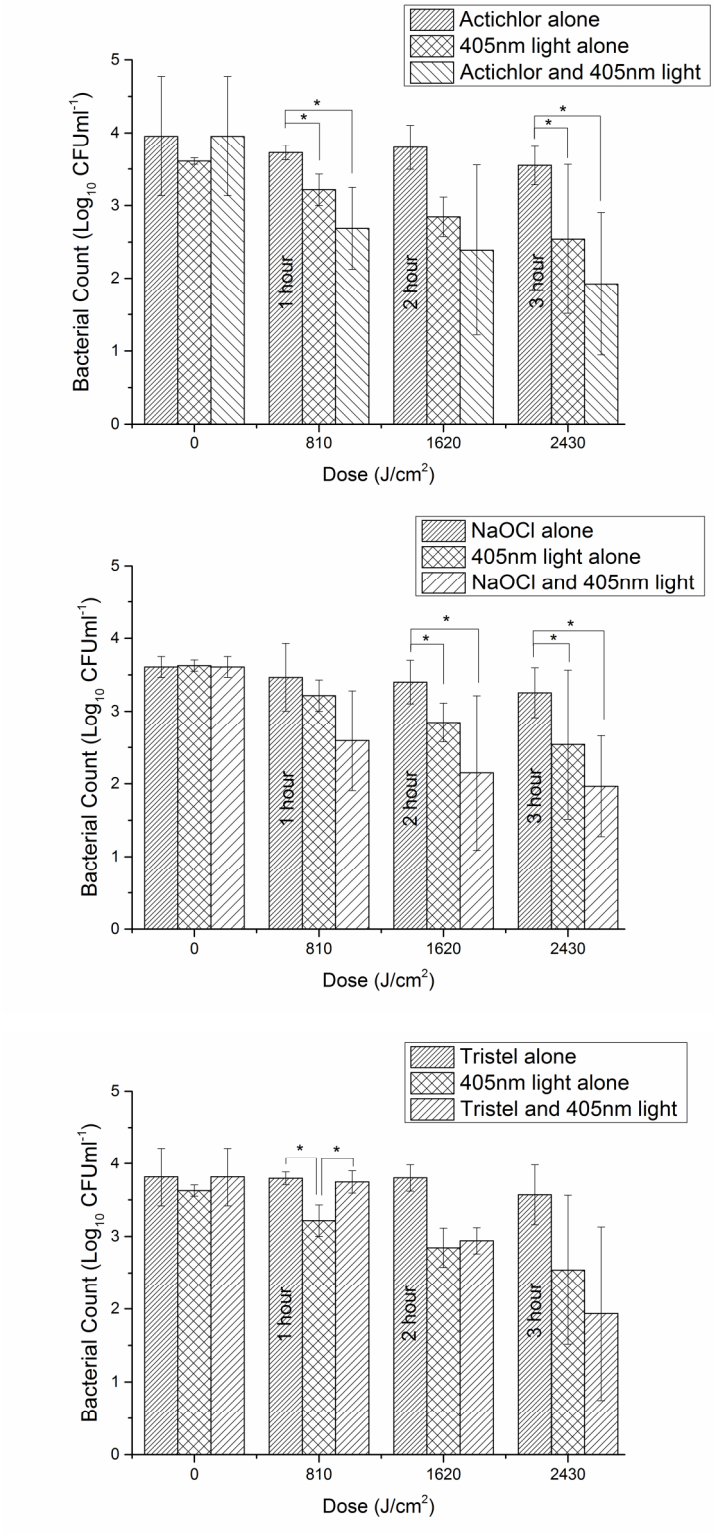

Figure 5.6 Spores on Stainless steel exposed to disinfectants (0.0001% Tristel, 0.1% sodium hypochlorite (NaOCl) and 0.001% Actichlor) in combination with 405nm light (225 mWcm^{-2}). Control samples were exposed to (i) 405 nm light alone and (ii) disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

When spores seeded on aluminium surfaces were exposed to disinfectants in combination with 405 nm light, all 3 disinfectants demonstrated enhanced sporicidal activity with 0.0001% Tristel exerting the greatest synergistic effect with a 57% increased spore inactivation in comparison to that achieved with 405 nm light alone. The results are shown in Figure 5.7.

When 405 nm light and 0.001% Actichlor were used in combination against spores on aluminium surfaces, slight synergy was observed, with a 0.8 $\log_{10}$ increased spore inactivation upon exposure to 2.43 $mWcm^{-2}$, as seen in Figure 5.7.

A significant sporicidal kill is observed following exposure to 810 Jcm^{-2} and 1.62 $kJcm^{-2}$ 405 nm light in the presence of 0.1% NaOCl, achieving spore reductions of 1.63 $\log_{10}$ and 2.44 $\log_{10}$ respectively, whilst controls showed a maximal 1 $\log_{10}$ reduction individually.

In the case of 0.0001% Tristel combined with a 2.43 $kJcm^{-2}$ 405 nm light a 2.33× increased reduction in spores was observed on aluminium surfaces, in comparison to controls exposed to 405 nm light alone.

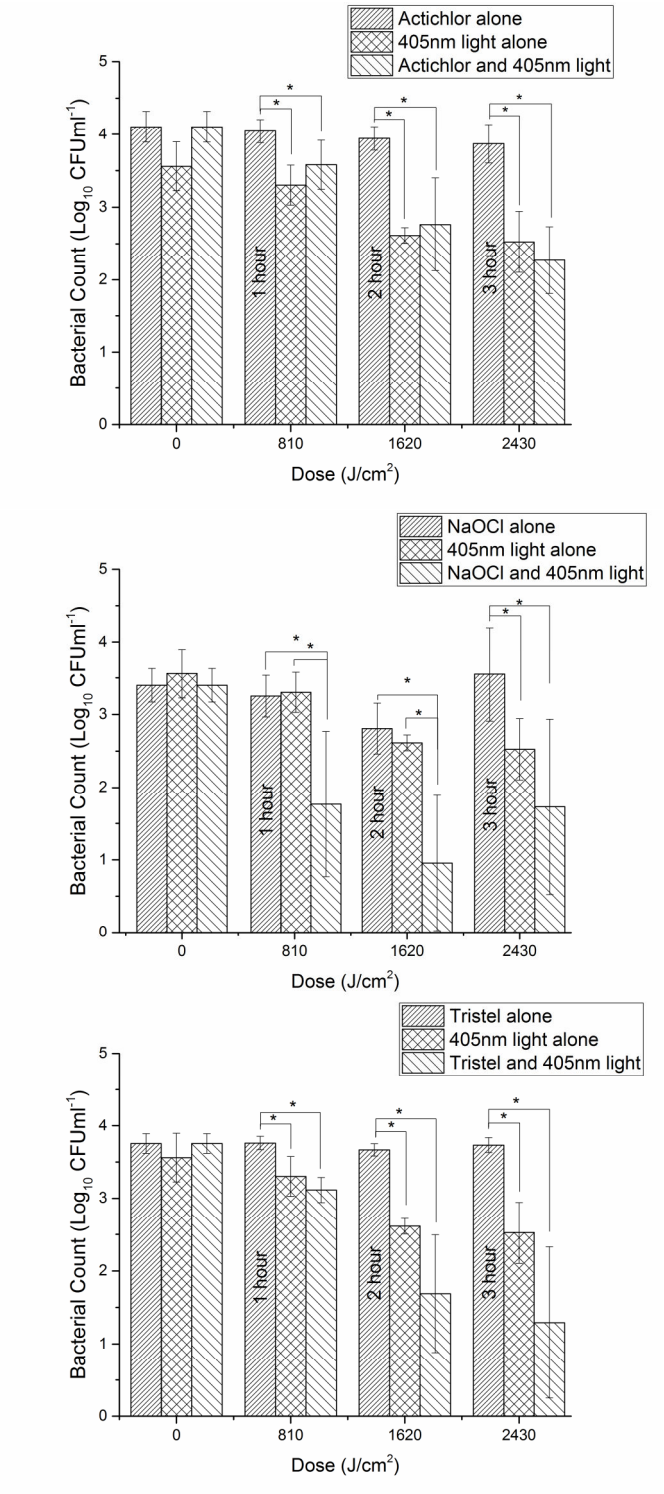

Figure 5.7 Spores on Aluminium exposed to disinfectants (0.0001% Tristel, 0.1% sodium hypochlorite (NaOCl) and 0.001% Actichlor) in combination with 405nm light (225 mWcm^{-2}). Control samples were exposed to (i) 405 nm light alone and (ii) disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

5.2.4.2. Low irradiance surface exposures

Figures 5.8, 5.9 and 5.10 show the results of the investigation into the possible synergistic effect when low irradiance 405 nm light is used alongside negligible concentrations of commonly used hospital disinfectants, previously demonstrated to exhibit synergy alongside high irradiance 405 nm light (225mWcm^{-2}) both in suspension and on surfaces. The light intensity used was ~0.4 mWcm^{-2}, and is representative of the typical level that would be used in a hospital setting for environmental decontamination. These investigations follow on from the initial proof-of-concept investigations, to provide a more realistic analysis of the possible synergy that could be obtained if used within the hospital alongside housekeeping to remove *C. difficile* spores from surfaces.

The results from this investigation demonstrated that, little to no synergy was observed as a result of the combinative effects of the low strength concentrations of disinfectants alongside the low irradiance 405 nm light levels used in this study. The greatest enhanced sporicidal effects were observed upon combination of low irradiance 405 nm light with Actichlor. As such, higher concentrations of disinfectants would need to be used to achieve greater spore reductions for suitable decontamination of surfaces within the clinical environment.

The results in Figures 5.8, 5.9 and 5.10 show that, firstly the negligible concentrations used had no sporicidal effects over the time periods used, and that, in Figures 5.8a, 5.9a, and 5.10a, a maximum significant reduction of only 0.97 $\log_{10}$ was observed on PVC over this time period using the 405 nm light alone.

On PVC surface coupons, as shown in Figure 5.8a, 0.001% Actichlor demonstrated the highest synergistic effect when used in combination with low irradiance light, with a 1.53 $\log_{10}$ reduction observed - a 0.84 $\log_{10}$ increased spore reduction compared to that achieved when exposed to 405 nm light alone. On PVC surface coupons, no enhanced sporicidal effects were seen upon combination with NaOCl and Tristel.

In Figure 5.8b it can be seen that, similar to that observed on PVC surfaces, on vinyl surfaces enhanced sporicidal effects were observed following combined exposure to 726 Jcm^{-2} and 0.001% Actichlor with a 0.6 $\log_{10}$ increase reduction in spores. On vinyl surfaces, synergy was only observed in the presenced of Actichlor, and not upon combination of low irradiance 405 nm light with NaOCl or Tristel at these concentrations.

Figures 5.8c, 5.9c and 5.10c demonstrate that on stainless steel surface coupons following combined exposure to 405 nm light at 726 Jcm^{-2} and all 3 disinfectants very little synergy was observed, with an increased bacterial reduction of 0.36 log_{10} (Figure 5.8c) and 0.61 log_{10} (Figure 5.9c) for 0.001% Actichlor and 0.0001% Tristel in comparison to the light exposed control, respectively, and no enhanced reduction in combination with 0.1% NaOCl (Figure 5.10c).

On aluminium surfaces, when low irradiance 405 nm light was used in conjunction with 0.001% Actichlor (Figure 5.8d), significant synergy was observed following exposure to a dose of 484 Jcm^{-2} ($P<0.001$) with an increased 1.85 log_{10} reduction.

On all surfaces investigated in the study, negligible synergy was observed between low irradiance light in combination with 0.1% NaOCl and 0.0001% Tristel, under the conditions of this study. Although synergy was not apparent under all test scenarios, this is likely due the use of very low concentrations of disinfectant, and the resilience of the spores when seeded onto surfaces. Although synergy was not observed under the conditions of the experiments, this does not indicate that synergy does not occur. To address this, experiments were repeated using increased levels of disinfectants in order to establish the critical concentrations of the different disinfectants that would be required to induce synergy.

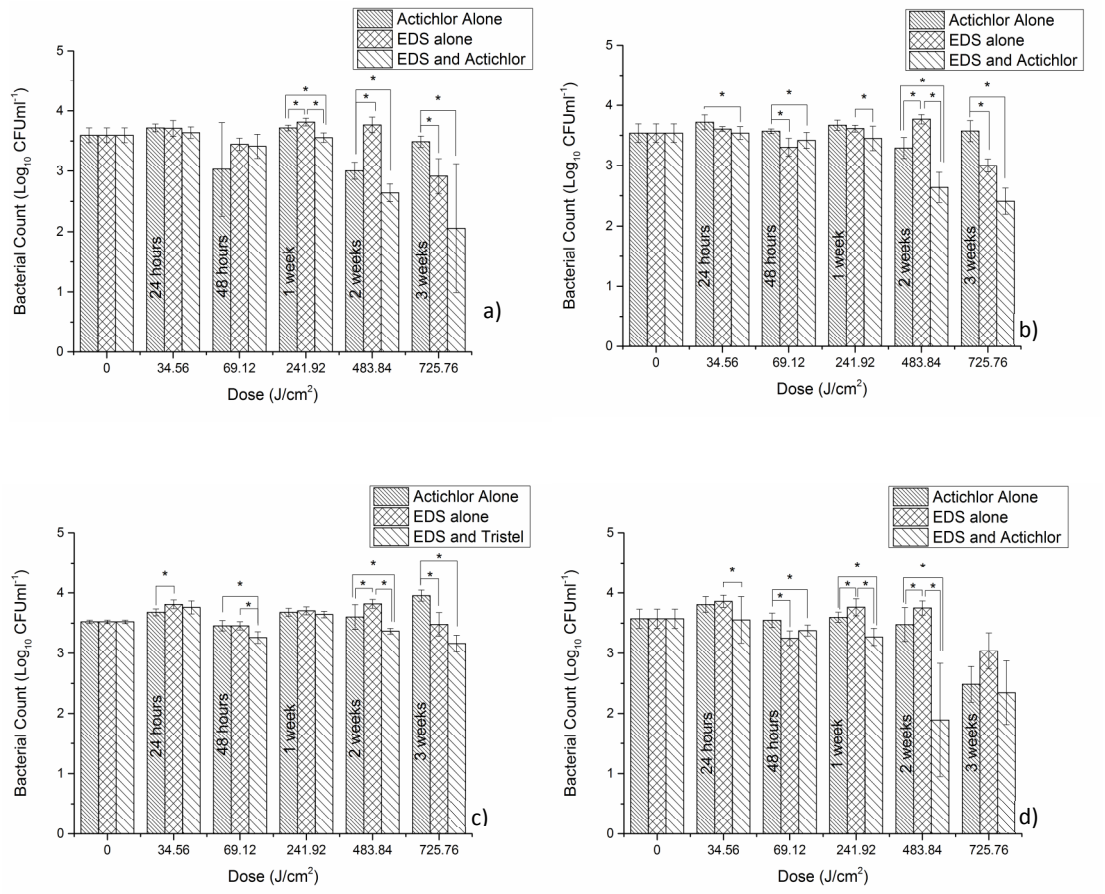

Figure 5.8 Spores seeded onto a) PVC b) Vinyl flooring c) Stainless steel and d) Aluminium exposed 0.001% Actichlor in combination with the low irradiance 405 nm light (0.4 mWcm^{-2}). Control samples were exposed to 405nm light alone, and disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

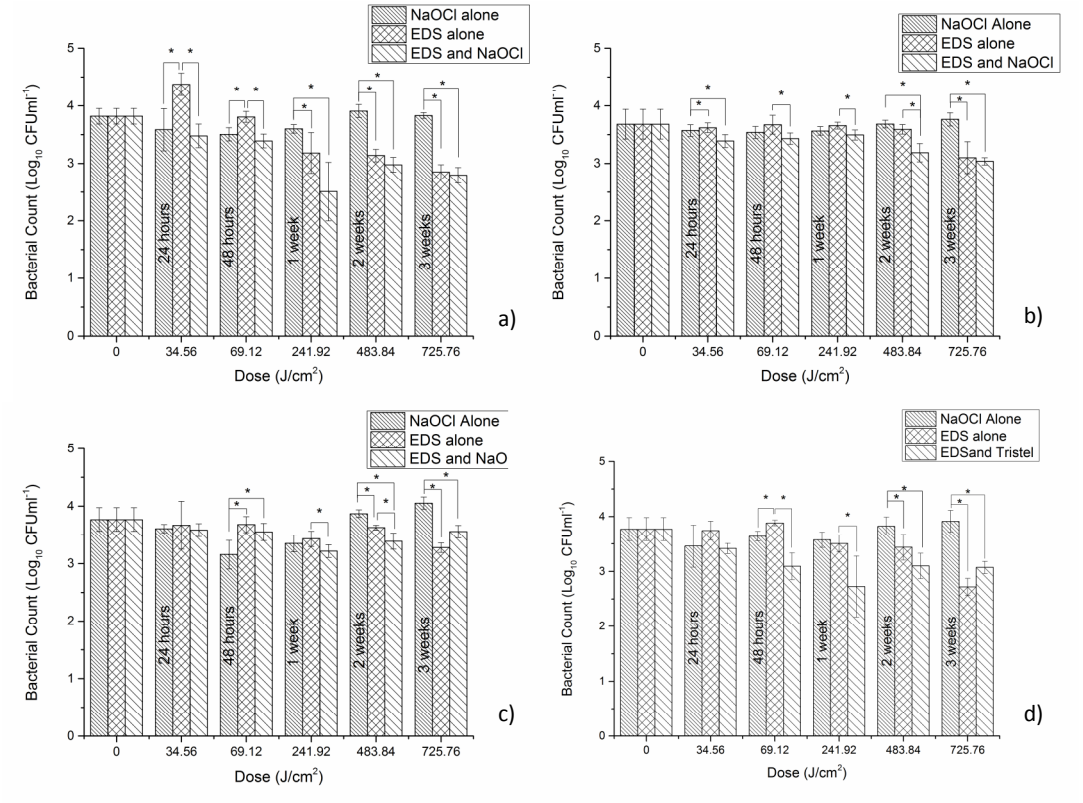

Figure 5.9 Spores seeded onto a) PVC b) Vinyl flooring c) Stainless steel and d) Aluminium exposed 0.1% NaOCl in combination with the low irradiance 405 nm light (0.4 mWcm^{-2}). Control samples were exposed to 405nm light alone, and disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

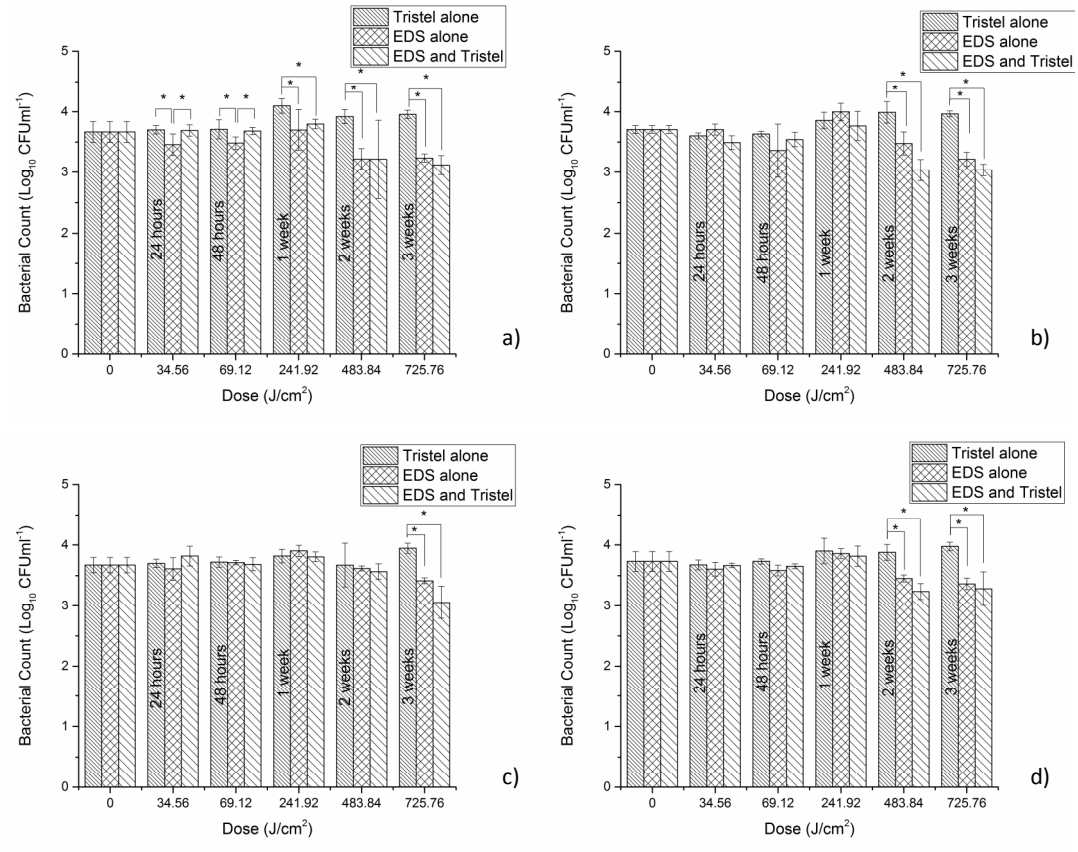

Figure 5.10 Spores seeded onto a) PVC b) Vinyl flooring c) Stainless steel and d) Aluminium exposed 0.0001% Tristel in combination with the low irradiance 405 nm light (0.4 mWcm^{-2}). Control samples were exposed to 405nm light alone, and disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

Table 5.3. The inactivation of *C. difficile* spores seeded onto clinically relevant surfaces using increasing doses of low irradiance light (~0.4mWcm^{-2}) and low concentrations of disinfectants. * represent significant bacterial inactivation compared to 405 nm light alone (P≤0.05). Each data point is a mean value (n=6).

Surface	Dose (Jcm^{-2})	0.0001% Tristel (CFUml^{-1})			0.001% Actichlor (CFUml^{-1})			0.1% NaOCl (CFUml^{-1})		
		405nm light alone ± SD	Tristel Alone ± SD	405nm light and Tristel ± SD	405nm light alone ± SD	Actichlor Alone ± SD	405nm light and Actichlor ± SD	405nm light alone ± SD	NaOCl Alone ± SD	405nm light and NaOCl ± SD
Vinyl Flooring	0	3.71 ± 0.06	3.71 ± 0.06	3.71 ± 0.06	3.54 ± 0.15	3.54 ± 0.15	3.54 ± 0.15	3.68 ± 0.25	3.68 ± 0.25	3.68 ± 0.25
	242	4.00 ± 0.14	3.90 ± 0.13	3.80 ± 0.24	3.61 ± 0.06	3.67 ± 0.08	3.45 ± 0.2	3.66 ± 0.05	3.56 ± 0.07	3.49 ± 0.08
	484	3.50 ± 0.19	4.00 ± 0.18	3.03 ± 0.18	3.76 ± 0.08	3.29 ± 0.17	2.63* ± 0.2	3.59 ± 0.08	3.68 ± 0.07	3.18* ± 0.16
	726	3.22 ± 0.11	3.96 ± 3.04	3.04 ±	3.00 ± 0.11	3.57 ± 0.17	2.41 ± 0.12	3.10 ± 0.28	3.76 ± 0.11	3.03 ± 0.06
PVC	0	3.67 ± 0.17	3.66 ± 0.17	3.66 ± 0.17	3.59 ± 0.12	3.59 ± 0.12	3.59 ± 0.12	3.82 ± 0.13	3.82 ± 0.13	3.82 ± 0.13
	242	3.67 ± 0.34	4.10 ± 0.12	3.80 ± 0.08	3.81 ± 0.06	3.72 ± 0.04	3.55 ± 0.08	3.18 ± 0.35	3.60 ± 0.07	2.51 ± 0.51
	484	3.21 ± 0.17	3.92 ± 0.12	3.21 ± 0.65	3.77 ± 0.12	3.00 ± 0.14	2.64 ± 0.14	3.34 ± 0.11	3.91 ± 0.11	2.98 ± 0.13
	726	3.23 ± 0.07	3.96 ± 0.07	3.11 ± 0.16	2.91 ± 0.29	3.49 ± 0.09	2.06 ± 1.07	2.85 ± 0.13	3.83 ± 0.05	2.79 ± 0.13
Stainless steel	0	3.67 ± 0.13	3.67 ± 0.12	3.67 ± 0.12	3.52 ± 0.03	3.52 ± 0.03	3.52 ± 0.03	3.77 ± 0.21	3.77 ± 0.21	3.77 ± 0.21
	242	3.90 ± 0.09	3.82 ± 0.11	3.8 ± 0.08	3.70 ± 0.06	3.68 ± 0.07	3.64 ± 0.05	3.43 ± 0.13	3.35 ± 0.15	3.22 ± 0.11
	484	3.62 ± 0.05	3.95 ± 0.08	3.06 ± 0.27	3.82 ± 0.07	3.60 ± 0.20	3.37* ± 0.05	3.63 ± 0.04	3.87 ± 0.06	3.39* ± 0.14
	726	3.41 ± 0.05	3.95 ± 0.08	3.06 ± 0.27	3.48 ± 0.20	3.95 ± 0.10	3.16 ± 0.14	3.28 ± 0.21	4.05 ± 0.12	3.56 ± 0.11
Aluminium	0	3.73 ± 0.16	3.73 ± 0.16	3.73 ± 0.16	3.57 ± 0.16	3.57 ± 0.16	3.57 ± 0.16	3.77 ± 0.21	3.77 ± 0.21	3.77 ±0.21
	242	3.86 ± 0.08	3.90 ± 0.21	3.82 ± 0.17	3.76 ± 0.14	3.60 ± 0.08	3.27* ± 0.14	3.51 ± 0.15	3.57 ± 0.14	2.72 ± 0.56
	484	3.45 ± 0.06	3.88 ± 0.13	3.24 ± 0.13	3.75 ± 0.12	3.48 ± 0.28	1.89* ± 0.94	3.44 ± 0.23	3.83 ± 0.16	3.10 ± 0.23
	726	3.36 ± 0.09	3.98 ± 0.07	3.28 ± 2.8	3.04 ± 0.30	2.48 ± 0.83	2.34 ± 0.53	2.71 ± 0.15	3.91 ± 0.20	3.07 ± 0.11

5.2.4.3. The synergistic effect of low irradiance 405 nm light and increased concentrations of disinfectants on clinically relevant surfaces

As very little synergy was observed when the low irradiance 405 nm light was used alongside the negligible concentrations of disinfectants previously discussed (Section 5.2.4.2), further investigations were carried out using higher concentrations of all 3 disinfectants, but still lower concentrations than recommended for use within the hospital. This is because synergy was observed on surfaces using high irradiance 405 nm light in Section 5.2.4.1, however in Section 5.2.4.2 which used a low irradiance 405 nm light, the lack of synergy may be explained by both the dramatic decrease in the irradiance of violet-blue used, alongside the negligible oxidative effects of the disinfectant solutions used.

The increased concentration of disinfectants selected for this section of the study, alongside the sporicidal effects observed following suspension of spores in these solutions for 7 days, are listed in Table 5.4. These experiments were carried out using the same methodology as discussed before in Section 5.2.3. As before, 50 µl of spore suspension was seeded onto surfaces using a pipette and dried. 100 µl of disinfectant was then pipetted onto the spore inoculum. The surface coupons were then immediately exposed to increasing durations of low irradiance 405 nm light. Post-exposure, spores were resuspended in 5 ml PBS and agitated using an L-shaped spreader. Samples were then spread plated onto BA plates and incubated at 37°C for 48 hours under anaerobic conditions. Samples were then enumerated and viable bacterial counts recorded as $\log_{10}$ $CFUml^{-1}$.

