Using Blue Light for Bacterial Inactivation

Miss Jodie Louise Barber BSc. Microbiology

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Supervisors: Professor Roger Pickup, Dr Glenn Rhodes

Declaration

The work presented in this thesis is the result of my own research. It has not been submitted in substantially the same form for the reward of a higher degree elsewhere. The majority of the bacterial strains, with the exceptions of the *Salmonella* and *Pseudomonas* mutants, were originally isolated and identified by Miss Katherine E. Aston as part of her Master's thesis. I have clearly referenced materials and results gained from other studies and cited accordingly.

Acknowledgements

Firstly, I would like to thank the company Marl International Limited for their collaboration with this project. Without their support and expertise, it would have been impossible to complete this project to the existing standard. Specifically, I would like to thank Mr Rob Woodhouse (Design Manager) for his time spent listening to my ideas and translating them into a purpose-built piece of equipment, along with the rest of the team members involved in its construction. A massive thank you to Mr Adrian Rawlinson (Managing Director) for his express interest in the project and for providing me with the opportunity to share the results gained from experiments with the valued customer Nichia, who were also the provider of the LEDs within the light module.

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Abstract

Aims. To investigate the inactivation of a range of recently isolated environmental and clinical bacteria using blue light from light emitting diodes (LEDs).
Specific objectives. To investigate the impact of the following on bacterial inactivation using a custom-built LED light module: i) exposure time; ii) sample distance; iii) bacterial concentration; iv) sample position on plate; v) incorporation of UV-resistance plasmids and also to conduct comparisons in inactivation efficacy of: i) pulsed versus continuous exposure to 405 nm light and ii) 405 nm versus UV-C.
Methods. Bacterial cultures were grown in Tryptone Soya Broth (TSB) and after processing were washed twice prior to re-suspension in Phosphate Buffered Saline (PBS). Following this, dilutions were made and exposed, on agar plates, to blue light (BL, 405 nm) or UV-C light (290 nm - 100 nm) in a variety of experiments.
Results. The results indicated that bacterial inactivation by BL is dose-dependent, the presence of a plasmid encoding UV-resistance genes did not provide protection, and no relationship between inactivation through BL or UV-C could be inferred.
Conclusions. Depending on the dose, 405 nm BL was shown to be effective at inactivating the majority of the bacterial species and strains tested. Exceptions were strains belonging to the genus *Enterobacter* and *Raoultella.* Overall, there was a broad array of sensitivities across the species used.
Significance and Impact of the Study. Results indicate a positive potential future for using BL to inactivate bacteria. More research is required to investigate this at different levels.

Research Background

The year 2014 marked the movement by the World Health Organisation (WHO) to address the current crisis affecting the globe report on the surveillance of antimicrobial resistance (AMR). Here, it was highlighted that a return to the pre-antibiotic era is a reality for the 21st Century if the problem is not properly addressed (WHO 2014). There are several approaches that may be taken in order to improve the situation. Among these are improved understanding of the mechanisms behind resistance development; heightened and more effective prevention strategies; the discovery of new antibiotics; and the development of novel technologies for the combating of microbial infections.

Following on from this, there were discoveries of alternative antimicrobial treatments prior to and in parallel with the advent of antibiotics, such as bacteriophage therapy (Wittebole *et al.,* 2014). However, these were overlooked due to the effectiveness of antibiotics at that time (Hamblin 2012). The current decrease in antibiotic efficiency allows for re-evaluation of the previously under-investigated strategies. Among these is antimicrobial photodynamic therapy (aPDT). This is an approach that utilizes the production of reactive oxygen species (ROS), via the excitation of photosensitizers, to inactivate microbes via the damage caused to a range of biomolecules. The photosensitizers may be exogenous or endogenous. This study focuses on the latter.

In recent years, research has developed and redirected from general use of aPDT (all light wavelengths) to a similar approach that utilises specific wavelengths of light. Of these, 405 nm light has shown strong potential for the reduction of a number of problematic microorganisms (McKenzie *et al.*,2013; Kim *et al.*,2016; Maclean *et al.*,2009; Murdoch *et al.*,2012; Guffey & Wilborn 2006; Maclean *et al.*,2010; MacLean *et al.*,2013). This study is a continuation of the existing research, and investigates a number of factors that may influence the effectiveness of 405 nm light at inactivating bacteria.

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

1.1 History of Photodynamic Therapy (PDT)

The first discovery that light can mediate a negative effect on microorganisms was made by Oscar Raab in the early 20th Century (Rajesh *et al.*, 2011). This was through observation that the combination of acridine red and light had a lethal effect on *Paramecium* spp. (Mitton & Ackroyd 2005) during experiments which were unrelated to this concept. Around the same time, a treatment for *Lupus vulgaris* using phototherapy was discovered by Neils Finsen (Rajesh *et al.*, 2011; Denis *et al.*, 2012), who was later awarded a Nobel Prize for the treatment of small pox using red light (Mitton & Ackroyd 2005). Despite this new-found knowledge, more research did not follow immediately, and it was not until decades later that experiments comprising the use of photosensitizers (PS), oxygen and light, a technique known as photodynamic therapy (PDT), made an emergence in the form of a book published by Thomas Dougherty *et al.*, (1978) (Rajesh *et al.*,2011). Interestingly, despite the previous discoveries made regarding the impact of light and PS on microorganisms, the focus of the book was on cancerous tumours. The successes of PDT led to its acceptance as a treatment of pre-cancerous skin lesions contained above neck level by the Food and Drug Administration in 1999 (Rajesh *et al.*,2011). To this day, a large sector of PDT is focussed on cancer treatments (Hamblin *et al.*, 2005) as opposed to microbial inactivation; a distinction between the two treatments is often made by referring to the latter as antimicrobial photodynamic therapy (aPDT). The emergence of multidrug-resistant bacterial pathogens worldwide has resulted in intense research efforts in novel antimicrobial technology, including aPDT and blue light therapy. However, extensive research needs to be performed on a large variety of bacteria, using numerous parameters, before the scope of using light for microbial inactivation can be fully determined.

1.1.2 Why move on from aPDT?

Although aPDT has been shown to successfully inactivate a diverse group of microorganisms, including bacterial species, viruses, fungi and mycoplasma (Hamblin *et al.,* 2005; Tavares *et al.,* 2010), developments of this strategy are being investigated that omit the use of externally applied photosensitizers (Maclean *et al.,* 2014; Maclean *et al.,* 2009; Enwemeka *et al.,* 2009; Bumah *et al.,* 2013; Song *et al.,* 2013). This is for a number of reasons: i) certain photosensitizers have been reported to have serious side effects on health (Bumah *et al.,* 2013); ii) the requirement of hospitals to put in place more efficient decontamination systems necessitates a method that is more practicable; whilst the effectiveness of PS in conjunction with light is impressive, it is not suitable for decontamination of large areas (a study by *Maclean et al*., (2010) successfully applied a blue light-system to disinfect large areas within a hospital room); iii) ideally a system that does not involve the use of light within the harmful ultraviolet region needs to be developed to allow continuation of daily activities whilst the treatment is being under-taken; iv) development of a cost-effective, easily accessible system – one that does not include highly specialist or complicated equipment; v) development of a decontamination method that does not permit the development of antimicrobial resistance; and finally, vi) development of a method that is versatile in application range. As a consequence of these requirements, studies on the use of blue light in place of aPDT for the inactivation of microorganisms have emerged in recent years. There is emphasis on the wavelength range 400 – 420 nm; of particular focus is the wavelength 405 nm (McKenzie *et al.,* 2013; Maclean *et al.,* 2009; Maclean *et al.,* 2014; Endarko *et al.,*). The paper by Ibbotson 2011 reviews the documented adverse effects of aPDT/PDT therapy. These range from mild short-term effects to more serious and medium- to long-term effects. These have been summarised within Table 1.1 below.

Table 1.1. Reported symptoms of aPDT/PDT on patients. The colour-coding refers to whether the effect is short-term (green), medium-term (orange) or long-term (red). Information was taken from the comprehensive review by Ibbotson (2011).

