INACTIVATION OF FOODBORNE PATHOGENIC AND SPOILAGE MICROORGANISMS BY 405 NM LIGHT: AN INVESTIGATION INTO POTENTIAL DECONTAMINATION APPLICATIONS

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

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ABSTRACT

The control of microbial contamination in the food industry is critical, as contamination of food produce, surfaces and equipment can lead to acquisition of foodborne infections. Microbial contamination can also result in food spoilage, which can cause both product and financial loss. Consequently novel decontamination technologies are being sought to help reduce contamination.

Initial investigations examined the efficacy of 405 nm light for inactivation of a range of common foodborne microorganisms, both in suspension and on agar surfaces. All exposed populations were significantly reduced following 405 nm light exposure. The hypothesised inactivation mechanism involves photoexcitation of endogenous porphyrin molecules within the microorganisms, resulting in production of reactive oxygen species, oxidative cell damage and microbial inactivation. This theory was investigated by exposing fungi to 405 nm light under both aerobic and anaerobic conditions. Results displayed significant reduction in inactivation rates under oxygen depleted conditions, highlighting the critical role of oxygen during 405 nm light inactivation.

This study also demonstrated inactivation of bacterial contamination and biofilms on a range of surfaces, demonstrating potential environmental decontamination applications. Further work highlighted the enhanced bacterial inactivation efficacy of 405 nm light when bacteria were exposed under sub-lethal environmental conditions, typical of those present in the food processing industry. Further studies also demonstrated the synergistic effect of TiO_2 with 405 nm light, thereby enabling significantly enhanced bacterial inactivation rates. Studies also investigated potential applications for food decontamination and preservation, with preliminary results highlighting successful prevention of spoilage on a range of food products and significant decontamination of *E. coli* on fresh fruit.

This study has confirmed the microbicidal efficacy of 405 nm light, whilst demonstrating a range of potential applications for use within the food industry for improved environmental decontamination. In conclusion 405 nm light has potential to be used safely and effectively as an additional decontamination technology in the food industry.

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CHAPTER 1

INTRODUCTION

The ubiquitous nature of foodborne pathogens is both a threat to public health and a continuous challenge to the food industry, with The World Health Organisation estimating approximately 1.8 million deaths annually from the consumption of contaminated food produce. Furthermore the associated financial implications of microbial contamination are vast, with respect to both medical and lost productivity costs, with the estimated annual cost of bacterial foodborne illness to the US economy being in excess of $70 billion (Scharff 2011).

Despite major attempts to reduce current contamination levels, foodborne infections are rising as a result of increased food production, processing and consumption. There are many current decontamination and sterilisation technologies available, however many present limitations, either in terms of their environmental suitability, their mechanistic features or their antimicrobial properties. Consequently the need for novel decontamination technologies is being continuously investigated, in order to help minimise the presence of microbial pathogens within the food industry.

The use of light for microbial inactivation has long been employed, with the microbicidal properties of ultraviolet (UV) light being well established. However, despite the effectiveness of UV-light, there are numerous limitations of this technology for use in large open areas (such as food processing and productions factories) with respect to limited penetrability, degradative properties and safety issues. More recently, the use of visible light and more specifically violet-blue light with a wavelength of 405 nm has demonstrated antimicrobial properties against a wide range of pathogens (Endarko et al., 2012; Murdoch et al., 2010; Maclean et al., 2009; 2008). Whilst the majority of research to date has focused on the use of 405 nm light for healthcare-related decontamination applications and infection treatment, little research has been conducted to evaluate applicability of 405 nm light for decontamination applications in the food industry. This will be the major focus of the current thesis.

Initial work in this thesis determined the sensitivity of a range of foodborne pathogens to 405 nm light both in liquid and on agar surfaces in order to establish inactivation kinetics. Following on from initial susceptibility studies, a series of experiments were conducted examining potential decontamination applications including investigating the efficacy of 405 nm light for decontamination of inert surfaces and bacterial biofilms. To further elucidate applications within the food industry, synergistic effects of 405 nm light combined with various other antimicrobial environmental stress conditions were investigated. Chemical enhancement was also investigated through the use of photocatalytic titanium dioxide.

In addition to environmental decontamination, it was important to ascertain the potential for food decontamination and preservation applications. Decontamination of artificially contaminated produce and prevention of natural spoilage was investigated on a range of food products.

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

Chapter 2 *(Background and Literature Review)* discusses the current problems associated with microbial contamination in the food industry and details a number of currently employed decontamination technologies, discussing the benefits and limitations of each and also highlighting the requirement for novel technologies. A large focus is given to the recent progress of visible light decontamination technologies.

Chapter 3 *(Microbiological Methodology)* details the various microorganisms, media, diluents, microbiological preparations, light arrays, enumeration methodology and biochemical tests used in experimentation.

Chapter 4 *(Investigation into the use of 405 nm light for Inactivation of Foodborne Bacteria)* investigates the bactericidal effects of high intensity 405 nm light for inactivation of several key foodborne bacteria both in suspension and on agar surfaces in order to generate inactivation kinetics. This section of work also examined bacterial inactivation using low irradiance 405 nm light to determine the efficacy of long periods of continuous exposure for environmental decontamination applications.

Chapter 5 *(Inactivation of Fungi using 405 nm light)* investigates the sensitivity of a range of fungal species to 405 nm light. Subsequent work examined inactivation under both aerobic and anaerobic conditions, in order to elucidate the role of oxygen during

405 nm light induced inactivation. This chapter also investigates the comparable susceptibility of dormant and germinating *Aspergillus niger* spores when exposed to 405 nm light.

Chapter 6 *(Disinfection of Contaminated Surfaces and Biofilms using 405 nm Light)* investigates the efficacy of 405 nm light for surface decontamination of glass, acrylic, stainless steel and various rubberised flooring materials contaminated with *Escherichia coli*. This chapter also demonstrates bacterial biofilm inactivation, investigating both single species and mixed species biofilms. Biofilm inactivation through transparent plastic and glass materials was also investigated.

Chapter 7 *(Synergistic Bactericidal Effect of 405 nm light when Combined with Sub-lethal Temperature, Osmotic and Acid Stress Conditions)* examines the synergistic inactivation of *Escherichia coli* and *Listeria monocytogenes* when exposed to 405 nm light under sub-lethal environmental stress conditions.

Chapter 8 *(Photocatalytic Enhancement of Antimicrobial 405 nm light using Titanium Dioxide Nanoparticles)* investigates the use of photocatalytic material TiO_2 in combination with 405 nm light for enhanced antimicrobial action. This chapter examines the enhanced bactericidal efficacy of 405 nm light, when combined with TiO_2, both in liquid suspension and on a range of commercially available surface materials.

Chapter 9 *(Potential Applications of 405 nm light for Food Related Decontamination Applications)* investigates the use of 405 nm light for prevention of natural spoilage of bread, fruit and dairy produce. This chapter also investigates bacterial decontamination of fresh fruit produce. The final section of this chapter examines the ability to achieve bacterial inactivation through plastic packaging commonly utilised within the food industry, demonstrating potential decontamination applications of packaged food produce.

Chapter 10 *(Conclusions and Recommendations for Future Work)* summerises the findings from each experimental chapter, highlighting potential decontamination applications of 405 nm light for use within the food industry. Recommendations for future work are also discussed.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.0 General

This literature review will discuss the contamination problems faced by the food industry, both in domestic and industrial settings, and the current physical and chemical technologies being employed to help minimise food related contamination and subsequent infection. This review also discusses the use of light for microbial inactivation, with particular focus directed at the use of 405 nm violet-blue light for microbial decontamination.

2.1 Microbial Contamination in Food Settings

Microorganisms exist throughout the environment where they can harmlessly reside on skin and inanimate objects, as well as in soils, crops, plants and water. However, despite in many cases the ability to exist as commensal organisms; many microorganisms can exert unfavourable and often harmful characteristics, through the ability to produce and release toxic by-products and to cause a variety of infectious diseases.

A summary of key food related pathogens is provided in Table 2.1. The presence of these pathogens in the food industry is of great concern and can lead to substantial contamination issues that can result in severe problems for both the consumer and industrial manufacturers.

When foods become contaminated two main problems can arise. Food spoilage can result, in which the presence of microorganisms can sometimes be detected through visual and sensory evaluation. However a large proportion of spoiled foods do not exhibit abnormal or unpleasant characteristics and this often leads to their consumption and subsequent acquisition of foodborne illness. The following sections discuss both food spoilage and food related infections

Table 2.1 *Microorganisms commonly associated with food spoilage and food related illness.*

Bacteria ***(Gram negative)***	**Bacteria** ***(Gram positive)***	**Fungi/ Yeasts**	**Parasitic Protozoa/ Worms**	**Viruses**
Escherichia coli [a]	*Staphylococcus aureus* [a]	*Aspergillus* spp [ab]	*Toxoplasmosis gondii* [a]	Norovirus[a]
Salmonella spp[a]	*Listeria monocytogenes* [a]	*Penicillium* spp [ab]	*Giardia lamblia* [a]	Hepatitis A[a]
Shigella sonnei [a]	*Enterococcus* spp [a]	*Zygomycetes* spp [ab]	*Trichella spiralis* [a]	Hepatitis E[a]
Campylobacter jejuni [a]	*Clostridium perfringes* [a]	*Fusarium* spp [ab]	*Cryptosporidium parvum* [a]	Rotavirus[a]
Pseudomonas spp[ab]	*Clostridium botulinum* [a]	*Sacchromyces* spp [b]	*Taenia* spp [a]	Enteric adenovirus [a]
Yersinia enterocolitica [a]	*Bacillus cereus* [a]	*Candida* spp [b]		
Vibrio spp [a]				

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

2.1.1 Food related illness

The World Health Organisation (WHO) have estimated that almost 1.8 million people die every year from the consumption of contaminated food and water (Buzby and Roberts, 2009; Newell et al., 2010). Recent studies have estimated that around 76 million people in the US alone will contract a food related illness every year, of which 128000 people will become hospitalised and approximately 5000 will die. Estimated costs from diagnostics and medical treatment are in excess of $35 billion annually (CDC, 2012; Hoffman et al., 2012; Buzby and Roberts et al., 2009; Mead et al., 1999).

Consumption of contaminated foodstuffs can result in food-related illness with symptoms that can range from mild nausea and fever to severe neurological complications that can often be fatal. The severity of infection is dependent on a multitude of factors including the species of the causative agent and the infective dose. For example, *Escherichia coli* infections depend largely on the acquired strain. In many cases mild gastroenteritis will ensue that will self-limit within a few days. However, upon acquisition of more virulent strains, conditions such as haemolytic uremic syndrome can develop where red blood cells are destroyed and renal failure may result. Table 2.2 highlights a range of common food-associated pathogens, medical symptoms and annual infection rates.

The immunological state of the infected individual is also a critical factor when addressing food related infections. Infection by *Listeria monocytogenes*, isolated traditionally in soft cheeses, pates and raw vegetables, can often go unnoticed, for example ingestion of this bacterium can be fatal for immunocompromised individuals, especially during pregnancy, where foetal birth defects can occur as well as spontaneous abortion and still birth.

Table 2.2 *Microbial pathogens commonly associated with food related infection, the associated foods, common symptoms and annual illness rates for United states. Adapted from CDC 2011 and FDA 2013.*

Organism	**Associated Foods**	**Medical Symptoms**	**Estimated Annual Illnesses**
Norovirus	Ready to eat meals	Nausea, vomiting, cramps, diarrhoea	5.5 million
Staphylococcus aureus	Improperly refrigerated meats and vegetables	Nausea, abdominal cramps, diarrhoea, vomiting	241000
Escherichia coli O157:H7	Uncooked beef, unpasteurized milk, raw fruit and vegetables	Diarrhoea, abdominal cramps, vomiting	73000
Campylobacter jejuni	Undercooked poultry, unpasteurised milk	Undercooked poultry, unpasteurised milk Fever, cramps, vomiting, diarrhoea (with/without blood)	43000
Shigella sonnei	Contaminated drinking water, undercooked and raw meats	Abdominal cramps, fever, diarrhoea with blood and mucus	14000
Salmonella spp. (nontyphoidal)	Raw/undercooked eggs, poultry, meat, unpasteurized milk	Diarrhoea, fever, abdominal cramps	7550
Listeria monocytogenes	Cold meats, dried meats, soft cheeses	Fever, gastrointestinal symptoms, headaches, loss of balance, convulsions	1600

2.1.2 Food Spoilage

Although many microorganisms can induce food related infections, some microorganisms are not responsible for inducing illness but instead induce food spoilage. Food spoilage is defined as a sensory change in a food product that renders it unsuitable or undesirable for consumption through altered smell, appearance or taste (Gram et al., 2002). These changes result when microorganisms grow and metabolise within the food product, resulting in undesirable characteristics within the food. Both physical and chemical changes can be observed, consequently unpleasant tastes and odours are often associated with the product, rendering it undesirable for consumers (Gram et al., 2002).

Generally microbial spoilage of food products can be caused by three types of causative agents: yeasts, moulds and bacteria. Yeasts often colonise foodstuffs with high sugar or salt content. Common yeasts identified on food products include *Saccharomyces* and *Candida* spp. *Saccharomyces* has a number of beneficial fermentation properties, but it can also induce spoilage of alcoholic beverages whereby turbidity and off-flavours can develop (Vriesekoop et al., 2013; Doyle et al., 2007). Similarly *Candida* spp. can spoil a wide variety of foods ranging from fresh fruit and vegetables to dairy produce (Doyle et al., 2007).

Mould growth from organisms such as *Aspergillus*, *Penicillium* and *Zygomycetes* can result in a dense filamentous layer that can, in many cases, be observed visually on a range of produce including fruits, vegetables, cereals, nuts, spices, jams and dairy products. Moulds, although not always toxigenic, are very diverse and some species can produce toxic and carcinogenic mycotoxins (Doyle, 2007). Mould spoilage in bread accounts for ~5% product loss every year resulting in approximately £600 million worth of bread deemed unsuitable for consumer sale (Needham et al., 2004; Sperber and Doyle, 2009).

A range of bacteria can colonise foodstuffs and induce spoilage. The presence of bacteria can often result in offensive odours that give clear indications of contamination. However, depending on the level of contamination, bacteria may not be detected and thereby be consumed and initiate a food related infection.

2.2 Sources of Contamination

Contamination and subsequent transmission of microorganisms occurs in both domestic kitchens and industrial settings, and can arise from a multitude of sources. A recent study has suggested that between 40-60% of cases of food related illness arise in private homes due to improper storage, insufficient cooking and cross contamination of microorganisms from raw foods to ready to eat foods (Jong et al., 2008; Fein et al., 1995; Partnership for Food safety Education, 2004). This section will detail a range of common sources where foodstuffs can become contaminated.

2.2.1 Raw food materials

Raw food production and processing, both in domestic and industrial settings is a key contributory factor for contamination and transmission of microorganisms. Both raw meats and poultry can carry a range of microbial contaminants that upon sufficient cooking will be inactivated rendering the food safe for consumption. However, during the processing stage the raw materials can be transferred to various other sources including hands, machinery, equipment and work surfaces, where contaminants can then reside and provide a continual source of contamination.

Identification of the initial source of contamination is often difficult. Due to increased global demand for food, much produce is imported from less economically developed countries where health and safety regimes are not as strictly implemented as those in Europe and America. The US Centre for Disease Control (CDC), estimates that around 16% of food consumed in the US is imported, and between 2005-2010, 39 outbreaks of foodborne illness were related directly to imported foods (CDC, 2012). High levels of microbial contamination on raw meat and poultry products have been linked to animal overcrowding during rearing, again due to increasing global demand. This allows for rapid spread of infection between animals mainly through oral/faecal transmission, meaning that upon slaughter the carcases already carry substantial microbial loads (Fredrick and Huda, 2011).

Furthermore, the increased demand for natural and organic produce is greatly limiting the use of antimicrobial pesticides on crops and vaccinations given to animals. A study

by Van Overbeke (2007) highlighted the increased prevalence of *Campylobacter* in organic chicken flocks compared to conventional breeds (Van Overbeke et al., 2007).

2.2.2 Contamination and transmission via work surfaces

The initial contamination of food contact surfaces can arise directly through contact with colonised raw products. Similarly, direct transfer from previously contaminated objects such as cloths, sponges and knives can give rise to and further spread surface contamination (Mattick et al., 2003; Scott, 1999). Aerial deposition of microorganisms can also act as a source from open windows and ventilation systems (Prendergast et al., 2004).

Contamination of food produce due to the residual presence of microorganisms adhered to surfaces, machinery and equipment is an increasing concern with around 40% of food related diseases estimated to arise from contaminated equipment (Le Gentil et al., 2010). Several studies have indicated that a number of bacterial species including *E. coli, S. aureus, L. monocytogenes* and *Salmonella* spp. can survive on various surface materials for months acting as a continuous inoculum for re-colonisation of microbes (Carpentier and Cerf, 2011; Kusumaningrum et al., 2007; Kramer et al., 2006). The presence and persistence of microbial spores in the food industry is also of major concern due to their highly resilient structure, with ability to withstand extreme environmental pressures, leading to severe contamination issues.

The adherence of microorganisms to surfaces varies greatly depending on the organism, the surface material and the environmental conditions, with initial attachment of planktonic cells often described as weak. Microbial attachment to surfaces under the correct environmental conditions can lead to biofilm formation. Biofilm formation is a well-recognised problem in the food industry, as biofilm presence can have detrimental consequences for food safety. These complex cellular structures form a transparent, enclosed, protective matrix for microorganisms and this provides extensive resistance to both chemical and physical cleaning agents, meaning facilitating their removal is often extremely challenging (Movassagh et al., 2010). The presence of biofilms and its subsequent removal will be discussed further in Chapter 6.

2.3 Current Preventative Decontamination Measures

Despite continual efforts to avoid and minimise contamination within food settings, widespread microbial presence still exists. Strict attention to good hygiene and manufacturing practice can help minimise contamination levels. A number of currently available methods used for environmental decontamination are discussed in the following sub-sections of this chapter.

2.3.1 Hazard Analysis Critical Control Points (HACCP)

HACCP is a globally recognised systematic preventative strategy aimed at identifying and minimising potential chemical, biological and physical hazards within foods during the entire manufacturing process (Caswell and Hooker, 1996). During each stage of production, processing and transportation, potential hazards are identified and preventative measures are implemented to help minimise to risk of safety issues. Despite continual efforts to try and rigorously enforce the use of this system, many manufacturers are still not complying, and this has subsequently led to a number of recent national incidences (Domenech et al., 2011). A number of recent reports have demonstrated incidences of food related infection acquired from contaminated food produce. In 2005, 85 individuals acquired a multidrug resistant strain of *Salmonella* from consumption of unpasteurised Mexican style cheese (CDC, 2010). Failure to comply with correct pasteurisation procedures led to the export of contaminated produce, which resulted in severe illness, although in this instance infection was not fatal (CDC, 2010).

2.3.2 Food preservation

A number of food preservation techniques are currently employed to reduce/prevent the growth of microorganism within foods; these are highlighted in Figure 2.1. During processing, foodstuffs may be treated with multiple preservation methods, applied either simultaneously or sequentially and this is known as hurdle technology. Hurdle technology has been applied globally to provide gentle but effective food preservation, in a synergistic manner that can target various cellular components within microbial populations (eg membranes, enzymes, DNA) (Gould, 1995; Leistner, 2000).

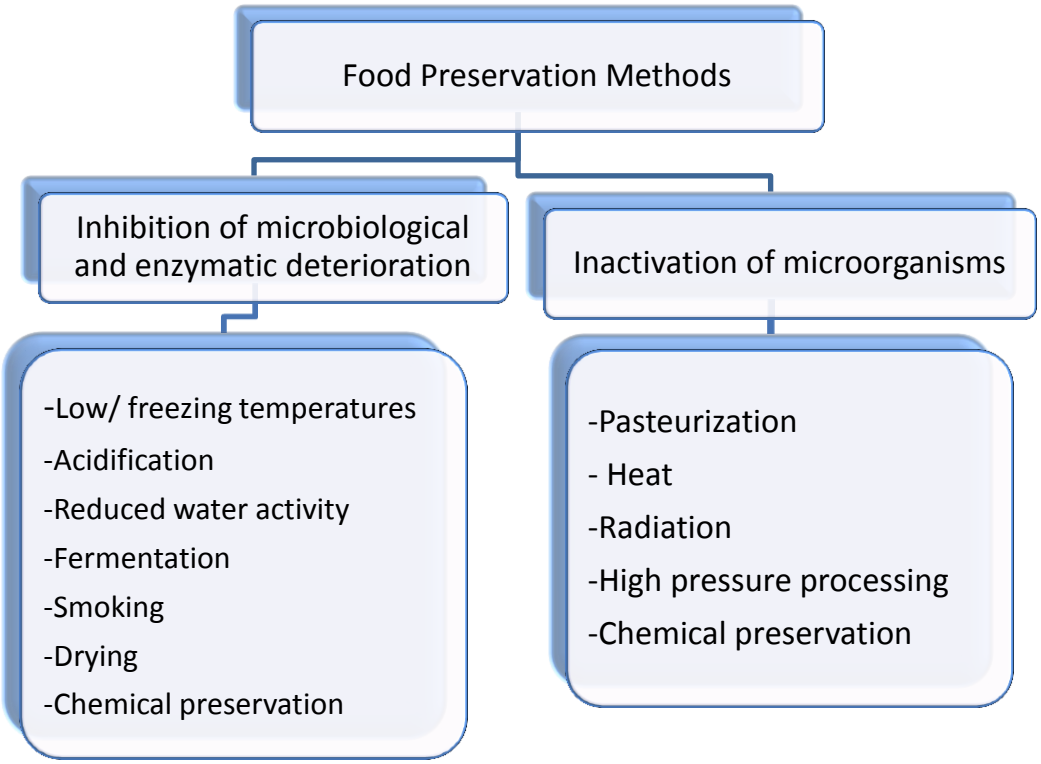

Figure 2.1 *Major food preservation methods employed for inhibition and inactivation of microorganisms.*

2.3.3 Use of chemical agents for decontamination

In the food processing industry, chemicals are routinely used to sanitize and disinfect work surfaces, machinery and equipment. It is vital that specific cleaning agents are identified and the frequency and standard of cleaning is maintained, in order to reduce the risk of cross contamination. The efficacy of a chemical agent for decontamination is determined by its ability to reduce microbial populations. The standard is generally accepted as a 5 $\log_{10}$ reduction in population in 30 seconds on food contact surfaces, and a 3 $\log_{10}$ reduction in population on non-food contact surfaces (e.g. floors and walls) (Schmidt, 2012; Association of Official Analytical Chemists., 2012).

A number of factors must be considered before selecting cleaning agents for decontamination purposes, for example; the microorganism, the types of residual food soil (blood/ grease) and the chemistry of its removal, the surface material and the

environmental conditions (e.g. temperature and humidity) (Schmidt, 2012). A large number of chemical cleaning agents must be applied to soil free surfaces, with the general recommended practice being rinse, clean, rinse, and sanitise/disinfect (Schmidt, 2012). However, the recommendation is generally to utilise a detergent prior to standard cleaning to assist the removal of soil from a surface prior to the sanitisation programme. Detergents possess surfactants, which allows for reduction in surface tension, allowing for efficient removal of soil material (Parker, 2007)

2.3.3.1 Chemical agents

There are various chemical agents available for different standards of cleaning and it is important to understand the different terminologies. Although specific definitions are described below, it is important to highlight that these terms are often used interchangeably throughout the literature. A few of the common terminologies are listed below:

- Sanitize: The reduction of microorganisms to a level that is considered safe for humans,
- Disinfect: This refers specifically to the removal of all vegetative cells,
- Sterilize: Removal and destruction of all living organisms (Schmidt, 2012).

In the food industry, chemical antimicrobial agents are most commonly used, with a large number of these products acting as oxidizing agents. Hypochlorites are the most commonly utilised chemical agents in both the food industry and the domestic kitchen, due to their low cost and high efficacy against a spectrum of microorganisms (Riazi and Matthews, 2011). Various other oxidizing agents including idophors, hydrogen peroxide, quaternary ammonium compounds and peroxyacetic acid are also utilised, but result in varying success depending on the target microorganism. Table 2.3 highlights a number of common chemical agents used throughout the food industry.

Table 2.3 *Common chemical agents used in the food industry, their mechanism of action and their effects on microorganisms and biofilms.*

Chemical Agent	***Mechanism of Action***	***Affected Microorganisms***	***Effective Against Biofilm***
Hypochlorites	Ability to attack cell membrane through strong oxidizing properties, reduces membrane permeability and results in cell lysis.	Bacteria, fungi, viruses	No
Iodophors	Penetrates the cell quickly and cause disruption of protein structure through direct attachment to the sulphurs of proteins. Nucleic acid synthesis can consequently become disrupted.	Bacteria, fungi, viruses	No
Hydrogen Peroxide	Strong oxidizing properties, with ability to destroy intracellular components, as well as cellular structure.	Bacteria, fungi, viruses	Yes
Peroxyacetic Acid	Disruption of thiol groups within the microbial cell membrane, results in oxidation of the cell membrane.	Bacteria, fungi, viruses	Yes
Quanterary Ammonium Compounds	Attraction of positively charged cations to negatively charged bacterial proteins, predominately in the phospholipid bilayer. This blocks the uptake of nutrients into the cell and prevents the release of waste.	Bacteria, fungi	Yes

Table adapted from: National Collaborating Centre for Environmental Health (Gaulin et al., 2011)

2.3.3.2 Limitations of chemical agents

Despite the efficacy of the aforementioned chemical cleaning agents, a number of limitations have been identified, hindering their continual use within the food industry. Repeated exposure to specific chemical agents may result in decreased sensitivity or even resistance to that specific agent. Microorganisms can develop resistance by a number of different mechanisms; these include cellular efflux, impermeable cellular membranes and the production of neutralizing intracellular enzymes (Davidson and Harrison, 2002; Bower and Daeschel, 1999). Resistance to chemical agents is largely conferred by innate structural features, such as impermeable outer membrane that helps prevent penetration of chemical agents (Bower and Daeschel, 1999; McDonnell and Russell, 1999). Less commonly, resistance can also occur through inactivator enzymes that are capable of deactivating various antimicrobial agents, for example catalase can neutralise hydrogen peroxide.

Toxicity and safety issues are also a concern with regards to chemical cleaning agents. Many oxidizing agents have been strongly associated with mild to severe skin and respiratory irritations, particularly chlorine based compounds such as sodium hypochlorite (Gaulin et al., 2011). A further limitation of chemical based cleaning agents is their ability to corrode a number of metal materials. Consequently, as a large proportion of work surfaces and machinery in the food industry are comprised solely or partly of metal based materials, this presents a significant issue.

2.3.4 Physical cleaning

Physical cleaning of both surfaces and equipment are routinely executed in the food industry for removal of microbial soils. Traditional techniques such dry brushing are still used, however cleaning techniques such as pressure washing and steam cleaning are being increasingly introduced into sanitation programmes. High pressure washing of floors, walls and large equipment is often a time efficient method of cleaning. However, pressurized spray can dissipate chemicals, microorganisms and other residual soils, creating undesirable aerosols (Gibson et al., 1999). A number of limitations have also been identified with steam cleaning, including high financial expense and associated physical hazards.

2.3.5 Use of biocidal materials

Self-disinfecting materials have become increasing popular in the clinical environment over recent years and are now showing great potential for use within the food industry. A number of heavy metals, including silver, gold, cobalt, copper iron, titanium and zinc, are all capable of exerting bactericidal and fungicidal effects. Copper has also shown virucidal efficacy (Warnes and Keevil, 2013). To date, silver and copper impregnated or coated surfaces have received the greatest attention.

2.3.5.1 Silver coating

Silver ions (Ag^{+}) possess the greatest level of antimicrobial properties of all the toxic heavy metal ions. The antimicrobial mechanism is thought to be based on the binding of silver ions to the disulphide and sulfhydral groups found in the proteins within the microbial cell wall, resulting in the disruption of metabolic processes and eventually cell death (Weber and Rutala, 2013). Surfacine (Surfacine Developmemt Company, Tyngsborugh, MA) is the only product to date that has been utilised for self-disinfecting surfaces in the clinical environment. A 3D polymeric network is immobilized onto the substrate surface, which is then coated with small (sub-micron) particles of silver that result in a silver/polymer complex. Silver is selectively transferred onto the microorganism following direct contact, until a toxic concentration has been taken up by the microorganism, resulting in toxic accumulation and cell death. Results have shown 3.3-4.3 $\log_{10}$ reduction of *S. aureus* and upto 4.8 $\log_{10}$ reduction of *Pseudomonas aeruginosa* just hours after application of Surfacine (Weber and Rutala, 2013).

2.3.5.2 Copper coating

The antimicrobial mechanism of copper differs from that of silver, in that copper ions can undergo oxidation and have the ability to generate reactive oxygen species. The production of copper radicals can then damage microbial lipids, proteins and nucleic acids, resulting in cell death (Elguindi et al., 2011; Samanovic et al., 2012). Copper coated surfaces have demonstrated antimicrobial effects on a wide range of pathogens, including *S. aureus,* and *E. coli* (Grass et al., 2011). Integration of copper into a range of products such as door handles, push panels, grab rails, toilet seats and light switches, has shown significantly lower microbial loads when compared to products made of standard materials (Karpanen et al., 2012). A recent study by Casey and colleagues

demonstrated that a copper coated toilet seat resulted in a 94%-98% reduction in total aerobic CFU count (Casey et al., 2010).

2.3.5.3 Altered surface topography

The development of altered topography has given rise to a new range of self-disinfecting surfaces. Sharklet Technologies (Alachua, Florida) have developed a material with altered surface properties, based on that similar to shark skin in order to reduce bacterial adhesion and biofilm formation. Results demonstrated that after 7 days standard surface material displayed early stage biofilm formation, whereas Sharklet™ material displayed no sign of colonization until 21 days (Chung et al., 2007). Information on the exact composition of the material is vague; however, knowing that it is a plastic material suggests that a major limitation is likely to be susceptibility to UV degradation.

2.3.6 Need for novel decontamination technologies

As described, there are currently numerous technologies available for decontamination within the food processing environment, each possessing benefits and limitations. No current method used for decontamination is 100% effective in all situations. To date most decontamination methods possess limitations with regards to environmental suitability or their antimicrobial properties, which has paved the way for the development of novel decontamination technologies. However, it is considered of greatest efficacy to use multiple antimicrobial approaches (hurdle technology) in order to reduce contamination levels and consequently new technologies are required not just to replace current methods, but to complement existing ones.

2.4 Light Based Technologies

Various strategies are being employed to overcome the problem of antimicrobial resistance, and most prominent amongst non-chemical based technologies is the use of light for microbial inactivation. The use of light has long been employed for its wide antimicrobial properties, and to date there are several types of light systems that have demonstrated positive inactivation results. These are listed below:

- Germicidal continuous ultra violet light,
- Pulsed Light (visible and UV),
- Visible light (Photodynamic Inactivation + exogenous photosensitisers),
- Violet-blue visible light (Photodynamic Inactivation +endogenous photosensitisers).

Figure 2.2 highlights where each light system appears on the electromagnetic spectrum (Adapted from Yin et al., 2013).

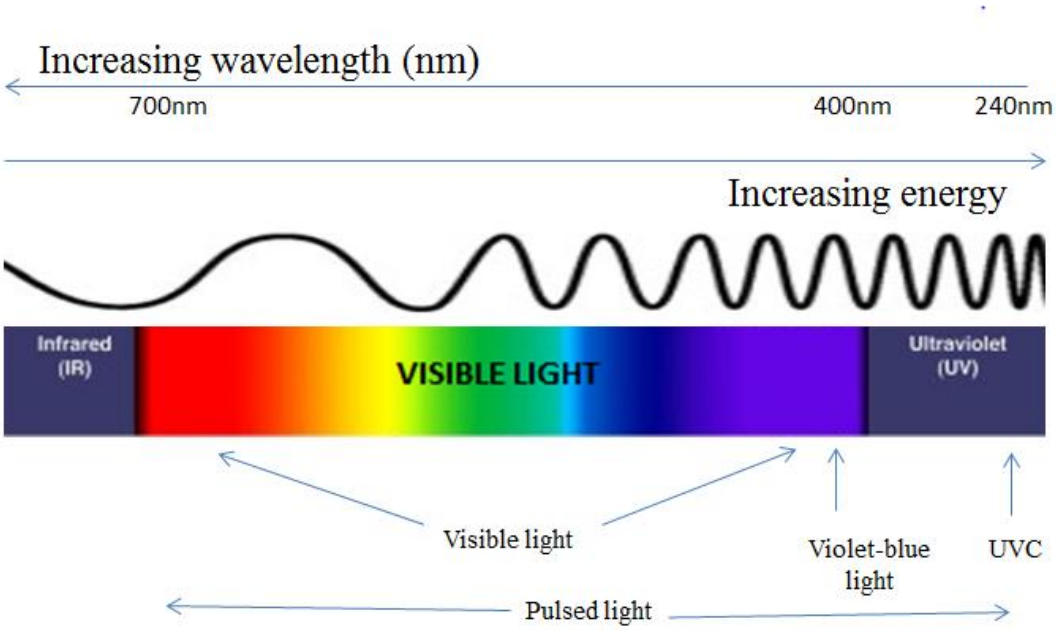

Figure 2.2 *Electromagnetic Spectrum highlighting the various wavelengths used by different antimicrobial light systems.*

2.4.1 Ultra violet light

Ultra violet (UV) (200-400 nm) light possesses wavelengths shorter than visible light but longer than X-rays. UV light can be divided into three distinct categories, based on the wavelengths and the subsequent effect on humans. UV-A (315-400 nm) wavelengths are mostly associated with melanin production and tanning, UV-B (280-315 nm) with sun burn and melanoma, and UV-C (200-280 nm) or germicidal UV which has known carcinogenic properties (Bucheli-Witschel et al., 2010; Bolton, 2001).

2.4.1.1 Inactivation mechanism of ultra violet light

UV light can be used to inactivate a wide range of microorganisms including viruses, bacteria, algae, moulds, yeasts and protozoa (Guerrero-Beltran and Barbosa-Canovas, 2004). UV-C light is the highly germicidal section of the UV spectrum, and causes damage to microorganisms through alterations in the microbial DNA and protein.

Exposure of microorganisms to UV-C light results in absorption of UV-C photons by microbial DNA or RNA pyrimidine bases (thymine or cytosine in DNA / uracil or cytosine in RNA), resulting in cross-linking of the two bases, forming chemical dimers, known specifically as pyrimidine dimers. These can be further categorised into cyclobutane pyrimidine dimers, including thymine dimers, and 6, 4 photoproducts. Formation of dimers inhibits cell replication and consequently results in microbial inactivation, through failure to replicate by mitosis (Bolton and Linden, 2003; Guerrero-Beltran and Barbosa-Canovas, 2004).

2.4.1.2 Applications and limitations of ultra violet light

UV light has profound antimicrobial properties, with disinfection applications for water, air, inanimate surfaces and food produce currently well established (Boyce et al., 2011; Davies et al., 2011; Ozen and Floros, 2001). UV light sources can provide rapid decontamination of a wide spectrum of microorganisms and this non-specificity also makes UV-C based technologies applicable for whole room or other large scale applications. In 2000, the US Food and Drug Administration (FDA) approved UV-C light as an alternative treatment to thermal pasteurization of fresh juice (Koutchma, 2008; US FDA., 2000). As well as food and liquid treatment, promising applications of UV-C Light have been associated with surface decontamination, where UV decontamination of surfaces and of equipment in bakeries, cheese and meat plants have been used alongside standard cleaning protocols (Koutchma, 2008). A study by Stermer et al. (1987) demonstrated 61% reduction of *S. typhimurium* on chicken breasts with a dose of only 86 mJ cm^{-2} UV-C light treatment. The efficacy of fully automated UV-C devices, such as the Tru-D Rapid Room Disinfection device (Lumalier, Memphis), have been demonstrated within clinical settings, where the system is placed in an empty room and controlled by wireless remote. A study by Nerandzic and colleagues demonstrated up to 3 log_{10} reduction in *Clostridium difficile* spores following a dose of 22 mJ cm^{-2}.

This technology has potential to be applied to the food industry where whole room decontamination is required (Nerandzic et al., 2010).

UV-C light based technologies are known to be particularly harmful to humans and can exert known carcinogenic effect (Bolton, 2001). For this reason, use of this technology must be contained within enclosed and carefully monitored environments. Due to the short wavelengths of UV light, penetration through opaque liquids and transparent materials is hindered demonstrating further limitations of UV light systems for decontamination purposes.

2.4.2 Pulsed light

Pulsed light (PL) has shown efficacy in food-related applications for decontamination of both food and environmental surfaces. PL systems, such as the Xenon flash lamp can inactivate a wide spectrum of microorganisms (Anderson et al., 1999) using short time pulses of an intense broad spectrum light, rich in UV-C wavelengths. The emitted light produces a high flash peak, consisting of wavelengths from 200-1100 nm (Gomez-Lopez et al., 2007; Dunn et al., 1997). The UV-C component of this system is regarded as the most important aspect for microbial inactivation, with Wang et al. (2005) confirming that the UV content of Xenon flash lamps (220-290 nm) provides the major contribution to microbial inactivation. Various studies have shown the efficacy of this technology, with Ghaseni demonstrating a 9 $\log_{10}$ reduction in *E. coli* following 100 pulses of light, each of which was 9 J cm^{-2} (Ghaseni et al., 2003).

The main advantages of PL systems are that they can provide rapid inactivation when compared to continuous wave UV-light (CW-UV), due to higher peak energy being delivered over shorter durations. Also, Xenon flash lamps are regarded as more environmentally friendly than CW UV lamps as they do not contain mercury (Gomez-Lopez et al., 2007; Wang et al., 2005). The main disadvantage associated with this technology is sample heating, which can directly affect food produce. As well as limited penetrability which is characteristic of all light based systems.

2.4.3 Photodynamic inactivation

Photodynamic inactivation (PDI) was discovered more than 100 years ago by Oskar Raab, following observation of inactivation of *Paramecia caudatum* after bright light

exposure (St Dennis et al., 2011; Raab., 1900). PDI requires three key elements; visible light, oxygen and a photosensitiser molecule (PS). The PS is taken up by the cell by penetration of the cell membrane (Gomes et al., 2013; Ramberg et al., 2003), where upon illumination, the PS becomes able to absorb light, resulting in excitation, electron transfer and the production of toxic chemical species that can subsequently induce oxidative damage and cell death (Hamblin and Hassan, 2004). Although the antimicrobial properties of PDI were well established in early literature, the introduction and progression of chemical antimicrobial agents in the 1940's supressed interest in the use of PDI. However, owing to the increasing problem of chemical antimicrobial resistance, PDI is currently receiving considerable amounts of interest as a 'novel' antimicrobial technology. The use of visible light for various medical purposes has since been established in the clinical field, with treatments for malignant and premalignant, and dermatologic conditions being introduced in the mid 1990's (Lyons et al., 2012; Kharkwal et al., 2011; Fayter et al., 2010; Charakida et al., 2004; Dougherty, 1984).

2.4.3.1 Inactivation mechanism of PDI

Following illumination, the photosensitiser can be energised by a specific wavelength of light, through absorption of photons that result in excitation from the ground singlet state to the excited singlet state, which is an unstable, short lived state (Maisch et al., 2004). The photosensitiser can then undergo inter system crossing to the triplet state that is lower energy but longer lived than the singlet state, and at this stage may interact with O_2 in two distinct processes (Hamblin and Hassan, 2004; Maisch et al., 2004).

The Type 1 process involves transfer of an electron from the triplet state photosensitiser molecule to an organic substrate, following illumination by visible light (400-700 nm), which subsequently can result in the production of radical ions and in turn the radical ions can interact with molecular oxygen (O_2) to produce a number of toxic products including superoxide, hydrogen peroxide and hydroxyl ions (Maisch et al., 2004; St Dennis et al., 2011). Alternatively the Type 2 process occurs through direct interaction between O_2 and the triplet state photosensitiser, resulting in electron transfer and singlet oxygen production ($^1O_2$). Singlet oxygen has strong oxidizing properties capable of

interacting with various cellular components. The Type 1 and Type 2 pathways for PDI are shown in Figure 2.3.

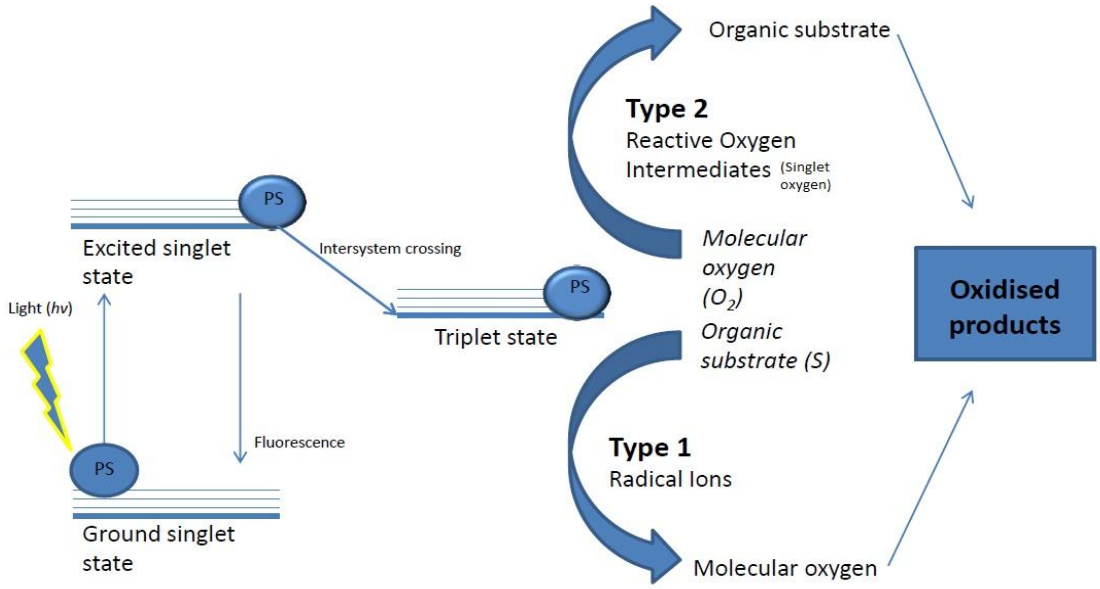

Figure 2.3 *Schematic illustration of photodynamic inactivation, via Type 1 and Type 2 pathways. The photosensitiser (PS) absorbs photons, which causes excitation from the singlet ground state to excited singlet state. Intersystem crossing results in triplet state formation. Type 1 and Type 2 PDI processes then occur via interactions with organic substrates or molecular oxygen. Adapted from Yin et al.(2013).*

For PDI to induce microbial inactivation either augmentation of endogenous photosensitisers must occur or exogenous photosensitisers must be added and readily taken up by the target cell, through diffusion prior to light treatment. Microbial cells vary in their susceptibility to photodynamic inactivation. Cellular structure and organisation can affect uptake and interaction of photosensitizing molecules, consequently varying the success of the photodynamic inactivation process. Differences in PDI susceptibility between Gram positive and Gram negative bacteria have been attributed to the obvious differences in cellular structure. Gram positive bacteria possess an outer-wall, approximately 15-80 nm thick, which can contain up to 100 peptidoglycan layers. The Gram positive cell wall promotes high porosity, allowing diffusion of macromolecules into the inner plasma membrane (Jori et al., 2006: Freidrich et al., 2000). Subsequently the cell wall present in Gram positive bacteria does

not act as a permeability barrier for a large proportion of commonly used photosensitisers. In contrast, Gram negative bacteria possess an additional outer membrane (10-15 nm thick) that consists of a complex peptidoglycan network alongside porin proteins, lipoproteins and lipopolysaccharides. In order to assist diffusion across the outer membrane, various molecules such as Tris-EDTA have been used for destabilisation (Hamblin and Hasan, 2004). However, a number of photosensitising molecules have now been identified that are capable of crossing the outer membrane without inducing destabilisation of the structure. The combination of positively charged groups and carbohydrate moieties within photosensitisers can result in increased cell recognition and water solubility, which improves microbial cell membrane penetration (Gomes et al., 2012). Specific examples include meso substituted cationic porphyrins and water soluble zinc phthaloyanines (Hamblin and Hasan, 2004; Maisch et al., 2005; Nitzan et al., 2004). The effects of photodynamic inactivation have also been documented in yeasts and fungi, but these structures are often difficult to inactivate owing to their larger cell size and complex cell wall composition, consisting of glucan, mannan, chitin and lipoproteins (Jori et al., 2006). Efficacy of PDI has also been demonstrated against a number viruses, including polymovirus and herpes simplex virus through the use of exogenous photosensitive molecules such as acridine orange and toluidine blue, with up-to a 7 $\log_{10}$ reduction achieved (Nims and Plavsic., 2013).

Exogenous photosensitisers used in PDI are typically phenothiazinium dyes such as Methylene blue and Toluidine blue O, as well as halogenated xanthenes such as Rose Bengal and acridines. These, in combination with light of the appropriate wavelength, have demonstrated the ability to kill a vast range of microorganisms (Hamblin & Hasan, 2004). Studies by Nitzan also demonstrated that it is possible to augment levels of endogenous photosensitiser molecules, known as poprphyrins (these will be described in greater detail in Section 2.4.4.2), by pre-incubation of bacteria with δ-aminolevulinic acid (ALA). δ-ALA is the first product in porphyrin biosynthesis, thereby addition of this molecule results in enhanced and accelerated production of naturally occurring endogenous porphyrin (Nitzan et al., 1999; 2004). By increasing levels of naturally occurring porphyrins, enhanced photosensitisation was observed in both Gram-positive and Gram-negative bacteria when exposed to narrow band visible blue light, between 407-420 nm (Nitzan et al., 2004).

2.4.4 Blue light photoinactivation

Environmental stimuli can trigger physiological responses in microorganisms. Recent discoveries concerning the genomes of photosynthetic and chemotropic bacteria and fungi have identified a number of blue light receptors, including photoactive yellow proteins (PYP) and blue light using FAD (BLUF) (Yin et al., 2013; Tschowri et al., 2012). Studies have highlighted that blue light can photo-regulate microbial lifestyle choices, influencing bacterial motility, initiating and suppressing biofilm formation and increasing microbial virulence (Gomelsky and Hoff., 2011). However studies have also demonstrated that blue light can promote microbial inactivation. Blue light microbial inactivation is known to have advantages when compared to UV based technologies. For example although not as germicidal, blue light has benefits relating to its higher safety and increased transmissibility due to possessing longer wavelengths (see Table 2.4).

2.4.4.1 Violet-blue light wavelength sensitivity

A more novel approach to PDI is through the use of violet-blue light. It is worth noting that throughout the literature and hence throughout this current study this visible light phenomenon is described largely as blue light inactivation. However the wavelengths for blue light are ~400-460 nm, but the wavelengths utilised and discussed in this work (~405 nm) are strictly violet-blue, and possess higher energy. Neverthe less for the purpose of this study, violet-blue light will be generally referred to as blue light.

During PDI studies it was hypothesised that microbial inactivation could be achieved without the addition of exogenous photosensitiser molecules. Previous work by Maclean investigated the bactericidal effect of visible blue light on a range of bacterial pathogens including *Staphylococcus aureus* (Maclean et al., 2008a; 2008b; 2009). Initial work by Maclean et al. (2008a) used broadband white light with a range of optical filters to identify optimal inactivation efficacy of *S. aureus*. These studies established that *S. aureus* can be photodynamically inactivated by light with the wavelength of around 405 nm (±5 nm) without the addition of exogenous or δ-ALA induced photosensitisers. In a similar study by Fukui et al. (2008) wavelength sensitivity of *Porphyromonas ginivalis* was investigated by exposure to a light source with a range of wavelengths (400-700 nm) leading to a conclusion that 400-410 nm was

most effective for bacterial inactivation. Subsequently, further work concluded *P. gingivalis* demonstrated greatest sensitivity to 405 nm light. A recent study by Endarko et al. (2012) analysed bandwidths between 400-500 nm for inactivation of *L. monocytogenes* and confirmed 405 nm (±10 nm) to be most effective for inactivation. Increasing research in this field has identified the presence of intra-cellular endogenous porphyrin molecules present within bacteria which have been accredited to being involved in the inactivation mechanism (Dai et al., 2013a; 2013b). Porphyrin molecules and their role in blue light photoinactivation will be detailed in the following section.

2.4.4.2 Porphyrins

Porphyrins are naturally occurring, highly pigmented, organic compounds, involved in numerous biological processes such as oxygen transport, pigmentation, photosynthesis and catalysis. They have applications in various fields ranging from fluorescence imaging to medicine (Josefsen and Boyle, 2006; Goldoni, 2002). Porphyrins are fundamental to a number of biological processes. One of the best known porphyrins is heme, the pigment present in red blood cells, which acts as a co-factor to haemogloblin, the protein responsible for oxygen transport in the blood.

Porphyrins are tetrapyrrolic molecules, consisting of four pyrrolic subunits interlinked through methine bridges (Goldoni, 2002). A number of common porphyrins have demonstrated a high absorption peak at wavelength of around 405 nm; which is termed the soret band (Goldoni, 2002). Additionally, weaker absorptions, termed Q bands, are associated with longer wavelengths having lower energy photons (450-700 nm) (Josefsen and Boyle, 2006). However, the exact absorption maxima can be altered and is dependent on the presence of other constituents, such as the binding of metal ions. Porphyrin molecules are divided into two groups, metal binding and non-metal binding. The size of a porphyrin molecule is ideal for binding a number of metals ions including copper, cobalt, iron nickel and zinc (Goldoni, 2002). For PDI reactions, non-metal bound porphyrins must be used, as any singlet oxygen produced through illumination would be quenched by the metal moiety. Schematic diagrams of metal and non-metal bound porphyrins are shown in Figure 2.4.

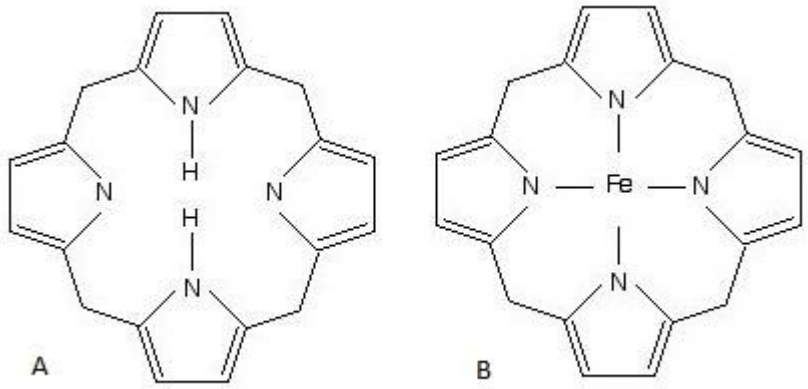

Figure 2.4 *Basic structure of porphrin molecule. A shows a non-metal bound porphyrin, and B shows metal bound porphyrin.*

Identification of endogenous porphyrins

The concentration and specific type of porphyrins present in microorganisms are known to vary. Previous studies investigating *Propioibacterium acnes* identified predominately the presence of corproporphyrin and protoporpyrin (IX) (PpIX) (Ashkenazi et al., 2003). A study by Hamblin identified the endogenous presence of both corproporphyrin and protoporpyrin (IX) in laboratory cultured *H. pylori* (Hamblin et al., 2005). *Staphylococcus aureus* has also been shown to contain varying types of porphyrins including corproporphyrin and uroporphyrin and these have also been identified in *E. coli* using high performance liquid chromatography (HPLC), a technique employed in analytical chemistry to separate a compound in order to identify specific components (Nitzan and Kauffman., 1999). A number of recent studies have also continued to focus on identification of endogenous porphyrin molecules. A recent study by Dai et al. (2013a) investigated the presence of endogenous porphyrin molecules in *P. aeruginosa* by fluorescence spectrophotometry. The presence of intracellular corproporphyrin III and uroporphyrin III were detected by spectral peaks highlighted at 613 and 667 nm, repectively.

2.4.4.3 Inactivation mechanism of blue light

The inactivation mechanism for blue light phototinactivation is theorised to be almost identical to that of standard photodynamic inactivation. However, unlike traditional PDI, where a photosensitizing agent is applied, the mechanism of blue light inactivation

occurs through excitation of endogenous porphyrin molecules within the microbial cells. These endogenous porphyrins act as photosensitisers, which can absorb photons of light, optimally at ~405 nm due to the peak absorption at this wavelength resulting in excitation from the ground singlet state to the triplet state (shown in Figure 2.3). It has been suggested that microbial inactivation by light occurs predominately by the Type 2 process, through interaction of the triplet state porphyrin with molecular oxygen, resulting in production of singlet oxygen (Yin et al., 2013; Cunliffe and Goulden., 2000; Omata et al., 2005). The exact mechanism by which reactive oxygen species interacts with microbial cells still remains unclear. Until fairly recently it was assumed that cellular damage was non-specific, with oxidation of various cellular constituents occurring. However, a recent study by Dai demonstrated disruption of the cytoplasmic contents and breakage of the cell wall of *S. aureus* following blue light treatment (425 nm), providing mounting evidence that blue light photoinactivation may induce initial and/or predominant oxidative destruction of the cell membrane structure (Dai et al., 2013).

2.4.5 Comparison of different light technologies

Table 2.4 provides a condensed summary of the various different light technologies discussed in the previous sections, highlighting key differences between each of the sources.

***Table 2.4**. Summary table highlighting various light technologies currently available, with typical wavelength, inactivation mechanism, and efficacy against microorganisms, advantages, disadvantages and current applications also highlighted*

Light	Typical Wavelength	Inactivation Mechanism	Efficacy Against Microorganisms	Advantages	Disadvantages	Typical Applications
UV-C	200-280nm (Bucheli-Witschel et al., 2010)	Absorption of UV-C photons by microbial DNA/RNA pyrimidine bases. Results in DNA damage (Bolton and Linden 2003; Guerrero-Beltran and Barbosa-Canovas 2004)	Bacteria, fungi, virus, parasite (Oguma et al., 2004)	Rapid decontamination. Broad antimicrobial spectrum (Oguma et al., 2004;)	Carcinogenic, poor penetrability through materials, resistance possible (Bolton 2001)	Surface ,food and water decontamination (Gailunas 2003; Nerandizc et al., 2010; Nerandzic and Donskey 2010; Rajikovic et al.,2010)
Pulsed Light	Broad spectrum 180-1100nm(Elmnasser et al., 2007)	Primarily UV damage, also photothermal damage and physical damage can occur (Elmnasser et al., 2007; Gomez-Lopez. et al., 2007; Wekhof 2000)	Bacteria, fungi, virus, parasite (Elmnasser et al., 2007; Rowan et al., 1999)	Broad antimicrobial spectrum (Elmnasser et al., 2007), rapid decontamination over continuous wave UV sources (Wang et al.,2005)	Poor penetrability through plastics and opaque liquids, thermal effects on food, (Elmnasser et al., 2007; Jun et al.,2003)	Decontamination of foods, water purification systems (Gomez-Lopez et al., 2007; Gomez-Lopez et al., 2005)
PDI	Visible light either at specific or range of wavelengths (400-700nm) (Al-Ahmed et al.,2013)	Photoexcitation of photosensitiser, results in oxidation of cells(Maisch et al., 2004)	Bacteria, fungi, virus, parasite (Al-Ahmed et al.,2013; Schagen et al., 1999; Escobar et al., 2006)	Increased penetrability compared to UV, safe for human exposure (Ericson et al., 2008)	Addition of exogenous photosensitiser prior to treatment, not as germicidal as UV	Decontamination of foods, medical applications (Brown et al., 2004; Luksiene et al 2008; Paskeviciute et al., 2008)
Violet/ Blue PDI	Visible light (405-420nm) (Maclean et al., 2008a; Endarko 2012)	Photoxcitation of endogenous porphyrin induces oxidation of cells (Hamblin and Hassan 2004;Maclean et al., 2008a)	Bacteria, fungi, antiviral activity not yet established(Maclean et al., 2009; Murdoch et al., 2013)	No exogenous photosensitiser, Increased penetrability compared to UV, safe for human exposure (Maclean et al., 2008a; McKenzie et al., 2013)	Not as germicidal as UV	Environmental surface decontamination; medical application (Askenzie et al., 2003 Maclean et al., 2010; Bache et al. 2010)

2.5 Blue light Research: Clinical Applications

The use of blue light in the region of 405 nm for microbial inactivation has promoted much research related to potential clinical applications. The next section of this chapter will detail current laboratory, animal and clinical studies utilising blue light, in the region of 405 nm.

2.5.1 Antimicrobial laboratory studies

Over recent years the antimicrobial profile of blue light (400-425 nm) has rapidly expanded, with various research groups demonstrating the antimicrobial efficacy of light in the region of 405 nm on a wide spectrum of microorganisms (Endarko et al., 2012; Murdoch et al., 2012; Maclean et al., 2012; Maclean et al., 2009; Enwemeka et al., 2008; Guffey and Wilborne., 2006; Hamblin et al., 2005). Several studies have demonstrated the successful inactivation of a wide range of problematic nosocomial pathogens, in liquid suspensions and on various surfaces, including methicillin-resistant *Staphylococcus aureus* (MRSA) (Maclean., 2006; Guffy & Wilborn, 2006; Maclean et al., 2008; Enwemeka et al., 2008; Maclean et al., 2009).

Maclean et al. (2009) investigated the inactivation of a range of 11 important nosocomial bacterial pathogens, exposed in liquid cultures, including Gram positive *S. aureus, S. epidermidis, E. faecalis, S. pyogenes, C. perfringens* and Gram negative *E. coli, A. baumannii, P. vulgaris, K. pneumonia and P. aeruginosa*, with results demonstrating significant inactivation of all bacteria tested. Results showed, in the most part, that Gram positive bacteria were much more susceptible to 405 nm light, with the exception of *E. faecalis*, which demonstrated least susceptibility.

Similar studies have demonstrated the susceptibly of a number of other key medically relevant bacteria, including *Propoionibacterium acnes, Helicobacter pylori* and *Chlamydia trachomatis* (Wasson et al., 2012; Hamblin et al., 2005; Ashkenazi et al., 2003). A recent study by Wasson and colleagues demonstrated the potential application of 405 nm light for treatment of *Chlamydia trachomatis*. (Wasson et al., 2012). With the ability to infect the conjunctival and reproductive tract mucosa, *C. trachomatis* is one the leading causes of both sexually transmitted infections and visual impairment globally (Gerbase et al., 1998). The effect of 405 nm LED lights on chlamydial growth

in endocervical epithelial HeLa cells (*in-vitro*) was also investigated and demonstrated a dose dependent response, with significant inhibitory effect achieved only at levels of 5 J cm^{-2}.

2.5.2 Animal studies

Skin and soft tissue infections (SSTI) resulting from bacterial colonisation in an area of skin abrasion affects millions of people per year (McCaig et al., 2006). Between 2001 and 2003, 11.6 million cases of *Staphylococcus aureus* related skin and soft tissue infections were reported in the US (McCaig et al., 2006). In particular MRSA strains are being increasingly identified as causative agents of skin and soft tissue infections, with the prevalence of community acquired MRSA (CA-MRSA) in intensive care facilities exceeding 50% (Viswanathan et al., 2012; Dai et al., 2013b; Witte et al., 2009). SSTI were once referred to as modest conditions that were easily treated with oral and topical antibiotics. However, owing to increased virulence of these strains and reduced susceptibility to current antimicrobial treatments, serious and often fatal conditions can now arise.

Extensive work has been carried out on the antimicrobial properties of blue light (400-420 nm) and the potential for wound decontamination has been discussed in numerous publications, however, little data existed until recently. A number of recent studies have been conducted by a leading group in blue light phototherapy from Harvard University, which have demonstrated the potential applications of blue light for treatment of wound infections in murine models.

A recent study by Dai and colleagues investigated the bactericidal properties of blue light (415 ±10 nm) for community acquired (CA-MRSA) infected mouse skin abrasions (Dai et al., 2013b). Abrasions were created by scrapping an area of skin (1.2 cm^2) with a scalpel to produce first degree skin abrasions, with the majority of the epidermis layer removed. *S. aureus* (USA300, CA-MRSA strain) was used to inoculate the wound. In order to determine bacterial inactivation of both acute and established infections, light was delivered 30 minutes (acute) and 24 hours (established) after initial inoculation of the wound. Bioluminescence imaging was used to detect bacterial infection in the wound. Results demonstrated a 2 $\log_{10}$ reduction in bacterial counts following an applied dose of ~20 J cm^{-2} and 96 J cm^{-2}, for acute and established infections,

respectively. However bacterial re-growth was established 24 hours after cessation of light treatment. Further investigation into this area is required before the full potential can be determined.

Burn wounds are particularly susceptible to bacterial colonisation and infection due to the destruction of cutaneous barriers. Over recent years Gram negative bacteria have been identified as a particular concern, with *P. aeruginosa* being extensively identified as a major causative pathogen for burn related infections. For *in vivo* studies mice were burnt with a preheated (~95°C) brass block to their dorsal surface for 3 seconds, resulting in full thickness third degree burns (1.2 cm^2). Following burn formation, 60 µl bacterial sample (3×10^6 CFU mL^{-1}) was topically applied to initiate infection. Infected burn wounds were treated with blue light 30 minutes after inoculation. Bioluminescence imaging was used to monitor bacterial presence pre and post treatment. Following a dose of 55.8 J cm^{-2} (62 minutes, 14.6 mW cm^{-2}), bacterial luminescence was completely eradicated. By comparison, the control, non-light treated burn wounds, resulted in the mice developing systemic infection that resulted in death 72 hours after initial inoculation (Dai et al., 2013a). Results demonstrate that blue light therapy may have potential applications for treatment of infected burn wounds, but further work is required to confirm the efficacy of this treatment.