Table 5.4 Exposure of *C. difficile* spores to increased concentrations of chlorine based disinfectants over a 7 day period

Disinfectant	Concentration	Surface	Initial Spore Count (log_{10} $CFUml^{-1}$ ± SD)	Surviving Spore Count (log_{10} $CFUml^{-1}$ ± SD)	% Inactivation
Sodium Hypochlorite	0.2%	PVC	3.15 ± 0.09	3.46 ± 0.43	0
		Vinyl	3.07 ± 0.22	3.26 ± 0.55	0
		Stainless Steel	3 ± 0.5	3.8 ± 0.08	0
		Aluminium	3 ± 0.07	3.8 ± 0.07	0
Actichlor	0.05%	PVC	3 ± 0.18	3.17 ± 0.16	0
		Vinyl	3 ± 0.08	3.42 ± 0.18	0
		Stainless Steel	3.23 ± 0.07	3.47 ± 0.15	0
		Aluminium	3 ± 0.07	2.79 ± 0.26	20%
Tristel	0.001%	PVC	3 ± 0.2	2.5 ± 0.17	63%
		Vinyl	3.11 ± 0.14	2.89 ± 0.1	43%
		Stainless Steel	3.3 ± 0.3	3.06 ± 0.6	0
		Aluminium	3.04 ± 0.11	3.6 ± 0.1	0

The results of this investigation have demonstrated that upon combination of higher strength disinfectants with low irradiance 405 nm light, the degree of spore inactivation as a result of synergistic oxidative effects is dependent on both the surface and the disinfectant. The greatest sporicidal effects were observed on aluminium surfaces upon combination of low irradiance light with 0.05% Actichlor, with an 11× increase in spore reduction in comparison to both controls, following exposure to 242 Jcm^{-2}. Enhanced sporicidal effects were observed with all disinfectant/ surface combinations with the exception of NaOCl on aluminium and PVC, and Tristel on aluminium.

In Figure 5.11a, when spores seeded on PVC were exposed to the combined action of 0.05% Actichlor and low irradiance 405 m light at 484 Jcm^{-2}, complete inactivation was observed (P=0.009). Comparatively, a 0.55 log_{10} and 1.08 log_{10} reduction was achieved with Actichlor and low irradiance light alone, respectively. As can be seen in Figure 5.12a, on PVC surfaces, the combined effect of 0.2% NaOCl gave similar results to that when NaOCl was used alone. Slightly enhanced sporicidal effects were observed on PVC coupons following combined exposure to 0.001% Tristel and low irradiance light at 484 Jcm^{-2}, with an increase of 1.37 log_{10} bacterial reduction than when exposed to 0.001% Tristel alone and no inactivation observed upon exposure to light alone.

On vinyl surfaces, significant synergy was observed upon combination of the low irradiance light and 0.001% Tristel, with complete inactivation achieved following exposure to a dose of 484 Jcm^{-2} (P=0.001) (Figure 5.13b), whilst no sporicidal activity was observed when exposed to light or 0.001% Tristel alone. Both 0.05% Actichlor and 0.2% NaOCl demonstrated a synergistic effect on vinyl surfaces with up to an 8.5× and 7× increase in spore inactivation in comparison to controls, respectively (Figure 5.11b and Figure 5.12b); however the results were not significant (P=>0.05).

All 3 disinfectants demonstrated a significant enhanced sporicidal effect on stainless steel, as demonstrated in Figures 5.11c, 5.12c and 5.13c, following exposure to a dose of 484 Jcm^{-2}, with a 2.24 log_{10}, 1.46 log_{10} and 1.47 log_{10} reduction (P<0.001) achieved in combination with 0.001% Tristel, 0.2% NaOCl and 0.05% Actichlor, respectively, in comparison to little to no inactivation when exposed to controls alone.

Enhanced sporicidal activity in the presence of 0.05% Actichlor following exposure to a dose of 242 Jcm^{-2} was observed on aluminium surface coupons, as previously discussed (Figure 5.11a), with almost complete inactivation observed (P<0.001). Upon combination of 0.2%

NaOCl and 0.001% Tristel with a dose of 484 Jcm^{-2} (Figure 5.12d and Figure 5.13d) the inactivation rates observed on aluminium surfaces were similar to that of the controls exposed to disinfectant alone.

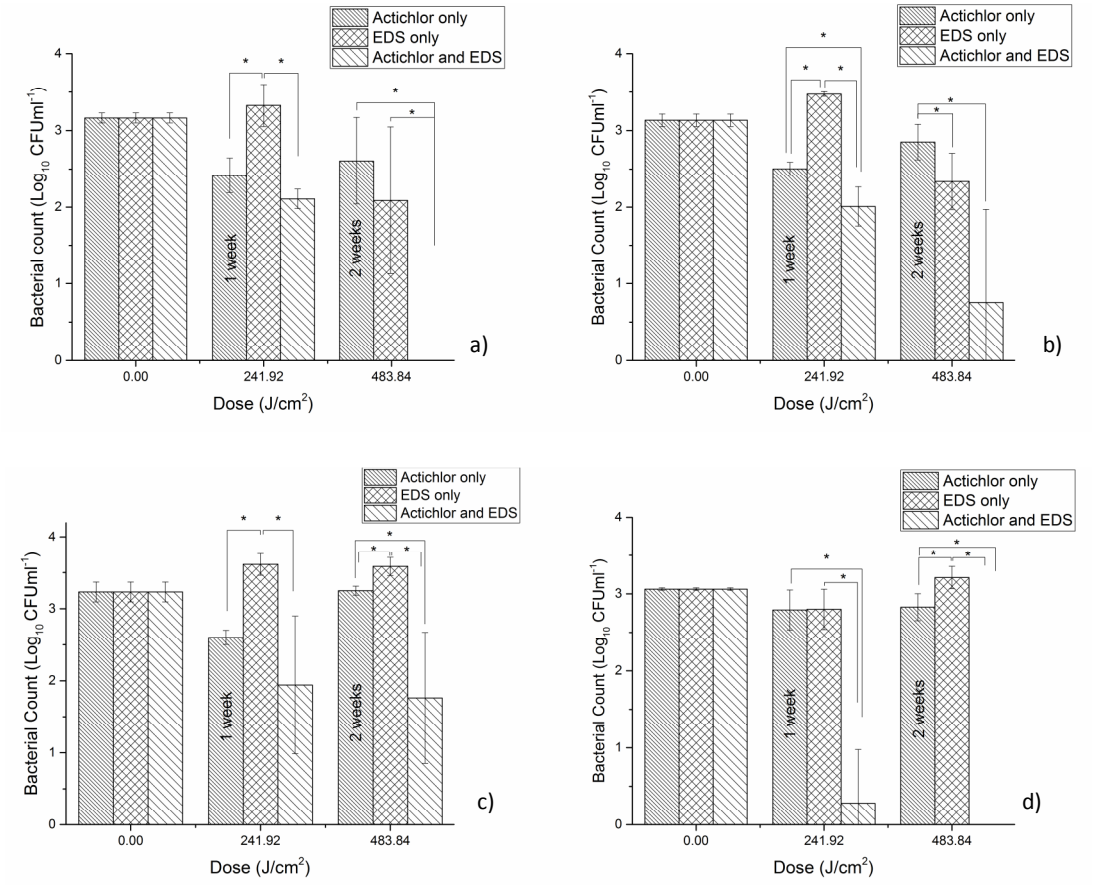

Figure 5.11 Spores seeded onto a) PVC b) Vinyl flooring c) Stainless steel and d) Aluminium exposed 0.05% Actichlor in combination with low irradiance 405 nm light (0.4 $mWcm^{-2}$). Control samples were exposed to (i) 405 nm light alone and (ii) disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represents significant bacterial inactivation compared to controls ($P \leq 0.05$). Each data point is a mean value ± SD (n=6).

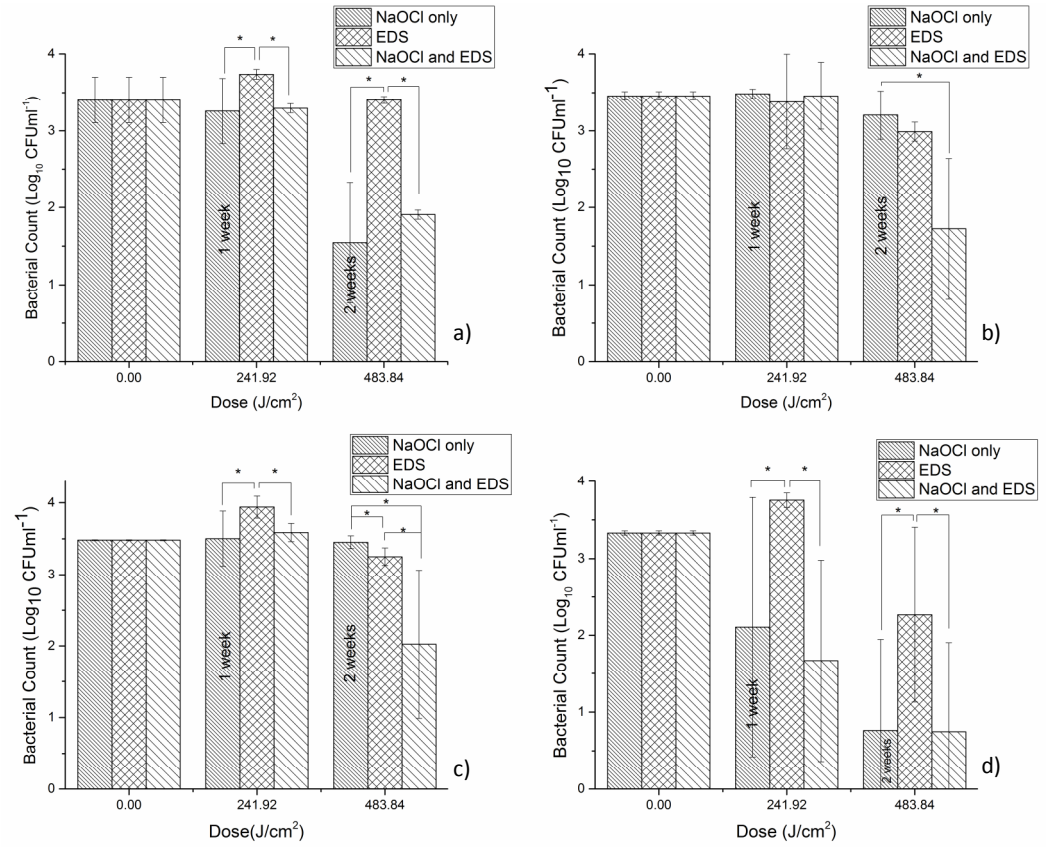

Figure 5.12 Spores seeded onto a) PVC b) Vinyl flooring c) Stainless steel and d) Aluminium exposed 0.2% NaOCl in combination with low irradiance 405 nm light (0.4 mWcm^{-2}). Control samples were exposed to (i) 405 nm light alone and (ii) disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represents significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

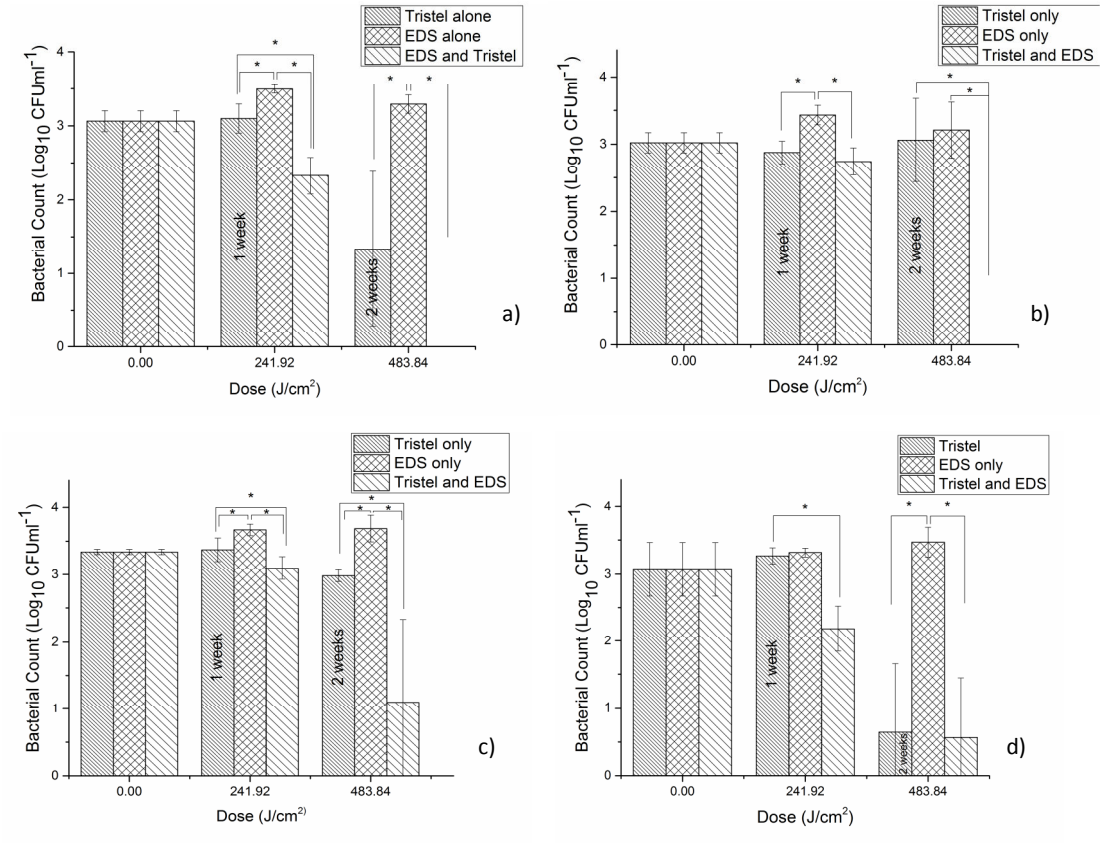

Figure 5.13 Spores seeded onto a) PVC b) Vinyl flooring c) Stainless steel and d) Aluminium exposed 0.001% Tristel in combination with low irradiance 405 nm light (0.4 mWcm^{-2}). Control samples were exposed to (i) 405 nm light alone and (ii) disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined. * represents significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

5.3. DISCUSSION

The investigation of the synergy between commonly used disinfectants and 405 nm light was carried out due to concerns associated with the ability of *C. difficile* to form highly resilient spores, which are able to survive in the environment for up to 5 months and withstand various treatments used to clean the clinical environment. In recent years, environmental contamination with spores has been shown to play a vital role in the transmission of *C. difficile* and as such there is a demand for novel technologies that can successfully decontaminate the clinical environment. The previous chapter has demonstrated that, although 405 nm light is capable of inactivating *C. difficile* spores, due to the high resilience of the spores a very high dose of 405 nm light is required for successful inactivation (2.43 $kJcm^{-2}$ required for a 3 $\log_{10}$ reduction). To achieve this dose using low irradiance light (of approximately 0.4 $mWcm^{-2}$), similar to the levels used by the HINS-light EDS for decontamination of the clinical environment, would require lengthy exposure periods, and whilst the system is designed to be used continuously, effective decontamination in a shorter timescale would be much more desirable. Therefore, to achieve sporicidal effects at doses similar to what would be used in the clinical environment the sporicidal effects of 405 nm light need to be enhanced. As such, inducing a synergistic effect could lead to enhanced sporicidal effects at lower irradiances of 405 nm light allowing for the successful decontamination of spores at lower irradiances safe for continuous human exposure.

Experiments were carried out to investigate if a synergistic effect could be achieved upon combination of 405 nm light and chlorine based disinfectants as both of these mechanisms result in bacterial inactivation via oxidation. It was hypothesised that combination of both inactivation mechanisms would result in accelerated bacterial inactivation. An advantage of using chlorine based disinfectants currently recommended for cleaning rooms of patients with CDI to enhance the sporicidal action of 405 nm light is that this would allow for the enhancement of current disinfection strategies, without replacing in house cleaning, with very little disruption to the daily routine of cleaning staff within the clinical environment.

Alongside resulting in enhanced decontamination of the patient environment without the disruption of current routines upon the implementation of this technology, there are concerns regarding the universal use of high strength chlorinated disinfectants and their inefficiency. As such, if a synergistic effect is observed upon combination of current

disinfectants with 405 nm light this could allow for the use of lower strength disinfectants whilst still resulting in effective sporicidal effects.

The results of this study support the hypothesis that the sporicidal efficacy of 405 nm light could be enhanced when used alongside hospital disinfectants recommended for use in the rooms of patients with confirmed CDI both in suspension and on a range of clinically relevant surfaces. The extent of the enhanced sporicidal activity is dependent on the disinfectant and the surface it is applied to.

5.3.1. Enhanced sporicidal activity of 405 nm light upon combination with disinfectants in suspension

Due to the ability of the highly resilient *C. difficile* spores to withstand inactivation by traditional cleaning detergents, high concentration chlorine based disinfectants are required for the cleaning of rooms of patients housing CDI. There are concerns associated with these high concentration disinfectants including health and safety issues alongside the potential for emerging resistance of pathogens to commonly used disinfectants. The use of lower concentrations of disinfectants due to the synergistic effect of 405 nm light could not only lead to enhanced spore inactivation at lower irradiances of 405 nm light, but could also allow for the use of lower strength disinfectants and, as such, minimise the health and safety problems associated with disinfectants.

Results of this study demonstrate that the use of 405 nm light in combination with the three selected disinfectants can lead to a considerable reduction in exposure time and concentrations of disinfectant required to eliminate *C. difficile* spores. Results demonstrated that in suspension 3-3.5 $\log_{10}$ reductions could be achieved following exposure to a dose of 1.62 $kJcm^{-2}$ (Figure 5.1, Figure 5.2 and Figure 5.3): an approximate 33% reduction in dose than that required when using 405 nm light exposure alone. Significantly, the levels of disinfectants that were used were deliberately selected so that they exerted negligible effects on the spores when applied alone – thus demonstrating that even low concentration disinfectants can induce the synergistic inactivation effect. Together, the oxidative effects exerted by both the disinfectants and the 405 nm light exposure when combined, prove to be more lethal than when applied independently, resulting in accelerated spore inactivation.

An important aspect for consideration in this study is the concentrations of disinfectants used to assess the synergistic activity with 405 nm light. As discussed, the concentrations

used were selected based on the concentrations that induced no significant effect on the spores over the maximum exposure period required to achieve complete inactivation of the *C. difficile* spores using 405 nm light alone, i.e. 3-hr (Table 5.1). These concentrations proved to be different for each disinfectant (0.1% NaOCl; 0.001% Actichlor; 0.0001% Tristel), and in all cases, were lower than the recommended concentrations for use within the clinical environment. For hospital use, concentrations of 0.1% sodium hypochlorite (1,000 ppm available chlorine), 0.1% Actichlor (1,000 ppm available chlorine), and 0.01% Tristel (100-120 ppm available chlorine) are recommended for use (Jarbis, 2007; Vohra & Poxton, 2011; Dawson et al., 2011). However, in the case of NaOCl, although a concentration of at least 1,000 ppm is currently recommended for high level disinfection, several reports have recommended concentrations of 1,000-100,000 ppm for high level disinfection and sporicidal activities and this is likely to be the case with other chlorinated disinfectants (Barbut et al., 2009; Heling et al., 2001; Gerding et al., 2008). Although the concentrations used in this study were up to 100 times more dilute than recommended for use in the clinical environment, when used in combination with 405 nm light significant sporicidal activity was achieved.

No previous studies have investigated the potential synergistic effect of visible light alongside disinfectants, with most synergistic studies focusing on UV light alongside chlorinated disinfectants. Cho et al. (2006) investigated the possible synergistic effect of chlorine and ozone as well as that of chlorine when used in conjunction with UV light. This study reported that accelerated inactivation of *B. subtilis* spores was observed due to the synergistic effect of free chlorine following treatment of spores with chlorine dioxide or ozone. The pre-treatment with chlorine dioxide/ozone resulted in synergy as chemical disinfectants must access the cortex and spore core through the spore coat in order to damage the vital components such as DNA and proteins; therefore the primary disinfectant accelerated inactivation by facilitating the subsequent transport of the secondary disinfectant due to alterations in the physical and chemical structures in the spore coat (Cho et al., 2006). However, when free chlorine was combined with UV light a synergistic effect was not observed. It is suggested that this is because UV light inactivates microorganisms by damage to DNA and RNA which are vital for the synthesis of key proteins and reproduction, whilst it has been suggested that the major mechanism of free chlorine and chlorine dioxide inactivation of *B. subtilis* spores is not by DNA damage and thus the differences in inactivation mechanisms led to the absence of a synergistic effect.

(Young & Setlow, 2003). An additive effect has been reported for UV light when it is used in conjunction with NaOCl (0.1 ppm), Chlorine dioxide (1 ppm) and peracetic acid (2.3 ppm), with synergy reported in conjunction with H_20_2 due to the production of hydrogen radicals produced in the presence of UV light (Vankerckhoven et al., 2011). Baylis and Waites demonstrated the synergistic effect of H_20_2 and far UV (254 nm) against spores of *B. subtilis*, with 30 seconds irradiation using UV alone achieving a 81% reduction in spores, whilst in the presence of H_20_2 (1 g/100 ml), a 99.99% reduction in spore population was achieved (Bayliss & Waites, 1979). This synergistic effect was further demonstrated with the spores of 13 further *Bacillus* species and the spores of *Clostridium sporogenes,* with up to a 2,000-fold greater inactivation achieved when UV light was combined with H_20_2 than when UV was used alone (Bayliss & Waites, 1979). In support of this, enhanced synergistic inactivation of *B. subtilis* spores was achieved in combination of hydrogen peroxide (0.5-1% W/V) and UV light at a wavelength of 254 nm, at concentrations of hydrogen peroxide which were not sporicidal in the absence of UV (Gardner & Shama, 1998). In addition to UV light, peracetic acid has also been found to have a synergistic effect when used alongside H_20_2, with enhanced killing of *B. subtilis* demonstrated upon combination of the two (Leggett et al., 2015). This is thought to be due to the damage to the spore coat by H_20_2 allowing for better penetration of peracetic acid into the spore (Leggett et al., 2015).

5.3.2. Comparison of the Sporicidal Efficacy of recommended Concentrations of Disinfectants in the Absence and Presence of 405 nm Light

Investigations were carried out to directly compare the synergistic effect that would be observed in the clinical environment upon exposure of spores to the current recommended concentration of disinfectants alongside 405 nm light. Spores were exposed to disinfectants prepared at concentrations recommended for sporicidal use, these investigations were carried out in suspension. Currently, Tristel is recommended at a concentration of 0.01% for an exposure time of 5 minutes, whilst 0.1% Actichlor is recommended for an exposure time of 10 minutes. Sodium hypochlorite is recommended for sporicidal used at a range of concentrations and length of exposures, as previously discussed in Section 2.4.2.1 and 5.3.1 (Gerding et al., 2008; Barbut et al., 2009; Heling et al., 2001). It is important to question if these extended time periods are representative of realistic cleaning expectations for the rooms of patients with CDI.

As shown in Table 5.2, Actichlor achieved a complete 3.13 log_{10} reduction in spores following 1 minute exposure – this is a likely contact time period in the hospital environment. 1% NaOCl also demonstrated high sporicidal efficacy, with a complete 3.42 log_{10} reduction following 3 minutes exposure. The results in Table 5.2 show that this was further enhanced by 405 nm light with a 88% reduction in spores observed after 1 minute following exposure to 1% NaOCl alone whilst in the presence of 405 nm light this was accelerated to a 97% inactivation of spores. Tristel was the least efficient disinfectant for the inactivation of *C. difficile* spores with a 1 log_{10} population remaining viable following exposure to the disinfectant for 10 minutes. After 7.5 minutes exposure to 0.01% Tristel a 95% reduction was observed. The inactivation of spores in the presence of Tristel was enhanced by 405 nm light with a 98% reduction in spore count observed following exposure for 7.5 minutes, whilst a complete 100% inactivation was observed following 10 minutes exposure to 0.01% Tristel in conjunction with 405 nm light.

5.3.3. Enhanced Sporicidal Activity of High Irradiance 405 nm Light Upon Combination with Disinfectants on Surfaces

Comparative surface tests were carried out as such tests are commonly preferred for evaluating sporicides, as suspension tests are often considered to be less rigorous than carrier tests. There is little published with regards to the direct comparison of the efficacy of sporicidal disinfectants on spores in suspension to that of spores seeded on surfaces. Perez et al. (2005) stated that nothing had been published with regards to the survival of *C. difficile* spores on inert surfaces following treatment with disinfectants, all that was available was a range of papers reporting a decrease in CDI as a result of the use of oxidative disinfectants (Perez et al., 2005). As the environment and surfaces can act as a constant reservoir for *C. difficile* spores, and with spores surviving on surfaces for up to 5 months, it is of vital importance to compare the efficacy of disinfectants on surfaces, as well as in suspension as some disinfectants that are effective in suspension may not be as effective on surfaces. This is especially important if the disinfectant is intended for use primarily on surfaces. For example, the effectiveness of chlorine dioxide against spores at doses as low as 100 ppm following 30 seconds was demonstrated in suspension whilst on surfaces chlorine dioxide was shown to be ineffective at doses as high as 600 ppm following 10 minutes exposure against spores (Perez et al., 2005).

In the present study the results in Figure 5.4, Figure 5.5, Figure 5.6 and Figure 5.7 demonstrate that *C. difficile* spores seeded onto surfaces demonstrated an increased

resilience to the combinative effects of 405 nm light and low-strength disinfectants in comparison to when in suspension (Figures 5.1-5.3). A dose of 2.43 $kJcm^{-2}$ achieved almost complete inactivation of spores in suspension in combination with all 3 disinfectants, whilst the same dose applied to surfaces (2.43 $kJcm^{-2}$) in combination with disinfectants did not achieve near inactivation of spores on all surfaces, with the extent of the enhanced sporicidal activity dependent on both the disinfectant and the surface it is applied to.

This increased resilience of spores to disinfectants when seeded on surfaces has previously been reported by Sagripanti et al. (2007) in which their study reported that *P. aeruginosa* was approximately 300-fold more resistant to disinfectants when inoculated on contaminated surfaces than in suspension. Oule et al. (2012) reported that, when exposed to a polyhexomethylene-guanidine hydrochloride based disinfectant (Akwaton), *B. subtilis* spores were more susceptible when in suspension with distilled water than those dried on stainless steel and glass surfaces (Oulé et al., 2012). The explanation for this may be that in suspension, spores are hydrated and therefore more sensitive to the immediate effects of disinfectants, whereas in the dry state spore hydration is required before inactivation of the components can occur. In support of this, studies have reported that sanitizers and biocides are more effective against microbes in suspension than on surfaces (Block, 2004; Block et al., 2000; Carballo & Araùjo, 2012). In contrast to this, a study by Wesgate et al. (2016) stated that there was no statistical differences in test performance between surface and suspension experiments (Wesgate et al., 2016). A study by Andre et al. (2012) compared the efficacy of a biocide against *Clostridium sporogenes, Geobacillus stearothermophilus* and *Moorella thermoautotrophica*, both in suspension and inoculated onto stainless steel surfaces with results demonstrating that the foam biocide successfully inactivated all 3 organisms in suspension, whilst on stainless steel surfaces it effectively inactivated *C. sporogenes* spores but had a reduced decontamination effect on other species. This supports the results of this study, demonstrating that the efficiency of disinfectants may differ in suspension or on surfaces and that for research with regards to disinfectants for cleaning hospital surfaces it is important that this is taken into consideration (Andre et al., 2012).