Symptom	Description and/or explanation	Reference
Pain	Unpredictable due to variation among individuals	Grapengiesser *et al.*,2002; Clark *et al.*,2003; Wang *et al.*,2001
	Caused by the generation of reactive oxygen species (ROS); this cannot differentiate between healthy patient tissue and site of treatment	Ibbotson 2011
	Predominantly experienced during the irradiation phase of treatment; stinging sensation may be experienced during application of photosensitizer onto broken/sensitized skin.	Clark *et al.*,2003
	The nature of the pain also varies during and after the irradiation treatment. Typically, the level of pain peaks during the second quarter of treatment and transitions from a stinging/burning sensation to one of throbbing. This may continue for numerous ours following treatment.	Valentine *et al.*,2011; Gholam *et al.*,2010
	The percentage of patients that find the pain to be severe varies among reports and papers; Ibbotson 2011 quotes ~20% of patients report extreme pain during treatment.	Ibbotson 2011; Grapengiesser *et al.*,2002; Sandberg *et al.*,2006; Moseley *et al.*,2006
Discomfort	Experienced by most patients. This can be due to a range of sensations, including stinging and burning	Morton *et al.*,2001; Clark *et al.*,2003; Wennberg 2005; Algermissen *et al.*,2003
Infection and pustules	The rate of infections is the lowest in comparison with alternative non-surgical techniques, including cryotherapy. This is likely because of the effects of inducing ROS species within infective agents themselves.	Morton *et al.*,1996; Dai *et al.*,2009; Yung *et al.*,2007
	Sterile pustule development commonly reported following PDT treatment for acne vulgaris	Stine Regin Wiegell & Wulf 2006; S R Wiegell & Wulf 2006
	Infrequent reports of PDT-induced cellulitis; this was shown in the study by Wolfe *et al.*,2007 to be caused by *S. aureus*.	Wolfe *et al.*,2007
Increased risk of ulceration or erosion at treatment site	This is linked to a number of factors, including infection and pustule formation. However, although the lower leg is a site featuring increased ulceration risk, it was shown that PDT elicits a lower risk than conventional therapies. Paper	Ibbotson 2011; Morton *et al.*,1996

	reports ulceration rate to be <1% in their studies.	
Purpura development	At treatment site. Observed for a small portion of the patients treatment by this paper. Factors that increase the risk of this event include the position of treatment site (below the knee) and venous stasis.	Ibbotson 2011
Oedema	Observed primarily where the treatment site is located on the temple or forehead area, resulting in swelling and/or bruising of the eye area.	Ibbotson 2011
Development of erosive pustular dermatosis	There was one report of this occurrence within the review by Ibbotson 2011; however, it is unclear whether the cause for this was the PDT or condition being treated (actinic damage). Furthermore, there are contradictory reports of successful treatment of erosive pustular dermatosis using PDT. Investigative work is therefore required to elucidate the mechanisms behind these phenomena.	Ibbotson 2011; Guarneri & Vaccaro 2009; Meyer *et al.*,2010
Reactivation of herpes simplex virus	This mainly concerns susceptible patients, where the treatment site is facial. Few reports of this occurrence.	Touma *et al.*,2004
Bullous pemphigoid development	So far only reported in Ibbotson *et al.*, could be due to immune-modulatory effects of PDT.	Ibbotson 2011
Immunosuppression	Immunosuppression is a side-effect of topical PDT. Reduction of epidermal Langerhans cells within animal models observed following PDT.	Ibbotson 2011
	Local and systemic immunosuppression results from ALA PDT in animal models.	Hayami *et al.*,2007; Kyagova *et al.*, 2011
	Immunosuppressive effect within humans was demonstrated in experiments on healthy subjects, where topical PDT was shown to reduce Mantoux reactions.	Matthews & Damian 2010
Dermatitis	May be due to: phototoxicity, irritation, sensitization, allergic contact dermatitis	Gniazdowska *et al.*,1998; Wulf & Philipsen 2004
	Reports of contact dermatitis to MAL following PDT; considered to be related to concentration.	Harries *et al.*,2007
Scarring	Atrophic or hypertrophic scarring may occur, although this is uncommon. Ibbotson 2011quotes the occurrence of significant scarring as <1% of cases. Therefore, PDT is considered a low-risk treatment regarding scarring, and has been recognised for superiority in patient preference and cosmetic outcome.	Ibbotson 2011
Photo-onycholysis	This was reported form a case where ALA leaked into the nails of a patient receiving PDT for actinic keratosis of the hands and fingers. The side-effects on the nails include extreme pain during treatment, discoloration and onycholysis. This was due to the combination of MAL and red light; onycholysis resolved within 3 months.	Hanneken *et al.*,2008

Milia or epidermoid cyst development	This is a reflection of the phototoxicity level, and occurs due to disruption of the dermo-epidermal junction. Although these generally resolve, they can present issues such as likeness to recurrent BCC (hence problematic diagnosis and persistence).	Ghaffar *et al.*,2007
Destruction of sebaceous glands	This can occur during treatment of *A. vulgaris* on the face. An approach to limit this is the reduction of irradiances used, since both the irradiance and total dose contribute to this happening.	Ibbotson 2011; Hongcharu *et al.*,2000
Pigmentation changes	Hypo- or hyperpigmentation may occur as a result of topical PDT. Although ALA and MAL can both result in pigmentation changes, the former is most often responsible.	Steinbauer *et al.*,2009
	PDT seems to activate melanocytes, resulting in higher numbers and thus increased pigmentation. This effect on pigmentation can continue during the two weeks following treatment.	Ibbotson 2011
	In one study, hyperpigmentation occurred in 2.2 % of cases.	Schroeter *et al.*,2007
	Hypopigmention, on the other hand, is much less documented. It is much less common than hyperpigmentation.	Ibbotson 2011
Hair growth alterations	Akin to pigmentation changes, alterations in hair growth due to PDT may be increased or decreased growth.	
Toxicity	PDT is both cytotoxic and genotoxic. Additionally, the impact of PDT varies according to the cell type – lymphocytes are an example of a susceptible cell type.	Chu *et al.*,2006; Calzavara-Pinton *et al.*,2007
Impact on DNA	Although the primary target of topical PDT is membranes, DNA-damage resulting from a variety of photosensitizers was demonstrated in *in vitro* studies. Single-strand breaks may also occur.	Ibbotson 2011

From the above table, it appears that the majority of side-effects of aPDT are short-term. Furthermore, the majority of adverse effects reported only occurred in a minority of cases. However, these effects are still an issue with aPDT/PDT. Therefore, there is a requirement to either find alternative photosensitizers or other adaptations to aPDT/PDT that reduce the likelihood of these occurring, or develop different phototherapy approaches altogether, where phototherapy approaches encompasses aPDT, blue light therapy, low-level light therapy and applications using ultraviolet (UV) light

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1.2 Using visible light to inactivate bacteria

There are an increasing number of studies that focus on the inactivation of bacteria using visible light (refer to Figure 1.1 for the visible and ultraviolet wavelength ranges). The phototoxicity of white light on *Staphylococcus aureus* has been demonstrated previously (Lipovsky *et al.*, 2009). The results of the study revealed that significant reductions in bacterial viability (up to 99.8 %) of some strains can be achieved using white light in the absence of exogenous photosensitizers. The effectiveness of this appears to depend on a variety of factors related to endogenous porphyrin content, pigmentation and bacterial response to oxidative stress. Figure 1.1 summarises the wavelength ranges of the ultraviolet and visible light sections within the electromagnetic spectrum (EMS).

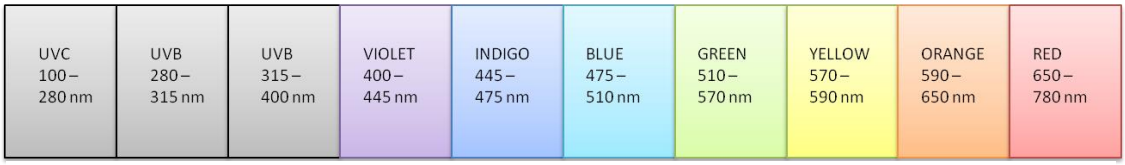


Figure 1.1. Ultraviolet and visible light wavelengths within the electromagnetic spectrum (EMS).

1.2.1 Blue Light for bacterial inactivation (BI)

A large portion of studies on visible light-mediated bacterial inactivation claim that the blue wavelengths (~400 – 500 nm) of visible light (~400 – 800) are the most effective (Lubart *et al.*, 2010). The primary focus of the study by Lipovsky *et al.*, (2010) was the level of reactive oxygen species (ROS) produced following exposure to different sets of wavelength within the visible region of the electromagnetic spectrum. These were 400-500 nm, 500-800 nm, 415nm and 455 nm respectively. The results indicated that the region that resulted in the highest production of ROS was the blue light (BL) region: 400-500 nm. Furthermore, within the BL spectrum, 415 nm was the most effective wavelength. This is similar to the inactivation results of other studies assessing the impact of BL on bacteria, such as the study conducted by Michelle Maclean *et al.*, (2008), in which the maximum inactivation of

Staphylococcus aureus was achieved through exposure to 405 nm within the range 400 – 420 nm. This range was also featured in the study by Tomb *et al.*, (2014), who additionally found 405 nm to be the most effective wavelength. Table 1.2 below lists the known bacterial species involved in studies assessing the inactivation efficiency of BL, along with the specific wavelength(s) within the spectrum.