2.5.3 Mammalian cell and tissue studies

With increasing promise towards the use of blue light for treatment of wound decontamination, it is important to assess the possible detrimental effects of blue light towards host cells and tissues. To date however, there are only a handful of studies that have investigated this area.

A number of recent studies have focussed on comparing the susceptibility levels between bacterial cells and mammalian cells when exposed to blue light, in order to determine whether microbial cells can be inactivated without detrimental effects being exhibited on mammalian cells. A study by Dai et al. (2013a) compared the susceptibility of the bacterium *P. aeruginosa* with human keratinocytes when exposed to blue 415 nm light. Keratinocytes are skin cells constituting approximately 90% of the outmost skin layer, known as the epidermis. Initial *in vitro* results highlighted significantly greater inactivation of *P. aeruginosa* than of human keratinocytes (P= 0.001) when treated with

an applied dose of ~110 J cm^{-2} (96 minutes of illumination at an irradiance of 19.5 mW cm^{-2}). Following an applied dose of ~110 J cm^{-2}, almost complete inactivation of the bacterial population was achieved (~7.6 log_{10} reduction), compared to 0.16 log_{10} loss in viability of human keratinocytes. This phenomenon was also evidenced in a study comparing the effects of 405 nm light on osteoblasts and bacterial isolates from infected arthroplasty patients, which found that osteoblasts could be exposed to bactericidal levels of light with no loss of osteoblast cell viability (McDonald et al., 2013).

A further study investigating the effects of blue light (420 nm) on keratinocytes demonstrated an 82% reduction of IL-1 α, a pro inflammatory cytokine (Shnitkind et al., 2006). Reduced concentration of IL-6, a proinflammitory cytokine associated with chlamydial infections was also observed in a later study by Wasson, (2012) following 405 nm illumination of endocervical epithelial cells. These results highlight the capabilities of blue light to interfere with and modulate the inflammatory process (Shnitkind et al., 2006). This has both potential benefits and detrimental implications. The inflammatory response is used to help clear bacterial infections; therefore interference from blue light could potentially interfere with the body's innate immunity. However, there is also the possibility that if blue light can interfere with and modulate inflammatory response, there may be potential for treating inflammatory medical conditions.

With regards to wound decontamination, an important factor for consideration is the effects of blue light on wound healing. An *in vitro* study by McDonald and colleagues investigated the effect of 405 nm light on fibroblast populated collagen lattices (FPCL), as a model of mammalian wound healing (McDonald et al., 2011). The effects on cell proliferation, morphological changes and α-smooth muscle actin (marker for fibroblast contractile activity) expression were investigated. Results also demonstrated that doses up to 18 J cm^{-2} were able to promote significant bacterial inactivation without inducing significant inhibitory effects on FCPL. Results demonstrated that detrimental effects of 405 nm light on FPCL were dose dependant (McDonald et al., 2011).

Kleinpenning et al. (2010) investigated the effect of blue light (380-440 nm, peak emission 420 nm) on healthy skin biopsies, to determine presence of photo-damage,

skin ageing and melanogenisis. Patients were exposed to irradiation (20 J cm^{-2}) for 5 consecutive days. Histological findings demonstrated that no inflammatory cells or sunburn were visible following light treatment. There was however a statistically significant increase in perinuclear vacuolisation of keratinocytes after 48 hours (P= 0.02); however this normalised after irradiation was ceased. The effect of blue light on photo-ageing was also investigated. Elastin fibres displayed normal appearance, both during and after blue light irradiation. Expression of MMP-1, a dermal protease involved in degradation of extracellular matrices, including fibronectin, collagen and elastin was present, but no significant increase was detected between illuminated and non-illuminated biopsies. Investigation into melanogenisis demonstrated minimal hyperpigmentation of the irradiated skin in only one volunteer, however histologically this was confirmed as a significant increase in melanain A positive cells. This decreased when blue light treatment was discontinued and it was concluded that short term use of blue light could be applied without promoting adverse effects on skin. In a later study by the same group investigating the efficacy of blue light treatment of psoriatic patients, results highlighted that 80% of treated patients demonstrated hyperpigmentation surrounding the psoriatic plaque (Kleinpenning et al., 2012).

In summary, it has been demonstrated that blue light at certain levels can cause phototoxic effects on mammalian cells. However, the comparative susceptibilities between microorganisms and mammalian cells to violet blue light have highlighted specific doses that are detrimental only to microbial cells. In conclusion, blue light phototherapy should utilise the optimal dose to selectively promote microbial inactivation, without inducing detrimental effect on surrounding mammalian cells.

2.5.4 Clinical studies

The use of broadband visible light plus the addition of a photosensitising molecule for medical purposes has long been established in the clinical field, with treatments of malignant, premalignant, dermatologic, dental, gastric and viral conditions being clinically approved (Lyons et al., 2012; Kharkwal et al., 2011; Fayter et al., 2010; Charakida et al., 2004; Dougherty, 1984). The interest in blue light without the addition of exogenous photosensitising molecule has more recently been investigated as an alternative method of phototherapy. Following the success of multiple *in vitro* studies

and animal studies, the use of blue 405 nm light as a treatment method for a range of medical conditions ranging from dermatologic conditions to wound and burn infections has sparked increasing interest. *In vitro* assessment has been conducted on various medically relevant pathogenic organisms. From this, studies have more recently progressed to clinical human trialing.

2.5.4.1 Blue light treatment for dermatological conditions

Following the success of various *in vitro* studies, the efficacy of blue light phototherapy on acne lesions has been investigated. Acne is a chronic inflammatory disease caused by colonisation of *Propoionibacterium acnes* in hair follicles and is most prevalent in adolescents; however it can persist until late adulthood (Williams et al., 2012). Approximately 80% of the population is affected by acne, with around 20% of cases resulting in permanent facial scarring. Consequently improved treatments are continuously being sought. A study by Ashkenazi and colleagues highlighted the presence of endogenous corpoporhyrin was sufficient to induce inactivation following exposure to intense blue light (407-420 nm) (Ashkenazi et al., 2003). Bacterial cultures treated with a single dose of 75 J cm^{-2} of blue light were reduced by between 1- 2 log_{10}. Greater inactivation (4 log_{10}) was achieved following two consecutive illuminations, 24 hours apart. In a similar study by Kawada et al. (2002), five strains of *P. acnes* were exposed to intense blue light (407-420 nm). Bacterial suspensions were directly exposed to and demonstrated an approximate 25% reduction following a dose of 324 J cm^{-2}. Results suggest the use of blue light may be used effectively for treatment of skin conditions.

A study by Faghihi et al. (2011) evaluated the comparative efficacy of blue light and topical erythromycin. The right hand side of the patients face was treated with topical antibiotics twice daily and the left by sunlight irradiation with a blue light portable filter (415 nm) held on the face for 15 minutes per day. Following a three month trial, a 20% improvement in acne lesions was achieved by blue light treatment in 46% of patients, slightly less than the overall improvement achieved by erythromycin (58%). The exact irradiance levels used were not detailed in this study; however it is likely that blue light phototherapy could be strengthened by increasing the applied dose. Drying of skin, erythema and skin irritation were monitored, with results concluding no significant

prevalence in side effects through blue light treatment when compared directly to topical erythromycin. Faghihi concluded the use of blue light for acne treatment was both safe and partially effective.

Elman et al. (2003) examined the effect of high intensity blue light (405-420 nm) on inflammatory acne lesions. Patients were exposed to an irradiance of 90 mW cm^{-2} for 15 minutes, twice a week for 4 weeks. Following completion of the trial, 77% of patients demonstrated a 59% reduction in inflammatory lesions, no side effects were reported (Elman et al., 2003). In a similar study by Shalita et al. (2001), *P. acnes* was cultured from patients foreheads, pre- and post- treatment. Patients underwent 6 bi-weekly treatments and were exposed to an irradiance of 55 mW cm^{-2} for 15 minutes (50 J cm^{-2}). A 90% reduction in bacteria was recorded in 60% of patients, with the remaining 40% presenting low initial levels of *P. acnes*, thus presenting no significant decrease following blue light treatment.

Recent work has also investigated the use of blue light phototherapy on psoriatic patients. The presence of protoporphyrin in psoriatic skin acts as an endogenous photosensitizing molecule to blue light therapy (Bissonnette et al., 1998). Kleinpenning et al. (2012) evaluated high dose blue light (420 nm) for treatment of psoriasis plaques, investigating both treatment efficacy and detrimental effects. Psoriatic lesions demonstrated significant clinical improvement. However, side effects were observed, with ~80% of patients demonstrating hyperpigmentation surrounding the psoriatic plaque.

2.5.4.2 Blue light treatment for *Helicobacter pylori* infections

Helicobacter pylori is a common Gram negative bacterium, capable of colonizing the mucosal lining of the stomach, and inducing a spectrum of conditions from gastritis to gastric adenocarcinoma (Suerbaum et al., 2002). Prevalence rates in the US are between 30-40%, and up to 70% in developing countries (CDC 2013). Many current oral antibiotics have acquired resistance, reducing their effectiveness, with 20% failure rate associated with current antibiotic therapy (Gisbert et al., 2008). Further limitations include poor patient compliance and undesirable side effects. A study by Hamblin et al. (2005) reported the use of narrow band violet-blue light for inactivation of *H. pylori in*

vitro, with results showing a 5 $\log_{10}$ reduction following an applied dose of around 16 J cm^{-2}.

A pilot study by Ganz et al. (2005) conducted a clinical assessment on 10 patients, evaluating the use of blue light (405 nm) to treat *H. pylori* infections endoscopically using a flexible optical fibre cable. Illumination of the mucosa was received over a 1 cm^2 area, with gastric biopsies taken pre and post light exposure to allow evaluation of treatment. Preliminary *in vitro* studies demonstrated that 32 J cm^{-2} was sufficient to reduce the bacterial population by 5 $\log_{10}$. *In vivo* assessment also demonstrated 90% reduction (1 $\log_{10}$) following irradiance of 40.5 J cm^{-2} (15 mW cm^{-2} for 4.5 minutes).

A later study by the same group investigated treatment of *Helicobacter pylori* infection using intra-gastric blue light wand (408 ±2 nm) phototherapy (Lembo et al., 2009). The dose delivered was dependent on the severity of infection. Eighteen female patients with *H. pylori* infection, confirmed by standard urea breath tests pre and post treatment, had a standard upper endoscope inserted to both, inspect and obtain biopsies from three sections of the stomach (antrum, body and fundus). A guide wire was fed through the endoscope, the endoscope was removed and the light wand was inserted into the catheter sheath and over the guide wire into the stomach. Gastric biopsies were taken pre- and post- illumination to allow quantification of microbial reduction in the antrum, body and fundus.

Results demonstrated significant reductions in *H. pylori* in both the body and the antrum, with 97%, 95% and 86% reductions noted for antrum, body and fundus, respectively. From both studies it was concluded that blue light phototherapy represents a novel approach for potential treatment of *H. pylori* infections. The use of blue light demonstrates potential not only for decontamination of surface wounds, but also for treatment of internal infection (Dai et al., 2013b; Lembo et al., 2009; Ganz et al., 2005). Further studies must be conducted in order to provide more detailed safety assessment of interactions between blue light and mammalian cells.

2.5.4.3 Blue light for environmental decontamination in hospitals

The antimicrobial properties associated with violet-blue light combined with the safety benefits for human exposure when compared to UV treatment, make for ideal use as an environmental decontamination technology within clinical settings. Research within

The Robertson Trust Laboratory for Electronic Sterilisation Technologies (ROLEST) at the University of Strathclyde has led to the design and development of a novel low irradiance 405 nm light based decontamination technology. The High Intensity Narrow Spectrum light Environmental Decontamination System (HINS-light EDS) is a ceiling-mounted light source for microbial inactivation. A number of recent studies have demonstrated the efficacy of the HINS-light EDS; a ceiling mounted lighting system consisting of a matrix of LEDs that provides continuous, low irradiance (~0.5 mW cm^{-2}) 405 nm illumination of the exposed environment. The HINS-light EDS has been designed with integration of white LED, to produce lighting that is predominately white and so blends well with standard room lighting. This system is described in greater detail in Section 3.3.4.

Studies have demonstrated the efficacy of the HINS-light EDS for use within the clinical environment. Evaluation of the technology has been carried out in isolation rooms within two main clinical areas: a Burns Unit and an Intensive Care Unit (ICU). Maclean et al. (2010) assessed the effectiveness of the HINS-light EDS on hospital isolation rooms used to treat burns patients. When the room was unoccupied, approximately 90% reduction of surface bacteria was achieved after continuous EDS illumination over a 24 hour period. A further study monitored bacterial reduction in occupied rooms of MRSA infected burns patients. Bacterial counts were taken by contact plate sampling and results demonstrated a significant reduction (56%-86%) in bacterial counts, over and above reductions associated with standard and routine cleaning procedures. Bache et al. (2012) assessed the efficacy of the HINS-light EDS in an inpatient isolation room and an out-patients clinic within Glasgow Royal Infirmary Burns Unit. Contact plates were collected and comparisons were made between environmental bacterial contamination levels recorded with and without the use of HINS-light EDS. Inpatient studies highlighted statistically significant reductions in environmental contamination following use of the HINS-light EDS exposure. Outpatient studies demonstrated a 61% reduction in contamination levels on sampled surfaces around the clinical room. A more recent study by Maclean et al. (2013) investigated continuous decontamination of an occupied ICU room. As with previous studies, the HINS-light EDS demonstrated significant efficacy with bacterial reductions

of up to 67% observed on various surfaces, including bin lids, sharps boxes and nurses trolleys (Maclean et al., 2013).

The successful results demonstrated in each of these studies highlight the potential decontamination applications of HINS-light EDS, not only in the clinical setting, but also in the food industry, where there are definite applications for whole room decontamination.

2.6 Violet-Blue Light Research: Food Industry Applications

To date the majority of research has tended to focus on the efficacy of 405 nm light for clinical use, with respect to both environmental decontamination and wound phototherapy applications for wound decontamination and other medical treatments. However, the wide antimicrobial properties of blue light indicate that it has potential to be applied to areas outwith the clinical setting, such as food processing, production and preparation areas. Currently the literature displays very few studies investigating the use of blue light for food related applications, however a small number of *in vitro* studies have demonstrated the susceptibility of bacteria associated with food related infection to 405 nm light.

2.6.1 Antimicrobial studies

Following from the success of blue light photoinactivation for nosocomial pathogens, increasing interest has been raised in respect to 405 nm light inactivation of food related pathogens for potential environmental decontamination applications within the food industry. Table 2.2 highlights a number of common food-associated pathogens that are primarily responsible for a large proportion of foodborne infections. A number of recent studies have investigated the efficacy of 405 nm light on several of these food-associated bacteria. Murdoch et al. (2012) demonstrated susceptibility of a number of key food organisms; *S. enterica* serovar enteritidis, *E. coli*, *S. sonnei*, and *L. monocytogenes* when exposed to 405 nm light in suspension.

To date results have demonstrated significant inactivation on all organisms tested. Murdoch et al. (2012) demonstrated greatest susceptibility was displayed by *L. monocytogenes* where a 5 $\log_{10}$ reduction was observed following exposure to

10 mW cm^{-2}, with an applied dose of 108 J cm^{-2}. In contrast, *S. enteritidis* demonstrated greatest resilience, with only a 3.5 log_{10} reduction observed following an applied dose of 288 J cm^{-2}. An earlier study by Murdoch et al. (2010) demonstrated the increased susceptibility of the key food pathogen *Campylobacter jejuni*, with a total dose of 18 J cm^{-2} being required to achieve a 5 log_{10} reduction when exposed in liquid culture, which was most likely due to the microaerophilic nature of this organism.

Work has also demonstrated inactivation of bacteria on nutritious surfaces, where 2 log_{10} populations were exposed to ~70 mW cm^{-2} of 405 nm light at increasing doses. Complete inactivation of *L. monocytogenes* was shown following 128 J cm^{-2}. As with liquid suspensions, inactivation of Gram negative bacteria (*E. coli*, *S. sonnei* and *S. enteritidis*) required a greater applied dose (~ 192 J cm^{-2}). The bactericidal properties of 405 nm light for surface decontamination on inert PVC and acrylic, representative of typical surface materials found within food settings have also demonstrated further potential of 405 nm light for decontamination applications. Significant surface inactivation of both *L. monocytogenes* and *S. enteritidis* following only 15 J cm^{-2} have been demonstrated (Murdoch et al., 2012).

Investigation into practical applications of blue light for use in food settings, has received little attention, despite the growing interest for medically relevant applications. A number of recent studies have investigated photodynamic inactivation of food packaging and food products with the addition of various photosensitising molecules. A study by Luksiene et al. (2009) investigated inactivation of artificially seeded *B. cereus* by photosensitisation using δ-ALA, to enhance endogenous porphyrins on food packaging surfaces using 400 nm light. A number of studies have discussed the use of blue light (400 nm) for decontamination of food produce; however these have also incorporated photosensitizing agents. Paskeviciute et al. (2009) investigated decontamination of tomatoes, lettuce, cabbage and nectarines using sodium chlorophyll based salt on artificially seeded *B. cereus*. Similarly, a later study by the same group used the same method to inactivate contaminated strawberries, seeded with *L. monocytogenes* (Luksiene and Paskeviciute, 2011). A summary of these studies is shown in Table 2.5. Although studies demonstrated significant microbial inactivation, their requirement for photosensitizing agents may be regarded as a limitation for direct

applications in industry. However to the best of our knowledge there have been no practical decontamination studies utilising only endogenous photosensitising molecules.

Table 2.5 *Examples of photodynamic inactivation used for microbial decontamination of food and food packaging.*

Food/ Packaging	Wavelength	Irradiance ($mWcm^{-2}$)	Photosensitiser	Microorganism	Dose ($J\ cm^{-2}$)	Log_{10} reduction
Necterines[a]	400nm	20	Sodium Chloropyll	*B. cereus*	6	2.33
Cherry tomatoes[a]	400nm	20	Sodium Chloropyll	*B. cereus*	6	2.88
Iceberg lettuce[a]	400nm	20	Sodium Chloropyll	*B. cereus*	6	1.68
Chinese cabbage[a]	400nm	20	Sodium Chloropyll	*B. cereus*	6	1.84
Strawberry[b]	400nm	12	Sodium Chloropyll	*L.monocytogenes*	14.4	1.8
Plastic trays (LINPAC)[c]	400nm	20	δ-ALA	*B. cereus*	4.0	4.0

a. Paskevicute et al., 2009; b. Luksiene et al., 2011; c Luksiene et al., 2009

The objective of this PhD was to investigate the antimicrobial efficacy of violet-blue 405 nm light on a range of food associated pathogens without the addition of exogenous photosensitive molecules, in order to determine whether this technology has possible applications for use within the food industry for microbial decontamination. Each subsequent chapter investigates specific applications in which 405 nm light could be applied to the food industy. These include surface and biofilm inactivation, chemical and environmental stress enhancements and preservation and decontamination of food produce during 405 nm light exposure.

CHAPTER 3

MICROBIOLOGICAL METHODOLOGY

3.0 Overview

This chapter will discuss the microorganisms used throughout this study, as well as the various culture media, chemicals, diluents, reagents and equipment used. Details of the standard microbiological techniques used for cultivation and enumeration are also provided.

3.1 Microorgansims

This section details the bacterial, yeast and fungal strains used throughout this study, as well as the cultivation procedures.

3.1.1 Microbial strains

The strains of microorganisms used are listed in Table 3.1. Cultures were obtained from the National Collection of Type Cultures (NCTC), (Collindale, UK), the Laboratorium voor Microbiologie, Universiteit Gent (LMG) and Mycotheque de l'Universite Catholique de Louvain (MUCL). LMG and MUCL are both part of The Belgian Co-ordinated Collections of Microorganisms, (BCCM) (Gent, Belgium).

3.1.2 Culture and maintenance of microorganisms

Microbial strains obtained from culture banks (NCTC, BCCM) were reconstituted, by inoculation into the appropriate broth medium for growth (Table 3.2). Once strains were proven viable and pure, they were transferred onto Microbank™ beads (Prolab Diagnostics) and kept at -70°C for long term maintenance. For experimental use of a strain, an inoculated bead was streaked onto an agar plate of the appropriate growth medium (shown in Table 3.2) and incubated. The growth requirements were dependent on the microorganism and these are highlighted in Table 3.2. For short term storage, the microorganism was sub-cultured from the agar plate onto an agar slope and refrigerated at 4°C. This was used as the regular source of inoculum for daily experimental work. All

microbial strains were re-streaked onto a new agar slope every four weeks, gram stained (Section 3.7.1) and viewed under the light microscope to ensure cultures remained pure.

Culturing bacteria and yeast microorganisms for experimental use involved extracting a loopful of the required microorganism from the agar slope and inoculating into 100 mL of broth medium. The broth was incubated under rotary conditions (125 rpm). The specific media, temperature and incubation time were dependent on the microorganism being cultivated. Specific requirements are detailed in Table 3.2.

In the case of cultivation of the filamentous fungus, *Aspergillus niger*, spores were collected using a sterile loop and sub-cultured onto a fresh agar slope and incubated at 30°C for 5 days to ensure sufficient spore production.

Table 3.1 *Microbial strains used during this study.*

Microorganism		Source	Collection Number
Staphylococcus aureus	Bacteria (Gram +)	NCTC	4135
Escherichia coli	Bacteria (Gram -)	NCTC	9001
Listeria monocytogenes	Bacteria (Gram +)	LMG	11994
Pseudomonas aeruginosa	Bacteria (Gram -)	LMG	9009
Shigella sonnei	Bacteria (Gram -)	NCTC	12984
Salmonella enteritidis	Bacteria (Gram -)	NCTC	4444
Aspergillus niger	Fungi (mould)	MUCL	38993
Sacchromyces cerevisae	Fungi (yeast)	MUCL	28749
Candida albicans	Fungi (yeast)	MUCL	29903

Table 3.2 *Microbial growth requirements*

Microorganism	Growth Medium	Incubation Time	Temperature (°C)
S. aureus	Nutrient Broth/Agar	18-24 hours	37
E. coli	Nutrient Broth/Agar	18-24 hours	37
L. monocytogenes	Tryptone soya Broth/ Agar	18-24 hours	37
P. aeruginosa	Nutrient Broth/Agar	18-24 hours	37
Sh. sonnei	Nutrient Broth/Agar	18-24 hours	37
Salm. enteritidis	Nutrient Broth/Agar	18-24 hours	37
A. niger	Malt Extract Broth/ Agar	5 days (spore production on agar slope)/ 3 days (sample plates enumeration)	30
S. cerivisae	Malt Extract Broth/ Agar	18-24 hours (broth)/ 48 hours (sample plates enumeration)	30
C. albicans	Malt Extract + Yeast Extract Broth/ Agar	18-24 hours (broth)/ 48 hours (sample plates enumeration)	37

3.2 Media

Media were prepared by weighing out exact quantities and dissolving in distilled water. Quantities used were dependent on the specific media; details are given in Section 3.2.1. To ensure sterility, all media were autoclaved at 121°C for 25 minutes. Post sterilisation, molten agar was held in a water bath at 48°C to allow cooling, prior to preparation of agar plates and slopes. Plates and slopes were incubated at 37°C overnight to ensure the agar was free from contamination prior to experimental use.

3.2.1 Agars and broths

Details of the broth and agar media used for microbial cultivation are detailed in Table 3.3. All media were obtained from OXOID Ltd,UK.

Table 3.3 *Broth and agar media used for microbial cultivation*

Media	Product Code	Quantity Used
Nutrient Broth	CM0001	13 g L^{-1}
Tryptone Soya Broth	CM0129	30 g L^{-1}
Malt Extract Broth	CM0057	20 g L^{-1}
Nutrient Agar	CM0003	28 g L^{-1}
Tryptone Soya Agar	CM0131	40 g L^{-1}
Malt Extract Agar	CM0059	50 g L^{-1}
Mannitol Salt Agar	CM0085	111 g L^{-1}
Violet Red Bile Agar	CM0107	38.5 g L^{-1}
Listeria Selective Agar Base	CM0856	55.4 g L^{-1}

3.2.2 Diluents and other media

- Phosphate Buffer Saline (Oxoid Ltd, UK) [BR0014G]- 1 tablet in 100 mL distilled water.
- Bacteriological peptone (Oxoid Ltd, UK) [LP0037] 1 g L^{-1}

- Yeast Extract (Oxoid Ltd, UK) [LP0021] - 0.1%
- Bile Salts (Oxoid Ltd, UK) [LP0055] 1.5 g L^{-1}
- Gram Stain Reagents: Crystal violet, Lugol's iodine, Ethanol and Safronin (Sigma Aldrich, UK)

Details on the concentrations of these diluents, reagents and supplementary media are highlighted in specific chapters.

3.3 405 nm light Exposure Systems

For 405 nm light exposure of microorganisms, narrowband light emitting diode (LED) arrays were used. Use of narrow spectral width light sources with a central wavelength of 405 nm (the wavelength upon which this study is focussed) allowed specific investigation into the antimicrobial efficacy of 405 nm light. It is important to note that although sources are referred to as 405 nm throughout this thesis; peak emissions were variable between different sources, as indicated in Figure 3.1. However, for convenience, sources will be referred to as 405 nm throughout this study.

The light emitted by the LED arrays was generated in the active region of an indium gallium nitride/ gallium nitride (InGaN/GaN) semiconductor junctions of the light emitting diodes. When voltage is applied, electrons in the conduction band move through semiconductors, and may recombine with positively charged holes located in the valence band. An energy gap which separates these two bands is known as the bandgap. In a normal diode, the resultant energy is emitted in the form of heat. However, in LED this recombination results in the release of higher energy photons of a specific wavelengths corresponding to the band gap energy. In the production of LED, different materials are used to give different band gaps and thus photons of different wavelengths are generated.

A number of different LED arrays were used throughout this study in order to provide a range of irradiances from approximately 0.5 mW cm^{-2} to 200 mW cm^{-2}. General details of each 405 nm LED system are discussed; however specific details of how the systems are used in the various experiments are detailed in subsequent chapters.

- Source A: ENFIS QUATTRO Mini Air Cooled Light Engine (ENFIS Ltd, UK)
- Source B: ENFIS PhotonStar Innovate UNO 24 (PhotonStar, UK)
- Source C: 405 nm 99 DIE LED array (OptoDiode Corp, CA, USA)
- Source D: High Intensity Narrow Spectrum Environmental Decontamination System (HINS-light EDS)

The optical emission spectra for the ENFIS QUATTRO Mini Air Cooled Light System, ENFIS PhotonStar Innovate UNO 24 and 99 DIE are shown in Figure 3.1, with peak wavelengths also displayed for each light source.

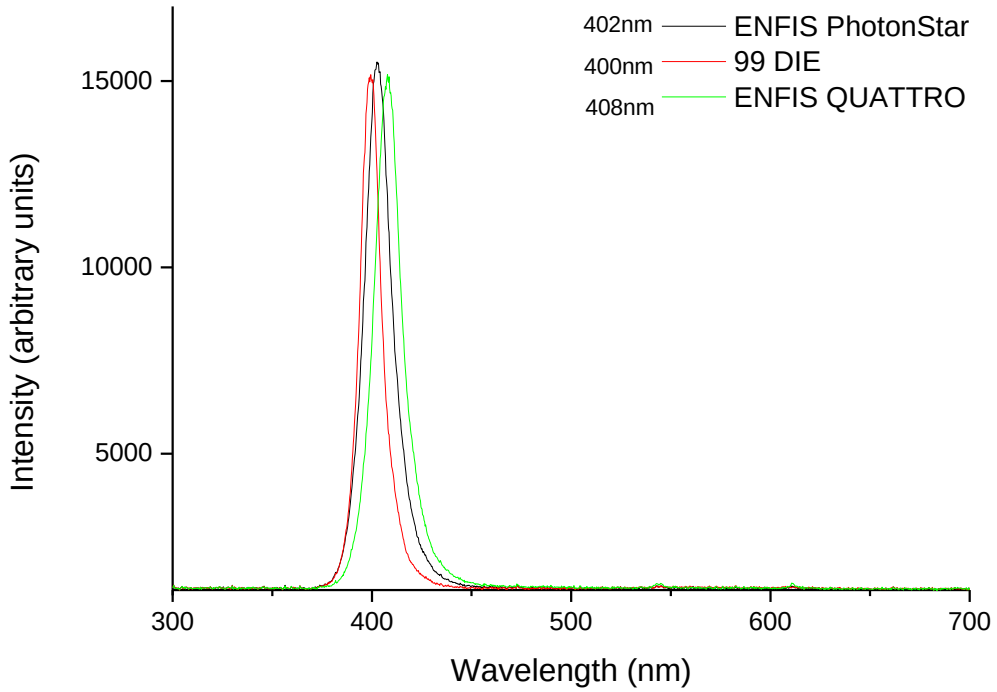

Figure 3.1 *Optical emission spectrum of ENFIS QUATTRO Mini Air Cooled Light System, ENFIS PhotonStar Innovate UNO 24 and 99 DIE OptoDiode Corp, with peak wavelengths as defined in the legend.*

3.3.1 ENFIS QUATTRO Mini Air Cooled Light Engine

The ENFIS QUATTRO Mini Air Cooled Light Engine system consists of a 4 cm^2 array of 144 (12×12) violet LEDs, with peak emission in the region of 405 nm (±5 nm) (Figure 3.2). Optical analysis of the output confirmed a peak wavelength of 408 nm and a bandwidth of 17 nm, this can also be defined as full width half maximum (FWHM).

The emission spectrum shown in Figure 3.1 was measured using an Ocean Optics HR4000 spectrometer and SpectraSuite computer software package.

The light engine was connected to an LED driver (ENFIS, UK), and powered by a 48 V power supply (Sunpower, USA). To minimise transfer of heat to the samples, a metal heat-sink was attached to the array to absorb and disperse heat. A fan was also attached, allowing continuous airflow, enabling cooling and ventilation (Figure 3.3). The light engine was mounted onto a clamp that was attached to a metal pole and base plate. This allowed the engine to be held in position above bacterial samples, and the distance between the array and the sample to be altered in order to adjust the output irradiance required for sample exposure. As standard, the distance between the array and the sample was set at 5 cm, giving an irradiance output of approximately 200 mW cm^{-2} directly below the array.

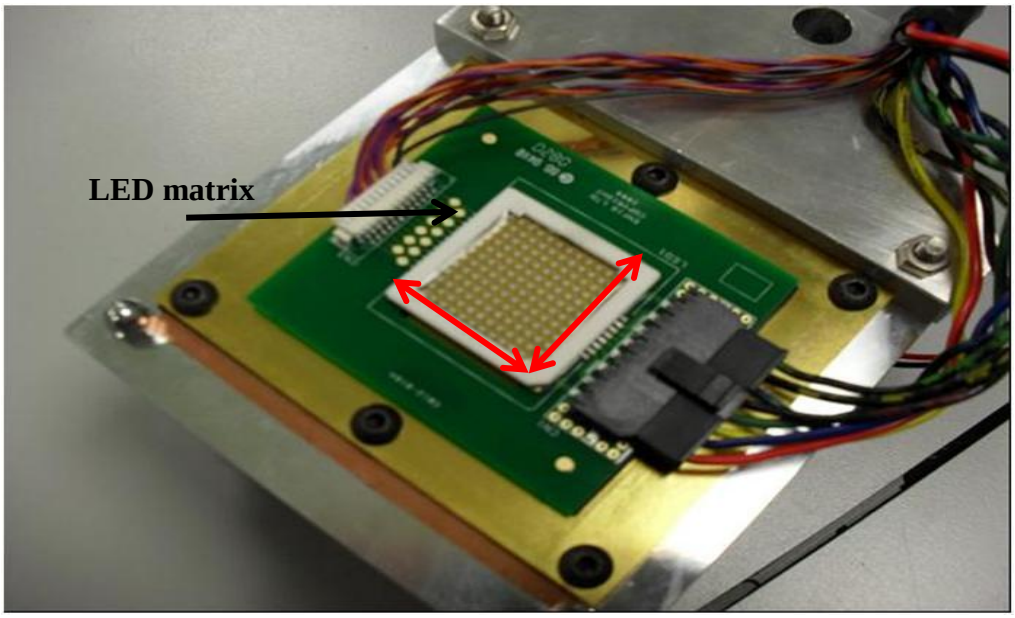

Figure 3.2 *ENFIS QUATTRO Mini Air Cooled Light System. Highlighted is the 4×4 cm array containing 144 light emitting diodes.*

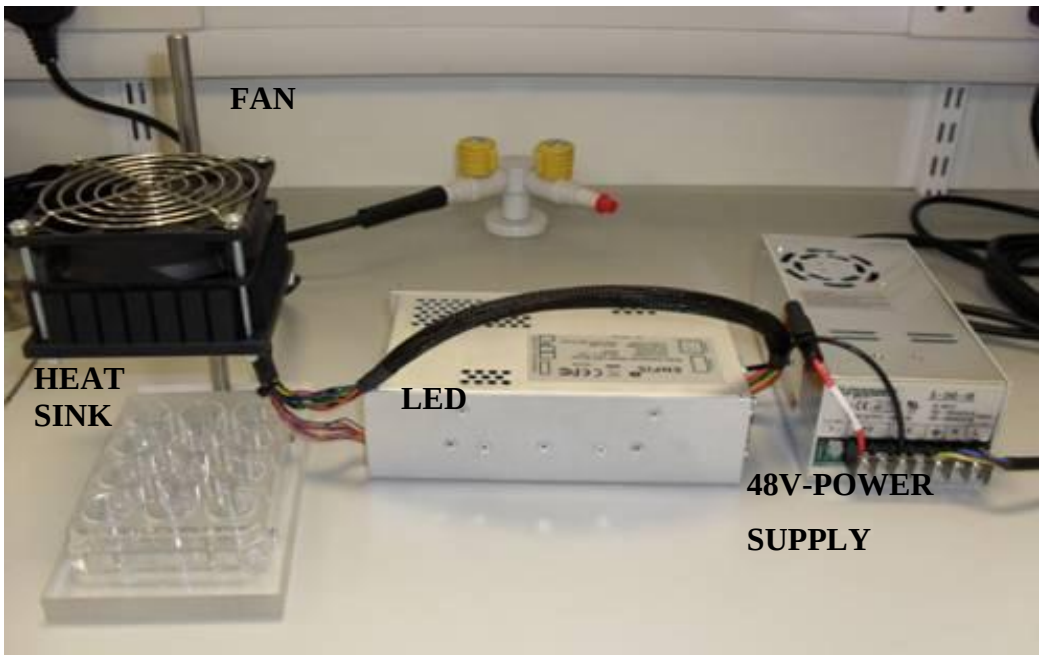

Figure 3.3 *ENFIS QUATTRO Mini Air Cooled Light System, mounted to allow positioning above sample.*

3.3.2 ENFIS PhotonStar Innovate UNO 24

The Innovate UNO 24 system contains 24 violet LEDs with a peak wavelength in the region of 405 nm. Optical analysis of the output confirmed a peak wavelength of 402 nm and a bandwidth of 17 nm, as shown in Figure 3.1.

The light engine was powered by 40 V Phillips Xitanium LED driver. As with the QUATTRO array, this source was also attached to a heatsink and fan to dissipate heat (Figure 3.4). The system was mounted similarly, allowing alterations in the distance between the array and the sample for the purpose of making irradiance adjustments. As standard, the distance between the array and the sample was set at 5 cm, giving an irradiance output of ~65 mW cm^{-2} directly below the array.

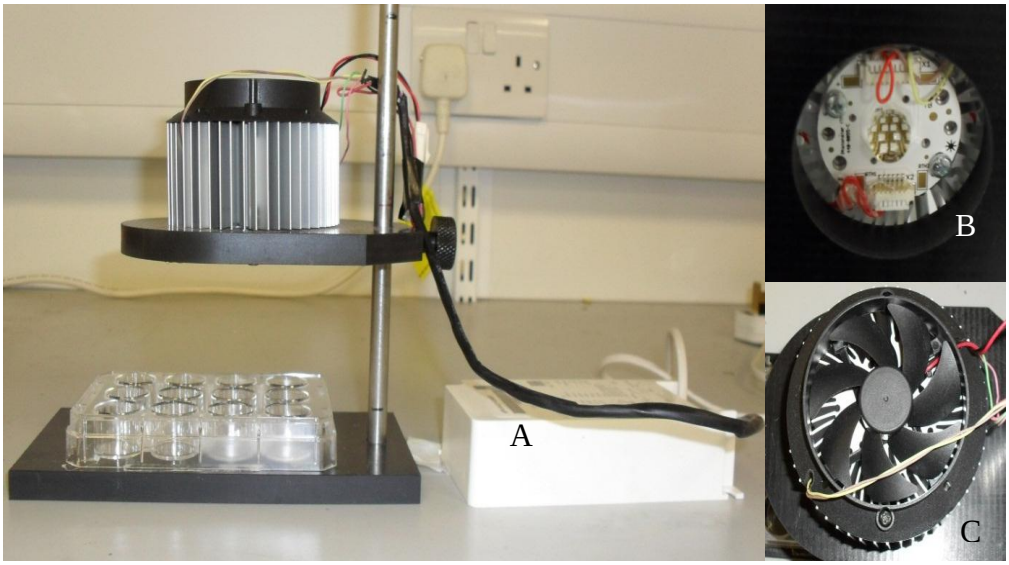

Figure 3.4 *PhotonStar Innovate UNO 24 light system, with Phillips LED driver (A) used for both liquid and surface seeded bacterial inactivation experiments. LED array, (B) and attached fan (C).*

3.3.3 405 nm 99-DIE LED array

These arrays were manufactured by OptoDiode Corp (California, USA). Optical analysis of the output confirmed a peak wavelength of 400 nm and a bandwidth of 13 nm as shown in Figure 3.1.

The LED arrays were attached to a heat sink and cooling fan, again to allow dissipation of heat away from test samples. The LED array was mounted to PVC housing, specifically designed to fit on top of a 12-well micro plate (NUNC, Denmark). Microbial samples were held in a central well of the plate and positioned directly under the array, at a distance of ~ 2cm. The array was powered by a variable DC power supply (0-20 A and 0-16 V). For experiments, the current and voltage were typically set at 0.6 A and 10.9 V, respectively, giving an approximate irradiance of 40 mW cm^{-2} at a distance of approximately 2 cm. Variation in these settings will be highlighted in subsequent chapters.

Figure 3.5 *Experimental arrangement using the 405 nm 99 DIE LED array, for light exposure of bacterial suspensions.*

3.3.4 HINS-light Environmental Decontamination System (HINS-light EDS)

The HINS-light Environmental Decontamination System (HINS-light EDS), as mentioned in Chapter 2, Section 2.5.4.3, is a ceiling-mounted light source which uses 405 nm light for microbial inactivation. The light source consists of 16 violet (405 nm) LEDs (OptoDiode Corp, USA) and 5 broadband white LEDs, as shown in Figure 3.6A. The emission spectra for both narrowband violet light and broadband white light are shown in Figure 3.7. Optical analysis of the output confirmed a peak wavelength of 403 nm and a bandwidth of 16 nm for the violet blue LED's. Another peak was depicted at 660 nm for the white LEDs.

White lights are incorporated to blend the colour output of the light source, to ensure the output appears predominately white. Arrays were mounted to an aluminium heat-sink (30×30×8.5 cm) in order to minimise the build-up of heat. A Fresnel lens and diffuser was positioned in front of the LED matrix, with the distance between the LED matrix and the lens/diffuser controlling the distribution of the light. A schematic representation of the optical arrangement is shown in Figure 3.6B. The diffuser and Fresnel lens were clamped onto a 59 ×59 cm PVC tile with circular window for light emission. The diffuser and lens were held by aluminium side panels in order to increase light

reflectance. The unit was designed so that it could be easily installed by replacing existing standard ceiling tiles. For operation, the arrays were driven by two low-voltage power supplies, one supplying 270 W (15 V at 18 A) to the violet LEDs, and the other supplying 15 W (10 V at 1.5 A) to power the white LEDs. These power supplies were located within an external control box.

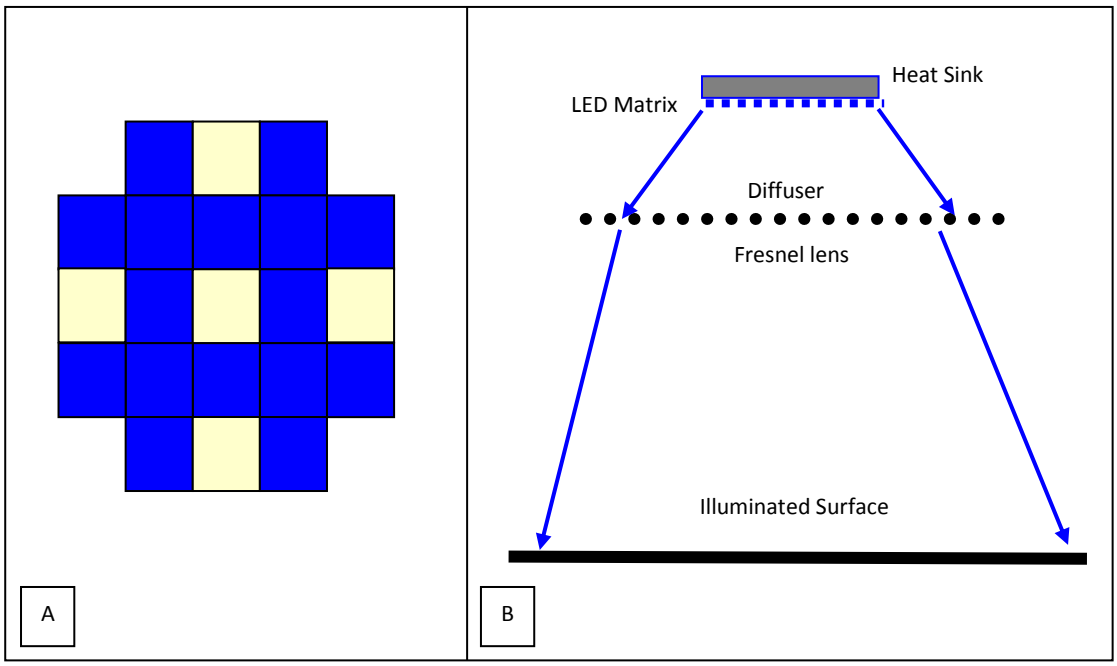

Figure 3.6 *Schematic examples of (A) a HINS-light EDS light source, and (B) optical arrangement for HINS-light EDS environmental illumination. Adapted from patent (Anderson et al., 2007).*

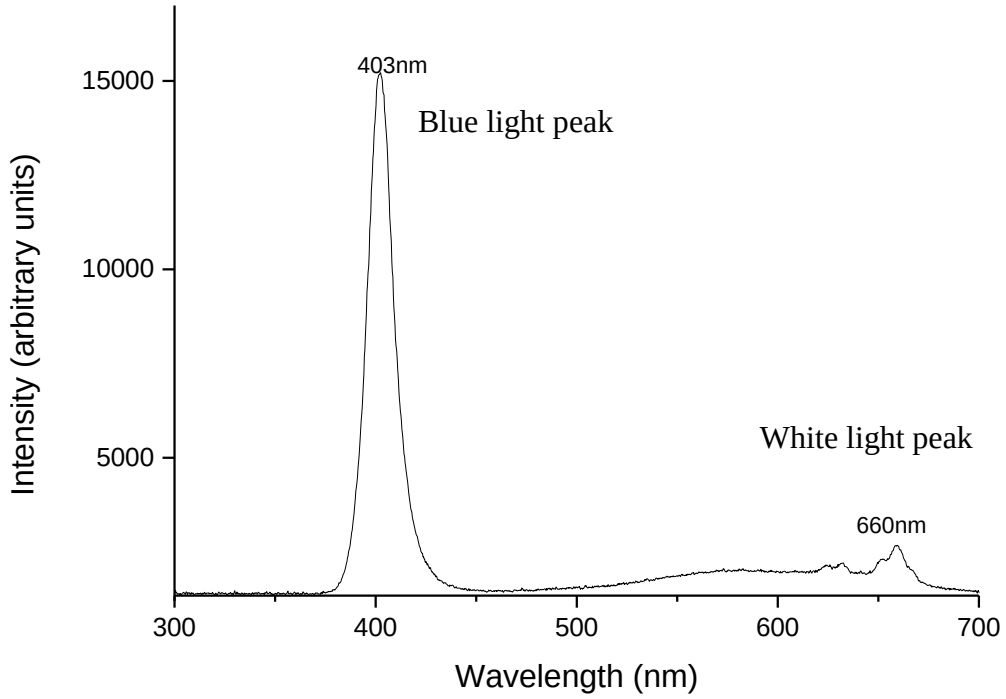

Figure 3.7 *Optical emission spectrum of HINS-light EDS, with peak wavelengths highlighted for both blue and white LED's.*

3.3.5 Comparison of different arrays

Table 3.4 provides a summary of the different properties associated with each of the arrays. Note in particular the variation in irradiance, which directly reflects why different systems were used in different studies.

Table 3.4 *Summary table of the LED array sources used throughout this study, highlighting the manufacturer, the voltage input, the peak wavelength, the bandwidth and the typical irradiance used.*

Name	Manufacturer	Voltage input	Peak wavelength	Bandwidth (FWHM)	Typical irradiance
ENFIS QUATTRO Mini Air Cooled Light Engine	ENFIS, UK	48 V	408 nm	17 nm	180 mW cm^{-2}
ENFIS PhotonStar Innovate UNO 24	PhotonStar, UK	40 V	402 nm	17 nm	65 mW cm^{-2}
405 nm 99 DIE LED array	OptoDiode Corp, CA	Variable (0-16 V)	400 nm	13 nm	40-70 mW cm^{-2}
HINS-light EDS	Prototype, Strathclyde University*	15 V	403 nm	16 nm	0.5 mW cm^{-2}

****Patent***. *J.G. Anderson, M. Maclean, G.A. Woolsey and S.J. MacGregor (2007). International Patent Application 0721374.7. "Optical Device for the Environmental Control of Pathogenic Bacteria", PCT application number WO 2009/056838. Patent filing date: October 2007*

3.4 Microbial Enumeration

When conducting microbial inactivation experiments, it is necessary to accurately enumerate the population densities both pre and post light treatment to allow calculation of achieved microbial reduction.

For population enumerations, suspension samples are typically plated onto agar and incubated as detailed in Table 3.2. Depending on the populations that were used in the

particular experiment, the method of plating and dilution were varied in order to ensure quantifiable results were obtained.

When microbial populations were expected to be high, suspensions were serially diluted prior to plating to ensure quantifiable results. Following incubation, agar plates were enumerated by counting colonies on the plates and microbial counts were recorded as colony forming units per millilitre (CFU mL^{-1}) or CFU plate depending on whether liquid samples or surface seeded samples were exposed to the light treatment.

The following sections provide details of the dilution, plating and enumeration techniques that were used throughout.

3.4.1 Re-suspension and serial dilutions

Following incubation, broths were centrifuged at 3939 ×*g* for 10 minutes, the supernatant was discarded and the resultant pellet was re-suspended in 100 mL PBS (pH 7.2). In order to achieve the desired starting population for experimental use a 1 mL volume was aseptically transferred from the 100 mL suspension into 9 mL PBS, to make a 10^{-1} CFU mL^{-1} dilution. Subsequent dilutions were made by transfer of 1 mL of this suspension into a further 9 mL PBS giving a 10^{-2} CFU mL^{-1} and so on, until the desired starting population was achieved. At each dilution stage each suspension was mixed using a Whirly mixer (Fisher) to ensure production of a homogenous suspension at each stage in the dilution process.

3.4.2 Plating and enumeration techniques

Various plating and enumeration techniques were used throughout this study, with the method used dependant on the number of microorganisms expected to survive following light treatment. Each of the techniques is detailed in the following sections.

3.4.2.1 Spiral plating

Spiral plating was used for samples that were expected to be between 10^3- 10^5 . A WASP 2 spiral plater (Don Whitley Scientific) (Figure 3.8A) was used to produce spiral plates as follows. Microbial samples were taken up by a syringe-like stylus, 50 µl sample volume was deposited onto a rotating agar plate producing a logarithmic Archimedes spiral. This deposition pattern meant that a greater volume of sample was

deposited in the centre of the spiral, with less deposited towards the outer edges (Figure 3.8 B). Samples were left upright to dry prior to incubation.

Following incubation, colonies were counted manually using a standard colony counter (Stuart Scientific, UK), with a counting grid held over the plate. The number of colony forming units in a specific section of the grid was referenced against supplied data sheets, which permitted the enumeration of the CFU mL^{-1}. Alternatively, the plates were counted automatically. The ACOLYTE software programme automatically counts using ACOLYTE version 1.19 computer software programme, which automatically enumerates the CFU mL^{-1}.

3.4.2.2 Spread plating

If a microbial population was expected to be < 10^3 CFU mL^{-1}, spread plates could also be prepared using the WASP 2 spiral plater. In this instance a 100 µl sample was taken up by the stylus and deposited in a linear spiral across the agar plate (Figure 3.7C). Manual spread plates could also be prepared by aseptically depositing 100 µl, 200 µl or 500 µl onto agar plates. The sample was then spread over the surface using a sterile L-shaped spreader until the sample had been absorbed. Following 18-24 hours incubation, plates were manually counted and then multiplied by the correct dilution factor, in order to calculate the microbial count in CFU mL^{-1}.

3.4.2.3 Pour plating

If samples were predicted to have microbial counts of less than ~200 CFU mL^{-1}, the pour plate method was used as the method of choice. For this, 1 mL sample was deposited into a sterile 90 mm petri dish then ~20 mL of molten agar (held at 48°C) was poured into the dish. To ensure an even distribution of the sample, the plate was rotated clockwise and anti-clockwise 10 times. Plates were then left to set on the open bench for ~10-15 minutes before incubation. Enumeration of the entire plate provided a direct CFU mL^{-1} count, as the sample deposited was 1 mL.

Use of these plating methods provided a detection limit of between 1 CFU mL^{-1} (pour plate method) and 10 CFU mL^{-1} (spread plate method).

Figure 3.8 *WASP 2 spiral plater (A), and agar plates highlighting the distribution pattern of the spiral (B) and spread plates (C).*

3.5 Statistical Analysis

Data points represent average results taken from a minimum of triplicate independent experiments, with a minimum of two samples plated for each experiment ($n \geq 6$), with an average standard deviation expressed at each data point. Significant differences were calculated at the 95% confidence interval using ANOVA (one way) with MINITAB software release 15. These were highlighted throughout this study using asterisk (*). Statistical analysis is highlighted in the results of each subsequent chapter.

3.6 Additional Equipment

This section gives a brief overview of the main equipment used. Further detail of the use of some of the more specialised/ key equipment will be detailed in subsequent chapters.

- *Autoclave:* A Kestral automatic autoclave (LTE Scientific, UK) and a bench-top autoclave (Dixons Surgical Instruments, UK) were used for sterilisation of media for experimental use and also for sterilisation of microbial waste prior to disposal.
- *Water Distiller*: A Merit 400 Distil was used to produce distilled water, free of impurities for preparation of media and diluents.

- *Measuring Scales:* OHAUS Navigator and OHAUS Adventurer digital balances were used for weighing out media and chemical reagents.
- *Incubators:* A rotary shaker incubator (New Brunswick Scientific, Germany) was used for broth cultivation of microorganisms. Temperature and revolutions per minute (rpm) could be adjusted manually. A static fan assisted incubator (LTE Scientific, UK) was used for plate cultivation of microorganisms. Temperature settings could be adjusted manually.
- *Centrifuge:* A Heraeus Labofuge 400R (Kendro Laboratory Products, USA) was used for centrifugation of microorganisms from their growth media. Samples were centrifuged at 3939×*g* for 10 minutes at room temperature.
- A Sciquip 1-15K centrifuge (Sigma, UK) was used for centrifugation of 1.5 mL Eppendorf tubes containing small volumes of microbial suspensions. Specific details of use will be given in subsequent chapters.
- *Pipettes*: Gilson pipettes (100 µL, 1 mL, 5 mL and 10 mL) were used for sterile transfer of liquids, both diluents and microbial suspensions. For transfer of chemical reagents, single use sterile pipettes were used.
- *Refrigerators:* Lee medical refrigerators (UK), maintained at 4°C, were used for storage of all microbial cultures on agar slopes, and storage of test kits and reagents that required refrigeration.
- *Water bath*: A Grant water bath (UK) was used for holding molten agar following sterilisation in the autoclave. Temperature was kept at 48°C, but had a manual control system that allowed the temperature to be adjusted if necessary.
- *Ultra sonic bath*: An Ultra BT Ultrasonic bath U100 (Ultrawave Limited, Cardiff) was used for sonication of TiO_2 nanoparticles in liquid suspension.
- *Stomacher:* A Mix 2 stomacher (DW Scientific, UK) was used to breakdown and liquify food samples. Time and pressure could be adjusted manually and these settings were dependent on the specific sample being stomached.
- *Microscope*: A Nikon Eclipse E400 Light Microscope (Nikon Instruments, Europe) was used for visualisation, identification and quantifying microbial cultures, with magnififcation up to × 1000. A Nikon Coolpix 4500 digital camera was used for capturing images of slide preparations.

- *Spectrophotometer:* A Biomate 5-UV-Visible Spectrophotometer (Thermo Scientific, USA) was used for measuring the transmission and absorption spectra of a range of samples, including bacterial suspensions, chemical suspensions, diluents and broths. Scans were conducted either at a single wavelength or a broader spectrum, dependent on the specific experimental requirements. Samples were placed in cuvettes alongside a blank sample to allow directly comparable results. The cuvette type used depended on the wavelength region being investigated: scans carried out in the UV and visible region required the use of quartz and plastic cuvettes, respectively.
- *Fluorescence spectrophotometer*: A Shimadzu spectrofluorophotometre (RF-5301-PC, America) was used for measuring the fluorescence of a range of samples, including bacterial suspensions. Scans were conducted either at a single wavelength or a broader spectrum; specific scans will be detailed in subsequent chapters.
- *Radiant power meter and photodiode detector:* A Power meter and photodiode detector (LOT Oriel, USA) was used to measure the irradiance output from the various 405 nm light sources.
- *Vortex*: A WhirliMixer (Fisherbrand, UK) was used for re-suspension of microbial cell pellets into PBS suspension and homogenous mixing of serially diluted suspensions.
- *Thermocouple:* A Kane May KM340 thermocouple (UK) was used for measuring the temperature of light exposed microbial samples.
- *Magnetic stirring plate:* An MSH basic stirring plate (Scientific Laboratory Supplies, UK) was used with magnetic followers (Fisher Scientific) to allow continuous agitation of microbial samples during experimental work.
- *Camera*: A Samsung ES60 digital camera was used to record visual images of equipment and experimental results.
- *Haeocytometer*: An improved Neubauer Haemocytometer (Hawksley Technology, England) was used for enumeration of fungal spore suspensions.

3.7 Microbiological Identification Tests

In order to ensure the identity of the microorganisms being used in experimental procedures it was critical to routinely conduct a number of microbiological tests to confirm that cultures were pure.

3.7.1 Gram staining

The Gram staining technique was used to differentiate between Gram positive and Gram negative bacteria. It is commonly the first procedure used for identification of unknown bacteria, or to check purity of a culture. Not only does this give the Gram status of the bacteria, but it can also show the morphology of the cell. The process is outlined below:

- *Preparation of smear for staining*

Using a sterile wire loop, a single isolated colony was collected from an agar plate (or a loopful of bacterial suspension if using liquid culture) and emulsified in a droplet of water in the centre of a glass microscope slide. After air drying, the sample was fixed to the slide by passing it through the blue flame of a Bunsen Burner 2-3 times.

- *Staining Procedure*

The sample was flooded with crystal violet for approximately 30 seconds. The sample was rinsed with Lugol's Iodine and then covered in a fresh layer of iodine and left for approximately 60 seconds. The sample was then rinsed with ethanol, until all violet colour had drained from the slide. The sample was then counterstained with safronin and left for a further 60 seconds. Finally, the sample was rinsed with water, blotted dry and viewed under the oil immersion microscope lens.

Identification of pink colonies represents a Gram negative organism, whilst blue-violet colonies represent Gram positive.

3.7.2 STAPHAUREX

Staphaurex (Remel, Thermo Fisher Scientific, UK) is a rapid latex test, used for detection of Protein A and clumping factor readily associated with *Staphylococcus aureus*. Agglutination of the test latex solution, followed by lack of agglutination of the control latex solution indicates a positive *S. aureus* result. The test procedure is described below:

- Shake the latex reagents vigorously prior to use.
- Place one drop of the test latex solution into a single circle on the reaction card, and one drope of the control latex solution into an adjacent circle.
- Using a supplied mixing stick, collect a single colony and mix well with the latex suspension.
- Repeat for control latex solution.
- Allow 30 seconds for agglutination reaction to occur.
- Positive result shows agglutination in test and not in control.

CHAPTER 4

INVESTIGATION INTO THE USE OF 405 NM LIGHT FOR INACTIVATION OF FOODBORNE BACTERIA

4.0 General

Microbial contamination of foodstuff can result in spoilage of food and acquisition of food related disease. The financial burden of microbial contamination of foods is estimated to be in the region of £1.5 billion and $35 billion for the UK and USA, respectively. However, these figures are quite conservative and true costs are predicted to be significantly higher (FSA, 2009; Buzby and Roberts, 2009).

Over recent years the use of violet-blue 405 nm light for microbial inactivation has generated much interest, owing to its bactericidal effects against a wide range of clinically significant pathogens, including *Methicillin resistant Staphylococcus aureus* (MRSA). Recent studies have demonstrated the use of 405 nm light for environmental disinfection of a hospital isolation room, with results demonstrating significant reductions (up to ~ 90%) in environmental bacterial counts following continuous 405 nm light exposure (Bache et al., 2012; Maclean et al., 2010) Successful results from these clinical studies have sparked interest in potential environmental disinfection applications for 405 nm light in the food sector, both in industry and in domestic kitchens.

As discussed in Chapter 2, 405 nm light inactivation of bacteria is thought to occur through excitation of intracellular porphyrin molecules, resulting in oxidative damage, promoting cellular damage and cell death. This inactivation mechanism has benefits over many other current chemical and technological antimicrobial treatments. The inactivation mechanism does not induce DNA mutations, making organisms less likely to develop genetic mutations and acquire resistance making it a it a safer alternative for human exposure. Furthermore the non-specific oxidative damage exerted on exposed microorganisms enables effective inactivation of a wide range of microbial species.

The work presented in this chapter focuses on evaluating the antibacterial efficacy of 405 nm light for inactivation of significant food-related bacterial pathogens under various conditions, and is divided into three main subsections as follows:

- Inactivation of a range of foodborne pathogens including *E. coli, S. enteritidis, L. monocytogenes* and *S. aureus* in liquid suspension, using high irradiance 405 nm light (65-200 mW cm^{-2}) in order to generate proof-of-principle inactivation kinetics.
- Inactivation of a range of foodborne pathogens including *E. coli, S. enteritidis, L. monocytogenes* and *S. aureus* seeded onto surfaces using high irradiance 405 nm light (65-200 mW cm^{-2}) in order to generate proof-of-principle inactivation kinetics.
- Inactivation of bacteria seeded onto surfaces using low irradiance 405 nm light (~0.5 mW cm^{-2}) using the HINS-light environmental decontamination system (EDS), in order to establish the efficacy of this system for decontamination of contaminated surfaces, and its potential for application within the food industry.

4.1 High Irradiance 405 nm-light Treatment of Foodborne Bacteria in Suspension

Previous studies have shown successful *in vitro* inactivation of hospital related bacteria when exposed to 405 nm light in liquid suspension. The aim of this study was to determine whether similar results could be achieved with bacteria commonly associated with food products.

4.1.1 Bacterial Preparation

The bacteria used in this section of experimental work were *S. aureus*, *E. coli*, *L. monocytogenes* and *S. enteritidis*. Bacteria were cultivated at 37°C overnight and centrifuged at 3939 × g for 10 minutes. The resultant pellet was then re-suspended in 100 mL PBS and serially diluted to achieve the desired starting population (as described in Section 3.4.1).

4.1.2 High Irradiance 405 nm light Treatment of Bacterial Suspensions

For inactivation of bacterial suspensions, initial experiments were conducted using the ENFIS QUATTRO light system (Source A), described in Section 3.3.1. However due to a technical fault, this system had to be replaced in later experiments with a lower power light source (ENFIS PhotonStar Innovate UNO 24 system; Source B) as described in Section 3.3.2.

A 3 mL volume of bacterial suspension, of either 10^2 or 10^5 CFU mL^{-1} population, was transferred into one of the central wells of a 12-well multi-dish. The LED array was fixed at a 5 cm distance from the sample surface giving an approximate irradiance output of 65 or 200 mW cm^{-2}, when using Source B or Source A, respectively. The plate was positioned directly below the array. Photographs of these experimental set-ups are shown in Figure 4.1. An identical control plate that received no 405 nm light exposure was also set up on the lab bench.

Test samples were exposed to increasing durations of 405 nm light, and from this, the applied dose of 405 nm light could be calculated using the equation:

$$E = P \times t$$

Where E = dose (or energy density) in J cm^{-2}, P = irradiance (or power density) in W cm^{-2}, and t = exposure time in seconds.

Post-exposure, samples (and non-exposed control samples) were plated onto agar, as detailed in Section 3.4.2 and incubated at 37°C for 18-24 hours. Samples were then enumerated and the viable bacterial counts recorded as $\log_{10}$ CFU mL^{-1}.