The results of this study demonstrated that the greatest synergy was observed with the combination of high irradiance 405 nm light with NaOCl and Actichlor on PVC surfaces. Enhanced sporicidal effects were further observed on all other clinically relevant surfaces,

with the exception of PVC and vinyl surface coupons when 0.0001% Tristel was used in combination with light.

Enhanced sporicidal activity was observed when high irradiance 405 nm light (225 $mWcm^{-2}$) was combined with 0.001% Actichlor and 0.1% NaOCl on both vinyl and PVC surfaces. On PVC surface coupons in the presence of 0.001% Actichlor complete inactivation was achieved at a dose 1.62 $kJcm^{-2}$, whilst complete inactivation was achieved following exposure to a dose of 810 Jcm^{-2} in the presence of 0.1% NaOCl. Comparatively, the light-only exposed control required 1.5× and 3× this dose, respectively, to achieve a 2.55 log_{10} reduction.

Following exposure to a dose of 1.62 $kJcm^{-2}$ and 2.43 $kJcm^{-2}$ alone on vinyl flooring spores demonstrated a 91% and 93% reduction. In comparison a 99% reduction was achieved upon combination with 0.1% NaOCl (1.62 $kJcm^{-2}$) and a 100% reduction (2.43 $kJcm^{-2}$) achieved in combination with 0.001% Actichlor.

Tristel demonstrated no synergy in combination with 405 nm light on PVC and vinyl, whilst all three disinfectants demonstrated only slight synergy on stainless steel. High resistance on stainless steel could be due to reflection of 405 nm light by stainless steel surfaces. Furthermore, the peak to valley height values of stainless steel may affect the rate of inactivation, due to bacterial entrapment within grooves which can minimise exposure of bacterial spores to both 405 nm light and disinfectants. The hydrophobic nature of stainless steel could further result in reduced surface area for exposure due to the contact angle on the surface due to droplet formation as opposed to spreading of the suspensions.

The only surface on which enhanced sporicidal effects were observed when 0.0001% Tristel was used in combination with 405 nm light was aluminium, with 10-100% increase in sporicidal activity in comparison to controls. 0.1% NaOCl also demonstrated enhanced sporicidal activity on aluminium with a ~2× increase in bacterial inactivation in comparison to the controls alone following exposure to a dose of 810 Jcm^{-2}.

The results of this study provide data indicating that different surface materials may result in altered bactericidal activity of disinfectants.

5.3.1. Enhanced Sporicidal Activity of Low Irradiance 405 nm Light Upon Combination with Disinfectants on Surfaces

On all surfaces, upon combination of the low concentration disinfectants (0.1% NaOCl, 0.001% Actichlor and 0.0001% Tristel) with low irradiance 405 nm light, as demonstrated in

Figure 5.8, Figure 5.9 and Figure 5.10, very little enhanced sporicidal inactivation was observed. As the initial proof of concept investigations used a much higher irradiance of 405 nm light (225 $mWcm^{-2}$ for both suspension and surface investigations), an explanation for this is likely to be that the much lower doses of the 405 nm light, in combination with the very low concentrations of disinfectants, were not enough to induce a synergistic oxidative effect. This irradiance of 405 nm light (0.4 $mWcm^{-2}$) was similar to those previously successfully utilised for environmental decontamination applications in the clinical environment and has been deemed safe for human exposure (Bache et al., 2012; Maclean et al., 2013; Maclean et al., 2010). To overcome the barrier of the very low irradiance used in comparison to the initial proof of concept studies, slightly raised concentrations of the disinfectants of interest were used. The concentrations of disinfectants used for the investigations, as can be seen in Figure 5.11, Figure 5.12 and Figure 5.13, were 0.2% NaOCl, 0.01% Tristel and 0.05% Actichlor – still lower than that recommended for sporicidal effects for housekeeping. These results have established that under conditions similar to that of the clinical environment, at low concentrations of all 3 disinfectants enhanced sporicidal inactivation was observed, surface dependant.

In the case of 0.05% Actichlor used in conjunction with the low irradiance 405 nm light, synergy was observed on all surfaces investigated, with the greatest synergy observed on aluminium surfaces. Following exposure to a dose of 242 Jcm^{-2} in combination with 0.05% Actichlor, almost complete inactivation was observed on aluminium surfaces, whilst little to no inactivation was observed for both controls. Combined exposure to 484 Jcm^{-2} achieved complete inactivation on PVC surface coupons, a 2.38 log_{10} reduction on vinyl surfaces and a 1.47 log_{10} reduction on stainless steel whilst controls showed a maximum spore reduction of 0.77 log_{10}.

0.2% NaOCl demonstrated synergy when combined with 484 Jcm^{-2} low irradiance light on vinyl flooring with a 96% inactivation in spores achieved, in comparison to 39-69% inactivation achieved by the controls. Similarly, enhanced inactivation on stainless steel was observed following exposure to the same dose with a 91% spore reduction observed, whilst controls achieved an 11-43% reduction in viable count.

0.001% Tristel combined with low irradiance light exhibited synergy on all 4 surfaces, however on aluminium surfaces this was only observed in combination with a dose of 242 Jcm^{-2}. On PVC and vinyl surface coupons complete spore inactivation was achieved following exposure to 484 Jcm^{-2}, with no reduction in spores in the case of the controls on

vinyl surfaces and a 1.73 log_{10} reduction when exposed to 0.001% Tristel alone on PVC surfaces. On stainless steel surface coupons a 2.24 log_{10} reduction was achieved upon combined exposure to 484 Jcm^{-2} low irradiance light (little to no reduction when observed for the controls).

The results in Section 5.2.4.3 demonstrated that lower doses of low irradiance 405 nm light achieved greater inactivation of *C. difficile* spores on many of the surfaces when combined with slightly higher strength chlorine based disinfectants in comparison to that observed previously upon combination with high irradiance light. Spores seeded onto surface coupons were exposed to a maximum dose of 484 Jcm^{-2} low irradiance 405 nm light in this section of the study, in comparison to 2.43 $kJcm^{-2}$ when using high irradiance exposures, yet both were capable of achieving up to 3 log_{10} reductions in spore inactivation. In combination with 0.01% Actichlor and high irradiance 405 nm light complete inactivation was observed on PVC 1.62 $kJcm^{-2}$, whilst 0.05% Actichlor combined with low irradiance exposure at a 3-fold lower dose achieved the same ~3 log_{10} $CFUml^{-1}$ reduction. On stainless steel low irradiance 405 nm light combination with 0.2% NaOCl achieved the same spore reduction as high irradiance light at a 5-fold lower dose. On vinyl flooring, the combinative effects of low irradiance light and 0.2% NaOCl resulted in only a 1 log_{10} lower reduction in spores than that was observed following exposure to a 70% higher dose of high irradiance 405 nm light in combination with 0.1% NaOCl.

The results in Table 5.5 show a summary of these findings that have been discussed, highlighting the surfaces on which a complete 3 log_{10} $CFUml^{-1}$ spore reduction was achieved, and the doses and disinfectants required to achieve complete inactivation. It is of significance to note that neither high irradiance nor low irradiance 405 nm light alone resulted in spore inactivation on any of the surfaces investigated at the doses applied in this study.

Table 5.6 then proceeds to demonstrate the increased spore reduction, in comparison to that achieved upon to 405 nm light alone, on all surfaces in combination with all disinfectants investigated.

Table 5.5 The dose of high irradiance or low irradiance 405 nm light required to achieve a complete 3 log_{10} $CFUml^{-1}$ reduction in *C. difficile* spores seeded onto clinically relevant surfaces. A dash represents that complete spore inactivation was not achieved

Dose of light required in combination with disinfectants to achieve complete spore inactivation (Jcm^{-2})						
Surface material	**High irradiance light (225 $mWcm^{-2}$)**			**Low irradiance light (0.4 $mWcm^{-2}$)**		
	0.1% NaOCl	**0.001% Actichlor**	**0.0001% Tristel**	**0.2% NaOCl**	**0.01% Actichlor**	**0.001% Tristel**
PVC	810	1620	-	-	484	484
Vinyl	-	2430	-	-	-	484
Stainless steel	-	-	-	-	-	-
Aluminium	-	-	-	-	484	-

Table 5.6 The % increased spore inactivation in comparison to that achieved upon exposure to 405 nm light alone, at doses previously shown in Table 5.5. If the dose is not stated in Table 5.5 complete spore inactivation was not achieved, and as such the highest dose used in the studies for each light source is to be assumed (2.43 $kJcm^{-2}$ and 484 Jcm^{-2} for high irradiance and low irradiance light, respectively)

% increase in spore reduction upon combination with disinfectants in comparison to 405 nm light alone						
Surface material	High irradiance light (225 $mWcm^{-2}$)			Low irradiance light (0.4 $mWcm^{-2}$)		
	0.1% NaOCl	0.001% Actichlor	0.0001% Tristel	0.2% NaOCl	0.05% Actichlor	0.001% Tristel
PVC	6	63	0	70	87	67
Vinyl	7	7	0	27	10	100
Stainless steel	7	14	10	48	94	97
Aluminium	0	3	5	47	100	99

This 3-5× increased dose required when high irradiance 405 nm light was used may be partly explained by the reduced time of exposure associated with high irradiance treatments (a maximum of 3 hours, in comparison to low irradiance treatments which were carried out for a maximum of 2 weeks). Thus other factors such as desiccation may play a role in the inactivation of spores, due to weakening of the spore coat by disinfectants over prolonged periods of time alongside the stresses as a result of the constant exposure to 405 nm light, resulting in enhanced inactivation over long periods of time. Additionally, higher concentration disinfectants were used - although still having minimal inactivation on the spores over prolonged periods of times - thus the enhanced synergistic oxidative effects will play a role in this enhanced inactivation at lower doses of violet-blue light.

A further possible explanation is that, when exposed to very high irradiances of 405 nm light, there is surplus light due to the limited amounts of porphyrins found naturally within the cells, and as such there is a threshold to the amount of photons that can be absorbed. Upon exposure to low irradiance light it is likely that all/the majority of the light photons are being absorbed. This demonstration that, at an irradiance safe for human exposure, spore inactivation can be achieved using 405 nm light when used in combination with disinfectants at concentrations lower than currently recommended for cleaning, is a significant finding. This is very important data with regards to the implementation of this technology in the clinical environment, as this decontamination system would be used throughout the day alongside normal hospital lighting.

5.3.2. CONCLUSIONS

Previous solutions for the decontamination problems associated with current in house strategies have suggested that either increasing contact times of disinfectants during cleaning procedures or using higher strength chlorine based disinfectants could lead to enhanced bacterial inactivation. However, during routine daily cleaning within the clinical environment long contact times are unlikely due to time constraints on cleaning. Furthermore increased contact time could result in damage to materials, therefore it is likely that increased chlorine content is a more efficient practise to minimise the *C. difficile* burden; however the aim for current *C. difficile* practices is to move away from higher chlorine content (Ungurs et al., 2011). The synergistic sporicidal effect demonstrated in this chapter could provide a solution allowing for reduced chlorine concentrations within recommended disinfectants, without the need for increased contact time of disinfectants on surfaces.

This demonstration that a novel decontamination system - that allows for the continuous cleaning of the environment in the presence of patients and staff without causing any disruptions within the clinical environment - can successfully inactivate *C. difficile* spores which have been shown to be resistant to various treatments and play a vital role in the transmission of disease within the hospital, could greatly improve the decontamination of the patient environment. This decontamination system would allow for the continuous killing of spores, even between cleaning. This is especially of importance following the shedding of vegetative cells and spores from the patient to the environment following bowel movements, reducing levels in the patient environment between cleaning and as such reducing the further propagation of the disease. Additionally this technology could compensate for inefficiencies in hospital cleaning, allowing for sporicidal action in areas where wiping may have been inefficient through the combinative oxidative effects previously demonstrated.

Employment of 405 nm light could also permit the use of lower strength disinfectants. In turn this could lead to reduced corrosion of surfaces within the hospital, which can harvest spores, and also reduce the harmful effects caused by high strength disinfectants on healthcare workers.

CHAPTER 6

Factors Influencing the Susceptibility of *C. difficile* Spores to 405 nm Light

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The previous two chapters have demonstrated the efficacy of antimicrobial 405 nm light for the inactivation of *C. difficile* vegetative cells and spores, and their enhanced susceptibility upon combination with low-strength chlorine-based disinfectants.

This chapter aims to investigate some of the factors that may influence spore susceptibility to 405 nm light. The chapter will be split into 3 subsections:

- The first section focuses on providing evidence of the mechanism of inactivation of 405 nm light, and the vital role of oxygen for photodynamic inactivation;
- The second section investigates the influence of triggering spore germination on spore susceptibility to 405 nm light; and
- The third section assesses how the germicidal efficacy of 405 nm light for spore inactivation compares to that of UV-C light.

6. GENERAL

The work in Chapter 4 demonstrated the efficacy of 405 nm light for the successful inactivation of both the vegetative cells and spores of *C. difficile* and *B. cereus*. This is of particular importance due to the problems arising as a result of spore resilience, alongside the many drawbacks of current decontamination technologies. As previously discussed in Chapter 2 and Chapter 4, it has been hypothesised that the inactivation of microorganisms upon exposure to 405 nm light is reliant on the presence of naturally occurring endogenous porphyrins and oxygen leading to the production of ROS, and ultimately cell death. This chapter investigates the requirement of oxygen for the inactivation of *C. difficile* spores, allowing for further insight into the mechanism of inactivation of 405 nm light. Furthermore, this chapter investigates the presence of naturally occurring porphyrins within *C. difficile* vegetative cells and spores to compare if there are any crucial differences within the cells that may cause the enhanced inactivation of vegetative cells; it is known that the type and amounts of porphyrin present result in differing photo-inactivation rates in different bacterial species (Yin et al., 2013).

6.1. METHODOLOGY

To assess the oxygen dependence of 405 nm light inactivation, spores were exposed to 405 nm light under three different conditions:

- aerobic conditions in the presence of reactive oxygen species (ROS) scavengers;
- anaerobic conditions in the absence of ROS scavengers; and,
- anaerobic conditions in the presence of ROS scavengers.

Ascorbic acid (Sigma Aldrich, UK) (30mM) was selected as the reactive oxygen species (ROS) scavenger for this study, and was used at a concentration of 30 mM. Ascorbic acid was selected for use as a scavenger as it is one of the strongest radical scavengers, reducing oxygen, nitrogen and sulphur based radicals and superoxide. Additionally, ascorbic acid was selected due to its previous use in experiments by Maclean et al. (2008) when investigating the role of oxygen in bacterial inactivation following exposure to 405 nm light, thus allowing for comparisons of the results obtained (Maclean et al., 2008; Niki, 1991).

6.1.1. TREATMENT METHOD

C. difficile spores were prepared as described in Section 3.3.2.1. The bacterial preparation was customised for each oxygen dependence experiment accordingly, as follows:

- **Exposed under anaerobic conditions in the absence of ROS scavengers**

Bacterial spores were suspended in 9 ml pre-reduced PBS that had been exposed to reduced conditions for 24 hours prior to bacterial preparation. Following this, serial dilutions were made using 9 ml volumes of pre-reduced PBS, to give a resultant 10^3 $CFUml^{-1}$ bacterial spore suspension. Following preparation, the bacterial suspension was transferred to a 12-well multi-plate which was transferred into an anaerobic atmosphere generation cabinet and placed under the ENFIS Photostar Innovate UNO 24 system at a distance of 5 cm as described previously in Section 3.5.1. The samples were then exposed to 405 nm light at an irradiance of 225 $mWcm^{-2}$ for increasing time durations. Controls were exposed to identical conditions but were not exposed to 405 nm light.

- **Exposed under anaerobic conditions in the presence of ROS scavengers**

Ascorbic acid was used in conjunction with the anaerobic cabinet to further deplete the oxygen levels within samples (to minimise the levels of any residual oxygen within the samples following preparation on the lab bench). For bacterial preparation, bacterial spores were resuspended into 9 ml pre-reduced PBS that had been exposed to reduced conditions for 24 hours prior to bacterial preparation. Following this, serial dilutions were made into 9 ml PBS containing 30 mM ascorbic acid, to give a resultant 10^3 $CFUml^{-1}$ bacterial suspension. Following preparation, the bacterial suspension was transferred to a 12-well multi-plate which was transferred into an anaerobic atmosphere generation cabinet and placed under an ENFIS Photostar Innovate UNO 24 system, as before. Controls were exposed to identical conditions but were not exposed to 405 nm light. A further control was resuspended in PBS and exposed to identical conditions but was not exposed to 405 nm light, allowing for a comparison of the populations of the controls suspended in 30 mM ascorbic acid to that of controls suspended in PBS to ensure that the ascorbic acid alone had no sporicidal effects on the suspension.

- **Exposed under aerobic conditions in the presence of ROS scavengers**

To investigate the effects of the ROS scavenger alone, exposures were repeated in the presence of ascorbic acid but on the lab bench under aerobic conditions. For this the bacterial suspensions were prepared as previously for the experiments using ascorbic acid

under anaerobic conditions, however the 12-well-plate was placed under the ENFIS Photostar Innovate UNO 24 system on the laboratory workbench and the 30 mM ascorbic acid suspension was not pre-reduced. The samples were then exposed to 405 nm light at an irradiance of 225 $mWcm^{-2}$, for increasing time durations. Control samples were exposed to identical conditions but not exposed to 405 nm light. A further control was resuspended in PBS and exposed to identical conditions but was not exposed to 405 nm light to ensure that ascorbic acid had no sporicidal effects on the suspension.

6.1.2. Plating and Enumeration

Following exposure to the desired dose, both the sample and the control were plated onto pre-reduced blood agar plates and incubated at 37°C for 48 hours under anaerobic conditions. Samples were then enumerated and viable bacterial counts recorded as log_{10} $CFUml^{-1}$, as discussed in Section 3.7.2. Experiments were repeated in triplicate.

6.1.3. Detection of endogenous porphyrin activity

Experiments were conducted in an attempt to detect the presence of naturally occurring endogenous porphyrins within both the vegetative cells and spores of *C. difficile.* For this, a 10^6 $CFUml^{-1}$ population of each sample was prepared, as described in Sections 3.3.1 and 3.3.2.3.

Porphyrin extraction

The vegetative cell and spore suspensions, prepared as previously described, in Sections 3.3.1 and 3.3.2.3, were centrifuged at 4300 rpm for 10 minutes and washed with PBS twice to ensure removal of all nutrient growth media which could possibly have an effect on the spectrophotometric readings. The supernatant was discarded and the resultant pellets were then resuspended in 1 ml 1% SDS – 0.1 M NaOH, vortexed and transferred into Eppendorf tubes. The samples were left for 18-24 hours covered in foil at room temperature to lyse the cells and allow for the release of intracellular porphyrins into the extracellular medium for analysis by fluorescence spectrophotometry. Following incubation, samples were centrifuged at 8000 ×g for 10 minutes. The supernatant was then analysed using a Shimadzu fluorescence spectrophotometer (RF-530-PC, America) using an excitation wavelength of 405 nm with the emission spectra measured between 500-800 nm.

The fluorescence spectrophotometer typically consists of a source of light, a monochromator to select the required frequency for excitation, a sample holder, a second

monochromator and a photomultiplier to analyse the fluorescence light (Rho, 1972) . With the fluorescent spectrophotometer, one can either fix the frequency of exciting light and measure the spectrum of fluorescence emitted by the sample, or set the fluorescence monochromator on the frequency of the fluorescence band of the substance and observe how the intensity of this fluorescence varies with the frequency of the exciting light used. The fluorescence spectrophotometer passes a beam through the suspension of interest, in this case at a wavelength of 405 nm, resulting in the excitation of porphyrins within the suspension. Porphin, the basic ring structure of porphyrins, contains conjugated double bonds, therefore in acid solution and in organic solvents, porphyrins are highly fluorescent in the red and infrared regions of the spectrum (Rho, 1972). Upon excitation of the endogenous porphyrin, following the absorption of a photon, an electron goes into a higher electronic state and the molecule is excited. The molecule remains in its electronic excited state for nanoseconds, and then it falls back to its ground state resulting in the emission of a photon, which has a longer wavelength than the photon used for excitation, this results in the emission of light which is then measured.

6.2. Results: The Investigation into the oxygen dependence of 405 nm light on *C. difficile* inactivation

6.2.1. Exposure of spores to 405 nm light under anaerobic conditions in the absence of ascorbic acid

When *C. difficile* spores were exposed to 405 nm light under anaerobic conditions in the absence of an oxygen scavenger, a significant 1.9 $\log_{10}$ reduction ($P<0.001$) was observed following exposure to a dose of 1.62 $kJcm^{-2}$, and this is shown in Figure 6.1. A complete reduction of 3 $\log_{10}$ was observed following exposure to a dose of 2.43 $kJcm^{-2}$ ($P<0.001$), this was similar to the 3.3 $\log_{10}$ reduction in inactivation under aerobic conditions previously demonstrated in Section 4.1.4.

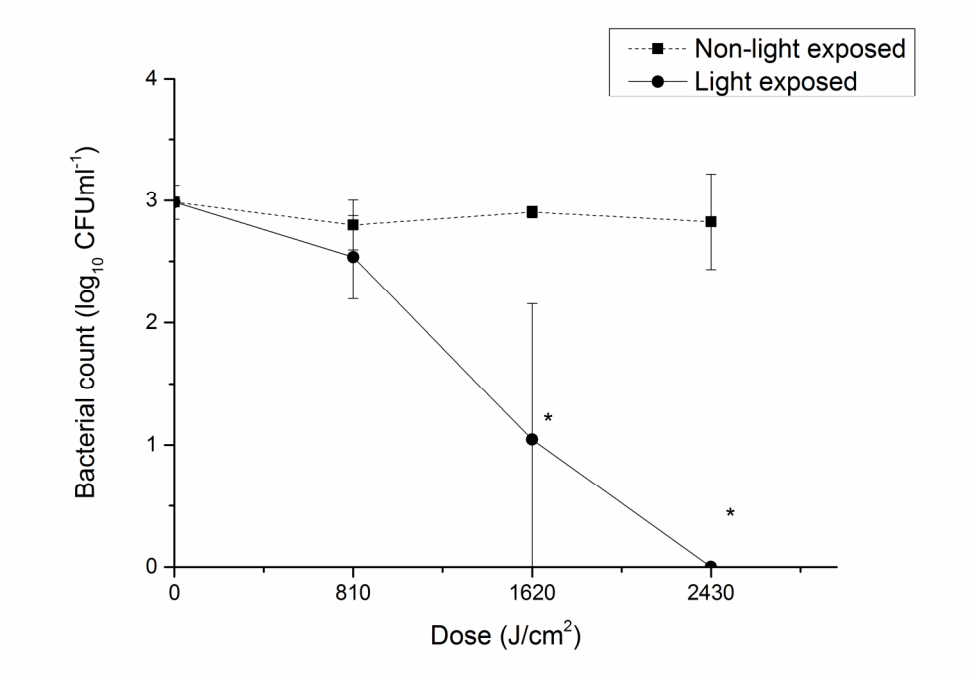

Figure 6.1 The inactivation of *C. difficile* spores exposed to 225 mWcm^{-2} 405 nm light under anaerobic conditions. Control samples were non-light exposed for the equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

6.2.2. EXPOSURE OF SPORES TO 405 NM LIGHT UNDER ANAEROBIC CONDITIONS IN THE PRESENCE OF ASCORBIC ACID

In order to ensure that there was no residual oxygen within the media upon exposure to 405 nm light under anaerobic conditions, an oxygen scavenger was added to the bacterial spore suspensions. The results from this experiment are shown in Figure 6.2. Following exposure to a dose of 2.43 kJcm^{-2}, a significant reduction of only 0.79 $\log_{10}$ CFUml^{-1} was observed under anaerobic conditions in the presence of an oxygen scavenger (P=0.03). Ascorbic acid alone had no detrimental effects on the spore suspension.

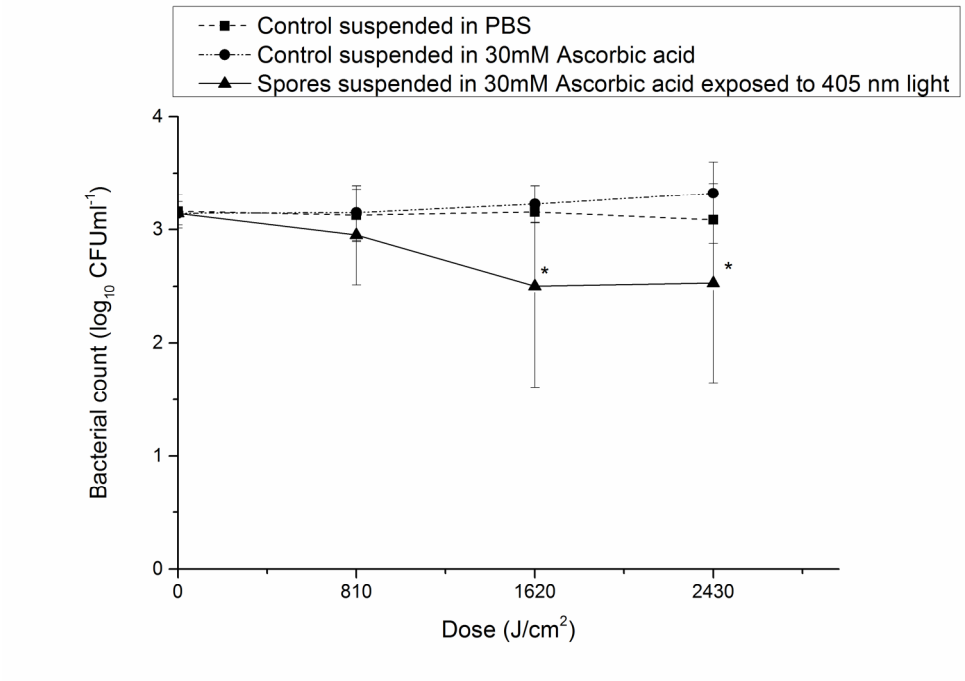

Figure 6.2 The inactivation of *C. difficile* spores exposed to 225 mWcm^{-2} 405 nm light under anaerobic conditions in the presence of 30 mM ascorbic acid ROS scavenger. PBS alone, and PBS with ascorbic acid were used as controls to demonstrate that 30 mM ascorbic acid had no effect on the spores. Control samples were non-light exposed for the equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to non-light exposed control suspended in 30mM Ascorbic acid (P≤0.05). Each data point is a mean value ± SD (n=6).

6.2.3. EXPOSURE OF SPORES TO 405 NM LIGHT IN AEROBIC CONDITIONS IN THE PRESENCE OF ASCORBIC ACID

In comparison to the results observed in 6.2.2, almost complete inactivation was observed when light exposure was conducted under aerobic conditions in the presence of ascorbic acid (Figure 6.3). This was carried out to assess to effects of ascorbic acid alone on the inactivation mechanism of 405 nm light. In the presence of the ROS scavenger under aerobic conditions, a significant 1.93 $\log_{10}$ (P=0.018) reduction was achieved following exposure to a dose of 1.62 kJcm^{-2}. In comparison to the inactivation kinetics of spores in anaerobic conditions exposed to 405 nm light in the absence of oxygen scavengers, the inactivation is similar to that of spores suspended in ascorbic acid whilst in aerobic conditions, with significant 3.3 $\log_{10}$ and 2.8 $\log_{10}$ (P<0.001) reductions achieved following exposure to a dose of 2.43 kJcm^{-2}, respectively - complete inactivation in both cases.