Table 1.2. List of known bacterial strains/species involved in studies using BL, along with the specific wavelength(s) used.

Bacterial strain/species	**Source/collection number**	**Wavelength(s) used (nm)**	**Reference**
Staphylococcus aureus	NCTC 4135	405	(Maclean *et al.*, 2009; McKenzie *et al.*, 2013)
MRSA	Clinical isolate 16a, GRI	405	(Maclean *et al.*, 2009)
Staphylococcus epidermidis	NCTC 11964	405	(Maclean *et al.*, 2009)
Streptococcus pyogenes	NCTC 8198	405	(Maclean *et al.*, 2009)
Enterococcus faecalis	NCTC 00775	405	(Maclean *et al.*, 2009)
Clostridium perfringens	NCTC 13124	405	(Maclean *et al.*, 2009)
Acinetobacter baumannii	NCTC 12156	405	(Maclean *et al.*, 2009)
Pseudomonas aeruginosa	NCTC 9009	405	(Maclean *et al.*, 2009)
Pseudomonas aeruginosa	LMG 9009	405	(McKenzie *et al.*, 2013)
Escherichia coli	NCTC 9001	405	(Maclean *et al.*, 2009; McKenzie *et al.*, 2013)
Escherichia coli serotype 0157:H7	NCTC 12900	405	(Murdoch *et al.*, 2012)
Proteus vulgaris	CN 329	405	(Maclean *et al.*, 2009)
Klebsiella pneumoniae	NCTC 9633	405	(Maclean *et al.*, 2009)
Salmonella enterica serovar enteritidis	NCTC 4444	405	(Murdoch *et al.*, 2012)

Shigella sonnei	NCTC 12984	405	(Murdoch *et al.*, 2012)
Listeria monocytogenes	NCTC 11994	405	(Murdoch *et al.*, 2012)
Listeria monocytogenes	LMG 19944		(McKenzie *et al.*, 2013)
Mycobacterium terrae	LMG 10394	405	(Murdoch *et al.*, 2012)
MRSA	IS-853	470	(Enwemeka *et al.*, 2009)
MRSA	US-300	470	(Enwemeka *et al.*, 2009)
Aggregatibacter actionmycetemcomitans	ATCC 33384	400-420	(Song *et al.*, 2013)
Fusobacterium nucleatum	ATCC 23726	400-420	(Song *et al.*, 2013)
Porphyromonas gingivalis	ATCC 33277	400-420* 380-520**	(Song *et al.*, 2013)* (Soukos *et al.*, 2005)**
Porphyromonas intermedia	ATCC 25611	380-520**	(Soukos *et al.*, 2005)**
Porphyromonas nigrescens	ATCC 33563	380-520**	(Soukos *et al.*, 2005)**
Prevotella melaninogenica	ATCC 25845	380-520**	(Soukos *et al.*, 2005)**
Streptococcus constellatus	ATCC 27823	380-520**	(Soukos *et al.*, 2005)**

*Refers to the light wavelengths used by Song *et al.*, 2013, whilst **refers to those used by Soukos *et al.*, 2005.

Table 1.2 holds a list of the bacterial species that have been involved in studies featuring blue light. This information illustrates that a diverse range of bacteria from different genera have been included in these studies; therefore, it can be concluded that there is a broad spectrum of recorded effects of blue light on bacteria. It is clear that the most popular wavelength within the BL part of the EMS is 405 nm; this will be discussed in detail below.

1.2.2 405 nm – the most popular BL wavelength for bacterial inactivation?

Although a range of wavelengths of BL is presented in Table 1.2, it is clear that the most commonly applied wavelength is 405 nm. In the majority of cases, this is because multiple studies have been conducted by the same researchers; subsequently, there has been a progression in specificity from visible light through to 405 nm due to successive experiments narrowing down the most effective wavelength range (Maclean *et al.,* 2008). However, some studies have furthered the existing research to determine the factors behind the efficacy of 405 nm. In accordance with the widely-accepted theory that the mechanism behind BL bacterial inactivation involves excitation of endogenous photosensitizers leading to production of reactive oxygen species (ROS), analysis of porphyrin levels is featured in a few studies (Soukos *et al.*,2005; Ashkenazi *et al.*,2003; Borelli *et al.*,2006) . High-performance liquid chromatography (HPLC) analysis of the bacterium *Propionibacterium acnes* revealed that coproporphyrin is the most abundant porphyrin within this organism (Ashkenazi *et al.,* 2003). In contrast to *S. aureus*, which appears to contain one type of porphyrin, other bacterial species can contain multiple types. This is demonstrated in Table 1.3 below.

Table 1.3. Porphyrin distribution among different bacterial species.

Bacterial Species	Identified Porphyrins	Reference
Porphyromonas nigrescens	Uroporphyrin Heptacarboxyl Porphyrin Protoporphyrin	(Soukos *et al.*, 2005)
Porphyromonas gingevalis	Coproporphyrin	(Soukos *et al.*, 2005)
Prevotella melaninogenica	Uroporphyrin Protoporphyrin Coproporphyrin	(Soukos *et al.*, 2005)
Porphyromonas intermedia	Protoporphyrin Coproporphyrin	(Soukos *et al.*, 2005)
Staphylococcus aureus	Coproporphyrin	
Propionibacterium acnes	Coproporphyrin Protoporphyrin Coproporphyrin I Coproporphyrin III	(Ashkenazi *et al.*, 2003) (Shu *et al.*,2013) (Borelli *et al.*, 2006) (Borelli *et al.*, 2006; Kawada *et al.*, 2005)

The porphyrins in the above table are not an exhaustive list of those found in the corresponding bacterial species. This is due to the fact that in some cases, such as for *P. acnes,* there is conflicting literature on the types and amounts of porphyrins present (Shu *et al.*,2013). Furthermore, it was previously found that environmental factors such, such as culture age, habitat and medium pH, can determine the porphyrin quantities present within *P. acnes* (Kjeldstad *et al.*, 1984). There is a significant gap in the current literature regarding porphyrins in bacteria that needs to be addressed.

1.3 Contradictory results: light-induced proliferation of bacteria

In contradiction to the results of the majority of studies featuring BL-inactivation of bacteria, the opposite was shown to occur to one of the *Staphylococcus aureus* clinical isolates in the study by Lipovsky *et al.*, 2009. Whilst BL resulted in a decrease in viability of strain 101, strain 500 displayed a 15.8 % increase following exposure to a BL dose of 72 J/cm². In addition to this, doses of 7.2 and 36 J/cm² caused proliferation of both strains of *S. aureus* (Lipovsky *et al.*, 2009). This proliferation effect was also observed in another study, involving the organisms *Staphylococcus aureus, Pseudomonas aeruginosa* and *Propionibacterium acnes* (Guffey & Wilborn 2006). The dose range in the study by Guffey & Wilborn (2006) was low: 1 – 15 J/cm². Subsequently, proliferation of *Staphylococcus aureus* following exposure to 405 nm at the lowest dose resulted in proliferation; all of the doses resulted in proliferation of *Propionibacterium acnes* at this wavelength; and no proliferation was observed for *Pseudomonas aeruginosa.* When the wavelength of 470 nm was applied using the same dosage range, similar results were observed. No proliferation was observed for *Pseudomonas aeruginosa,* every dose resulted in proliferation of *Propionibacterium acnes* and proliferation of *Staphylococcus aureus* occurred up to doses of 10 J/cm². These results demonstrate that whilst high intensities of BL result in bacterial inactivation, presumably through production of ROS, the opposite occurs when the intensity is too low. There is therefore a requirement to determine the minimum thresholds for the inactivation of a range of bacterial strains, so that a general minimum intensity for bacterial inactivation may be established.

1.4 Mechanisms of action of aPDT/PDT and BL

The mechanism(s) of action for both aPDT and BL-inactivation are the same in the existing literature. The difference between the two approaches is the nature of the photosensitizers and the range of wavelengths used. For aPDT, exogenous photosensitizing agents are applied to bacteria prior to exposure to broadband visible light (Tavares *et al.*,2010). For BL-inactivation, it is thought that some bacteria possess endogenous photosensitizers, mostly in the form of porphyrins. This is therefore exploited by applying wavelengths corresponding to the peak absorbance of

porphyrins. Ultimately, it is the production of reactive oxygen species (ROS), in both instances, that is responsible for the detrimental effects (Rajesh *et al.*, 2011) that occur. This phenomenon was demonstrated by Lipovsky *et al.*, (2010). Furthermore, a correlation between ROS level and extent of phototoxicity was also found by Lipovsky *et al.*, (2008;2009). The role of ROS, and therefore the presence of oxygen, in phototoxicity has been demonstrated through experiments that counteract the presence of ROS and oxygen. This has been achieved by incorporating ROS scavengers, or altering the conditions such that oxygen is absent (Ginsburg *et al.*, 2005). Furthermore, the requirement of oxygen in photodynamic inactivation was illustrated by von Tappeiner and Jodlbauer in 1904 (Mitton & Ackroyd 2005).