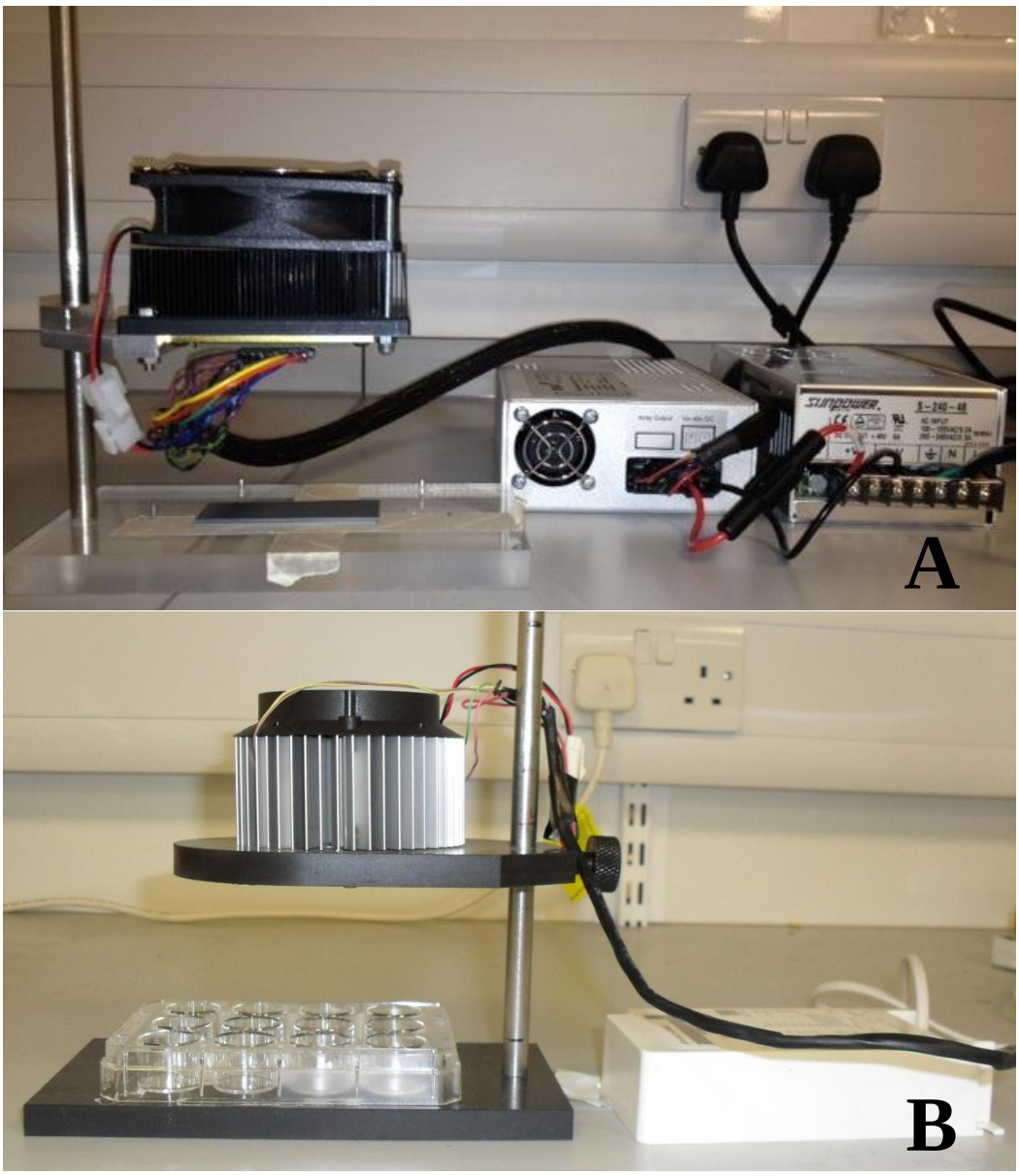

Figure 4.1 Experimental setup displaying ENFIS QUATTRO Mini Air Cooled Light System (A) and PhotonStar Innovate UNO 24 light system (B) used throughout this chapter for suspension and agar surface inactivation.

4.1.3 Measurement of temperature change in bacterial suspension

In order to measure the potential temperature rise in samples exposed to high irradiance light, temperature measurements of 3 mL PBS suspensions exposed to 180 mW cm^{-2} 405 nm light were recorded every 10 minutes over a 60 minute duration, using a thermocouple (Kane May KM340). A maximum increase of 6°C was recorded

throughout the test period. This was to ensure that inactivation of the bacteria during light exposure experiments was not resulting from a heating effect.

4.1.4 Results: Bacterial inactivation in suspension using high irradiance 405 nm light

Bacterial suspensions exposed to high irradiance 405 nm light demonstrated significant inactivation when compared to control samples. Figure 4.2 highlights inactivation of *E. coli, S. aureus, L. monocytogenes* and *S. enteritidis* when exposed to high irradiance 405 nm light.

Results highlight that *L. monocytogenes* was the most susceptible of the organisms tested. Although minimal inactivation was achieved following an applied dose of ~20 J cm^{-2}, a sharp decrease in bacterial population was observed between 20 and 30 J cm^{-2}, with a 3.1 log_{10} reduction (P= 0.00) achieved following 30 J cm^{-2} . Following exposure with 39 J cm^{-2}, a further 1.9 log_{10} reduction, and complete inactivation, was observed.

Complete inactivation of a 10^5 CFU mL^{-1} population of *S. aureus* required 109 J cm^{-2}. Reductions of 0.4 and 1.2 log_{10} were achieved following exposure to 54 and 81 J cm^{-2}, respectively. Greatest inactivation was recorded between 81 and 109 J cm^{-2}, where an 3.8 log_{10} reduction in bacterial population was observed, resulting in complete bacterial inactivation. Significant inactivation was noted at all data points following a given dose of > 54 J cm^{-2}, where in all cases P= 0.00.

Inactivation of the Gram negative bacteria, *S. enteritidis* and *E. coli* required significantly higher doses than used for the Gram positive bacteria. Significant inactivation of *S. enteritidis* was observed following an applied dose of 234 J cm^{-2}, whereby a 0.56 log_{10} reduction was recorded, with a 1.33 log_{10} reduction achieved after exposure to 292 J cm^{-2}. Complete inactivation of *S. enteritidis* was achieved after 351 J cm^{-2}.

Results demonstrated that complete inactivation of *E. coli* required the highest dose of all tested organisms. For *E. coli*, a dose of 163 J cm^{-2} was required before a significant decrease in bacterial population was observed (P= 0.01). Exposure to doses of 218 and 327 J cm^{-2} a resulted in 0.5 and 2.5 log_{10} reductions, respectively. Complete inactivation

of the 10^5 CFU mL^{-1} population was achieved following exposure to a total dose of 436 J cm^{-2}.

It is important to note that, in all cases, control data remained consistent throughout with no significant decreases in control populations over the duration of the experiment (P>0.05).

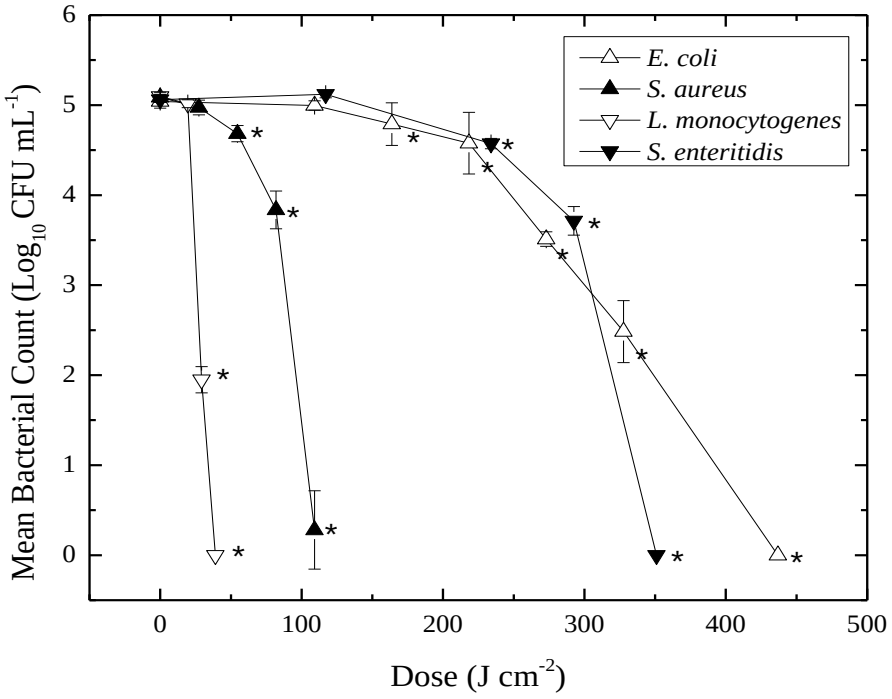

Figure 4.2 *Inactivation of E. coli, S. aureus, L. monocytogenes and S. enteritidis in liquid suspension (10^5 CFU mL^{-1}) using high irradiance 405 nm light, displayed as a function of dose. * represent significant bacterial inactivation when compared to associated non-exposed control samples (P≤0.05). Control data for each organism showed no significant change (P>0.05).*

Figures 4.3 and 4.4 detail inactivation results of *S. aureus* and *E. coli* in suspension at two different population densities (10^2 and 10^5 CFU mL^{-1}), giving comparative inactivation kinetics for the different bacterial populations. It is important to note that at both population densities, similar inactivation kinetics were observed. As with the higher population densities, *S. aureus* was inactivated at a much lower dose than the

equivalent population of *E. coli*. Significant inactivation (0.97 $\log_{10}$ reduction) of the 10^2 CFU mL^{-1} population of *S. aureus* was achieved following an applied dose of only 27.3 J cm^{-2}, with complete inactivation achieved following 54.6 J cm^{-2}. Inactivation of the 10^2 CFU mL^{-1} population of *E. coli* required a much greater dose, with significant inactivation (0.6 $\log_{10}$) achieved following 109 J cm^{-2}. Near complete inactivation (<5 CFU mL^{-1} surviving) was achieved following 218 J cm^{-2}.

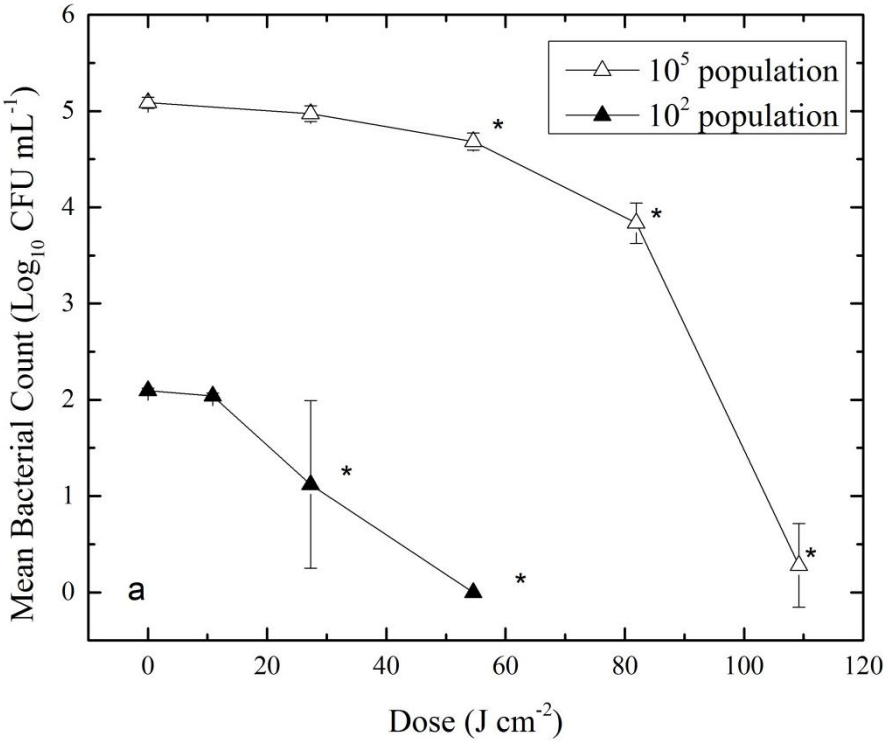

Figure 4.3 *Comparative inactivation of 10^5 and 10^2 CFU mL^{-1} populations of S. aureus in liquid suspension using high irradiance 405 nm light (180 mW cm^{-2}), displayed as a function of dose. * represent significant bacterial inactivation when compared to associated non-exposed control samples ($P \leq 0.05$).*

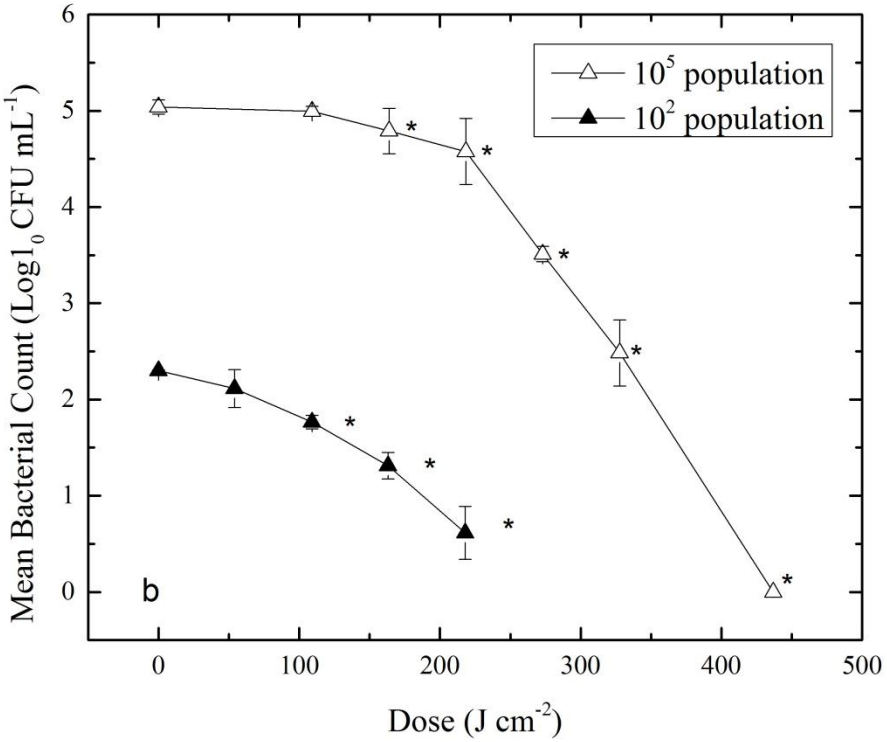

Figure 4.4 *Comparative inactivation of 10^5 and 10^2 CFU mL^{-1} populations of E. coli in liquid suspension using high irradiance 405 nm light (~180 mW cm^{-2}), displayed as a function of dose. * represent significant bacterial inactivation when compared to associated non-exposed control samples ($P \leq 0.05$).*

4.1.5 Evidence of Sub-lethal damage induced by 405 nm light

Following on from these experiments, which demonstrated inactivation of total viable counts, an investigation into the sub-lethal effects of 405 nm light was conducted using *S. aureus* as the model organism.

Bacteria that are sub-lethally damaged/ injured are less likely to grow on selective media than non-selective media due to the selective pressures exerted on the bacteria when on the harsher growth environment of selective media. Therefore this principle can be used to estimate the level of sub-lethal damage within a bacterial population (Carson et al., 2002)

Evidence of sub-lethal injury was ascertained by plating the light-exposed *S. aureus* samples onto both nutrient agar (non-selective) and mannitol salt agar (selective for Staphylococci), and the sub-lethally damaged populations were quantified from the difference in counts between the growth on the selective and non-selective media. As

can be seen from Figure 4.5, 405 nm light can induce sub-lethal damage to bacteria. Following a dose of 29 J cm^{-2}, significant differences in inactivation rates were observed between samples plated onto selective and non-selective agars (P=<0.05). Following a higher dose of 58.5 J cm^{-2}, a significant difference is observed between the counts on the selective (5 log_{10} reduction) and non-selective (1.86 log_{10} reduction) media, with the difference between these two counts highlighting a substantial sub-lethally damaged population of 3.10 log_{10} CFU mL^{-1}.

The major constituent of MSA is sodium chloride (NaCl; salt) with a concentration of 7.5%. In order to try and determine whether salt alone was the constituent inhibiting growth of sub-lethally damaged populations on selective agar, 7.5% NaCl was added to nutrient agar. Results in Figure 4.5 show that inactivation on MSA and salt-enriched NA are statistically similar. Inability of *S. aureus* to grow on salt-rich environments is indicative of membrane damage (Carson et al., 2002), suggesting that the damage induced by 405 nm light results in sub-lethal membrane damage prior to complete lethality. This will be further discussed in Section 4.4.

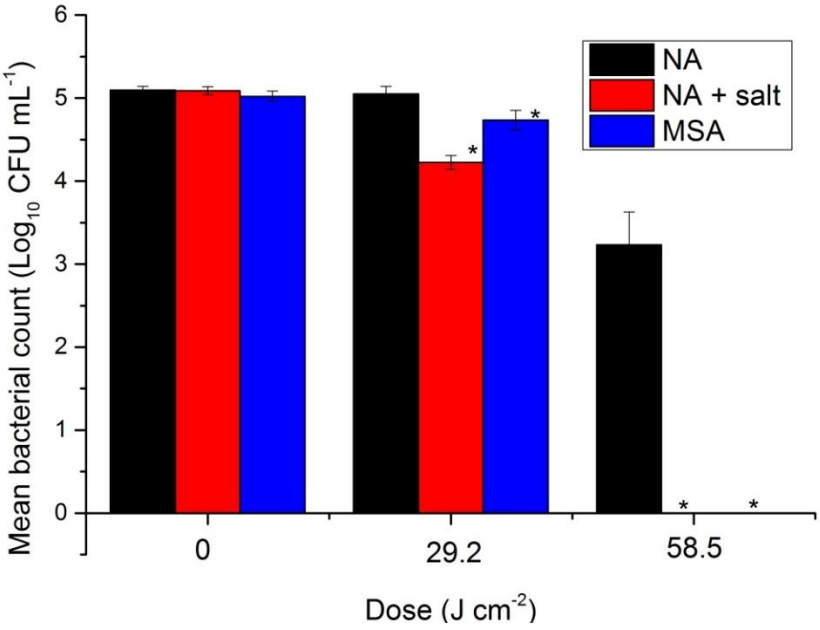

Figure 4.5 *Comparative inactivation of S. aureus on selective and non-selective media demonstrating lethal and sub-lethal damage of S. aureus following exposure to 405 nm light at an irradiance of ~65 mW cm^{-2}. * represent significant bacterial inactivation on selective agar (MSA and NA+salt), when compared to associated non-selective (NA) counts (P≤0.05).*

4.2 Inactivation of bacteria-seeded agar using high irradiance 405 nm light

Once the basic inactivation kinetics for bacteria in liquid suspension were established, experiments were conducted to determine the inactivation effects of 405 nm light on bacteria when exposed on surfaces, using agar plates as the test surface. This first series of experiments were conducted using a high irradiance light source (~70 mW cm^{-2}) in order to evaluate the efficacy of 405 nm light treatment for rapid decontamination. Low levels of bacteria were seeded onto the plate to represent the levels of surface contamination typically found around the environment (Maclean et al., 2010). This low-level surface contamination was also comparable with the low population densities exposed in liquid suspension (Figure 4.3-4.4).

4.2.1 Calculation of average power across an agar plate

Unlike bacterial suspension studies, this section of work involved direct exposure of a much larger surface area (90 mm vs. 22 mm diameter for a suspension well). Consequently, it was important to accurately quantify irradiance over the exposed surface.

In order to determine the average irradiance across the 90 mm agar plate, the ENFIS QUATTRO LED array unit (Source A) was set at a height of 5 cm above the surface of the plate, and irradiance measurements were taken every 5 mm over an *x* - *y* grid that encompassed the peri dish using a radiant power meter. Measurements showed that the irradiance peak at the centre of the plate was 180 mW cm^{-2}, gradually decreasing towards the outer edges shown in Figure 4.6. From this data, the average irradiance across the plate surface was calculated to be 71.2 mW cm^{-2} using Origin Pro software.

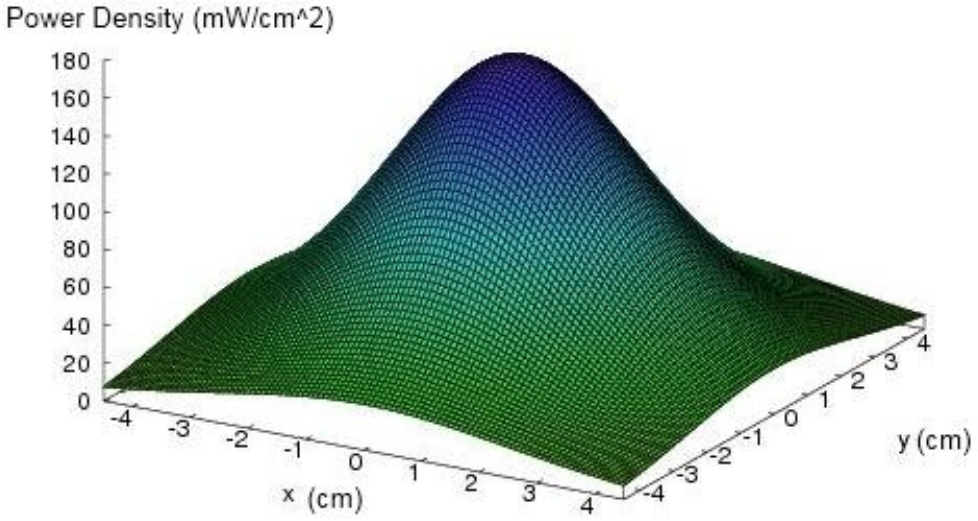

Figure 4.6 *3D model of power distribution across 9 cm petri dish at a height of 5 cm from array produced using Origin Pro software.*

4.2.2 Bacterial Preparation

Bacterial suspensions of *E. coli*, *S. enteritidis*, *S. sonnei*, *L. monocytogenes* and *S. aureus* were prepared as described in Section 3.4.1 and diluted to a population of 10^3 CFU mL^{-1}.

4.2.3 Methods: Bacterial inactivation on agar using high irradiance 405 nm light

To prepare bacterial seeded agar surfaces for exposure, a 100 µL volume of bacterial suspension was spread plated (Section 3.4.2) onto an agar plate surface, giving a seeding population of approximately 100-200 CFU per plate. Plates were then positioned under the light source and exposed to 71 mW cm^{-2} 405 nm light. Test samples were exposed to increasing doses of 405 nm light, and non-exposed control samples were set up simultaneously to allow comparison. Post exposure, test and control samples were incubated at 37°C for 18-24 hours and then enumerated. Bacterial counts were recorded as CFU per plate.

4.2.4 Results: Bacterial inactivation on agar using high irradiance 405 nm light

Results from Figure 4.7- 4.11 highlight inactivation of *S. aureus, L. monocytogenes, S. sonnei, E. coli and S. enteritidis* respectively, on agar surfaces. Results are expressed as percentage reduction due to slight variation in starting populations, thereby allowing direct comparison between the different organisms. Results demonstrated that as with suspension work, inactivation of Gram positive bacteria was achieved at much lower doses of 405 nm light when compared to Gram negative bacteria.

Rapid inactivation of *S. aureus* following high irradiance 405 nm light exposure is shown in Figure 4.7. Following an applied dose of 10.5 J cm^{-2}, no significant inactivation was observed. However a significant 96% population reduction was achieved after exposure to 21 J cm^{-2} and complete inactivation was achieved following 31 J cm^{-2} of 405 nm light treatment.

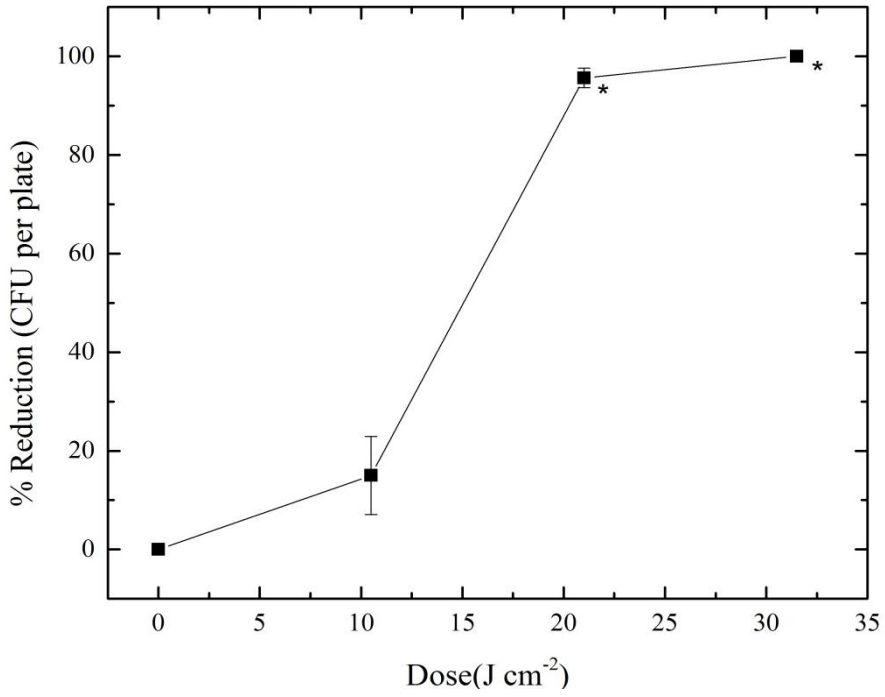

Figure 4.7 *Inactivation of S. aureus, on nutrient agar surfaces upon exposure to high irradiance (70 mW cm^{-2}) 405 nm light, expressed as a function of dose. * represent significant bacterial inactivation when compared to the associated non-exposed control samples ($P \leq 0.05$).*

Following 42 J cm^{-2} of 405 nm light exposure, a 51% reduction in *L. monocytogenes* was observed, but this reduction was not significant (P= 0.053) when compared to control populations. A significant decrease in bacterial population was observed following an applied dose of 84 J cm^{-2}, where complete inactivation was achieved.

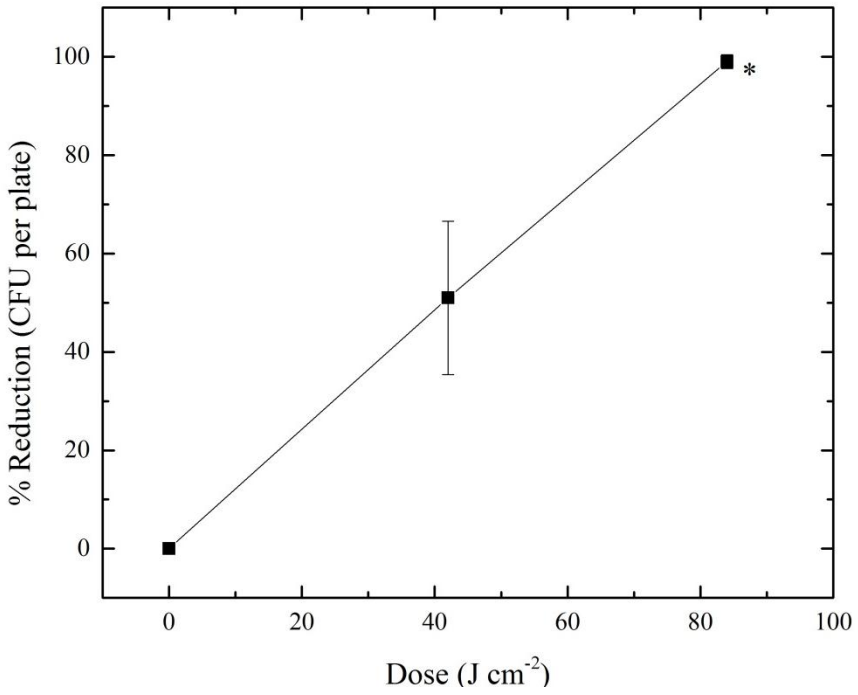

Figure 4.8 *Inactivation of L. monocytogenes, on tryptone soya agar surfaces upon exposure to high irradiance (70 mW cm^{-2}) 405 nm light, expressed as a function of dose. * represent significant bacterial inactivation when compared to the associated non-exposed control samples ($P \leq 0.05$).*

Inactivation of *S. sonnei* exposed to 405 nm light on nutrient agar demonstrated a rapid decrease in bacterial count, shown in Figure 4.9, with a 68% reduction observed following an applied dose of 42 J cm^{-2}. However this was not statistically significant (P= 0.078) due to the large standard deviations recorded. Treatment with 84 J cm^{-2} generated a 96% reduction, with complete inactivation achieved following exposure to 126 J cm^{-2}.

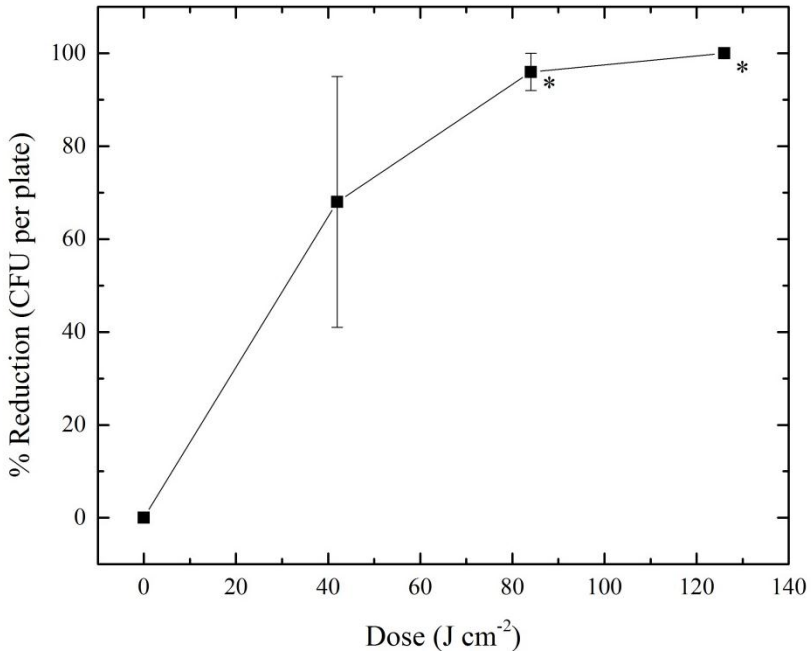

Figure 4.9 *Inactivation of S. sonnei, on nutrient agar surfaces upon exposure to high irradiance (70 mW cm^{-2}) 405 nm light, expressed as a function of dose. * represent significant bacterial inactivation when compared to the associated non-exposed control samples (P≤ 0.05).*

Significant inactivation (P=0.05) of *E. coli* was achieved following an applied dose of 42 J cm^{-2}, with ~35% reduction in bacterial population recorded. Further exposure demonstrated that after 84 J cm^{-2} an 85% reduction in bacterial count was achieved, with 100% reduction observed following a dose of 126 J cm^{-2}. Results are shown in Figure 4.10.

Inactivation of *S. enteritidis* was shown to require greatest exposure for complete inactivation. The data shown in Figure 4.11 shows an 18% reduction in bacterial count following 42 J cm^{-2}, but this was not significant (P= 0.286) when compared to control samples. Similarly, following 84 J cm^{-2}, non-significant reduction was achieved (P= 0.204). Upon exposure to 126 J cm^{-2}, a 62% decrease in bacterial population was observed, producing statistically significant inactivation levels (P= 0.002), with near complete inactivation (97%) achieved following 168 J cm^{-2}.

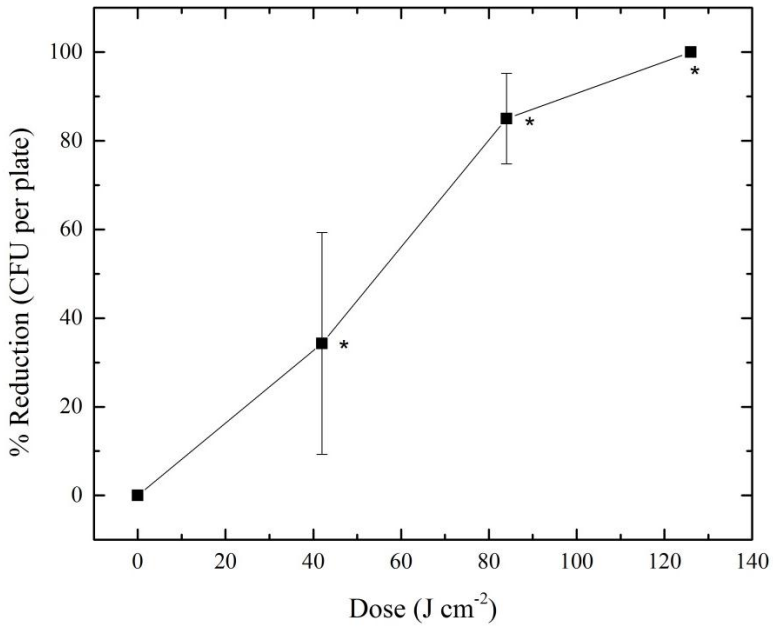

Figure 4.10 *Inactivation of E. coli, on nutrient agar surfaces upon exposure to high irradiance (70 mW cm^{-2}) 405 nm light, expressed as a function of dose. * represent significant bacterial inactivation when compared to the associated non-exposed control samples (P≤ 0.05).*

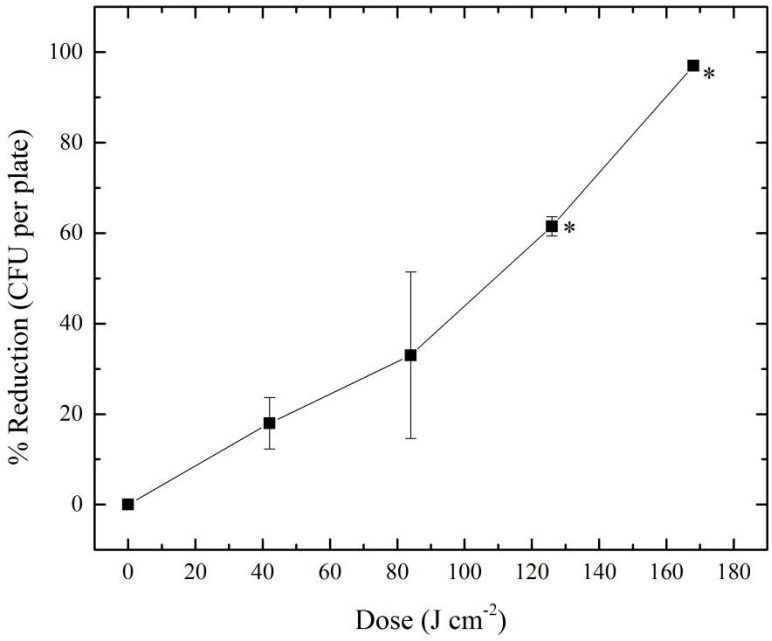

Figure 4.11 *Inactivation of S. enteritidis, on nutrient agar surfaces upon exposure to high irradiance (70 mW cm^{-2}) 405 nm light, expressed as a function of dose. * represent significant bacterial inactivation when compared to the associated non-exposed control samples (P≤ 0.05).*

4.3 Inactivation of bacteria-seeded agar using low irradiance 405 nm light.

Following successful inactivation of bacteria in suspension and when seeded onto agar surfaces using high intensity 405 nm light exposure it was of great interest to determine the efficacy of low irradiance 405 nm light (using the HINS-light EDS) for inactivation of foodborne pathogens. In particular, inactivation of low bacterial populations, which are more representative of natural environmental surface contamination levels was investigated. Also, previous work has assessed the use of high irradiance 405 nm light over relatively short exposure periods. However, for environmental applications it was critical to assess the efficacy of longer continuous exposures, using low irradiance light, in order to evaluate the potential use of this technology for environmental decontamination applications within the food industry.

4.3.1 Bacterial Preparation and Low Irradiance Treatment Method

Bacterial suspensions were prepared and deposited onto agar as described in Section 4.2.2. For low irradiance exposures, the HINS-light Environmental Decontamination System (EDS; Source D) was used. Prior to each experiment, irradiance measurements of both the blue light alone and of the combined blue and white LEDs were taken. The irradiance for blue light alone was found to be ~0.5 mW cm^{-2} (±0.03 mW cm^{-2}). Although slight variation was recorded across the grid, for the purpose of dose calculations, a standard irradiance of 0.5 mW cm^{-2} was used.

A gridded table, positioned directly below the EDS system, shown in Figure 4.12, represents the typical experimental set up. The table was divided into 35 square sections, allowing placement of multiple agar plates for simultaneous exposure over an extended time period. Agar plates were placed on a table, directly under the light source and exposed to increasing durations of 405 nm light (up to 11 hours), at a distance of ~1.5 meters from the light source. Experiments were conducted with plate lids removed, to allow direct exposure of bacterial on the agar surface to the 405 nm light. Control samples were set up simultaneously but received no 405 nm light exposure. Following treatment the samples were incubated overnight at 37°C and enumerated manually. Counts were recorded as CFU per plate.

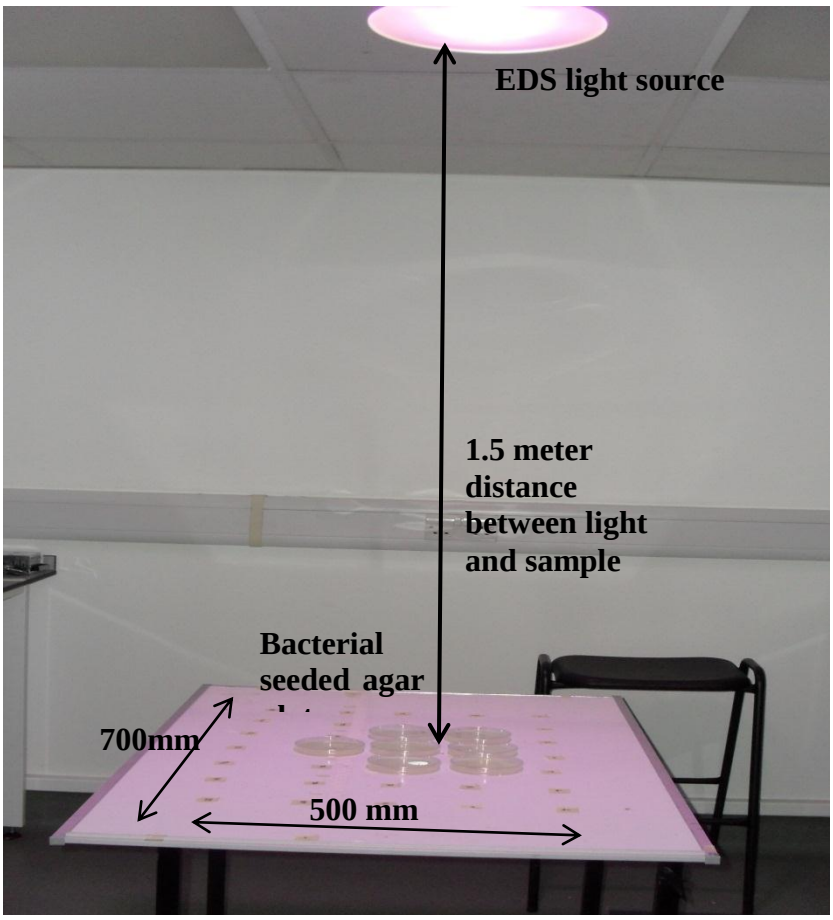

Figure 4.12 *Experimental set up for exposure of bacterial-seeded agar surfaces to the HINS-light EDS (~0.5 mW cm^{-2} 405 nm light).*

4.3.2 Results: Bacterial inactivation on agar using low irradiance 405 nm light

The following section details the inactivation results following exposure of surface-seeded bacteria to low irradiance 405 nm light. Results are expressed as % reduction to allow convient comparison between different organisms.

The results for inactivation of *S. aureus* on NA when exposed to low irradiance 405 nm light are shown in Figure 4.13. Significant inactivation was shown after 1 hour exposure, with a 51% reduction in bacterial count recorded. Treatment with increasing durations of 405 nm light continued to promote increasing inactivation with 82%, 92% and 95% reductions achieved following 2, 3 and 4 hours, respectively. Complete bacterial inactivation was achieved following 7 hours treatment.

Figure 4.14 highlights the inactivation kinetics of *L. monocytogenes* on tryptone soya agar by exposure to the EDS. Statistically significant inactivation was noted after 4 hours exposure and longer. After 5 hours expoure a 44% reduction was observed. A sharp decrease in bacterial population was observed following a 6 hour exposure period, with 88% reduction recorded. Near complete inactivation (97% reduction) was achieved after 7 hours exposure.

Figure 4.15 shows inactivation of *E. coli* seeded onto nutrient agar by exposure to low irradiance 405 nm light. As can be seen, significant inactivation (P= 0.012) was achieved following only 1 hour exposure, with each subsequent dose also displaying significant inactivation (P≤ 0.05). Steady inactivation was achieved between 1 and 3 hours, where a 20% reduction was observed in bacterial count. Following 4 hours exposure, a 48% reduction in bacterial count was observed, with a 64% reduction observed following 6 hours. Complete inactivation was achieved after 8 hours exposure.

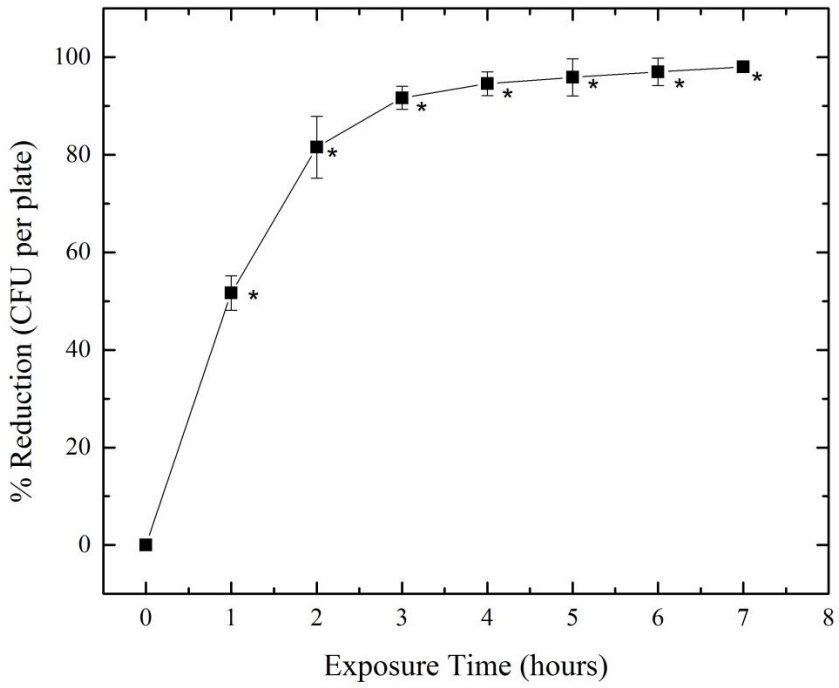

Figure 4.13 *Inactivation of S. aureus seeded on nutrient agar upon exposure to low irradiance 405 nm light (~0.5 mW cm^{-2}) using the HINS-light EDS (source D). * represent significant bacterial inactivation when compared to the associated non-exposed control samples (P≤ 0.05).*

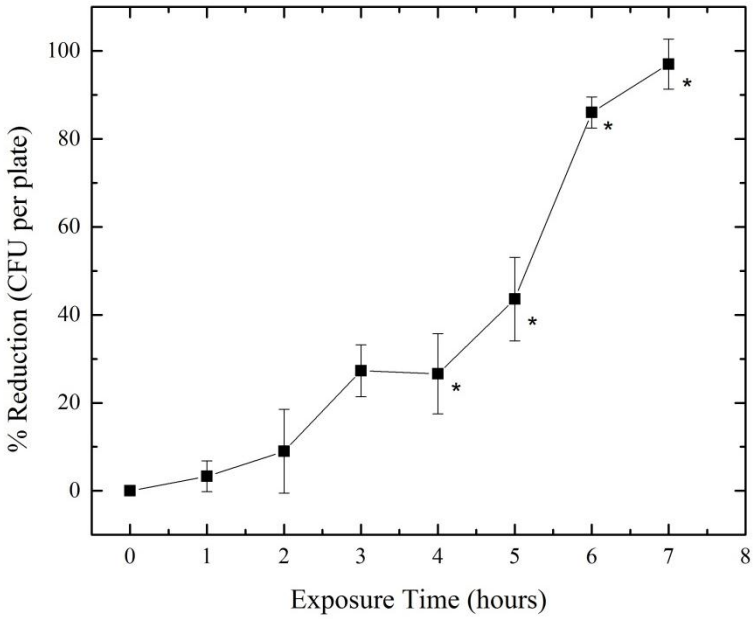

Figure 4.14 *Inactivation of L. monocytogenes seeded on tryptone soya agar agar upon exposure to low irradiance 405 nm light (~0.5 mW cm^{-2}) using the HINS-light EDS (source D). * represent significant bacterial inactivation when compared to the associated non-exposed control samples (P≤ 0.05).*

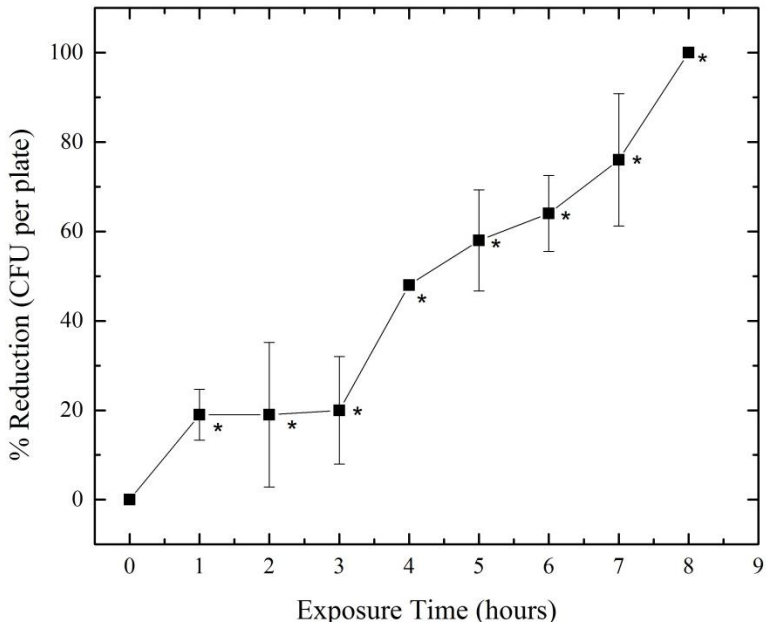

Figure 4.15 *Inactivation of E. coli seeded on nutrient agar upon exposure to low irradiance 405 nm light (~0.5 mW cm^{-2}) using the HINS-light EDS (source D). * represent significant bacterial inactivation when compared to the associated non-exposed control samples (P≤ 0.05).*

In the case of *S. sonnei* (Figure 4.16), significant inactivation was observed following 4 hours exposure, with a 24% decrease, followed by 60% inactivation after 5 hours. 94-100% inactivation was achieved following 7-8 hours exposure.

Inactivation of *S. entertidis* on agar surfaces is shown in Figure 4.17. Significant inactivation was achieved following 4 hours exposure, where a 28% reduction in bacterial count was achieved. Bacterial reduction was shown to continue at a relatively linear rate, with complete inactivation observed following 11 hours exposure. In order to achieve complete inactivation during EDS exposure experiments, *S. enteritidis* required the greatest exposure period of 405 nm light of all bacteria tested.

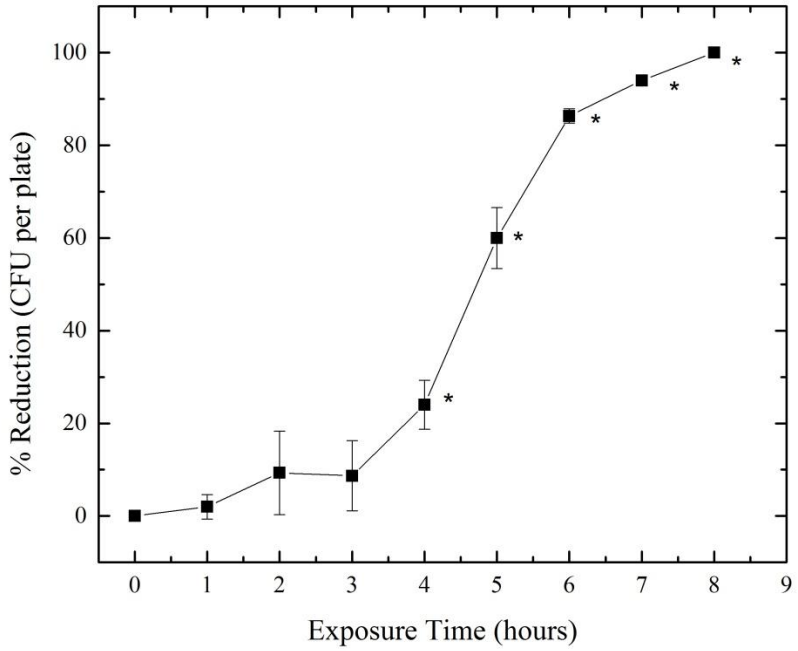

Figure 4.16 *Inactivation of S. sonnei seeded on nutrient agar upon exposure to low irradiance 405 nm light (~0.5 mW cm^{-2}) using the HINS-light EDS (source D). * represent significant bacterial inactivation when compared to the associated non-exposed control samples (P≤ 0.05).*

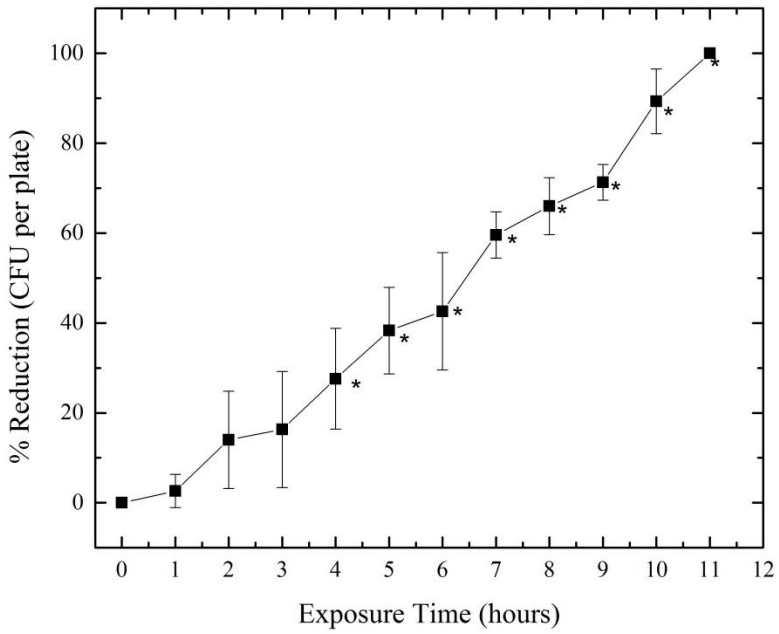

Figure 4.17 *Inactivation of S. entertidis seeded on nutrient agar upon exposure to low irradiance 405 nm light (~0.5 mW cm^{-2}) using the HINS-light EDS (source D). * represent significant bacterial inactivation when compared to the associated non-exposed control samples (P≤ 0.05).*

Data shown in Table 4.1, summerises the required doses necessary to promote an approximate 50% and 100% reduction for all species investigated. Results in Table 4.1 highlights differences in the doses and times required to induce a 50% reduction in the bacterial species. For complete/near complete inactivation, there was very little variance between the doses/times required for effective kill of all organisms, with an applied dose of between 12.6 and 14.4 J cm^{-2}, sufficient to generate ~100% reduction in bacterial count, with the exception of *S. enteritidis*. All applied doses were considerably less than that required to inactivate the same population density exposed to high irradiance 405 nm light detailed in Section 4.2.4, where the dose required was between 31 and 168 J cm^{-2}, depending on the specific bacteria.

Table 4.1 *Doses (J cm^{-2}) required to induce bacterial inactivation on agar surfaces following extended periods of low irradiance 405 nm light exposure (0.5 mW cm^{-2}) from the HINS-light EDS.*

	~50% Reduction		**~100% Reduction**	
	Time (h)	**Dose (J cm^{-2})**	**Time (h)**	**Dose (J cm^{-2})**
S. aureus	1	1.8	7	12.6
L. monocytogenes	4	7.2	7	12.6
E. coli	4	7.2	8	14.4
S. sonnei	5	9.0	8	14.4
S. enteritidis	7	12.6	11	19.8

Due to the long exposure times used in these experiments, and the fact that the seeded plates were exposed without their lids, there was the possibility that the bacterial populations may have been stressed due to potential desiccation of the agar surfaces over the exposure period. To first investigate this, weight measurements of agar plates were taken pre- and post- exposure that showed a 13% decrease in the weight of 'lid off' samples, compared to a 0% decrease in those where the lid remained in place. This suggested that a desiccation effect may be occurring. Following on from this, the inactivation experiment using *S. aureus* was repeated, with the transparent petri dish lids left on the agar plates during exposure. Figure 4.18 highlights the transmission spectra through the plastic petri lids, demonstrating that 405 nm light can successfully transmit through this material. However, an ~28% loss in transmission occurs. To ensure that the same irradiance of light was used for sample treatment, the distance between the light and the table was reduced to ~ 1.1 meter to maintain the irradiance transmitting through the plastic lid remained at ~0.5 mW cm^{-2}. Results enabled comparison of the inactivation rates of surface-seeded *S. aureus* exposed with 'lid on' and 'lid off', in order to determine whether bacteria were more susceptible when exposed directly, likely enhanced due to the added desiccation stress on the organism.

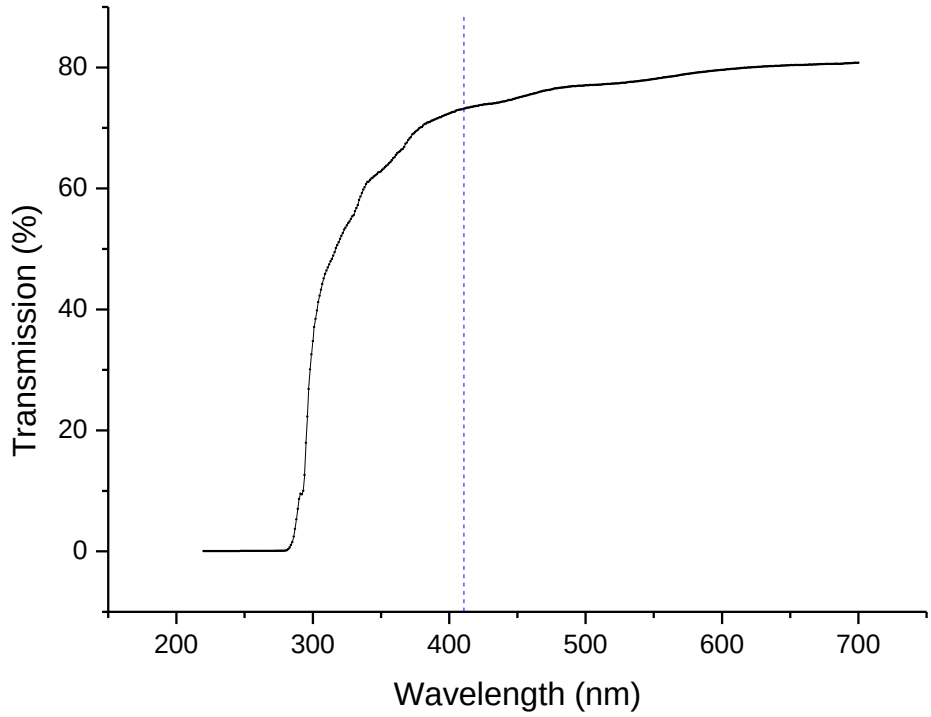

Figure 4.18 *Transmission spectra through plastic petri dish lid, with transmission highlighted at 405 nm.*

Results in Figure 4.19 shows inactivation of *S. aureus* when exposed to low irradiance 405 nm light, with and without plastic lids. Although results, in general, show a similar trend, with complete inactivation achieved following 7 hours exposure in both cases, initial inactivation is shown to be significantly greater for bacterial contamination directly exposed to the light. Following 1 hour exposure, a 50% reduction was observed in ’lid off’ tests compared to an approximate 30% for ‘lid on’ tests. Similarly, 80% and 50% reductions were observed after 2 hours with ‘lid off’ and ‘lid on’ samples, respectively. Significantly enhanced kill was also recorded after 3 hours. However following 4, 5, 6 and 7 hours exposure similar levels of inactivation were recorded for both sets of tests, with no significant differences recorded.

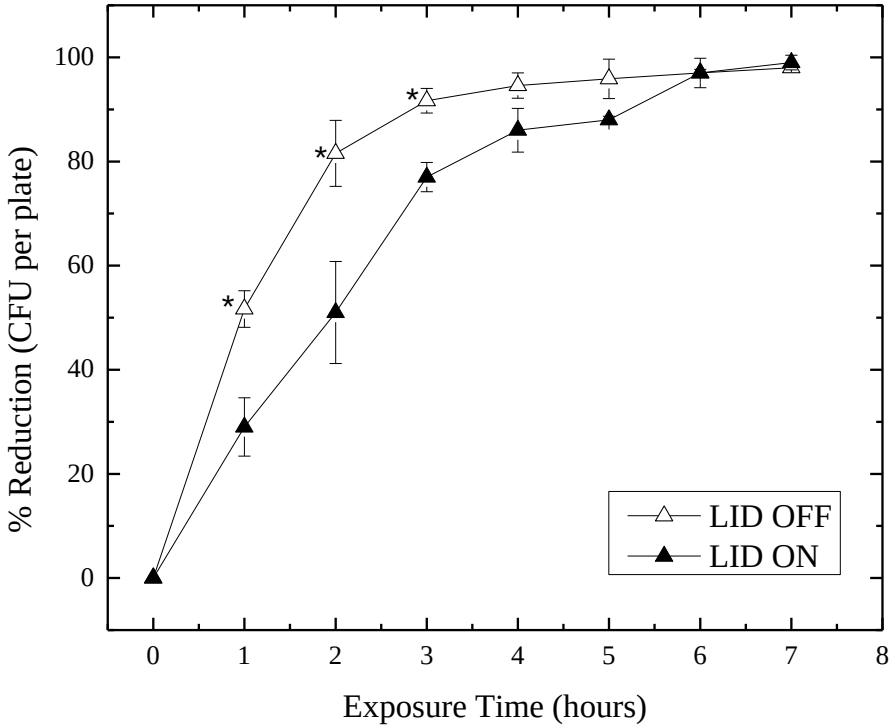

Figure 4.19 *Inactivation of S. aureus seeded on nutrient agar surfaces upon exposure to low irradiance (~0.5 mW cm^{-2}) 405 nm light, using the HINS-light EDS (Source D). Bacteria were exposed with plastic lids on and off. * represent significant bacterial inactivation of lid off tests when compared to corresponding lid on tests (P≤ 0.05).*

4.4 Discussion

Results detailed in this chapter have successfully demonstrated inactivation of a range of food-related bacterial pathogens in both liquid suspension and on seeded agar surfaces by 405 nm light exposure.

The most susceptible bacteria tested in liquid suspension was shown to be *L. monocytogenes,* which demonstrated complete inactivation of a 5 $\log_{10}$ population following a dose of 39 J cm^{-2}. This was followed by *S. aureus,* which was completely inactivated after a treatment with 109 J cm^{-2}. *E. coli* and *S. enteritidis* could be successfully inactivated but were found to be much less susceptible, with similar reductions requiring doses of up to 436 J cm^{-2} - approximately 11 and 4 times more than the doses required for inactivation of *L. monocytogenes* and *S. aureus*, respectively.

Investigation into the inactivation of low population densities of *S. aureus* and *E. coli* also demonstrated significant inactivation, with *S. aureus* again displaying far greater susceptibility than the equivalent population density of *E. coli*. It is important to note that previous studies have demonstrated successful inactivation of bacteria at much greater population densities than those demonstrated in this current work. A study by Maclean et al., (2009) presented significant inactivation of *S. aureus*, with 7 and 9 $\log_{10}$ reductions shown following doses of approximately 180 and 220 J cm^{-2}, respectively. It is interesting to note that a 9 $\log_{10}$ reduction of *S. aureus* required approximately half the dose necessary to promote a 5 $\log_{10}$ reduction of *E. coli* in the current study.

The inactivation kinetics shown in Figures 4.2, 4.3 and 4.4 are consistent with previous studies, with an initial phase of low bactericidal activity followed by rapid inactivation of the population. These kinetics suggest there may require to be a build-up of reactive oxygen species within the organism before lethal cell damage occurs. (Maclean et al., 2009; Maclean et al., 2008).

The results from the present study demonstrate an obvious variation in susceptibility to 405 nm light between Gram positive and Gram negative bacteria, with Gram positive species proving to be more susceptible to 405 nm light treatment. This data correlates well with previous work by Murdoch et al. (2012 and 2010) and Maclean et al. (2009) where in general, Gram positive bacteria proved to be more sensitive to 405 nm exposure. However a notable exception to this trend has been observed with the Gram negative microaerophilic organism *Campylobacter jejuni*, which displayed extremely high levels of susceptibility during 405 nm light exposure (Murdoch et al., 2010), accredited to its requirement for reduced environmental oxygen.