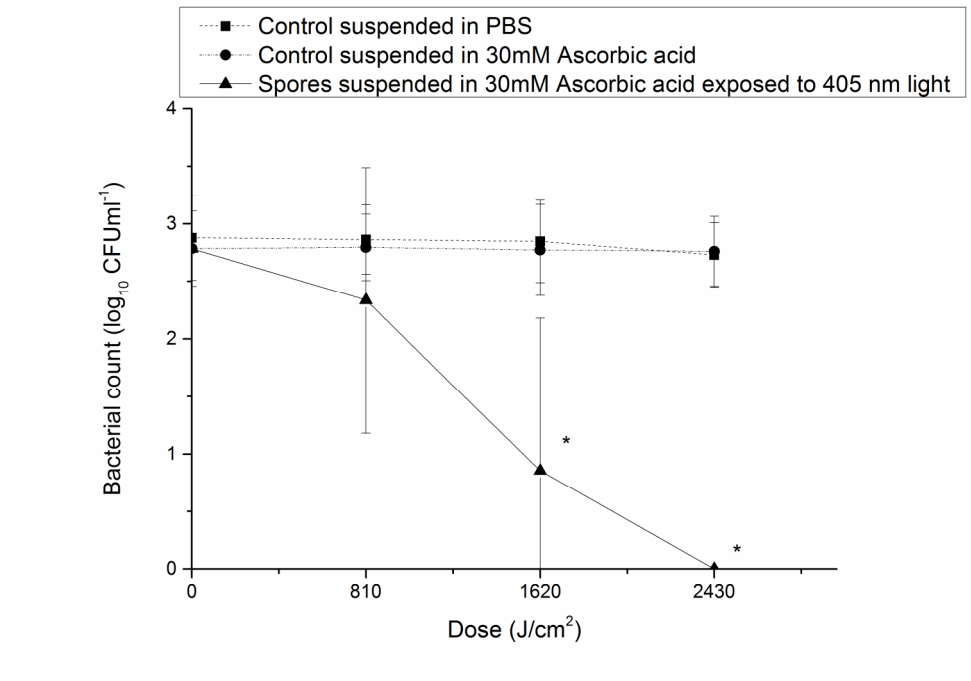

Figure 6.3 The inactivation of *C. difficile* spores exposed to 225 mWcm^{-2} 405 nm light under aerobic conditions in the presence of 30 mM ascorbic acid as a ROS scavenger. PBS alone, and PBS with ascorbic acid were used as controls and were not exposed to 405 nm light, to demonstrate that 30 mM ascorbic acid had no effect on the spores. Control samples were non-light exposed for the equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to non-light exposed control suspended in 30mM Ascorbic acid (P≤0.05). Each data point is a mean value ± SD (n=6).

6.2.4. DETECTION OF ENDOGENOUS PORPHYRIN ACTIVITY

The porphyrin content of both the spore suspension and vegetative cell suspension was analysed using a fluorescent spectrophotometer. A 10^6 CFUml^{-1} population was used to analyse both suspensions, with the results shown in Figure 6.4. As shown in Figure 6.4, no peaks indicating the presence of porphyrins were observed following excitation of the sample with wavelength of 405 nm.

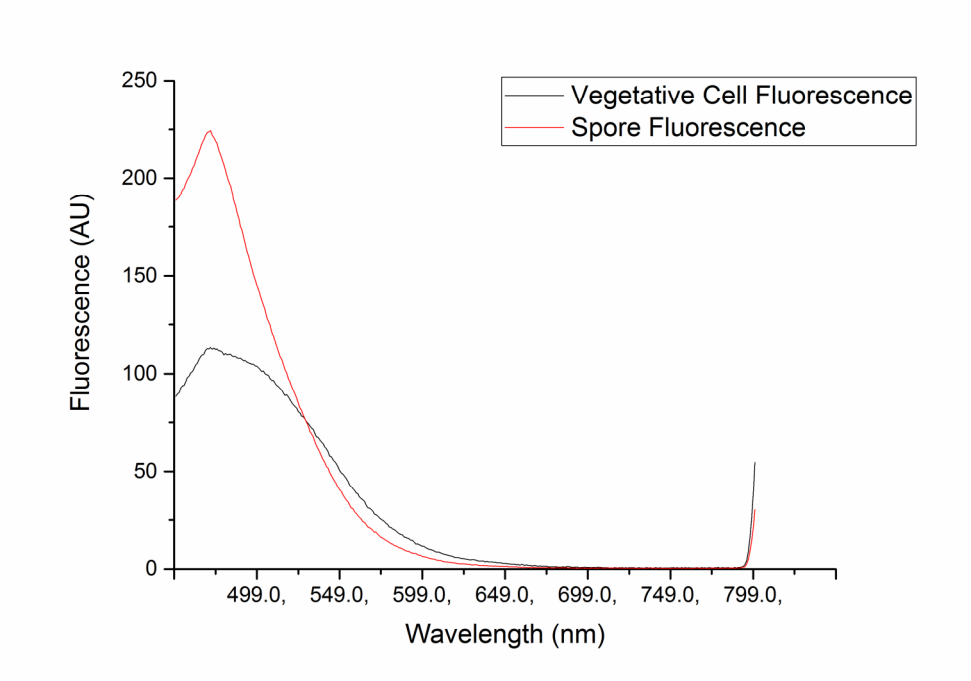

Figure 6.4 Fluorescence spectra of a *C. difficile* vegetative and spore suspension. Fluorescence emission spectra were detected from spore suspension preparations dissolved in NaOH-SDS, using an excitation wavelength of 405 nm. Emission spectra are shown between 500-800 nm.

6.3. DISCUSSION

These experiments were carried out to investigate the hypothesis that oxygen plays a vital role in photodynamic inactivation via the production of ROS during the type I and type II pathway resulting in cell death. The results in Figure 6.2 demonstrate that under anaerobic conditions in the presence of an oxygen scavenger, although significant inactivation is observed, this is very low with only a 0.79 $\log_{10}$ reduction, in comparison to a ~3.5 $\log_{10}$ reduction observed under aerobic conditions. These results support the theory that photodynamic inactivation requires visible light and oxygen, and indicates that this is due to the presence of porphyrins, for inactivation to occur and the importance of oxygen for this process (Hamblin & Hasan, 2004; Murdoch et al., 2013).

In a similar study by Maclean et al. (2008) exposure of *S. aureus* to visible light, with a 400 nm long-wave pass filter to prevent UV light transmission, in the presence of oxygen scavengers significantly reduced bacterial inactivation. These results are in line with further studies demonstrating the oxygen dependence of photodynamic inactivation, with the results demonstrating that the presence of oxygen scavengers could protect bacteria and fungi from inactivation by 405 nm light (Murdoch et al., 2013; Ramakrishnan et al., 2016). A study by Abad-Lozano demonstrated the susceptibility of *B. subtilis* spores to visible light

with only 13% survivors after 24 hours of exposure, whilst no inactivation was observed under anaerobic conditions upon exposure for up to 48 hours, further supporting the results observed in this study (Abad-Lozano and Rodriguez-valera, 1984).

The use of ascorbic acid in this study demonstrates the importance of singlet oxygen ($^1O_2$) in the photodynamic inactivation process as ascorbic acid acts as a $^1O_2$ quencher and radical scavenger (Maclean et al., 2008; Maclean et al., 2008; Niki, 1991). Slight inactivation was observed in anaerobic conditions in the presence of the ROS scavenger, and this may be explained by the fast binding of the highly reactive singlet oxygen and the inability of the ascorbic acid to bind fast enough to protect the spores (Feuerstein et al., 2005). Furthermore, the partially inefficient access to ROS generated within the cells may also have resulted in inactivation, as ascorbic acid cannot scavenge lipophilic radicals within the lipid compartment itself, but acts in synergy with tocopherol for the reduction of lipid peroxyl radicals (Feuerstein et al., 2005; Maclean et al., 2008; Niki, 1991).

This importance of singlet oxygen, produced via the dominant type II pathway, in the photodynamic inactivation process has previously been demonstrated in a study by Tavares et al. in which sodium azide, acting as a singlet oxygen quencher, was observed to protect the bacteria from photodynamic inactivation (Tavares et al., 2011). Furthermore in this study by Taveres et al., L-cysteine, used as a free radical scavenger, was observed to confer protection. This was assumed to be due to the singlet oxygen quenching by the sulfanyl group (–SH), not its radical scavenger ability, as D-mannitol used as a free radical scavenger only resulted in slight protection presumably against ROS produced in the Type I pathway (Tavares et al., 2011). Singlet oxygen generated out with the spore has been shown to be ineffective, likely due to the short life of singlet oxygen rendering it unable to penetrate the spore coat in time to have an adverse effect (Abad-Lozano and Rodriguez-Velera 1984).

A previous study, by Ramakrishnan et al. demonstrated the importance of H_2O_2 in the inactivation of bacteria using 405 nm light, with H_2O_2 scavenging sodium pyruvate providing protection for *S. epidermidis*, and with the combined use of three oxygen scavengers (dimethyl thiourea, an intracellular H_2O_2 scavenger, sodium pyruvate, an intracellular ·OH scavenger and catalase, which detoxifies extracellular H_2O_2) providing higher levels of protection than when used individually, indicating that a range of ROS are likely to play a role in the inactivation process to some extent (Ramakrishnan et al., 2016).

Detection of endogenous porphyrin activity

The aim of these experiments was to elucidate the types and levels of porphyrin molecules present within *C. difficile* spores and vegetative cells, and to detect if there is a difference in the porphyrin content in vegetative cells compared to that of spores. Both Coproporphyrin and Protoporphyrin IX (PpIX) can be excited with blue light to produce fluorescence in the red region of the light spectrum, with distinguishing fluorescence maxima observed at 635 nm and 618 nm, respectively, for PpIX and coproporphyrin (Seo et al., 2009). Unfortunately, the results in Figure 6.4 do not detect any porphyrin content following excitation. Limitations in obtaining higher population numbers are likely to explain why no detectable porphyrin emission peaks were observed upon analysis of the samples. However obtaining high *C. difficile* spore concentrations was a problem, as detailed in Section 3.3.2.3. In addition no higher than a 10^6 $CFUml^{-1}$ vegetative cell population could be obtained in the absence of spore formation.

In conclusion this section has demonstrated the importance of oxygen for inducing cell death of the vegetative cells and spores of *C. difficile* following exposure to 405 nm light, and in particular the importance of singlet oxygen, with very little inactivation observed under anaerobic conditions in the presence of anaerobic scavengers to ensure the absence of oxygen in the media. Additionally it was confirmed that ascorbic acid at the concentrations used for this study did not have a detrimental effect on the spores, even in combination with 405 nm light, and as such was not responsible for any of the inactivation observed.

Part 2 - Enhanced Susceptibility of *C. difficile* spores to 405 nm light upon the Induction of Spore Germination

6.4. GENERAL

Spore germination is defined as the irreversible loss of spore properties. This process allows for the growth of vegetative cells, which are capable of producing toxins, resulting in disease. Germination results in the loss of the highly resilient features of the spore, resulting in increased susceptibility to stressors (Barra-Carrasco et al., 2013). Whilst *C. difficile* spores are highly resilient to disinfectants and play a major role in the transmission of disease, vegetative cells are extremely sensitive to stresses such as oxygen, the gastric barrier and a wide range of decontamination procedures.

Similarly to *Bacillus subtilis*, *C. difficile* germination is thought to follow a three-stage process, however much is still yet to be learned with regards to the germination and outgrowth process. The three stages include:

(i) the binding of germinants to specific receptors,

(ii) the release of monovalent cations, and,

(iii) hydrolysis of the spore peptidoglycan cortex by cortex-lytic enzymes, core rehydration and initiation of metabolic activity and replication (Moore et al., 2013; Peter Setlow, 2003).

Spore germination is induced by the binding of germinants to a germinant receptor (GR). In the case of *C. difficile*, germinants include bile salts such as cholate, taurocholate, glycocholate and deoxycholate (Dembek et al., 2013; Heeg et al., 2012; Moore et al., 2013; Sorg & Sonenshein, 2008). Unlike many other sporulating bacteria, *C. difficile* does not have any germinant receptors. Work by Francis et al. (2013) identified that the catalytically inactivated serine protease CspC acts as a taurocholate GR and is essential for *C. difficile* spore germination in the presence of taurocholate. It is presumed that this serine protease acting as a GR is located at the interface of the spore PG cortex and spore coat. Following binding to the 'germinant receptor' a series of irreversible biochemical and biophysical reactions occur, including full rehydration of the spore core, enzymatic degradation of the cortex peptidoglycan and hydrolysis of small acid soluble spore proteins (SASPs) to amino acids (Dembek et al., 2013; Olguín-Araneda et al., 2015; Setlow, 2003). The dormant spores

lose their phase bright appearance and their impermeability to water, resulting in a water influx and loss of Ca^{2+}-DPA. This allows for spore rehydration, resumption of metabolism and results in a gradual increase in spore volume. Following these initial stages (0-60 minutes), the germinating spores then undergo DNA replication and symmetric cell division (60-180 minutes) (Dembek et al., 2013).

CspC is required for Ca-DPA release, however it is unclear if CspC reacts with Ca-DPA (Barra-Carrasco et al., 2013; Francis et al., 2013). It is presumed that CspC activates CspB following sensing the bile salt germinant. This activated CspB is then thought to activate the SleC cortex hydrolase, however the precise signalling pathway activating the *C. difficile* cortex hydrolysing machinery remains unclear (Paredes-Sabja et al., 2014).

C. difficile differs from most spore forming bacteria as it possesses different cortex hydrolytic machinery which is formed by two cortex lytic enzymes, SleC and SleM. In early germination pro-SleC is cleaved by the serine protease CspB, belonging to the subtilisin family (i.e. Csp proteases) and this converts pro-SleC to SleC (Barra-Carrasco et al., 2013). Previous studies have demonstrated that inactivation of SleC results in spores that are unable to degrade their peptidoglycan cortex, therefore cannot proceed with germination beyond stage I (Burns et al., 2010b). SleM is synthesised as a mature enzyme and is likely to degrade muropeptides derived from the peptidoglycan of the cell wall that are generated by SleC during spore germination (Barra-Carrasco et al., 2013). *C. difficile* further differs from other spore forming bacteria, such as *B. subtilis*, which typically release Ca-DPA fragments first followed by cortex hydrolysis, as cortex hydrolysis appears to precede Ca-DPA release during *C. difficile* germination – it is thought that Ca-DPA release may be dependent on cortex hydrolysis (Sorg, 2015). Hydrolysis of the spore cortex occurs and allows for the uptake of water leading to core expansion and metabolism resumes. This germination pathway is shown in Figure 6.5.

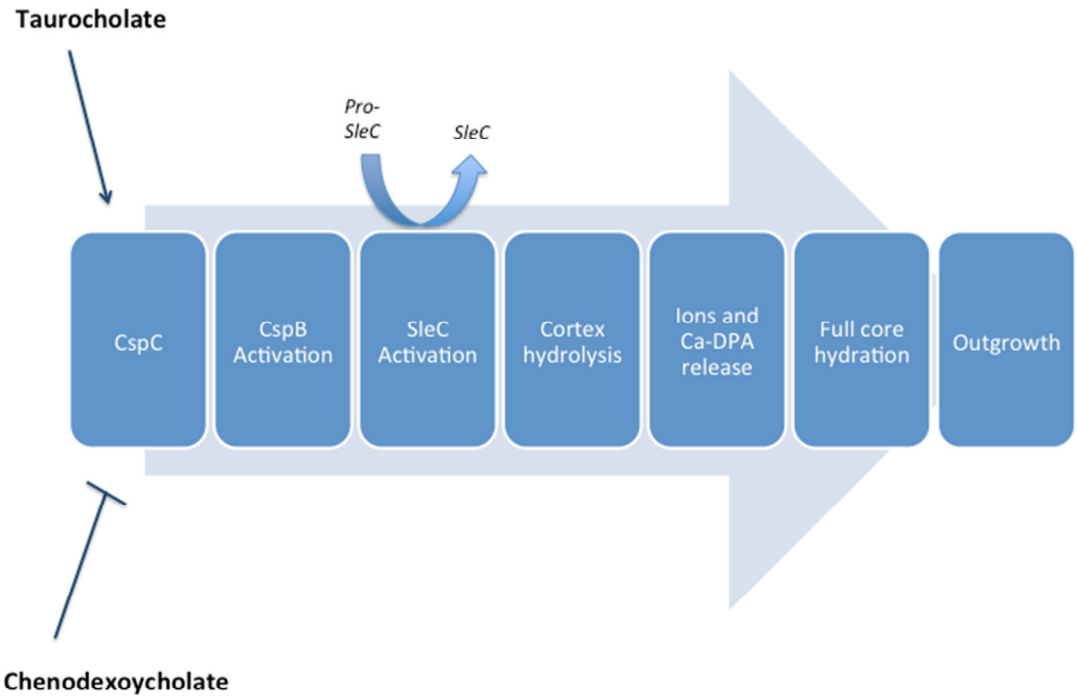

Figure 6.5 The hypothesised *C. difficile* germination outgrowth pathway following the interaction of germinants with germination receptors (adapted from Paredes-Sabja et al. 2014).

Taurocholate is found in the intestinal tract of both humans and animals and, as a result of the aerobic environment in the small intestine, the presence is restricted. Chenodeoxycholate is a bile salt which has been shown to inhibit spore germination, enabling regulation of *C. difficile* sporulation, and it appears to interact more tightly with *C. difficile* spores than taurocholate (Heeg et al., 2012; Sorg & Sonenshein, 2009). In healthy individuals deoxycholate, a secondary bile salt metabolised from cholate derivatives escaping the enterohepatic circulation, prevents spore colonisation and outgrowth. Spores germinate in the jejunum, these then pass through to the ileum reaching the aerobic environment of the cecum. Primary bile salts are metabolised by the normal microflora to secondary bile salts, thus preventing the vegetative growth of spores; in healthy individuals a small amount of germination is of no consequence in the aerobic environment of the cecum (Rineh & Kelso, 2014). However, as a result of antibiotic treatment the colonic microbiota suffers dysbiosis, resulting in increased availability of taurocholate in the anaerobic environment and secondary bile salts such as deoxycholate are not produced and vegetative cell growth is not prevented, enabling disease to occur in

susceptible individuals (Burns et al., 2010a; Olguín-Araneda et al., 2015; Sorg & Sonenshein, 2008).

Germination is classically measured as a decrease in optical density of the spore suspension, occurring alongside the release of DPA, rehydration of the core and degradation of the cortex (Dembek et al., 2013). This is then followed by an increase in OD_{600} correlated with outgrowth and cell division as the cells enter logarithmic growth phase.

As spores are generally significantly more resistant to inactivation and decontamination processes than their corresponding vegetative counterparts, the induction of germination may lead to enhanced susceptibility of spores to inactivation by various decontamination methods.

6.5. METHODOLOGY

Sodium taurocholate has previously been demonstrated to be the choice germinant for *C. difficile*, with taurocholate and its metabolites - cholate and desoxycholate - demonstrating enhanced germination of *C. difficile* spores (Wilson, 1983). Comparison of sodium taurocholate with other bile salts, sodium cholate and sodium desoxycholate, found sodium taurocholate to be the only bile salt that enhanced germination whilst having no adverse effect on vegetative cells (Wilson, 1983). In a recent investigation by Sorg and Sonenshein (2008) which studied the effects of several bile salts on the germination of *C. difficile* spores, sodium taurocholate was the only bile salt to induce germination of *C. difficile* spores in solution. Sorg and Sonheinshein further reported that glycine and taurocholate act as co-germinants to induce spore germination.

To investigate the optimal bile salt for use in this study to induce spore germination a range of bile salts were used including:

- 0.5% sodium taurocholate,
- 0.5% bile salts (containing sodium taurocholate and sodium glycocholate) and,
- 0.5% bile salts in combination with 0.5% glycine.

Spores were inoculated into 9 ml volumes of germination media and were incubated under anaerobic conditions at room temperature (20°C) for 48h, with samples taken at a range of time points. Samples were then plated onto blood agar and incubated at 37°C under anaerobic conditions for 48 hours. Samples were also heat treated to determine the spore

population, at 80°C for 10 minutes followed by plating onto blood agar and incubated at 37°C under anaerobic conditions for 48 hours. Additionally spores were suspended in H_20 as a control over this study to allow for demonstration of the spore population when not suspended in germination medium.

The results of this scoping study, shown in Table 6.1, show that all three bile salt combinations induced similar results; however 0.5% bile salts resulted in the highest decrease in spore count – likely due to the greatest outgrowth of spores to vegetative cells. As such, brain heart infusion (BHI) broth supplemented with 0.5% w/v bile salts was chosen to be the germination medium for this study.

Table 6.1 The measurement of germination of *C. difficile* spores upon incubation (at room temperature) in BHI supplemented with a range of germinants: sodium taurocholate, bile salts and bile salts in combination with glycine. Heat treated (HT) samples demonstrate the proportion of the population that remain as spores in the total population (non-HT samples).

Incubation time in germination medium	Number of spores present in the Germination media ($CFUml^{-1}$)					
	0.5% Sodium Taurocholate		0.5% bile salts		0.5% bile salts and 0.5% glycine	
	Non HT	HT	Non HT	HT	Non HT	HT
0	4.95	3.97	4.77	3.57	4.94	3.98
10	5.71	3.96	5.09	3.65	5.10	3.91
20	5.75	3.91	5.77	3.54	4.64	3.88
30	5.77	4.08	5.54	3.90	5.57	3.99
60	5.87	3.62	5.41	3.72	5.41	3.89
90	5.82	3.45	5.59	3.47	5.37	4.84
180	5.70	3.63	5.54	2.92	5.56	3.90

6.5.1. BACTERIAL PREPARATION

C. difficile spores were prepared as detailed in Section 3.3.2.2. 1 ml was then transferred into 9 ml BHI supplemented with 0.5% w/v bile salts. The growth of the bacterial population, under anaerobic conditions at room temperature, was recorded over a period of 3 hours. A control sample was inoculated in H_20 to demonstrate the spore population remains constant when not inoculated in germination medium. At each time point (0 minutes, 1, 2 and 3, 24 and 48 hours), 500 µl was removed from the germinating sample and heat treated at 80°C for 10 minutes to get an estimate of the spore population present in the suspension.

6.5.1.1. 405 nm light exposure of germinating suspensions

The initial experiment exposed the germinating suspension to 405 nm light immediately after inoculation of spores into the germination medium with light exposure conducted on the laboratory bench in the aerobic environment. Further investigations pre-incubated the spores at room temperature in the germination medium for 1 hour, 3 hours, and 24 hours under anaerobic conditions prior to exposure to 405 nm light in the aerobic environment.

For all experiments, following suspension in the germination medium for the allocated period of time, 2 ml of the suspension was transferred to a 12-well multi-plate and exposed to 405 nm light at an irradiance of 225 $mWcm^{-2}$ for increasing time durations. The control suspension was exposed to the exact same conditions, however was exposed to standard laboratory lighting. A further control was suspended in sterile distilled water to demonstrate the non-germinating spore suspension population. Following exposure to the desired dose, both the sample and the controls were plated onto blood agar plates and incubated at 37°C for 48 hours under anaerobic conditions. Samples were then enumerated and viable bacterial counts recorded as $\log_{10}$ $CFUml^{-1}$.

6.6. RESULTS

The results in Figure 6.6 demonstrate the change in vegetative cell and spore count over a period of 48 hours when suspended in germination media under anaerobic conditions at room temperature. The results demonstrated that, immediately following inoculation into the germination media the vegetative cell population immediately increased to a 4.3 $\log_{10}$ population, with an eventual increase to a 6.8 $\log_{10}$ population following 48 hours incubation period. A 0.67 $\log_{10}$ reduction in the spore count was observed, with a 3.22 $\log_{10}$

CFUml^{-1} starting spore population at 0 minutes, and a 2.55 log$_{10}$ CFUml^{-1} population following 48 hours incubation.

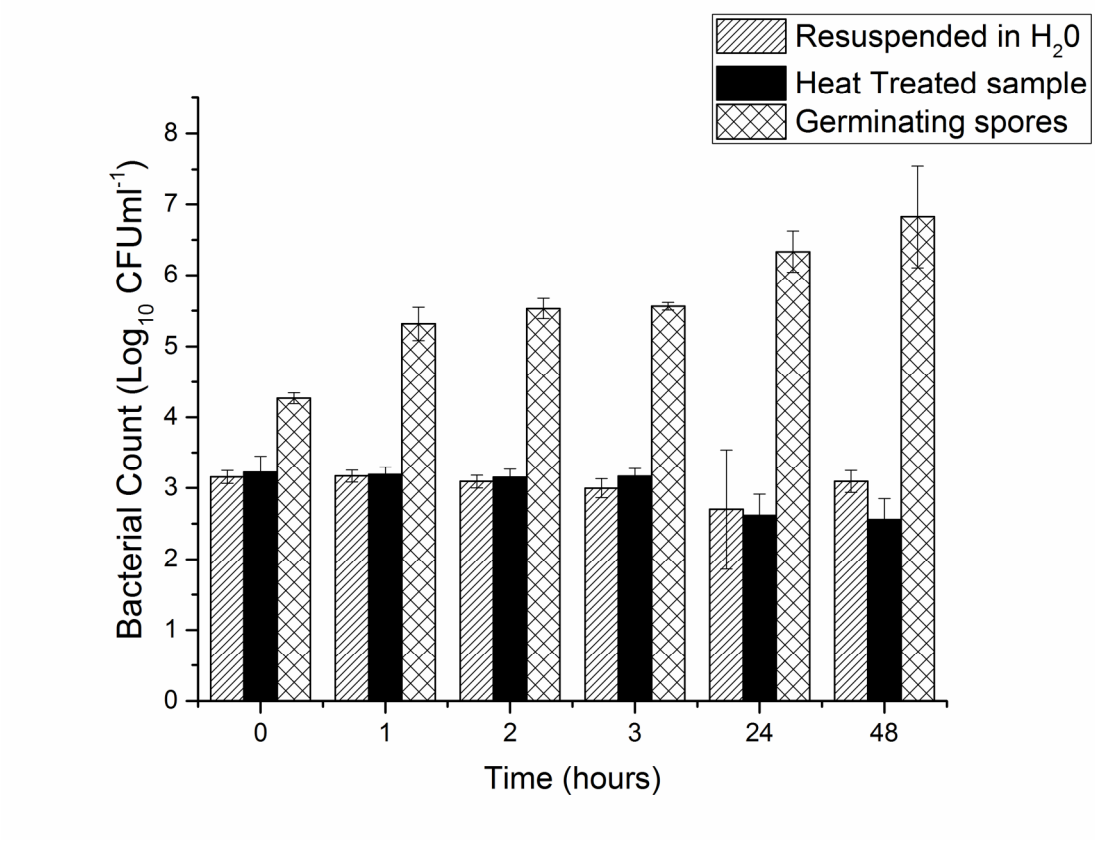

Figure 6.6 The growth rate of germinating *C. difficile* spores when resuspended in BHIS w/v 0.5% bile salts under anaerobic conditions room temperature over 48 hours. The suspension was heat treated at 80°C for 10 minutes to determine the population of spores present in the suspension during germination. Spores were also resuspended in water as a control to demonstrate the population when not suspended in germination media. Each data point is a mean value ± SD (n=6).