1.4.1 The Type I and Type II (photochemical) reactions

Following the excitation of photosensitizer (PS) molecules, whether endogenous or exogenous, the transition from the PS ground state to an excited state leads to one or both of two photochemical reactions (De Lucca *et al.*, 2012). The excited triplet state PS may:

i) React with a substrate, resulting in the production of lipid-derived or hydroxyl radicals (OH●) through electron transfer. In turn, these result in production of reactive oxygen species (ROS) through reaction with endogenous molecular oxygen (Type I Reaction) (Koshi *et al*, 2011).

ii) Contribute to the production of singlet state oxygen due to energy transfer from the transition of the excited triplet state PS to ground state (triplet) molecular oxygen. Biomolecule oxidation and cell death ensue (Type II Reaction) (Fila *et al.*, 2013).

Both of the above pathways induce the oxidative stress response in bacteria. Although both pathways are capable of causing damage, the antioxidant enzymes contained by bacteria, including catalase and superoxide dismutase, are unable to counter-effect the actions of singlet oxygen (Almeida *et al.*,2011), making this the most detrimental type of ROS.

In an attempt to determine the pathway of most importance in inactivation of Gram positive and Gram negative bacteria, Hamblin *et al.*, (2012) conducted a series of experiments to investigate the contribution to killing by singlet oxygen and hydroxyl radicals. The markers used to study these components' individual contribution were singlet oxygen sensor green (SOSG) and hydroxylphenyl fluorescein (HPF) respectively. The results gained from this study indicated that Gram Positive and Gram Negative bacteria exhibit different sensitivities to the products of the type I and II pathways of aPDT. Whilst Gram positive bacteria appear to be more susceptible to singlet oxygen, hydroxyl radicals appear to be more detrimental towards Gram negative bacteria. In either case, it has been postulated that the multi-target attack of ROS is attributed to the inactivation of bacteria, such as *E. coli* (Mitoraj et al. 2007).

1.4.2 Reactive Oxygen Species

The toxicity of reactive oxygen species (ROS) has been investigated for decades, following the discovery by Carlioz & Touati (1986) that superoxide dismutase (SOD) is essential for the correct growth of *E. coli* aerobically. Oxidation of biomolecules, such as amino acids, is of restricted efficiency by molecular oxygen due to the molecular orbital structure of oxygen (Cabiscol *et al.*, 1999). Since the outer shell contains two spin-aligned, unpaired electrons, it is only possible for a single electron to be transferred from a molecule containing spin-paired electrons. In addition to this, oxygen molecules and the majority of organic molecules are weak univalent electron acceptors and donators respectively (Imlay 2003). ROS, on the other hand, have greater reduction potential and hence are stronger univalent oxidants than oxygen itself (Imlay 2003).

1.4.2.1 Intracellular ROS production

ROS are produced continuously within aerobically growing microorganisms (Morgan *et al.*, 1986) due to univalent reduction of molecular oxygen (Gonzalez-Flecha & Demple 1995; Cabiscol *et al.*, 1999; Morgan *et al.*, 1986) and as by-products of adventitious oxidation of redox electrons by molecular oxygen (Imlay 2003). In *E.*

coli , Cabiscol *et al.*, 1999 showed that 87% of total H_2O_2 was generated via the respiratory chain. This has also been highlighted as an internal source of O_2^-. This is because the enzymes involved in the respiratory chain are effective at univalent redox reactions, a required property of enzymes for the successful transferral of electrons to oxygen (Imlay 2003; Gonzalez-Flecha & Demple 1995; Imlay 1995). Subsequently, bacteria (particularly aerobes and facultative aerobes) have developed mechanisms to maintain the ROS level to an acceptable level (Tamarit *et al.*, 1998). For example, in the organism *E. coli*, there is a continuous system in place whereby the by-products of autoxidation of the redox enzymes, such as superoxide and hydrogen peroxide, go on to be degraded by enzymes. These include superoxide dismutase, catalase and catalase-peroxidase (Keith & Valvano 2007).

It is when the level of ROS increases beyond the capacity of the cell to counteract this that oxidative stress occurs (Cabiscol *et al.*, 1999; Farr & Kogoma 1991; Tamarit *et al.*, 1998). The acceleration of mutagenesis and enzymic damage are two examples of biological events that occur due to elevated intracellular levels of oxidants within bacteria (Imlay 2015a). It is speculated that numerous external stressors are responsible for this elevation; however, there is conflicting evidence on this topic (Imlay 2015a). The paper by Imlay (2015) lists 30 events that have been suggested to trigger increased oxidant levels in bacteria, including near-UV radiation. Antibiotics are an example of a high-controversy subject regarding increased ROS production.

1.4.2.2 Impact of ROS on biomolecules

ROS are suggested to irreversibly oxidise cellular macromolecules, including DNA, RNA, lipids, proteins, unsaturated triacyl glycerols and cholesterols (Calzavara-Pinton *et al.*, 2007; Alves *et al.*, 2013). This results in loss of function and structural changes (Alves *et al.*, 2013). The impact is therefore multi-target and non-specific (Almeida *et al.*,2011). This was demonstrated by the damage to *E. coli* from non-coherent visible light, within the range 408-750 nm. Affected areas include the membrane, metabolism, mutagenesis and loss of culturability (Arana *et al.*, 1992; Webb & Malina 1967). However, despite this existing knowledge, the precise details of what is required for cell death to occur have not been clearly established (Ruiz-González *et*

al., 2012). For example, it is unclear how important PS location is in PDT (Ruiz-González *et al.*, 2012). Further insight has been provided using electron microscopy has demonstrated that damage to the cell envelope occurs progressively during exposure to irradiation (Ruiz-González *et al.*, 2012).

1.4.2.3 Oxidising potential of ROS

Despite the potential for damage that can occur, there are also restrictions on certain ROS that limit their oxidising potential and reactivity. For example, interference in the oxidation of electron-rich molecules by O_2^- occurs due to its anionic charge. The reactivity of H_2O_2 is limited by the stable nature of the oxygen-oxygen bond (Imlay 2003). Neither of these events apply to the hydroxyl radical; it is therefore the ROS species that can cause the most damage (Imlay 2003; Cabiscol *et al.*, 1999).

1.4.2.4 ROS and Lipid Peroxidation

Lipid peroxidation has been attributed to cell death through oxidative stress (Maness *et al.*, 1999). There are two modes of attack of ROS on lipids. These are:

i) **Direct effects.** Lipid peroxidation occurs when unsaturated fatty acids are oxidised. This results in alteration of membrane properties due to decreased membrane fluidity, in addition to disruption of membrane-bound proteins (Cabiscol *et al.*, 1999).

ii) **Indirect effects.** Furthermore, the generation of further highly-reactive products, such as aldehydes, can result (Cabiscol *et al.*, 1999). These are longer-lived than ROS; therefore, they are able to exert damaging effects over a prolonged amount of time.

A lipidomic approach was used by Alves *et al.*, 2013 to determine the impact of photodynamic inactivation on membrane phospholipids in *E. coli*. Since cytoplasmic membranes are primarily composed of phospholipids, this is an important site of ROS-mediated damage during photoinactivation of bacteria (Alves *et al.*, 2013). This

is reflected by the 97.5 % increase in lipid hydroperoxides in bacterial cells subjected to photosensitization in the study by Alves *et al.,* 2013.

1.4.2.5 ROS and Enzymes

Another effect of ROS is the inactivation of enzymes. This can occur due to the conversion of amino acids into derivatives; these are responsible for the inactivation (Flahaut S, Laplace JM, Frère J 1997). Following a 1 hour exposure to 2 mM H_2O_2, losses of 80% and 25% of enzymatic activity by alcohol dehydrogenase E and enolase in *E. coli* were observed (Tamarit *et al.,* 1998).