Differences in susceptibilities are not yet fully understood, but it has been theorised that organism-specific differences in porphyrins, with respect to different types and/or concentrations of porphyrins within the cells, may influence susceptibility. A study by Nitzan et al. (2004) previously demonstrated that the presence of intracellular coproporphyrin could be responsible for increased inactivation in Gram positive bacteria. The levels of coproporyphrin in Gram positive bacteria were between two and three times that found in Gram negative bacteria suggesting that greater susceptibility may be a direct result of the presence of coproporphyrin. A number of other studies

have identified varying types of porphyrins present amongst different bacterial species (Dai et al., 2013; Buchovec et al., 2010; Hamblin et al., 2005). However, these studies have simply identified specific porphyrin types, without quantifying exact porphyrin levels within the cells, nor have they analysed or concluded a relationship between porphyrin type, its presence within Gram positive and negative cells, and its subsequent sensitivity to 405 nm light treatment.

It has also been suggested that the physiological differences between the two cell types may account for the variation in susceptibility. A number of PDI studies utilising exogenous photosensitizing molecules have suggested that morphological differences in the membrane structure may promote differences in susceptibility (Malik et al., 1992). Demidova and Hamblin. (2005) also suggested that variations in inactivation levels may be accredited to cellular structure, where penetration of light through more structurally complex cells is reduced. This theory relates well to results achieved throughout this chapter, whereby inactivation of Gram positive bacteria is achieved at a much greater rate when compared to more structurally complex Gram negative species.

As mentioned in Chapter 2, the mechanism of violet-blue light inactivation is thought to involve non-specific destruction of the cellular target by oxidative damage. However, as previously discussed, recent work by Dai et al. (2013b) visually demonstrated membrane damage of *S. aureus*. Results shown in Section 4.1.4 and 4.1.5 demonstrate that 405 nm light can successfully induce both lethal and sub-lethal damage. These results support the visual data provided by Dai and colleagues (2013b) where loss of salt tolerance by *S. aureus* is indicative of structural damage, more specifically membrane damage (Carson et al., 2002). Sub-lethal injury of microbial cell membranes may alter their permeability and affect their ability to osmoregulate the cell sufficiently and/or to expel toxic materials- in this case ROS (Gibson 1984). It is therefore likely that 405 nm light can successfully induce membrane damage, which first promotes sub-lethal effects, most likely leakage of cellular components, followed by complete lysis of the cell, resulting in cell death. It is possible that simultaneous internal damage is occurring during light exposure, but to date this has been overlooked due to investigation into structural integrityof the cell.

Generally, the results from this study correlate well with previous photoinactivation studies. A recent study by Dai et al. (2012) investigated inactivation of (MRSA) following treatment with 415 nm blue light. Results demonstrated that ~5 $\log_{10}$ reduction was observed after exposure to a dose of 115 J cm^{-2}. These results were almost identical to the results shown in Figure 4.2, where 5 $\log_{10}$ reduction of methicillin sensitive *S. aureus* (MSSA) was achieved following a dose of 108 J cm^{-2}. Similar levels of susceptibility between MSSA and MRSA have also been previously demonstrated by Maclean and colleagues (2009). The current problems associated with resistant bacterial strains means there is a continuous challenge for development of effective antimicrobial treatments and is therefore extremely important to determine whether or not resistant strains are equally susceptible. The results from both studies indicate comparable levels of inactivation, suggesting antibiotic resistant strains are similarly susceptible to violet-blue light treatment.

A recent study by Murdoch et al. (2012) investigated the 405 nm light susceptibility of a range of bacteria, using an irradiance of ~10 mW cm^{-2}. The study demonstrated a 5 $\log_{10}$ reduction of *L. monocytogenes* following an applied dose of 108 J cm^{-2}. This dose was more than double that required in this current study. In addition, Murdoch reported that complete inactivation of *E. coli* (10^5 population) was achieved following ~300 J cm^{-2}, a much lesser dose when compared with that applied in this current study. Although variations are shown to occur between the two studies, there are a number of factors that may account for this. Both the light system and the irradiance levels utilised were different between the two studies, therefore this may suggest that inactivation is not only dose dependent, but also depends on the way in which dose is applied (i.e, high irradiance for short exposure times vs low irradiance for longer times). This is discussed later in the chapter. Another possible reason that may account for the variations in inactivation is the differences in peak wavelengths. Whilst Murdoch utilised a source that produced a peak at 400 nm, the source in this current work displayed a peak at 408 nm. Consequently this may indicate differences in the absorption peaks of different bacteria.

In addition to demonstrating the susceptibility of foodborne pathogens in liquid suspension, a major focus of this chapter was investigating the efficacy of 405 nm light for inactivation of low population densities seeded on to surfaces, using agar plates as

the test surface. Although reductions of >5 $\log_{10}$ are typically viewed as the standard benchmark for effective decontamination (Schmidt 2012) – and indeed this level of antimicrobial activity was successfully demonstrated with the suspension experiments – surface experiments were conducted utilising low population densities (10^2 CFU per plate) in order to mimic the typical levels of bacterial contamination commonly found on environmental surfaces (Maclean et al., 2010).

High irradiance 405 nm light treatment of contaminated surfaces demonstrated significant inactivation of *L. monocytogenes, S. aureus, E. coli, S. enteritidis and S. sonnei*, with complete kill achieved using doses between 31 and 168 J cm^{-2}, depending on the organism. Again, the Gram negative species displayed increased tolerance to the 405 nm light. Interestingly, when comparing the doses with those required for inactivation of similar populations in suspension, significant differences were observed, with a greater dose required for inactivation in suspension: ~54 vs. 31 J cm^{-2} for *S. aureus* and ~218 vs. 126 J cm^{-2} for *E. coli*. This is likely due to direct exposure on surfaces, whilst in suspension, penetration of light through a 3 mL sample is required.

Conversely, it is interesting to note that, when using high irradiance light, a 2 $\log_{10}$ reduction of *L. monocytogenes* on nutrient agar required more than double the dose necessary for a 5 $\log_{10}$ reduction in PBS suspension. A possible explanation for this may be the availability of nutrients present in the exposed agar (which are not present in PBS). These may aid survival during exposure but it is not yet clear why this is the case for only for *L. monocytogenes*. A possible explanation may be due to the presence of Sigma B, (σ^B), a general stress response sigma factor that has been identified in *L. monocytogenes*, which has been shown to help cell survival under stressful environmental conditions. It may be that exposure on a surface (stressful conditions) may enhance regulation of sigma B and consequently help protect against damage. The role of sigma B in regards to protection of *L. monocytogenes* will be discussed further in Chapter 7.

Previous studies demonstrating the bactericidal effects of 405 nm light have typically involved exposure of high bacterial population densities in PBS, or low population densities seeded onto surfaces, using high irradiance light. Therefore it was of great interest to expand this knowledge and investigate the bactericidal efficacy of low

irradiance 405 nm light on low bacterial population densities, typical of the levels associated with environmental contamination. Previous studies by Maclean et al. (2010; 2013) and Bache et al. (2012) investigated the general disinfection efficacy of low irradiance 405 nm light *in situ* within the clinical environment, with results demonstrating up to 90% reduction in bacterial contamination in unoccupied rooms and up to 86% when the room was occupied by an MRSA patient. However, despite these successful clinical results there is currently no published quantitative laboratory data demonstrating the efficacy of low irradiance 405 nm light (<1 mW cm^{-2}) against specific bacterial species. This data is critical for providing a greater understanding of the exposure levels required to promote effective environmental decontamination.

Work in this chapter which utilised the HINS-light EDS to expose bacterial contamination seeded onto agar surfaces to ~0.5 mW cm^{-2} light demonstrated successful inactivationof all the organisms tested. *S. aureus* was shown to be most susceptible to low irradiance 405 nm light, whereby a 90% reduction was achieved following only 3 hours exposure. Figures 4.14 and 4.15 show complete inactivation of Gram positive *S. aureus* and *L. monocytogenes* following 7 hours exposure, whilst Gram negative bacteria were shown to require greater exposure with *E. coli* and *S. sonnei* requiring 8 hours and *S. enteritidis* requiring greatest exposure of all bacteria tested in order to achieve complete inactivation (11 hours). Overall, both Gram positive and Gram negative bacteria displayed much more similar inactivation kinetics when exposed to low irradiance 405 nm light, when compared to studies investigating high irradiance exposure, such as those detailed in Sections 4.1.4 and 4.2.4. For example, inactivation of bacteria using low irradiance 405 nm light required between 12.6-19.8 J cm^{-2}, whilst for high irradiance treatment doses ranged from 39-436 J cm^{-2}, depending on the species. The similarity in inactivation kinetics displayed by both Gram positive and Gram negative species when exposed to low irradiance light is advantageous with respect to general environmental disinfection applications, as the exposure time should induce uniform decontamination regardless of the mixed bio-burden present in the environment – an effect that was seen in the studies demonstrating the use of low irradiance light for disinfection of hospital isolation rooms (Maclean et al., 2013).

A major difference between the results obtained in the low irradiance study compared to high irradiance inactivation results, both from this study and previously published work,

is the doses of 405 nm light required for bacterial inactivation. It was extremely interesting that the dose required to promote a 2 log_{10} reduction on surfaces, using low irradiance light, was significantly less than all previous high irradiance studies. For organisms exposed to low irradiance 405 nm light, significant population reductions were achieved following 1.8- .2 J cm^{-2} (1-4 hours at 0.5 mW cm^{-2}), and 2 log_{10} reductions were typically achieved by 12.6-19.8 J cm^{-2} (7-11 hours). In comparison, the results detailed earlier in the chapter showed that an equivalent 2 log_{10} population exposed to high irradiance light on agar surfaces required significantly greater doses of 405 nm light. A comparison of this is shown in Figures 4.20 and 4.21 for *S. aureus* and *E. coli*, respectively. For *S. aureus,* complete inactivation using the EDS system (0.5 mW cm^{-2}) was achieved following only 12.6 J cm^{-2}, 60% less than the 31.5 J cm^{-2} dose required when using high irradiance light (70 mW cm^{-2}). This is even more pronounced in the case of *E. coli*, with 89% less dose required for inactivation when using low irradiance light.

Previous studies involving liquid suspensions indicated the dose-dependent nature of the inactivation reaction (Murdoch et al., 2012; Endarko et al., 2012). However, current results suggest there may be a more complex reaction at work. This was partly demonstrated by the work of Endarko et al. (2012), which showed significant differences in inactivation of *L. monocytogenes* when using irradiance levels of 8.6 mW cm^{-2} and 85.6 mW cm^{-2}. It is perhaps the case that the reactions are, to an extent, dose-dependent when irradiance levels are in the same order of magnitude. However, when there are substantial differences in the irradiance levels being applied, dose dependence is no longer applicable, possibly due to the differences in microbial responses to short bursts of high irradiance light, compared to longer but less intense periods of exposure. It may also be possible that, when exposed to high bursts of irradiation, only a critical amount of light photons can be absorbed by the bacterial porphyrins and the rest is essentially wasted energy.

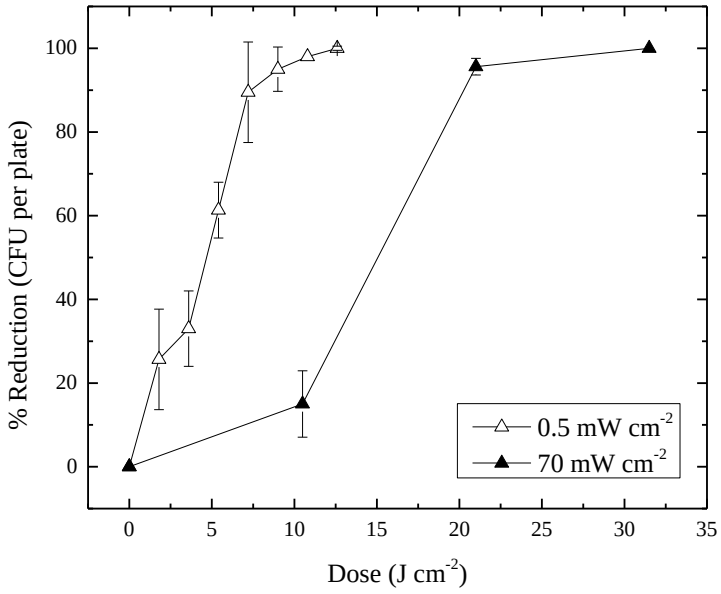

Figure 4.20 *Comparison of the inactivation kinetics of S. aureus on agar surfaces using low versus high irradiance 405 nm light. The low and high irradiances levels used were 0.5 and 70 mW cm^{-2}, respectively.*

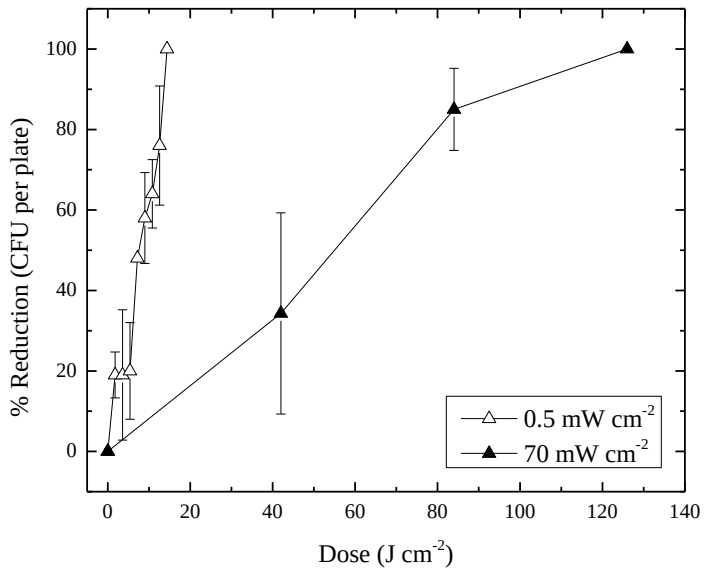

Figure 4.21 *Comparison of the inactivation kinetics of E. coli on agar surfaces using low versus high irradiance 405 nm light. The low and high irradiances levels used were 0.5 and 70 mW cm^{-2}, respectively.*

To help indicate if any environmental issues were contributing to this phenomenon, it was important to investigate whether prolonged exposure of bacteria on surfaces during low irradiance experiments was influenced by any desiccation effect that may have aided inactivation and thus partly explain why significantly lower doses were required for kill when compared to those under high irradiance light. Comparison of the inactivation kinetics of bacteria exposed directly or in closed plates demonstrated a significantly enhanced rate of inactivation, although the doses required for complete inactivation were similar. Therefore, although dessication appears to be a contributing factor, there appears to be other factors influencing this difference in dose requirements. Further work is required elucidate the mechanism and understand the differences in inactivation rates.

Overall, the work of this chapter has demonstrated the susceptibility of a number of significant foodborne pathogens to 405 nm light inactivation. Successful inactivation was achieved using high irradiance light for decontamination of bacteria in suspensions and on surfaces. Also, importantly, inactivation of these pathogens was achieved using low irradiance (~0.5 mW cm^{-2}) light. This is very significant as this low irradiance, and the light system used, have proved effective for environmental decontamination applications within healthcare facilities (Maclean et al., 2013b; Bache et al., 2012; Maclean et al., 2010). Evidence of the efficacy of both low and high irradiance 405 nm light against foodborne pathogens highlights the potential of this system for both environmental decontamination and other disinfection applications within the food industry. However, in order to fully establish the potential of 405 nm light as a decontamination technology, further microbial studies must be conducted to demonstrate its full antimicrobial effects and properties. A range of these important factors will be investigated in the subsequent chapters.

Chapter 5

INACTIVATION OF FUNGI USING 405 NM LIGHT

5.0 General

Work in Chapter 4 demonstrated susceptibility of a range of food-related bacterial pathogens to 405 nm light. This chapter progresses to investigate the effects of 405 nm light on a range of spoilage and disease-causing fungi, including *Saccharomyces cerevisiae*, *Candida albicans* and spore forming *Aspergillus niger*. An overview of each species is provided below, with microscopy images shown in Figure 5.1.

Sacchromyces cerevisiae is a dimorphic yeast known commonly as brewers or bakers yeast and is routinely used throughout the food industry for the brewing of alcohol and production of breads. Although ubiquitous in nature, *S. cerevisiae* has the ability toinduce undesirable spoilage effects, typically on sugar rich foods such as concentrated juices and condiments. Ingestion of *S. cerevisiae* can cause serious infections, particularly in immunocompromised individuals, and can be a major cause of invasive fungal infections (Lund and O'Brien 2011; Enache-Angoulvant and Hennequin 2005).

Candida albicans is a polymorphic yeast, with the ability to switch between budding cells and filamentous cells. It is considered one of the most prevalent yeasts associated with human infection, able to induce a spectrum of conditions, ranging from mild oral candidiasis, thrush and vaginitis, to potentially life threating infections (Cormick et al., 2009; Mean et al., 2008). Entry into the human body is largely gained from indwelling medical devices; however, environmental colonisation on surfaces can generate biofilm production providing potential sources of contamination (Bandara and Weerasekara 2008).

Aspergillus niger is a spore-forming filamentous fungi used in fermentation for production of citric acid and extracellular enzymes, such as amylases, proteases and lipases (Schuster et al., 2002; Roukas 2000). This fungus is also commonly found in the environment and frequently causes food spoilage. *A. niger* infections are rare and are

generally associated with those of suppressed or lowered immunity (Schuster et al., 2002).

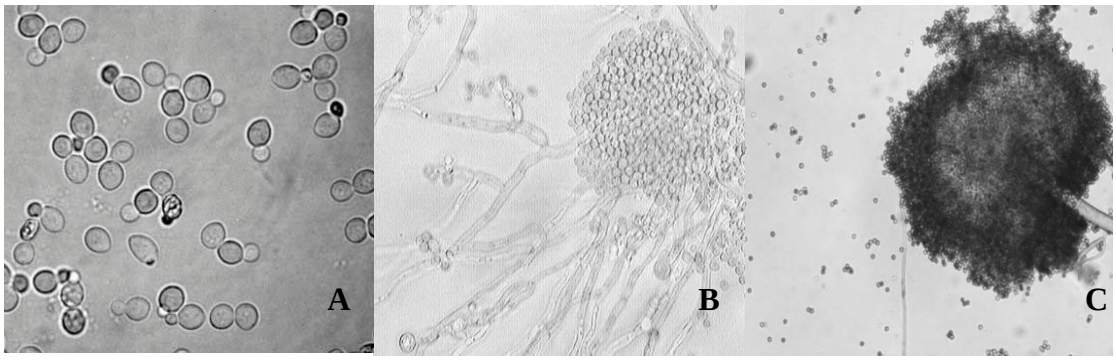

Figure 5.1 *Microscopic visualisation of (A) S. cerevisiae, (B) C. albicans and (C) A. niger by wet mount light microscopy, highlighting the morphologies of each of the cell types.*

The presence of fungi in the food industry is a major concern with respect to both spoilage and infection. This section of work was split into two sections;

- The first investigating the inactivation of the yeasts *S.cerevisiae* and *C. albicans* under aerobic and anaerobic conditions as well as investigating the role of oxygen during 405 nm light photoinactivation, and
- The second investigating the inactivation of *A. niger* spores under aerobic and anaerobic conditions and their enhanced susceptibility to 405 nm light during germination, when compared to dormant spores.

5.1 Investigation into the Oxygen-Dependence of 405 nm light Yeast Inactivation

As discussed in Chapter 4, evidence of the inactivation mechanism of 405 nm light highlights the prominent role of endogenous porphyrin molecules, with inactivation being widely accepted as an oxygen-dependent process (Maclean et al., 2008a). Work within this study, conducted in collaboration with Murdoch, aimed to demonstrate the requirement of oxygen for 405 nm light inactivation of *S. cerevisiae* and *C. albicans,* to further elucidate the mechanism of inactivation of 405 nm light. To assess the requirement of oxygen for 405 nm light inactivation of yeasts, exposure experiments,

comparative to those by Murdoch (2010), were conducted under oxygen depleted conditions, through the use of both reactive oxygen species scavengers (ROS) and anaerobic environmental conditions.

5.1.1 Previous investigation of 405 nm light for yeast inactivation

Previous work by Murdoch (2010) demonstrated the susceptibility of *S.cerevisiae* and *C. albicans* to 405 nm light exposure, as shown by Figure 5.2. As can be seen from graph (a), inactivation of *S. cerevisiae* occurs at a substantially faster rate than when compared to *C. albicans* (graph b), with a significant 1 $\log_{10}$ reduction achieved following an applied dose of only 36 J cm^{-2}. Following exposure to 72 J cm^{-2}, a 1.77 $\log_{10}$ reduction was observed, with complete inactivation (5.1 $\log_{10}$) achieved following 288 J cm^{-2}.

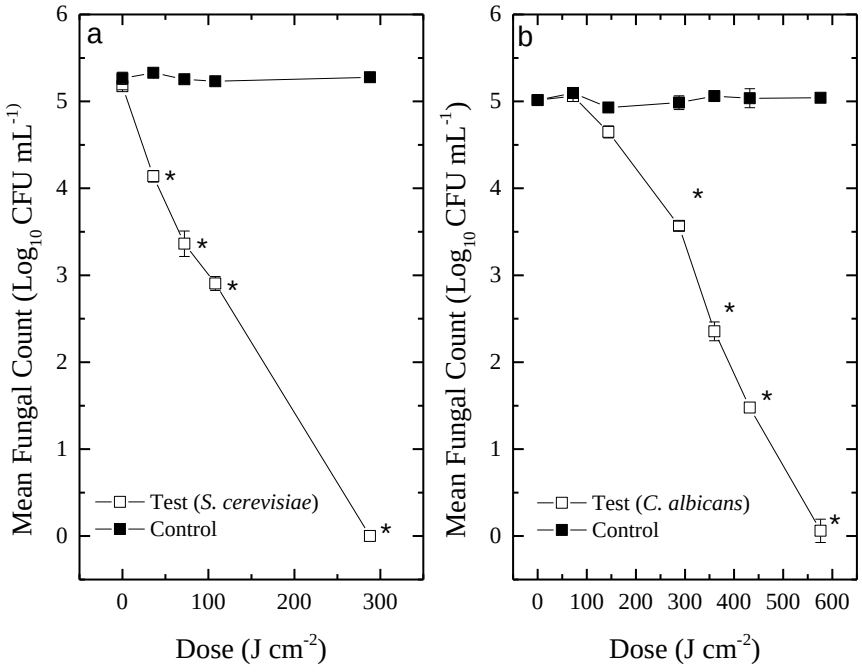

Figure 5.2 *Inactivation of S. cerevisiae (a) and C. albicans (b) in liquid suspension by exposure to 405 nm light at an irradiance of ~40 mW cm^{-2} under aerobic conditions.* represent statistically significant differences between exposed and non-exposed samples. Adapted from Murdoch (2010).*

C. albicans demonstrated significantly less susceptibility, with only a 1.5 $\log_{10}$ reduction observed following a dose of 288 J cm^{-2}. Complete inactivation was achieved following 576 J cm^{-2} of 405 nm light exposure. No significant change was observed in the control samples for both *S. cerevisiae* and *C. albicans*.

These inactivation kinetics, generated by Murdoch (2010), provided the baseline data for subsequent experiments to investigate the 405 nm light yeast inactivation mechanism carried out as part of the research presented here.

5.1.2 Yeast preparation

S. cerivisae and *C. albicans* were prepared as described Section 3.1.2. Broths were centrifuged and re-suspended in PBS as described in Section 3.4.1. The resultant cell densities were recorded as 10^7 and 10^8 CFU mL^{-1} for *S. cerivisae* and *C. albicans*, respectively. Yeast suspensions were serially diluted to give approximate starting populations of 2.0×10^5 CFU mL^{-1} for experimental purposes.

5.1.3 Treatment method

Three millilitre volumes of yeast suspension were pipetted into a central well of a 12-well multi-dish, giving a liquid depth of ~10 mm. The sample was agitated during exposure by adding a micro-magnetic stirrer and placing the sample dish onto a magnetic stirring plate. The 405 nm 99 DIE LED array (Source C) was used for all fungal tests, as described in Section 3.3.3. The irradiance from the array was measured to be 40 mW cm^{-2} at the surface sample (2 cm distance). Test samples were exposed to increasing doses of 405 nm light to determine inactivation kinetics. Control samples were set up which were identically treated but received no 405 nm light illumination. Post exposure, samples were plated and incubated as detailed in Chapter 3, Table 3.2. Results were enumerated and recorded as CFU mL^{-1}.

5.1.4 Oxygen dependence experiments

Oxygen dependence experiments were split into three sections, where light exposure of yeast samples was applied under various conditions as described below:

1. Exposed within an anaerobic atmospheric cabinet: Samples exposed to 405 nm light in an anaerobic cabinet were first prepared under anaerobic conditions using PBS

that had been exposed to reduced conditions in the anaerobic environment for at least 1 hour prior to 405 nm light exposure. Following preparation, samples were then exposed to 405 nm light as described in Section 5.1.3 whilst held in an anaerobic cabinet. Controls were also held under anaerobic conditions.

2. Exposed within an anaerobic cabinet + ROS scavenger: Oxygen levels within the anaerobic cabinet were further depleted by adding ascorbic acid (Sigma Aldrich, UK) into the yeast suspensions to act as an oxygen scavenger. Ascorbic acid was selected as the reactive oxygen species scavenger (ROS scavenger) for use in these experiments due to its previous use by Maclean et al (2008a) in studies investigating the role of oxygen in bacterial inactivation by 405 nm light exposure. Yeast suspensions were prepared and diluted as described in Section 3.4.1, to produce 10^6 CFU mL^{-1}; 1 mL was then transferred into a 9 mL volume of PBS containing 30 mM concentration of ascorbic acid, to give the required 10^5 CFU mL^{-1} starting population. Samples were then exposed in an anaerobic cabinet, as described in Section 5.1.3. Control samples (yeast suspended in 30 mM ascorbic acid and held under anaerobic conditions) were also set up but not exposed to 405 nm light.

3. Exposed aerobically on bench + ROS scavenger: The effects of ascorbic acid alone, as a reactive oxygen species scavenger, under aerobic conditions was also investigated. For this, yeasts were prepared in 30 mM ascorbic acid as previously described and exposed to 405 nm light under aerobic conditions (on the laboratory benchtop). Control samples (yeast suspended in 30 mM ascorbic acid and held on the laboratory bench) were also set up but not exposed to 405 nm light.

All test samples were exposed for the maximum dose required to achieve complete inactivation under aerobic conditions (288 J cm^{-2} for *S. cerevisiae* and 576 J cm^{-2} for *C. albicans*), as determined by Murdoch (2010), shown in Figure 5.2.

5.1.5 Results: Oxygen dependence of yeast inactivation during 405 nm light exposure

Table 5.1 details inactivation results of *S. cerevisiae* and *C. albicans*, under both aerobic and anaerobic conditions when exposed to 405 nm light, with and without ROS scavengers.

Results demonstrated reduced inactivation of *C. albicans* under anaerobic conditions, with only a 1.8 $\log_{10}$ reduction observed, compared to ~5 $\log_{10}$ achieved under aerobic conditions. However inactivation of *S. cerevisiae* did not appear to be as oxygen dependent, with a 4.4 $\log_{10}$ reduction achieved when exposed in the anaerobic environment, compared to 5.2 $\log_{10}$ reduction recorded during aerobic exposures. Although less prominent for *S. cerevisiae*, statistical analysis confirmed significant differences in the inactivation rates of both yeasts when exposed aerobically and anaerobically.

In order to further examine oxygen dependence, ascorbic acid (a scavenger of ROS) was added to the yeast suspensions prior to 405 nm light exposure in the anaerobic cabinet. Addition of the scavenger provided an additional measure to minimise the residual dissolved oxygen in samples. Results exposing scavenger-supplemented suspensions to 405 nm light under anaerobic conditions are shown in Table 5.1. Results show ascorbic acid successfully inhibited inactivation of *C. albicans*, with only a 0.03 $\log_{10}$ reduction observed following 288 J cm^{-2} during anaerobic exposure of 405 nm light. The presence of ascorbic acid also reduced inactivation of *S. cerevisiae*, with only a 2 $\log_{10}$ reduction observed under scavenger-supplemented anaerobic conditions, compared to a 4.3 $\log_{10}$ reduction when anaerobically exposed in the absence of a scavenger.

In order to assess the effect of the ROS scavenger alone on the 405 nm light inactivation mechanism, exposures of yeast suspensions supplemented with ascorbic acid under aerobic conditions were also conducted. Results (Table 5.1) demonstrate that under aerobic conditions, inactivation of both *S. cerevisiae* and *C. albicans* is significantly reduced (P=0.01 and P=0.00, respectively) when the suspending media is supplemented with ascorbic acid, compared to results obtained for standard aerobic conditions, where 2.7 $\log_{10}$ and 3.2 $\log_{10}$ reductions were recorded for *S. cerevisiae* and *C. albicans*, respectively.

Overall, although differences between the organisms are observed, results demonstrate that for both yeast species, greatest inactivation was achieved when exposed under aerobic conditions in the absence of scavengers. Anaerobic exposure within the cabinet and aerobic exposure with a scavenger provided varying results, with reduction in inactivation rates appearing to be dependent on the specific organism.

Table 5.1. *Mean population reductions of fungal test species after exposure to 405 nm light under aerobic and anaerobic conditions. Doses used for exposure were those required to achieve a 5 log_{10} reduction of each organism under aerobic conditions: 288 J cm^{-2} for S. cerevisiae and 576 J cm^{-2} for C. albicans.*

Microorganisms	Mean Reduction in Microbial Numbers (Log_{10} CFU mL^{-1})			
	Light-exposed		Light-exposed + Scavenger	
	*Aerobic	**Anaerobic	*Aerobic	**Anaerobic
C. albicans	5.02 (±0.1)	1.76 (±0.3)	3.25 (±1.6)	0.03 (±0.0)
S. cerevisiae	5.18 (±0.1)	4.37 (±0.8)	2.72 (±0.3)	2.03 (±0.1)

* yeast exposed on benchtop, ** yeast exposed within anaerobic cabinet

5.2 405 nm light Inactivation on Dormant and Germinating *A. niger* Spores

The presence of spores, or conidia, in the food industry is a major concern primarily because of their common presence as aerial contaminants and their resistant structure. Conidia are specialised structures responsible for extreme environmental tolerance. Their persistence within the environment, ability to colonize various crops and their resilient nature against numerous fungicides have led to growing interest in the use of light-based inactivation technologies in order to control and inactivate fungi in the environment. The following sections describe investigations into the susceptibility of both dormant and germinating *A. niger* spores to 405 nm light.

5.2.1 Previous investigation of 405 nm light inactivation of dormant *A. niger* spores

Previous work by Murdoch (2010) investigated the inactivation efficacy of 405 nm light on dormant *A. niger* spores. Results shown in Figure 5.3 demonstrate the significant resilience of the dormant spores, with no reduction in population evident after application of 454 J cm^{-2} – a dose which was capable of inducing complete/ near complete inactivation of the two yeast species, *C. albicans* and *S. cervisiae*. Application of greater doses did, however, result in a steady decrease in the spore population, with 1, 2.5 and 5 log_{10} reductions achieved following exposure to 907, 1360, and 2268 J cm^{-}

^{2}, respectively. No significant change was noted in the non-exposed control samples over the duration of the experiment.

Following from the work conducted by Murdoch, two main aspects of the inactivation efficacy of 405 nm light on spores were investigated:

- The oxygen dependence of the spore inactivation mechanism, and,
- The inactivation of germinating *A. niger* spores, in order to determine whether germinating spores demonstrated greater susceptibility than dormant spores.

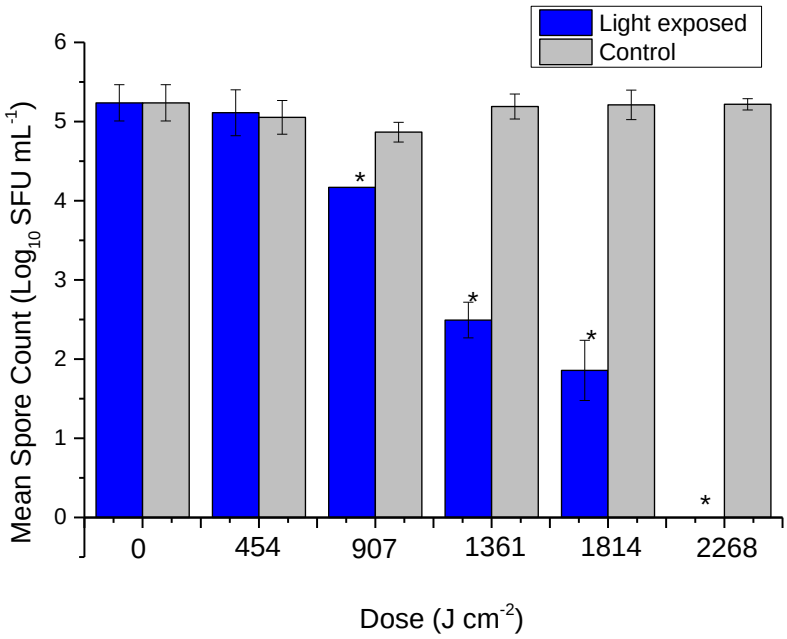

Figure 5.3 *Inactivation of dormant Aspergillus niger spores in liquid suspension, by exposure to 405 nm light, expressed as a function of dose. * represent light exposed populations which were significantly different to non-exposed control population (P≤0.05). Adapted from Murdoch (2010).*

5.2.2 Methodology for preparation and enumeration of spores

A. niger spores were prepared as described in Section 3.1.2. Slopes were flooded with sterile PBS, containing 1% Tween-80, in order to prevent aggregation of spores. An L-shaped spreader was used to gently remove spores from the slope, and the slope was

gently inverted several times to produce a heavily populated spore suspension. One–mL of the resultant spore suspension was transferred into 9 mL PBS prior to enumeration of spore population by use of an Improved Neubauer haemocytometer (Weber Scientific International, UK). Once the population had been established, spore suspensions were diluted to ~2×10^5 spore forming units per millilitre (SFU mL^{-1}) for experimental use. Post exposure, samples were plated onto malt extract agar and incubated as detailed in Chapter 3, Table 3.2. Results were enumerated and recorded as SFU mL^{-1}.

5.2.2.1 Principle of haemocytometer

The haemocytometer is a device used for counting cells and other microscopic organisms. A diagrammatic representation is shown in Figure 5.4. It is a specialised thick glass slide containing a rectangular indentation. This forms the chamber used for holding liquid suspension (depth 0.1mm). The glass is etched with a counting grid, which is visible only under microscopic view. The grid is divided into 9 large squares, each 1 mm × 1 mm (Figure 5.5), for experimental purposes, the four outer quartiles (labelled Q1-Q4) were used for counting cellular populations. An average of the 4 outer quartiles is used to calculate the spore population (spores mL^{-1}).

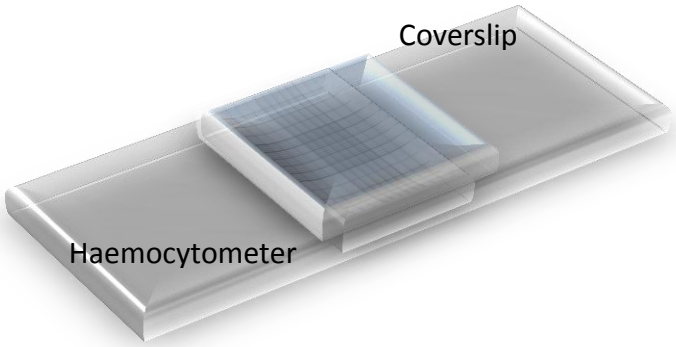

Figure 5.4 *Diagrammatic representation of an Improved Neubauer Haemocytometer, demonstrating the chamber counting grid, over-layed with coverslip.*

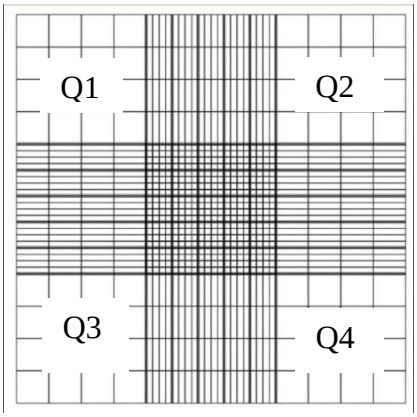

Figure 5.5 *A diagrammatic representation of counting grid etched on an Improved Neubauer Haemocytometer, with the 4 quartiles used for counting highlighted.*

5.2.2.2 Haemocytometer protocol

To use the haemocytometer for enumeration of spore populations, the following protocol was followed:

1. The haemocytometer and specialised cover slip were cleaned using 70% ethanol.
2. The coverslip was moistened (with exhaled breath) and affixed to the haemocytometer. Correct fixing was identified by visualisation of 'Newton's rings' (rainbow-like rings under the cover-slip) which indicate that the cover slip has adhered to the haemocytometer via suction. This generates a chamber with sufficient depth and capillary action.
3. A 10µl sample of spore suspension was pipetted into the bottom edge of the coverslip, filling the haemocytometer chamber by capillary action.
4. The counting grid was visualised using microscopy (400× magnification), shown in Figure 5.6.
5. The four outer quartiles were counted and averaged to give an accurate representation of the spore population in the sample.
6. A calculation was then performed using the equation $C = \tilde{N} \times 10^4$ where C is the average number of cells per ml, $\tilde{N}$ is the average number of spores counted in the chamber, and 10^4 is the volume conversion factor for 1 mm^2

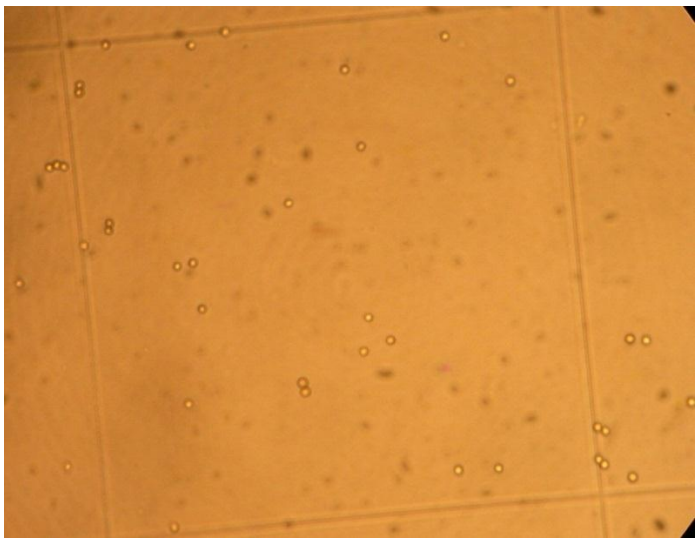

Figure 5.6 *A. niger spores on a haemocytometer counting grid, viewed under a light microscope using the ×40 lens (400× magnification).*

5.2.3 Investigation of the oxygen dependence of the spore inactivation mechanism

As with the yeasts, it was important to establish the involvement of oxygen in the inactivation process of *A. niger* spores. The following section of work investigated the requirement of oxygen to establish it's significance during 405 nm light inactivation of fungal spores.

5.2.3.1 *Aspergillus niger* spore treatment

For *A. niger* oxygen dependence experiments, samples were prepared as described in Section 5.2.2. For oxygen-dependent experiments, all preparations were conducted in an anaerobic cabinet as described in Section 5.1.4. Following preparation, a 3 mL sample (10^5 SFU mL^{-1}) was exposed to ~ 63 mW cm^{-2} 405 nm light (Source C) whilst held in an anaerobic cabinet. Controls were also held under anaerobic conditions.

5.2.3.2 Results: Oxygen dependence of fungal spores during 405 nm light exposure

Figure 5.7 compares the effects of 405 nm light on *A. niger* spores when exposed under aerobic and anaerobic conditions. Results indicate that following an applied dose of 2.3 kJ cm^{-2}, complete inactivation was successfully achieved (~ 5 $\log_{10}$). However, results in Figure 5.7 also highlight that depletion of environmental oxygen by exposure

within an anaerobic cabinet almost completely inhibits spore inactivation, with only a 0.1 $\log_{10}$ reduction observed. Due to this significant inhibition of inactivation under anaerobic conditions, further reduction of oxygen by the addition of ROS scavengers (as carried out with *S. cerevisiae* and *C. albicans*) was not required.

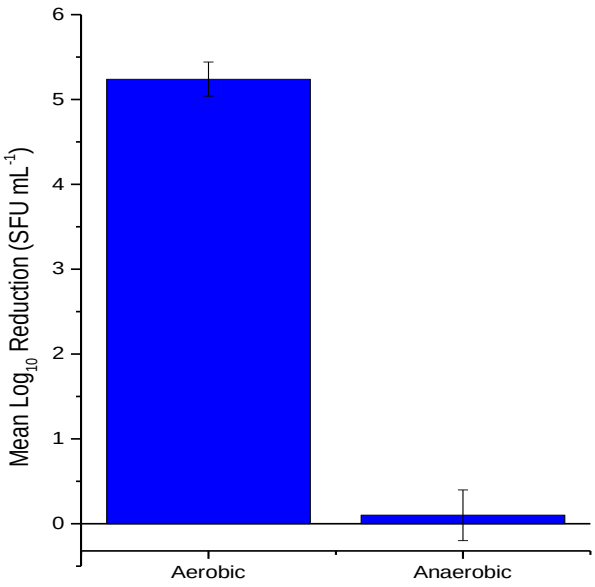

Figure 5.7 *Mean population reductions of A. niger spores after exposure to 405 nm light under aerobic and anaerobic conditions. Doses used for exposure were those required to achieve a 5 log_{10} reduction under aerobic conditions (2.3 kJ cm^{-2}).*

5.2.4 Investigation of 405 nm light inactivation of germinating *A. niger* spores

Following on from the work conducted by Murdoch (2010), investigation into the inactivation efficacy of 405 nm light on germinating *A. niger* spores was carried out in order to determine whether germinating spores were more susceptible than dormant spores. Analysis of results obtained by Murdoch demonstrated that when exposed to 454 J cm^{-2}, dormant spores were not affected (Figure 5.3). Subsequently, this dose was selected for exposure of germinating spores to determine whether they showed increased sensitivity when compared to their dormant counterpart.

5.2.4.1 Germination of *A. niger* spores

A. niger spores were prepared as previously described in section 5.2.2. A 1 mL volume of 10^6 SFU mL^{-1} was re-suspended in 9 mL of chemically defined germination medium rather than PBS, to produce a starting population of ~2×10^5 CFU mL^{-1}. The chemically defined germination medium consisted of 1.0 g KH_2PO_4, 0.25 g $MgSO_4$, 0.2 mg $CuSO_4$, 6.0 mg $FeSO_4$, 4.0 mg $MnCl_2$, 50 mg $CaCl_2$, 2.0 g NH_4SO_4, 10.0 g Sucrose, 5.0 g monosodium glutamate per litre of sterile water . This germination medium was used as it was clear in colour and allowed transmission of the light through the sample without any attenuation.

After the spore suspension was added to the germination medium, 10 mL volumes were dispensed into sterile petri dishes and held at 30°C for up to 8 hours under static conditions (to prevent clumping during germination an effect that occurs more readily under shaken flask cultivation conditions). At 2 hour intervals, 3 mL volumes of germinating spore suspension were removed and exposed to 405 nm light for 2 hours at an irradiance of ~63 mW cm^{-2} (dose of 454 J cm^{-2}). Non exposed control samples were set-up simultaneously with light exposed samples. Results then allowed direct comparison between dormant conidia and those undergoing germination.

5.2.4.2 Results: Inactivation of germinating *A. niger* spores using 405 nm light

Figure 5.8 demonstrates the susceptibility of *A. niger* spores to 405 nm light during increasing stages of germination. Results highlight that when *A. niger* spores are pre-incubated in germination medium and then exposed to 454 J cm^{-2} of 405 nm light, significantly enhanced inactivation is observed compared to that shown with the dormant spores at the same dose. Significant reduction in spore count was observed following 405 nm light exposure after a 2 hour incubation period in the germination medium (P= 0.00). When the period in germination media prior to light exposure was extended (up to 8 hours), reductions in spore counts progressively increased from 0.8 to 2.5 log_{10} SFU mL^{-1}. No significant reduction in SFU count was recorded with dormant conidia exposed to the same dose of 454 J cm^{-2} (PBS control), demonstrating that once germination of conidia is initiated, increased susceptibility entails, with sensitivity increasing as germination progresses.

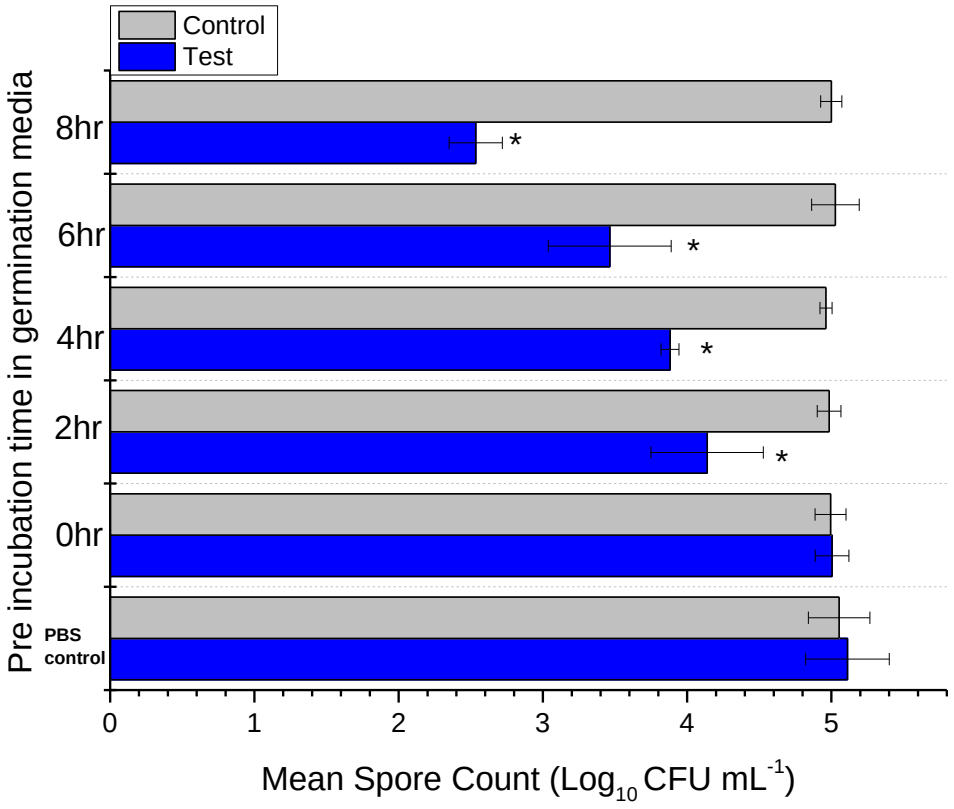

Figure 5.8 *Demonstration of the increasing susceptibility of A. niger conidia to 405 nm light when exposed at various stages of germination. All conidia were exposed to a dose of 454 J cm^{-2}. * represents light exposed populations which were significantly different to non-exposed control population (P≤0.05).*

5.2.4.3 Morphological changes of germinating *A. niger* exposed to 405 nm light

To investigate whether 405 nm light exposure induces morphological changes during germination, spores were incubated in germination media for 6 hours at 30°C, exposed to 454 J cm^{-2} as detailed in Section 5.4.2.1, and then incubated in germination media for a further two hours (8 hour total incubation). A germination period of 6 hours was selected as this was the first stage at with outgrowth of germ tubes was visible, thereby confirming germination (Figure 5.9 B). Although germination was initiated prior to this, after 2 and 4 hours negligible differences in cell morphology were visible. Microscopic examination of the light-exposed fungal samples and the non-exposed control samples, which had been incubated continuously for 8 hours in germination media with no 405 nm light exposure, enabled a visual comparison of whether light exposure induced

a morphological change during germination. For visualisation purposes fungal samples were centrifuged and re-suspended in $1/10^{th}$ of the volume to effect cell aggregation and increase the cell density in the field of view.

Typical *A. niger* conidial germination has been previously described in a study by Anderson and Smith (1970). Dormant conidia were shown to be approximately 3.5 µm in diameter and possess rough, dark pigmented spore coats. During germination an increase in diameter by a process of both imbibition and spherical growth swelling was observed, resulting in conidia that were approximately 6.5-7 µm. This was followed by single or double germ tube outgrowth. Microscopic examination of conidia in this study, following 0, 6, 8 and 10 hours incubation in the germination medium showed a similar process, with the dark pigmented spores (Figure 5.9A) increasing in diameter and displaying germ tube outgrowth (Figures 5.9 B-D)

The morphological effects of 405 nm light when applied during the germination process can be clearly seen in Figure 5.10. Significant differences in the extent of germination can be observed between conidia exposed to 454 J cm^{-2} of 405 nm light following a 6 hour period of germination, followed by a further 2 hour post exposure incubation in germination media (Figure 5.10B), and non-exposed conidia after an equivalent 8 hour germination period, demonstrating normal germination (Figure 5.10C). These results show that exposure to 405 nm light during germination has an inhibitory effect on the germination process, whereby 'normal' germination does not continue following a period of 405 nm light exposure. Obvious differences are apparent in the germ tube morphology, where there is a distinct difference in the visual appearance, in respect to both the quantity and size of the germ tubes.

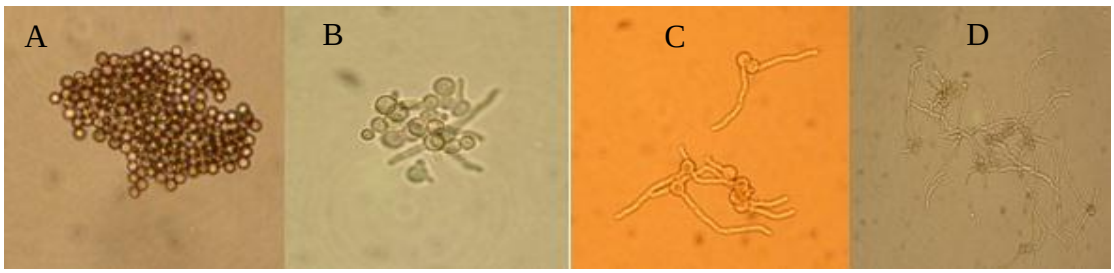

Figure 5.9 *Microscopic visualisation of germination process of A. niger following (A) 0 hours, (B) 6hours, (C) 8hours and (D)10hours in germination media with no 405 nm light exposure. Cells were viewed under ×400 magnification.*

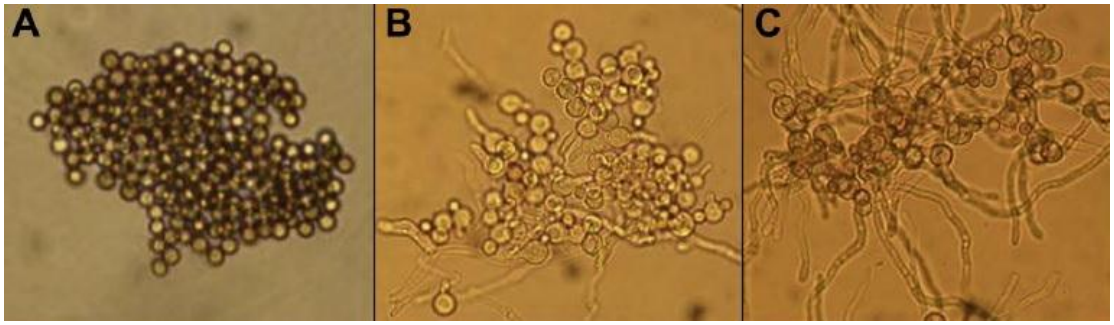

Figure 5.10 *Microscopic visualisation of (A) dormant conidia, (B) conidia exposed to 405 nm light (454 J cm^{-2}) after 6 hour germination period followed by a further 2 hour post-exposure incubation in germination media, showing abnormal germination, and (C) non exposed conidia after an equivalent 8 hour germination period, showing normal germination. Cells were viewed under ×400 magnification.*

5.3 Detection of Endogenous Porphyrin Molecules within Fungi

As discussed in Section 2.4.4.2, the inactivation mechanism of 405 nm light on bacteria is based on the presence of endogenogenous porphyrin molecules. This section of work aimed at determining and identifying the presence of endogenous porphyrin molecules present within the fungal species tested throughout this chapter, by use of fluorescence spectrophotometry, whereby specific fluorescence signals are associated with endogenous porphyrin molecules.

5.3.1 Preparation and treatment

C. albicans, S. cerevisiae and *A. niger* cultures were prepared as previously described. Following the required incubation period, broths were centrifuged and re-suspended in PBS. Cells were washed twice to ensure complete removal of all nutrient growth medium, as this could potentially affect spectrophotometry readings. The supernatant was disposed and the resultant pellets were suspended in 1 mL of 0.1 M NaOH- and 1% sodium dodecyl sulphate (SDS) for 24 hours under dark conditions in order to lyse cells and allow release of intracellular porphyrin content into the extracellular medium for analysis by fluorescent spectrophotometry. Following 24 hours incubation, cell suspensions were then centrifuged and the supernatant was analysed using a fluorescence spectrophotometer to determine fluorescence of specific endogenous poprphyrin molecules. Excitation of each of the samples was carried out at 405 nm and the emission spectra for each organism were recorded between 500 and 800 nm.

5.3.2 Results: Identification of endogenous porphyrin molecules within fungi

Results in Figure 5.11 highlight the fluorescence emission spectra of suspension preparations of the three fungal species suspended and dissolved in NaOH-SDS. Excitation of the cell supernatants at 405 nm displayed peak emissions at 611 nm and 608 nm for *S. cerevisiae* and *C. albicans*, respectively. No peaks were detected for *A. niger* spores when excited under identical conditions, which is likely due to the dark pigmentation of the spores, interfering with fluorescent spectrophotometry measurements. As a result, analysis of *A. niger* mycelia was conducted, indicating a slight peak at 607 nm. The greatest emission peak was observed in *S. cerivisae*, followed by *C. albicans* and then *A. niger*.

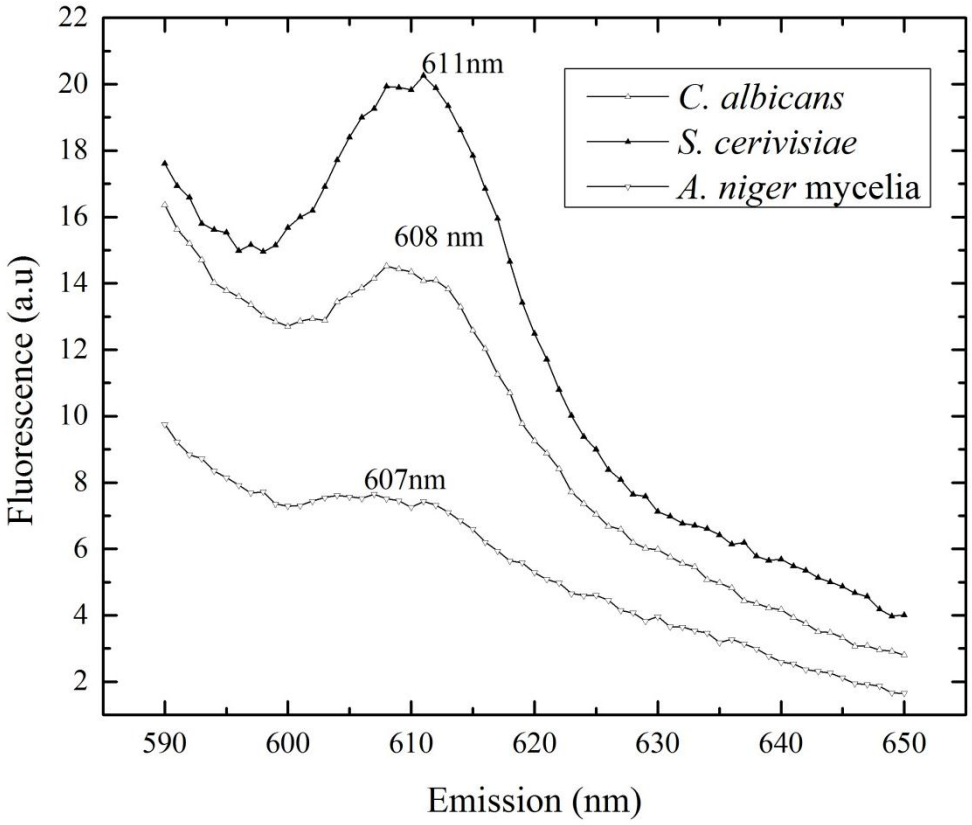

Figure 5.11 *Fluorescence spectra of S. cerevisiae, C. albicans and A. niger mycelia. Fluorescence emission spectra were detected from suspension preparations of the three species dissolved in NaOH-SDS, using an excitation wavelength of 405 nm. Emission spectra are shown between 590-650 nm.*

5.4 Discussion

Results from this study have shown that *S. cerevisiae, C. albicans* and both dormant and germinating *A. niger* spores can be successfully inactivated by exposure to high intensity 405 nm light. Initial results from Murdoch demonstrated that of the two yeast species investigated, *S. cerevisiae* was more sensitive than *C. albicans* with the dose levels required to promote a 5 $\log_{10}$ reduction being ~288 J cm^{-2} and ~ 576 J cm^{-2}, respectively. Dormant *A. niger* conidia were shown to display greatest resistance to 405 nm light inactivation, with a dose of approximately 2.3 kJ cm^{-2} required to achieve an equivalent 5 $\log_{10}$ reduction in population- approximately four and eight times higher than that required for *C. albicans* and *S. cerivisae*, respectively.

Despite dormant conidia of *A. niger* requiring high doses of 405 nm light for inactivation, results shown in Figure 5.8 highlight that, upon germination, susceptibility to 405 nm light significantly increased. Inactivation kinetics over an 8 hour germination period demonstrated increased susceptibility and a statistically significant 1 $\log_{10}$ reduction in population after only a 2 hour germination period, indicating that the initial changes undergone by germinating spores are sufficient to increase their susceptibility. The reason for this increased susceptibility over the course of the germination period could be related to morphological/structural changes, such as stretching or fracture of the dense pigmented spore coat, which could contribute to increased light penetration of the spore (Russell 2003). It could also be speculated that increased susceptibility may be due to increased metabolic vulnerability to light-induced ROS during the germination process, or indeed a combined effect from both aforementioned mechanisms.

This data indicates that initiation of germination could represent a novel method to enhance 405 nm light efficacy for inactivation of fungal spores. It is also interesting to note that similar results have been displayed with bacterial spores upon exposure to ultraviolet (UV) radiation. A study by Nerandzic and Donskey (2010) investigated the efficacy of UV-C irradiation for inactivation of germinating *Clostridium difficile* spores. They applied a germination solution onto a range of common surfaces seeded with *C. difficile* spore, and administered UV-C light by an automated UV device. Results demonstrated significantly enhanced inactivation of germinating *C. difficile* spores using UV-C radiation when compared to exposed dormant spores. However, to date no studies have investigated the enhanced efficacy of violet-blue light on germinating bacterial spores.

Microscopic analysis of conidia exposed to 405 nm light at a dose of 454 J cm^{-2} during germination demonstrated that light exposure interfered with germinating conidia, with hyphal development appearing stunted when compared to non-exposed controls, as shown in Figure 5.10 (B) and (C), respectively, indicating that 405 nm light exposure during germination can negatively affect both germ tube and hyphal growth processes. It is interesting to note that conidia exposed to 405 nm light (454 J cm^{-2}) following 6 hours incubation in germination media followed by a further 2 hours, post-exposure (total 8 hours), displayed very similar visual appearance to conidia incubated for 6

hours, rather than those incubated consistently for 8 hours, strongly suggesting that 405 nm light has inhibitory effects on the germination process.

The resistance displayed by dormant *A. niger* spores when exposed to 405 nm light is not surprising given their ability to survive extensive periods exposed to solar UV radiation during aerial dispersal. Numerous studies have demonstrated the strong resistant properties of various fungal spores, including *A. niger, Fusarium culmorum*, *Mycosphaerella pinodes* and *Botrytis cinerea* (Anderson et al., 2000; Rotem and Aust., 2008). *A. niger* spores are known to be particularly difficult to inactivate by light due the their multi-layered pigmented spore coats that contain aspergillin, a black coloured melanin like compound (Ray and Eakin 1975). A recent study by Esbelin et al. (2013) demonstrated that *A. niger* spores with fawn and white pigmentation were more sensitive to both pulsed and continuous UV-C radiation than wild type strains possessing dark pigmentation. A study by Anderson et al. (2000) used acetone to extract aspergillin from *A. niger* conidia. Light absorbance measurements from the resultant suspension demonstrated that aspergillin absorbs around 5-8 times more light in the UV wavelengths than at 405 nm. This suggests that *A. niger* spores may have less protection against 405 nm light when compared directly to UV radiation.

When investigating the susceptibility of fungi to 405 nm light, it is interesting to compare the results from this current study with previously published data on bacteria. Bacteria have also displayed considerable variability in susceptibility, as discussed in the previous chapter. Comparison between values required for fungal inactivation and bacterial inactivation would typically suggest that fungal species may be somewhat more resistant to 405 nm light treatment. It is interesting to note that inactivation of *C. albicans* and other yeasts have been shown to be slightly more resistant to photodynamic antimicrobial chemotherapy (PACT) than Gram positive bacteria, thereby requiring higher drug concentrations and greater doses of light (Zeina et al., 2002; Rosseti et al., 2013). As described previously, many fungal species are protected by the presence of carotenoid and melanin like pigments that can harden the cell wall and successfully absorb harmful and damaging wavelengths of light (Free 2013). Fungi also present thick cellular walls, containing various constituents including chitin, glucan, mannan and various other lipids that may also protect from direct ROS mediated damage (Donnelly et al., 2008). Another possible explanation is that the

inactivation effect in prokaryotic cells (bacteria) appears to result from a 'single hit process' whereby all cellular targets are equally susceptible to ROS, and if damaged, cell death entails. In comparison, eukaryotic cells (yeast and fungi) require a 'multi-hit process' where saturation of multiple targets is required prior to complete inactivation. These differences are most likely due to the variance in size between bacterial and fungal cells, with *Candida* species being approximately 25-50 times larger than bacteria cells and subsequently containing a much greater number of target organelles (Calzavara-Pinton et al., 2005; Zeina et al., 2002).