In order to determine if susceptibility of spores to 405 nm light was increased as soon as the spores were suspended in the germination medium, spores were inoculated into germination media and then transferred immediately to a 12-well multi-dish and exposed to 405 nm light at an irradiance of 225 mWcm^{-2} for increasing doses.

Results, shown in Figure 6.7, demonstrate the significant 0.66 log$_{10}$ population decrease ($P<0.001$) achieved following exposure to a dose of 810 Jcm^{-2} in comparison to the starting population at 0 minutes. In comparison to the exposed sample, the unexposed control continued to grow with a population increase of 0.93 log$_{10}$, indicating that germination continued. The results of the non-light exposed and 405 nm light exposed germinating samples at 810 Jcm^{-2} were significantly different to one another ($P<0.001$).

When comparing the results of the inactivation of the 10^4 $CFUml^{-1}$ population in the germination medium to the kinetics for the same population density of spores in PBS (Figure 6.8), results showed that following exposure to a dose of 4.86 $kJcm^{-2}$ only a 0.97 log_{10} reduction was achieved in the germination solution ($P<0.001$) in comparison to a 3.33 log_{10} reduction in PBS following 2.43 $kJcm^{-2}$($P= 0.005$). Complete inactivation of the spores suspended in germination medium was observed following exposure to a dose of 9.72 $kJcm^{-2}$ – a 4-fold higher dose than that required for the spores suspended in PBS.

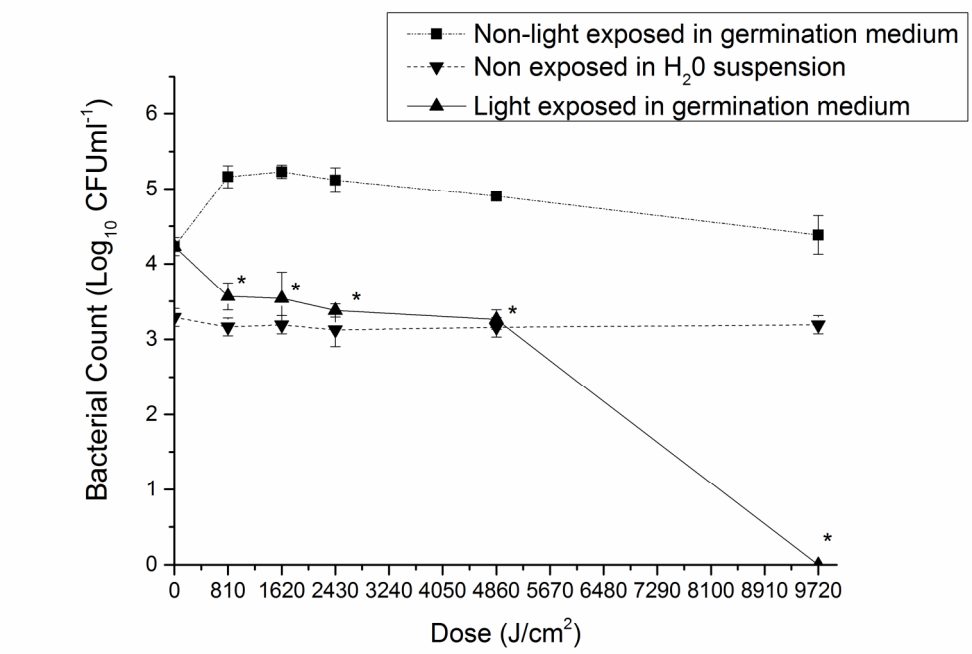

Figure 6.7 Exposure of germinating spores to 405 nm light (225 $mWcm^{-2}$) immediately after inoculation into germination media (BHI w/v 0.5% bile salts). Control samples were non-light exposed for the equivalent time periods (0, 1, 2, 3, 6 and 12 hours) therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. A further control was inoculated into H_20 to demonstrate the population sample, when not germinating, this was not exposed to 405 nm light and as such was exposed for equivalent time periods and is represented by a dotted line. * represent significant bacterial inactivation compared to the non-exposed control ($P\leq0.05$). Each data point is a mean value ± SD (n=6).

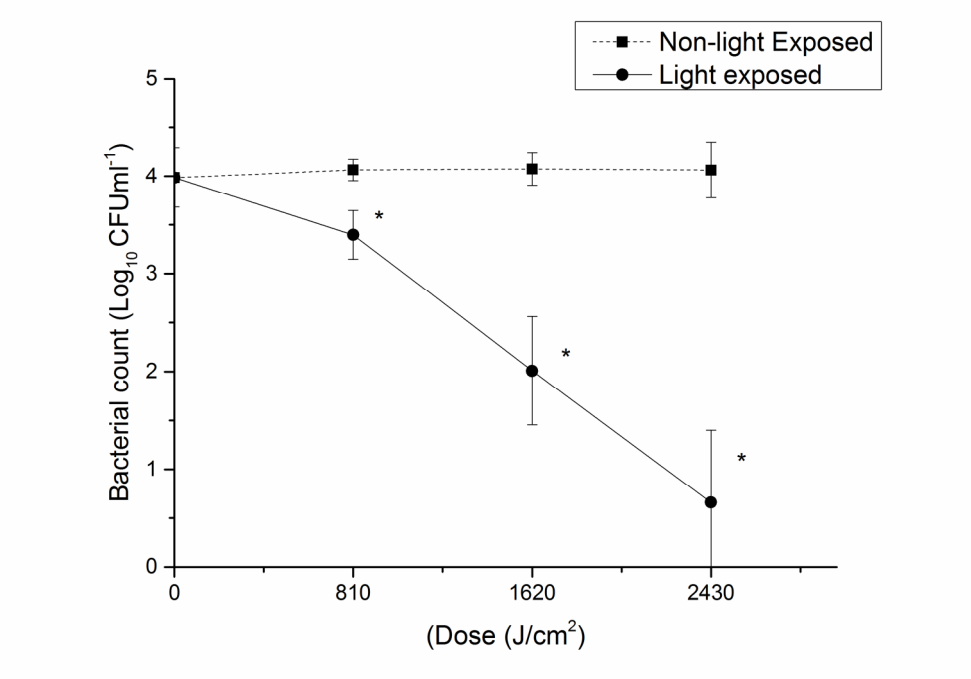

Figure 6.8 Inactivation kinetics of 10^4 CFUml^{-1} *C. difficile* population suspended in PBS when exposed to 225 mWcm^{-2} 405 nm light. Control samples were non-light exposed for the equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation as compared to the control (P≤0.05). Each data point is a mean value ± SD (n=6).

As can be seen in Figure 6.9 the transmission of light through BHI supplemented with 0.5% bile salts is 23.4% in comparison to 100% transmission through a PBS suspension

Following demonstration of the lowered transmission of 405 nm light through the BHIS germination suspension, the irradiance transmitting through the 2 ml PBS and germination medium suspensions in the 12-well plate was then measured to get an accurate reading of how much light was being absorbed by the different suspending media. Irradiance measurements were taken by positioning the radiant power meter detector directly below the well of the sample dish and measuring the amount of light passing through the empty well (to account for any loss through the base of the well), and through the 2 ml PBS and germination medium samples. Results showed that through the PBS, there is no loss of the 225 mWcm^{-2} irradiance, however only an irradiance of 50 mWcm^{-2} transmits through the BHIS suspension containing 0.5% bile salts. The light through the PBS was not reduced, whilst there was a loss of light through the plate base. An explanation for this is that the PBS focuses the light. This reduction in the transmission of 405 nm light through BHIS w/v 0.5% bile salts may offer an explanation to the decreased spore inactivation, even following the induction of germination, when spores are suspended in germination media.

This provides justification for the results in Figures 6.7 and 6.8, as the reduced levels of 405 nm light transmission through the germination medium will contribute to lower inactivation rates.

As a result of this, 50 $mWcm^{-2}$ will be used in the analysis when calculating the exposed dose for spores suspended in germination medium.

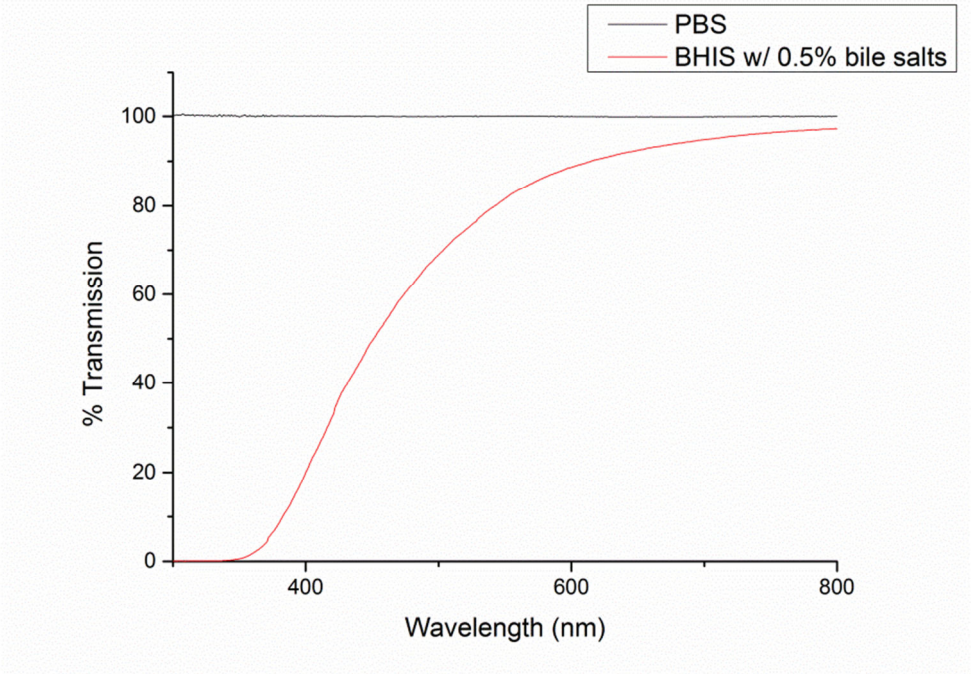

Figure 6.9 Comparison of the light transmission through PBS and BHIS w/v 0.5% bile salts.

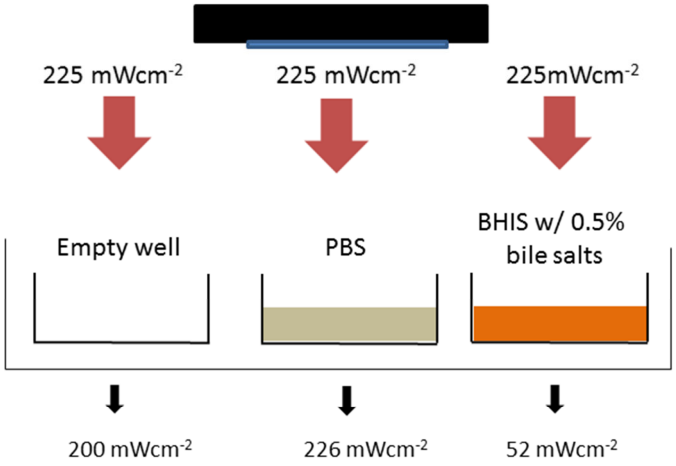

Figure 6.10 Measurement of the irradiance of 405 nm light passing through 2 ml samples of suspending media (PBS and BHIS w/v 0.5% bile salts suspensions) in the well of a 12 well multi-plate.

The inactivation data for spores in germination media (Figure 6.7) using the actual transmitted irradiance of 50 $mWcm^{-2}$ are shown in Figure 6.11 and compared to that of a 10^4 $CFUml^{-1}$ population suspended in PBS. This demonstrates the much more comparable doses required for inactivation. Analysis of the data in fact shows that there is enhanced susceptibility of *C. difficile* spores immediately following exposure to germination medium in comparison to spores suspended in PBS, with complete inactivation observed after exposure to a dose of 2.16 $kJcm^{-2}$ and an 11% lowered dose required to achieve a complete reduction (10^4 $\log_{10}$ $CFUml^{-1}$).

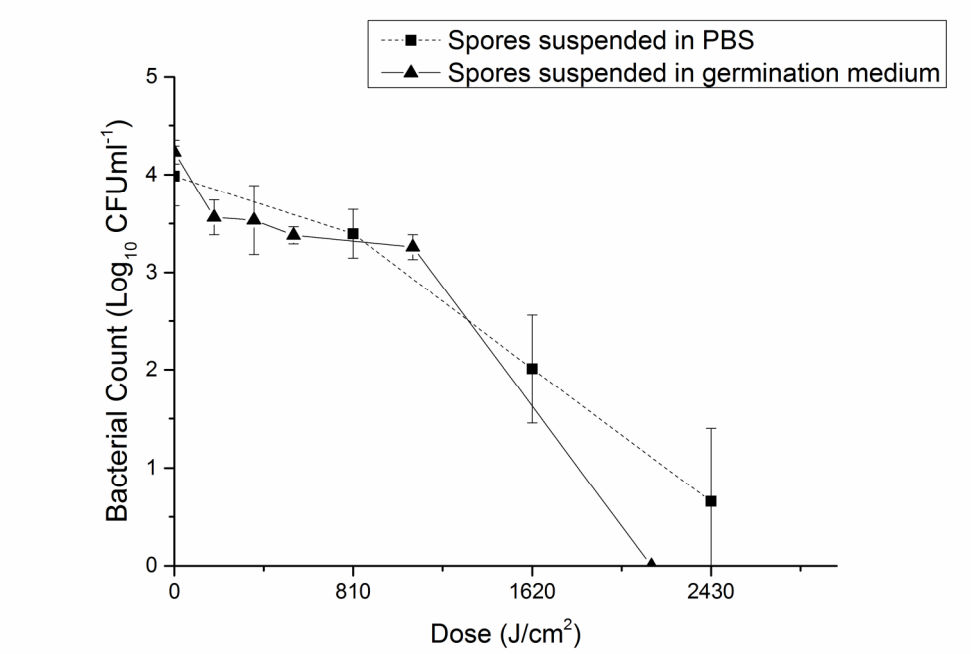

Figure 6.11 Comparison of the inactivation kinetics of *C. difficile* spores when suspended in germination medium (BHIS w/v 0.5% bile salts) and PBS following exposure to 405 nm light. The applied irradiance was 225 $mWcm^{-2}$, but for calculation of dose this was adjusted to 50 $mWcm^{-2}$ in the case of the germination medium due to the reduced transmission through the sample.

Investigations then proceeded to determine the inactivation kinetics for germinating *C. difficile* spores following incubation in the germination medium for 1 hour under anaerobic conditions (Figure 6.12). The results of this investigation demonstrated an initial decrease, followed by an increase, in spore population despite the application of an increased dose from 180 Jcm^{-2} to 360 Jcm^{-2}. This may be due to spores re-sporulating in the aerobic environment. After 1 hour germination, it is unlikely that complete germination has

occurred, with Shen (2015) reporting that germination takes up to 2 hours to complete following germinant sensing (Shen, 2015). Therefore exposure to the aerobic environment may have prevented any further germination taking place, whilst also resulting in germinating spores re-sporulating. Furthermore, as complete germination of spores is unlikely to have occurred, many germinating spores may still maintain several protective properties of spores at this stage, providing protection from damage by the ROS produced by 405 nm light.

To investigate if germinating spores had re-sporulated upon exposure to the aerobic environment, spores were suspended in BHIS w/v 0.5% bile salts under anaerobic conditions, as before, for 1 hour followed by exposure to the aerobic environment for 1 hour. At both time points the sample was plated, and a sample heat treated at 80°C for 10 minutes sample was plated – this was to count both the vegetative cell counts and the spore count, to determine if there was any fluctuation in either to determine for germination and sporulation events occurring in the germination medium and upon prolonged exposure to the aerobic environment. The results in Figure 6.13 demonstrate that following germination, when the suspension was exposed to the aerobic environment for 1 hour there was a decrease in the vegetative cell count indicating that some of the germinating cells may undergo sporulation again.

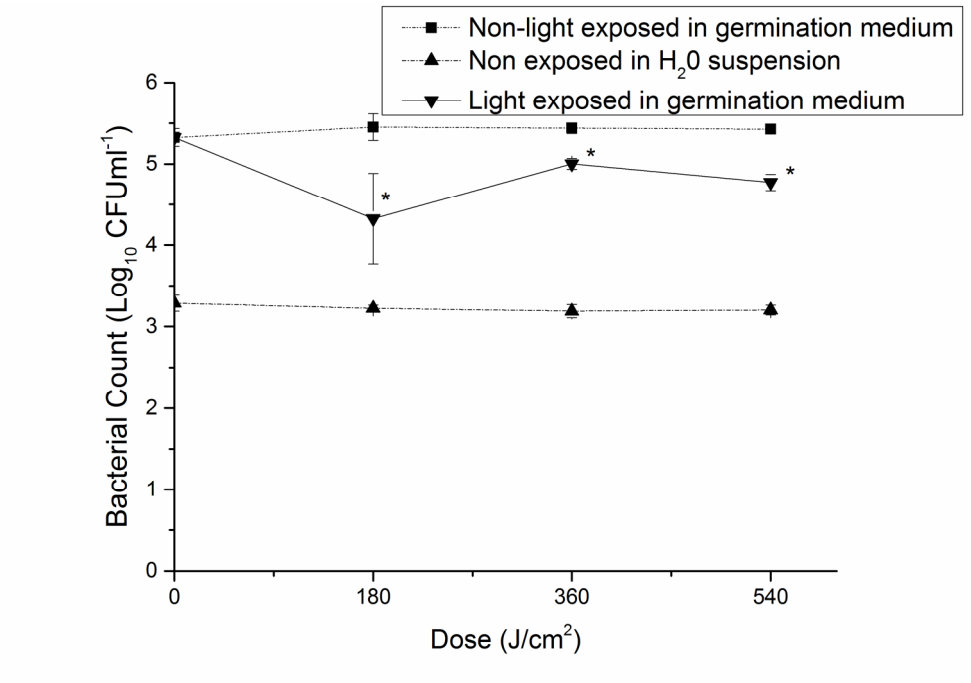

Figure 6.12 Exposure of germinating spores to 225 mWcm^{-2} 405 nm light following 1 hour germination in BHIS w/v 0.5% bile salts under anaerobic conditions. Control samples were non-light exposed for the equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation compared to the non-light exposed control (P≤0.05). Each data point is a mean value ± SD (n=6).

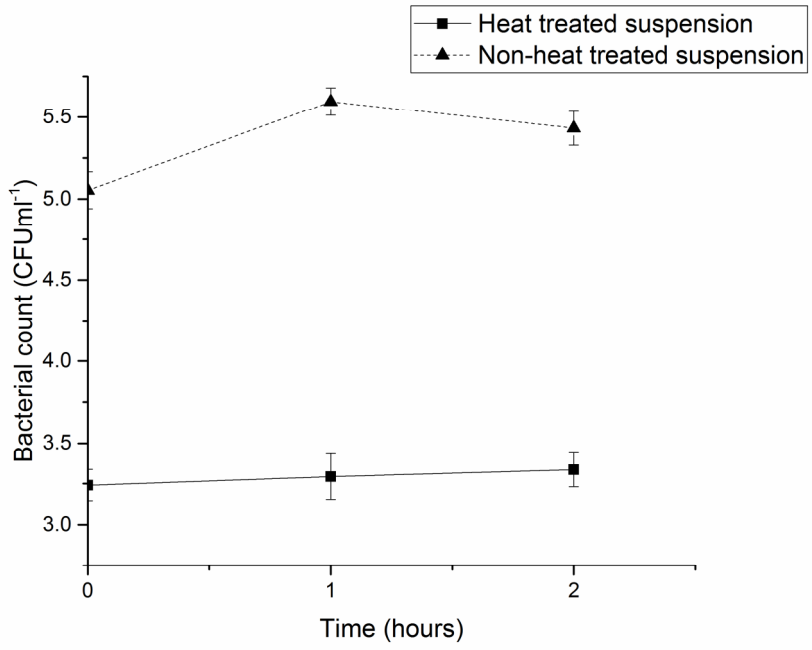

Figure 6.13 *C. difficile* spores suspended in germination media under anaerobic conditions for 1 hour, followed by exposure to the aerobic environment for one hour to investigate if germinated spores re-sporulate upon exposure to the aerobic environment. This graph demonstrates the vegetative and spore population of the suspension, with the first hour demonstrating the increased vegetative cell population as a result of germination of the spores under anaerobic conditions in BHIS w/ 0.5% bile salts, and the second hour demonstrating the reduction of vegetative cells as a result of re-sporulation following exposure of the suspension to the aerobic conditions.

To investigate if longer incubation in the germination medium under anaerobic conditions would lead to enhanced inactivation due to further outgrowth of the spores to vegetative cells, *C. difficile* spores were inoculated into the germination medium and incubated under anaerobic conditions for 3 hours. Following 3 hours, germinating spores were exposed to 405 nm light for increasing doses (Figure 6.14) with the results demonstrating a much greater susceptibility of germinating spores to 405 nm light upon prolonged exposure to germinants under anaerobic conditions. A significant reduction of 2.33 log_{10} was achieved following exposure to a dose of 180 Jcm^{-2} (P=0.01) and a further 0.7 log_{10} reduction was achieved following a dose of 540 Jcm^{-2} (P=0.001). This was then compared to the inactivation kinetics of a 10^5 $CFUml^{-1}$ spore population suspended in PBS, with the results in Figure 6.15 demonstrating that enhanced inactivation was observed following exposure to germinants for 3 hours under anaerobic conditions with a 4.5× increase in dose required to achieve a similar 5 log_{10} reduction in comparison to germinating spores.

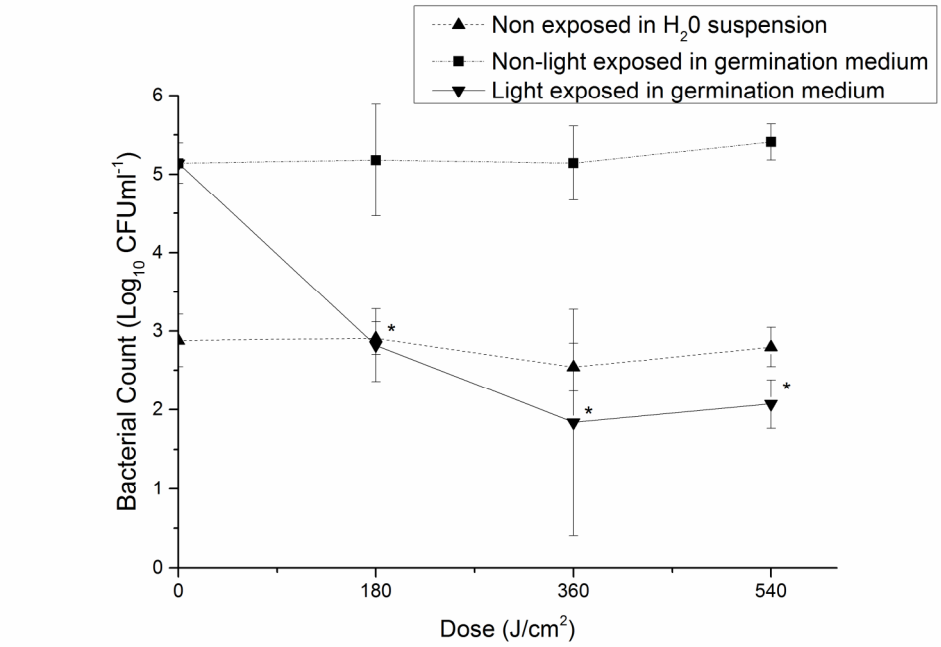

Figure 6.14 Exposure of germinating spores to 225 $mWcm^{-2}$ 405 nm light following 3 hour germination in BHIS w/v 0.5% bile salts under anaerobic conditions. Control samples were non-light exposed for the equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. A further control was inoculated into H_20 to demonstrate the population sample, when not germinating, this was not exposed to 405 nm light and as such was exposed for equivalent time periods and is represented by a dotted line. * represent significant bacterial inactivation compared to non-exposed controls (P≤0.05). Each data point is a mean value ± SD (n=6).

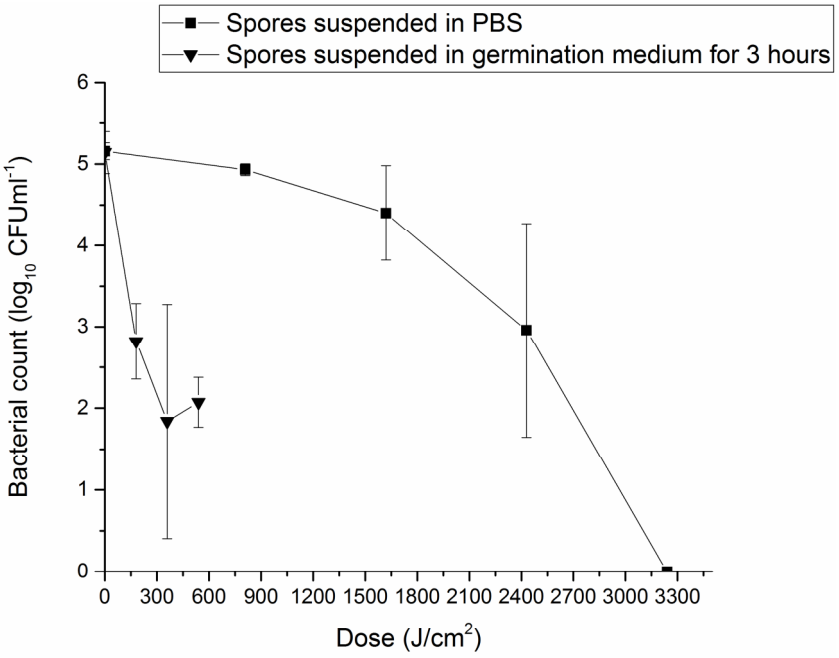

Figure 6.15 Comparison of the inactivation kinetics of *C. difficile* spores following incubation in germination medium (BHIS w/v 0.5% bile salts) under anaerobic condition for 3 hours and PBS. The applied irradiance was 225 mWcm^{-2}, but for calculation of dose this was adjusted to 50 mWcm^{-2} in the case of the germination medium due to the reduced transmission through the sample.

Following on from the previous results in Figure 6.15 demonstrating enhanced sensitivity to 405 nm light following prolonged germination under anaerobic conditions, studies then proceeded to investigate if 24 hour incubation of spores in the germination medium could further enhance susceptibility to 405 nm light. These results demonstrate a similar inactivation rate to that of the spores germinated for 3 hours, despite the log increase in population size. This indicates that the sensitivity of spores, despite the longer germination time, does not increase substantially and it is likely that full outgrowth has occurred following 3 hours germination. As can be seen in Figure 6.16, a complete 6.1 $\log_{10}$ kill was observed following exposure to a dose of 720 Jcm^{-2} ($P<0.001$). A significant 2.9 $\log_{10}$, 2.46 $\log_{10}$ and 3.88 $\log_{10}$ reduction was observed following exposure to doses of 180, 360 and 540 Jcm^{-2}, respectively ($P<0.001$).