1.4.2.6 ROS and Protein oxidation

The cellular metabolism of cells is disrupted by oxidation of proteins (Cabiscol *et al.,* 1999). This is due to the structural alteration and changes in the function as a consequence of oxidation (Cabiscol *et al.,* 1999); this can be caused by oxidation of cysteine-bound sulphur atoms and methionine (Kashmiri & Mankar 2014). Subsequently, alteration of membrane properties and decreased membrane fluidity can result (Kashmiri & Mankar 2014).

Furthermore, disruption of protein activity can occur due to changes in the cytosol reducing environment; this has implications for cytosolic proteins that have evolved such that the cysteines are maintained in the reduced form (Cabiscol *et al.,* 1999). The formation of carbonyl groups in amino acid residues may occur (Tamarit *et al.,* 1998), leading to protein unfolding (Kashmiri & Mankar 2014). Indeed, successful measurement of oxidative stress in tissue, protein and cell samples is enabled by the presence of these carbonyl groups (Tamarit *et al.,* 1998). The major protein targets within *E.coli* in the study by Tamarit *et al.,* 1998 were involved in diverse, but vital, functions. These included protein synthesis (EF-G), chaperone-function (DNA-K), glucose catabolism (alcohol dehydrogenase E) and outer-membrane protein OmpA (Tamarit *et al.,* 1998). However, not all proteins are susceptible to oxidation. An

example of such was discovered by Tamarit *et al.*, 1998: the outer membrane protein OmpC.

1.4.2.7 Oxidative DNA damage

The sugar and base moieties of DNA and RNA are a primary target, leading to blockage of replication due to lesion formation. However, in contrast to UV radiation, the DNA damage incurred by aPDT is not thought to be a substantial factor in the killing of cells. Furthermore, DNA repair via specialist proteins can occur, limiting the effectiveness of this as a mechanism. Damage to DNA can occur due to particular ROS. Furthermore, ROS that unable to directly cause damage, such as superoxide, are able to indirectly cause damage. In the case of superoxide, this is because of its involvement in the production of a ROS that directly damages DNA, hydroxyl radicals (Imlay 2015b). DNA repair enzymes of importance include exonuclease III, DNA polymerase I, RecBC nuclease and RecA protein (Morgan *et al.*, 1986).

1.4.2.8 Oxidative stress regulons in *Escherichia coli* and *Salmonella typhimurium*

Bacteria possess systems that are able to sense adiverse conditions and elicit a response accordingly (Storz *et al.*,1990). Following on from this, mechanisms for the response and damage-limitation of reactive oxygen species (ROS) are possessed by all aerobic organisms (Farr & Kogoma 1991). In the case of *Escherichia coli* and *Salmonella typhimurium*, some of these mechanisms are controlled through two known regulons: SoxRS and OxyR (Storz *et al.*,1990; Christman *et al.*,1989; Morgan *et al.*,1986; Farr & Kogoma 1991). In addition to these, there is the well-known SOS response in bacteria, which was the first discovered DNA-repair system in *E. coli* (Janion 2008). However, OxyR will be the primary focus of this study regarding regulons and oxidative stress in bacteria.

1.4.2.8.1 OxyR: Overview

OxyR belongs to the LysR family of regulatory proteins (Storz *et al.*,1990; Christman *et al.*,1989; Morgan *et al.*,1986; Farr & Kogoma 1991), and has been shown to negatively regulates it's expression whilst positively regulating genes within the OxyR regulon (Storz *et al.*,1990; Christman *et al.*,1989; Morgan *et al.*,1986; Farr & Kogoma 1991). The OxyR regulon is specific to hydrogen peroxide (Chiang & Schellhorn 2012; Storz *et al.*,1990). However, it also provides some protection against other stresses, such as those imparted by singlet oxygen, near-UV, heat and lipid peroxidation (Chiang & Schellhorn 2012).

1.4.2.8.2 Oxidised and reduced forms of OxyR

The OxyR protein exists in two structurally different forms (Chiang & Schellhorn 2012). This is due to the hydrogen peroxide-mediated oxidation of the conserved Cys199 residue containing a sulphur residue, which is converted into sulphuric acid as a consequence of the aforementioned oxidation (Chiang & Schellhorn 2012). The formation of a reversible disulphide bond between the Cys199 and Cys208 residues follows. The resulting structural differences in the regulatory domain of OxyR contribute to the differential binding properties and regulatory behaviour of the oxidised and reduced forms of OxyR. In the absence of hydrogen peroxide, it exists in the reduced form, where it negatively regulates genes, including negative autoregulation (Christman *et al.*,1989).

1.4.2.8.3 OxyR regulon

The OxyR regulon consists of over 30 genes (Christman *et al.*,1989; Chiang & Schellhorn 2012; Farr & Kogoma 1991; Wei *et al.*,2012), that may be positively or negatively regulated by OxyR (Chiang & Schellhorn 2012). These encode defensive products, including catalase, thioredoxins, hydrogen peroxidases, and alkyl hydroperoxide reductase (Chiang & Schellhorn 2012). The rate of synthesis for the different gene products was previously shown to be divided into immediate/early

synthesis and late synthesis. This is on the basis that maximum synthesis rates occurred within 30 minutes and after 30 minutes respectively, within the recorded 60 minute interval following exposure of *Salmonella typhimurium* cells to a low dose of hydrogen peroxide (Farr & Kogoma 1991).

1.4.2.8.4 Distribution of OxyR and OxyR analogues within bacteria

OxyR has been most extensively investigated within *E. coli* and *S. typhimurium.* However, it has been reported that there is conservation of the regulation of catalase and alkyl hydroperoxide reductase homologues by OxyR among the gammaproteobacteria (Chiang & Schellhorn 2012). Furthermore, identification of OxyR homologues within a variety of bacteria within the phylum *Proteobacteria* have been identified (Wei *et al.*,2012). However, there are significant differences in the homologues, such as composition of the conserved cysteines and transcriptional regulation, compared to the *E. coli* OxyR protein (Wei *et al.*,2012).

1.5 Iron and oxidative stress

Despite the fact that ferrous irons can lead to ROS production via the Fenton reaction, iron is also a cofactor with some of the enzymes employed to remove ROS, such as certain superoxide dismutases (Cornelis *et al.*, 2011). There is, therefore, a significant link between the networks for iron homeostasis and oxidative resistance (Cornelis *et al.*, 2011). An example of this is the control of *dps.* Whilst this has a ferritin role, storing and sequestering iron, it is also implicated in oxidative resistance due to the protection of DNA from ROS, and the utilization of H_2O_2 to oxidise Fe^{2+} with water as a by-product in place of ROS (as in the Fenton reaction). Free iron may be released into the cell due to the damage to Fe-S protein centres caused by superoxide anion. These Fe-S clusters can also become oxidised by H_2O_2.

1.6 The Viable but non-culturable (VBNC) state in bacteria

An issue that can arise when assessing bacterial viability is that it is not always concomitant with culturability. Cells that appear dead due to loss of culturability may reside in a state referred to as the 'viable but non-culturable (VBNC) state (Heim *et al.*, 2002; Signnoretto C., Lleo M., Tafi M.C. 2000). A variety of bacterial species, both Gram negative and Gram positive (Signnoretto C., Lleo M., Tafi M.C. 2000), are able to enter the VBNC state. This permits them to remain dormant and regain their infectiveness once favourable conditions arise (Signnoretto *et al.*, 2000) VBNC cells retain metabolic activity (Signnoretto *et al.*, 2000) In fact, it is metabolic activity that differentiates between VBNC and dormant cells (Ramamurthy *et al.*, 2014). Subsequently, studies using solely culturing techniques for the assessment of the efficacy of BL at killing bacteria are flawed in this respect.

1.6.1 VBNC state theories and conclusions

There are at present two theories regarding the true definition of the VBNC state. These were summarised by Signnoretto *et al.*, (2000)and are as follows:

- The VBNC state is a precursor state to cell death; effectively an intermediary phase between cell life and cell death.
- A distinct physiological state in which bacteria may reside in upon exposure to conditions that are not conducive to normal growth and functioning, upon which bacteria may exit once more favourable conditions arise.

Despite this being a topic that is still under much scientific debate, the VBNC state has generally been accepted as a distinct physiological state in bacteria (Ramamurthy *et al.*, 2014). However, since there is evidence that some bacterial species are able to remain pathogenic in the VBNC state (Ramamurthy *et al.*, 2014), it is of the utmost significance within healthcare and industrial food settings regardless of the correct theory. Therefore, the inclusion of bacteria in this state within studies investigating antimicrobial technologies is not only desirable, but a necessity.