It is also interesting that the relatively high doses required for inactivation of dormant *A. niger* spores were similar to those reported by Maclean et al. (2013) for the inactivation of bacterial endospores. Results from this study highlighted that a 5 $\log_{10}$ reduction of dormant *A. niger* conidia required a dose of ~2.3 kJ cm^{-2}, whereas Maclean et al. (2013) reported that approximately 1.7 kJ cm^{-2} was required to achieve a 3.6 $\log_{10}$ reduction of *Bacillus cereus* endospores.

The mechanism of action involved in fungal inactivation has not yet been fully investigated, however, it is considered extremely likely that the inactivation mechanism in fungal species involves photoexcitation of free porphyrin molecules within the exposed cells, as indicated for bacterial inactivation, promoting production of singlet oxygen and other ROS which cause damage to fungal cells (Dai et al., 2012; Donnelly et al., 2008). Previous PDI studies investigating fungal inactivation combined with the addition of exogenous photosensitisers suggest the sites of damage in fungi during light exposure is typically associated with the plasma membrane and cell wall (Gonzales et al., 2010; Donnelly et al., 2008; Lambrechts et al., 2005; Strakovskyaya et al., 1999). Both the cell wall and plasma membrane possess a negative electric charge, which attract the binding of positively charged photosensitizers such as methylene blue and toluidine blue (Gonzales et al., 2010). The fact that singlet oxygen is short lived and can only diffuse short distances, indicates thatdamage is most likely to occur in sites close to initial production (Donnelly et al., 2008; Hamblin and Hassan 2004; Kalka et al., 2000; Bertolini et al., 1987).

Results from fluorescent spectrophotometry were used to confirm the general presence of porphyrins within exposed fungal organisms. However, location and quantification of

porphyrin levels were not investigated. The fluorescence emission spectra of the three organisms tested indicated similar peaks in the region of 605-615 nm and although the results are qualitative, there were distinct differences in the intensity of the detected peaks. The similarity in these peaks indicates that the three species are likely to contain the same predominant intracellular porphyrin, possibly corproporphyrin due to the similarity in its emission peaks when excited at 405 nm (Hamblin et al., 2005; Dai et al., 2013a). However, further analysis would be necessary in order to provide full identification. The use of high performance liquid chromatography (HPLC) could potentially both identify and quantify various porphyrin molecules with microbial cells but due to time constraints, this was outwith the scope of this study.

Limitations in the extraction method used during fluorescence spectrophotometry are likely to explain why no detectable porphyrin emission peaks were observed upon analysis of *A. niger* spores. It is possible, that due to the persistence of some dark spore pigment extract in the supernatant, this masked the fluorescence detection of porphyrins. In order to generate fluorescence data for *A. niger* to allow comparison with the other fungal species tested during the study, *A. niger* mycelia was utilised as an extraction source.

Upon comparison of the fluorescence emissions and the inactivation rates of the three species investigated, it was interesting to note that *S. cerevisiae*, which was the most susceptible to 405 nm light inactivation, displayed the highest intensity of intracellular porphyrin, and *A. niger* which was least susceptible displayed the lowest. This suggests that the levels of intracellular porphyrins detected may have a direct influence on the susceptibility of the organism.

Further investigation into the mechanism of 405 nm light inactivation of fungal species also demonstrated that, as with bacterial species, oxygen plays a critical role in the inactivation mechanism, further supporting the hypothesis that the inactivation involves photoexcitation of endogenous porphyrins, an oxygen dependent reaction (Hamblin and Hassan 2004). The use of an anaerobic cabinet and ROS scavengers demonstrated that reducing oxygen and ROS significantly decreased 405 nm light-induced inactivation. The use of ascorbic acid as the ROS scavenger provided some important information with regards to the mechanism of action. Ascorbic acid has been described as one of the

strongest radical scavengers, which can be taken up by the microbial cell and act as a $^1O_2$ quencher and scavenger for hydroxyl radicals (Granot et al., 2003; Maclean et al., 2008; Niki., 1991), thus suggesting these radicals act as key contributory factors to the inactivation process. However, the fact that inactivation was not completely inhibited with the addition of ROS scavengers may be explained by the likelihood that various other radical species are involved in the inactivation process, such as superoxide anions and hydrogen peroxide.

Table 5.1 highlights the variability in results when comparing the inactivation of the two yeast species under anaerobic conditions. Reduced inactivation of *S. cerevisiae* was observed under anaerobic conditions, and this was further reduced when combined with ascorbic acid. However, a notable level of inactivation was still observed (2 $\log_{10}$ CFU mL^{-1}), suggesting increased susceptibility of the organism or the potential involvement of other factors in the inactivation mechanism for this organism. Interestingly, in the presence of ascorbic acid, *C. albicans* was shown to be more susceptible to 405 nm light inactivation than *S. cerevisiae*, whilst exposure of *A. niger* spores under anaerobic conditions demonstrated almost no inactivation (0.1 $\log_{10}$). These differences in results between the three species tested suggest that the roles of O_2 and ROS during inactivation are variable amongst species. Although fluorescence spectrophotometry suggested similarities in the porphyrin content between the two yeasts, albeit at different concentrations, it is extremely unlikely that this is the sole reason for differences detected during inactivation under anaerobic conditions, and it is very possible that additional chromophores contribute to the inactivation of eukaryotic cells, however, elucidation of this was outwith the scope of this study.

Other groups studying the effects of light to control fungal microorganisms have traditionally used UV wavelengths, which are known to be much more germicidal than 405 nm light. A study by Begum et al. (2009) treated various fungi, including *A. niger* with UV-C light at 254 nm. They found that following only 180 seconds exposure a 2 $\log_{10}$ reduction of *A. niger* spores was observed following a dose of ~0.5 J cm^{-2}. Upon comparison with 405 nm light inactivation results, for the same 2 $\log_{10}$ reduction, spores required 6 hours exposure at 63 mW cm^2, at a dose of ~ 1.3 kJ cm^{-2}. A similar study by Anderson et al. (2000) studied the effect of pulsed UV light on *A. niger*. Agar plates seeded with fungi were exposed to a Xenon flashlamp with high UV content. Following

1000 pulses, a 3-4 $\log_{10}$ reduction was observed. Similar results have also been shown with yeasts. A recent study by Dai et al. (2011) demonstrated sensitivity of UV-C irradiation on *C. albicans*, with a 3 $\log_{10}$ reduction achieved after a dose of only 20 mJ cm^{-2}, again significantly lower energy than the 454 J cm^{-2} required for the same population reduction using 405 nm light. Although it is well established that UV light is substantially more biocidal than 405 nm light, irradiance levels could be increased in applications where more rapid fungal inactivation is required.

To summarise, results shown in this section have successfully demonstrated the fungicidal properties of 405 nm light on *Sacchromyces cerevisiae, Candida albicans,* and on both dormant and germinating spores of *Aspergillus niger*. Results also highlight that as with bacteria, the mechanism of action of 405 nm light appears to be oxygen dependent, with the presence of intracellular porphyrins being detected through fluorescent spectrophotometry. Evidence of the antifungal effects of 405 nm light presented in this chapter opens up the possibility of developing disinfection technologies for air or surface decontamination in the food industry where the reduction of fungal contamination is either desirable or essential, in order to reduce environmental contamination. Work in subsequent chapters will investigate the use of 405 nm light for potential surface decontamination applications.

CHAPTER 6

DISINFECTION OF CONTAMINATED SURFACES AND BIOFILMS USING 405 NM LIGHT

6.0 General

Surfaces and equipment within food processing, catering and domestic environments are a major source of contamination, with microbial presence increasing the risk of cross contamination of microorganisms to food produce (Carpentier and Cerf, 2011; de Jong et al., 2009; van Asselt et al., 2008; Kusumaningrum et al., 2003).

Traditionally, stainless steel surfaces were used for the production and preparation of foodstuffs, with stainless steel being regarded as the material of choice due to its mechanical strength, corrosive resistance and longevity (Kusumaningrum et al., 2003). However, over recent years, aesthetics have greatly influenced the integration of other materials such as polycarbonate, mineral resins and treated woods. Although more aesthetically pleasing, these materials do not retain the same degree of resistance to abrasion as stainless steel and are consequently more susceptible to impact damage during daily domestic life (Stevens and Holah, 1993; Holah and Thorpe, 1990). This in turn has created serious hygiene issues, with microorganisms able to reside in small abrasions and cause recontamination of hands, equipment and food produce (Carpentier and Cerf, 2011; de Jong 2008). Transmission of microbial contaminants from work surfaces onto foodstuffs and food packaging, and vice versa, is still one of the leading sources of bacterial contamination, resulting in human infection and spoiled food produce.

A number of studies have indicated that various bacterial species including *E. coli*, *S. aureus*, *L. monocytogenes* and *P. aeruginosa* can survive on surfaces from hours to days following initial contamination (Carpentier and Cerf, 2011; Kusumaningrum et al., 2003; Scott and Bloomfield 1990; Jiang and Doyle 1999). Studies have highlighted the ability of bacteria to persist on both cooking utensils and cleaning equipment such as

cloths and sponges, with the ability to readily promote cross contamination (Scott and Bloomfield 1990). A number of other studies have demonstrated the extent of bacterial survival during cross contamination between kitchen surfaces and foods, indicating that up to 100% transfer of bacterial contamination can occur (Chen et al., 2001).

Bacterial attachment to surfaces under the correct environmental conditions often leads to biofilm formation. Biofilms are defined as microbial communities attached either to a surface or to one another, which are enclosed within a complex self-produced protective matrix of hydrated extracellular polymeric substances (EPS) (Ramage et al., 2011; Flemming and Wingender, 2010; Donlan 2002). EPS are comprised mainly of polysaccharides, proteins, nucleic acids lipids and water, providing a complex three-dimensional polymer network. This matrix is known to account for up to 90% of the biofilm mass and is responsible for the macroscopic appearance of biofilms, which are often referred to as slime (Flemming and Wingender, 2010). The EPS not only functions as a protective barrier for enclosed cells, but has several other roles including: adhesion, retention of water, nutrient source and exchange of genetic information.

Formation of a biofilm is a stepwise process consisting of various stages, as shown in Figure 6.1. The structure of a biofilm can range from simple monolayers to vast complex multicellular structures, comprised either of single or mixed microbial species. Biofilm cells present much greater resistance than their planktonic counterparts, to both physical stresses, such as flowing water and UV exposure and to chemical stresses, such as disinfectants (Stewart and Costerton, 2001; Stoodley et al., 2001; O'Toole and Kaplan, 2000; Gibson et al., 1999). Biofilm formation is a well-recognised problem within the food industry, due to its presence having detrimental effects on food safety. Biofilms are considered an emergent public health concern, being increasingly associated with contamination within various food industries including brewing, seafood, dairy, meat and poultry processing (Fleming and Ridgway 2009; Shikongo-Nambabi 2011; Chmielewski and Frank 2003; Harvey et al., 2007; Sofos and Geornaras 2010). Food production premises provide an ideal environment for biofilm formation. These, often moist, environments have a continuous supply of nutrients from various food products, as well as vast surface areas for attachment and a continuous supply of inoculum to initiate biofilm formation.

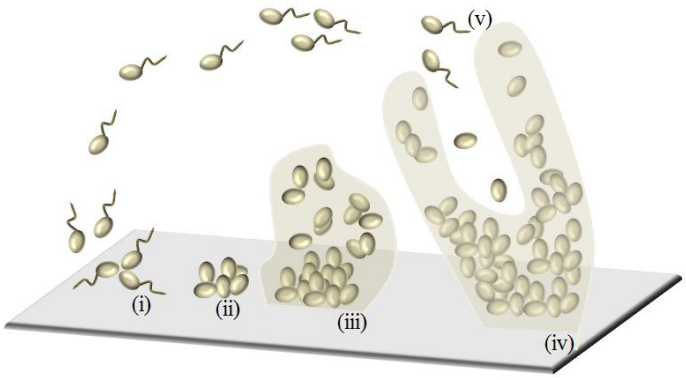

Figure 6.1 *Diagrammatic representation of biofilm development as stepwise process consisting of various stages, (i) initial attachment, (ii) irreversible attachment, (iii) early development of biofilm (iv), maturation and (v) dispersion.*

Both chemical and physical disinfection are routinely used for removal of microbial contamination in food processing/preparation areas. However, poor penetrability of disinfectants through biofilms is a major limitation, facilitating bacterial survival, re-dispersal and recontamination (Agostinho et al., 2011; Otto et al., 2011; Stewart and Costerton 2001). Also, the use of chemical agents at sub-lethal concentrations has been associated with acquisition of bacterial resistance (Patel 2005). Genetic adaptations in many microbial species have also led to the development of resistance against numerous antimicrobial cleaning agents, consequently preventing successful disinfection (Burmølle et al., 2006; Patel 2005). Mechanical cleaning methods, such as scrubbing and brushing, are also routinely employed in food preparation areas but these also present limitations in respect to spread and recolonisation of microorganisms (Pajkos et al., 2004).

The efficacy of many current physical and chemical treatments for surface decontamination is limited a fact that has paved the way for the development of novel decontamination technologies. This chapter investigates the efficacy of 405 nm light for surface decontamination, describing inactivation of bacteria attached to a range of surfaces commonly located in both domestic kitchens and industrial food environments, including glass, acrylic, stainless steel and various rubberised flooring materials.

Section 6.1 will investigate inactivation of bacterial contamination seeded onto surfaces while Section 6.2 will investigate the efficacy of 405 nm light for inactivation of bacterial biofilms, of varying complexities on plastic, glass and stainless steel surfaces.

6.1 Decontamination of Inert Surfaces using 405 nm light

For surface decontamination experiments, *E. coli* was used as the model organism. This organism was selected for two main reasons: firstly, because of the significance of *E. coli* as a food-related contaminant and human pathogen, and secondly because the greater resilience of this organism to 405 nm light (as shown in Chapter 4) means that if successful decontamination results could be achieved, the antimicrobial effects should be easily replicated with other food-related pathogens.

6.1.1 Bacterial preparation

E. coli was prepared as described in Section 3.1.2. Bacterial suspensions were serially diluted in PBS to ~10^7 CFU mL^{-1} for experimental use.

6.1.2 Surface preparation and seeding

Coupons of glass, acrylic, Polyflor homogenous Pur 2000 flooring (Polyflor, UK), Tarkett wet room flooring and Wood FX flooring (Tarkett, UK) (~70mm × 25mm) were used to represent a range of common materials (work surfaces and flooring) located in both domestic kitchens and industrial settings, on which bacterial contamination may occur. The materials were cleaned with ethanol (70%) and suspended fully in 10^7 CFU mL^{-1} bacterial suspensions (~125 mL) for 30 minutes at room temperature under rotary conditions (120 rpm). Surface coupons were then removed from the suspension and placed in a laminar flow cabinet for 20 minutes to allow for drying. This methodology was adapted from that by Buchovec et al. (2010).

6.1.3 405 nm light exposure system

The light source used for these experiments was the ENFIS PhotonStar described in Section 3.3.2. As shown in Figure 3.4, the light source was mounted at a distance of 5 cm from the base. To calculate the average irradiance that the seeded coupons would be exposed to, the irradiance profile across the dimensions of the coupons at this

distance was measured. As can be seen in Figure 6.2, irradiance decreased gradually from the centre (~67 mW cm^{-2}) to the outer edges of the coupon (~35 mW cm^{-2}). The average irradiance across the surface at the 5 cm distance was calculated, to be ~60 mW cm^{-2}. Irradiance measurents were taken across the *x* and *y* axes at 5 mm, using a radiant power meter. Average irradiance was calculated by inputting data into Origin-Pro software to produce 3D profile.

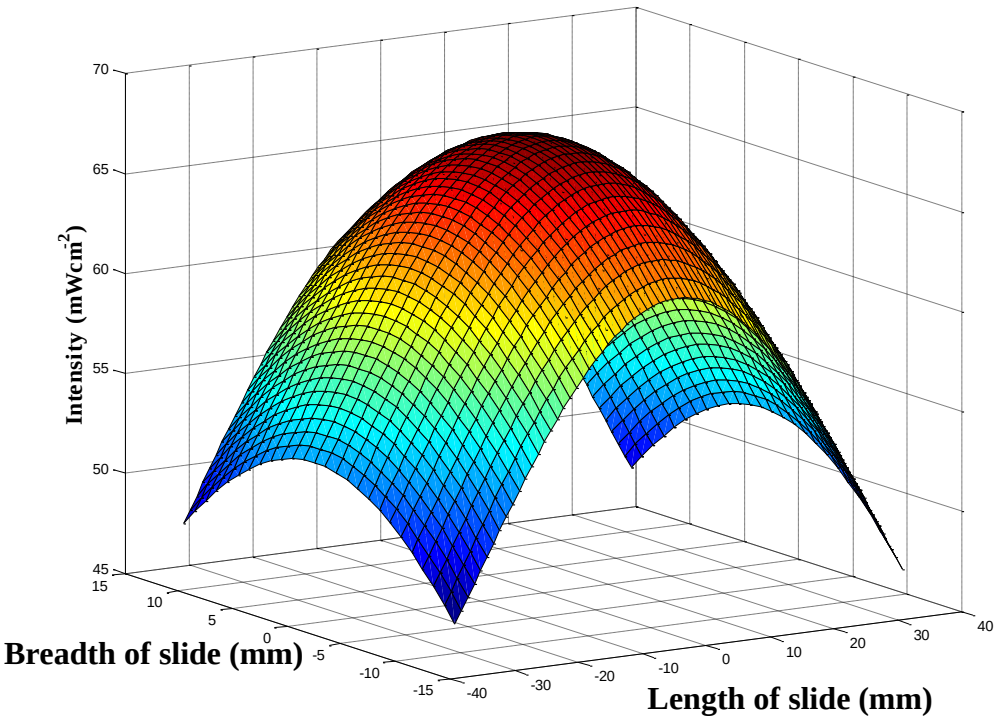

Figure 6.2 *3D model of 405 nm irradiance distribution across the coupon surface giving an average irradiance of ~ 60 mW* cm^{-2}*, using Origin Pro software.*

6.1.4 Surface treatment method and bacterial recovery

Seeded coupons were placed directly under the light source and exposed to increasing durations of 405 nm light treatment. The coupons were raised ~1.5 cm above the base (whilst maintaining the 5 cm distance from the light source) to allow airflow underneath the coupon and prevent any significant increase in temperature as shown in Figure 6.3. Following exposure, bacteria were recovered from the surface using a PBS-moistened sterile cotton-tipped swab. The light-exposed surface was swabbed continuously for two minutes to ensure maximum recovery of bacteria. The swab was then immersed in 10 mL PBS and vortexed for 1 minute to allow re-suspension of bacteria from swab into

suspension. Serial dilutions were prepared as described in Section 3.4.1 to ensure quantifiable results were produced. For enumeration, samples were plated using the pour plate method, with 1 mL sample volumes over-laid with nutrient agar (as detailed in Section 3.4.2.3). Results are reported as CFU count per millilitre (log_{10} CFU mL^{-1}). Non-exposed control samples were prepared simultaneously but remained on the work bench, exposed only to natural room lighting, for the duration which test samples were exposed to 405 nm light.

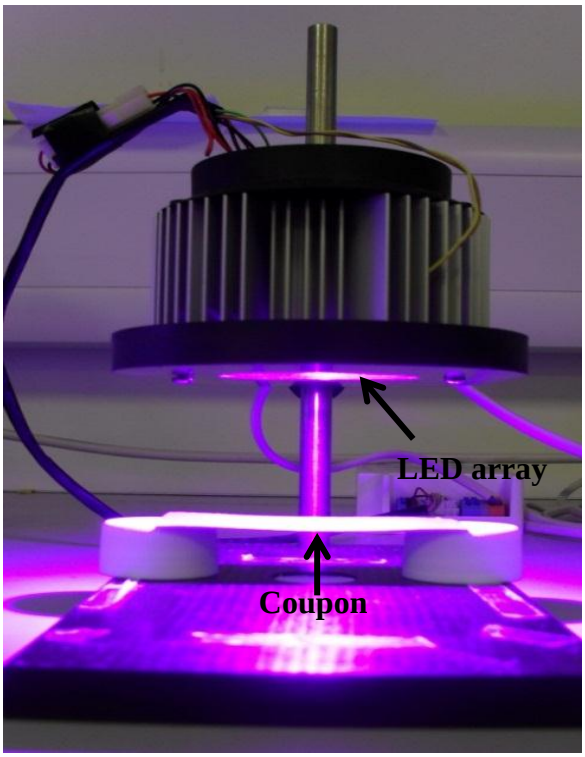

Figure 6.3 *Experimental set up during 405 nm light surface decontamination, where coupons were raised to allow air flow and minimise temperature increase.*

6.1.5 Results: Decontamination of surfaces using 405 nm light

Data shown in Table 6.1 highlight results of the successful decontamination of a range of surfaces seeded with *E. coli*. It can be seen that, under the seeding conditions used in this study, similar levels of bacterial attachment (in the region of 4.6-4.8 log_{10} CFU mL^{-1}) were achieved on the glass, Polyflor Pur homogenous flooring, Polyflor Wood flooring and Tarkett wet room flooring. Acrylic demonstrated the lowest levels of attachment with only 3 log_{10} CFU mL^{-1} population detected on the surface.

Table 6.1 *Inactivation and log_{10} reduction of E. coli on a range of surfaces following 36 J cm^{-2} 405 nm light exposure using an average irradiance of ~60 mW cm^{-2}. * indicates significant differences in exposed and non-exposed bacterial populations (P≤0.05).*

Surface	Starting population (±st dev)	Non Exposed (±st dev)	Exposed (±st dev)	Log_{10} reduction	P value
Acrylic	3.12 (±0.02)	3.06 (±0.05)	0.93 (±0.75)	2.19*	0
Glass	4.61 (±0.01)	4.7 (±0.06)	0 (±0)	4.61*	0
Polyfloor Pur homogenous	4.66 (±0.03)	4.69 (±0.08)	0.21 (±0.33)	4.45*	0
Tarkett Wet room	4.64 (±0.06)	4.27(±0.19)	0.85 (±0.22)	3.79*	0.002
Polyflor Wood Fx	4.77 (0.03)	4.67 (0.16)	4.32 (± 0.18)	0.45*	0.03

Following an applied dose of 36 J cm^{-2}, the only surface that demonstrated complete bacterial inactivation was glass, giving a 4.61 log_{10} CFU mL^{-1} reduction. Investigation into the inactivation rates on commercially available flooring generated mixed results. Bacterial inactivation on Polyflor Pur homogenous flooring demonstrated a 4.45 log_{10} CFU mL^{-1} reduction. Tarkett wet room flooring showed similar levels of inactivation, with a 3.79 log_{10} reduction recorded after treatment with 36 J cm^{-2}. Inactivation of *E. coli* on Polyflor Wood flooring appeared to be least successful of all the tests, with only a 0.45 log_{10} CFU mL^{-1} reduction observed after 36 J cm^{-2}. However, despite variance being recorded between the different materials, statistical analysis highlighted significant reduction in all cases (P≤0.05) following treatment with 36 Jcm^{-2} 405 nm light. No significant reduction in bacterial population was recorded for non-

exposed control coupons, demonstrating that any inactivation that occurred was a direct result of 405 nm light exposure.

6.2 Inactivation of Bacterial Biofilms by 405 nm light exposure

Following successful decontamination of *E. coli* on a range of surface materials, the next stage investigated decontamination of bacterial biofilms using 405 nm light. This section of work investigated the efficacy of 405 nm light for inactivation of bacterial biofilms, with varying complexities, on plastic, glass and stainless steel surfaces.

6.2.1 Bacterial preparation

The bacteria used in this study were: *E. coli*, *S. aureus*, *L. monocytogenes*, and *P. aeruginosa*. Bacteria were prepared as described in Section 3.1.2. Bacterial solutions were diluted to a population density of 10^7 CFU mL^{-1} for experimental use.

6.2.2 Biofilm formation

Biofilms were prepared on glass, acrylic and stainless steel coupons (~70 × 25 mm). These materials were selected for various reasons: (i) they represent typical environmental surfaces within the food industry, (ii) glass and acrylic represent hydrophilic and hydrophobic surfaces, respectively, and (iii) transparency of glass and acrylic allowed transmission of the 405 nm light. All coupons were cleaned with 70% ethanol to sterilise and remove grease. For development of single species biofilms, coupons were fully immersed in 125 mL 10^7 CFU mL^{-1} bacterial suspension for 1 hour to facilitate initial attachment. The bacterial suspension was then discarded and replaced with growth media (1.0 g bacteriological peptone and 0.7 g l^{-1} yeast extract in sterile distilled water) in which the slides were left for a predetermined period to allow development of the biofilm. For development of monolayer biofilms, coupons were left in the growth media for a 4-hour period, while mature biofilms were generated using increasing time periods of 24, 48, or 72 hours (method adapted from Gibson et al., 1999). Due to time restraints, 'mature' biofilm populations were developed on glass and acrylic surfaces only. Initial bacterial populations for *E. coli* biofilms developed on glass, acrylic and stainless steel are highlighted in Table 6.2.

Table 6.2 *Initial bacterial biofilm populations following development over 4, 24, 48 and 72 hours on glass, acrylic and stainless steel surfaces.*

Bacteria	**Surface Material**	**Development Period**			
		4 hr	**24 hr**	**48hr**	**72 hr**
E. coli	Glass	3.55 (±0.02)	5.72 (±0.07)	7.82 (±0.05)	7.17 (±0.02)
	Acrylic	4.11(±0.05)	4.69 (±0.07)	5.13 (±0.11)	-
	Stainless Steel	3.64 (±0.03)	-	-	-
P. aeruginosa	Glass	3.58 (±0.03)	-	-	-
L. monocytogenes	Glass	4.14 (±0.27)	-	-	-
S. aureus	Glass	5.36 (±0.07)	-	-	-

6.2.3 405 nm light exposure system

The light source used for these experiments was the ENFIS QUATTRO described in Section 3.3.1 (Figure 3.3). It was predicted that inactivation of bacteria concealed within a protective EPS matrix would be considerably more difficult than bacteria simply seeded onto a surface; consequently a higher irradiance light source was used for this experimental series.

The light source was mounted at a distance of 5 cm from the base. To calculate the irradiance that the seeded coupons would be exposed to, the irradiance profile across the dimensions of the coupons at this distance was measured. As can be seen in Figure 6.4, irradiance decreased gradually from the centre (~200 mW cm^{-2}) to the outer edges of the coupon (~40 mW cm^{-2}). The average irradiance across the surface at the 5 cm distance was calculated to be 141 mW cm^{-2}. Irradiance measurements were taking across the entire surface material using a radiant power meter at 5 mm intervals, and a 3D model was produced using Origin Pro software.

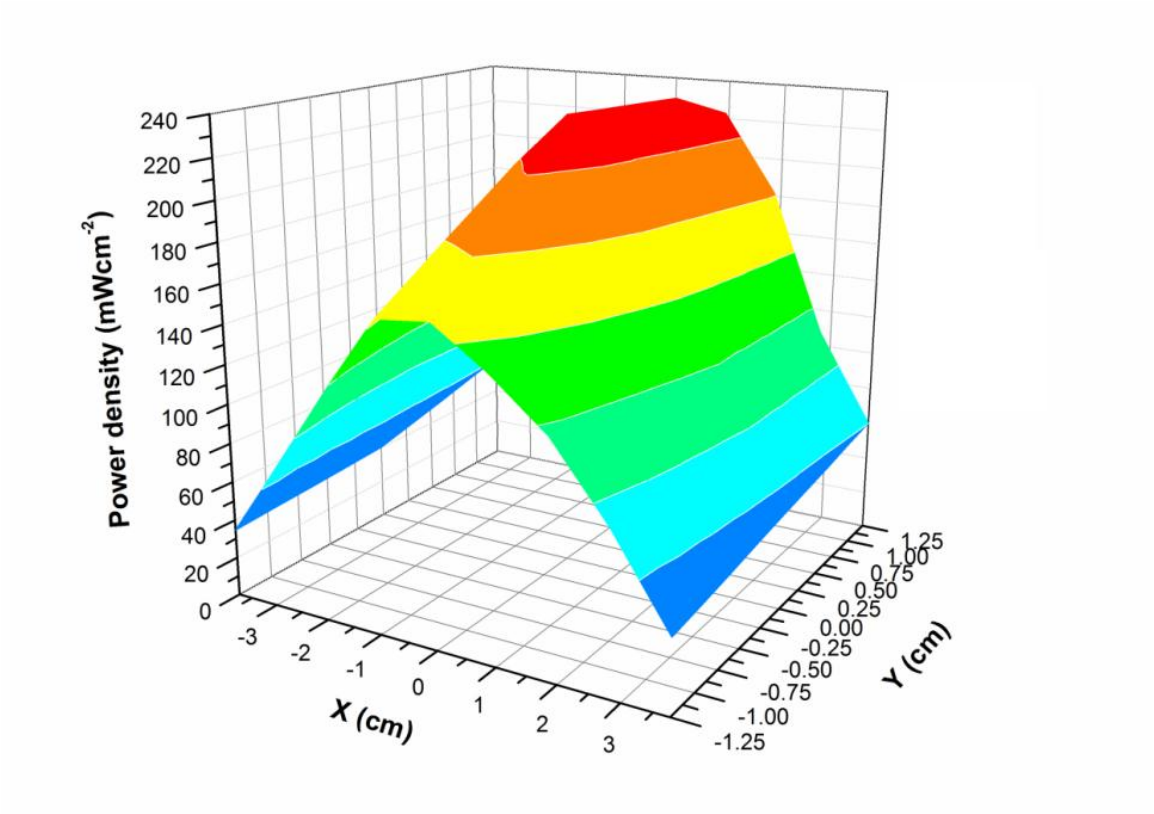

Figure 6.4 *3D model of 405 nm irradiance distribution across the coupon surface giving an average irradiance of ~ 141 mW cm^{-2} using Origin Pro software.*

6.2.4 Treatment method and bacterial recovery

Following biofilm development, coupons were placed directly under the light source at an irradiance of 141 mW cm^{-2} and exposed to increasing doses of 405 nm light, as described in Section 6.1.4. The coupons were raised (~1.5 cm) at either end of the slide, as previously described, to allow air flow and prevent heat build-up. Following light exposure, surviving bacteria were recovered from the coupon using the swabbing method described in Section 6.1.4. The swab was then immersed in a 10 mL volume containing 9 mL PBS and 1 mL 3% Tween-80 suspension and vortexed for 1 minute to allow re-suspension of bacteria from swab into suspension. Suspensions were serially diluted and plated as described previously in Section 6.1.4. Results were reported as CFU count per millilitre ($\log_{10}$ CFU mL^{-1}). Non-exposed control samples were prepared simultaneously.

6.2.5 Temperature measurements

During light exposure, temperature measurements were taken across the surface of the coupons in order to ensure that there was not a build-up of heat on the materials that could contribute to bacterial inactivation. For each material, temperatures were measured after 0, 20, 40 and 60 minutes light exposure, using a Kane May KM340 thermocouple, on three separate locations across the slide. The thermocouple was gently held againt the surface material for a few seconds to allow temperature measurements to be recorded.

6.2.6 Transmission of 405 nm light through glass and acrylic surfaces

In addition to directly exposing biofilms, the potential for 405 nm light to transmit through glass (0.1 cm) and acrylic (0.2 cm) materials and inactivate biofilms on the underside of the coupons was also investigated. To do this, it was important to determine the transmissibility of the light through these materials. This was measured by placing the power meter detector head on the underside of the exposed coupon, prior to any bacterial biofilm development. Transmission of 405 nm light through glass and acrylic was found to result in an approximate 4% loss in irradiance for both materials.

6.2.7 Analysis of the inactivation behaviour of biofilms: Weibull Analysis

The inactivation behaviour of monolayer bacterial biofilms grown on glass and acrylic surfaces was analysed using the Weibull statistical approach, which can help in the identification of potential differences in inactivation dynamics. The Weibull distribution has been employed for the analysis of previous microbial inactivation kinetics, including PDI studies (Schenk et al., 2008; Van Boekel 2002).

Weibull analysis was conducted to analyse the inactivation behaviour of monolayer bacterial biofilms developed on glass and acrylic surfaces, using the equation:

$$\log_{10}[S(D_c)] = -0.4343\alpha D_c^{\beta}, \quad (1)$$

where α and β are parameters of the Weibull distribution. In the Weibull's approach, the 405 nm light dose, D_c, which is required to kill a single microorganism from an entire population is considered as a measure of resistance of this organism to the light.

It is also assumed that D_c is Weibull distributed. The survival rate of microorganisms which form biofilms, $S(D)$, is defined as the number of microorganisms surviving at a specific 405 nm light dose, $N(D)$, divided by the initial number of microorganisms, N_0:

$$S(D) = N(D)/N_0 \quad (2)$$

6.2.8 Results

This section presents inactivation results of monolayer and mature biofilms on various surface materials.

6.2.8.1 Inactivation of *E. coli* monolayer biofilm on glass, acrylic and stainless steel surfaces

Results have demonstrated that *E. coli* biofilms on glass, acrylic and stainless steel surface materials can be successfully inactivated by 405 nm light exposure. Figure 6.5 shows inactivation of *E. coli* monolayer biofilms. Following 4 hours development, biofilm populations were ~3.55, 4.11 and 3.64 $\log_{10}$ on glass, acrylic and stainless steel surfaces, respectively.

Significant inactivation of *E. coli* monolayer biofilms was shown after only 84.6 J cm^{-2} exposure to 405 nm light on all surfaces, with a 2.52, 0.42 and 0.75 $\log_{10}$ CFU mL^{-1} reduction recorded for glass, acrylic and stainless steel, respectively. Monolayer biofilms on stainless steel surfaces demonstrated significantly greater inactivation than inactivation on acrylic surfaces (P= 0.023), but significantly less than glass (P=0.012) following a dose of 84 J cm^{-2}. Complete inactivation (3.55 $\log_{10}$ CFU mL^{-1}) was achieved on glass surfaces following an applied dose of 168 J cm^{-2}, with near complete inactivation achieved on both acrylic and stainless steel (less than 10 CFU mL^{-1}). Reductions in monolayer biofilm populations on each material following 168 J cm^{-2} were all statistically similar. Control biofilm populations on non-exposed coupons showed no significant decrease (P= >0.05).

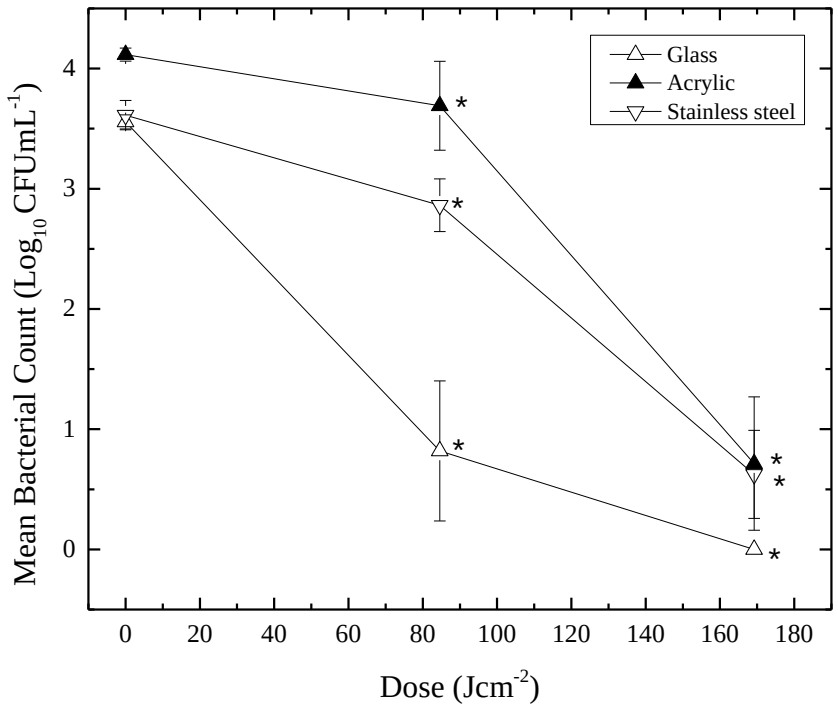

Figure 6.5 *Inactivation of E. coli monolayer biofilms on glass, acrylic and stainless steel surfaces following exposure to 405 nm light with an irradiance of ~140 mW cm^2, given as a function of dose. Biofilms were allowed to develop for 4 hours before light exposure. * Indicates statistically significant differences when compared to control samples ($P \leq 0.05$).*

6.2.8.2 Inactivation of mature *E. coli* biofilms on glass and acrylic surfaces

Following 24 hours development, bacterial biofilm populations increased to ~5.72 and 4.69 CFU mL^{-1} on glass and acrylic coupons, respectively, as shown in Table 6.2. For glass, minimal inactivation was observed following treatment with 84 J cm^{-2}. However, following applied doses of 168, 254 and 338 J cm^{-2}, inactivation occurred at a relatively linear rate, with the reductions of 2.27, 4.41 and 5.7 log$_{10}$ CFU mL^{-1}, respectively, shown in Figure 6.6a. Biofilms developed over 48 and 72 hours on glass had increased cell densities, with populations of between 7 and 8 log$_{10}$ CFU mL^{-1} prior to light exposure (shown in Figure 6.6a). The rate of inactivation for both 48 and 72 hour biofilms were similar, with a 3 -3.5 log$_{10}$ CFU mL^{-1} reduction achieved on both surfaces following 168 J cm^{-2}, and a further approximate 2 log$_{10}$ CFU mL^{-1} reduction following a

338 J cm^{-2} dose. Following an applied dose of 508 J cm^{-2}, 7.4 and 7.0 log_{10} reductions were achieved with the 48 and 72 hour biofilm populations, respectively, with less than 10 CFU mL^{-1} surviving in each case.

Results shown in Figure 6.6 (b) highlight that, following 24 hours of biofilm development, populations were ~4.7 log_{10} CFU mL^{-1} on acrylic surfaces. Biofilm inactivation was shown to occur at a steady and consistent rate following increasing 405 nm light exposure, with 2.3, 3.07, 3.67 and 4.69 log_{10} CFU mL^{-1} reductions recorded following doses of 84, 168, 254 and 338 J cm^{-2} respectively. Development of biofilms over 48 hours generated a bacterial population of ~5.1 log_{10} CFU mL^{-1} on the acrylic surface. As with 24 hour biofilms, inactivation was shown to occur at a steady rate, with 1.2, 2.6, 3.7 and 5.09 log_{10} CFU mL^{-1} reductions observed following 84, 168, 254 and 338 J cm^{-2} 405 nm doses of light, with near complete inactivation achieved following a dose of 508 J cm^{-2} (<1 CFU mL^{-1} surviving). Bacterial biofilm formation on acrylic surfaces after 72 hours in growth media demonstrated no significant increase in bacterial count from that recorded after the 48 hours growth period (P= 0.06). Therefore,biofilms grown for 72 hours on acrylic surfaces were not investigated.

The population densities of all non-exposed control biofilm samples, on both glass and acrylic, remained consistent throughout, with no significant differences recorded over the duration of the experiment, indicating that inactivation was a direct result of 405 nm light exposure. It is likely that the reason for no loss of viability in the control populations was due to the relatively short periods involved (up to 1 hour) as well as the protective effect of the biofilm structure.

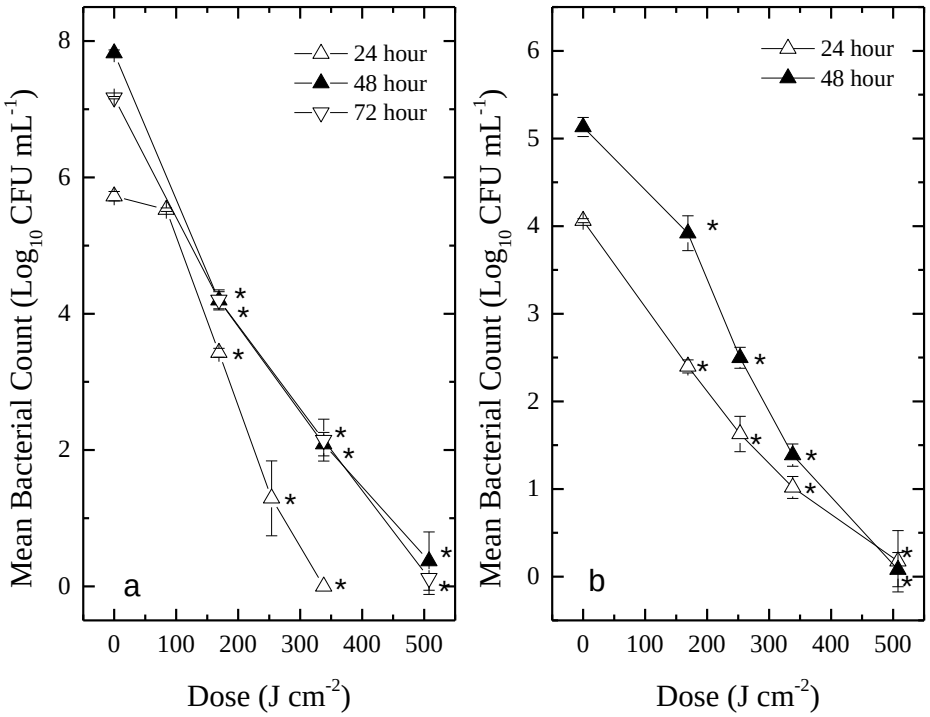

Figure 6.6 *Inactivation of E. coli biofilms on glass (a) and acrylic (b) surfaces following exposure to 405 nm light with an irradiance of ~140 mW cm^2, given as a function of dose. Biofilms were allowed to develop for 24, 48 and 72 hours before light exposure. * Indicates statistically significant differences when compared to control samples ($P \leq 0.05$).*

6.2.8.3 Temperature variation on surfaces during biofilm inactivation.

The temperature across the glass and acrylic coupons was monitored to ensure that prolonged exposure to 405 nm light (up to 60 minutes, dose of 508 J cm^{-2}) did not cause significant heating of the coupon materials, and consequently contribute to inactivation. No significant temperature build up was observed on test materials during light exposure. The maximum temperature recorded was 33°C (8°C increase), indicating that bacterial kill was not a result of negative thermal effects. The recorded temperature measurements are highlighted in Table 6.3. It is important to note that, although an average irradiance across the coupons has been calculated for experimental use, Figure 6.4 indicates that there is greater irradiance at the centre of the coupon,

explaining why greater temperature increase is observed in this area of the material, when compared to the outer edges (left and right).

Table 6.3 *Temperature measurements taken on the left, centre and right side of glass and acrylic coupons following 405 nm light exposure (~140 mW cm^{-2}).*

Exposure Time (min)	**Dose (J cm^{-2})**	**Glass Temperature (°C)**			**Acrylic Temperature (°C)**		
		Left	Centre	Right	Left	Centre	Right
0	**0**	25	25	25	25	25	25
20	**169**	28	31	27	29	29	30
40	**338**	28	32	28	30	33	31
60	**508**	28	33	30	31	33	31

6.2.8.4 Inactivation of *E. coli* monolayer biofilms through transparent materials

Experiments were carried out to establish whether the 405 nm light could transmit through the glass and acrylic materials and inactivate biofilms on the underside of the coupons. For 'indirect' biofilm exposure, after removal from the growth medium, the upper side of the slide was wiped clean with 70% ethanol to ensure biofilm formation was only on the underside of the slide. The inactivation data for these 'indirectly' exposed biofilms is presented in Figures 6.7a and 6.7b in comparison with the directly exposed biofilm results from Figure 6.5.

The glass coupons have high transmittance in the visible light region at around 405 nm. As a result, there were negligible differences between the inactivation for both direct and indirectly exposed biofilms, as can be seen from Figure 6.7a. There was no significant difference (P= 0.738) between the direct and indirect $\log_{10}$ CFU mL^{-1} reductions following an applied dose of 84 J cm^{-2}, and complete inactivation (<1 CFU mL^{-1} survivors) was achieved for both samples following treatment with

169 J cm^{-2}. Similarly, Figure 6.7b illustrates the similar inactivation curves for both direct and indirect exposure of biofilms on acrylic, with no significant difference in the population reductions achieved with direct and indirect following 84 J cm^{-2} (P= 0.421) and 168 J cm^{-2} (P= 0.507) indicating that 405 nm light can transmit through acrylic materials.

As noted, irradiance measurements determined that transmission of the 405 nm light through the glass and acrylic slides resulted in an approximate 4% reduction in irradiance. However, results showed that this reduction was insufficient to cause significant differences in the inactivation rates of the directly and indirectly exposed biofilms on either the glass or the acrylic surfaces.

It was interesting to note that, despite similar levels of inactivation being observed on glass and acrylic following an applied dose of 168 J cm^{-2}, there was a significant difference in inactivation rates following 84 J cm^{-2} (P=0.002). In order to analyse differences in the inactivation mechanism on different surfaces Weibull analysis was conducted. The coefficient β, which determines the shape of the Weibull distribution, was obtained for both surfaces by both direct and indirect light exposure. Figure 6.7d highlights that on acrylic surfaces, the shape parameter β is greater than 1, indicating that with increased 405 nm light dose, microorganisms within the biofilm become increasingly damaged. In the case of glass surfaces (Figure 6.7c), the shape parameter β was shown to be less than 1. When β is less than 1 means that the rate of inactivation is higher at lower 405 nm light doses, and this rate decreases with an increase in light dose. Potentially such inactivation behaviour may indicate that remaining (surviving) microorganisms in the biofilm become more resistive to 405 nm light.

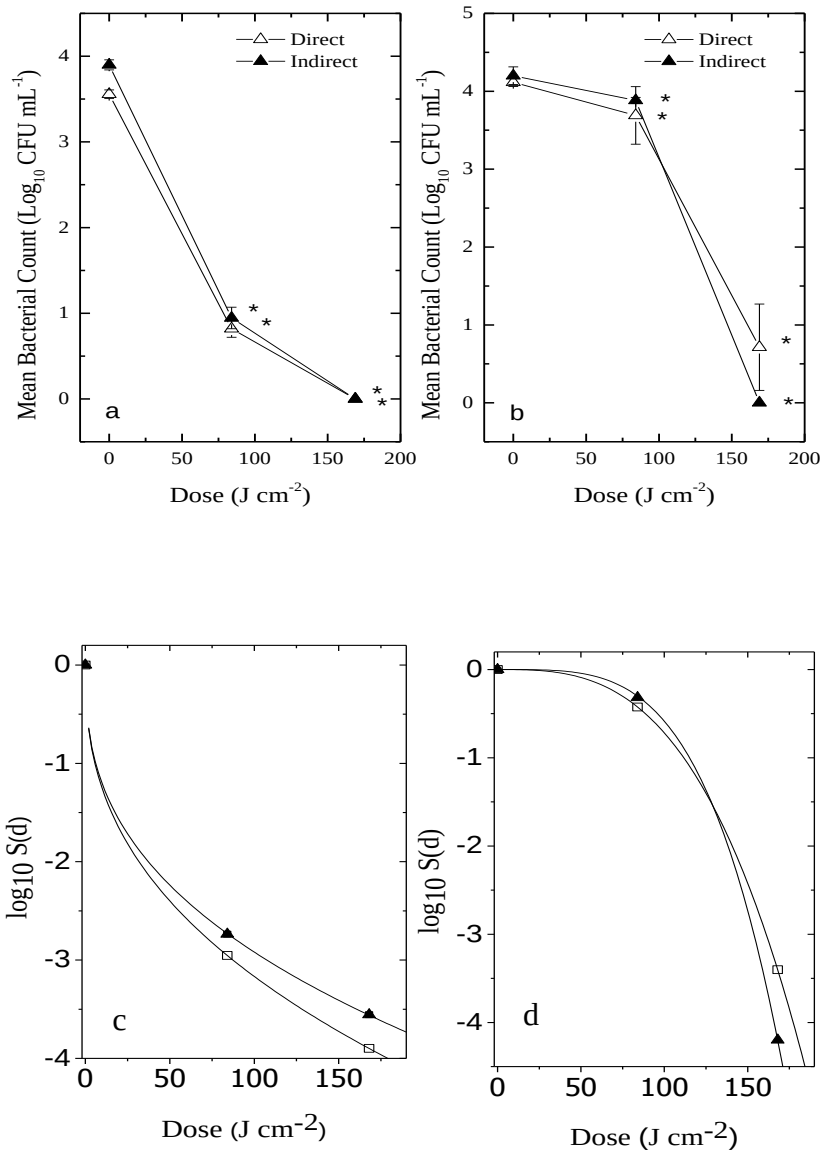

Figure 6.7 *Inactivation of E. coli monolayer biofilms on (a) glass and (b) acrylic, by direct and indirect exposure to 405 nm light. The average irradiance of 405 nm light across the slides was approximately 140 mW cm^{-2}. *Indicates a statistically significant difference when compared to control samples ($P \leq 0.05$). No significant difference was observed between direct and indirect inactivation on both surfaces. Weibull analytical fit lines are shown as (c) glass and (d) acrylic and were obtained using equation (1).*

6.2.8.5 Inactivation of different bacterial monolayer biofilms on glass surfaces

As a comparison to *E. coli*, the bactericidal efficacy of 405 nm light was tested against a range of other bacterial biofilms. *S. aureus*, *L. monocytogenes* and *P. aeruginosa* were selected due to their association with contamination in the food industry and also due to their known association with biofilm formation.

Monolayer biofilms attached to glass surfaces were exposed to 42, 84 and 168 J cm^{-2} of 405 nm light to determine the comparative levels of bacterial inactivation. As can be seen from Table 6.4, it was found that the initial starting populations varied considerably between the different bacterial species, with higher populations found for the Gram-positive species.

Results in Table 6.4 show that successful bactericidal effects were recorded with all the tested monolayer biofilms. Significant reductions were observed in all cases ($P \leq 0.05$), with the exception of 42 J cm^{-2} for *E. coli*. Inactivation of *E. coli* monolayer on glass, demonstrated only a 0.19 $\log_{10}$ CFU mL^{-1} following an applied dose of 42 J cm^{-2}. However, following 84 J cm^{-2}, a 2.5 $\log_{10}$ CFU mL^{-1} reduction was observed, with complete inactivation following 168 J cm^{-2}.

Initial inactivation of *P. aeruginosa* following 42 J cm^{-2} was substantially greater than that recorded for *E. coli*, with a 1.5 $\log_{10}$ CFU mL^{-1} reduction noted. Following 84Jcm^{-2}, *P. aeruginosa* population was reduced by 2.43 $\log_{10}$ CFU mL^{-1}, with complete inactivation achieved after treatment with 168 J cm^{-2} (3.72 $\log_{10}$ CFU mL^{-1}).

As mentioned previously, the initial starting population of the two Gram positive bacterial counts were higher. A *L. monocytogenes* population of 4.14 $\log_{10}$ was reduced by 0.61 $\log_{10}$ CFU mL^{-1} following 42 J cm^{-2} 405 nm light, with a 1.09 $\log_{10}$ CFU mL^{-1} reduction recorded after 84 J cm^{-2}. Following an applied dose of 168 J cm^{-2}, unlike both Gram negative organisms, complete inactivation was not achieved but a 2.48 $\log_{10}$ CFU mL^{-1} reduction was observed.

The initial *S. aureus* monolayer biofilm was the most densely populated of the four bacterial species tested 5.36 $\log_{10}$ CFU mL^{-1}. Significant reductions ($P \leq 0.05$) were recorded following doses of 42, 84 and 168 J cm^{-2}, with 0.61, 1.87 and 2.75 $\log_{10}$ CFU mL^{-1} reductions, respectively.

Overall, the population reductions achieved following a dose of 168 J cm^{-2} were similar between the two Gram-negative bacteria (~3.6 log_{10} CFU mL^{-1}), with which complete inactivation was achieved, and between the two Gram-positive bacteria (~2.6log_{10} CFU mL^{-1}). However, it is likely that these differences were directly related to the variations in starting populations, since the Gram positive bacterial populations were significantly more numerous.

Table 6.4 *Inactivation of bacterial monolayer biofilms on glass surfaces following exposure to 405 nm light with an average irradiance of 140 mW cm^{-2}. * represent statistically significant bacterial reduction when compared to associated non-exposed control samples.*

Bacterial species	**Time (min)**	**Dose (Jcm^{-2})**	**Non-exposed sample (mean log$_{10}$ CFU mL^{-1})**	**Exposed sample (mean log$_{10}$ CFU mL^{-1})**	**Log reduction (* P≤0.05)**	**P value**
E. coli	0	0	3.55(±0.02)	3.55 (±0.02)	0	
	5	42	3.52(±0.05)	3.33(±0.05)	0.19	0.07
	10	84	3.39(±0.07)	0.89(±0.58)	2.50*	0.002
	20	168	3.41(±0.11)	0(±0)	3.41*	0.00
P. aeruginosa	0	0	3.58(±0.03)	3.58(±0.03)	0	
	5	42	3.47(±0.01)	1.97(±0.05)	1.5*	0.00
	10	84	3.59(±0.05)	1.16(±0.06)	2.43*	0.00
	20	168	3.72(±0)	0(±0.29)	3.72*	0.00
L.monocytogenes	0	0	4.14(±0.27)	4.14(±0.27)	0	
	5	42	4.10(±0.08)	3.49(±0.57)	0.61*	0.047
	10	84	4.24(±0.16)	3.15(±0.08)	1.09*	0.002
	20	168	4.14(±0.27)	1.66(±0.41)	2.48*	0.001
S. aureus	0	0	5.36(±0.07)	5.36(±0.07)	0	
	5	42	5.32(±0.33)	4.75(±0.06)	0.61*	0.004
	10	84	5.89(±0.9)	3.45(±0.33)	1.87*	0.00
	20	168	5.36(±0.07)	3.14(±0.09)	2.75*	0.002

6.3 Inactivation of Mixed-species Biofilms using 405 nm light

In a real working environment, biofilms generally form as complex mixed species structures consisting of various different organisms. Consequently, following use of single-species monolayer and mature biofilms, the next step was to investigate the efficacy of 405 nm light for decontamination of mixed-species biofilms.

6.3.1 Mixed-species biofilm formation

For development of mixed species biofilms, cleaned glass coupons were immersed in 125 mL PBS suspension containing 62.5 mL 10^7 CFU mL^{-1} *E. coli* and 62.5 mL 10^7 CFU mL^{-1} *S. aureus* for 1 hour. Slides were then placed in growth media for a further 24 hours to allow mature biofilm formation. After biofilm development, slides were aseptically removed from the growth media and left to dry for 10 minutes in sterile conditions at room temperature prior to light exposure.

6.3.2 Treatment method and bacterial recovery

For mixed species biofilms, slides were exposed to 405 nm light at an irradiance of 141 mW cm^{-2} for 30 minutes (total dose 254 J cm^{-2}). This exposure was selected as it had proved to cause significant inactivation of both *E. coli* and *S. aureus* biofilms when exposed as single species. Following exposure, slides were swabbed as described in Section 6.2.4 and samples were plated onto a range of agars. For enumeration of the total viable count (TVC) of bacteria in the mixed species biofilm, 1 mL samples were pour-plated using nutrient agar (as described in Section 6.1.4). In addition to this, specific counts of the *S. aureus* and *E. coli* populations in the biofilm were obtained using selective agar. Bacterial samples (100 µl-500 µl) were plated onto mannitol salt agar (MSA) and violet red bile agar (VRBA), for selective isolation of *S. aureus* and *E. coli*, respectively. Plates were then incubated at 37°C for 18-24 hours. Plates were enumerated and results recorded as CFU count per millilitre (log_{10} CFU mL^{-1}).

6.3.3 Results: Inactivation of mixed species biofilms

In addition to investigating single-species biofilms, tests were carried out to assess the antimicrobial effect of 405 nm light on mixed-species biofilms. Mixed-species biofilms

containing both *E. coli* and *S. aureus* were prepared on glass coupons. Confirmation of the mixed population was obtained by microscopic analysis (Figure 6.8) of a biofilm slide which had been Gram-stained in order to visualise the presence of both *E. coli* (pink rods) and *S. aureus* (purple cocci).

Table 6.5 displays inactivation data of *E. coli, S. aureus* and mixed (*E. coli* and *S. aureus)* biofilms developed on glass coupons for 24 hours, following an applied dose of 254 J cm^{-2}. As with monolayer population, *S. aureus* grew to a significantly higher population than *E. coli* when developed for 24 hours, with starting populations recorded as ~6.3 and 5.7 log_{10} for *S. aureus* and *E. coli*, respectively. Results in Table 6.5 show that, following a dose of 254 J cm^{-2}, significant inactivation was achieved in all cases. Exposure of single-species biofilms induced a 4.4 log_{10} CFU mL^{-1} reduction in *E. coli* and a 2.97 log_{10} CFU mL^{-1} reduction in *S. aureus* biofilms following an applied dose of 254 J cm^{-2}. Successful inactivation was also observed in the case of the mixed biofilm population, with a 2.19 log_{10} CFU mL^{-1} reduction in TVC, following 254 J cm^{-2}. Analysis of the pre- and post-exposure biofilm populations using VRBA and MSA selective media demonstrated significant inactivation of both bacterial species present in the biofilm (P= 0.00), with approximately 1.2 and 1.7 log_{10} CFU mL^{-1} reductions in *E. coli* and *S. aureus*, respectively, being observed.

After a 24 hour growth period, the population of mixed species biofilm was lower than the populations achieved when compared to the single species biofilms of *E. coli* and *S. aureus.* Analysis of mixed biofilm population using VBRA and MSA selective media highlighted that although the total population was ~5 log_{10} CFU mL^{-1}, the ratio between *S. aureus* and *E. coli* was uneven, with *S. aureus* being the dominant coloniser (as shown in Table 6.5). This is possibly a direct result of interactions between the bacterial species and competition for attachment.

Table 6.5 *Inactivation of E. coli, S. aureus and mixed (E. coli and S. aureus) bacterial biofilm, grown for 24 hours on glass surface and exposed to 405 nm light (254 J cm^{-2}). Counts are provided for total viable counts of S. aureus and E. coli. Selective counts of S. aureus and E. coli have been provided using selective media (MSA and VRBA). * represent statistically significant bacterial reduction when compared to associated non-exposed control sample.*

Biofilm Species	**Media**	**Exposed biofilm (log_{10} CFU mL^{-1})**	**Non-exposed biofilm (log_{10} CFU mL^{-1})**	**Log_{10} Reduction (*P≤0.05)**
E. coli	NA	1.29	5.72	4.43*
S. aureus	NA	3.34	6.31	2.97*
Mixed	NA	3.09	5.29	2.19*
	MSA	2.63	4.30	1.67*
	VRBA	1.10	2.29	1.19*

MSA(Mannitol Salt Agar), VRBA(Violet Red Bile Agar), NA (Nutrient Agar)

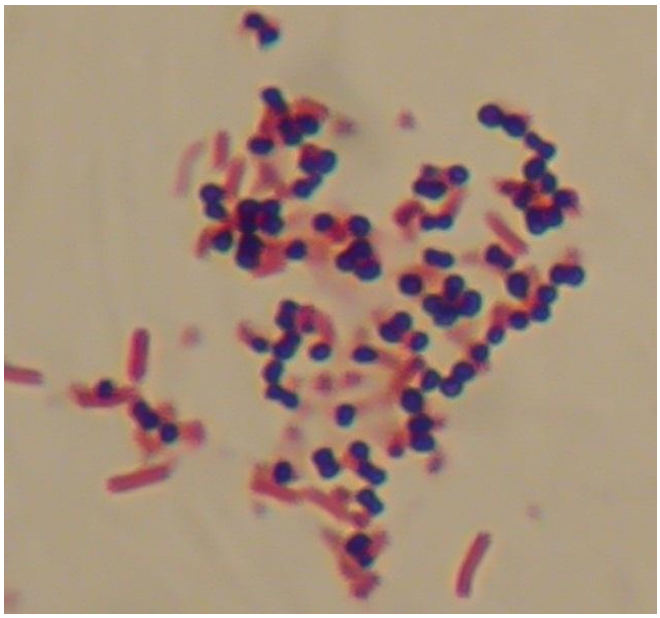

Figure 6.8 *Microscopic visualisation of a Gram stain of a mixed species biofilm consisting of S. aureus and E. coli after 24 hour development. Cells were viewed under oil immersion at ×1000 magnification.*

6.4 Discussion

Despite the development of new antimicrobial agents and novel sterilisation and disinfection technologies, bacterial contamination of surfaces and subsequent biofilm formation remains a significant problem in the food industry. Results from this chapter have demonstrated bactericidal capability of 405 nm light for surface decontamination and have shown, for the first time, the bactericidal effects of 405 nm light on bacterial biofilms, with results demonstrating successful inactivation of biofilms on glass, acrylic and stainless steel surfaces, all of which are used throughout the food industry.