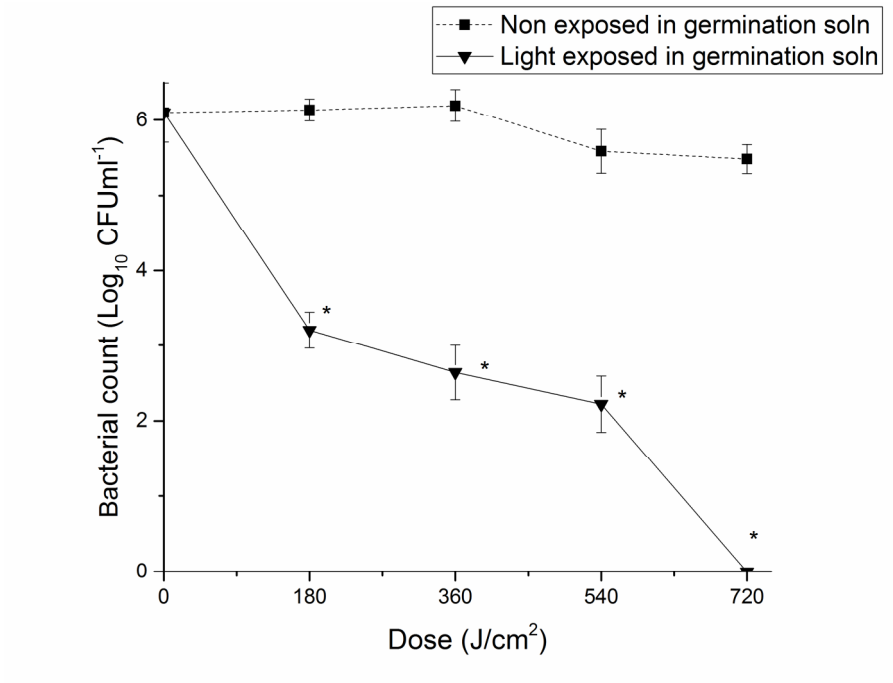

Figure 6.16 Exposure of germinating spores to 225 mWcm^{-2} 405 nm light following 24 hour germination in BHIS w/v 0.5% bile salts under anaerobic conditions. Control samples were non-light exposed for the equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light exposure only. * represent significant bacterial inactivation (P≤0.05). Each data point is a mean value ± SD (n=6).

To compare this with the inactivation kinetics of vegetative cells, due to the assumption that following 3-24 hours germination the dominating cell types in the suspension were spores undergoing germination and vegetative cells, investigations were conducted to determine the inactivation kinetics of vegetative cells at a population of 10^6 CFUml^{-1}. As seen in Figure 6.17, upon exposure of the 10^6 CFUml^{-1} vegetative cell population to 405 nm light (70 mWcm^{-2}), exposure to doses of 756 Jcm^{-2} and 1008 Jcm^{-2} achieved bacterial reductions of 2.74 $\log_{10}$ and 3.8 $\log_{10}$, respectively (P<0.001). Comparatively, germinating spores required an applied dose of 720 Jcm^{-2} to achieve a 6.1 $\log_{10}$ reduction, a 28% lowered dose than that required to achieve a 3.8 $\log_{10}$ reduction in vegetative cells.

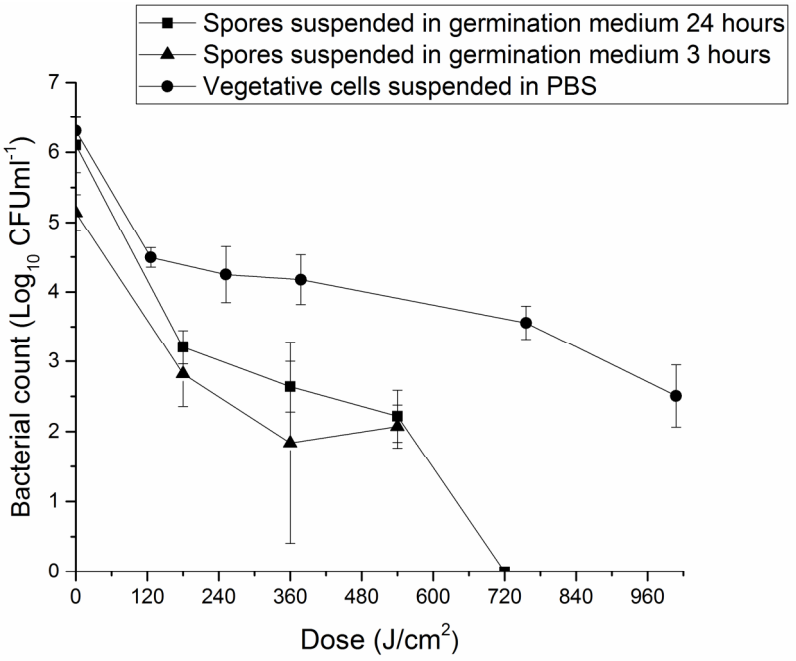

Figure 6.17 Comparison of the inactivation of *C. difficile* vegetative cells, at a population of 10^6 CFUml^{-1}, with a similar population density of germinating *C. difficile* spores suspended in germination medium (BHIS w/v 0.5% bile salts) for 3 and 24 hours. Vegetative cells in PBS were exposed to 405 nm light at 70 mWcm^{-2} for increasing doses. Germinating cell suspensions were exposed to 225 mWcm^{-2} with 50 mWcm^{-2} used to calculate dose due to the reduced transmission through the medium.

6.7. DISCUSSION

In order to determine if the sensitivity of *C. difficile* spores to 405 nm light was increased upon outgrowth of vegetative cells, spores were incubated in germination medium for increasing time periods under anaerobic conditions prior to exposure to 405 nm light. To determine that germination was occurring, spores were inoculated into the germination media (BHIS w/v 0.5% bile salts) and samples were taken over increasing time durations and plated onto blood agar. Samples were also treated at 80°C for 10 minutes and plated onto blood agar, allowing for enumeration of both the total bacterial count and the spore count – as vegetative cells are killed following heat treatment.

As seen in Figure 6.6, following suspension in germination medium a rapid surge in the spore population was observed. It is unlikely that this is due to immediate germination at 0 minutes. A possible explanation for this flux in the bacterial count is that the spores may take up the nutrients from the germination medium, and although immediately taken from the sample and plated onto agar, once transferred to optimal anaerobic conditions any residual nutrients from the germination medium that are still within the spore may then result in non-viable spores germinating once incubated under ideal conditions. This is supported by the previous reporting that the presence of physical or chemical agents may

result in the enhanced recovery of viable spores by mechanisms not directly involving germination, for example by supporting growth of the developing vegetative cells (Wilson, 1983).

Following on from initial germination scoping, germinating spores were then exposed to 405 nm light at different stages of spore germination. This was initially done immediately following inoculation of spores into germination medium to investigate if any immediate increase in the susceptibility of highly resilient spores was observed. Although significant inactivation of the germinating population exposed to 405 nm light immediately following inoculation was observed following exposure to 405 nm at increasing doses (Figure 6.7), the enhanced inactivation was not that which was expected, with only a 1 $\log_{10}$ reduction achieved following exposure to a dose of 4.86 $kJcm^{-2}$, whilst spores suspended in PBS were completely inactivated at half this dose. This may be due to the extreme stress being exerted on the germinating spores, including exposure to the aerobic environment alongside the stress of the violet-blue light, resulting in the inhibition of germination. However, this is most likely explained by the transmission of light through the suspensions, as seen in Figure 6.9 and Figure 6.10, with 100% transmission of 405 nm light through PBS suspensions and 23% transmission through BHIS w/v 0.5% bile salts, and only an applied dose of 50 $mWcm^{-2}$ penetrating through the spores suspended in the germination medium. Once this applied dose was accounted for, the enhanced susceptibility of spores upon induction of germination was able to be compared, with a complete 4 $\log_{10}$ reduction observed following exposure to a dose of 2.16 kJ cm^{-2}, in comparison to a 3 $\log_{10}$ spore reduction in PBS upon exposure to a dose of 2.43 $kJcm^{-2}$. Enhanced susceptibility upon germination was hypothesised as spores have protective layers protecting the inner core from ROS damage, upon germination the protective coat is removed and hydrolysis of the spore core likely makes the spore more susceptible to ROS (Banerjee et al., 2012).

The reduction in the control bacterial count, as seen in Figure 6.7 and Figure 6.11, over the 12 hour time period is likely due to the formation of spores as a result of prolonged exposure of the vegetative cells formed following germination to the aerobic environment, and the competition for nutrients as a result of the increased population, minimising germination and constraining bacterial growth resulting in bacterial death and re-sporulation. This potential re-sporulation upon prolonged exposure to the aerobic environment was then investigated, with spores inoculated into germinating medium under anaerobic conditions for 1 hour followed by exposure to the suspension to the aerobic

environment for 1 hour, as shown in Figure 6.13. As can be seen in Figure 6.13 it appears that the germinating spores re-sporulate upon removal from the anaerobic environment and during prolonged exposure to the aerobic environment, and this may provide an explanation as to why upon exposure immediately after inoculation into germination medium the spores do not exhibit as great a susceptibility as may have been hypothesised due to restricted outgrowth under aerobic conditions.

To investigate if longer germination times under anaerobic conditions could enhance spore susceptibility, spores were incubated in germination medium for 1, 3 and 24 hours prior to exposure to 405 nm light at an irradiance of 225 $mWcm^{-2}$. Enhanced susceptibility was observed upon longer incubation periods of spores in germination medium of between 1 hour and 3 hours, with a 72% inactivation achieved following exposure to 540 Jcm^{-2} following 1 hour germination of spores in comparison to a 99.9% bacterial kill following 3 hour germination of *C. difficile* spores, in comparison to a 76% spore reduction achieved when spores where suspended in PBS following exposure to a 33% increased dose of 810 Jcm^{-2}. This increased sensitivity to 405 nm light following germination is likely due to the loss of spore protection properties, such as the spore coat and rehydration of the spore, as a result of outgrowth to vegetative cells following contact with germinants.

Upon comparison of the inactivation kinetics following pre-incubation in germination medium for 3 hours and 24 hours, the sensitivity of the germinating spores to 405 nm light is not significantly enhanced with a 99.9% and 100% bacterial reduction observed, respectively, following exposure to a dose of 540 Jcm^{-2} 720 Jcm^{-2}. An explanation for this, as previously discussed, is that full outgrowth of the spore has occurred after 3 hours exposure to the germinants.

As the spore population pre-incubated in germination medium for 3 or 24 hours likely contained a high population of vegetative cells, as a result of spore outgrowth following germination, the inactivation kinetics were further compared to that of a 10^6 $CFUml^{-1}$ vegetative cell population suspended in PBS (Figure 6.17). Investigations further compared the inactivation kinetics of the germinating spores to that of a high population *C. difficile* spore suspension (10^5 $CFUml^{-1}$) upon exposure to 405 nm light (Figure 6.17). Surprisingly, a 10^6 $CFUml^{-1}$ population of *C. difficile* vegetative cells appeared much more resilient to 405 nm light exposure than the spores undergoing germination suspended in the germination media, with complete inactivation of the germinating spores (5.2 log_{10} and 6.1 log_{10}) observed following exposure to a dose of 720 Jcm^{-2} whilst a similar dose of 756 Jcm^{-2} only

achieved a 2.74 $\log_{10}$ reduction in vegetative cell counts. A possible explanation for this is that the germinating spores suspended in the germination media upon exposure to 405 nm light, whilst exposed to stressful aerobic conditions, are in the presence of otherwise apt conditions resulting in lesser stressors resulting in sporulation of the vegetative cells in comparison to that of the vegetative cell population exposed to 405 nm light in PBS. As such, it is likely that a higher population of the vegetative cells suspended in PBS are undergoing sporulation. In comparison, the spores which have been exposed to germination media for 3+ hours under anaerobic conditions may be unable to undergo complete re-sporulation due to the continued exposure to germinants preventing full development of the spore protection mechanisms and thus leaving the bacteria unable to protect themselves from the 405 nm light. A further explanation may be that bile salts contain porphyrin or photo reactive molecules, which upon excitation produce exogenous ROS that cannot cross the spore coat to cause enhanced inactivation. However, upon the induction of germination, due to the loss of spore protection mechanisms and degradation of the spore cortex these ROS may result in additional damage to cells. As such, further investigations should be carried out to determine if, upon excitation with 405 nm light, bile salts produce ROS.

In comparison to the inactivation kinetics of a 10^5 $CFUml^{-1}$ spore population suspended in PBS, requiring a 3.24 $kJcm^{-2}$ exposure dose to achieve complete inactivation, the results in Figure 6.14 and Figure 6.16 demonstrate the much greater susceptibility of the germinating spore population following pre-incubation in germination medium for 3 and 24 hours, requiring a 78% lower dose to achieve similar or greater reductions.

Nerandzic & Donskey (2010) previously reported increased susceptibility of *Clostridium* spores to UV-C radiation following the induction of germination. The enhanced inactivation was found to range between *C. difficile* strains, ranging from 0.85 $\log_{10}$ to 2.15 $\log_{10}$ inactivation, suggesting that different strains may not become as susceptible as others to stressors following the induction of germination (Nerandzic & Donskey, 2010). Stimulation of germination of *C. difficile* spores further resulted in reduced survival on surfaces in room air. Therefore, stimulation of germination could potentially be beneficial as a strategy to reduce the burden of spores in the environment even in the absence of UV-C treatment (Nerandzic & Donskey, 2010). A previous study by De Sordi et al. demonstrated that light delivered at a dose of 14.4 Jcm^{-2} in the presence of 5 mM methylene blue could not induce any killing of *C. difficile* spores. However, following the induction of germination treatment

with red light at a wavelength of 665 nm at a dose of 0.24 Jcm^{-2} for 10 seconds, resulted in significant reduction in *C. difficile* germinating spores (De Sordi et al., 2015).

The enhanced inactivation of spores in relation to longer exposure to germinants has also previously been demonstrated. In a study by Nerandzic & Donskey (2010) *C. difficile* spores were incubated in germinating medium for increasing periods of time, and following incubation the germinating suspension was then exposed to ethanol. The results of this study demonstrated that, as the length the spores were left to germinate under anaerobic conditions, so did the susceptibility to ethanol (Nerandzic & Donskey, 2010). This is further supported by a study in which Murdoch et al. demonstrated the increased susceptibility of *Aspergillus niger* spores to 405 nm light at a dose of 454 Jcm^{-2} upon increased incubation in germination medium from 2-8 hours incubation, obtaining a 0.8 log_{10} and 2.5 log_{10} reduction respectively (Murdoch et al., 2013). Increased susceptibility of the conidia (yeast spores) to 405 nm light was evident after only a 2 hour germination period, indicating that the initial changes in the spore coat are required in order to increase bacterial susceptibility (Murdoch et al., 2013). Additionally, germinating *B. subtilis* spores exposed to 405 nm light demonstrated enhanced susceptibility to photodynamic inactivation with the highest sensitivity of the germinating spores observed following 90 minutes of spore outgrowth; upon interrupting outgrowth at early stages enhanced susceptibility of spores was still observed (Abad-Lozano and Rodriguez-Velera, 1984).

A previous study further demonstrated that the induction of germination, resulting in a loss of *B. cereus* spore protection properties can lead to enhanced spore susceptibility, especially to treatments that previously had no adverse impact on the spore (Pol et al., 2001). *B. cereus* demonstrated resistance to nisin and/or PEF treatment in a study carried out by Pol et al. however following the induction of germination the nisin resistance of spores appears lost, with susceptibility of the spores observed in the very early stages of germination, suggesting that access to the membrane early in germination is important.

In conclusion, this research has demonstrated significant findings that upon inducing spore germination, the susceptibility of these highly resilient spores to 405 nm light is significantly increased, requiring 78% lowered doses to achieve complete inactivation. Furthermore, this study has demonstrated that upon triggering germination the germinating spore is more susceptible to inactivation than its vegetative cell counterparts. This is key information

demonstrating a mechanism to greatly enhance the susceptibility of both spores and vegetative cells of the highly infectious *C. difficile*, and as such could suggest the introduction of the use of germinants to increase inactivation of *C. difficile* under a range of settings to a wide range of technologies: in this case, 405 nm light for use in the clinical setting.

Part 3 - Comparison of the Germicidal Efficacy of 405 nm light and UV-C for the Inactivation of *C. difficile* spores

6.8. GENERAL

C. difficile spores are generally much more resilient than their vegetative counterparts due to the change in DNA conformation as a result of small acid soluble proteins (SASPS) binding to DNA upon sporulation, the presence of dipicolonic acid within the spore core, spore dehydration and the presence of pigments in the spore coat that absorb radiation (Setlow, 2006). Due to the resistance of spores to disinfectants used within the clinical environment and the ability of *C. difficile* spores to survive in the environment for up to 5 months, 'no touch' systems such as those using ultraviolet (UV) light (as discussed in Section 2.5.2.3) are becoming more commonly used as a method of decontamination within the hospital.

The most germicidal wavelength of UV light (termed UV-C) are in the region of 200-280 nm, and exposure to these results in DNA and RNA damage following photon absorption, causing the formation of thymine dimers between the bases thymine and cytosine, interrupting replication, therefore inhibiting reproduction and growth (Anderson et al., 2013). UV light has been used extensively for the treatment of contaminated air alongside its applications for surface and water sterilization, and is able to effectively kill a range of bacteria, fungi and viruses. There are several commercially available UV-C devices undergoing clinical trials for hospital decontamination, many of which are automatic non-touch disinfection systems, ensuring operator safety. However, the drawbacks associated with the use of UV-C light - such as its low penetrability and degradative properties - results in limiting factors with regards to the use of the decontamination devices. Furthermore, due to the carcinogenic effects caused by UV-C light, the use of these devices requires the rooms of use to be completely vacated, which may limit the amount of times the device can be used in a busy hospital, and also resulting in longer turnaround times between patients (Maclean et al., 2015; Yin et al., 2013).

Although less germicidal than UV light, violet-blue 405 nm light has the advantage that it can be used at levels which provide an antimicrobial effect whilst being non-detrimental to

exposed individuals (Hamblin & Hasan, 2004; McDonald et al., 2013; Yin et al., 2013). In addition to this, violet-blue light does not induce material degradation, which is a known problem with UV light (Irving et al., 2016). The increased safety aspects coupled with the antimicrobial efficacy of 405 nm light make it favourable for day to day use for infection-control alongside current housekeeping protocols. As discussed in Section 2.6.3, recent studies carried out in hospital isolation rooms have demonstrated the use of this technology for continuous decontamination of occupied environments, with reductions in the levels of bacterial contaminants by up to 80%, over and above that achieved with standard cleaning and infection control procedures alone (Maclean et al., 2010; Bache et al., 2012; Maclean et al., 2015; Maclean et al., 2014). This coupled with successful inactivation observed when *C. difficile* spores were exposed to 405 nm light and enhanced inactivation in the presence of recommended sporicidal disinfectants (Moorhead et al., 2016) has indicated the potential for the use of 405 nm light for the successful inactivation of *C. difficile* within the hospital environment.

Previous studies have demonstrated the efficacy of UV-C light for the inactivation of spores, which will be discussed further later in this chapter. With the previous chapters demonstrating the susceptibility of *C. difficile* spores to 405 nm light, this chapter compares the efficacy of both UV-C light and 405 nm light for the inactivation of *C. difficile* spores and weighs the advantages and disadvantages of both decontamination techniques.

6.9. METHODOLOGY

6.9.1. TREATMENT METHOD

C. difficile spores were cultivated as previously described in Section 3.3.2.1. Exposure of bacterial suspensions was conducted using the high irradiance ENFIS Photostar Innovate UNO 24 system or the UV-C light source, as previously detailed in Sections 3.5.1 and 3.6, respectively.

For exposure of suspensions a 2 ml volume of the bacterial suspension was transferred to a 12-well multi-dish. This was then placed 5 cm below the light source for 405 nm light exposures and 1 cm below the light source for UV-C light source, with the plate placed on a stand to allow adequate air flow around the dish, preventing any potential heat to build-up at the base of the multi-dish.

For surface exposures 50 µl of the spore suspension was inoculated onto PVC, Vinyl flooring, aluminium or stainless steel and dried for 15-20 minutes at 50°C. These were then

exposed to an irradiance of 1.51 $mWcm^{-2}$ at a distance of 1 cm for UV-C light treatment, and 225 $mWcm^{-2}$ at a distance of 5 cm for 405 nm light treatment, for increasing durations.

Controls were exposed to the same laboratory conditions, however were not exposed to UV-C light or 405 nm light. Samples were then enumerated and viable bacterial counts recorded as log_{10} $CFUml^{-1}$, as discussed in Section 3.7.2. Experiments were repeated in triplicate.

6.10. RESULTS

6.10.1. COMPARISON OF THE EFFICACY OF 405 NM LIGHT AND UV-C FOR THE INACTIVATION OF *C. DIFFICILE* SPORES IN SUSPENSION

C. difficile spores were exposed to shortwave UV light (UV-C) and 405 nm light for a comparison of the inactivation kinetics of the two germicidal regions of light. Figure 6.18 demonstrates the efficacy of UV-C light for the inactivation of *C. difficile* spores suspended in PBS, requiring only 30 minutes exposure to an irradiance of 1.51 $mWcm^{-2}$ (a dose of 2.7 Jcm^{-2}) to achieve a 3.5 log_{10} reduction ($P<0.001$). In comparison, 405 nm light required a dose of 2.43 $kJcm^{-2}$ to achieve a 3.3 log_{10} reduction, as seen in Figure 6.19. UV-C light is 900× more efficient at inactivating *C. difficile* spores, however as previously mentioned there are many drawbacks associated with UV-C light for use as a decontamination method.

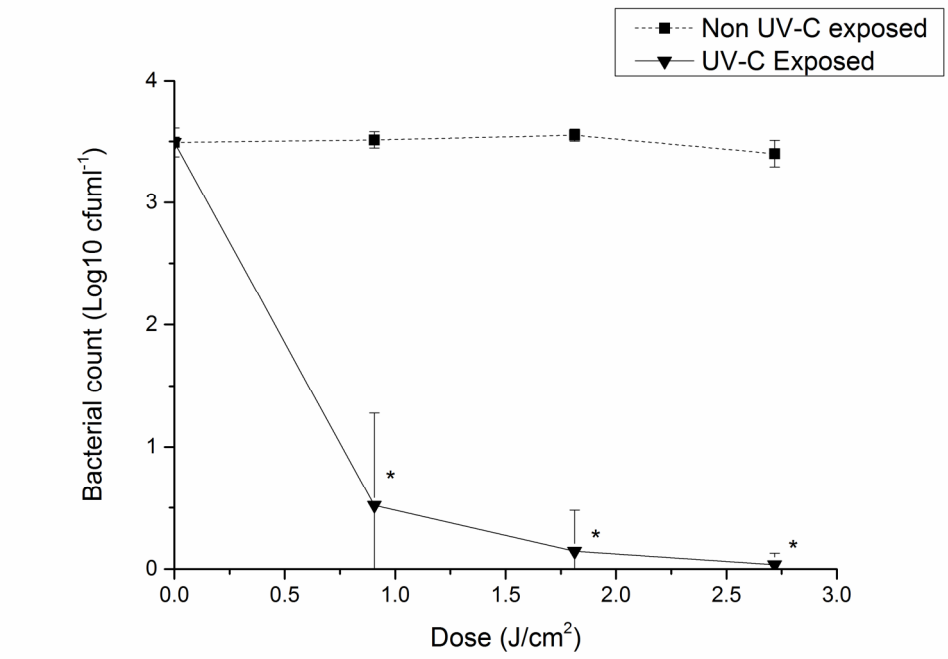

Figure 6.18 The effect of increasing dose of UV-C light (1.51 mWcm^{-2}) on *C. difficile* spores suspended in PBS. Control samples were non-light exposed for the equivalent time periods (0, 10, 20 and 30 minutes), therefore are represented as dotted line trends. The dose given refers to 405 nm light / UV-C exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

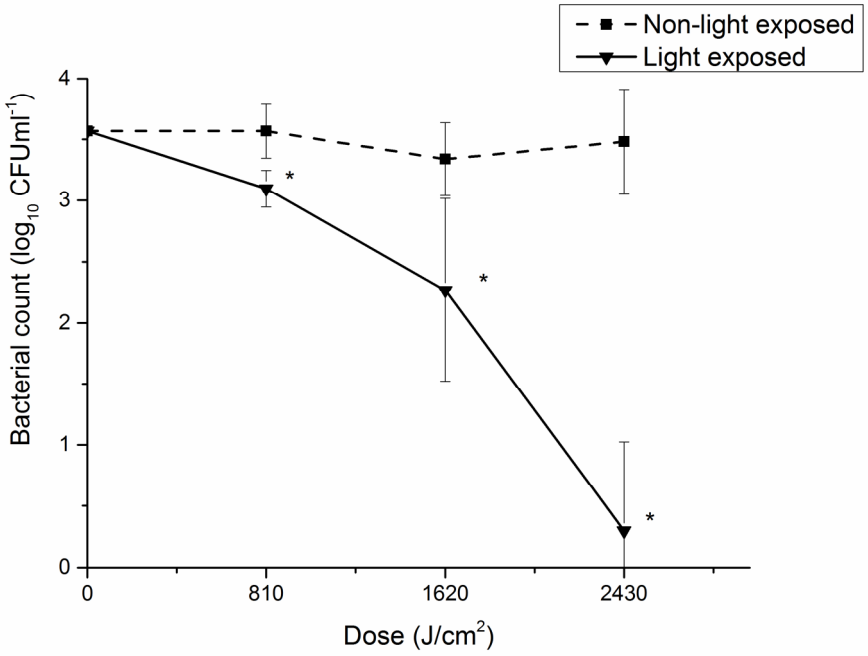

Figure 6.19 The effect of increasing dose of 405 nm light (225mWcm^{-2}) on *C. difficile* spores suspended in PBS. Control samples were non-light exposed for the equivalent time periods (0, 1, 2 and 3 hours), therefore are represented as dotted line trends. The dose given refers to 405 nm light / UV-C exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

6.10.2. COMPARISON OF THE EFFICACY OF 405 NM LIGHT AND UV-C FOR THE INACTIVATION OF *C. DIFFICILE* SPORES ON CLINICALLY RELEVANT SURFACES

Similar to the results observed in the suspension experiments (Figure 6.18 and Figure 6.19), UV-C light was demonstrated to be much more sporicidally efficient than 405 nm light for decontamination of all surfaces.

On PVC surface coupons upon exposure to 405 nm light (2.43 $kJcm^{-2}$) (Figure 6.20a, Figure 6.21a, Figure 6.22a and Figure 6.23a) almost complete inactivation was observed with a 2.55 log_{10} reduction achieved, whilst on all other surfaces a 1.46 log_{10} - 1.63 log_{10} inactivation was observed. Comparitively, *C. difficile* spores seeded onto surfaces coupons exposed to UV light demonstrated much greater susceptibility, with a 99.7% lowered dose than that used for 405 nm light achieving spore reductions of 3.44 – 3.71 log_{10} (Figure 6.20b, Figure 6.21b, Figure 6.22b and Figure 6.23b).