1.6.2 Role of Hydrogen Peroxide in inducing the VBNC state

Furthermore, the study by Arana *et al.,,* (1992) indicated that hydrogen peroxide promoted the transition into the VBNC state in *Escherichia coli*. In this study, visible light was addressed as a significant abiotic factor involved in decreased numbers of *E. coli* in natural systems. Since hydrogen peroxide is a ROS, it is reasonable to hypothesise that a percentage of the bacteria involved in experiments involving the production of ROS could have entered the VBNC state and hence were mistakenly deemed inactivated by the study. This is of particular relevance to both the food and healthcare domains within society, since in the past outbreaks that could not be otherwise explained were attributed to bacteria in the VBNC state (Andrews *et al.,,* 2013). They are therefore a threat to the public health and should be addressed accordingly.

1.6.3 Distinguishing the VBNC state from other physiological states

However, the VBNC state is not to be confused with the starvation response. This can occur due to nutrient deprivation or exhaustion. The main difference between the two states is that cells undergoing the starvation response retain culturability despite the adverse conditions (Heim *et al.,* 2002).

1.6.4 *Enterococcus faecalis* and the VBNC state

It has been demonstrated previously that *Enterococcus faecalis* transitioned into the VBNC state upon release into the environment, and therefore undesirable conditions, since this organism is primarily an enteric organism and hence is not well adapted to environments outside of the mammalian host (Ta *et al.,* 1999; Heim *et al.,* 2002). This could explain the results gained from the study by Maclean *et al.,* (2009), whereby *Enterococcus faecalis* disrupted the general trend that Gram positive bacterial require lower doses of BL for inactivation. Conversely, required the highest

dose out of all the bacterial species, with an inactivation of 2.6 log10 following 216 J/cm2 BL.

1.6.5 Alterations in the cell physiology of VBNC cells

In this state, there are distinctions in the cell wall structure compared to cells in the stationary and exponential states (Canepari *et al.*,, 2000). An example is increased cross-linking and resistance to mechanical stresses (Canepari *et al.*,, 2000), in addition to the size and shape of the cells. Size reduction and transition from rods to cocci are among the changes that Gram negative cells in the VBNC state undergo (Signnoretto *et al.*, 2000) Signnoretto C., Lleo M., Tafi M.C. 2000).

1.6.5.1 Cell envelope changes

Cell envelope alterations also occur in the VBNC state (Signnoretto C., Lleo M., Tafi M.C. 2000). Scanning electron microscopy revealed formation of polymer-like filaments, associated with starved *Vibrio parahaemolyticus* in the study by Chai & Jiang 1996. This could have implications in protection of cells in the VBNC state when exposed to blue light. In addition to this, bleb formation between the inner and outer membrane can occur, and within *Vibrio cholera* the presence of polymer-like filaments has been recorded (Signnoretto *et al.*, 2000) The formation of blebs could have led to the misinterpretation of localised envelope damage due to ROS in studies using electron microscopy for evaluation of the impact of BL on bacteria.

1.6.5.2 Cell Wall Chemical Composition

(Signnoretto *et al.*, 2000) investigated the cell wall chemical composition of *E. faecalis* and made direct comparison with cells in the stationary and exponential phases, along with cells that had been killed by UV. These investigations revealed

that the mechanical resistance of cells in the VBNC state was twice that of cells in the other states described.

1.6.5.3 Peptidoglycan chemical composition

Furthermore, the peptidoglycan chemical composition of VNBC cells was analyzed. All muropeptide families exhibited significantly different changes in comparison to cells in the stationary and exponential phases. The greatest increase was seen for the higher oligomer family at 95 %. Subsequently, the degree in cross-linking was substantially greater in VBNC cells.

1.6.6 Interference of VBNC state bacteria within studies

A concern regarding the efficacy of the phototherapy used in the existing studies is that the majority employ viable counts as a means of assessing the effectiveness of light, whether in the form of aPDT, BL or PL therapy, for bacterial inactivation. However, it is well-known that a variety of bacteria are able to exist in a state that permits them to remain dormant and regain their infectiveness once favourable conditions arise (Signnoretto C., Lleo M., Tafi M.C. 2000). Although initially this behaviour was predominantly displayed by Gram negative bacteria, there are cases of Gram positive bacteria eliciting this behaviour (Signnoretto C., Lleo M., Tafi M.C. 2000). Whilst in this state, however, they appear dead or inactivated using traditional enumeration techniques due to their loss of culturability. It is important to note that despite this, the cells are still metabolically active. This phenomenon is referred to as the viable but non-culturable (VBNC) state (Heim *et al.*, 2002; (Signnoretto *et al.*, 2000) . Subsequently, the question of accuracy must be raised in regard to the studies that solely employed viable counts within their studies. Heim *et al.*, 2002 inferred that the VBNC state is a survival strategy employed by bacteria that are unable to endosporulate. In addition to this, a reference to the lack of success in culturing the majority of environmental isolates could be a reflection of these organisms residing in the VBNC state. Therefore, when assessing the effectiveness of BL as a biological

control method, the development of a method/technique that evaluates the effectiveness on all physiological states, including the VBNC state, is integral for an all encompassing study.

1.7 Practical Applications of BL

The purpose of this study was to determine if using BL within a range of applications, such as medical, environmental and within the food industry, is a possibility. The literature contains other studies that have investigated one or more of these themes using light within the blue region. The findings of these studies will be discussed below, whilst the findings and conclusions of this study will feature within the discussion section.

1.7.1 Suitability of using blue light technology on humans

Whilst investigating the impact of blue light on bacteria and the mechanisms involved, it is important to address the impact of humans and mammalian cells. Whilst blue light is in the region of visible light, and therefore not considered as dangerous as ultraviolet wavelengths, the intensities used in these experiments are not typical of the average exposure gained from natural light. This is because all the energy is focussed into a narrow spectrum high intensity set of wavelengths with a peak of 405 nm. Furthermore, since this region of blue light is within close proximity of the UV wavelengths, it is technically violet light. There is also the possibility of overlap into the UV section of the electromagnetic spectrum.

1.7.1.1 Effect of 405 nm light on mammalian cells

Whilst the quantity of scientific publications regarding the effect of 405 nm on microbial cells vastly outweighs that for mammalian cells, there are an increasing number of investigations into the effect of 405 nm light on mammalian cells due to the success of the applications of this wavelength within investigations involving

microorganisms. So far, these studies have been limited to using animal models; however, careful consideration has been given to the choice of model so that the effects can be related to human cells as closely as is practicable. A direct comparison of the impact of 405 nm on bacteria and oesteoblasts was performed in the study by (Ramakrishnan *et al.*,2014). The findings of the experiments indicated that in parallel to microbial cells, the effects of 405 nm are dose-dependent. The same trend was also found to be true for fibroblasts, with significant declines in fibroblast viability occurring following a dose of 54 J/cm² (McDonald *et al.,* 2011). This dose was also shown to negatively impact oesteoblast viability (McDonald *et al.,* 2012) by the same leading author. Following the observation that following a dose of 54 J/cm², physiological changes indicative of preservation and early onset of apoptosis led the authors to state that to identify if sub-lethal damage occurs to mammalian cells due to exposure to 405 nm light, there is a requirement for studies that investigate this. Furthermore, studies on the impact on mammalian DNA are needed. Since the sensitivity of the oesteoblasts was lower than that for the bacteria (Ramakrishnan et al. 2014), this allowed determination of a dose range that was effective for microbial inactivation in the absence of detrimental effects on the oestoblasts. In the case of the study by (Ramakrishnan et al. 2014), exposure to 36 J/cm² had no impact of the functioning, morphology, viability or proliferation of the oesteoblats. Conversely, this dose resulted in inactivation rates between 99.5 and 100 % for a range of bacterial pathogens, including *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli, Acinetobacter baumannii* and *Klebsiella pneumonieae* (Ramakrishnan et al. 2014).

1.7.1.2 Light-mediated ROS production in mammalian cells

In direct contrast to the results following exposure of oesteoblasts and fibroblasts in the above section, contradictory evidence is presented in the paper by Hockberger *et al.,* 1999 regarding the effect of BL on mammalian cells. It is stated that BL exerts toxic effects on mammalian cells, with mutations and even death occurring as a consequence of exposure to doses as low as 2-6 J/cm². The type of mammalian cells here has not been specified; therefore, this cannot be representative inclusively of all

mammalian cell types. As the above section clarifies, the effects of BL on certain mammalian cells have been shown to be dose-dependent. It can therefore be assumed that production of ROS does occur in mammalian cells, but at higher thresholds than for bacteria. The production of H_2O_2 within peroxisomes and mitochondria within 3T3 cells was shown to result from exposure to light with a wavelength range of 450-490 nm. However, the dose parameters applied were very high – an irradiance of 6.3 W/cm² for 20 minutes (7.56 kJ/cm²) as opposed to the irradiance of 1.25 mW/cm² for 240 minutes (18 J/cm²) that was deemed to have no negative effects on oesteoblasts in the study by (Ramakrishnan et al. 2014) mentioned in the above section. Hence there is need for a study that determines ROS production in a variety of mammalian cell types at the doses previously reported to be non-detrimental.