Results displayed in Table 6.1 highlight bacterial inactivation of *E. coli* seeded onto various surfaces, including laboratory standard materials and commercially available flooring surfaces. Attachment of bacteria to each of the materials was shown to be variable, with populations between 3.1 and 4.7 $\log_{10}$ observed. Variations in attachment and in subsequent inactivation may be due to direct interactions between the bacteria and the surface materials- these will be further discussed later in this chapter. Greatest adhesion was displayed by Polyflor Wood flooring and however this surface displayed least inactivation, with only 0.45 $\log_{10}$ CFU mL^{-1} reduction observed following an applied dose of 36 J cm^{-2}. However, it is important to note that when converting data shown in Table 6.1 into absolute bacterial counts, rather than $\log_{10}$ counts, a 0.45 $\log_{10}$ reduction of a 4.7 $\log_{10}$ population is significantly greater than an ~2 $\log_{10}$ reduction of a 3.2 $\log_{10}$ population. Complete inactivation was achieved only on glass, with a ~4.6 $\log_{10}$ reduction obtained following 36 J cm^{-2}. However, in all cases, inactivation of *E. coli* on all surface materials were shown to be significant.

A recent study by Murdoch et al. (2012) investigated the susceptibility of *L. monocytogenes* and *S. enterica* (~2- 2.3 $\log_{10}$ population) when exposed to 405 nm light (45 J cm^{-2}) on acrylic and PVC surfaces. Results by Murdoch demonstrated lower bacterial adherence levels than those observed in the current study, whicis likely due to two factors: (1) the different bacteria used between the two studies and their ability to adhere to different materials (*E. coli* vs *S. enteritidis* and *L. monocytogenes*), and (2) the different methodology used to seed the acrylic surfaces with bacteria, whereby Murdoch aerosolised bacteria using a nebuliser, in contrast to the suspension method employed in this current study (described in Section 6.1.2).

Results shown by Murdoch demonstrated significant inactivation of both bacteria, with 1.46 and 2.19 $\log_{10}$ CFU mL^{-1} reductions observed for *S. enterica* on acrylic and PVC, respectively and 0.21 and 0.9 $\log_{10}$ CFU mL^{-1} reductions achieved for *L. monocytogenes* on acrylic and PVC following treatment with 45 J cm^{-2}. Although Murdoch presented successful inactivation, as with the current study, attachment and subsequent inactivation were variable amongst different bacterial species and surface materials. These results were not directly comparable with the current study due to the variations in initial starting populations. However, it is interesting to note that despite initial populations in the current study being considerably higher than those displayed by Murdoch (3-5 $\log_{10}$ vs. ~2 $\log_{10}$), a lesser dose (36 J cm^{-2}) was required to promote significant inactivation on all surfaces, with this dose being sufficient to induce complete inactivation (~4.6 $\log_{10}$) of *E. coli* on glass.

Despite variations in adhesion levels and inactivation rates, results from this current study correspond well with previous data produced by Murdoch, confirming that 405 nm light can be used successfully to decontaminate a range of surfaces contaminated with various different bacteria. It is therefore envisaged that this method of inactivation could be successfully applied to a range of microorganisms on various different surface materials.

It is interesting to compare inactivation data on inert surfaces from this chapter investigating inactivation on inert surfaces with previous work investigating inactivation on nutrient rich surfaces, where an applied dose of 36 J cm^{-2} was sufficient to induce a 3 $\log_{10}$ CFU mL^{-1} reduction on glass compared to 126 J cm^{-2} that was required to inactivate an ~2 $\log_{10}$ population on nutrient agar (Section 4.2.4). This suggests that bacteria present on inert surfaces are more susceptible to 405 nm light. It is also possible that bacteria exposed on inert surfaces are sub-lethally stressed, possibly by a desiccation effect and this may make them more susceptible to 405 nm light exposure. Further work is required to determine exactly why susceptibility varies. However in terms of practical applications, the fact that greater inactivation can be achieved on inert surfaces is favourable in respect to environmental decontamination applications.

Following from successful decontamination of surfaces, it was of great interest to investigate the efficacy of 405 nm light for biofilm decontamination. A number of

studies have highlighted that bacterial biofilms can readily form on both glass and plastic surfaces, with production of an extracellular matrix in as little as 4 hours (Gibson et al., 1999; Donlan 2002; Habimana et al., 2009; Koseoglu et al., 2006; Shanks et al., 2005). Although generally any surface is susceptible to biofilm formation, adhesion is dependent upon the physiochemical properties of the surface, such as texture, temperature and hydrophobicity. Some studies have reported stronger initial adhesion between bacteria and hydrophobic surfaces, such as plastics, compared to that of hydrophilic materials (including glass) which may account for initial variations in *E. coli* monolayer biofilm populations on the glass and acrylic. Experimental data from this study highlighted that after 1 hour, *E. coli* attachment to acrylic was greater when compared to that on glass surfaces, However statistical analysis showed this to be insignificant (P= 0.098). Investigation into the 405 nm light inactivation of *E. coli* monolayer biofilms on glass and acrylic demonstrated successful results, with near complete inactivation observed following an applied dose of 168 J cm^{-1}, highlighting the susceptibility of monolayer biofilms to 405 nm light.

Comparison between inactivation efficacies of 405 nm light on *E. coli* seeded surfaces and biofilms has demonstrated, as expected, increased resistance of bacterial biofilm cells. Results in Table 6.1 highlight that, following an applied dose of 36 J cm^{-2}, complete inactivation of a 4.6 log_{10} population was achieved on glass surfaces. In comparison, inactivation of *E. coli* monolayer biofilms grown on glass coupons was considerably more difficult, where minimal reduction was observed following an applied dose of 42 J cm^{-2}, as shown in Table 6.4. Complete inactivation of ~3.55 log_{10} population was achieved after 168 J cm^{-2}, more than four times the dose required for surface inactivation. This result is not surprising due to the presence of the protective matrix around the biofilm cells, which provides physical protection to the enclosed population.

In addition to monolayer biofilms, results demonstrated the successful inactivation of more mature *E. coli* biofilms on both on glass and acrylic surfaces. Population densities of these biofilms ranged from approximately 10^4–10^8 CFU mL^{-1}, with the more densely populated biofilms requiring increasing exposure periods for complete inactivation. Biofilms generated on acrylic surfaces over a 24 hour time period required increased exposure time for complete inactivation when compared to those on glass surfaces,

despite having significantly lower starting bacterial populations (~1.5 $\log_{10}$ CFU mL^{-1} lower). This may be an artefact of the physical adhesive properties between bacteria and specific materials. Chmieleweski and Frank reported that although initial bacterial adherence to hydrophobic surfaces is likely to be stronger, greater maximum bacterial adhesion is achieved on hydrophilic surfaces with high free surface energy allowing for generation of denser biofilm populations (Chmielewski and Frank 2003; Hyde et al., 1997; Blackman and Frank 1996). This information correlates with data shown in this study, with results demonstrating greater adhesion and increased biofilm formation on hydrophilic glass surfaces, following the development of mature biofilm structures. Results highlighted that mature biofilms developed over 24 and 48 hour periods had bacterial densities of approximately 4.5 $\log_{10}$ versus 6 $\log_{10}$ CFU mL^{-1} and 5 $\log_{10}$ versus 8 $\log_{10}$ CFU mL^{-1} for hydrophobic acrylic and hydrophilic glass surfaces, respectively.

Bacterial biofilms generated on both glass and acrylic surfaces over 48 and 72 hours appeared to have similar population densities, suggesting that after the 48 hour growth period, bacterial attachment was maximised and had consequently plateaued. This may be attributed to a lack of nutrients present in the growth media after extended time periods, suggesting that these media must be replenished to generate increased biofilm populations.

Successful inactivation of bacterial biofilms on the underside of the glass and acrylic surfaces was also shown, demonstrating the ability of the 405 nm light to transmit through these transparent materials whilst maintaining its antimicrobial activity. With regards to the results of the Weibull analysis, the difference in the inactivation behaviour of monolayer biofilms could potentially be attributed to the different degree of adhesion of microorganism to these acrylic and glass surfaces, with the hydrophobic and hydrophilic interactions between the monolayer biofilms and the surfaces to some extent influencing the inactivation behaviour. However, mature, 24 h, 48 h, and 72 h biofilms did not show similar tendencies; their inactivation curves, shown in Figure 6.6, demonstrate almost linear behaviour and cannot be fitted with the Weibull's curves. Therefore, it is possible to conclude that, with an increase in biofilm 'age' (biofilm thickness and/or number of microorganisms) the influence of substrate material on the inactivation process becomes significantly reduced or disappears completely. The

present work is aimed only at identification of potential differences in the inactivation behaviour and does not involve a full-scale statistical analysis which requires larger number of experimental data points (further data would be needed in order to validate the proposed statistical model).

A variety of methods have been used for biofilm sampling in previous studies including sonication and swabbing (Patted et al., 2013; Gibbert et al., 2010; Schaule et al., 2000; Gibson et al., 1999). Despite all being successful and well-utilised methods for recovering microorganisms, each presents its own limitations. Swabbing is a widely used and recognised method for sampling bacterial contamination within health care and food industrial settings as well as for recovery for bacterial biofilms in laboratory experiments (Patted et al., 2013; Gibbert et al 2010; Gibson et al., 1999). This method was used throughout this study as a viable and effective technique for bacterial biofilm removal from all surfaces. Regardless of the limitations (ie. incomplete removal of all microorganisms) associated with swabbing for bacterial removal, exposed and non-exposed samples in this study were recovered identically using a standard swabbing technique, allowing for directly comparable results.

The methodology for biofilm formation used in this study was adapted from previous work by Gibson and colleagues (Gibson et al., 1991). The possibility of including a rinsing stage prior to light exposure to ensure all non-attached microorganisms were removed was investigated. Following the required time period in growth media, slides were submerged into sterile water, removed and the process repeated again in fresh sterile water, to ensure complete removal of non-attached cells. Results demonstrated that there was no significant difference between biofilm populations on rinsed and non-rinsed slides, for both monolayer and more mature biofilms ($P>0.08$). Rinsing of test surfaces provided conclusive evidence of biofilm formation, as non or weakly attached cells would have been removed during the rinsing stage. Non-significant reduction in bacterial count following rinsing suggested that bacteria were protected, most likely by the presence of an exopolysaccharide matrix, which has been shown to protect cells concealed within biofilm layers from harsh environmental conditions such as flowing water (Schwartz et al., 2003).

Looking at the results in Table 6.4, for the inactivation of the four different bacterial species, it can be seen that successful inactivation was achieved with all organisms, with approximate reductions of 3.6 and 2.6 $\log_{10}$ CFU mL^{-1} for Gram-negative and Gram-positive biofilm populations, respectively. However it is difficult to directly compare the efficacy of 405 nm light for the inactivation of the different bacterial species due to the differences in starting populations observed within the monolayer biofilms. The methodology for preparing the biofilms was consistent for the different bacterial species, but resulted in the generation of varying populations, possibly reflecting differences in propensity for attachment and/or the rate of multiplication of the attached populations. Variance in bacterial inactivation between the Gram positive and Gram negative species, as shown in Tables 6.4 and 6.5, is likely to have been a direct effect of the increased adherence of the Gram-positive cells causing increased starting populations, and consequently requiring a greater exposure for complete inactivation, compared to the lower populated Gram negative biofilms. However, recent data published by Murdoch et al. (2012) showed that even at similar starting populations, surface-seeded Gram negative *Salmonella enterica* was inactivated 30% more effectively than Gram-positive *L. monocytogenes* when exposed to 405 nm light whilst seeded onto plastic surfaces (Murdoch et al., 2012).

When the mixed-species biofilms were exposed to 405 nm light, successful inactivation was achieved, with a 2.2 $\log_{10}$ CFU mL^{-1} reduction in total population demonstrated. Use of selective media also allowed assessment of the specific populations of *E. coli* and *S. aureus* within the mixed-species biofilm. VRBA and MSA were chosen for this purpose as they facilitated the selective isolation of *E. coli* and *S. aureus*, respectively (Brisdon 1998). Microscopic examination of the mixed-species biofilm (Figure 6.8) highlighted that biofilm distribution was not linear across the entire surface area, but instead displayed many large cellular communities with individual cells dispersed randomly. Results demonstrated that *S. aureus* was the predominant organism to colonise the biofilm, however, successful inactivation of both bacterial species was achieved, demonstrated by significant population reductions for both species. It is interesting to note that, although *S. aureus* was shown to be the predominant coloniser, initial colonisation appeared dominated by *E. coli* cells, with attachment of *S. aureus* largely present on top of previously colonised *E. coli* populations. This observation

suggested that *E. coli* may act as a primary coloniser during biofilm development, highlighting possible roles of cellular interactions during biofilm formation. Although little is understood about intracellular signalling in mixed-species biofilms, it is clear that cell-cell communication (sometimes referred to as quorum sensing) plays a major role in biofilm development. A study by Davies et al. (1998) demonstrated that *P. aeruginosa* deficient in the quorum sensor inducing gene *lasI* produced biofilms only 20% as thick as those produced by wild type strains (Davies et al., 1998). It is likely that bacteria, in this case *E. coli*, are able to communicate with other bacteria and recruit them for a more densely populated, heterogeneous biofilm. The reason as to why *E. coli* can act as an initial coloniser over *S. aureus* is not yet apparent. However, as mentioned previously, the physiochemical properties of the surface play a significant role in bacterial attachment, therefore adhesion ability may be variable between species, depending on each of their independent structures.

As discussed, the antimicrobial activity of 405 nm light can be attributed to excitation of porphyrin molecules within the cell, leading to production of ROS and oxidative cellular damage (Maclean et al., 2008a; Hamblin and Hassan 2004). Recent studies have suggested oxidative damage may directly affect cellular membranes, resulting in reduced membrane stability (Wasson et al., 2012). Interference with the cell membrane and its components may consequently reduce biofilm stability, leading to biofilm degradation through bacterial inability to remain attached to surfaces. It may also be plausible that following 405 nm light exposure, alterations in structural membrane components may possibly prevent cellular attachment and thus inhibit biofilm formation. Although this study simply investigated the effects of 405 nm light on the viability of bacterial biofilms once attached to glass and acrylic surfaces, it would be of great interest to investigate the degradative properties of 405 nm light on bacterial biofilms as well as investigating the specific effects of 405 nm light on cellular adhesion. A previous study by Mussi et al. (2010) investigated the use of longer blue light wavelengths (462 nm) for inhibition of biofilm formation. Studies have shown that many bacteria possess blue light receptors capable of causing photo-regulated behaviour upon exposure to blue light, and it has been suggested that direct interactions between blue light and their receptors may have inhibitory effects on biofilm formation (Dai et al., 2012; Mussi et al., 2010). However, Mussi and colleagues stated that the

effect of light on biofilm formation was inconclusive; highlighting that further work is required to identify any relationship between blue light, blue light receptors and biofilm formation.

The light irradiance produced by the LED array used in this study has a Gaussian distribution, as with many LED-based light systems (Molerno and Sun 2008). The highest irradiance is found directly below the centre of the LED array distributing gradually towards the outer edges. Irradiance over the entire test slide was measured (as shown in Figure 6.3) and the average irradiance was calculated. Although there was a large difference in the measured irradiance at the centre and the outer edges of the slide, the fact that (i) complete inactivation of the biofilm populations could be achieved and (ii) swabs were used to recover bacteria from the entire slide not just the centre point, demonstrated that the inactivation effect was achieved across the entire slide, regardless of the non-uniformity of the light exposure.

The use of light for biofilm decontamination has been extensively investigated, with particular focus on UV-light due to its highly bactericidal properties (Li 2011; Andersen et al., 2006). Although UV-A is partially transmissible through glass and plastic materials, short wavelengths in the UV spectrum (200-280 nm) possesses poor penetration ability, when compared to light in the visible spectrum (Black 2012). Evidence has suggested that UV-C radiation is largely absorbed by organic materials such as biofilms, resulting in poor penetration of light and insufficient decontamination (Andersen et al., 2006; Matsumura and Ananthaswamy 2004). This is unlike 405 nm light treatments, which can better penetrate through the extracellular matrix to inactivate the enclosed bacterial populations, due to the longer wavelength of the photons.

Regular and thorough disinfection is regarded as a key control measure to help prevent cells irreversibly attaching to surfaces and inhibit biofilm formation. As discussed in Chapter 2, coating surfaces with antimicrobial agents can reduce bacterial adhesion and prevent biofilm formation (Knetsch and Koole 2011; Srey et al., 2013). Also, a number of studies have shown that pre-conditioning surfaces with a surfactant can prevent bacterial adhesion (Chen 2012; Choi et al., 2011). Various antimicrobial chemicals agents have been used in the biofilm disinfection process; but their efficacy is often limited by the presence of organic materials including fat and blood. There are currently

numerous types of disinfecting agents that are used in the food industry to help control biofilms, including hydrogen peroxide, chlorine, ozone and peracetic acid (Chmielewski and Frank, 2007). Hydrogen peroxide (H_2O_2) is one of the most widely-used disinfectants for biofilm treatment due to its high oxidizing capabilities that can directly affect not only the enclosed bacteria but the biofilm matrix (de Carvalho 2007). H_2O_2 is also known to be capable of inhibiting biofilm formation at relatively low concentrations (0.05%) and can also inactivate mature biofilms (5-7 $\log_{10}$ CFU mL^{-1}) at concentrations of ~0.1-0.2% (Shikongo-Nambabi 2010).

Much research has also demonstrated the use of ozone against biofilms, again due to its high oxidation potential (Tachikawa et al., 2009). However, there are a number of limitations associated with this technology. For example, ozone is known to be very corrosive so that corrosion resistant surfaces such as stainless steel must be in place. Toxicity and irritation to workers is another major concern. Furthermore the cost of treatment can be high with respect to both capital investment and power consumption.

The use of sonication for control of biofilms has also been extensively investigated (Oulahal- Lagsir et al 2000). Ultra-sound treatment involves applying high frequency sound waves (>20 kHz) to agitate and dislodge particles from surfaces, in this case microorganisms. Sonication can cause bacteria to cavitate, whereby a vacuum can form in the liquid causing denaturation of proteins and disintegration of bacterial cells (Black 2012). However despite the fact that sonication can reduce cell viability, it is unlikely bacteria can solely be eliminated using ultrasonic technologies and therefore other treatments must be applied in order to create a synergistic effect.

In summary, this study has demonstrated the use of 405 nm light for successful decontamination of bacteria on a range of surface materials. This study has also demonstrated, for the first time, the use of 405 nm light for biofilm decontamination. Biofilms of varying maturity and also of varying bacterial species have been shown to be susceptible to inactivation, demonstrating the ability of the 405 nm light to inactivate even densely populated biofilm communities. The ability to inactivate bacterial populations present on inert surfaces using 405 nm light is of great significance. The use of 405 nm light in industrial and domestic settings may have potential to help reduce microbial populations on surfaces help prevent biofilm formation. However, it must be

emphasised that physical cleaning is still required to remove residual microbial contamination. The role of 405 nm light in the food industry would-be supplementary and must not be expected to replace current cleaning protocol, with the combined synergy of 405 nm light and chemical cleaning should be advantageous for enhanced microbial inactivation. Synergistic bactericidal effects will be discussed in subsequent chapters.

CHAPTER 7

SYNERGISTIC BACTERICIDAL EFFECT OF 405 NM LIGHT WHEN COMBINED WITH SUB-LETHAL TEMPERATURE, OSMOTIC AND ACID STRESS CONDITIONS

7.0 General

The requirement for foods to present longer shelf lives has led to increased preservation, processing and decontamination methods being utilised for ensuring minimal microbial presence (Loretz et al., 2009). During food processing, microorganisms are exposed to a vast array of physical and chemical stresses including various temperatures, pH and osmotic concentrations. In many instances these altered conditions can be regarded as sub-lethal stress factors, often referred to as hurdles (Dykes and Withers., 1999; Lou and Yousef., 1997).

Hurdle technology is a global system employed by the food industry for ensuring the safety of foods through reduction or elimination of microorganisms. During food processing microbial populations may be exposed to single or multiple stresses, either simultaneously or sequentially, including both high and low temperatures, water availability, acidity, competitive microorganisms and preservatives such as sodium chloride, sulphite and sorbate (Leistner 2000).

Application of single hurdles may cause only a bacteriostatic effect, preventing further replication and growth of an established bacterial community. Under extreme stress conditions bactericidal effects can occur, causing complete destruction of bacterial populations. However, such extremities of a single stress may alter or compromise the nutritional value and organoleptic attributes of food stuffs (Beales 2003). Consequently, the production of safe foods currently employs multiple hurdles throughout the food production process, resulting in multi-target action on the cell, whereby each is critical

for the elimination of microbial populations, without inducing detrimental sensory properties.

Surviving bacteria within food processing environments may be in a stressed sub-lethally injured state, and it is important to understand how these bacteria, as well as non-stressed bacteria, will respond to 405 nm light treatment. This chapter will investigate the synergistic inactivation effects of 405 nm light on the significant food pathogens, *Escherichia coli* and *Listeria monocytogenes*, when exposed under environmental stress conditions (temperature, osmotic and heat).

7.1 405 nm light Inactivation of Non-stressed Bacteria

Prior to inactivation under stress conditions, it was necessary to establish inactivation kinetics of both *E. coli* and *L. monocytogenes* under normal non-stressed conditions, where bacteria were exposed in PBS (pH 7.2 and 0.8% salt concentration) at room temperature (~22°C).

7.1.1 Bacterial preparation

E. coli and *L. monocytogenes* were used throughout this series of work. *E. coli* and *L. monocytogenes* were cultured in 100 mL of nutrient broth and tryptone soya broth, respectively, at 37°C for 18-24 hours and then centrifuged at 3939 ×g for 10 minutes in order to produce a bacterial pellet (Section 3.0.2 and 3.3.1). The resultant pellets were re-suspended in 100 mL PBS and diluted to a starting population of ~1x10^5 CFU mL^{-1}.

7.1.2 Treatment method

A 3 mL volume of 10^5 CFU mL^{-1} bacterial suspension was transferred into a central well of a 12-well multi-dish. A 99 DIE 405 nm LED Array (light source C) was used for all experimental work in this section, with an irradiance of approximately 70 mW cm^{-2} used for all exposures. The LED array was mounted onto a PVC housing designed to fit over the micro plate, with the array sitting directly above the sample at an approximate distance of 2 cm. Test samples were exposed to increasing doses of 405 nm light. Post-exposure, samples were plated onto nutrient agar or tryptone soya agar, for *E. coli* and *L. monocytogenes*, respectively, and enumerated as described in

Section 3.3.2. Control samples were set up simultaneously under identical environmental conditions but without 405 nm light exposure.

7.1.3 Results: 405 nm light inactivation of non-stressed *E. coli* and *L. monocytogenes*

The inactivation data for *E. coli* and *L. monocytogenes* under non-stressed conditions are shown in Figure 7.1. Significant inactivation of *L. monocytogenes* was observed following an applied dose of 63 J cm^{-2}, where an approximate 0.6 log_{10} CFU mL^{-1} reduction was observed (P=0.00). Complete inactivation of *L. monocytogenes* was observed following 84 J cm^{-2} of 405 nm light (20 minutes at 70 mW cm^{-2}). Inactivation of *E. coli* required a much greater dose than *L. monocytogenes*. Significant inactivation of *E. coli* was not observed until exposure to a dose of 189 J cm^{-2} (0.6 log_{10} reduction; P=0.00). Inactivation continued with increased doses of 405 nm light, and complete inactivation of a 5 log_{10} CFU mL^{-1} population was achieved following treatment with 378 J cm^{-2} (90 minutes at 70 mW cm^{-2}). This data provided baseline inactivation curves for non-stressed populations.

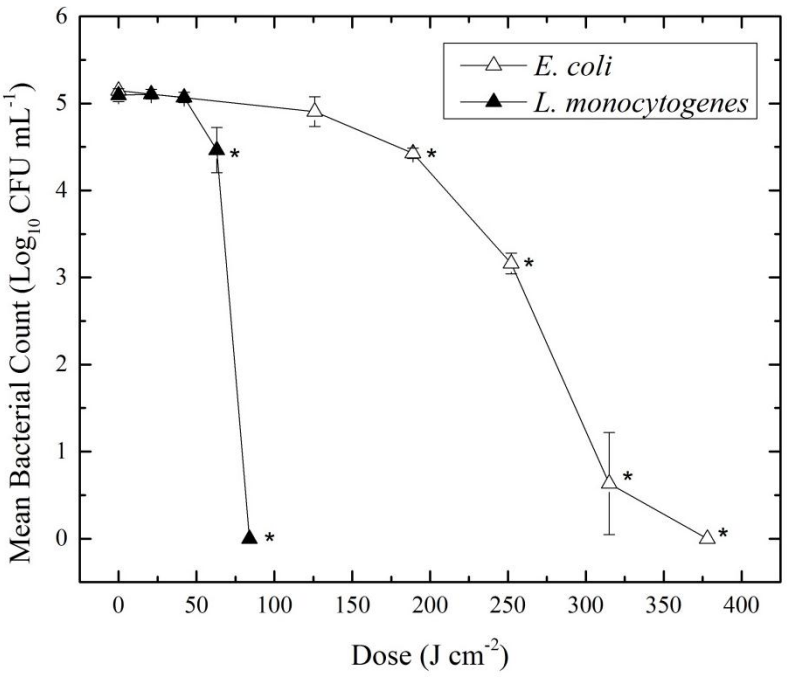

Figure 7.1 *Inactivation of non-stressed E. coli and L. monocytogenes by exposure to 405 nm light (70 mW cm^{-2} irradiance). Bacteria were exposed in PBS (pH 7.2 and 0.8% salt concentration) at room temperature. * indicates statistically significant differences when compared to control samples (P≤0.05)*

7.2 Assessment of Sub-lethal Stressing of Bacterial Population

To investigate the degree of stress induced by temperature, osmotic and acid conditions on *L. monocytogenes* and *E. coli*, experiments were conducted to expose bacterial suspensions to various conditions (without 405 nm light treatment). Bacterial suspensions were held under the altered environmental conditions for the maximum time required to cause complete bacterial inactivation when exposed to 70 mW cm^{-2} 405 nm light under non-stressed conditions (i.e. 20 minutes for *L. monocytogenes* and 90 minutes for *E. coli*), as shown in Figure 7.1.

The aims of this were to:

1. Identify environmental conditions which would cause no significant effect on the bacterial populations, then;
2. Quantify the level of the sub-lethal damage caused by these stresses.

Environmental conditions which induced sub-lethal stress on the bacterial populations will be utilised in later light-exposure experiments (Sections 7.3 and 7.4) to investigate the synergistic effects of 405 nm light with these sub-lethal temperature, osmotic and acid stresses.

7.2.1 Temperature stress: Determination of non-lethal stress levels

The temperatures selected for investigation were 4°C and 45°C, and are representative of temperatures used in food production and storage (Charalampopoulos and Webb 2013; Pereira et al., 2011). 3 mL samples of 10^5 CFU mL^{-1} bacterial suspensions were prepared and held at 4°C (in a refrigerator) and 45°C (in an incubator) for 20 and 90 minutes, for *L. monocytogenes* and *E. coli*, respectively. Samples were then plated onto NA/TSA for *E. coli* and, *L. monocytogenes*, respectively, and incubated overnight as described in Section 3.4.2.

Results demonstrated that neither temperature resulted in significant decrease of the bacterial populations (P=0.00). Both 4°C and 44°C were selected for use in future experimental work.

7.2.2 Acid Stress: Determination of non-lethal stress levels

The concentration of citric acid selected for investigation was representative of those typically utilised for preservation purposes within the food industry. To identify acid-stress conditions which caused non-lethal effects on the bacterial populations, 3 mL samples of bacteria were suspended in PBS acidified to pH 2.5, 3, 3.5, and 4, using 1% citric acid (Sigma Aldrich, UK). The pH was measured using a pH meter and probe Samples, with a population density of 10^5 CFU mL^{-1}, were left at room temperature for 20 and 90 minutes (for *L. monocytogenes* and *E. coli*, respectively) to determine the bactericidal effects of the acid alone. Samples were plated onto (NA/TSA for *E. coli* and, *L. monocytogenes*, respectively) and incubated overnight as described in Table 3.2.

Results shown in Table 7.1 show that negligible inactivation occured when both bacterial species were held at pH 2.5, pH 3 and pH 3.5. Consequently pH 3 and pH 3.5were selected for acid stresses.

7.2.3 Osmotic Stress: Determination of non-lethal stress levels

Osmotic conditions investigated were selected to represent the upper concentration of salt used during food processing (Albarracin et al., 2011; Knorr 1998). To identify appropriate osmotic concentrations which caused non-lethal effects on the bacterial populations, bacteria were suspended in water at various salt (NaCl; Sigma Aldrich, UK) concentrations: 0% (distilled water), 0.8% (PBS), 1%, 10% and 15%. Samples with a population density of 10^5 CFU mL^{-1} were held at room temperature for 20 and 90 minutes (for *L. monocytogenes* and *E. coli*, respectively) to investigate the effects of osmotic stress alone. Samples were plated onto NA/TSA for *E. coli* and, *L. monocytogenes*, respectively and incubated overnight as described in Table 3.2.

Results showed that no significant decrease in bacterial population was observed for any of the five salt concentrations investigated (Table 7.1). Therefore, the two highest concentrations, 10% and 15%, were selected for further use.

Table 7.1. *Bacterial populations (~5 log_{10} CFU mL^{-1}) held at various salt and acid stress conditions for 90 minutes (E. coli) and 20 minute (L. monocytogenes). These time periods represent the exposure times required to achieve complete inactivation of each organism when exposed to 405 nm light (~70 mW cm^{-2}) under non stressed conditions. * represents significant inactivation where P≤0.05. Conditions selected for experimental use are highlighted as shaded rows.*

Stress		***E.coli*** **(Log_{10} CFU mL^{-1})**	***L. monocytogenes*** **(Log_{10} CFU mL^{-1})**
		90 mins stress exposure	**20 mins stress exposure**
Temperature	22° C [a]	5.06(±0.09)	5.01(±0.03)
	4° C	5.08(±0.07)	5.05(±0.05)
	45° C	5.05(±0.07)	5.09(±0.02)
Salt (%)	0	5.05(±0.07)	5.06(±0.07)
	0.8[a]	5.06(±0.03)	5.09(±0.07)
	1	5.04(±0.1)	4.96(±0)
	10	5.08(±0.14)	5.04(±0.01)
	15	5.11(±0.1)	5.03(±0)
Acid (pH)	7.2[a]	5.06(±0.03)	5.03(±0.07)
	4	5.06(±0.11)	5.02(±0.08)
	3.5	5.08(±0.05)	5.04(±0.06)
	3	5.0(±0.07)	4.79(±0.01)*
	2.5	1.38(±0.12)*	2.49(±0.15)*

[a] Bacteria exposed at standard conditions (PBS and room temperature)

7.2.4 Identification of sub-lethally damaged bacteria

Results in Table 7.1 demonstrate that the conditions selected did not induce lethal damage to the bacterial population. However, in order to demonstrate whether the stress conditions selected above induced sub-lethal damage, bacterial suspensions were held under the selected temperature, osmotic and acidic stress conditions for 45 and 90 minutes (*E. coli)* and 10 and 20 minutes (*L. monocytogenes),* and were plated onto both non-selective and selective agars. As discussed in Section 4.1.4, evidence of sub-lethal injury can be ascertained from the difference in counts obtained from growth on selective and non-selective media, where sub-lethally damaged bacteria are unable to grow on selective media. This will be further discussed in Section 7.5.

7.2.5 Results: Sub-lethal damage of *E. coli* and *L. monocytogenes* exposed to various temperature, osmotic and acid conditions

During preliminary studies, temperature, salt and acidity conditions that exerted no significantly detrimental effects on populations of *E. coli* and *L. monocytogenes* over the respective exposure times were determined (Table 7.1). This subsequent study investigated the extent of sub-lethal damage which was exerted onto the microbial populations under the same conditions. Confirmation of sub-lethal damage was evidenced by the significantly reduced counts shown by the stressed populations when plated onto selective media (Violet red bile agar for *E. coli* and *Listeria* selective agar for *L. monocytogenes*), shown in Figures 7.2 and 7.3, respectively. Results shown in Figure 7.2 for the sub-lethal damage of *E. coli* highlight significant differences in bacterial counts between selective and non-selective media in all cases when exposed under all temperature, acid and osmotic conditions. During osmotic stress at 15% salt *E. coli* demonstrated the greatest level of sub-lethal damage, with ~ 0.6 $\log_{10}$ CFU mL^{-1} reduction shown after only 45 minutes, and ~0.82 $\log_{10}$ CFU mL^{-1} difference observed when comparing selective and non-selective counts, following 90 minutes treatment. Results also show that when held at 4°C, *E. coli* demonstrated least sub-lethal damage when compared to all other conditions, with only a 0.4 $\log_{10}$ CFU mL^{-1} difference observed between selective and non-selective populations after 90 minutes.

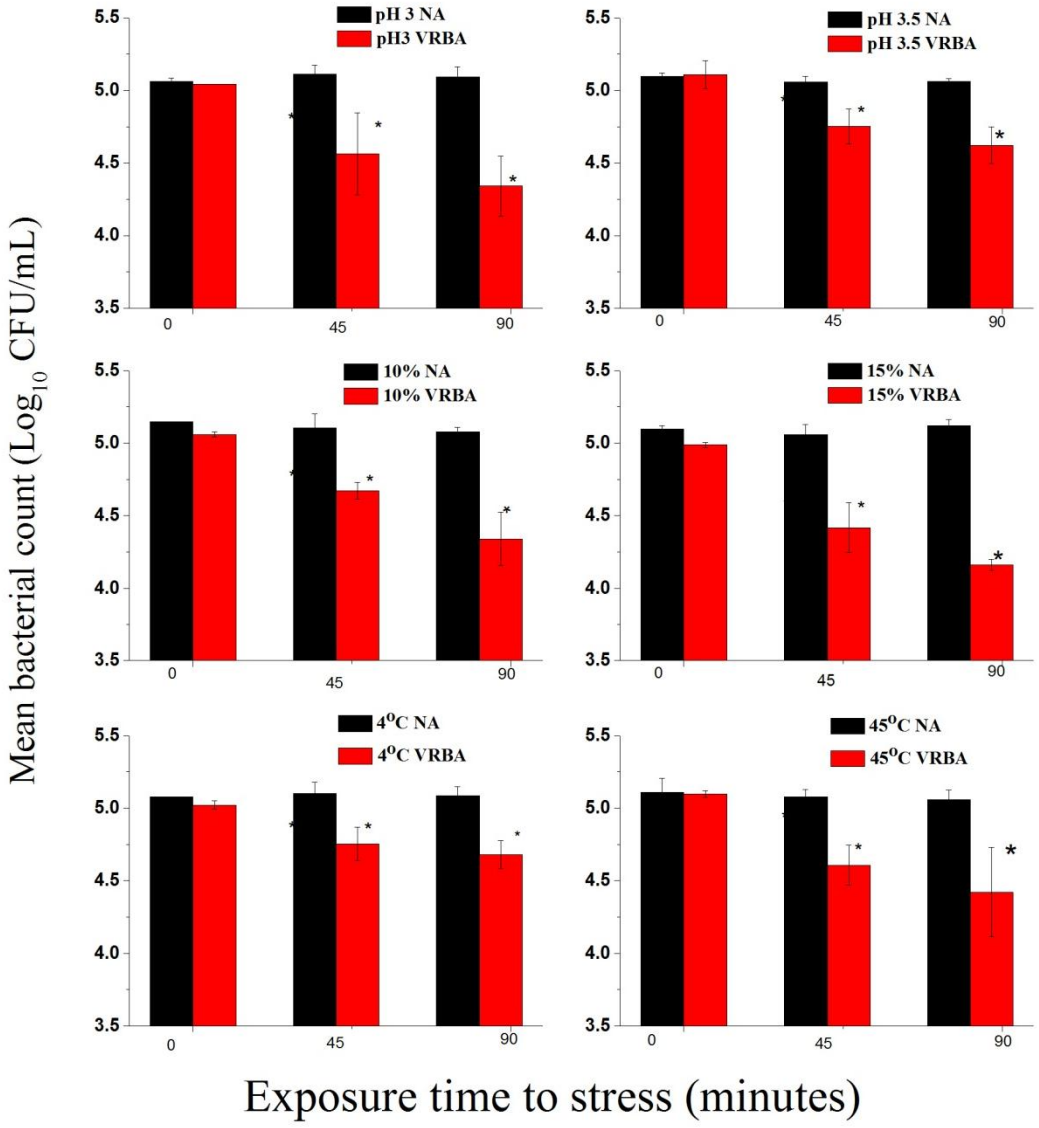

Figure 7.2. *Identification of sub-lethally damaged E. coli populations following exposure to various environmental conditions. Sub-lethal damage was quantified by comparing bacterial counts on selective media (violet red bile agar) and non-selective media (nutrient agar). *indicates statistically significant differences (P≤0.05) between bacterial populations on selective and non-selective media.*

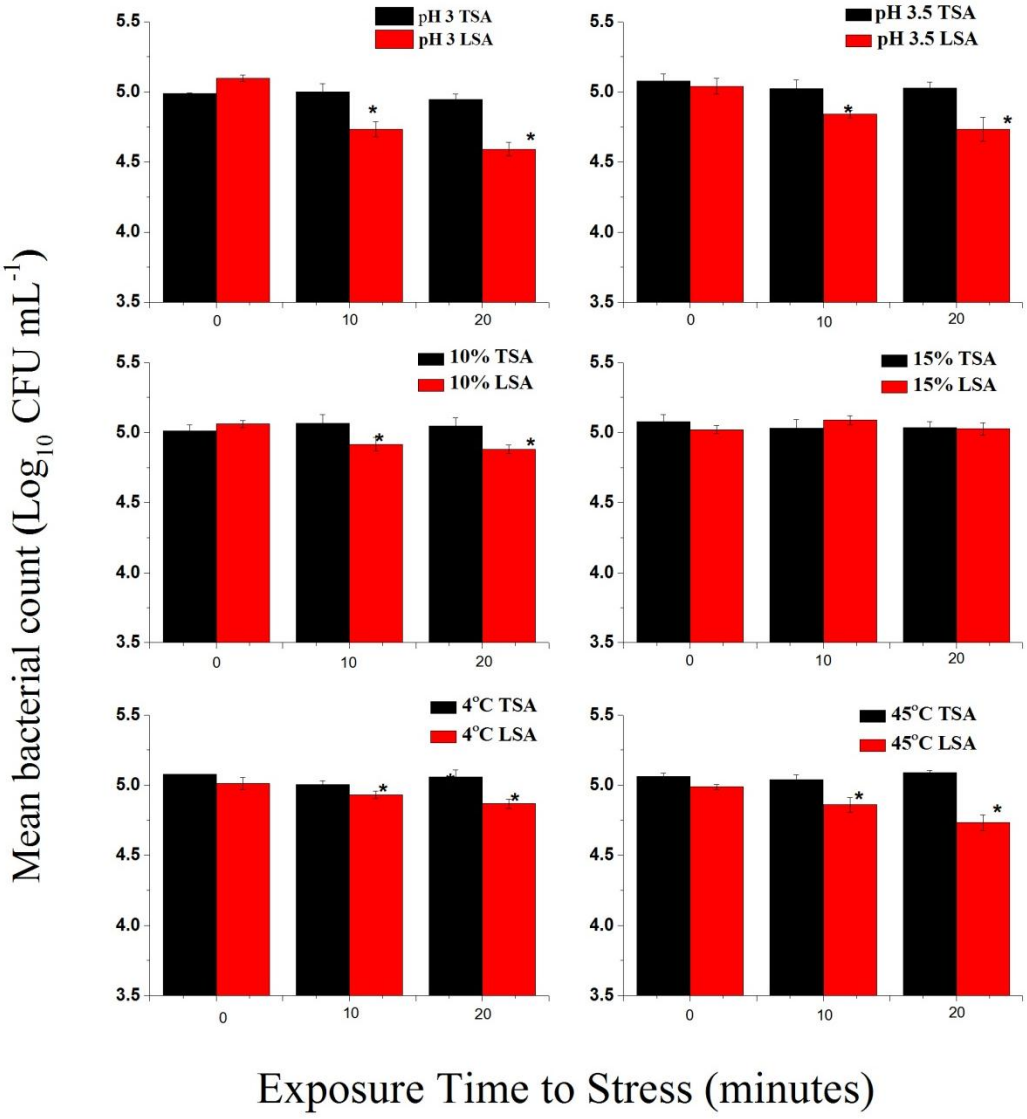

Figure 7.3. *Identification of sub-lethally damaged L. monocytogenes populations following exposure to various environmental conditions. Sub-lethal damage was quantified by comparing bacterial counts on selective media (violet red bile agar) and non-selective media (nutrient agar). *indicates statistically significant differences (P≤0.05) between bacterial populations on selective and non-selective media.*

Results in Figure 7.3 show sub-lethal damage of *L. monocytogenes* under various temperature, osmotic and acidic conditions. When compared to the results achieved for *E. coli*, it is apparent that sub-lethal damage in *L. monocytogenes* is less prominent;.

However, significant differences between selective and non-selective counts is shown for all stress conditions, demonstrating a sub-lethal effect, with the exception of 15% osmotic stress, which displayed no significant differences between counts. Greatest sub-lethal damage was observed following 20 minutes under acidic conditions (pH 3), whereby a 0.5 $\log_{10}$ CFU mL^{-1} difference was recorded.

Figure 7.4 illustrates variation in the bacterial counts when plated onto selective and non-selective media following exposure to acid stress (*L. monocytogenes*) and salt stress (*E. coli*). Figure 7.4 provides a general representation of the differences observed throughout the study when plating onto selective and non-selective media.

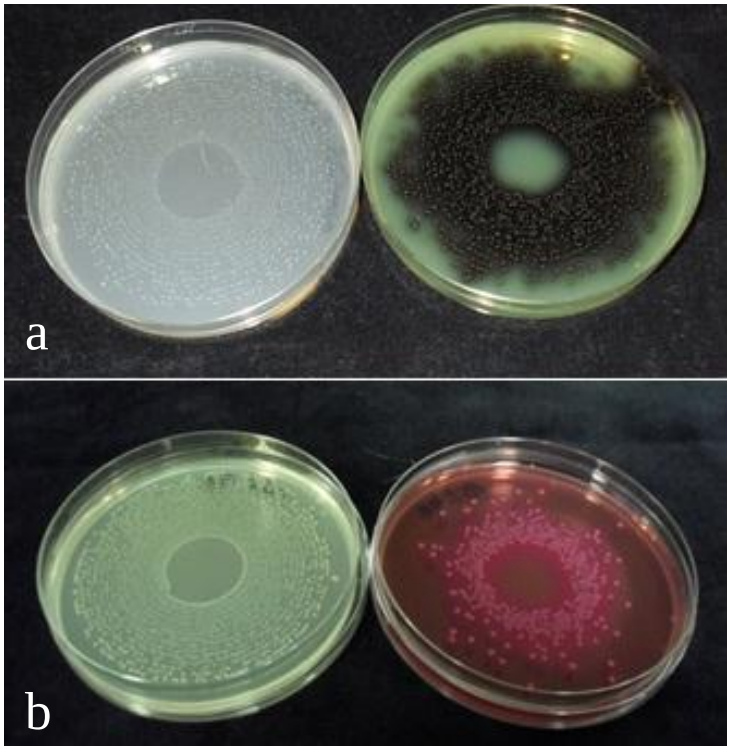

Figure 7.4 *Visual representation of variations in (a) L. monocytogenes and (b) E. coli populations when plated on to selective (right) and non-selective (left) agar following stress treatment. Listeria selective agar for selective isolation of L. monocytogenes displayed pale green colonies surrounded by black zones, due to hydrolyses of aesculin, Selective violet red bile agar for the selective growth of E.coli produced pink colonies due to fermentation of lactose. Sub-lethal damage is evidenced by the decreased bacteria counts evident on the selective media.*

7.3 405 nm light Inactivation of Bacteria under Sub-lethal Stress

Following assessment of the sub-lethal damage induced by various temperatures, osmotic and acidic conditions, *E. coli* and *L. monocytogenes* were simultaneously exposed to these sub-lethal conditions whilst exposed to 405 nm light treatment in order to assess the synergistic bactericidal efficacy of 405 nm light for bacterial inactivation under sub-lethal environmental conditions.

7.3.1 Treatment method

Stressed bacterial suspensions were prepared and exposed to 405 nm light under the following conditions:

- Temperature stress: 4°C and 45°C
- Acid stress: pH 3 and pH 3.5
- Osmotic stress: 0%, 10% and 15%

For light treatment, 3 mL volumes of 10^5 CFU mL^{-1} bacterial suspension were transferred into the central well of a 12-well multi-dish and exposed to increasing doses of 405 nm light as described in Section 7.1.2.

To investigate the synergy between 405 nm light and temperature stress, samples were exposed either in a refrigerator at 4°C, or in an incubator set at 45°C. Samples were exposed to 405 nm light and kinetics were then compared to inactivation kinetics of non-stressed bacteria exposed at room temperature (Figure 7.1).

For acid stress, bacterial suspensions at pH 3 and pH 3.5 were exposed to increasing doses of 405 nm light and results compared to the inactivation curves for non-stressed organisms exposed in PBS at pH 7.2 (Figure 7.1).

In the case of osmotic stress, bacterial suspensions in 0%, 10% and 15% salt solution were exposed to 405 nm light and results were compared to the kinetics for non-stressed organisms exposed in PBS at 0.8% salt concentration (Figure 7.1).

Control samples were set up for each experiment under identical environmental conditions, but without 405 nm light illumination. Post exposure, bacterial samples

were collected and plated onto nutrient agar (*E. coli*) and tryptone soya agar (*L. monocytogenes*) as detailed in Section 7.1.2.

7.3.2 Results: 405 nm light inactivation of sub-lethally stressed bacteria

The following section details the inactivation results of sub-lethally stressed bacterial populations simultaneously exposed to 405 nm light.

7.3.2.1 405 nm light inactivation of bacteria under temperature stress conditions

Tests were carried out on *E. coli* (Figure 7.5a) and *L. monocytogenes* (Figure 7.5b) to determine if cells held under temperature stress conditions were more susceptible to 405 nm light inactivation than non-stressed cells. With both species, inactivation rates were significantly greater at the 4°C and 45°C stress temperatures than at the non-stress temperature of 22°C. *E. coli* was shown to be most susceptible to 405 nm light treatment when exposed at a lower temperature of 4°C. Results in Figure 7.5a show that complete inactivation of *E. coli* was achieved following an applied dose of 252 J cm^{-2}, whereas for non-stressed bacterial populations an applied dose of 378 J cm^{-2} was required to promote complete inactivation. Results demonstrated similar trends in inactivation between tests conducted at 4°C and 45°C up to 189 J cm^{-2}, both displaying enhanced inactivation when compared to non-stressed populations. However, complete inactivation of tests conducted at 45°C was not achieved until 378 J cm^{-2}.

The temperature-enhanced light inactivation results were particularly striking for *L. monocytogenes* (Figure 7.5b). Under temperature stress conditions inactivation rates significantly increased. Complete inactivation at 45°C required approximately 50% less dose than that required for complete inactivation at 22°C (42 J cm^{-2} as compared with a dose of 84 J cm^{-2}). Although less evident than at 45°C, significantly enhanced inactivation of *L. monocytogenes* was also achieved at 4°C following 63 J cm^{-2}.

Population densities of non-exposed control samples held at the sub-lethal stress temperatures of 4°C and 45°C demonstrated no significant reduction when plated onto non-selective media, over the duration of the experiment for both *E. coli* and *L. monocytogenes* (P=0.39, 0.32 and 0.17, 0.23 for each bacteria respectively, at 4°C

and 45°C), indicating inactivation was a direct result of 405 nm light exposure and that the temperature stresses alone were non-lethal.

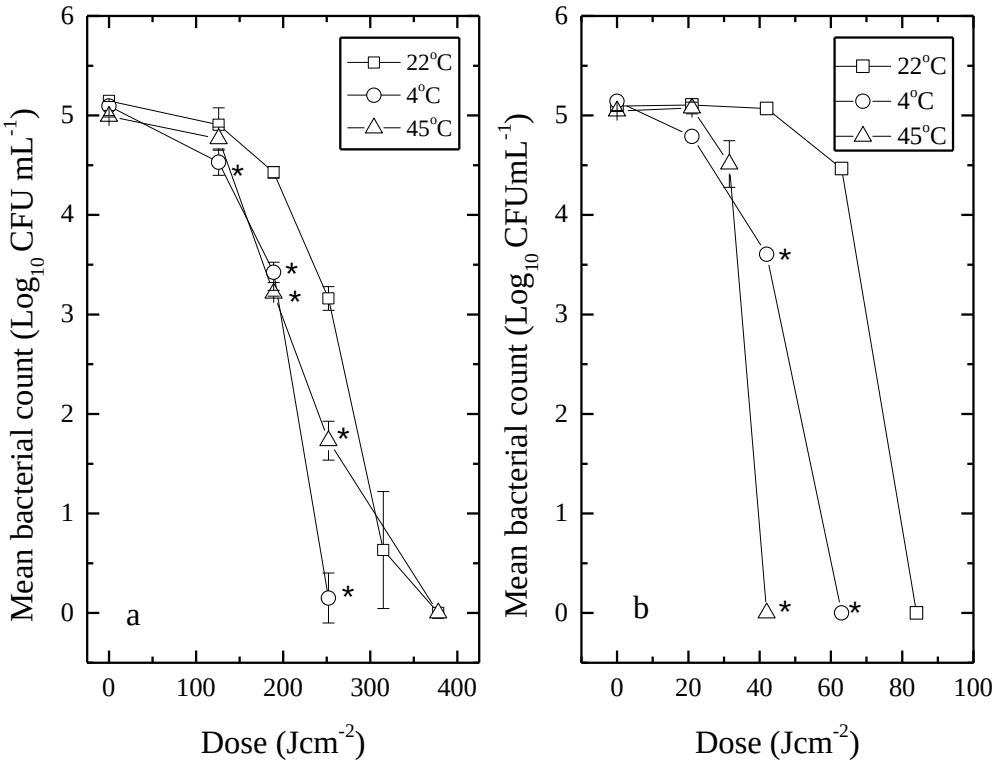

Figure 7.5 *Inactivation of (a) E. coli and (b) L. monocytogenes by exposure to 405 nm light (70 mW cm^{-2}) combined with temperature stress. Bacterial populations were suspended in PBS and exposed to 405 nm light at 4°C and 45°C. Data for exposure at 22°C (room temperature) provided a non-stressed comparison. * indicates data points which have significantly increased bacterial inactivation (P≤0.05) compared to non-stressed populations (22°C). No significant changes were observed in non-light exposed control samples (P=>0.05).*

7.3.2.2 405 nm light inactivation of bacteria under acid stress conditions

The results in Figure 7.6 demonstrate the enhanced inactivation of both *E. coli* and *L. monocytogenes* when exposed to 405 nm light under sub-lethal acid conditions. Results for *E. coli* (Figure 7.6a) show that inactivation rates were greatly increased, with a complete inactivation of *E. coli* at pH 3.5 achieved following a dose 200 J cm^{-2} (approximately 50% less than the dose necessary for inactivation of similar levels of non-acid stressed *E. coli)*. When acidity was increased to pH 3, the susceptibility of

E. coli to 405 nm light was further increased, with complete inactivation of the 5 $\log_{10}$ population achieved after 84 J cm^{-2} (50% of the dose required for complete inactivation at pH 3.5 and 25% of that necessary for complete inactivation of non-acid stressed *E. coli*).

The results for *L. monocytogenes* (Figure 7.6b) demonstrate similar synergistic effects, to those observed for *E. coli*, albeit at considerably reduced light dose levels. Significantly greater inactivation of *L. monocytogenes* occurred at pH 3.5 with complete inactivation achieved after only 63 J cm^{-2} (under non-stressed conditions (pH 7) only an approximate 0.5 $\log_{10}$ CFU mL^{-1} reduction was observed at the same dose). When acidity was further increased to pH 3, inactivation rates were further enhanced, with complete inactivation achieved following exposure to 42 J cm^{-2}. This dose was significantly less than the doses required for complete inactivation of both non-stressed cells (pH7) and cells exposed at pH 3.5 (P=0), with populations of 5 $\log_{10}$ CFU mL^{-1} and 3 $\log_{10}$ CFU mL^{-1} remaining after exposure to 42 J cm^{-2}, respectively. The results demonstrate that non-stressed *L. monocytogenes* requires 50-100% greater applied dose of 405 nm light to achieve complete inactivation compared to populations exposed to 405 nm light under acid stress at pH 3.5 and 3.

Non-light exposed control samples that had been sub-lethally stressed with acid demonstrated no significant reduction over the duration of the experiment, when plated onto non-selective media. P values were noted as 0.209 and 0.408 for *E. coli*, and 0.713 and 0.502 for *L. monocytogenes*, at pH 3 and pH 3.5, respectively.

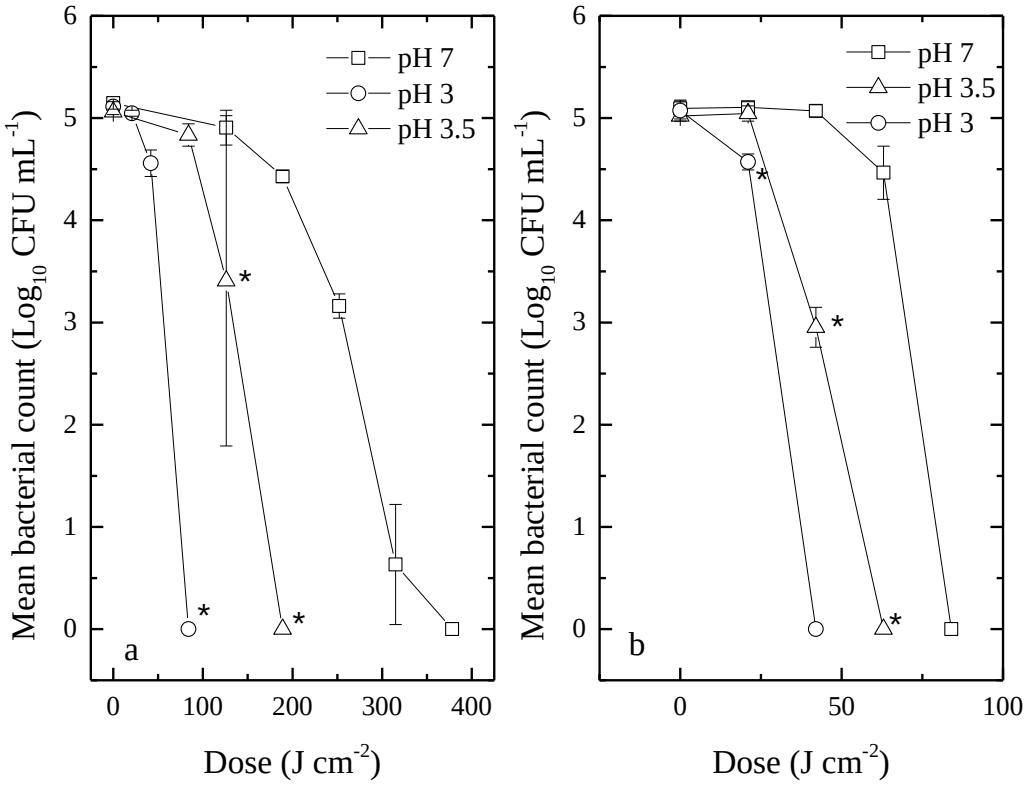

Figure 7.6 *Inactivation of (a) E. coli and (b) L. monocytogenes by exposure to 405 nm light (70 mW cm^{-2} irradiance) combined with acid stress. Bacterial populations were suspended in PBS at pH 3 and pH 3.5 and exposed to 405 nm light. Inactivation kinetics for exposure at pH 7 (PBS without acid) provide a non-stressed comparison. * indicates data points which have significantly increased bacterial inactivation (P≤0.05) compared to the non-stressed populations (pH7). No significant changes were observed in non-light exposed control samples (P=>0.05).*

7.3.2.3 405 nm light inactivation of bacteria under osmotic stress conditions

As shown in Figure 7.7a, 405 nm light inactivation of *E. coli* is enhanced when cells are exposed to sub-lethal salt concentrations of 10 and 15%. It was also found that the light inactivation curves of *E. coli* at 0.8% salt concentration (i.e. non-stressed in PBS) and in 0% salt followed similar trends.

When the salt concentration was increased to 10%, the *E. coli* inactivation rate increased significantly and complete inactivation (5 $\log_{10}$ CFU mL^{-1}) was achieved with exposure to a dose of 252 J cm^{-2} - significantly greater than the 1.2 and 2 $\log_{10}$

reductions observed with 0% and 0.8% salt suspensions respectively ($P \leq 0.05$). Increasing the salt concentration to 15% further enhanced *E. coli* inactivation with inactivation rates significantly greater at all applied doses when compared with 0%, 0.8% and 10% salt concentrations (P=0.00). Complete inactivation was achieved with a dose of 189 J cm^{-2}, giving a 5 log_{10} reduction compared to the 3 log_{10} reduction achieved when at 10% salt, 0.1 log_{10} for 0%, and only 0.5 log_{10} for (non-stressed populations at 0.8% salt concentrations.

Figure 7.7b illustrates the 405 nm light inactivation curves of *L. monocytogenes* when held at varying salt concentrations (0%, 0.8%, 10% and 15%). Bacterial inactivation at light doses of 21 J cm^{-2} and 42 J cm^{-2} was negligible at all salt concentrations tested. At dose levels above 42 J cm^{-2} the inactivation trends were approximately similar for cells held at salt concentrations of 0%, 0.8% and 10%, with complete inactivation achieved following exposure to 84 J cm^{-2} of 405 nm light. The inactivation rate for *L. monocytogenes* held at 15% salt was similar to that of 0.8% salt (PBS) until 63J cm^{-2}, after which the inactivation rate decreased relative to other salt concentrations and complete inactivation at 15% salt required a 50% greater dose (126 J cm^{-2}) than for cells at all other salt concentrations. Again, control data of non-light exposed samples plated onto non-selective agar, demonstrated no significant reduction in bacterial population when exposed to various osmotic concentrations over the entire duration of the experiment. P values were shown to be 0.70, 0.81, 0.70 (*E. coli*) and 0.726, 0.448, 0.576 (*L. monocytogenes*) for 0%, 10% and 15% salt concentrations, respectively.

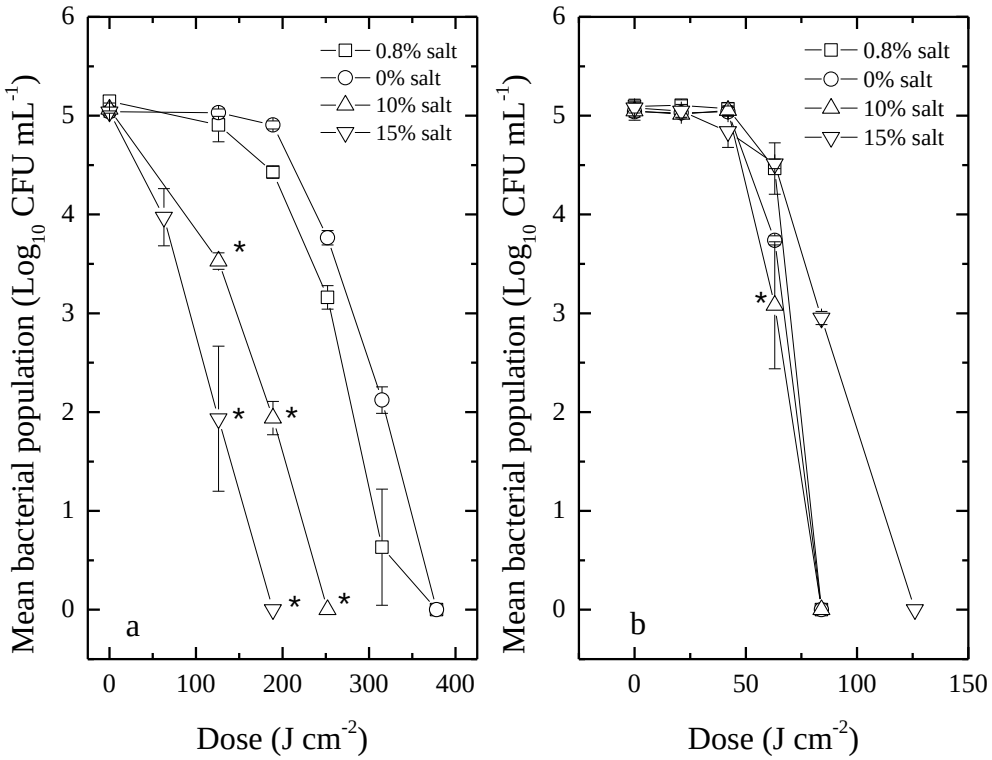

Figure 7.7 *Inactivation of (a) E. coli and (b) L. monocytogenes by exposure to 405 nm light (70 mW cm^{-2} irradiance) combined with osmotic (salt) stress. Bacterial populations were suspended in PBS with a salt concentration of 10% and 15% and exposed to 405 nm light. Inactivation kinetics for exposure at 0% (water) and 0.8% (PBS) provide non-salt stressed comparisons. * indicates data points which have significantly increased bacterial inactivation (P≤0.05) compared to the non-salt stressed populations (0% and 0.8%). No significant changes were observed in non-light exposed control samples (P=>0.05).*

7.4 405 nm light Inactivation of Sub-lethally Stressed Bacteria on Surfaces

Previous work in this chapter has investigated the effect of 405 nm light on sub-lethally stressed bacterial suspensions. Following successful results in bacterial suspension, the next step was to test the effects of 405 nm light for inactivation of sub-lethally damaged bacterial populations present on inert surfaces, which would be typical of the conditions found in food processing environments.