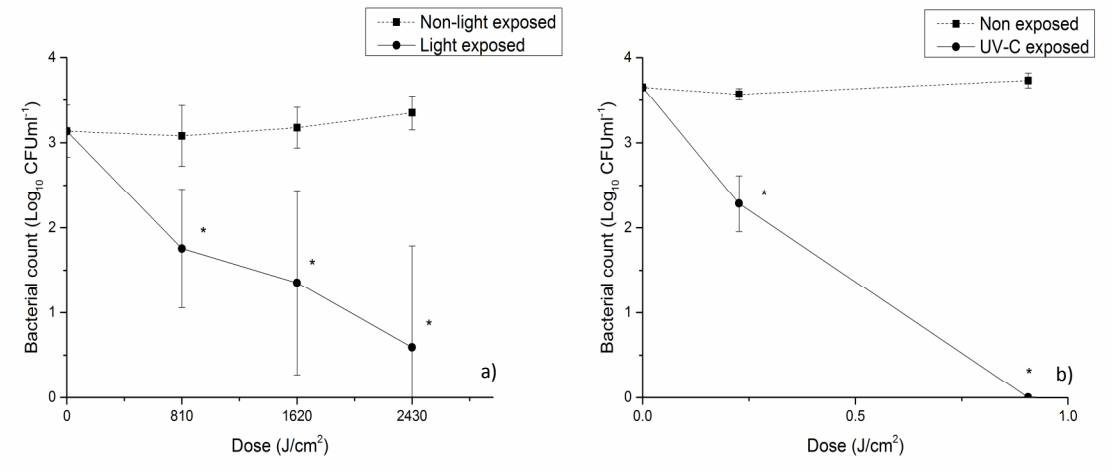

Figure 6.20 Comparison of the inactivation kinetics of *C. difficile* spores on PVC surfaces exposed to (a) 405 nm light at an irradiance of 225 $mWcm^{-2}$ and (b) UV-C light with an irradiance of 1.51$mWcm^{-2}$. Control samples were non-light exposed for the equivalent time periods (0, 2.5 and 10 minutes), therefore are represented as dotted line trends. The dose given refers to 405 nm light / UV-C exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

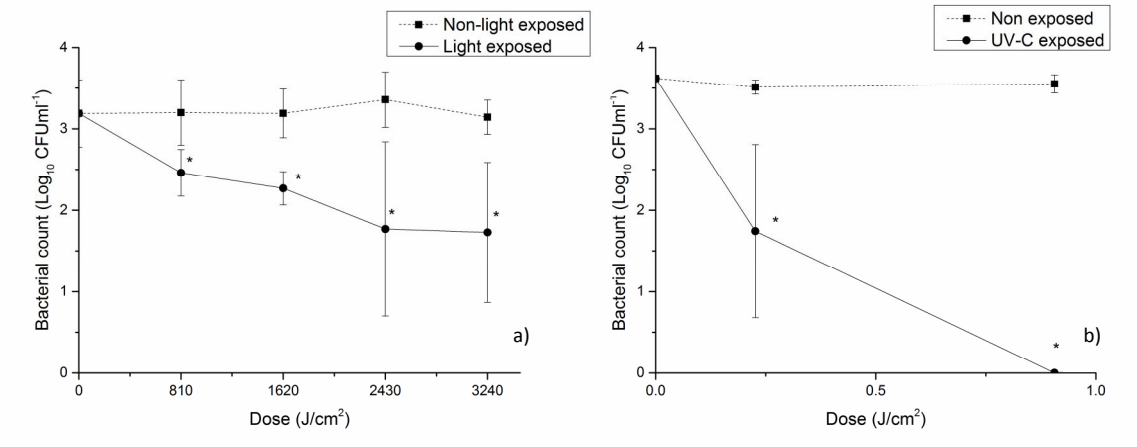

Figure 6.21 Comparison of the inactivation kinetics of *C. difficile* spores on vinyl flooring exposed to (a) 405 nm light at an irradiance of 225 mWcm^{-2} and (b) UV-C light with an irradiance of 1.51mWcm^{-2}. Control samples were non-light exposed for the equivalent time periods (0, 2.5 and 10 minutes), therefore are represented as dotted line trends. The dose given refers to 405 nm/ UV-C light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

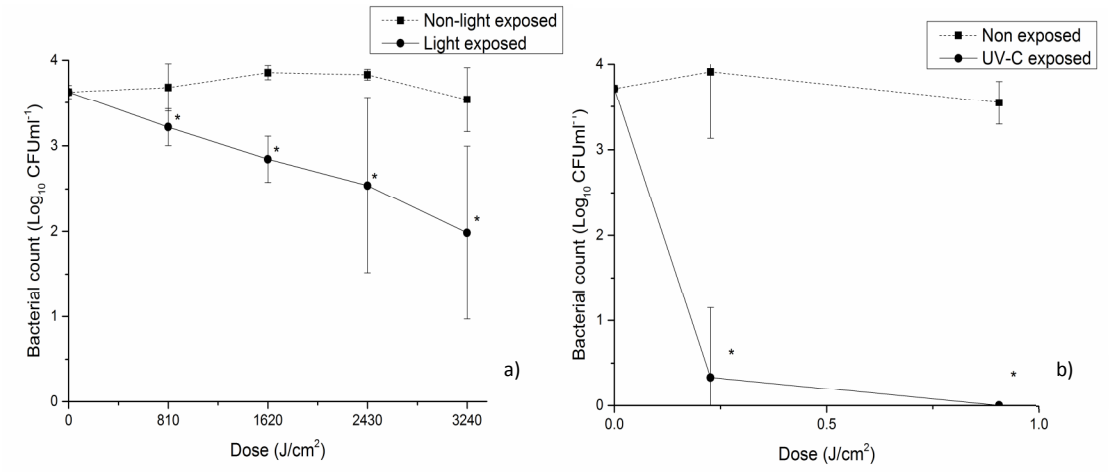

Figure 6.22 Comparison of the inactivation kinetics of *C. difficile* spores on stainless steel surfaces exposed to (a) 405 nm light at an irradiance of 225 mWcm^{-2} and (b) UV-C light with an irradiance of 1.51mWcm^{-2}. Control samples were non-light exposed for the equivalent time periods (0, 2.5 and 10 minutes), therefore are represented as dotted line trends. The dose given refers to 405 nm light / UV-C exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

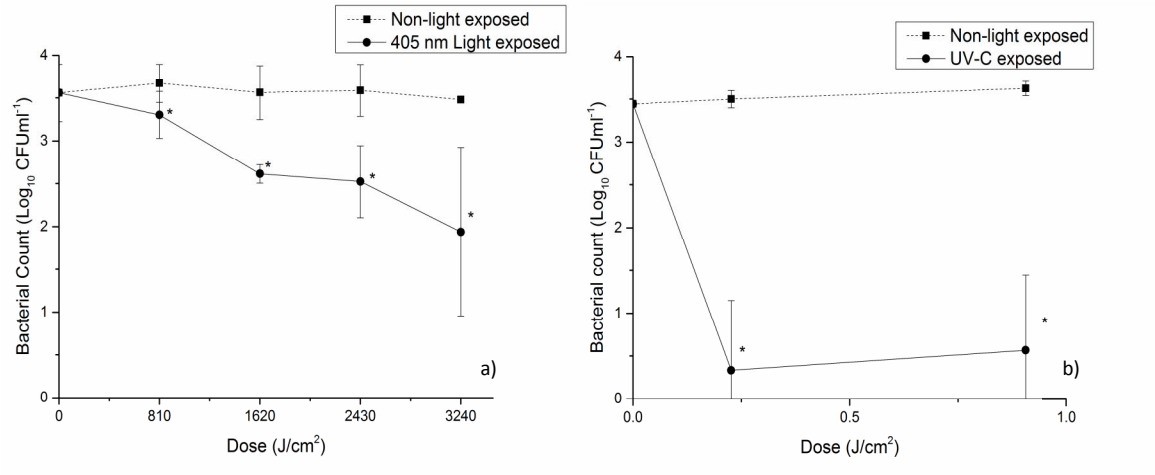

Figure 6.23 Comparison of the inactivation kinetics of *C. difficile* spores on aluminium surfaces exposed to (a) 405 nm light at an irradiance of 225 mWcm^{-2} and (b) UV-C light with an irradiance of 1.51mWcm^{-2}. Control samples were non-light exposed for the equivalent time periods (0, 2.5 and 10 minutes), therefore are represented as dotted line trends. The dose given refers to 405 nm / UV-C light exposure only. * represent significant bacterial inactivation compared to controls (P≤0.05). Each data point is a mean value ± SD (n=6).

The germicidal efficacy of 405 nm light and UV-C light for the inactivation of *C. difficile* spores was then compared to allow for a direct comparison of the efficacy of both germicidal light sources for sporicidal decontamination. As seen in Table 6.2, UV-C light is significantly more efficient for the inactivation of *C. difficile* spores, with 300-fold greater sporicidal efficiency observed in suspension. On clinically relevant surfaces UV-C light was 4,030-8,000-fold more efficient than 405 nm light. Spores seeded onto PVC surfaces exposed to UV-C light demonstrated the highest level of inactivation, whilst spores seeded onto vinyl stainless steel and aluminium surfaces exposed to 405 nm light exhibited the lowest germicidal efficiency.

Table 6.2 Comparison of the germicidal efficiency of 405 nm light and UV-C light for the inactivation of *C. difficile* spores in suspension and on a range of clinically relevant surfaces.

Suspension/ Surface type	Requirements for inactivation						Germicidal efficiency of UV light vs. 405 nm light
	UV-C			*405 nm light*			
	Dose (Jcm^{-2})	Log_{10} reduction	Germicidal efficiency (Log_{10} reduction/ Dose Jcm^{-2})	Dose (Jcm^{-2})	Log_{10} reduction	Germicidal efficiency (Log_{10} reduction/ Dose Jcm^{-2})	
Suspension	2.7	3.5	0.8	2430	3.3	0.001	×300
Surfaces							
PVC	0.906	3.65	4.03	2430	2.54	0.001	×4030
Vinyl	0.906	3.62	4	3240	1.46	0.0005	×8000
Stainless steel	0.906	3.71	3.86	3240	1.65	0.0005	×7720
Aluminium	0.906	2.87	3.17	3240	1.62	0.0005	×6340

6.11. DISCUSSION

Investigations were carried out the compare the efficacy of 405 nm light and UV-C light for the inactivation of *C. difficile* spores both in suspension and on clinically relevant surfaces. The results in Figure 6.18 demonstrate the high susceptibility of *C. difficile* spores in suspension to UV-C light with complete inactivation observed when exposed to a dose of 2.7 Jcm^{-2} in comparison to a dose of 2.43 $kJcm^{-2}$ 405 nm light for complete inactivation: ~900× that required for UV-C light.

On surfaces *C. difficile* spores were completely inactivated by UV-C light following exposure to a dose of 0.906 Jcm^{-2}, with the exception of aluminium surfaces on which a 99.5% reduction in *C. difficile* spores was observed (Figure 6.20- Figure 6.23). In comparison, 405 nm light requires much higher doses to obtain bacterial reduction on all the surfaces exposed, with almost complete inactivation observed only on PVC surfaces following exposure to approximately a 2,682-fold higher dose than that of UV-C light. On Vinyl flooring, aluminium and stainless steel surfaces only a 93-95% reduction in spore population was observed following exposure to 405 nm light at a dose of 3.24 Jcm^{-2} – 3576× the dose of UV-C light achieving complete inactivation of spores. Comparative vegetative cell inactivation kinetics upon exposure to 405 nm light and UV-C could not be obtained in the present study as the doses of UV-C light required for the inactivation of highly resilient *C. difficile* spores were so low, taking only minutes to achieve complete inactivation. However, it can be anticipated that at dosages obtaining sporicidal effects vegetative cells are fully inactivated.

In comparison to the maximum 3.71 log_{10} reduction in *C. difficile* spores following exposure to a dose of 0.906 Jcm^{-2} UV-C light (1.51 $mWcm^{-2}$) in the present study (Figure 6.22b), results from a study by Nerandzic et al. (2010) demonstrated a 2-3 log_{10} reduction in *C. difficile* counts following exposure to UV-C a constant dose of 59.4 Jcm^{-2} (22 $mWcm^{-2}$); this investigation was carried out using the Tru-D™ Rapid Room Disinfection device. A possible explanation for the higher doses of UV required in the study by Nerandzic et al. in comparison to that of the current study is that as the study by Nerandzic et al. was carried out in a room with the light source further away from test surfaces, whilst in the current study the contaminated surfaces were placed directly below the UV light source. As such,

more energy would be used in the whole room study to ensure adequate coverage of all surfaces.

The Tru-D UV-C emitting device, emitting continuous wave UV (CW-UV) light (wavelength 254 nm), is programmed to deliver light at an intensity of 22,000-36,000 $\mu Wscm^{-2}$ for spores, with a median 45 minute cycle for spores. Several studies have reported the success of Tru-D for decontamination of clinically relevant surfaces, with a study by Boyce et al. reporting a 1.7-2.9 log_{10} reduction in *C. difficile* spores when a population of 10^5 $CFUml^{-1}$ was inoculated onto stainless steel carrier disks and placed in several areas within a hospital room, both in the direct and indirect line of UV-C and exposed to a light at an intensity of 22,000 $\mu Wscm^{-2}$ for 67.6 minutes (Boyce et al., 2011). On Formica laminate sheets a 10^4-10^5 log_{10} reduction in *C. difficile* spores was observed following exposure to a light at an intensity of 36,000 $mWscm^{-2}$, with a 99.8% reduction observed after 50 minutes (Rutala et al., 2010). The efficacy of this device for spore decontamination is further supported by a study reporting a 41% reduction in CDI contamination in the rooms of patients following implementation of the Tru-D device (Anderson et al., 2013). The Pathogon continuous UV-C emitting device has been shown to be equally as effective as the Tru-D device for reducing contamination levels of *C. difficile*, MRSA and VRE (Nerandzic et al., 2014). A further continuous UV-C emitting device, Clinell UV-360, emits a wavelength of 254 nm, similar to that of the Tru-D device previously discussed, and has been shown to reduce *C. difficile* endospore contamination by 99.995% from a distance of 12 feet following an 8 minute exposure (Clinell UV-360, n.d.).

Lowered airborne contamination has also been demonstrated as a result of UV-C devices with a 40% reduction in bacterial counts demonstrated on settle plates in patient bathrooms, following exposure to the Sanuvox Aseptix 1 UV-C system, however these results were not statistically significant (P=0.31) (Hunt et al., 2016).

Pulsed xenon UV light, unlike continuous UV-C devices, emits intense broad spectrum light, combining UV-A, UV-B and UV-C, in short, high energy pulses, providing rapid decontamination, much faster than that achieved with continuous UV light. A recent study by Levin and colleagues reported that three 7 minute exposures of the Xenex PX-UV following terminal cleaning contributed to a 53% reduction in hospital acquired *C. difficile*

infections (Levin et al., 2013). In support of this, a 40-45% decrease in *C. difficile* infection rates was further demonstrated following implementation of PX-UV in a range of differing settings facility wide (Vianna et al., 2016). PX-UV is reported to inactivate *C. difficile* spores within 5 minutes of exposure, whilst CW-UV can range from 5-45 minutes for sporicidal inactivation (Stibich, 2017). In contrast, pulsed xenon sources have been shown to be less germicidal than UV-C, with UV-C demonstrating bacterial reductions of 1 $\log_{10}$, 3 $\log_{10}$ and 3.5 $\log_{10}$ in comparison to that of PX-UV achieving 0.5 $\log_{10}$, 1.8 $\log_{10}$ and 0.6 $\log_{10}$ reductions in *C. difficile*, MRSA and VRE contamination, respectively, with each light source delivered for a time of 10 minutes at a distance of 4 feet (Nerandzic et al., 2015). These conflicting reports indicate that, whilst both PX-UV and CW-UV devices have demonstrated their potential for sporicidal decontamination in the clinical environment, there is still much research to be done with regards to their efficacy within the hospital environment. PX-UV is considered to be more environmentally friendly than continuous wave UV, as these devices do not contain mercury, however, the Surfacide continuous wave UV-C device - although containing pelleted mercury - contains a lamp that lasts on average 20x longer than that of competitors, thus making it environmentally efficient (Veenhuis, 2017).

In a study using a handheld device that generates UV light in the far UV spectrum (185-230 nm) a 4.4 $\log_{10}$ reduction in *C. difficile* spores was reported following exposure to 100 $mJcm^{-2}$ for 5 seconds (20 $mWcm^{-2}$) (Nerandzic et al., 2012). A further handheld wand, the Verilux CleanWave Sanitizing Wand, emitting UV-C at a wavelength of 265 nm has further been demonstrated to inactivate a range of bacterial spores, including *C. difficile*, within a few seconds of exposure to an irradiance of 5.5 Wcm^{-2} at a distance of 12.5 mm with a 90% reduction of viable organisms achieved within 40 seconds (Petersson et al., 2014).

Although both continuous UV-C and pulsed-xenon sources have both been demonstrated to be highly successful for the inactivation of *C. difficile* spores and there is great potential for these devices to lower *C. difficile* contamination levels in patient rooms, there are several drawbacks associated with the use of UV-C. These disadvantages include:

- the necessity for the exclusion of personnel from treatment zones due to health risks;
- the low penetrability of UV-C light wavelengths;

- the decontamination effect is only short term as it cannot be used continuously (Maclean et al., 2015).
- degradation of surface materials can result in cracks in materials which can allow for bacteria – especially *C. difficile* spores which can live for up to 5 months - to avoid contact with cleaning disinfectants (Landelle et al., 2014; Wheeldon et al., 2008). It has previously been confirmed that 405 nm light does not result in photodegredation of materials (Irving et al., 2016; Yin et al., 2013).

A further drawback associated with the use of UV light is the potential for microbial resistance to develop. As the mechanism of inactivation of 405 nm light is non-specific oxidative damage, it is unlikely that resistance will develop. This has been demonstrated previously with PDT, where following the repeated exposure of bacteria to sub lethal doses of 670 nm light in the presence of methylene blue, resistance did not develop (Pedigo et al., 2009). Tomb et al. (2016) confirmed that resistance in unlikely to develop following repeated sub lethal exposure of *Staphylococcus aureus* to 405 nm light.

There is no specific information in the current literature with regards to the ability of *C. difficile* to repair sub-lethal damage caused by exposure to UV light. As such, the dose of UV-light applied for the treatment of *C. difficile* must result in complete kill of the pathogen, if not, photorepair could occur. As discussed in Section 2.5.2.2, any light between 300 – 500 nm can result in photorepair, including standard artificial lighting within the clinical environment.

It has also previously been reported that UV-C light is less effective at killing bacteria in clinical settings compared with experimental, non-clinical conditions. A study by Anderson et al. showed that UV-C light exposure decreased MRSA, VRE, *C. difficile*, and *Acinetobacter* populations by 3-4 $\log_{10}$ under laboratory conditions, whereas a 1.07 $\log_{10}$ reduction in total CFUs and a 1.35 $\log_{10}$ reduction in targeted pathogens was observed in the clinical environment (Anderson et al., 2013). However, in this particular study cultures were obtained before and after the application of UV light in rooms that had not undergone standard cleaning and disinfection, therefore it is possible that the efficacy of UV-C was affected by the continued presence of dirt and debris on surfaces and equipment (Rutala et al., 2010; Anderson et al., 2013).

Although the results of the present study highlight that much higher exposure doses of 405 nm light are required for the inactivation of spores in comparison to that of UV-C light, it is

important to note that 405 nm light does not pose the same threat to human safety (Yin et al., 2013), and can be utilised at levels that are safe for continuous human exposure. Therefore violet-blue 405 nm light can be used in the clinical environment throughout the day alongside normal hospital lighting, providing continuous disinfection of the clinical environment and enabling higher doses of 405 nm light to be emitted throughout the day, whilst UV-light requires complete clearance of patient rooms while cleaning is underway,. Therefore, despite the efficiency of UV-light for sporicidal activity being much greater than that of 405 nm light, the safety advantages for 405 nm provide distinct advantages for continuous treatment of patient-occupied areas.

6.12. OVERALL CONCLUSIONS

Overall, this chapter has investigated some key factors influencing the susceptibility of *C. difficile* spores to 405 nm light and compared its sporicidal efficacy to that of UV-light.

This chapter has successfully provided evidence supporting the mechanism of inactivation of 405 nm light, showing oxygen to play a vital role in the inactivation of *C. difficile* vegetative cells and spores. Additionally the enhanced inactivation of *C. difficile* spores upon triggering germination was demonstrated, with key results demonstrating significantly lower doses of 405 nm light required to achieve complete inactivation of spore populations upon germination of spores. The results demonstrated that, following suspension in germination medium for 24 hours, a 78% lower dose of 405 nm light was required to achieve a complete 6 $\log_{10}$ reduction in comparison to that which achieved a complete 5 $\log_{10}$ reduction observed for non-germinated spores.

Finally, this chapter progressed to compare the sporicidal efficacy of 405 nm light with that of UV-C light, with results demonstrating UV's enhanced germicidal efficacy when compared to that of 405 nm light. UV light demonstrated a 300× greater germicidal efficacy for the inactivation of *C. difficile* spores in comparison to 405 nm light in suspension and ranging from 4030 – 8000× on clinically relevant surfaces. Germicidal UV light sources are becoming increasingly popular for whole-room decontamination within the hospital environment, however due to some of the safety issues that have been discussed earlier, these technologies tend to be better suited for terminal cleaning, with 405nm light having distinct advantages for continuous decontamination applications.

CHAPTER 7

Conclusions and Future work

7. GENERAL

C. difficile is a major cause of antibiotic associated diarrhoea and pseudomembranous colitis, causing chronic and relapsing diarrhoea with a recurrence rate of 5 – 47% and a mortality rate as high as 17% (Bartlett & Gerding, 2008; Dubberke & Wertheimer, 2009; Pépin et al., 2005; Zilberberg et al., 2014). Additionally, CDI results in increased hospital stays and increased hospital costs. From the literature, it is clear that *C. difficile* spores play a significant role in the transmission of disease, with the environment harbouring these highly infectious resilient spores for up to 5 months (Shaughnessy et al., 2011). Furthermore, current disinfection strategies and technologies that have demonstrated sporicidal effects have several drawbacks including safety concerns, increased turnover times of patient rooms and inefficiency for the effective decontamination of the clinical setting with spores remaining in the environment upon admission of subsequent patients.

This study has investigated the efficacy of 405 nm violet-blue light for the inactivation of *C. difficile* with research focusing on the fundamental interactions of vegetative cells and spores with light. Previous studies have proven this technology to be effective for continuous decontamination of the hospital environment, however studies have focused on staphylococci as the indicator organisms, with studies measuring the total viable counts (TVC) (Bache et al., 2012; Maclean et al., 2010; Maclean et al., 2013), and as yet no testing has been conducted to evaluate the effects on *C. difficile* specifically in the clinical environment. The work of this body of research has enhanced this by investigating aspects that could have implications for clinical use, including potential mechanisms to enhance sporicidal effects, such as the use of disinfectants and the effects of soilage. This chapter summarises the overall findings of this study, and also discusses suggestions for future work to broaden and continue the research in this important area.

7.1. CONCLUSIONS

7.1.1. THE EFFICACY OF 405 NM LIGHT FOR INACTIVATION OF *C. DIFFICILE*

The present study investigated the efficacy 405 nm light for the inactivation of *C. difficile* vegetative cells and spores, both in suspension and seeded onto clinically relevant surfaces. The findings of this study demonstrated the successful inactivation of both vegetative cells and spores in suspension (up to a 5 $\log_{10}$ CFUml^{-1} reduction), with spores demonstrating an enhanced resilience requiring ×10 the dose (2.42 kJcm^{-2}) in comparison to their vegetative counterparts (252 Jcm^{-2}) to achieve a comparable 3 $\log_{10}$ CFUml^{-1} reduction.

Additionally, on clinically relevant surfaces, vegetative cells demonstrated an increased susceptibility to 405 nm light when seeded onto PVC and vinyl flooring in comparison to those in suspension. Spores, however, demonstrated an enhanced resilience to 405 nm light when seeded onto surfaces.

Results in Section 4.4 further demonstrated the successful inactivation of additional strains of *C. difficile*, and compared the inactivation kinetics of both vegetative cells and spores to that of *Bacillus cereus*, another spore-forming bacterium. Both *C. difficile* strains NCTC 11209 and ribotype 027 demonstrated susceptibility to 405 nm light, however demonstrated enhanced resilience in comparison to the inactivation kinetics of NCTC 11204, requiring a 25% increased dose to achieve complete inactivation. *B. cereus* vegetative cells exhibited a much greater susceptibility to 405 nm light than *C. difficile* vegetative cells, with a complete 3.5 $\log_{10}$ reduction observed at a 50% lower dose, whilst *B. cereus* spores demonstrated similar inactivation kinetics to that of *C. difficile* spores.

7.1.2. ORGANIC FAECAL MATTER ENHANCES SPORE SUSCEPTIBILITY

These investigations were carried out to investigate the efficacy of 405 nm light against *C. difficile* spores in the presence of organic faecal matter, as spores are known to be found in the environment associated with patient bowel movements (Rineh & Kelso, 2014).

Results have shown that inactivation of spores can be enhanced when suspended in faecal matter, requiring a 33% lower dose to achieve a similar 3 $\log_{10}$ CFUml^{-1} reduction. This may have positive implications for enhancing decontamination using 405 nm light in clinical environments, with potential for use within the bathrooms of patients with gastrointestinal infections alongside current cleaning and infection control measures. Enhanced inactivation is likely to be a result of the photoexcitation of components within the simulant faecal matter, such as yeast, miso and psyllium, upon exposure to light in the region of 405 nm.

Due to time constraints, vegetative cell inactivation kinetics when suspended in faecal matter were not investigated. However, due to the greater susceptibility of vegetative cells than spores, as previously demonstrated in Section 4.1.4, it is assumed that this enhanced inactivation of spores when suspended in faecal matter could translate to vegetative cells, but using much lower dose levels. Enhanced 405 nm light inactivation of vegetative cells could lead to increased decontamination of vegetative cells within patient faecal environmental contamination, using shorter exposure times, and as a result, minimise formation of spores within the patient environment.

However, enhanced inactivation of spores was not observed on surfaces. This may be as a result of the lowered transmission of 405 nm light through the faeces in comparison to PBS. However, in the case of faecal excreta, as previously discussed in Section 2.3.1, 10× more metabolically active vegetative cells are present in faeces in comparison to spores, and as such if these can be inactivated following shedding, this could potentially allow for a reduction in environmental contamination of spores. However, further work is required to elucidate this. At present, more work is needed to establish the potential for enhanced sporicidal effects of 405 nm light against surface-seeded contamination in the presence of faeces, and this will be discussed further in Section 7.2.

7.1.3. A SYNERGISTIC EFFECT IS ACHIEVED UPON COMBINATION OF 405 NM LIGHT AND CHLORINE-BASED DISINFECTANTS

Following demonstration of the successful inactivation of *C. difficile* using 405 nm light, experiments proceeded to investigate if a synergistic oxidative effect could be achieved upon combination of 405 nm light with recommended chlorine-based sporicidal disinfectants. If a synergistic effect could be achieved, this could allow for enhanced sporicidal effects using lower concentrations of chlorine-based disinfectants, at lower irradiances and shorter exposure times of 405 nm light. As previous results demonstrated an enhanced resilience of spores when seeded onto surfaces this was carried out both in suspension, for initial proof-of-concept, and on clinically-relevant surfaces to determine if spore inactivation could be achieved.

The results of this study demonstrated enhanced sporicidal activity upon the combined treatment of high intensity (225 $mWcm^{-2}$) 405 nm light and low strength chlorine-based disinfectants in suspension, with 33% lower doses of 405 nm light required when used in combination with all 3 selected disinfectants to achieve a similar 3 log_{10} $CFUml^{-1}$ reduction

as that achieved with 405 nm light alone. The results in Sections 5.2.4.1 and 5.2.4.3 further highlighted the importance of taking into account different surfaces when determining the sporicidal efficacy of disinfectants, as when seeded onto surfaces, sporicidal inactivation as a result of synergy was dependant on both the surface and the disinfectant used. This stresses that although a cleaning method may be successful on one surface, this must be demonstrated on a range of relevant surfaces.