1.7.1.3 BL for wound-healing

An area of particular interest within the variety of possible applications of BL microbial inactivation is wound healing. Despite the toxic effects of BL on microbial cells, the impact of BL on mammalian cells is deemed to be lesser than that on the microbial counterparts (Tomb *et al.*, 2014). The difference in sensitivity of microbe and mammalian cells has therefore been used to suggest development/application of intensities that are detrimental to microbes but safe for mammalian cells. Furthermore, a study that investigated the effects of a blue LED (470 nm) on the healing of excision wounds in rat models. The light exposure using the blue LED in these experiments was based on the established technique low level light therapy (LLLT), founded by (Adamskaya et al. 2011). BL was shown to successfully reduce repair time for the excision wounds through enhanced epithelialisation.

1.7.1.4 Blue light and acne

Perhaps one of the most widely known applications of blue light as a component of medical treatment is treatment of acne. This is extremely common in adolescents, with a reported 70-80% of members of this group experiencing acne (Papageorgiou *et*

al., 2000; Ashkenazi *et al.*, 2003). The main causal agent of this condition is the bacterium *Propionibacterium acnes*. A minimum of 40% of *P. acnes* has demonstrated antibiotic resistance (Ashkenazi *et al.*, 2003), leading to the search for non-antibiotic therapies. Furthermore, there have been reports that the wide use of antibiotics in treating acne was responsible raising the selective pressure for drug resistance among important nosocomial pathogens (Dawson 1998). This results that led to this collected from a study that took place at the Skin Research Centre within the University of Leeds between 1991 and 1997 (Dawson 1998). It was found that people in close contact with acne patients taking antibiotics on a long-term basis possessed higher levels of antibiotic resistant *Staphylococci*, and in greater population densities compared to controls (Dawson 1998). Consequently, suggested measures featured in the interim report prior to the decision for national guidelines included:

- Prescription of antibiotics only when strictly necessary
- Good compliance with antibiotic usage
- Restricted length of antibiotic treatment
- Use of benzoyl peroxide

This was said to be due to the transmission of antibiotic resistance genes from the acne patient. Therefore, the application of non-antibiotic acne treatments was desirable. This has resulted in studies using visible light. Wavelengths within the red and blue regions of the electromagnetic spectrum are the most commonly used for this application (Ashkenazi *et al.*, 2003; Dai *et al.*, 2012). Due to the natural production of intracellular porphyrins by this bacterium, it is possible to treat it with phototherapy in the absence of exogenous photosensitizers.

1.7.1.5 Dental applications of photodynamic therapy

An area where photoinactivation has advantages over traditional therapy is dentistry. This was highlighted in the study by Doukas et al 2007. For example, although conventional antibiotics exert antimicrobial effects, they also present a number of undesirable side effects (Feuerstein *et al.*, 2006), in addition to increasing antibiotic resistance (Song *et al.*, 2013). In addition to this, a traditional approach for the treatment of periodontal disease is mechanical debridlement of bacterial biofilms;

however, this is unable to completely eradicate the bacteria (Song *et al.*, 2013). This is partly because mechanical instrumentation is unable to reach all of the affected sites, due to the structural complexity of the root canal (Foschi *et al.*, 2007), in addition to the persistent nature of biofilms (Tennert *et al.*, 2014). Therefore, a therapy that is able to inactivate a higher percentage of pathogens, with minimal side effects, is required. Whilst studies treating periodontal pathogens with aPDT have generated positive results, there are a number of periodontic pathogens that contain high numbers of endogenous porphyrins. These include black-pigmented bacteria (BPB), *Porphyromonas gingevalis* and *Fusobacterium nucleatum.* In dentistry, composite resins are cured using blue light (Song *et al.*, 2013). Since this light is known to stimulate endogenous porphyrins within bacteria (Song *et al.*, 2013; Tennert *et al.*, 2014), the spectrum of uses for this light could potentially be expanded to encompass photoinactivation.

1.7.2 Environmental disinfection using BL

1.7.2.1 Bacterial spores

A small portion of bacterial species is able to transition from the vegetative cell state into what are known as endospores. These are structures that are able to withstand highly unfavourable conditions that would kill cells in the vegetative state. Hence, spores are one of the most resistant biological agents worldwide (St Denis *et al.*,2012). The fraction of bacterial species capable of sporulation is located in a sub-section of the Gram positive Firmicutes (St Denis *et al.*,2012; MacLean *et al.*,2013). An interesting fact to note is that spores are unable to conduct repair operations due to their state of dormancy, which comprises of very low metabolic rate and minimal to no enzyme activity (Setlow 2006). Therefore, a range of mechanisms have been developed for the protection of these microbial structures during dormancy, making them extremely resistant to a multitude of antimicrobial treatments.

1.7.2.2. Existing procedures for bacterial spore decontamination

Previous methods of decontaminating bacterial spores include heat treatment, high-level disinfectants, high-pressure processing and irradiation with ionizing radiation such as ultraviolet and gamma-rays (St Denis *et al.,* 2012). However, spores are many times more resistant to all of these treatments than vegetative cells. Following on from this, reports of the inactivation of bacterial spores by UV irradiation revealed that resistance is 50-fold greater than for vegetative cells (MacLean *et al.,* 2013). Due to their structure, traditional disinfectants, such as peroxides, are of limited effectiveness. This is also true for antibiotics. Therefore, a strategy for decontaminating these structures is essential for the maintenance of safe environments within the food and healthcare industries.

1.7.2.3. Using BL to inactivate bacterial spores

Unlike their vegetative cell counterparts, the spores of some bacterial species were shown to not be sensitive to certain aPDT conditions (Demidova and Hamblin 2005). This is due to a number of factors that heighten the resistance of bacterial spores to BL compared to their vegetative cell counterparts. These include thymidyl-thymidine adducts, spore-specific photoproducts and high-level DNA repair mechanisms contributing to the altered photochemistry of spores (St Denis *et al.,* 2012). However, inactivation of bacterial spores through various phototherapies, including BL, has been shown to occur regardless of the spore-specific defences (Dai *et al.,,* 2013). Specifically, in the study by MacLean *et al.,* 2013, inactivation of *Clostridium* and *Bacillus* spores was investigated. This is because of the treat that spores of these species present to clinical and food environments respectively (MacLean *et al.,* 2013). Whereas the dose required for a 3.5 $\log_{10}$ reduction of vegetative *Bacillus cereus* (*B. cereus*) cells was approximately 100 J/cm², the dose required for the same degree of inactivation of *B. cereus* endospores was approximately 1.3 kJ/cm². In addition to the significant different in dose required for inactivation between the two physiological states of *B. cereus,* the inactivation kinetics were also different. The inactivation rate for the endospores was more linear than that for vegetative cells. As with vegetative

cells, BL-inactivation was shown to be variable in a species-dependent manner. Inactivation data for the endospores of *Bacillus cereus, B. subtilis, B. megaterium* and *Clostridium difficile* was generated. This demonstrated that *B. subtilis* was the most sensitive to inactivation, whilst *C. difficile* was the most resistant.

1.7.2.4. BL-inactivation of bacterial biofilms

Another problem posed by bacteria regarding BL-inactivation is the ability to form biofilms. These are often responsible for heightened resistance of bacteria to disinfection (Bridier *et al.*, 2011). This is a persistent issue within food and clinical environments that needs to be addressed (Van Houdt & Michiels 2010). In the biofilms state, bacteria are protected from adverse environmental conditions due to their incorporation within an extracellular matrix. Bacterial phenotypes of cells within a biofilm differ from their sessile counterparts. Subsequently, increased resistance to disinfection is a common trait of bacteria within a biofilm (Bridier *et al.*, 2011). Subsequently, when investigating the effectiveness of BL-inactivation of bacteria, it is essential to include bacterial biofilms. In the study by McKenzie *et al.*, 2013, the inactivation of biofilms from a range of clinically important bacterial species using 405 nm light from an LED was assessed. The study incorporated factors such as biofilm maturity, substrate and single/multispecies biofilm communities. The results indicated that successful inactivation of bacterial biofilms can be achieved using BL. The effectiveness of inactivation was shown to be affected by the biofilm substrate material; biofilms on acrylic surfaces required higher doses of BL compared with those grown on glass surfaces. This was attributed to the adhesion properties of bacteria to the different surfaces. However, it was also found that with increased age, the influence of substrate on inactivation decreased. Finally, increased biofilm density illustrated dose-dependent behaviour, with greater doses required for higher biofilm densities.