7.4.1 Acid stressed bacterial preparation

Due to time constraints, only acid stressed tests were conducted on surfaces. Acid stress at pH3 was selected for use as it had proved to possess the greatest synergistic inactivation effect on both *E. coli* and *L. monocytogenes,* when combined with 405 nm light.

Due to the method employed for surface exposure in this series of experiments, acid stress and 405 nm light treatment were applied sequentially rather than simultaneously (as had been the case with the suspension experiments). For surface inactivation of sub-lethally stressed bacterial populations, bacterial suspensions acidified to pH 3 were prepared (as described in Section 7.2.2) and left on the laboratory bench for 30 minutes in order to produce an acid stressed population. This time period demonstrated significant sub-lethal damage ($P \leq 0.05$) of both bacteria when plated on selective media. In order to attain a suitable population for enumeration on the inert surface, bacteria were diluted in 9 mL volumes of PBS to 10^1 CFU mL^{-1}, as described in Section 3.4.1, resulting in a 10 mL volume of PBS containing a population density of ~ 150-200 CFU per 10 mL.

7.4.2 Seeding acid-stressed bacteria onto membrane filter surfaces

Unlike the surface work conducted in Chapter 6, sub-lethally stressed bacteria were not exposed to 405 nm light on glass or plastic surfaces. This was due to the possibility that the acid suspension could interfere with the propensity of bacterial attachment on these surfaces. Therefore, in order to achieve consistent reproducible results, acid stressed bacteria were seeded onto nitrocellulose membrane filters for exposure to 405 nm light. Figure 7.8 shows the experimental set-up used to filter acid-stressed bacterial populations onto the nitrocellulose surface. A nitrocellulose filter with a 45 mm diameter and a pore size of 0.47µm was used to ensure collection of microorganisms during filtration. The protocol used was as follows:

1. Funnel was removed from filtration system, cleaned with ethanol and left to dry.
2. Filter was aseptically transferred onto the porous disk of the filtration unit.
3. Funnel was positioned over the filter and held in place by a magnetic base, ensuring no leakage of sample.
4. 10 mL volume of 10^{1} CFU mL^{-1} bacterial suspension was poured onto the filter, followed by 10mL volume of PBS for washing.
5. The vacuum pump was switched on to allow drainage of the sample into the conical discard flask.
6. Upon completion of filtration, the funnel was removed.
7. The seeded filter was aseptically removed and placed onto an appropriate agar for use in light exposure experiments.

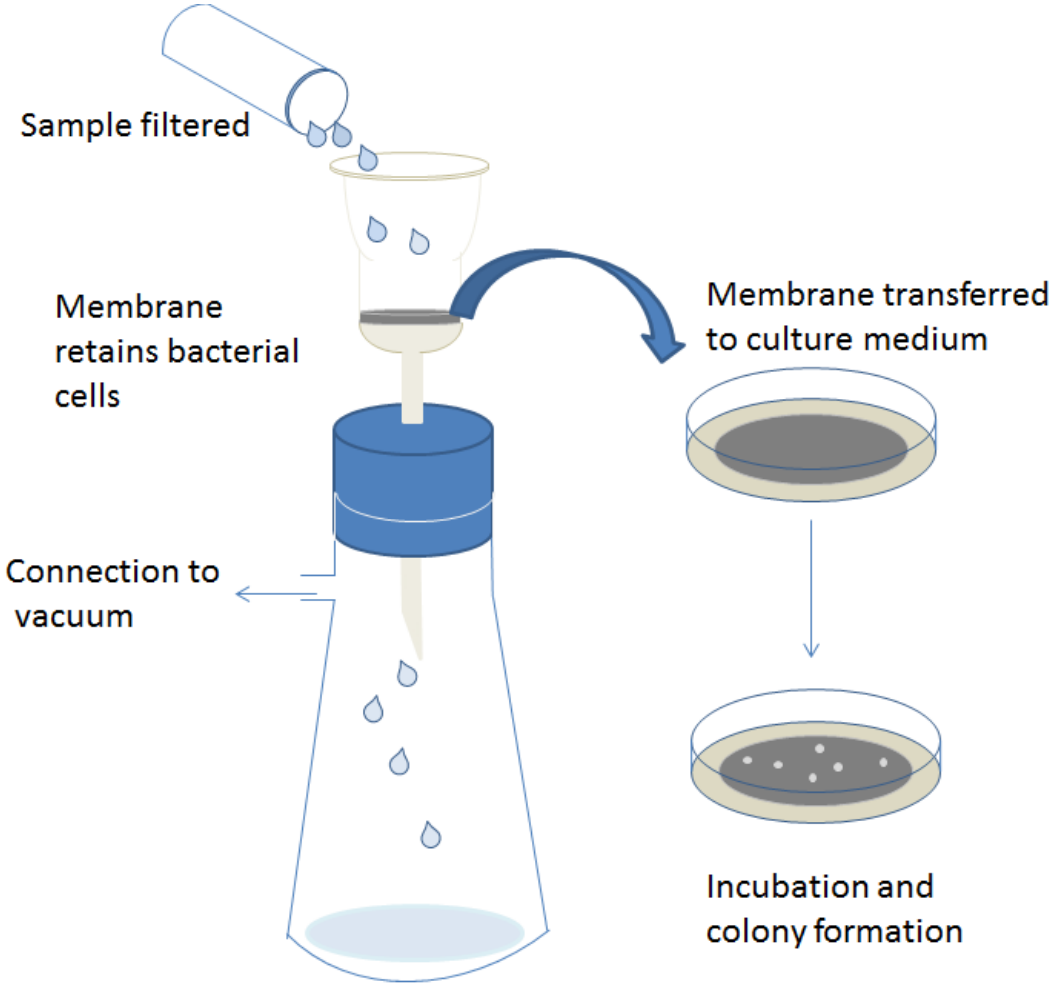

Figure 7.8 *Experimental set-up for filtration process for transfer of acid-stressed bacteria onto nitro cellulose membrane filter.*

7.4.3 Treatment method

Following filtration, membrane filters (placed on NA/TSA for *E. coli* and, *L. monocytogenes*, respectively) were positioned directly under the 405 nm LED array (light source B) at a distance of approximately 5 cm, with an average irradiance of 60 mW cm^{-2} over the membrane filter surface. All samples were exposed to 36 J cm^{-2} of 405 nm light (10 minutes exposure). Control samples were set up simultaneously under identical environmental conditions but without 405 nm light illumination. Post exposure samples were incubated and the viable CFU were enumerated as CFU/surface. Figure 7.9 shows bacterial growth on nitrocellulose membrane following filtration.

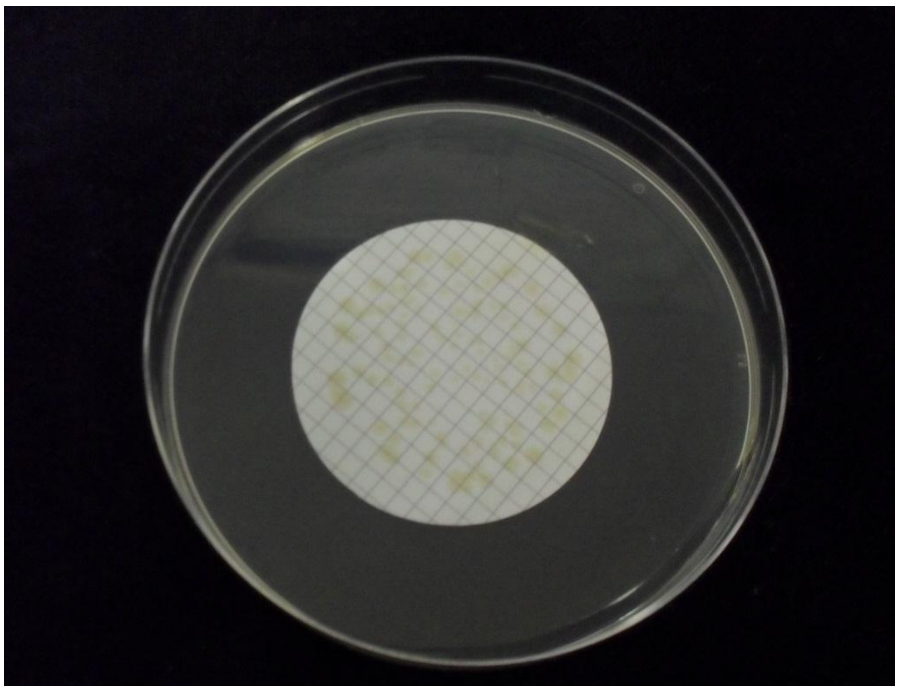

Figure 7.9 *Bacterial growth on nitrocellulose membrane following filtration and incubation.*

7.4.4 Results: 405 nm light inactivation of acid stressed bacteria on an exposed surface

To determine if environmental stress factors enhance the bactericidal effectiveness of 405 nm light for inactivation of bacteria on surfaces, a comparison was made of the inactivation rates of acid-stressed and non-stressed bacteria seeded onto nitrocellulose membrane surfaces. These experiments differed in one respect from the liquid suspension tests in that bacteria were acid-stressed prior to filtration and deposition on

the membrane surface, rather than simultaneous with light exposure, as was the case with the suspension tests.

Data in Table 7.2, shows the synergistic inactivation effects of 405 nm light and acid stressed of *E. coli* and *L. monocytogenes* on surfaces compared to non-stressed populations. Results for *E. coli* demonstrate a significant 95% reduction (P=0.00) in stressed bacteria was achieved when exposed to 36 J cm^{-2} of 405 nm light, compared to a 26% reduction for non-stressed bacteria. Similarly, inactivation of acid-stressed *L. monocytogenes* was enhanced when exposed to 405 nm light on the test surface. Following an applied dose of 36 J cm^{-2}, non-stressed *L. monocytogenes* demonstrated a 13% reduction in bacterial count compared to a significant 99% reduction (P=0.00) when bacteria were sub-lethally stressed with acid. Results show in both cases that it is the combined effect of environmental stress with 405 nm light that induces the lethal effects on bacterial populations. Results demonstrate that neither acid stress alone, nor 405 nm light exposure at 36 J cm^{-2}, is sufficient to induce significant bacterial inactivation (P≥0.05).

Table 7.2 *Inactivation of non-stressed and acid stressed (pH 3) E. coli and L. monocytogenes on nitro cellulose surfaces following exposure to 36 J cm^{-2} 405 nm light (10 minutes at 60 mW cm^{-2}). Results are expressed as CFU counts per surface. * Indicates where acid stressed sample reduction was statistically significant from non-acid stressed sample reduction (P≤0.05 calculated at a 95% confidence interval).*

Bacterial Species	**Acid Stress (pH 3)**	**Bacterial Surface Contamination (CFU per surface (±SD))**		**% reduction**
		Non-exposed (Control)	**Light-exposed (Test)**	
E. coli	No	148(±3.6)	110(±5.5)	26
	Yes	159(±13.1)	9(±8.7)	95*
L. monocytogenes	No	181(±21.2)	156(±16.5)	13
	Yes	205(±7.8)	3(±4.6)	99*

7.5 Discussion

Previous results have highlighted the bactericidal properties of 405 nm light, however it was of great interest to establish whether exposure to 405 nm light under sub-lethal environmental stress conditions could enhance bactericidal efficacy. Investigation into the combined bactericidal effects of 405 nm light and sub-lethal stresses, including temperature, osmotic and acidic stress, has established that a synergistic bacterial inactivation can be achieved when compared to 405 nm light exposure alone.

Light exposure of optimally grown *E. coli* and *L. monocytogenes* established the inactivation kinetics of non-stressed populations. These non-stressed populations were exposed to 405 nm light whilst suspended in PBS (0.8% salt at pH7.2) at room temperature (22°C). Results demonstrated that complete inactivation of 5 $\log_{10}$ populations could be achieved by exposure to 84 J cm^{-2} and 378 J cm^{-2} for *L. monocytogenes* and *E. coli* respectively. These findings are consistent with results of previous studies which have demonstrated higher sensitivity of *L. monocytogenes* to 405 nm light compared to *E. coli* (Endarko et al., 2012; Maclean et al., 2009).

Manipulation of temperature, salt and acidity are amongst the most important parameters that can be used to control microbial populations within the food industry. The precise temperature, salt concentrations and acidity conditions chosen for use in this series of work were those that were experimentally established to induce only sub-lethal stress on the test organisms. Once specific stress conditions were selected, confirmation that each stress induced sub-lethal damage on bacterial populations was necessary. Sub-lethal injury of bacteria implies a degree of damage to cell structure and or function, without inducing cell death (Wesche et al., 2008). In order to demonstrate the sub-lethal effect of each of the chosen stress conditions, bacterial populations were plated onto selective and non-selective media, prior to any 405 nm light exposure, in order to confirm the presence of sub-lethally damaged cells. The differences in selective and non-selective CFU counts were indicative of the extent of sub-lethally damaged bacterial populations, as cells that had been structurally and/or metabolically damaged were not able to grow on selective media.

It is clear that stressing the bacterial populations using the environmental conditions employed in this study was not adequate to induce cell death; however, these sub-lethal

stresses were sufficient to significantly enhance the susceptibility of these organisms to inactivation by 405 nm light exposure. Previous studies have highlighted that each of the stresses investigated (temperature, acidic, osmotic) have the capability to induce detrimental effects on cellular populations through various mechanisms. Although each stress in this study appears to present only sub-lethal effects, each has the potential to induce lethal effects on microbial populations. For example, studies have shown that bacterial exposure to low temperatures can result in alterations in fatty acid composition, altering membrane fluidity from a fluid to gel-like state. These changes are known to have detrimental effects on protein function, directly impacting on cellular signalling and regulation (Beales 2003; McMahon et al., 2006). Furthermore, when bacteria are exposed to low temperatures, reduction in growth rate can result through reduced metabolic and enzymatic activity (Beales 2003; Herbert 1989). Similarly, both acid and salt stress have the ability to induce cellular inactivation under the correct concentrations. Increasing salt concentration is still a major technique used for preservation throughout the food industry. When water availability is lowered by increased salt presence, dehydration can result, causing both structural and metabolic damage, which may result in microbial death (Beales 2003). Studies have also shown that osmotic stress in *E. coli* greatly inhibits transport of carbohydrates across the cell, preventing many energy-dependent processes essential for cell survival (Roth et al., 1985; Lucht and Bremer 1994).

When looking at the effects of the temperature, osmotic and acid stresses used in synergy with 405 nm light, results demonstrate that sub-lethal stress alone caused no significant effect on bacterial populations, as shown by data in Section 7.2.5. However, when bacteria were exposed to 405 nm light in combination with specific sub-lethal stresses, successful inactivation occurred in all cases, with inactivation rates significantly enhanced when compared to 405 nm light inactivation kinetics of non-stressed bacterial populations. One exception to this was the decreased susceptibility of *L. monocytogenes* whilst suspended in 15% salt; this will be discussed in greater depth later in this section.

Enhanced cellular inactivation upon combination of stress and 405 nm light may be a direct result of multi-target inactivation, supporting the principle of hurdle technology, whereby the bacteria are targeted at multiple sites, by multiple stresses, enhancing the

rate of inactivation. It is more likely however, that due to the lack of inactivation in the absence of 405 nm light, bacterial inactivation is occurring predominantly through oxidative damage from exposure to the light source. Undoubtedly susceptibility to 405 nm light is increased under stressed conditions. It is likely that some form of structural and/or metabolic stress is being induced whilst the bacteria are being exposed to sub-lethal stresses that alone is not enough to induce cellular inactivation, but is sufficient to increase susceptibility to oxidative damage during 405 nm light exposure. Previous studies have identified that oxidative damage is non-specific to cellular components. However, a recent study has suggested the production of reactive oxygen species (ROS) may directly affect the cell membrane (Dai et al., 2013; Wasson et al., 2012). It is worth noting that exposure to various environmental stresses can directly affect cellular structure and induce changes in the cellular membrane (Beales 2003) and it is therefore possible that bacteria exposed to a sub-lethal stress may have weakened or damaged membranes, making them increasingly susceptible to oxidative damage when exposed to 405 nm light.

Interestingly, exposure of *L. monocytogenes* to 405 nm light under osmotic stress did not exhibit such obviously enhanced inactivation kinetics. Figure 7.7 highlights that exposure of *L. monocytogenes* in 10% salt suspension appeared to give significantly enhanced inactivation after a dose of 63 J cm^{-2}, however, by 84 J cm^{-2}, complete inactivation was found with *L. monocytogenes* in 10, 0.8 and 0% salt suspensions. Interestingly, *L. monocytogenes* suspended in 15% salt displayed reduced sensitivity to 405 nm light inactivation, with complete inactivation not achieved until 50% greater dose, demonstrating the possible osmotolerance of *L. monocytogenes*. It would be assumed that increasing the salt concentration would enhance bacterial inactivation, or at least remain consistent with results at lower concentrations. However, the fact that for *L. monocytogenes*, susceptibility to 405 nm light was decreased with increased osmotic concentration suggests that an adaptive cellular protective mechanism may be involved. Previous studies have suggested that stress reactions may be non-specific, thereby a response to a particular stress may provide "cross protection" to other applied stresses (Leistner 2000, Koutsoumanis et al., 2003). This phenomenon is known as stress hardening. Previous studies highlighting stress hardening have shown microbial adaptations resulting from continued exposure to lethal and sub-lethal environmental

conditions allowing for partial or complete resistance against particular stress factors (Koutsourmanis et al., 2003; Lou and Yousef 1997). Stress hardening is a major concern in the food industry, whereby bacterial ability to develop increased tolerance or resistance to multiple environmental challenges may compromise food safety standards.

Of the three environmental stress conditions tested, low pH interacted with 405 nm light exposure to produce the greatest synergistic enhancement of light-induced inactivation. Inactivation under the most acidic conditions (pH 3), demonstrated the greatest synergy, with a 77% and 50% reduction in inactivation dose required for *E. coli* and *L. monocytogenes*, respectively, when compared to non-stressed populations exposed solely to 405 nm light. Results highlighted that although both species demonstrated increased susceptibility when compared to 405 nm light under acidic conditions, *E. coli* may have increased susceptibility when compared to *L. monocytogenes*. Similarly, under increased osmotic condition, *E. co*li was shown to be far more susceptible, whilst *L. monocytogenes* displayed resistant characteristics. This data highlights the apparent greater resilience of *L. monocytogenes* under both acidic and osmotic stresses compared to that of *E. coli*, which may be attributed to the presence of sigma B (σ^B) in *L. monocytogenes*, as mentioned in Chapter 4. σ^B is a general stress response alternative sigma factor that has been identified in various Gram positive bacteria and has been shown to contribute to survival under stress conditions including low pH, growth at low temperatures and acid stress (Kazmierczak et al., 2013; O'Byrne and Karatzas., 2008; Chaturongakul and Boor 2006; Ferreira et al., 2001). Survival of *L. monocytogenes* at high salt and acid concentrations has been largely attributed to the presence of σ^B dependent genes, including *ctc*, *betL* and *gadB*. Studies which these genes have been depleted or disrupted have shown reduced tolerance to specific stresses (van Schik and Abee 2005; Beumer et al., 1994; Garden et al., 2003; Kazimierczak et al., 2003; Cotter et al., 2001).

Although this study focused largely on determining the inactivation characteristics of sub-lethally stressed bacteria in suspension, tests were also conducted to investigate the synergistic effects on stressed bacteria of 405 nm light whilst exposed on an inert surface, in order to simulate scenarios representative of surface decontamination applications. For these tests, acid stressed (pH 3) bacterial populations were seeded onto a nitrocellulose membrane surface and exposed to 36 J cm^{-2} of 405 nm light.

Nitrocellulose membrane filters were selected as the surface to use in this series of work. As previously discussed, it was speculated that acid suspensions may interfere with the propensity of bacterial attachment on inert glass and plastic surfaces (Mafu et al., 2011; Herald and Zottola 1988), yielding inconsistent, non-reproducible results. Herald and Zottola seeded stainless steel surfaces with *L. monocytogenes* suspended in TSB and they found that by reducing the pH of the TSB, lesser adhesion of *L. monocytogenes* to stainless steel surfaces was observed. A study by Stanley also concurred with these results, suggesting that the range in which bacteria promote greatest metabolic activity (pH 7-8) is also the range in which greatest bacterial attachment to surfaces occurs (Stanley 1983). Consequently, the simplest method to demonstrate acid stressed bacteria on a surface was to pre-stress the bacteria in acid, filter the suspension and then expose to 405 nm light. The results demonstrate that significantly enhanced reduction of both *E. coli* and *L. monocytogenes* was achieved on the test surface when cells were sub-lethally acid-stressed prior to 405 nm light exposure, when compared to non-acid stressed tests. This finding demonstrates that the enhanced susceptibility of sub-lethally stressed bacteria to 405 nm light, observed when bacteria were present in liquid suspension, also applies to bacteria when treated on exposed surfaces. These surface inactivation results reinforce the previously published data by Murdoch et al. (2012) that have demonstrated the application of 405 nm light for inactivation of bacteria on inert surfaces. Results obtained in this study indicate that in most cases synergistic microbial inactivation would be achieved under 'real' environmental conditions when compared to laboratory cultivated experiments, suggesting that enhanced inactivation of many other microbial species, spores, biofilms and contaminated surfaces may be achieved in *in situ* environmental settings.

In summary, the results presnted in this chapter demonstrate the synergistic bactericidal effects of 405 nm light photoinactivation when combined with various environmental stresses. Although the results reported were conducted with only two species, *E. coli* and *L. monocytogenes*, it is anticipated that the key findings of this study will apply to a wide range of pathogenic and problematic bacteria. It is also reasonable to speculate from the results obtained that this enhanced inactivation effect will not be restricted to the specific temperature, salt and acidity conditions tested but will represent a more general phenomenon whereby the bactericidal properties of 405 nm light will be

enhanced when target cells are simultaneously subjected to a variety of environmental stress conditions. The results of the current study support the idea that 405 nm light could potentially have decontamination applications within the food processing industry and may have vast potential for integration into existing decontamination protocols, where the potential synergy of various technologies is favourable in respect to the hurdle concept. However, for commercial food related applications, larger scale studies are required.

CHAPTER 8
PHOTOCATALYTIC ENHANCEMENT OF ANTIMICROBIAL 405 NM LIGHT USING TITANIUM DIOXIDE NANOPARTICLES

8.0 General

Titanium dioxide (TiO_2) is non-toxic material used as a white pigmentation within various products such as cosmetics, foods and pharmaceuticals. Besides this, however, TiO_2 is being utilised for a number of applications due to its low cost, low toxicity and high photocatalytic activity (Ede et al., 2012; Chawengkijwanich and Hayata 2008; Gamage and Zhang 2010). Photocatalytic inactivation, or photocatalysis, of microorganisms through the combined use of light and exogenous nanoparticles has generated much interest for enhanced microbial inactivation. In 1985, Matsunaga and colleagues demonstrated, for the first time, the microbicidal properties of TiO_2 through UV activation. Extensive subsequent work has highlighted the photocatalytic effects of TiO_2 on a wide range of bacteria, viruses, fungi and algae (Matsunaga et al., 1985; Huang et al., 2000; Li et al., 2011; Pillai et al., 2012). Since then, work on photocatalysis has established that metal oxide semiconductors, including titanium dioxide, are most effective for photocatalytic microbial inactivation (Lipovsky et al., 2011; Fu et al., 2005; Huang et al., 2000).

The photocatalytic mechanism of TiO_2 is well understood. Illumination of TiO_2 promotes excitation of electrons from the valance band (VB) to the conductance band (CB), provided the energy absorbed from the photon is equal to or more than that of the associated band gap (a diagrammatic representation is shown in Figure 8.1), where *hv* is the photon energy (equal to Planks constant × frequency). Movement of electrons (e^-), which are negatively charged, from the valence band to the conductance band results in the formation of a positively charged hole (h^+) in the VB and a free electron (e^-) in the CB. Following this, electron-hole pairs either recombine or migrate to the surface of the TiO_2 molecule where they exhibit high oxidation potential, shown by the equation:

$$\mathbf{TiO_2 + } h\nu \rightarrow \mathbf{TiO_2\ (e^-_{cb} + h^+_{vb})}$$

Migration of positively charged holes to the semiconductor surface can directly result in oxidation of organic compounds (R), causing the formation of carbon dioxide and water. Alternatively, upon contact with water, the positively charged holes can induce breakdown of the water molecule, inducing formation of hydrogen (H^+) and hydroxyl ions (OH^-); OH^- can then react with h^+ to form hydroxyl radicals ($OH^{\cdot}$) (Kodom et al., 2012; Petrov et al., 2013; Hu et al., 2009; Mitoraj et al., 2006; Hoffman et al., 1994):

$$\mathbf{h^+_{vb} + R \rightarrow CO_2 + H_2O}$$

$$\mathbf{H_2O + h^+_{vb} \rightarrow OH^{\cdot} + H^+}$$

In the presence of molecular oxygen, electrons can attach to oxygen (O_2), generating superoxide ions. In some cases these can interact with H^+ to form hydroxyl radicals ($OH^{\cdot}$) and the hydroperoxyl radical $HO_2^{\cdot}$:

$$\mathbf{O_2 + e^-_{cb} \rightarrow O_2^{\cdot -}}$$

Previous studies had suggested that due to the wide band gap possessed by TiO_2, the use of visible light was limited due to the lower energy of it'sphotons as compared with UV photons, and may require doping, with either exogenous metal or non-metal elements in order to reduce the band gap, which would consequently increase visible light photocatalytic performance (Mitoraj et al., 2006; Lipovsky et al., 2011; Karunakaran et al., 2010; Seery et al., 2007). Photoactivity of doped-TiO_2 is largely dependent on the material. However, in most cases doping can narrow the band gap, which subsequently requires less energy to be transferred to an electron in order to move it from VB to CB, and thus is able to absorb visible light for photoactivation. Specific examples that have been shown to help enhance visible light photocatalytic performance include copper and nitrogen (Zaleska 2008).

Until recently it was widely accepted that photocatalytic activation of TiO_2,without doping, could be achieved only through UV illumination due to TiO_2 possessing a relatively wide band gap (3.0-3.2 eV). However, a number of groups have recently demonstrated the capabilities of visible light, at specific wavelengths, for photocatalytic activation of TiO_2 without the need for doping (Petrov et al., 2013; Chen et al., 2012; Peng et al., 2010; Lu et al., 2010). TiO_2 exists in various mineral forms, with the

combination of mixed phase anatase and rutile used preferentially during visible light treatments. Anatase form has a band gap of 3.2 eV corresponding to a UV wavelength of 385 nm. In contrast the rutile form has a smaller band gap of 3.0 eV, with excitation wavelengths that extend into visible wavelengths (~410 nm) (Hurum et al., 2003).

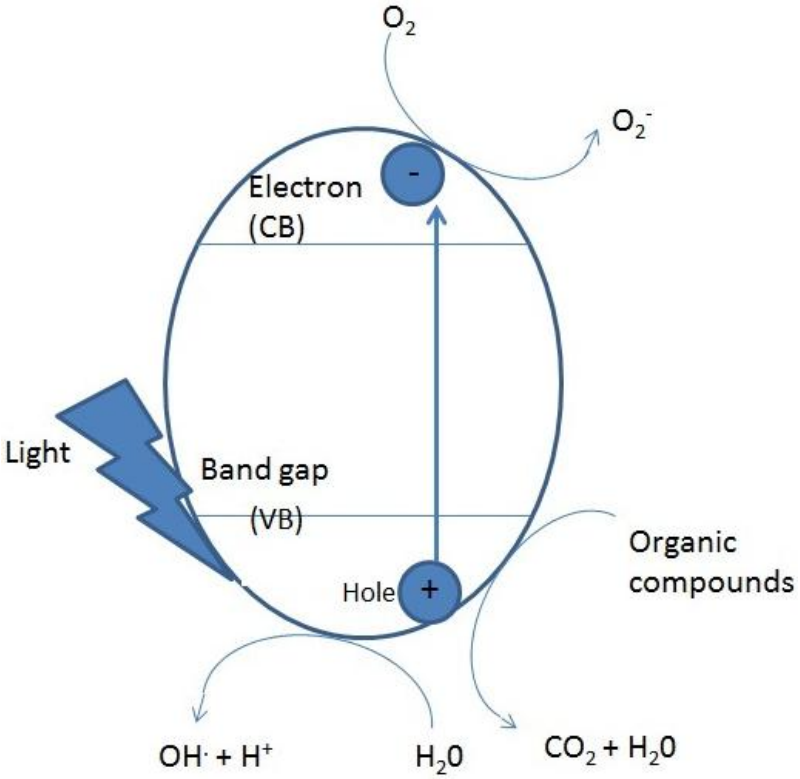

Figure 8.1 *Schematic diagram highlighting the microbicidal action of TiO_2 when exposed to light (hv). Upon illumination, movement of electrons from valence band (VB) to conductance band (CB) results in the formation of positively charged holes (h^+) and negatively charged electrons (e^-), which possess high oxidation potential. Microbial inactivation can also occur by direct contact with e^-, resulting in formation of CO_2 and H_2O. Adapted from Zaleska (2008).*

While previous work has demonstrated the antimicrobial efficacy of 405 nm light, however enhancement of the inactivation rate is desirable, and the potential for TiO_2 to be used to provide a photocatalytic effect to improve the efficacy of these violet-blue wavelengths for microbial kill is advantageous. This chapter investigates the use of non-doped TiO_2, at differing concentrations, for enhanced efficacy of 405 nm light inactivation of bacteria, in both liquid suspension and on surfaces. The significant

foodborne pathogen, *E. coli*, was selected as the model organism for this work. Due to the resilience of this organism against 405 nm light (as displayed in previous chapters), it is anticipated that if enhanced inactivation is observed in the presence of 405 nm light, similar trends in enhanced inactivation could be achieved for other bacterial species, although larger studies will be required to confirm this.

8.1 Enhanced Photocatalytic Inactivation of Bacteria in Liquid Suspension

The first section of this study focused on the enhanced photocatalytic inactivation of *E. coli* when exposed to 405 nm light in suspension in the presence of TiO_2 nanoparticles.

8.1.1 TiO_2 Preparation

TiO_2 (Aeroxide® P25 Degussa powder, 79%: 21% anatase: rutile,) (Evonik Industries, Germany) was used throughout this study. TiO_2 in concentrations of 1 g L^{-1} and 10 g L^{-1} was added to sterile water to produce 0.1% and 1% concentrations respectively. Suspensions were mixed thoroughly and treated with 30 kHz ultra-sonication (Ultrawave Ltd, Cardiff, UK) for 5 min at room temperature to ensure even dispersal of TiO_2 nanoparticles throughout the liquid (Huang et al., 2000).

8.1.2 Optical Transmission Measurements

The optical transmission spectrum of each of the three samples was measured using a Biomate 5-UV-Visible Spectrophotometer (Thermo Scientific, UK). A 3 mL sample of each TiO_2 concentration (0%, 0.1% and 1% TiO_2) was transferred into a plastic cuvette and transmission of light was measured between 380-450 nm. Data presented in Figures 8.2 and 8.3, shows the results obtained, with specific transmission at 405 nm highlighted.

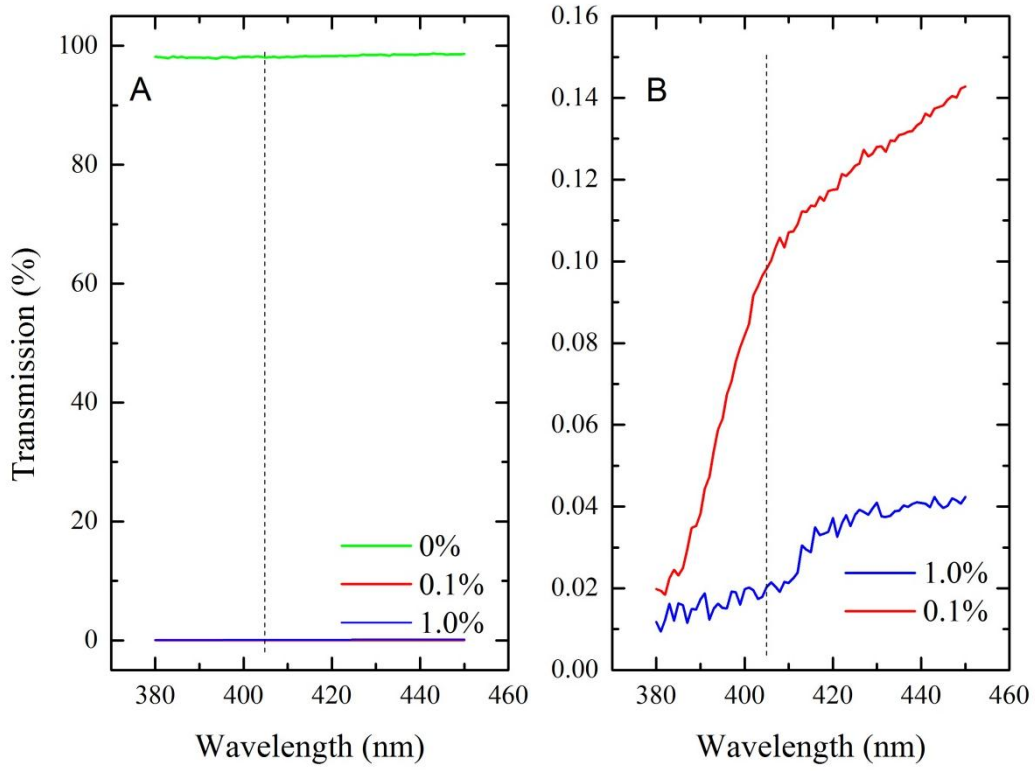

Figure 8.2 *Transmission spectra of TiO_2 suspensions (0%, 0.1% and 1%), with transmission at 405 nm highlighted (A). Figure (B) provides a detailed transmission spectrum of 0.1% and 1% TiO_2 suspensions.*

8.1.3 Bacterial Preparation

E. coli was used throughout this series of experimental work. Bacterial suspensions were prepared as described in Section 3.4.1. To prepare bacterial samples in 0% TiO_2, bacterial suspensions were diluted in PBS to ~10^5 and 10^2 CFU mL^{-1}; these were used to establish baseline inactivation kinetics. For tests involving TiO_2, bacterial suspensions were diluted to 10^6 or 10^3 CFU mL^{-1} in PBS and then the final dilution added to 0.1% or 1% TiO_2 suspension, giving resultant populations of 10^5 and 10^2 CFU mL^{-1}, respectively for experimental use. Bacterial populations were transferred to TiO_2 suspension after ultra-sonication, to ensure bacterial cells were not subjected to sonication.

8.1.4 Treatment Method

A 3 mL sample of bacterial suspension was transferred into one central well of a 12-well multi-dish. Samples were placed directly under the ENFIS QUATTRO array (light source A) at a distance of approximately 5 cm, giving an approximate irradiance of 180 mW cm^{-2}, as described in Section 3.3.1. Test samples were exposed to increasing doses of 405 nm light. Control samples were set up simultaneously, but were without 405 nm light exposure. Post-exposure, samples were collected and plated onto nutrient agar as described in Section 3.4.2. Plates were incubated at 37°C for 18-24 hours then enumerated, with results recorded as CFU mL^{-1}.

8.1.5 Results: Inactivation of *E. coli* in suspension using TiO_2 and 405 nm light

Figures 8.3 highlights the 405 nm light inactivation of 10^5 CFU mL^{-1} populations of *E. coli* suspended in a range of TiO_2 concentrations. In the absence of TiO_2 (0%), initial inactivation was slow, with only a 0.47 log_{10} reduction observed after exposure to 218 J cm^{-2}. However, complete inactivation of the 5 log_{10} population was achieved when the dose was doubled to 436 J cm^{-2}.

Addition of TiO_2 at 0.1% resulted in enhanced inactivation rates. Inactivation was enhanced significantly at all data points ($P \leq 0.05$), with 1.64 and 3.32 log_{10} reductions observed with doses of 109 and 163 J cm^{-2} which caused negligible change for the samples suspended in 0% TiO_2. Complete inactivation was achieved following exposure to a dose of only 218 J cm^{-2}: 50% less than the dose required for inactivation in the absence of TiO_2.

When the TiO_2 concentration was increased to 1%, inactivation was also shown to be enhanced when compared to tests conducted in the absence of TiO_2, and displayed similar inactivation kinetics as observed in the case of 0.1% TiO_2. A 2.1 log_{10} decrease in bacterial population was observed following a dose of 163 J cm^{-2} and complete inactivation achieved with 273 J cm^{-2}. However, although significantly greater inactivation was achieved compared to when exposed in 0% TiO_2, the extent of inactivation with doses of 54 and 163 J cm^{-2} was found to be significantly less than that achieved in 0.1% TiO_2 ($P \leq 0.05$).

Results in Figure 8.4 highlight inactivation of low population densities of *E. coli* (10^2 CFU mL^{-1}) suspended in the same TiO_2 concentrations. In the absence of TiO_2 (0% TiO_2) inactivation was shown to require the greatest applied dose, with complete inactivation requiring 327 J cm^{-2}. As with previous results, addition of TiO_2 enhanced the inactivation rates, with similar kinetics observed with use of 0.1% and 1% TiO_2. Addition of TiO_2 at 0.1% demonstrated significant enhancement when compared to 0% tests, with enhanced inactivation observed following 109 J cm^{-2} and 163 J cm^{-2}. Further increase of TiO_2 concentration to 1% again demonstrated enhanced inactivation when compared to 0% tests, however significantly less inactivation (0.46 log_{10}), when compared to 1.14 log_{10} reduction achieved at 0.1% tests, following an applied dose of 109 J cm^{-2}.

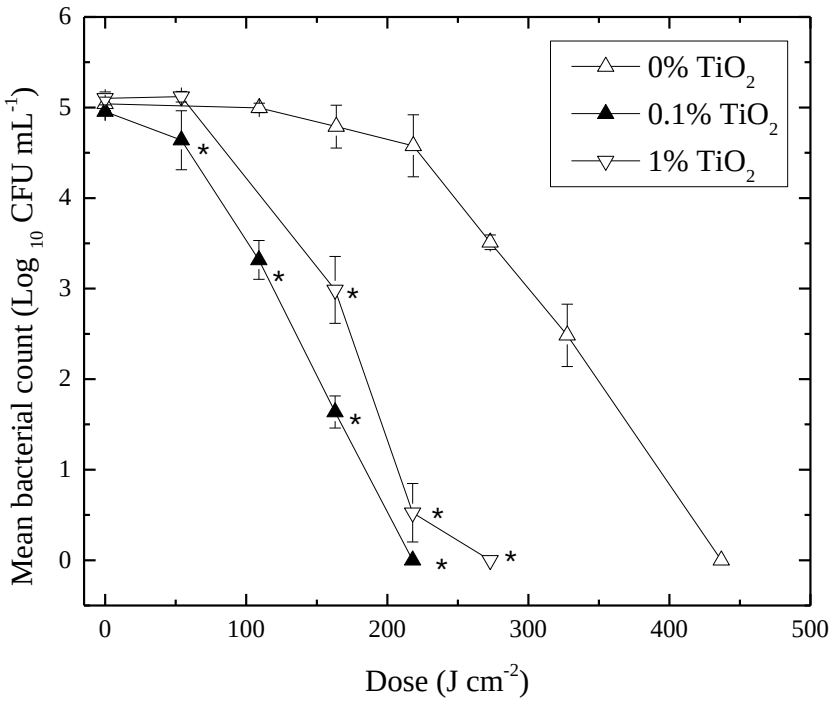

Figure 8.3 *Inactivation of 10^5 CFU mL^{-1} E. coli suspended in various concentrations of TiO_2 (0%, 0.1% and 1%) by exposure to increasing doses of 405 nm light, with an irradiance of 180 mW cm^{-2}. * represents statistically significant difference when compared to the samples exposed in 0% TiO_2 ($P \leq 0.05$).*

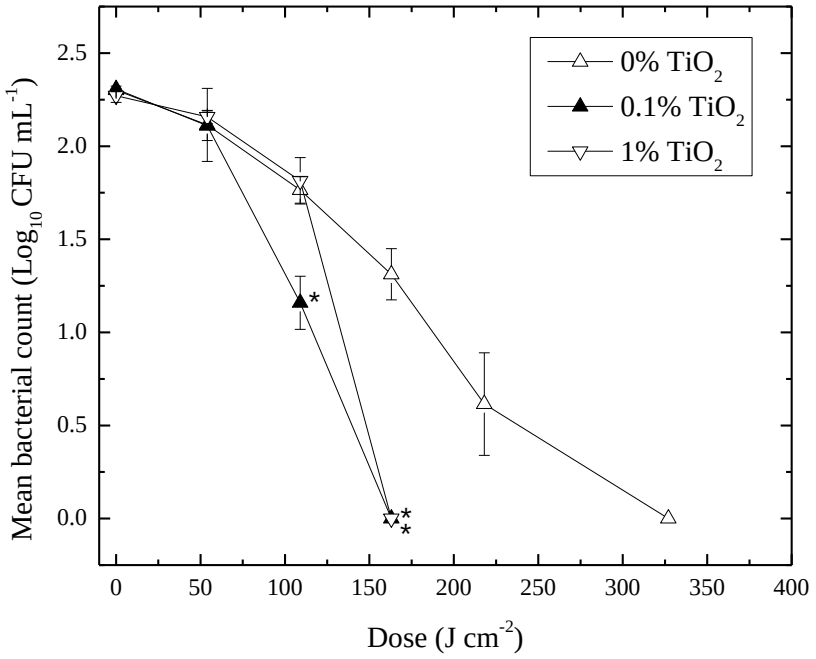

Figure 8.4 *Inactivation of 10^2 CFU mL^{-1} E. coli suspended in various concentrations of TiO_2 (0%, 0.1% and 1%) by exposure to increasing doses of 405 nm light, with an irradiance of 180 mW cm^{-2}. * represents statistically significant difference when compared to the samples exposed in 0% TiO_2 ($P \leq 0.05$).*

8.2 Enhanced Photocatalytic Inactivation of *E. coli* on TiO_2 coated surfaces

Following successful demonstration of the photocatalytic properties of TiO_2 with 405 nm light for inactivation of bacterial contamination in liquid suspension, the next step was to investigate the photocatalytic action of 405 nm light and TiO_2 for surface decontamination. This was done by investigating the inactivation efficacy of 405 nm light for decontamination of bacteria seeded onto surfaces coated with TiO_2. This section will detail how this work was conducted and the results obtained.

8.2.1 Bacterial Preparation

E. coli was used throughout this section of experimental work. Bacterial suspensions were prepared as described in Section 3.4.1. Suspensions were serially diluted in PBS to a population density of 1×10^7 CFU mL^{-1}.

8.2.2 Surface Preparation and Seeding

The test surfaces (70 × 25 mm coupons) used in these experiments were:

- glass slides
- Polyflor pur 2000 homogenous flooring (Polyflor, UK)
- Tarkett Wet room flooring (Tarkett, UK)

Coupons were cleaned using 70% ethanol. They were then fully suspended in a 10^7 CFU mL^{-1} suspension (either with or without 0.1 % TiO_2) for 30 minutes at room temperature under rotary conditions (120 rpm). Coupons were then removed from the suspension and placed in a laminar flow cabinet for drying for 20 minutes.

8.2.3 Treatment Method

Coupons were placed directly under the 405 nm LED array (ENFIS PhotonStar, UK/Source B) at a distance of ~5 cm, and exposed to increasing doses of 405 nm light. This light source provided an average irradiance of ~60 mW cm^{-2} across the entire surface area. As with previous surface exposure experiments (Chapter 6), the coupons were raised slightly during exposure to allow airflow and prevent a build-up of heat across the surface. Following exposure, bacteria were recovered from the surface using a moistened sterile cotton tipped swab (Section 6.1.4). For enumeration, samples were diluted and plated using the pour plate method, with 1 mL sample volumes over-layed with nutrient agar. Pour plates were incubated at 37°C for 18-24 hours and enumerated manually, with counts recorded as CFU mL^{-1}. Control were set up simultaneously, where *E. coli* was seeded onto surfaces, and exposed to 405 nm light but without TiO_2.

8.2.4 Inactivation of *E. coli* on TiO_2 coated surfaces

Table 8.1 details the results of exposing *E. coli* seeded onto TiO_2 coated surfaces to 18 J cm^{-2} (5 minutes) 405 nm light. After exposure to 18 J cm^{-2} on glass, complete

inactivation (4.36 $\log_{10}$ reduction) was achieved, compared to a 0.79 $\log_{10}$ reduction of *E. coli* on non-TiO_2 coated glass.

Exposure of bacteria seeded on polyflor pur 2000 homogenous flooring also demonstrated complete bacterial inactivation (4.96 $\log_{10}$ reduction) after an applied dose of 18 J cm^{-2}. In this case, the control data also exhibited a significant reduction in bacterial population; however, this was significantly less than the inactivation achieved on the TiO_2 coated surfaces (P= 0.00). Table 8.1 also highlights an increased reduction in bacterial count on Polyflor pur 2000 when exposed solely to 405 nm light. This surface has independently-tested antimicrobial properties, which have been shown to inhibit the growth of bacteria. The antimicrobial properties of this surface are clearly demonstrated in the control data (no TiO_2) in Table 8.1, where the bacterial population after an applied dose of 18 J cm^{-2} was significantly less ($P<0.05$) than that of the other two test surfaces.

The third surface investigated was Tarkett wet room flooring. Table 8.1 highlights a 3.6 $\log_{10}$ reduction of *E. coli* on TiO_2 coated surfaces: again significantly greater than the 0.84 $\log_{10}$ reduction recorded for inactivation of *E. coli* on non-TiO_2 coated surfaces.

Of the three surfaces tested, the Tarkett wet room flooring was the only one that did not demonstrate complete inactivation following 18 J cm^{-2} exposure. However, following an increased dose of 36 J cm^{-2}, complete inactivation was achieved on the TiO_2 coated surfaces.

It is important to note that no significant reductions were observed between the starting populations and the non-exposed control populations on the three surfaces, highlighting that inactivation was a direct result of 405 nm light exposure and not due to desiccation and/or any toxic effect from the TiO_2.

Table 8.1 *Inactivation of E. coli on 0.1% TiO_2 coated and non-TiO_2 coated surfaces by exposure to 405 nm light at a dose of 18 J cm^{-2}(5 minutes at 60 mW cm^{-2}). * represents statistically significantly reduced bacterial count on TiO_2 coated surface compared to non-coated surfaces.*

Surface	0.1% TiO_2	Start population	Control pop (±st dev)	Test pop (±st dev)	Log reduction
Glass	+	4.45 (±0.66)	4.36 (±0.12)	0 (±0.00)	4.36*
	_	4.64 (±0.06)	4.73 (±0.04)	3.82 (±0.08)	0.91
Polyflor pur 2000	+	4.78 (±0.04)	4.96 (±0.09)	0 (±0.00)	4.96*
	_	4.66 (±0.02)	5.00 (±0.09)	1.98 (±0.57)	3.02
Tarkett Wet room	+	5.27 (±0.06)	5.26 (±0.06)	1.42 (±1.1)	3.84*
	_	4.64 (±0.06)	4.92 (±0.09)	4.08 (±0.10)	0.84

8.3 Photocatalytic Oxidation of Indigo Carmine Dye

In order to demonstrate oxidative properties of TiO_2, photo-degradation of indigo carmine dye was investigated. Photodegradation occurs with the attack of organic substances by reactive oxygen species, such as hydroxyl radicals. In order to provide chemical confirmation of the oxidation activity of TiO_2 during 405 nm light illumination, the decolourisation of indigo carmine was investigated. This method has been utilised in previous literature to demonstrate photo-degradation (Li et al., 2012; Subramani et al., 2007). The photocatalytic degradation of indigo carmine dye was used to help confirm the chemical action of both 405 nm light and TiO_2 photocatalysis.

8.3.1 Preparation of indigo carmine suspension

Indigo carmine dye (Sigma-Aldrich, UK) (0.01%) was added to 200 mL of sterile water and wrapped in tin-foil to prevent degradation from room lighting. Another suspension was prepared identically, but with the addition of 0.1% TiO_2. This concentration was selected due to it demonstrating greatest photocatalytic effects in Section 8.1.5 and 8.1.6.

8.3.2 Treatment Method

Three millilitre samples of indigo carmine, with and without 0.1% TiO_2, were transferred to a 12-well multi-dish and positioned under the LED array (ENFIS PhotonStar/ Source B) at a distance of ~5 cm. Samples were exposed to an irradiance of 65 mW cm^{-2} for 25 minutes, giving a dose of ~98 J cm^{-2}. Control samples were also set up which and stored under dark conditions for the duration of the experiment.

8.3.3 Results: Photocatalytic oxidation effects of TiO_2 and 405 nm light on indigo carmine dye

The decolourisation of indigo carmine when exposed to 405 nm light in the presence of TiO_2 is shown in Figure 8.5. Figure 8.5 (A) highlights the control results where no decolourisation was observed in the absence of any light exposure. Results demonstrate that 405 nm light exposure can successfully induce an oxidization reaction – shown by the decolourisation of indigo carmine dye (B). However, greatest decolourisation was observed in the solution containing 0.1% TiO_2 following exposure to 405 nm light (C). This highlighted that TiO_2 can successfully accelerate 405 nm light photo-degradation of indigo carmine dye by oxidation.

Transmission measurements were recorded for each of the three samples shown in Figure 8.6, using a Biomate 5-UV-Visible Spectrophotometer (Thermo Scientific, USA). It would be expected that due to decomposition of indigo carmine dye, transmission measurements would increase from sample A to sample C. However due to the presence of TiO_2 in sample C, almost 0% transmission was recorded due to the opacity of the suspension. Therefore although sample C demonstrated the greatest visually observed degradation of indigo carmine dye, this was not highlighted through the transmission spectra shown in Figure 8.6.

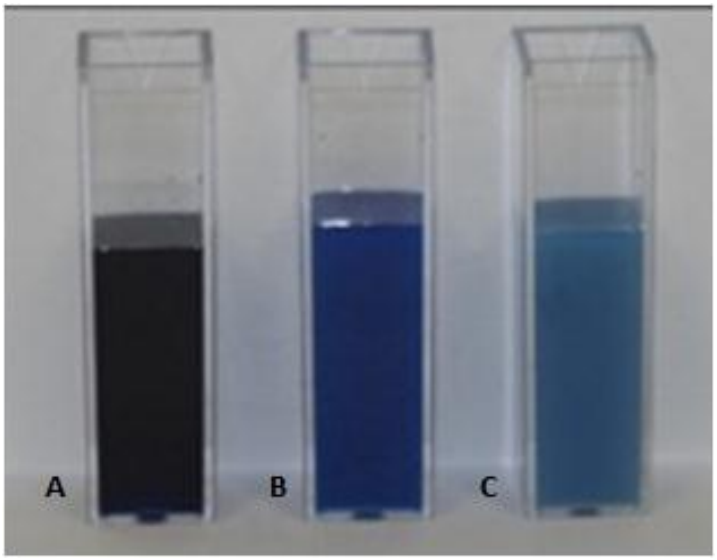

Figure 8.5 *Visual representation of decolourisation of indigo carmine dye due to oxidation by 405 nm light and TiO_2. Sample (A) represents a dark control where no 405 nm light exposure was given, (B) 405 nm light exposure (~98 J cm^{-2}) without TiO_2, and (C) 405 nm light exposure (~98 J cm^{-2}) in the presence of TiO_2.*

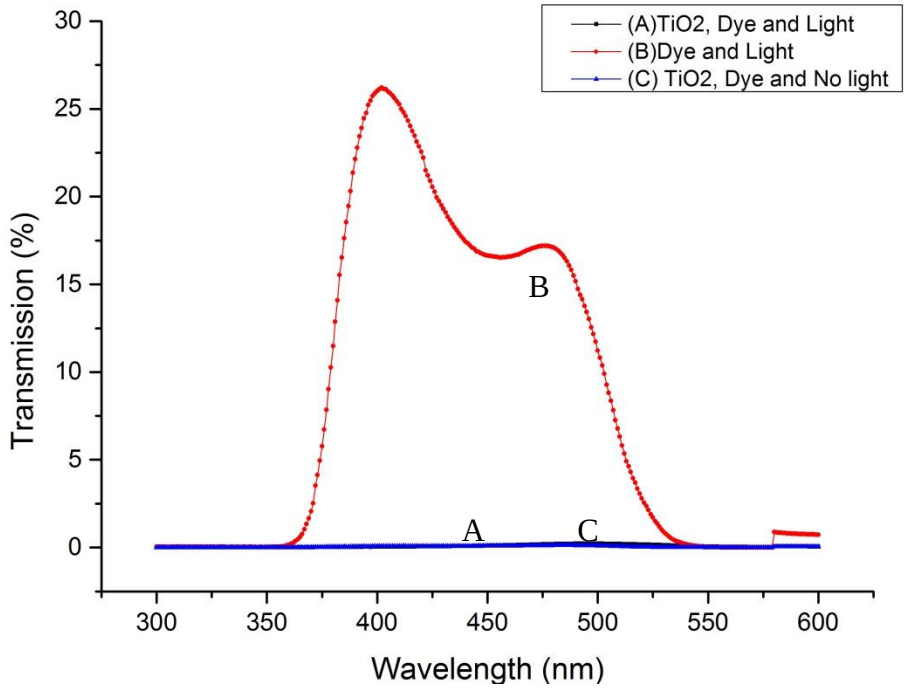

Figure 8.6 *Transmission spectra of Indigo Carmine Dye when exposed to (A) 405 nm light and TiO_2, (B) 405 nm light alone, and (C) TiO_2 alone.It is important to note that samples A and C are almost indistinguishable due to the presence of TiO_2.*

8.4 Discussion

The requirement to further enhance the microbicidal action of 405 nm light is key for the progression of decontamination applications in both clinical and food industries. The use of TiO_2 in combination with 405 nm light is advantageous due to its low cost and high photocatalytic properties, allowing for simple, safe and effective decontamination. This section of work has successfully demonstrated the enhanced efficacy of 405 nm light when combined with TiO_2 nanoparticles, both in suspension and on inert surfaces.

Previous studies had suggested that due to the wide band gap possessed by TiO_2 (3.0-3.2 eV), the use of visible light in photocatalytic applications was limited and may require doping in order to narrow the band gap. However a number of recent studies have since emerged showing the efficacy of light in the region of 405 nm for TiO_2 photocatalysis without doping (Petrov et al., 2013; Chen et al., 2012). This study has illustrated successful results for enhancing the bactericidal properties of 405 nm light through the addition of TiO_2 photocatalyst in both bacterial suspensions and on a range of surfaces.

Inactivation of *E. coli* in suspension was significantly enhanced in the presence of 0.1% TiO_2, with complete inactivation achieved with half the dose required when in the absence of TiO_2, highlighting the efficacy of TiO_2 as a photocatalyst when used in conjunction with 405 nm light. Results in Figures 8.3 and 8.4 also demonstrated enhanced inactivation of *E. coli* exposed to 405 nm light at 1% TiO_2 concentration, however, a lower rate of inactivation was achieved with 1% when compared to tests conducted at 0.1% TiO_2 concentration. This reduced antimicrobial efficacy at the higher TiO_2 concentration was a result of a reduction in 405 nm light transmission through the increasingly opaque TiO_2 suspensions, as highlighted in Figure 8.2.

Previous studies investigating the photocatalytic effects of TiO_2 combined with visible light have shown varying levels of success. A study by Tuchina et al. (2010), investigated inactivation of *E. coli* and *S. aureus* (10^3 CFU mL^{-1}) using 405 nm light at an irradiance of ~31.5 mW cm^{-2}, with 0.02% TiO_2. Tuchina reported complete resistance of *E. coli* to 405 nm light alone and only minimal inactivation in the presence of TiO_2, providing contradictory results to those obtained in this current study. Multiple

studies have demonstrated the bactericidal properties of 405 nm light on *E. coli* (Murdoch et al., 2012; Murdoch et al., 2010; Maclean et al.,2009). It is most likely that failure of bacterial inactivation reported by Tuchina resulted from a number of factors: Firstly, the applied dose of light exposure was insufficient, and secondly, the concentration of TiO_2 was too low to induce significant photocatalytic effects. Tuchina and colleagues also demonstrated photocatalytic inactivation of *S. aureus*, with 60% inactivation after an applied dose of only 9.5 J cm^{-2}. Although not investigated in this study, as mentioned previously, it is anticipated that photocatalytic inactivation using TiO_2 would be applicable to a range of microbial species.

An earlier study by Mitoraj et al. (2007) investigated the bactericidal effects of both UV-vis at 385 nm and visible 455 nm (200 mW cm^{-2}) light sources combined with a 0.1% concentration of TiO_2 on *E. coli* and *S. aureus*. They demonstrated that when exposed to UV-vis, 100% inactivation (6 $\log_{10}$ reduction) was achieved with 720 J cm^{-2}. However, with visible light they reported only a 20% reduction in *E. coli* population after an applied dose of ~1.44 kJ cm^{-2} and minimal *S. aureus* reduction (data not provided), demonstrating the greater efficacy of UV-vis light. Previous work by Maclean et al. (2008a) demonstrated the limited bactericidal properties of light over 440 nm. It is therefore reasonable to suggest that the source used by Mitoraj would be unlikely to possess direct bactericidal properties, but instead induce bacterial inactivation by activation of TiO_2. Furthermore, it is also not surprising that the dose required for UV-Vis (385 nm) was considerably higher than that used in this current work (1.44 kJ cm^{-2} vs. 273J cm^{-2}). This is likely attributable to the poor penetrative ability of 385 nm light through opaque TiO_2 solutions. This is illustrated in Figure 8.2, where transmission of ~380 nm light is less than 0.02%. This comparison highlights the importance of wavelength sensitivity for ensuring maximum bactericidal effects.

A number of previous studies have investigated the susceptibility of *S. aureus* as the model organism, during visible light photocatalysis. A recent study by Petrov et al. (2013) investigated the efficacy of 0.02% TiO_2 nanoparticles for inactivation of *S. aureus* when exposed to 405 nm light. They demonstrated that 56 J cm^{-2} 405 nm light alone caused 30% reduction of a 10^5 CFU mL^{-1} population, and this was increased to 67% using a dose of 28 J cm^{-2} when exposed in the presence of 0.02% TiO_2.

Chen and colleagues also investigated the use of ~2.1 kJ cm^{-2} 405 nm light (200 mW cm^{-2} for 3 hours) combined with TiO_2 for photocatalytic inactivation of *S. aureus*. Chen reported that 405 nm light alone demonstrated no inactivation of *S. aureus*, and when combined with 0.1% TiO_2 attained only an approximate 10% inactivation efficacy. These results differ significantly from results obtained in this work investigating TiO_2 photocatalytic effects combined with 405 nm light. It is surprising that no 405 nm light inactivation was achieved by Chen considering the dose applied was more than 20 × greater than the dose applied for *S. aureus* inactivation in previous chapters of this thesis. A major difference between the two studies was the volume of sample being exposed; Chen exposed 10 mL volumes, which may present attenuation issues. Another major difference between the two studies was that Chen exposed *S. aureus* in nutrient broth rather than PBS. It may be argued that providing the cells with a supply of nutrients during exposure can protect them by allowing them to metabolise and replicate whilst being exposed. Previous research by Murdoch investigated the variation in inactivation when exposing *S. aureus* in nutrient broth and PBS and found that inactivation in nutrient broth required greater doses than when exposed in PBS (216 J cm^{-2} vs. 144 J cm^{-2} for 5 log_{10} reduction), most likely due to the reduced transmission (Murdoch, 2011). Irrespective of this, at a dose of 2.1 kJ cm^{-2}, it is hard to understand why no inactivation was observed.

In addition to suspension inactivation, results from this chapter have demonstrated that significantly enhanced inactivation of surface contamination can be achieved by the addition of TiO_2 on to the exposed surfaces. Glass slides were initially used as a model surface to ensure the methodology allowed sufficient adhesion of both TiO_2 and bacteria. Following successful results on glass, various other surfaces were then tested. Results in Table 8.1 show both bactericidal effects of 405 nm light alone and the enhanced effects when combined with TiO_2 on a range of surfaces. Results highlight significant inactivation of *E. coli* on TiO_2-coated Tarkett wet room flooring and complete inactivation on both coated glass and Polyflor Pur 2000 flooring after an applied dose of 18 J cm^{-2}, when compared to non-coated surfaces, demonstrating the enhanced efficacy of 405 nm light for surface decontamination when using TiO_2 as a photocatalyst. This further highlights that synergistic treatments may lead to increased efficacy of decontamination and sterilisation technologies.

Previous studies have shown the effects of TiO_2-coated glass and plastic surfaces whilst exposed to UV and visible spectrum lighting (Pillai et al., 2012; Sunada et al., 2003; Chawengkijwanich and Hayata 2008; Mitoraj et al., 2007; Chen et al., 2012). However, to the best of our knowledge, this is the first study that has combined TiO_2 and 405 nm light with commercially available surfaces to demonstrate potential environmental surface decontamination applications. To date, although a small number of studies have been conducted using visible light, the majority of studies have utilised UV light for TiO_2 photocatalysis on surfaces. Pillai et al. (2012) exposed contaminated water samples in TiO_2 coated glass bottles to natural sunlight to try and promote water sterility. Positive results were found, with a 4 $\log_{10}$ reduction following 1.5 hours exposure. However, when a UV filter was added, inactivation was significantly reduced giving only a 1 $\log_{10}$ reduction after 2 hours exposure. Furthermore, Chawengkijwanich et al. (2008) and Sunada et al. (2003) have both demonstrated the bactericidal effects of TiO_2 coated surfaces when used in conjunction with UV light.