In addition to the high intensity light treatments, a low irradiance system (~0.4 $mWcm^{-2}$) was used for the exposure of spores seeded on clinically-relevant surfaces. This low irradiance light was more indicative of what would be used in the clinical setting if used for the continuous decontamination of rooms housing patients with CDI (Maclean et al. 2013; Maclean et al. 2010). Results demonstrated, that upon combination with chlorine-based disinfectants, and concentrations lower than that currently recommended for sporicidal cleaning, enhanced sporicidal effects were observed on all surfaces with up to 100% increased spore reductions achieved in comparison to that achieved when low irradiance 405 nm light was used alone.

Although a synergistic sporicidal effect was demonstrated upon the combination of disinfectants with 405 nm light on a range of clinically relevant surfaces, there is still much work to be done to gain a better understanding of the range of results observed on all surfaces upon using different disinfectants and irradiances of 405 nm light.

These results demonstrate that 405 nm light could successfully be employed for use alongside current in-house cleaning to facilitate the enhanced decontamination of vegetative cells and spores on clinical surfaces, and could allow for the use of lower strength disinfectants. This is turn could minimise the drawbacks associated with these currently recommended disinfectants, such as the release of irritating vapours that are harmful to healthcare workers. The use of lower strength disinfectants could further minimise spore transmission, as corrosion may potentially play a role in harvesting of bacterial spores in the environment, and as such the use of chlorinated disinfectants at lower strength concentrations could minimise corrosion as a result of frequent cleaning.

7.1.4. Oxygen is vital for successful 405 nm light inactivation

Chapter 6 investigated aspects related to the mechanism of 405 nm light inactivation of *C. difficile*, and demonstrated the requirement of oxygen for achieving successful photodynamic inactivation. In Section 6.2 a series of experiments were conducted in which

spores were exposed to 405 nm light under both aerobic and anaerobic conditions and the inactivation kinetics were compared. Results demonstrated that under anaerobic conditions, in the presence of the oxygen scavenger ascorbic acid, minimal sporicidal effects were observed. In comparison, maximal sporicidal effects were observed under aerobic conditions. This highlights the key role oxygen plays in the 405 nm light inactivation process.

7.1.5. TRIGGERING GERMINATION INCREASES SPORE SUSCEPTIBILITY TO 405 NM LIGHT

Results in Section 6.4 demonstrated the enhanced susceptibility of *C. difficile* spores upon induction of spore germination, with a 4.5-fold lower dose of 405 nm light (225 $mWcm^{-2}$) achieving a similar 5 log_{10} reduction following exposure of spores to germinants for 3 hours under anaerobic conditions. This is a significant finding, as this could potentially lead to the use of germinants within the clinical environment to allow for increased susceptibility of highly resilient spores to enhance their susceptibility to a range of cleaning strategies. The potential use of germinants to enhance *C. difficile* susceptibility in the aerobic environment, in conjunction with the previously demonstrated synergistic oxidative effect of 405 nm light and chlorine-based disinfectants, could allow for a multi-faceted approach to achieve maximal sporicidal effects, whilst minimising detrimental effects associated with current in-house cleaning, allowing for a cleaner hospital environment and consequently reduced spore transmission.

7.1.6. 405 NM LIGHT IS LESS EFFICIENT THAN UV, HOWEVER HAS SEVERAL SAFETY ADVANTAGES

A comparison of the germicidal effects of UV-C and 405 nm light (Sections 6.10.1 and 6.10.2) demonstrated the greater sporicidal efficacy of UV-C light, with 900x and 3576x lower doses required for similar population reductions (3.5 log_{10} $CFUml^{-1}$) in suspension and on surfaces, respectively. Despite the greater sporicidal activity of UV-C light, 405 nm light has some distinct operational advantages over UV-C light. These include its enhanced safety, allowing its use for continuous decontamination of the clinical environment, and also its ease of use. With these advantages of 405 nm light taken into consideration in addition to the enhanced sporicidal effects of 405 nm light when used in combination with currently used disinfectants, as discussed previously, could allow for the implementation of this technology over UV-C technologies.

As UV-C light can only be used for terminal cleaning following the vacation of rooms housing patients with CDI, another advantage of 405 nm light is the potential for continuous decontamination throughout the day. Currently, cleaning is typically carried out twice a day with no current sporicidal treatment for patient bathrooms in between cleaning procedures. Additionally, most novel technologies under development are focusing on terminal cleans. 405 nm light is a novel technology providing a solution for continuous decontamination of the patient environment, allowing for the maintenance of low levels of contamination in patient rooms and with an emphasis on being the only technology to date that could allow for maintaining low contamination following use of commodes.

7.2. FUTURE WORK

Although the current research has demonstrated the efficacy of 405 nm light for the inactivation of spores, and mechanisms to enhance sporicidal activity, there is still more fundamental research to be conducted in relation to further understanding the sporicidal effects of these wavelengths of light. These would include (i) identifying the naturally occurring porphyrins present in vegetative cells and spores, (ii) further investigations into the germination and sporulation cycles of the bacterium and (iii) investigation of the ROS produced as a result of photo-excitation and the cellular targets. Additionally, it is of vital importance to translate this research into the clinical environment, demonstrating the capability of this technology to reduce the *C. difficile* bio-burden in hospital rooms housing patients with CDI. As such, I suggest that future research moves forward in two strands, as detailed below.

7.2.1. THE FUNDAMENTAL INTERACTIONS OF 405 NM LIGHT WITH *C. DIFFICILE*

Although the work of the current study has gained insight into the mechanism of inactivation of 405 nm light on the spores and vegetative cells of *C. difficile*, and possible mechanisms to enhance inactivation, there is still much to be established in this area. As such, this section will focus on continuing to establish the fundamental aspects of inactivation as a result of exposure to 405 nm light.

7.2.1.1. Further Elucidation of the Mechanism of Inactivation

As attempted in Section 6.2.4, the intracellular porphryin content of vegetative cells and spores can be measured using a fluorescence spectrophotometer. Furthermore, high performance liquid chromatography (HPLC) could be used to specifically determine the levels of differing porphyrins, and as such identify if there is any correlation observed

between the rate of inactivation and the presence of specific porphryin molecules. HPLC was previously used to distinguish the porphyrin content of *E. coli* and *S. aureus* following incubation in δ-Aminolaevulinic acid, which naturally increases the synthesis of uroporphyrin, coporphyrin and protoporphyrin IX (Nitzan & Kauffman, 1999). No current studies have used this method to analyse the porphyrin content of *C. difficile*. As such, the methodology use by Nitzan & Kauffman would be adapted for this study. HPLC is much more specific than fluorescence spectrophotometry due to its ability to separate, identify and quantify each component within the solution of interest. In comparison, the fluorescence spectrophotometer measures the emission of light following excitement of the molecule of interest with a specific wavelength, and the output is then compared to that of known porphyrins within the literature.

These analytical methods would require a higher spore population to be obtained to allow for the analysis of intracellular components as the currently obtained populations are too low to allow for accurate detection and measurements of the porphyrin content using standard laboratory equipment. As such obtaining higher spore populations is a further aspect of great importance in future studies. As detailed in Table 3.2 several methodologies were attempted in order to attain a high spore titre, however these were unsuccessful. As such, further attempts to obtain a spore population > 10^8 CFUml^{-1} would allow for a greater understanding of the naturally occurring endogenous porphyrins.

Another area of interest with regards to further elucidating the mechanism of inactivation of *C. difficile* spores upon exposure to 405 nm light is to determine the specific damage caused by the production of ROS, following activation of the type I and type II pathways. Scanning electron microscopy and transmission electron microscopy could be employed for these studies to achieve a greater understanding of the structural damage induced by violet-blue light, and the ROS generated as a result of photo-excitation. Electron microscopy has previously been used successfully to demonstrate the disruption of the cytoplasmic contents and damage to the membrane of bacterial cells following blue light treatment, indicating the primary target site of violet-blue light is the cell membrane (Zhang et al., 2014; Dai et al., 2013; Ashkenazi et al., 2003).

7.2.1.2. Investigation of the Effects of 405 nm light on Sporulation

Currently there are major areas lacking in the literature with regards to the sporulation cycle of *C. difficile*. As this research area continues to expand, it would be of interest to

investigate the effects of 405 nm light on the sporulation cycle of *C. difficile* and to determine at what stage of sporulation *C. difficile* becomes resilient to 405 nm light. Further investigations should also investigate if 405 nm light could influence the sporulation cycle. An aspect of this research could include measuring the gene expression of light exposed and non-light exposed sporulating populations, at different stages of sporulation, to elucidate the effects of exposure to 405 nm light on the ability of vegetative cells to form dormant, resilient spores.

Babikhani et al. (2012) previously measured the effect of sub-minimum inhibitory concentrations of vancomycin, rifaximin, and metronidazole on the sporulation kinetics and expression of sporulation genes of different *C. difficile* strains. This was done by measuring the production of heat resistant spores and using quantitative reverse-transcription PCR analysis to assess the effect of drug treatment on sporulation gene expression. The methods used in this study could be adapted for the future work of interest.

This could potentially lead to a novel treatment of patient rooms preventing the sporulation of vegetative cells present in the environment (after excretion in patient faeces), with an emphasis on the novelty of this decontamination systems ability to be used continuously throughout the day, if 405 nm light can successfully inhibit or prevent sporulation.

7.2.2. Investigation of the Practical Application of 405 nm Light for Decontamination in Hospitals

It is also of clinical importance to develop the research in terms of the clinical applications of the novel decontamination light system. This will now be discussed.

Previous studies have reported that 405 nm HINS-light is capable of inactivating a wide range of organisms, including *C. difficile*. A ceiling-mounted lighting system (HINS-light EDS), which utilises 405 nm light to provide continuous decontamination of the air and exposed contact surfaces within occupied hospital wards and rooms has been developed (Bache et al., 2012; Maclean et al., 2013). Due to it using visible-light wavelengths, the system can be operated continuously in occupied rooms to provide on-going environmental decontamination without disruption of normal day-to-day hospital procedures. Studies have been conducted using this decontamination system in the clinical environment, showing reductions of up to 86% in staphylococcal contamination levels (Maclean et al.,

2010), however there has been no research with regards to monitoring *C. difficile* levels in the clinical environment.

Importantly, the laboratory-generated results of this study have highlighted the sporicidal efficacy of 405 nm light. Although extremely high doses were required for successful inactivation (e.g. 2.4 $kJcm^{-2}$ for a 3.5 log_{10} $CFUml^{-1}$ reduction in spores), results have demonstrated that enhanced sporicidal effects can be achieved when 405 nm light is used in combination with chlorine-based disinfectants. Studies used disinfectants recommended for in-house cleaning of rooms of CDI patients, at low strength concentrations, in suspension and on a range of clinically relevant surfaces. Results showed that enhanced sporicidal effects were observed both in suspension, and on a range of clinically relevant surfaces upon exposure to low concentration disinfectants in combination with both high irradiance and low irradiance 405 nm light. However, more research is required to fully determine how this would translate for application in the clinical environment.

Future work should assess the efficacy of 405 nm light for the enhanced decontamination of isolation rooms housing patients with CDI using 405 nm EDS prototype systems. To do this, the 405 nm EDS unit could be operated continuously during daylight hours in occupied rooms and used as a complementary disinfection procedure, under conditions where normal clinical care and infection-control measures are implemented. Investigations should evaluate the *C. difficile* levels in rooms of patients with CDI, and in the rooms of patients occupying the room following discharge of CDI patients, with and without the EDS. Environmental sampling could be performed using cellulose sponges on a range of surfaces most frequently contaminated with *C. difficile*, using methodologies similar to that in current literature (Biswas et al., 2015; Dubberke et al., 2007; Otter et al., 2009). Following this the samples should be cultured and enumerated to evaluate the decontamination efficacy. This would be repeated in periods without the light system in use in order to compare contamination rates.

Additionally, the decrease in airborne *C. difficile* vegetative cells and spores following patient bowel movements and the resultant contamination of surfaces surrounding patient toilets should be investigated using the 405 nm light EDS. Best et al. (2010; 2012) reported that toilet flushing may be a major source of environmental contamination in the bathrooms of patients due to the production of aerosols following flushing, with an estimated $1 \times 10^4 - 1 \times 10^7$ of *C. difficile* per 1 g of faeces (Best et al., 2012; Best et al.,

2010b; Dawson et al., 2011). It is likely that vegetative cells are excreted in the faeces, forming their more resilient spore counterparts once exposed to the aerobic environment.

Future work could investigate the efficacy of 405 nm light for the inactivation of vegetative cells and spores released into the environment as a result of shedding following patient excrement. This could be done using an air sampler to test the viable bacterial counts in the air of the patient commodes at different periods following the use of the toilet, sampling of surfaces surrounding the toilet using cellulose sponges and using agar settle plates in various areas of the patient bathroom. This would be done using a methodology similar to that used by Best et al. (2010) using an impactor to impact samples directly onto *C. difficile* selective agar plates (Best et al., 2010a). As mentioned, if the vegetative cells are exposed to 405 nm light before forming spores, this could result in much faster kill of *C. difficile* and as a result lower environmental contamination.

7.3. Overall Conclusions

Overall, this thesis has highlighted the problems associated with *C. difficile* in hospitals and has investigated the sporicidal activity of violet-blue 405 nm light, and subsequently discussed its potential for use as a novel optical decontamination technology for decontamination of *C. difficile* contamination within the patient environment. This is a novel area of research, and as such the work of this study has focused on investigating the fundamental aspects of the interactions of violet-blue 405 nm light with *C. difficile* vegetative cells and spores, and also demonstrated mechanisms for enhancing susceptibility of the highly resilient spores.

Having demonstrated the efficacy of this novel light decontamination technology for the inactivation of *C. difficile* cells and spores, and also successful mechanisms for enhancing microbial susceptibility, this work forms an excellent platform to continue to develop this research in order to gain a full understanding of its fundamental efficacy against *C. difficile*, and also with the aim of implementing this novel technology for use in the rooms of patients with CDI in the clinical environment, consequently reducing the risk of patient cross-infection from environmental sources.

APPENDIX A

PEER REVIEWED JOURNAL PUBLICATIONS

- **S. Moorhead**, M. Maclean, J.E. Coia, S.J. MacGregor, J.G. Anderson (2015) Synergistic efficacy of 405 nm light and chlorinated disinfectants for the enhanced decontamination of *Clostridium difficile* spores. Anaerobe, **37**: 72-77. doi: 10.1016/j.anaerobe.2015.12.006

- M. Maclean, K. McKenzie, **S. Moorhead**, R.M. Tomb, J.E. Coia, S.J. MacGregor, J.G. Anderson (2015) Decontamination of the Hospital Environment: New Technologies for Infection Control. *Current Treatment Options in Infectious Diseases*, **7**(1):39-51. doi:10.1007/s40506-015-0037-5

- **S. Moorhead**, M. Maclean, S.J. MacGregor, J.G. Anderson (2016) Comparative sensitivity of *Trichophyton* and *Aspergillus* conidia to inactivation by violet-blue light exposure. *Photomedicine and Laser Surgery*, **34(1):**36-41. [* Work carried out during Undergraduate project]. doi: 10.1089/pho.2015.3922

CONFERENCE PUBLICATIONS

- **S. Moorhead**, M. Maclean, J.E. Coia, S.J. MacGregor, J.G. Anderson. Antimicrobial efficacy of 405nm light against *Clostridium difficile*: evidence of enhanced sporicidal activity when combined with disinfectants. [Poster] Healthcare Infection Society, 2014 Nov 16-18; Lyon, France.

- **S. Moorhead**, M. Maclean, J.E. Coia, S.J. MacGregor, J.G. Anderson. Inactivation of *C. difficile* by 405 nm HINS-light. [Poster] 8th annual Environmental and Technology conference, 2014 June 26; Glasgow, UK. * Invited presentation.

- **S. Moorhead**, M. Maclean, J.E. Coia, S.J. MacGregor, J.G. Anderson. Synergistic efficacy of 405nm light and hospital disinfectants for the enhanced decontamination of *C. difficile* on clinically relevant surfaces. [Poster] 5th International *Clostridium difficile* symposium, 2015 May 19-21; Lake Bled, Slovenia.

- **S. Moorhead**, M. Maclean, J.E. Coia, S.J. MacGregor, J.G. Anderson. Sporicidal efficacy of the combined use of 405nm light and disinfectants for inactivation of *Clostridium difficile* on clinically relevant surfaces. [Poster] Action on Infection conference, 2015 Nov 21-23; Glasgow, UK.

- **S. Moorhead**, M. Maclean, J.E. Coia, S.J. MacGregor, J.G. Anderson. Enhanced decontamination of *C. difficile* spores on surfaces via the synergistic action of 405nm light and disinfectants. [Poster] Microbiology Society Annual Conference, 2016 Mar 21-24; Liverpool, UK.

- **S. Moorhead**, M. Maclean, J.E. Coia, S.J. MacGregor, J.G. Anderson. Enhanced visible light inactivation of *Clostridium difficile* and Norovirus within simulated faecal contamination. [Poster] Microbiology Society Annual Conference, 2017 April 3-6; Edinburgh, UK.

AWARDS/ NOMINATIONS

- My poster presentation was awarded poster prize and selected for a short oral at the Healthcare Infection Society.
- My poster presentation was awarded poster prize and selected for a short oral at Action on Infection conference.
- Following my poster presentation I was nominated for 'Sir Howard Dalton young microbiologist of the Year' at The Microbiology Society Annual Conference.

Healthcare Infection Society, 2014 Nov 16-18; Lyon, France

Antimicrobial efficacy of 405nm light against *Clostridium difficile*: evidence of enhanced sporicidal activity when combined with disinfectants

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Background

Clostridium difficile can cause major contamination problems due to its ability to form highly infectious and resilient spores which can survive in the environment for prolonged periods. Recent work has demonstrated the use of antimicrobial 405nm light for environmental decontamination within hospitals, however further information relating to efficacy against spores is required.

Aims

The aim of this investigation was to establish the efficacy of 405nm light for inactivation of *C. difficile* vegetative cells and spores, and to establish whether spore susceptibility can be enhanced by the combined use of 405nm light with low concentration chlorinated and non-chlorinated disinfectants.

Method

C. difficile vegetative cells and spore suspensions were exposed to increasing doses of 405nm light (70-225mW/cm^2) to establish sensitivity. Exposures were repeated with spores suspended in a range of routine hospital disinfectants at varying concentrations.

Results

A 99.9% reduction in vegetative cell population was demonstrated with a dose of 252J/cm^2, however spores demonstrated higher resilience, with a 10-fold increase in dose required. Enhanced sporicidal activity was achieved when spores were exposed in the presence of low concentration disinfectants, with 50% increase in susceptibility when exposed in the presence of 0.1% sodium hypochlorite.

Conclusion

C. difficile vegetative cells and spores can be successfully inactivated using 405nm light, and the sporicidal efficacy can be significantly enhanced when exposed in the presence of low concentrations of disinfectants. Further research may lead to potential use of 405nm light decontamination in combination with hospital disinfectants to enhance *C. difficile* cleaning and infection control procedures.

SYNGERGISTIC EFFICACY OF 405 NM LIGHT AND HOSPITAL DISINFECTANTS FOR THE ENHANCED DECONTAMINATION OF *C. DIFFICILE* ON CLINICALLY RELEVANT SURFACES

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The ability of *Clostridium difficile* to form highly infectious and resilient spores which can survive in the environment for prolonged periods causes major contamination problems.

Antimicrobial 405 nm light is capable of inactivating a wide range of organisms, including endospore-forming bacteria, and is being developed for environmental decontamination within hospitals, however further information relating to its efficacy against spores is required. This study aims to establish the efficacy of 405 nm light for inactivation of *C. difficile* vegetative cells and spores, and to establish whether spore susceptibility can be enhanced by the combined use of 405 nm light with low concentration chlorinated and non-chlorinated disinfectants, in both liquid suspension and on surfaces.

C. difficile vegetative cells and spore suspensions were exposed to increasing doses of 405 nm light (using irradiances of 70-225 mW/cm^2) to establish sensitivity. Exposures were repeated with spores suspended in a range of routine hospital disinfectants at varying concentrations. Controls were exposed to 405 nm light in the absence of disinfectants, and disinfectants in the absence of 405 nm light, to establish the sporicidal activity of each agent alone, and to demonstrate the synergistic effect when combined. These experiments were repeated with spores seeded onto a range of relevant inert surfaces.

A 99.9% reduction in vegetative cell population was demonstrated with a dose of $252J/cm^2$, however spores demonstrated higher resilience, with a 10-fold increase in dose required. Enhanced sporicidal activity was achieved when spores were exposed to 405 nm light in the presence of low concentration disinfectants, with a 50% increase in susceptibility when exposed in the presence of 0.1% sodium hypochlorite. Synergy was also noted with the other disinfectants used.

In conclusion, *C. difficile* vegetative cells and spores can be successfully inactivated using 405nm light, and the sporicidal efficacy can be significantly enhanced when exposed in the presence of low concentrations of disinfectants. Further research may lead to potential use of 405nm light decontamination in combination with selected hospital disinfectants to enhance *C. difficile* cleaning and infection control procedures.

Action on Infection conference, 2015 Nov 21-23; Glasgow, UK.

Sporicidal efficacy of the combined use of 405nm light and disinfectants for inactivation of *Clostridium* difficile on clinically relevant surfaces

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The ability of *Clostridium difficile* to form highly infectious and resilient spores which can survive in the environment for prolonged periods causes major contamination problems. Antimicrobial 405 nm light is capable of inactivating a wide range of organisms, including endospore-forming bacteria, and is being developed for environmental decontamination within hospitals. Currently, there are several drawbacks associated with the chlorinated disinfectants recommended for surface decontamination, including their corrosive nature and the release of irritating vapours affecting healthcare workers. This study aims to establish whether spore susceptibility to low concentration chlorinated disinfectants can be enhanced when used in conjunction with 405 nm light.

Spores were spot inoculated and dried onto PVC, vinyl flooring and stainless steel. Samples were then simultaneously exposed to disinfectants (0.0001% Tristel, 0.01% Actichlor and 0.1% sodium hypochlorite (NaOCl)) and 405 nm light at an irradiance of 225 $mWcm^{-2}$. Control samples were exposed to 405 nm light alone, and disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined.

Results showed that spores exposed to 405 nm light alone were reduced by 1-2 log_{10} on all surfaces after a dose of 2.4 $kJcm^{-2}$. On PVC, complete inactivation was achieved following a dose of 0.8 $kJcm^{-2}$in the presence of NaOCl, and >2 log_{10} reduction was achieved following exposure to 0.8 $kJcm^{-2}$and 1.6 $kJcm^{-2}$ in combination with Actichlor and Tristel, respectively. Sporicidal activity was significantly enhanced in the presence of all three disinfectants on vinyl, with a 2.5-3 log_{10} reduction achieved following exposure to a dose of 1.6 $kJcm^{-2}$ in the presence of both Actichlor and NaOCl. However on stainless steel, spores demonstrated higher resilience to the combined oxidative effects of 405 nm light and disinfectants, with a dose of 2.4 $kJcm^{-2}$ required to achieve a 1.5-2 log_{10} reduction for all 3 disinfectants.

In conclusion, the sporicidal efficacy of commonly used chlorinated hospital disinfectants can be enhanced on a range of clinically relevant surfaces when used alongside 405 nm light. However, the extent of the enhanced sporicidal activity is dependent on the disinfectant and the surface it is applied to. This has the potential to lead to a considerable reduction in exposure time and concentrations of disinfectant required to eliminate *C. difficile* spores. Further research may lead to potential use of 405 nm light decontamination in combination with selected hospital disinfectants to enhance *C. difficile* cleaning and infection control procedures.

Microbiology Society Annual Conference, 2016 Mar 21-24; Liverpool, UK.

Enhanced decontamination of *C. difficile* spores on surfaces via the synergistic action of 405nm light and disinfectants

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The ability of *C. difficile* to form spores which can survive for prolonged periods causes significant environmental contamination problems. 405nm light has wide antimicrobial activity against vegetative bacteria, and is being developed for environmental decontamination within hospitals. As expected, spores are more resilient to inactivation. This study aims to establish whether spore susceptibility can be enhanced by combining 405nm light with low concentration chlorinated disinfectants: sodium hypochlorite, Actichlor and Tristel.

Spore suspensions were seeded onto surfaces including PVC, stainless steel and vinyl flooring. Disinfectant was added to the surface, and the samples were then exposed to 405nm light at irradiances of ~0.2-225 $mWcm^{-2}$. Control samples were exposed to 405nm light alone, and disinfectants alone, to establish the sporicidal activity of each agent, and to demonstrate the synergistic effect when combined.

Results demonstrated increased sporicidal activity of 405nm light and low-concentration sodium hypochlorite and Actichlor against *C. difficile* seeded on vinyl flooring and PVC surfaces, with approximately 3-log_{10} reductions achieved with up to 66% lower doses than achieved with light alone. Tristel demonstrated limited synergy on vinyl and PVC, whilst all three disinfectants demonstrated minimal synergy on stainless steel. Results are also reported for lower intensity light, as used in the clinical environment.

In conclusion, the sporicidal efficacy of 405nm light is enhanced when used alongside chlorinated disinfectants. Further research could potentially lead to the use of lower strength chlorinated disinfectants in combination with 405nm light to provide enhanced decontamination of *C. difficile* spores in the clinical environment.

Microbiology Society Annual Conference, 2017 April 3-6; Edinburgh, UK.

Enhanced visible light inactivation of *Clostridium difficile* and Norovirus within simulated faecal contamination

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Frequent bowel movements associated with Norovirus and *Clostridium difficile* infection, and shedding the causative microbes in faeces, results in environmental contamination and increased infection transmission. This study demonstrates the efficacy of antimicrobial 405nm light – which has demonstrated success for environmental decontamination – for the enhanced inactivation of Norovirus and *C. difficile* spores when in the presence of faecal matter.

C. difficile spores and feline calicivirus (FCV; used as a norovirus surrogate) suspended in artificial faeces, were exposed to high intensity 405 nm light using irradiances of >150 $mWcm^{-2}$, and the dose response kinetics observed.

Results demonstrate the enhanced sporicidal and viricidal effects of 405nm light against *C. difficile* spores and FCV when in the presence of artificial faecal matter. For FCV, >4log_{10} reduction was achieved when suspended in artificial faeces using 1.4$kJcm^{-2}$: 50% less dose than required for inactivation in PBS. A similar enhancement was observed with *C. difficile,* with a 33% reduction in dose achieving inactivation in faeces compared to PBS (2.4$kJcm^{-2}$).

Analysis of the suspending media showed reduced transmission of 405nm light through the faeces compared to PBS, but organic matter within the artificial faeces may be predisposed to photosensitization, thus enhancing the inactivation process. Overall, results have shown that inactivation of these key organisms can be enhanced when suspended in faecal matter, and this may have positive implications for enhancing decontamination using 405nm light in clinical environments, with potential for use within the bathrooms of patients with gastrointestinal infections alongside current cleaning and infection control measures.

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I would like to dedicate my thesis to my parents, Kim and Johnny. Thanks to both of you for everything you have done for me, and for always supporting and encouraging me.

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