It is interesting to note the variation in results between suspension tests and surface tests. Results shown in Figure 8.3 show complete inactivation of *E. coli* in suspension (10^5 $\log_{10}$ CFU mL^{-1}) following a dose of 240 J cm^{-2}. However, a dose of only 18 J cm^{-2} was required to induce inactivation of the same *E. coli* population density on a range of surfaces. This difference may be credited to the fact that, when on surfaces, the bacterial cells are more evenly dispersed over a larger surface area, meaning the bactericidal effects of the light are not hindered by the opacity of the suspension or the depth in which the light had to penetrate.

All experimental work conducted in this study utilised Degussa P25 mixed phase TiO_2. As previously discussed, this mixture is composed of two mineral forms, anatase and rutile, each possessing different band gaps of which are sensitive to specific wavelengths. There is much controversy surrounding why this exact form possess greatest photoactivity. However, increasing research has suggested a number of possible reasons (Hurum et al., 2003):

1. The smaller band gap of rutile extends the useful range of photoactivity into the visible region, when combined with anatase.
2. The transfer of electrons from rutile to a lower energy anatase lattice leads to a more stable charge separation, which reduces recombination, whereas pure rutile generates rapid rates of recombination between h^+ and e^-.
3. The small size of rutile crystallites facilitates this transfer.

Previous work has demonstrated the bactericidal mechanism of TiO_2 photocatalysis (Haung et al., 2000; Sunada et al., 2003). Work investigating the mechanistic action of TiO_2 has suggested the initial target as the cell wall. In 1992, Saito and colleagues suggested that TiO_2 caused disruption of both the cell wall and cell membrane; this was shown by leakage of intracellular K^+ ions that paralleled with microbial death. Further evidence of membrane damage was reported by Sunada et al. (1998). When studying the bactericidal activity of TiO_2 on *E. coli*, they found that the endotoxin (LPS), a component of the outer membrane, was destroyed during photocatalytic treatment with TiO_2. Maness et al. (1999) reported that lipid peroxidation and subsequent breakdown of the cell membrane structure resulted in cell death. They concluded that the loss of membrane structure and therefore loss of membrane functions is the cause of cell death. Other studies have suggested direct photochemical oxidation of intracellular coenzyme A (CoA). Oxidation of CoA was shown to inhibit cell respiration and subsequently induce cell death (Matsunaga et al., 1988). However, a study by Huang et al. (2000), demonstrated that TiO_2 was able to diffuse through the outer membrane following TiO_2 induced membrane damage, enabling rapid destruction of the inner membrane and peptidoglycan layer, followed by progressive destruction of intracellular components (Huang et al., 2000; Gamage and Zhang 2010). Diffusion into the cell may be dependent on the size of the nanoparticles and how well clusters of TiO_2 particles are broken down. It is therefore most likely that the combination of initial cell membrane damage followed in some cases by further intracellular oxidative attack, ultimately results in cell death.

To help elucidate the inactivation mechanism and prove that an oxidation reaction was occurring by both 405 nm light alone and combined 405 nm light and TiO_2 treatment, suspensions were mixed with indigo carmine dye, in order to demonstrate photodegradtion. Degradation of indigo carmine occurs during oxidation, where the

breakdown of this large organic molecule results in decolourisation (Subramani et al., 2007). Decolourisation shown in Figure 8.5 demonstrates the breakdown of indigo carmine dye, both by 405 nm light and by combined 405 nm light and TiO_2 treatment. Results demonstrate that greater oxidation occurred in the presence of TiO_2 and 405 nm light when compared to 405 nm light alone. This suggests that greater bactericidal efficacy will occur in the presence of TiO_2, confirming this as a positive method for enhanced bacterial inactivation. Enhanced oxidation of the indigo carmine dye would have occurred by various mechanisms. Firstly, light alone would have a direct oxidation effect. The 405 nm light source would also interact with TiO_2, resulting in various oxidation processes described previously. This principle can also be applied to bacteria, where oxidative damage is being generated from both the light alone, from ROS produced by TiO_2 and by direct interaction with positively charged holes present in the surface of the TiO_2 nanoparticles, resulting in degradation of organic molecules.

In terms of potential applications, there are at present several companies manufacturing TiO_2 coated surfaces, such as MCH nano solutions and Titan Shield that do not interfere with durability/ composition of surface materials. However to date the use of TiO_2 on surfaces is based on the premise that either solar, standard room lighting or UV lighting is used for photocatalyisis. Although bactericidal properties of UV light are substantial, both alone and when combined with TiO_2, the implications for human health need to be considered, with safer, more favourable alternatives lying with visible light technologies. As previously discussed, the use of UV light has several limitations with respect to safety and degradation. Also, solar or standard room lighting contains only a small percentage (3-5%) of UV and a similarly small percentage of effective visible light for photoactivation of TiO_2 (Zhang et al., 2013). Therefore a combination of specific antimicrobial 405 nm lighting with TiO_2 coated surfaces presents significant decontamination applications for use within the food sector and also clinical environments. Results have shown in the current study that high-irradiance lighting can successfully induce photocatalytic effects of TiO_2. Results shown in Chapter 4, combined with previous environmental decontamination studies conducted within clinical settings (Maclean et al., 2010; 2013; Bache et al., 2012) using the EDS system, have demonstrated successful bacterial inactivation using low irradiance levels (~0.5 mW cm^{-2}). It would therefore be of great interest to investigate the efficacy of the

EDS system when used in conjunction with TiO_2 coated surfaces, and this would give a far clearer indication of potential environmental decontamination applications.

In conclusion, results from this chapter have demonstrated the synergistic effects of TiO_2 and 405 nm light for significantly enhanced bacterial inactivation, both in suspension and on a range of commercially available surfaces. Combining 405 nm light and TiO_2 has been shown to a promote a photocatalytic effect, whereby 405 nm light acts not only directly as a bactericidal light source but indirectly, by promoting activation of TiO_2 for photocatalysis, enhances the production of reactive oxygen species for microbial destruction. The combined action of 405 nm light and TiO_2 is likely to enhance bactericidal efficacy for areas where sterilisation and disinfection are required and this combination of antimicrobial effects may have great potential for environmental decontamination applications, with significant scope for the use of low irradiance 405 nm light for decontamination of TiO_2 coated surfaces.

CHAPTER 9
POTENTIAL APPLICATIONS OF 405 NM LIGHT FOR FOOD-RELATED DECONTAMINATION APPLICATIONS

9.0 General

With increasing global demand for food, the requirement for fresh, non-contaminated produce is continually increasing. Products such as bread, cheese and fruits are highly susceptible to mould and fungal spoilage. It has been estimated that as much as 5% of the world's total bread stock is lost annually due to mould spoilage, resulting in approximately £600 million financial loss (Needham *et al*., 2004; Sperber and Doyle 2009).

Due to changes in consumer demands over recent years, there has been a large shift from the use of chemicals for food decontamination and preservation. Consequently, various physical methods of decontamination have been increasingly employed. Despite the use and availability of treatments such as low temperature storage and modified atmospheric packaging (Nielsen and Leufven 2008), there have been a number of reports stating that many currently available technologies employed by the food industry are not effective enough, and in many instances have presented a negative impact on the quality of food produce (Luksiene and Brovko 2013). However, there is an obvious need for development of novel food decontamination treatments that are effective against a spectrum of microorganisms without altering the organoleptic properties and diminishing the nutritional value.

Until now, the work of this thesis has largely focused on the antimicrobial effects of 405 nm light on foodborne pathogens and spoilage organisms under various conditions, and also its potential application for environmental decontamination within the food environment. An area that has not yet been investigated is direct inactivation of microorganisms on fresh food produce. A number of studies have been conducted investigating the efficacy of violet-blue light for microbial decontamination of various

fruits; however, these studies have employed the use of exogenous photosensitising agents (Luksiene and Paskevicuite., 2011; Paskevicuite et al., 2009; Luksiene et al., 2009). The use of 405 nm light alone, without additional exogenous photosensitisers, for both preservation and microbial decontamination of food stuffs is an interesting idea, whereby continual exposure to 405 nm light may help to better preserve foods and in turn reduce both illness rates and financial losses.

This chapter firstly investigates the use of 405 nm light for preservation purposes, looking specifically at whether light exposure can reduce the development of natural spoilage of foods, including bread, fruit and dairy produce. The second section investigates bacterial decontamination of food stuffs. Finally, this chapter investigates bacterial inactivation in situations where the light must pass through various plastic packaging materials commonly used within the food industry.

9.1 Prevention of Food Spoilage using 405 nm light

The following section of work investigated the preservation efficacy of 405 nm light on three food types: bread, tomatoes and cheese, in order to assess whether spoilage of these products could be reduced or prevented if food-stuffs are stored under 405 nm light illumination.

9.1.1 Food Produce

Fresh produce was purchased prior to the start of a new experiment. For this series of work, no microbial preparations were required, as only natural spoilage was being investigated. Three food types (bread, cheese and tomatoes) were selected, due to their well-known susceptibility to spoilage. The food produce was prepared for experimental use as follows:

- Bread (Hovis, UK): a fresh loaf was purchased at the start of a new experiment. For exposure, a single slice was removed and placed into a transparent sandwich bag, to prevent the bread from drying during exposure.
- Tomato (Sainsburys, UK): fresh tomatoes were purchased at the start of each experiment. Tomatoes were removed from cellophane packaging and a single fruit was exposed directly to each light source.

- Cheese (Philadelphia, UK): individual Philadelphia cheese pots were used for cheese exposure tests. For exposure, the foil cover was removed from the container to allow exposure of the cheese to the light source.

9.1.2 Treatment method

Food-stuffs, prepared as above were placed directly under an ENFIS 405 nm light array (light source A) at a distance of ~20 cm, giving approximate irradiance of 3 mW cm^{-2} at the surface of the foodstuff. The light exposure unit was comprised of four arrays separated into exposure compartments by dividers, thus allowing four exposure tests to be carried out simultaneously. Food samples were placed under individual light sources, as shown in Figure 9.1. Once positioned, food samples were exposed continuously, at room temperature, for 7-9 days (depending on the degree of spoilage observed in each case). Samples were only removed from the light source for short durations (<5 minutes), to allow photographic evidence of the varying stages of spoilage to be recorded. Control samples were set up simultaneously, but were exposed to only standard laboratory lighting. Following exposure, control and test samples were compared visually, with qualitative results being recorded.

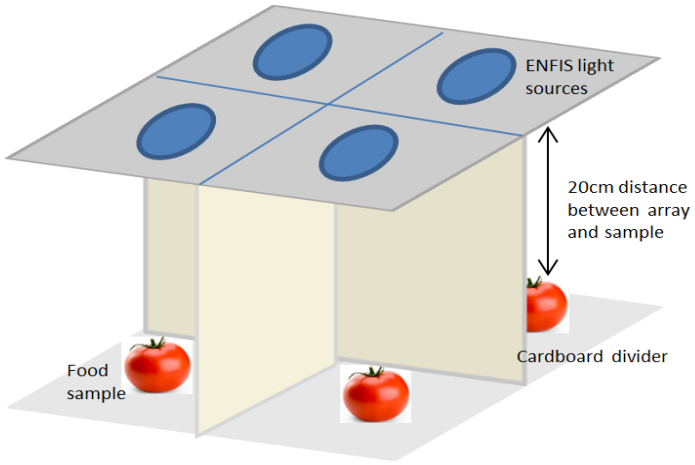

Figure 9.1 *Diagrammatic experimental set up used for simultaneous exposure of foods to individual 405 nm light arrays. The system was divided into four compartments, with an individual array used for illumination of each section, permitting exposure of four replicate samples simultaneously. Samples were exposed to ~3 mW* cm^{-2} *light at the surface of the foodstuffs, directly below the light source.*

9.1.3 Results: Use of 405 nm light for Prevention of Food Spoilage

Figure 9.2 illustrates the reduced spoilage of bread exposed continuously to 405 nm light for 9 days. As can be seen from Figure 9.2, non-exposed control samples (left) show substantial presence of mould across the entire surface area. In comparison, bread exposed to 405 nm light continuously (right) demonstrated virtually no mould growth on the directly exposed surface, highlighting that 405 nm light can effectively inhibit naturally-occurring spoilage. It is important to note that, although no spoilage is shown on the exposed surface of the test sample, the underside which received no light treatment, developed spoilage similar to that of the control sample, meaning that 405 nm light is only effective on directly exposed food surfaces.

Figure 9.2 *Photographic comparison of 405 nm light exposed bread (right), showing qualitative preservation effects of 405 nm light following continuous exposure at an irradiance of 3 mW* cm^{-2} *for 9 days. Non-light exposed controls demonstrated natural spoilage (left).*

In the case of tomatoes, Figure 9.3 highlights the obvious difference between exposed (right) and non-exposed tomatoes after 7 days. Results show that tomatoes exposed directly to 405 nm light showed no signs of spoilage, and both texture and colour

remained consistent, compared to non-exposed tomatoes which displayed reduced turgor, wrinkling of the skin, colour change and a large area that had developed soft and mushy texture (highlighted in Figure 9.3).

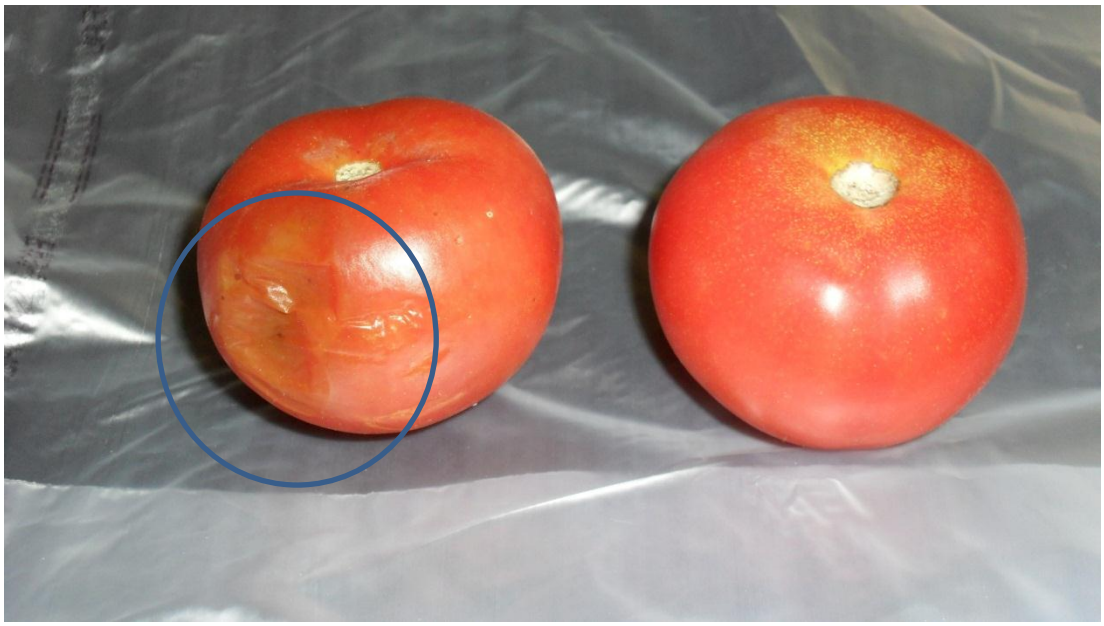

Figure 9.3 *Photographic comparison of 405 nm light exposed tomato (right), showing qualitative preservation effects of 405 nm light following continuous exposure at an irradiance of 3 mW cm^{-2} for 9 days. Non-light exposed tomatoes (left) demonstrated natural spoilage, which is highlighted in the photo.*

Figure 9.4 shows both 405 nm light exposed and non-exposed cheese samples after 7 days. As can be seen from Figure 9.4, no signs of mould spoilage were detected in either exposed or non-exposed samples. However, what can be observed is the fact that there is significant shrinkage in both cases, likely due to loss of water and subsequent drying as a result of the cheese being openly exposed without any protective film or covering. This loss of moisture is likely to have significantly affected the growth of microorganisms. Although spoilage was not detected, it is interesting to note the colour differences observed between light-exposed and non-exposed samples. As can be seen in Figure 9.4, the light exposed samples (bottom) have developed a distinct colour

change from non-exposed samples, indicating that 405 nm light has induced this colour change. This will be discussed further in Section 9.4.

Figure 9.4 *Photographic comparison of 405 nm light-exposed Philadelphia cream cheese (bottom), following 7 days continuous exposure at an irradiance of 3 mW cm^{-2}. Non-light exposed controls were simultaneously set up (top).*

9.2 Decontamination of *E. coli* Seeded Raspberries

Over recent years there have been a number of outbreaks of *E. coli* linked with contamination of various different fruits and vegetables, with small fruits becoming increasingly associated with foodborne illness (Bialka and Demirci 2008; Harris et al., 2003; Sivapalasingam et al., 2004). Consumption of contaminated fresh fruit produce is now regarded as the 2nd highest cause of foodborne illness in the US (CSPI 2006).

The following section details experiments which investigated the efficacy of 405 nm light for decontamination of small fresh fruit produce, using raspberries artificially seeded with *E. coli* as the test foodstuff. Raspberries were intentionally selected due to their non-uniform surface making decontamination significantly more challenging when compared to smooth-surfaced fruits.

9.2.1 Bacterial Preparation

E. coli was used throughout this series of experimental work. Bacterial suspensions were prepared as described in Section 3.4.1 and serially diluted in PBS to ~10^7 CFU mL^{-1} for experimental use.

9.2.2 Inoculation of *E. coli* onto the Surface of Raspberries

Raspberries were purchased at a local Marks and Spencer's supermarket and were used in experiments within 2 days of purchase. Raspberries were stored at 4°C when not in use. For each experiment, 15 g of raspberries were used. The berries were placed in a small container and the prepared inoculum (described above), was poured over the berries and left for 20 minutes at room temperature to allow bacterial attachment to the surface of the fruit. Methodology was adapted from Luksiene and Paskeviciute (2011).

9.2.3 Treatment Method

Following inoculation, the berries were placed in a laminar flow cabinet for 30 minutes to allow drying. Prior to light-exposure each berry was halved to allow direct exposure of the entire inoculated external surface. Raspberries were placed in a petri dish directly below the light source (Figure 9.1). Samples were exposed to increasing doses of 405 nm light. Non-exposed controls were prepared simultaneously, but these remained on the laboratory bench, exposed only to natural room lighting.

9.2.4 Bacterial Recovery

Post-exposure, raspberry samples were mixed with PBS (135 mL) and homogenized in a sterile Grade bag using a MIX2 stomacher for 60 seconds (DW Scientific, AES laboratories, UK). For enumeration, suspensions were serially diluted, plated as described in Section 3.4.2, and incubated at 37°C for 18-24 hours.

9.2.5 Results: Decontamination of *E. coli* seeded Raspberries

Results in Figure 9.4 demonstrate inactivation of *E. coli* contamination artificially seeded onto raspberries. Significant inactivation was achieved following an applied dose of only 10.8 J cm^{-2}, with an approximate 0.4 log_{10} reduction achieved. Following increasing doses of 405 nm light, inactivation of *E. coli* continued at a relatively linear rate, with an approximate 1.5 log_{10} reduction observed following 43.2 J cm^{-2}. Although

some inactivation was achieved, Figure 9.5 highlights limited bacterial inactivation. Control samples showed no significant reduction in bacterial contamination over the duration of the experiment.

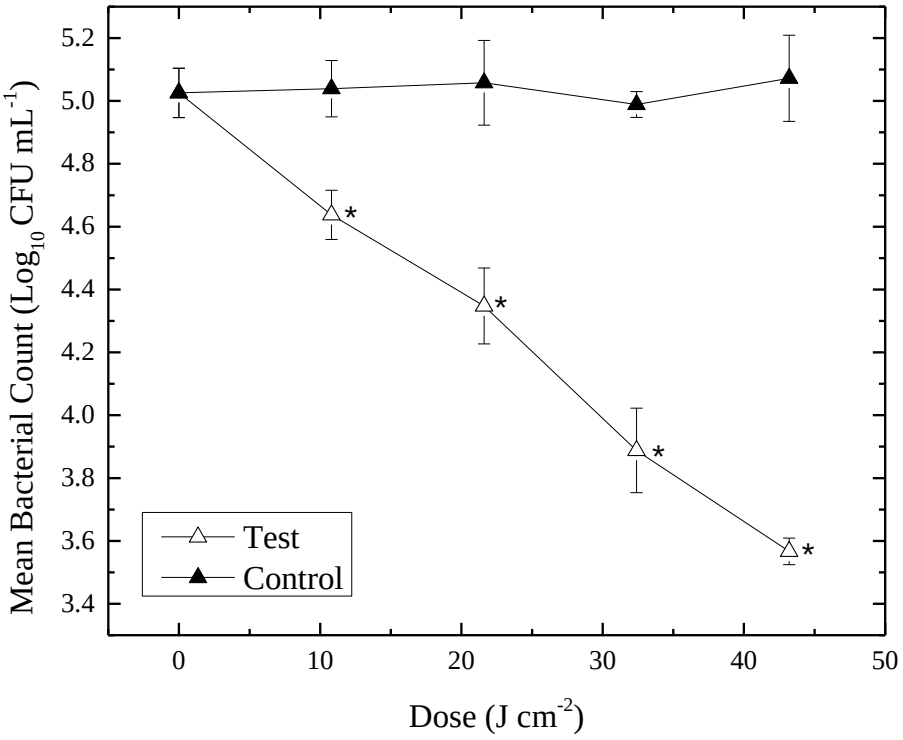

Figure 9.5 *Inactivation of E. coli seeded onto raspberries, following exposure to increasing doses of 405 nm light at an irradiance of ~3 mW cm^2. * represent significant bacterial inactivation when compared to the associated non-exposed control (P≤0.05).*

9.3 Inactivation of Bacteria through Packaging Material

This section of work investigated the penetrability and inactivation efficacy of 405 nm light through commercially available plastic packaging material used within the food industry.

9.3.1 Packaging Materials

Two types of commercially available plastic films, used for packaging and protection of fresh foods, were provided by LINPAC (West Yorks, UK). Lintherm VacD and Lintop are both used as transparent barrier films for protection of fresh produce such as meats,

poultry and fruits. Both materials act as oxygen barriers and are reported to extend product shelf life.

9.3.2 Optical Transmission Measurements

The transmission spectrum through each of the two plastic film materials was measured. Sample materials were cut to size, placed inside a plastic cuvette and transmission was measured between 200-600 nm. Figures 9.6 and 9.7 show the transmission spectra, with specific transmission at 405 nm highlighted: 73% transmission through Lintop, and 70% through Lintherm VacD. Direct measurement of the 405 nm light irradiance levels transmitted through the plastics were also recorded, and in accordance with the spectrophotometric analysis, demonstrated reduced light transmission. Irradiance was measured at a 3 cm distance from the array, giving an approximate irradiance of 70 mW cm^{-2}. Plastic films were then placed over the power meter and the irradiance remeasured, to determine the loss of irradiance through the plastics. Results are shown in Table 9.1.

It is also interesting to note that the loss of irradiance through each of the plastic films, shown in Table 9.1, corresponds well to the loss of % transmission displayed in Figures 9.6 and 9.7. For Lintop (0.093 mm thick) and Lintherm (0.194 mm thick), transmission measurements were shown to be reduced by 20 - 30 %, respectively. This corresponds well with the data in Table 9.1, which highlights a 27% and 30% reduction in irradiance when measured through the plastic films.

Table 9.1 *Irradiance measurements through LINPAC plastics and calculation of irradiance loss through materials, compared to direct irradiance (70mW* cm^2*).*

Material	**Transmissible Irradiance (mW cm^{-2})**	**% Loss of Irradiance**
Lintop	51	27
Lintherm VacD	49	30

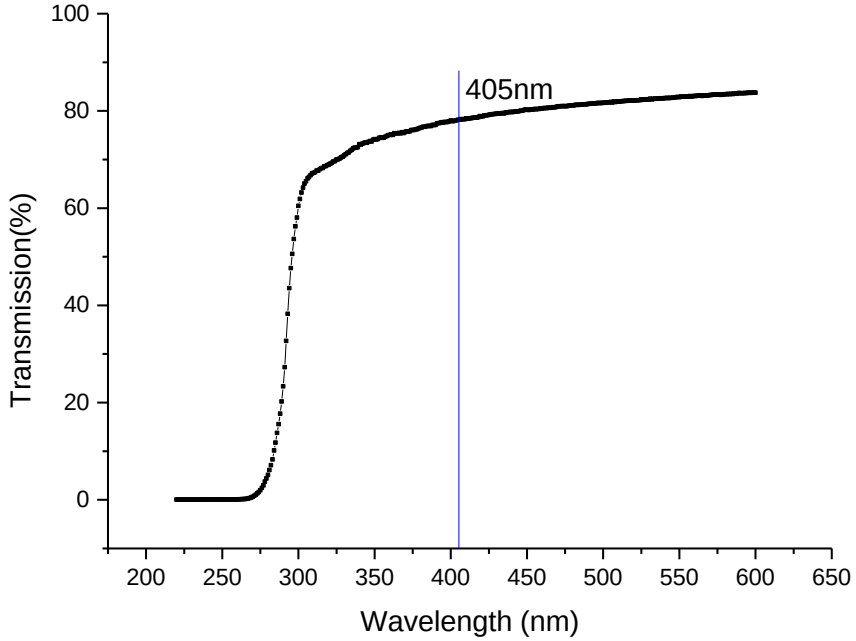

Figure 9.6 *Transmission spectrum of Lintop plastic film, with transmission at 405 nm highlighted.*

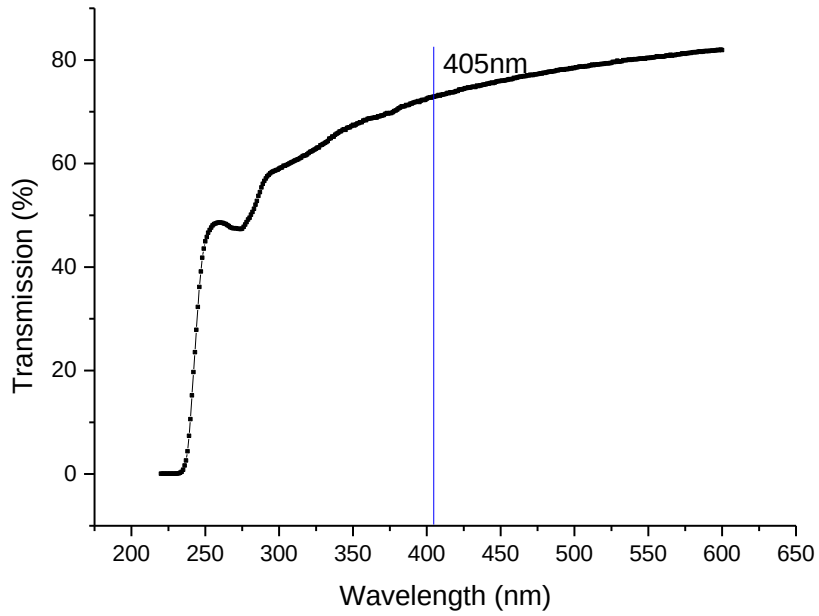

Figure 9.7 *Transmission spectrum of Lintherm VacD plastic film, with transmission at 405 nm highlighted.*

9.3.3 Bacterial Preparation

E. coli was used throughout this series of experiments. Bacterial suspensions were prepared as described in Section 3.4.1.

9.3.4 Treatment Method and Bacterial Recovery

A 3 mL volume of bacterial suspension with a population density of 10^5 CFU mL^{-1} was transferred into one of the central wells of a 12-well multi-dish. A layer of the plastic material was then placed over the dish. Samples were then exposed to 405 nm LED (light source C) as described in Section 3.3.3. The array was positioned directly above the sample, at a distance of ~ 3 cm, giving approximate irradiances of 51 mW cm^{-2} and 49 mW cm^{-2} through Lintop and Lintherm VacD plastics, respectively. Test samples were exposed to increasing doses of 405 nm light to determine the inactivation kinetics. Control samples were set up on the laboratory bench, but received no 405 nm light exposure. Both test and control samples were plated on to NA as detailed in Section 3.4.2. Samples were then incubated at 37°C for 18-24 hours and viable bacterial counts were recorded as $\log_{10}$ CFU mL^{-1}.

9.3.5 Results: Inactivation of *E. coli* through Plastic Packaging

Results in Figures 9.8 and 9.9 demonstrate inactivation of *E. coli* (10^5 populations) when exposed to increasing doses of 405 nm light through transparent plastic films. Results demonstrate in both cases that 405 nm light can penetrate the transparent plastic material and successfully inactivate *E. coli* populations.

Results in Figure 9.8 demonstrate inactivation of *E. coli* through Lintop plastic film, with results showing no inactivation up to 91.8 J cm^{-2}. Following an applied dose of 137.7 J cm^{-2}, a 1.6 $\log_{10}$ reduction was recorded. Although the standard deviation is relatively large at this data point, bacterial inactivation was shown to be significant when compared to the associated control samples. Following an applied dose of 183.6 J cm^{-2}, near complete inactivation (<1 CFU remaining from a 10^5 CFU mL^{-1} population) was achieved.

Similarly, 405 nm light exposure of *E. coli* through Lintherm VacD plastic demonstrated significant inactivation. Following a dose of 86.4 J cm^{-2}, a 1 $\log_{10}$ reduction in *E. coli* population was observed. This was shown to be significant when

compared to non-exposed control samples. Little inactivation was demonstrated between 86.4 and 129.6 J cm^{-2}, however, following a dose of 172.8 J cm^{-2}, a further 3.2 log_{10} reduction was recorded. Control populations remained consistent over the duration of the experiment, with no significant decrease in population observed.

It is worth noting that temperature measurements were recorded to ensure that covering the sample with a plastic film didn't result in a detrimental heating effect. The largest increase in temperature recorded was 8°C (21°C - 29°C) which would be insufficient to promote any detrimental effects on the bacterial population. Therefore, any inactivation that occurred was a direct result of 405 nm light exposure.

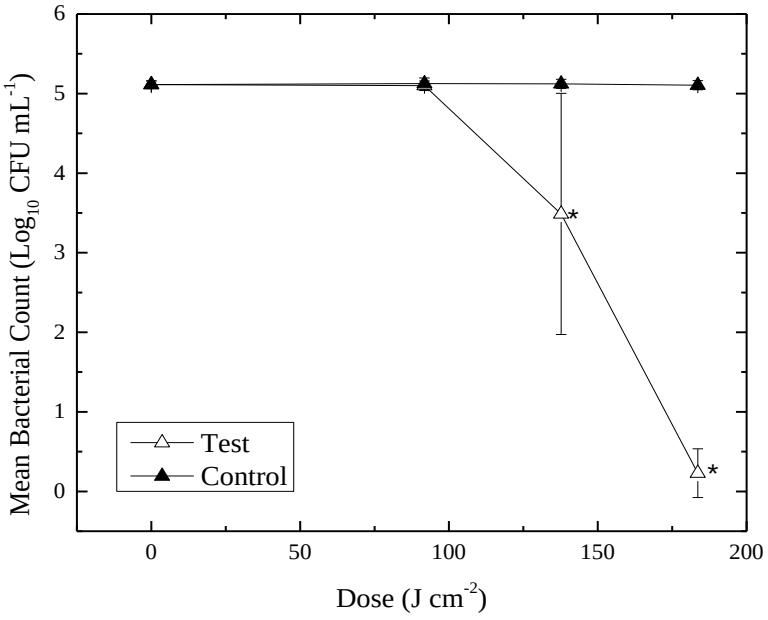

Figure 9.8 *Inactivation of E. coli in liquid suspension by exposure to 405 nm light through Lintop plastic film, at an irradiance of ~51 mW* cm^{-2}*. * represent significant bacterial inactivation when compared to the associated non-exposed control (P≤0.05).*

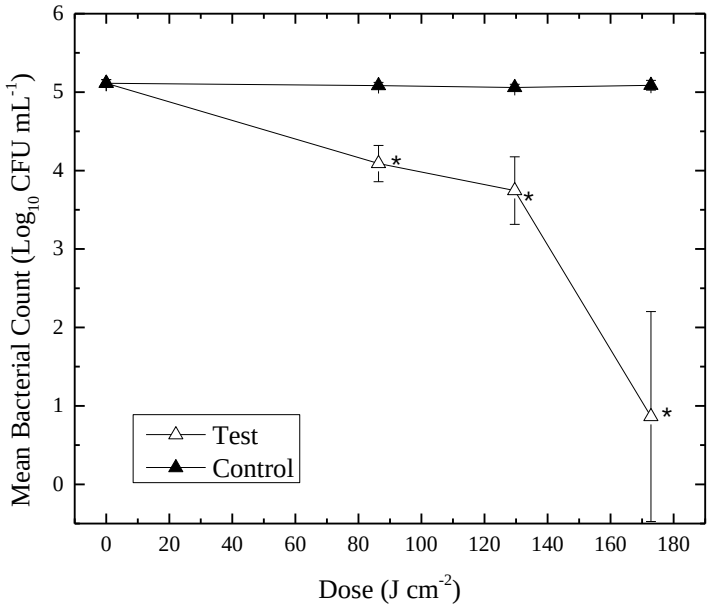

Figure 9.9 *Inactivation of E. coli in liquid suspension by exposure to 405 nm light through Lintherm VacD plastic film, at an irradiance of ~49 mW cm^{-2}. * represent significant bacterial inactivation when compared to the associated non-exposed control (P≤0.05).*

9.4 Discussion

Results detailed in this chapter have illustrated a number of possible practical applications for the use of 405 nm light within the food industry. Experiments have shown, for the first time, that 405 nm light may have potential not only for environmental decontamination, but may also have possible applications for food decontamination and prevention of spoilage. Results have demonstrated:

- Successful decontamination of food produce artificially inoculated with bacteria, following 405 nm light exposure;
- Prevention of naturally occurring spoilage by storage under 405 nm light illumination;
- Bacterial inactivation through commercially available plastic packaging materials, demonstrating that 405 nm light could potentially be used for decontamination of packaged foods or extension of product shelf life.

In order to demonstrate transmission of 405 nm light through transparent plastic films, bacterial suspensions were exposed to 405 nm light with plastic films placed over the sample dish. Bacterial suspensions were used for this study in order to provide relatively high bacterial populations to ensure sufficient inactivation was occurring. It is interesting to compare these results to previous data discussed in Section 4.1.4, where complete inactivation of *E. coli* (10^5 CFU mL^{-1}) required 436 J cm^{-2}, more than double the dose required for an approximate 5 log_{10} reduction in this study, despite having no plastic covering. It is worth noting that different arrays were used in this study and that of Chapter 4. It is important to highlight the differences in peak wavelengths between the two systems whereby the system used in Chapter 4 produced a peak wavelength at 408 nm, whilst the array in this chapter was shown to produce a peak at 400 nm. It is therefore likely that wavelength sensitivity is important to achieve maximum bactericidal effects.

High irradiance inactivation of bacterial suspensions was utilised to prove the principle that 405 nm light can transmit through transparent plastics and inactivate microbial populations. However in terms of practical applications, it is unlikely that such high irradiances would be employed. It is more plausible that a system developed for decontamination and/or preservation of fresh food produce would utilise irradiance levels similar to that of the EDS which is designed for environmental decontamination. The light intensity range required for both food processing and retail settings is 0.3-0.54 mW cm^{-2} (Luksiene and Brovko 2013). Therefore, a low-irradiance system would be ideal, as this irradiance is not only suitable for illumination of food settings, but has also been proven to be sufficient for inactivation of a range of microorganisms (Maclean et al., 2010; 2013; Bache et al., 2012).

Results in Figures 9.6 and 9.7 highlight the relatively high transmission of 405 nm light through the plastic films used in the study. These results further support data produced in Chapter 6, indicating that bacterial inactivation can successfully be achieved through various different plastic materials. Analysis of the transmission spectra demonstrates the increased transmissibility of 405 nm light when compared to shorter wavelength UV light. These results once again highlight the limitations of UV light; showing that 405 nm light could be successfully incorporated into food settings for decontamination of packaged food produce.

Until now, the use of 405 nm light has focused largely on environmental decontamination. However, the idea that 405 nm light could provide a novel method for food decontamination is extremely interesting. Little work has been conducted in this area, to date, with the majority of studies employing exogenous photosensitising agents to small fruits and vegetables (such as those described in Table 2.4) prior to light exposure at 400 nm. A number of studies by Luksiene and Paskeviciute have demonstrated that both *B. cereus* and *L. monocytogenes* can be significantly inactivated on the surface of fruit and vegetables when exposed to 400 nm light (6-14 J cm^{-2}) in the presence of photosensitising chlorophyll salt solutions. It is interesting to note that strawberries required the greatest dose of all produce tested, with 14 J cm^{-2} required to promote an approximate 2 log_{10} reduction, which was achieved for all other foods following an applied dose of only 6 J cm^{-2} (Luksiene and Paskeviciute 2011; Paskeviciute and Luksiene 2009). This is likely due to the irregularity of the strawberry surface, meaning bacteria are not evenly illuminated.

Results in this current study demonstrate that 405 nm light can successfully reduce *E. coli* contamination on the surface of raspberries without the requirement of exogenous photosensitising agents, although raspberry surfaces and other fruits, may well have photo-activatible compounds on them. Results in Section 9.2.5, demonstrated a 1.5 log_{10} reduction following 43 J cm^{-2} 405 nm light. A previous study by Bialka and Demirci (2008) demonstrated the efficacy of pulsed UV light for decontamination of *E. coli* on raspberries. Results demonstrated that at an applied dose of 44 J cm^{-2} a 1.5 log_{10} reduction was achieved, similar to the applied dose and bacterial reductions achieved in this current study, demonstrating that although pulsed UV light can be applied much more rapidly, the bactericidal efficacy for this particular application is not dissimilar to that of 405 nm light.

Although the dose utilised in Section 9.2.5 of this chapter was greater than those reported by Luksiene and Paskeviciute (2011), it is important to note a number of differences between the studies. Firstly, the fact photosensitising agents were employed by Luksiene and Paskeviciute is likely to be the primary reason for differences in inactivation. Furthermore the wavelength of light used in the two studies were different (400 vs. 405 nm) and also raspberries demonstrate even greater surface irregularity than

strawberries, possessing numerous crevices which may have promoted shadowing across the surface, interfering with direct illumination.

The use of light for microbial decontamination of foods appears to be effective. However, it was beyond the scope of this study to determine any light-induced effects on both organoleptic and/or nutritional properties. Work by Luksiene and Paskeviciute (2011) investigated the photosensitisation induced effects on a number of key quality attributes of treated berries. They found that strawberries exposed to 400 nm light exhibited no negative effects with respect to antioxidant activity, colour and taste. One of the main beneficial properties of fresh fruit and vegetables has been attributed to their natural antioxidants. Recently, phenolic compounds have received increasing attention due to their antioxidant properties, which are able to stimulate carcinogen detoxifying enzymes and counteract inflammatory processes (Parr and Bolwell 2000). Similarly anthocyanins, present in fruit, are known to help prevent cardiovascular disorders (Zafra- Stone et al., 2007). Investigation into whether photosensitisation reduced antioxidant content found minimal reduction following light exposure (Luksiene and Paskeviciute., 2011). However, it is surprising that no change in anthocyanin content was detected, considering the mechanism of inactivation employed by violet blue light, as many factors, including both light and oxygen, are capable of inducing anthocyanin degradation (Wang and Xu., 2007). A study by Tiwari et al. (2009) investigated the effect of ozone on strawberry juice anthocyanins and found ozone treatment reduced their concentration by 98%. Odriozola-Serrano et al. (2008) also demonstrated the negative nutritional effects of pulsed electric field treatment on treated strawberries. Luksiene and Paskeviciute (2011) also conducted a preliminary study in which 21 volunteers participated in a sensory evaluation of treated and non-treated strawberries. Eighteen of the 21 volunteers reported no sweetness or firmness changes in exposed and non-exposed berries. Based on the results obtained from Luksiene, it is anticipated that the use of 405 nm light, without photosensitising agents, has potential decontamination applications for food produce, such as ready to eat fruits. However, despite promising results, much greater evaluation of food quality and organoleptic properties following light exposure is required.

In addition to soft fruits, decontamination of other foodstuffs has also been investigated. A recent study by Haughton et al. (2012) investigated the susceptibility of *C. jejuni*

contaminated chicken to high intensity 395 nm light. Following artificial inoculation of *C. jejuni* onto raw chicken-breast, samples were exposed to up to 18 J cm^{-2} (10 minutes at 30 mW cm^{-2}). Results demonstrated an approximate 2.5 log_{10} reduction in bacterial counts when compared to non-exposed samples. The effect of light treatment on the quality of the chicken breast fillet was also investigated, and demonstrated that exposure to 18 J cm^{-2} was sufficient to induce significant colour lightening of the sample. The authors discussed that although colour was significantly affected, by altering a number of parameters such as distance between the sample and light source, and reducing the irradiance, negligible colour changes could be achieved in the chicken fillet. However these changes significantly reduced the level of bacterial inactivation achieved. Therefore further work is required to determine optimal operating procedures that could provide effective decontamination whilst presenting minimal detrimental attributes on food quality.

Despite positive food decontamination results being achieved, not only in this current study, but by various different groups, the extent to which 405 nm light may be utilised for direct food exposure may be severely limited. Endogenous photosensitiser molecules within foods such as riboflavins, myoglobins, chlorophyll and synthetic colorants are able to absorb energy from light and transfer it to triplet state oxygen to form singlet oxygen (Min and Boff 2002; Afonso et al., 1999; Lledias and Hansberg 2000). This can consequently result in the oxidation of food produce, which can influence nutritional and flavour quality, affecting consumer acceptability. Whang and Peng (1988) identified both myoglobin and oxymyoglobin as the primary photosensitisers of lipid oxidation in meats. They reported that peroxide values for both pork and turkey exposed to light were significantly higher than meats stored under dark conditions. Proteins have also shown to be susceptible to oxidation, capable of producing a range of off-flavours (King 1996).

A number of researchers have shown that exposure to visible light wavelengths between 365 nm and 500 nm can promote a significant increase in light oxidation in milk (Webster et al., 2009; Hansen and Skibsted 2000; Bradfield and Duthie 1965). Riboflavin has been implicated as the main photosensitising agents in dairy produce such as milk and cheese, known to be present in cheeses at levels of 0.3-0.6 mg per 10 g (Saxholt 1996). Light-induced degradation of lipids, proteins and vitamins can occur,

resulting in the formation of off-flavours and colour changes, which impair the product quality and nutritional value, and reduce its marketability (Webster et al., 2008; Mortensen et al., 2003). Colour changes in cheese associated with visible light exposure are highlighted in Figure 9.4. These qualitative images highlight the negative impact associated with direct exposure of cheese products to 405 nm light. It is therefore likely that 405 nm light could not be successfully used for decontamination and/or preservation of dairy products.

Despite inconclusive results with cheese samples, Figures 9.2 and 9.3 highlighted successful prevention of natural spoilage on both bread and tomatoes. Figure 9.2 highlighted significant inhibition of mould spoilage compared to non-exposed samples. With respect to inhibiting fungal growth, results in Chapter 5 demonstrated that exposure to 405 nm light both inactivated mould organisms and also interfered with the germinating process, resulting in stunted growth of hyphae. It is possible that in this case, direct exposure to 405 nm light may have either caused complete inactivation of any natural fungal contaminants, and/or induced stunted hyphal development, meaning mould growth was not visible. Further research is required to determine whether 405 nm light can induce detrimental oxidation effects on bread produce, similar to those reported with dairy and meats. To date, very little has been published on this subject area; however, Min and Boff (2002) reported that carbohydrates appear relatively insensitive to singlet oxygen oxidation, suggesting that light exposure for prevention of mould spoilage on breads may be a more plausible application.

Overall, results in this study have demonstrated that there are potential applications for 405 nm light in the decontamination and preservation of food produce. However, much research is still required to investigate both the physical and chemical effects of 405 nm light on food products. The use of hyper-spectral imaging for detection of changes in food products may determine detrimental effects induced by 405 nm light exposure. Overall, it appears that success may be dependent on the specific food group, but greater analysis must be conducted in order to establish this.

CHAPTER 10
CONCLUSIONS AND FUTURE WORK

10.0 General

This study examined the use of 405 nm light for inactivation of microorganisms associated with food spoilage and foodborne infection. Potential applications of 405 nm light for use within the food industry were also investigated. The findings of these investigations are concluded in this chapter. Also discussed are suggestions for future work that could help facilitate a greater understanding of the inactivation mechanism and further develop 405 nm light as a decontamination technology for use within the food industry.

10.1 Conclusions

10.1.1 Investigation into the use of 405 nm light for inactivation of foodborne bacteria

This study was conducted to demonstrate the bactericidal efficacy of 405 nm light on a range of bacteria commonly associated with foodborne infection, in both liquid suspension and on agar plates.

The inactivation efficacy of high intensity (up to 200 mW cm^{-2}) 405 nm light on *E. coli*, *S. enteritidis*, *S. aureus and L. monocytogenes* suspensions was first investigated. Inactivation was achieved for all bacteria following 405 nm light exposure. The rate of inactivation was variable amongst the different bacteria, with Gram positive bacteria being significantly more susceptible than Gram negative bacteria, in accordance with results in other published studies using hospital acquired infection-related bacteria (Maclean et al., 2009).

Following experimental work on inactivation of bacterial suspensions, low bacterial population densities were exposed to high intensity 405 nm light (~70 mW cm^{-2}) on agar plates in order to investigate the effectiveness of 405 nm light for decontamination of a test contaminated surface. Results again demonstrated the effective bactericidal

properties of 405 nm light, with significant reductions of surface populations in all cases with up-to 100 % reduction achieved.

In addition to the high intensity studies, a low irradiance system, the HINS-light Environmental Decontamination System (~0.5 mW cm^{-2}), was also used for exposure of contaminated agar plates. This combination of low irradiance light and low bacterial seeding populations was more indicative of the scenarios that would be found if utilising 405 nm light for decontamination of occupied room environments and premises, as demonstrated in clinical studies in hospital isolation rooms (Maclean et al., 2013; 2010; Bache et al., 2012). Results demonstrated that even at low irradiance levels, 405 nm light can successfully inactivate or inhibit a range of foodborne bacteria, when exposed on surfaces.

Results in Chapter 4 highlighted the antibacterial efficacy of both high and low irradiance 405 nm light for inactivation of foodborne bacterial pathogens, with initial results providing proof of principle inactivation kinetics.

10.1.2 Inactivation of fungi using 405 nm light

Following on from successful bacterial inactivation studies, the fungicidal effects of 405 nm light on a range of fungi, including *Saccharomyces cerevisiae*, *Candida albicans* and spore forming *Aspergillus niger* was investigated. Significant inactivation was achieved for all fungi. Photodynamic inactivation requires both oxygen and photosensitive molecules. Previous bacterial studies have provided evidence of the 405 nm light inactivation mechanism, demonstrating both the requirement for oxygen and presence of porphyrins (Maclean et al., 2008a; Hamblin et al., 2005; Nitzan and Kauffman 1999), however, to date this has not been evidenced with fungi. The first section of work in this chapter investigated the involvement of oxygen during 405 nm light inactivation. A series of experiments were conducted comparing fungal inactivation under aerobic and anaerobic conditions, confirming the role of oxygen in 405 nm light inactivation. Results demonstrated that under anaerobic conditions, inactivation was significantly reduced in all organisms, with greatest reduction observed with *A. niger* spores. In order to further deplete oxygen levels fungi (in an anaerobic cabinet) were exposed to 405 nm light in the presence of ROS scavengers and this was

shown to further reduce fungal inactivation. Overall, although differences were observed, results demonstrated that greatest inactivation was achieved when exposed under aerobic conditions in the absence of scavengers. These results demonstrated that reducing oxygen and ROS could reduce inactivation rates by up-to 5 $\log_{10}$, demonstrating the key role of oxygen during fungal inactivation by 405 nm light.

To provide further evidence supporting the inactivation mechanism of 405 nm light, the presence of porphyrins was investigated. Fluorescence spectrophotometric analysis of microbial suspensions was conducted to determine the presence of endogenous porphyrins. Fluorescence peaks in the region of 605-615 nm were detected suggested the presence of intracellular corproporphyrin, due to the similarity in its emission peaks when excited at 405 nm (Hamblin et al., 2005). These results suggest that inactivation of fungi involves photoexcitation of endogenous porphyrins within the exposed cells, as indicated for bacterial inactivation.

A significant finding of Chapter 5 was the demonstration of the enhanced susceptibility of *A. niger* spores to 405 nm light during germination, when compared to dormant spores. Results proved that, during germination, susceptibility to 405 nm light and subsequent inactivation significantly increased. Microscopic analysis confirmed that 405 nm light exposure of conidia during germination negatively affected both germ tube and hyphal growth processes. This suggests that efficacy of 405 nm light, and indeed other decontamination technologies, may be improved by induction of cellular processes such as germination that can make highly resistant spores much more susceptible to inactivation.

These results emphasise the complexity of the 405 nm light inactivation mechanism. Although results suggested the presence of porphyrin molecules and demonstrated the role of oxygen in fungal inactivation, further work is needed to identify specific roles of other contributing factors that may account for the variations observed between the inactivation responses of different species.

10.1.3 Disinfection of contaminated surfaces and biofilms using 405 nm light

This section of experimental work significantly built upon the previous agar surface inactivation data, and progressed to provide a detailed description of the inactivation

efficacy of 405 nm light for inactivation of *E. coli* seeded onto a range of inert surfaces including glass, acrylic, stainless steel and various rubberised flooring materials, indicative of surfaces commonly located in both, domestic kitchens and food processing environments. Despite variances in inactivation rates, significant inactivation was shown on all exposed surfaces with between 2 - 5 $\log_{10}$ reductions achieved, demonstrating the potential application of 405 nm light for surface decontamination.

Following inactivation of surface-seeded *E. coli*, a series of experiments were conducted investigating the inactivation efficacy of 405 nm light on single species and mixed-species biofilms on various surface materials. Variations in inactivation rates were observed, however this was considered likely to be an artefact of the variance of initial attachment levels. In all cases, significant reduction in bacterial counts were observed with up-to 8 $\log_{10}$ reductions recorded, demonstrating that 405 nm light can be successfully applied for decontamination of bacterial biofilms. Biofilm decontamination through transparent plastic and glass materials was also achieved, highlighting the penetrative properties of 405 nm light.

Results demonstrated that 405 nm light can be successfully used for disinfection of contaminated surfaces and biofilms on various surface materials. Work in this study also highlighted the penetrability properties of 405 nm light whereby biofilm grown on the underside of an exposed surface was significantly inactivated, demonstrating that 405 nm light decontamination is not limited to direct exposure, a major limitation of UV light technologies.

10.1.4 Synergistic bactericidal effect of 405 nm light when combined with sub-lethal temperature, osmotic and acid stress conditions

All experimental work in this thesis up to this stage had investigated microbial inactivation under essentially non-stressed conditions. However, bacteria within the food processing environment are likely to be exposed to a range of sub-lethal stress conditions and it was considered important to understand how such bacteria will respond to 405 nm light treatments. This section of work firstly investigated the synergistic effect of 405 nm light on *Escherichia coli* and *Listeria monocytogenes* in suspension when exposed to temperature, osmotic and acid stress conditions. These

environmental conditions were set at a level that would induce non-lethal stress (referred to as environmental sub-lethal stress conditions) and 405 nm light was then applied to determine if synergistic inactivation occurred. Results demonstrated that the inactivation efficacy of 405 nm light could be significantly enhanced when bacteria were exposed under sub-lethal stress conditions. Greatest enhanced inactivation was shown when bacteria were acid-stressed, with up-to 70% increased inactivation of bacteria when exposed simultaneously to acid and 405 nm light. Results also demonstrated that similar enhanced inactivation could be achieved when bacteria were exposed on surfaces, demonstrating that in 'real' environmental settings, 405 nm light inactivation is likely to be far greater than anticipated from initial surface inactivation results.

Although this study focused on only two species, *E. coli* and *L. monocytogenes*, it is predicted that a synergistic effect will be applicable to a wide range of bacteria. It is also reasonable to speculate that enhanced inactivation will not be restricted to the specific conditions employed in this work, but likely that any sub-lethally stressed bacterial populations exposed simultaneously to 405 nm light will undergo an enhanced inactivation effect.

Results obtained in this study support the use of 405 nm light in food preparatory and processing industries, demonstrating that enhanced inactivation is obtainable when bacteria are exposed under non-optimal conditions. Larger scale studies are required to determine the specific degree of microbial inactivation when exposed under other sub-lethal environmental conditions.

10.1.5 Photocatalytic enhancement of antimicrobial 405 nm light using TiO_2 nanoparticles

The use 405 nm light combined with TiO_2 nanoparticles to provide photocatalytically enhanced inactivation of *E. coli* was investigated, both in suspension and on inert surfaces. Significantly enhanced inactivation was observed upon combined treatment when exposed in liquid suspension, with 50% greater inactivation achieved with the addition of 0.1% TiO_2. Results investigating surface inactivation also demonstrated significantly enhanced inactivation of bacteria exposed in the presence of TiO_2. These results highlight that 405 nm light can not only induce bactericidal effects, but can be

used to successfully induce photocatalytic properties of TiO_2 for microbial destruction. In terms of practical applications, further work is required to establish both optimum concentrations of TiO_2 nanoparticles and 405 nm irradiances levels. However, exposure of TiO_2 coated surfaces to 405 nm light has potential to promote enhanced environmental decontamination.

10.1.6 Potential applications of 405 nm light for food-related decontamination applications

The final chapter of this thesis was split into 3 sections. The first investigated the efficacy of 405 nm light for prevention of natural food spoilage. Results in this section demonstrated that 405 nm light successfully prevented spoilage of both bread and fruits, when compared to non-exposed control samples. Interestingly, exposure of dairy produce highlighted negative effects on food quality, likely due to oxidation of food components, demonstrating that 405 nm light treatment is likely to be applicable to only certain food groups. Results in this section were only qualitatively assessed by visual inspection and further work is required to obtain quantitative results.

The second section of this study investigated the inactivation efficacy of 405 nm light for decontamination of artificially contaminated raspberries. Results highlighted 1.5 $\log_{10}$ reduction of *E. coli* on light exposed berries following only 45 J cm^{-2}, when compared to non-exposed controls. These results highlighted the potential treatment of foodstuffs with 405 nm light for microbial decontamination. However further research is required to ensure that organoleptic and nutritional properties are not altered during exposure.

The final section of work investigated the inactivation efficacy of 405 nm light through transparent plastic packaging films commonly used in the food industry. Results again confirmed the penetrative properties of 405 nm light, demonstrating significant inactivation of bacteria (5 $\log_{10}$) when exposed through the packaging film, indicating that the same method of treatment could be applied directly to packaged food stuffs for decontamination applications. It is important to highlight however that inactivation may be limited within modified atmospheric packaging due to the low levels of molecular oxygen available.

Importantly, results in this study have highlighted that 405 nm light may also have potential applications for direct food treatment, for both preservation and decontamination. However, more research is required to determine whether 405 nm light induces any detrimental changed in food quality and also further work is necessary to develop scaled up light delivery systems that could be practically deployed in the food processing industry.

10.2 Future Work

10.2.1 Further Microbial Inactivation Studies

An area in which, to date, very little research has taken place concerns the potential use of violet-blue light for viral inactivation. Norovirus is one of the leading pathogens associated with food-related infection (CDC 2011), and consequently the development of novel and effective technologies to reduce contamination levels is required. Due to the hypothesised mechanism of inactivation involving excitation of endogenous porphyrins, it is suspected that viral inactivation will be limited owing to the absence of endogenous porphyrin molecules. However, future work may be conducted investigating inactivation efficacy of viruses under various environmental conditions, whereby the presence of natural and/or synthetic exogenous photosensitizing molecules may help promote inactivation.

Furthermore future work investigating molecular mechanisms and systems biology must be investigated.

10.2.2 Inactivation Mechanism of 405 nm light

The examination of intracellular porphyrin molecules involved in 405 nm light inactivation can be conducted using high performance liquid chromatography (HPLC). As discussed previously, HPLC is a method employed to separate components of a mixture in order to analyse, identify and quantify specific components. Although a number of studies have utilised fluorescent spectrophotometry to identify porphyrins, these have simply identified emission peaks and correlated them with previous porphyrin data. More specific analysis is desirable in order to determine specific levels of differing porphyrins between different species and this may help ascertain differences

in inactivation susceptibilities. Also, identifying conditions that promote porphyrin production would also be useful.

An increasing number of studies have highlighted disruption of the cytoplasmic contents and breakage of the cell wall, suggesting that the primary target site of violet-blue light is the cell membrane. Ashkenzi et al. (2003) employed electron microscopy to demonstrate membrane damage. A more recent study by Dai et al. (2013) again demonstrated membrane damage of *S. aureus* treated with 425 nm light, strongly suggesting that blue light treatment may induce initial and/or predominant damage to the cell membrane. Results that have been presented in Section 4.1.5 also suggest 405 nm light may induce membrane damage. As discussed in Chapter 4, the loss of salt tolerance of *S. aureus* following 405 nm light exposure is indicative of membrane damage. Following membrane damage, inadvertent protein leakage would likely occur from the cell. Therefore, protein leakage from the cell into the extracellular environment should be investigated.

A number of methods are discussed throughout the literature for measuring protein leakage including LDH assay, Bradford assay and measuring leakage of UV absorbing material from the microbial cells. These assays could be applied to 405 nm light treated samples and compared to non-treated controls in order to quantify protein leakage from the cell which would help further elucidate the inactivation mechanism of 405 nm light exposed microorganisms.

10.2.3 Food Related Applications

Although results have demonstrated that 405 nm light is capable of inactivating foodborne pathogens in liquid, on nutritious and non-nutritious surfaces, there are several other considerations that need to be addressed prior to direct food decontamination. It is reasonable to suggest that 405 nm light treatment could be applied only for decontamination of food surfaces, meaning applications for whole-food decontamination may be limited. However, where surface spoilage is a particular concern, for example on fruits and breads, the use of 405 nm light may still be applicable. Much work is needed to ensure 405 nm light treatment of food produce does not induce any undesirable characteristics in respect to organoleptic and/or nutritional properties. Hyperspectral imaging has been regarded as an emerging analytical tool for

food quality and safety control, with recent applications being applied to testing the quality of various products such as fresh fruit and vegetable, meat and fish (Kelman et al., 2013; Mehl et al., 2004). Previous studies have utilised hyperspectral imaging to detect food spoilage by measuring biochemical changes in beef due to the presence of microorganisms (Peng et al., 2011). This imaging technique analyses the physical characteristics of the interaction between hyperspectral spectroscopy and the sample material, such as reflectance, transmittance, absorbance and fluorescence. Also, changes in key food components such as C-H, O-H and N-H molecular bonds can be detected (Kelman et al., 2013). Analysis of 405 nm light exposed samples could potentially reveal changes in chemical composition of the food, which may directly highlight any changes in organoleptic and nutritional properties. Imaging is described as a non-destructive, reliable and rapid technique, suggesting this method may provide relatively straightforward analysis.

Various assays may be employed to detect changes in internal chemical and antioxidant levels such as those investigated by Luksiene and Paskeviciute (2011). Sensory evaluation may also be conducted to assess both taste and texture changes. Therefore extensive and stringent testing would be necessary to ensure 405 nm light treatment of foods was safe and did not induce any toxic effect that would be harmful to consumers.

10.2.4 Potential Applications

The use of 405 nm light as an environmental decontamination system, has been successfully employed in clinical settings (Maclean et al., 2013; 2010; Bache et al., 2012). In terms of food-related applications, the EDS units could be installed into processing and production industries in order to reduce both air and surface contamination. On a domestic scale, 405 nm light may be integrated into under-unit kitchen lighting. Various other potential applications are also feasible, with a major option being directed at integration of 405 nm lights in refrigerators, at both domestic and industrial level. However, this would again involve prior evaluation of the effects of 405 nm light on food produce.

Based on results detailed in this thesis, development and integration of 405 nm light systems into domestic and industrial settings has clear potential to promote significant microbial decontamination. The use of 405 nm light within the food industry represents

a novel method for reducing microbial contamination that in turn, should potentially lead to a reduction in microbial transmission and subsequent foodborne infections.

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APPENDIX A

PEER REVIEWED JOURNAL PUBLICATIONS

- McKenzie K., Maclean M., Timoshkin I.V., MacGregor S.J and Anderson J.A. (2014). Enhanced inactivation of *Escherichia coli* and *Listeria monocytogenes* by exposure to 405 nm light under sub-lethal temperature, salt and acid stress conditions. *International Journal of Food Microbiology* **170**, 91-98.

- McKenzie K., Maclean M., Timoshkin I.V., Endarko E., MacGregor S.J and Anderson J.A. (2013). Photoinactivation of bacteria attached to glass and acrylic surface by 405 nm light: potential application for biofilm decontamination. *Photochemistry and Photobiology* **89**, 927-935.

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- Murdoch L.E., McKenzie K.., Maclean M., MacGregor S.J and Anderson J.G. (2013). Lethal effects of high intensity violet 405 nm light on *Sacchromyces cerevisiae, Candida albicans* and on dormant and germinating spores of *Aspergillus niger*. *Fungal Biology* **117,** 519-527.

CONFERENCE PUBLICATIONS

- McKenzie K., Maclean M., Anderson J.A., Timoshkin I.V and MacGregor S.J. Disinfection of contaminated surfaces and biofilms using violet-blue light: potential application for improved food safety? *The International Committee on Food Microbiology and Hygiene, 24th International Conference, Nantes France September 2014.* (POSTER).

- McKenzie K., Maclean M., Anderson J.A., Timoshkin I.V and MacGregor S.J. Titanium dioxide enhanced photodynamic inactivation of *S. aureus* and *E.coli*. *Society for General Microbiology, 169th Meeting, University of Warwick September 2012*. (POSTER).

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