1.7.2.5 Disinfection of hospital environments using BL

The effectiveness of a decontamination system termed 'high-intensity narrow-spectrum light environmental decontamination system'(HINS-light EDS) within a hospital environment was investigated by Maclean *et al.,* (2010). The peak wavelength of this system is 405 nm; operation is of a continuous and supplementary disinfection nature. It was mounted from the ceiling, allowing the disinfection of the air and environment. Three studies were carried out by Maclean *et al.,* (2010); these investigated:

- **Study A**. Impact of HINS-light EDS on surface levels of bacteria within an unoccupied isolation room
- **Study B**. Impact of an extended treatment phase of HINS-light EDS within a occupied room - MRSA-positive patient present
- **Study C**. Comparison of levels of bacterial contamination of an occupied room in the presence and absence of the HINS-light EDS

Significant reductions of plate counts (up to 91 %) were achieved for Study A. There was also no significant increase in plate counts after the HINS-light EDS was switched off. The results from Study B demonstrated that in the presence of a source of MRSA, reductions of up to 86 %. Finally, the results of Study C revealed that whilst HINS-light EDS was in operation, reductions in plate counts reached 62 %; however, once it was switch off, recovery of bacterial counts to over 100 % occurred. In summary, this study provided evidence that the use of BL within a hospital environment is able to effectively provide disinfection of surfaces within a hospital. It should not be the sole treatment, and effects are temporary when the room is occupied by an infected patient.

1.8 Summary of BL-inactivation of bacteria

This introduction can be summarised in the following points:

- BL-inactivation of bacteria is considered to be due to the production of ROS through excitement of endogenous photosensitizers (porphyrins) within bacteria
- Not all bacteria contain porphyrins, which could be a possible explanation for the differences in effectiveness of BL-inactivation of bacterial species
- Since ROS elicit a non-specific attack on biomolecules, resistance development is considered low-risk for BL-inactivation
- 405 nm appears to be the most effective wavelength within the BL region of the EMS
- Bacteria display a dose-dependent inactivation behaviour by BL; the higher the dose, the greater the extent of inactivation
- Establishment of minimum doses of BL for the inactivation of a range of bacterial species needs to occur for development of novel products incorporating BL technology
- The VBNC state needs to be addressed – proof that BL inactivates bacteria and that they do not enter the VBNC state must be achieved to ensure the accuracy and reliability of BL-inactivation results
- Inactivation of bacterial spores and biofilms, both of which display increased resistance to other disinfection technologies, can be achieved using high doses of 405 nm BL
- Applications of BL for inactivation of microorganisms are already in place in clinical environments (acne treatment and the HINS-light EDS system)

1.9 Aims and Objectives of this project

This project had a number of objectives within the general aim to investigate the inactivation of a range of recently isolated environmental and clinical bacteria using blue light from light emitting diodes (LEDs). These were to investigate the following on bacterial inactivation using a custom-built LED light module:

- Exposure time
- Sample distance
- Bacterial concentration
- Sample position on plate
- Incorporation of UV-resistance plasmids in *Pseudomonas putida*
- Impact of different OxyR mutations within *Salmonella enteric* serovar Typhimurium strain LT2.

And finally, in addition to the above objectives, to conduct comparisons in inactivation efficacy of: i) pulsed versus continuous exposure to 405 nm light and ii) 405 nm versus UV-C.

Chapter 2: Materials and Methods

2.1 Bacterial strains

Table 2.1 lists the bacterial strains taken from the previous library of strains created by Aston (2012; Lancaster MSc thesis), whilst Table 2.2 contains the *Salmonella typhimurium* mutant strains kindly provided by Dr John Roth (University of California). Lastly, Table 2.3 contains the *Pseudomonas putida* strains and constructs provided by Dr Glenn Rhodes (CEH, Lancaster). The identification, API profile and VNTR profiles were obtained by Aston (2012; Lancaster MSc thesis) previously by using the system provided on apiweb™ base (https://apiweb.biomerieux.com) and through sending the isolates to the Committee on Antimicrobial Resistance and Healthcare Associated Infection (ARHAI) for variable number tandem repeat (VNTR) Profiling.

Table 2.1 Bacterial strains extracted from the strain library by Aston (2012**;** MSc thesis, Lancaster University). **(**CITU – cardiac intensive care unit; MWC - MAINS WATER CITU)

Bacterial species/strain	API number	VNTR Profile	Site	Abbreviation
Escherichia coli	5144572	-	URINE	C1
Citrobacter freudii	1604573	-	URINE	C4
Citrobacter spp.	3604713	-	URINE	C8
Acinetobacter spp.	0000071	-	BLOOD	C10
Enterobacter cloacae	3304573	-	BLOOD	C13
Enterobacter sakazakii	3305175	-	URINE	C25
Enterobacter spp.	3305173	-	SPUTUM	C27
Enterobacter aerogenes	5305773	-	SPUTUM	C29
Serratia liquifaciens	1304763	-	SPUTUM	C30
Acinetobacter baumannii	0005042	-	SPUTUM	C31
Acinetobacter spp.	0.000005	-	WOUND	C38
Serratia liquifaciens	1304763	-	SPUTUM	C39
Acinotobacter baumannii	0004042	-	SPUTUM	C43
Raoultella ornithinolytica	5355773	-	URINE	C44
Serratia marcescens	5307761	-	THROAT	C45
Pseudomonas aeruginosa	-	10,3,5,5,-,1,3,7,7	MWC	E19
Pseudomonas aeruginosa	-	12,5,5,3,1,1,15,4,13	CITU	P1
Pseudomonas aeruginosa	-	12,5,5,3,1,1,15,4,-	CITU	P16
Pseudomonas aeruginosa	-	12,5,1,5,2,2,9,2,11	MWC	E24
Pseudomonas aeruginosa	-	3,4,5,2,3,5,2,9	MWC	E28

Table 2.2. *Salmonella enterica* serovar Typhimurium LT2 mutant strains provided by Dr John Roth (University of California).

***Salmonella enterica* serovar Typhimurium strain LT2 mutants**	**Abbreviation**
TT22481 sty(LT2) nadB499::MudJ oxyR::Tn10d-tet	S1
TT19390 sty(LT2) oxyR::Tn10 $COM	S2
TT22480 sty(LT2) oxyR::Tn10	S3
TT19137 sty(LT2) oxy1 del (oxyR-argH)	S4
TT19138 sty(LT2) oxy2 del (oxyR-argH)	S5
TT19388 sty(LT2) zii-614::Tn10 oxyR1 $COM	S6
TT19389 sty(LT2) zii-614::Tn10 oxyR2 $COM	S7
TT24008 sty(LT2) TR10000 oxyR::Tn10 $COM was MPC412	S8

Table 2.2 lists the OxyR mutant strains provided by Dr John Roth (University of California). $COM denotes strains that were provided by Gisela Storz (University of California). Tn10 is a transposable element. Therefore, where oxyR is followed by ::Tn10, this indicates a Tn10 insertion within the oxyR coding sequence. Deletion mutants were symbolised by 'del'. The information in brackets following this details the start and end points of the deletion. Strains S6 and S7 have Tn10 insertions near the different mutant *oxyR* genes (oxyR1 and oxyR2 respectively). Strain S8 is a Tn10 insertion transduced into the wild type strain, TR10000. And finally, strain S1 strain has two mutations — an insertion of phage-Mu derived MudJ element into the nadB499 gene causes a nutritional requirement that can be satisfied by either nicotinamide or nicotinic acid. The inserted element includes a lac operon that is expressed from the nada promoter. The second mutation is an insertion of Tn10d into oxyR — this Tn10d is defective for transposition. The reason for including OxyR mutants within the experiments is to investigate the involvement of OxyR in mediating the cellular response to the predicted increased levels of ROS, and hence induction of oxidative stress in bacterial cells exposed to blue light. Since there are two types of reaction that can occur during ROS-mediated oxidative stress, namely the Type I and Type II reactions, incorporating OxyR mutants is also a way of deducing which reaction pathway is of most importance, since the OxyR regulon is only capable of addressing the ROS products that arise from the Type I reaction (refer to section 1.4.1).

Table 2.3. *Pseudomonas putida* strains provided by Dr Glenn Rhodes (CEH, Lancaster).

Strains/constructs	**Abbreviation**
***P. putida* PaW340**	pseud1
***P. putida* PaW340 (pWW0ΔrulAB::KmR)**	pseud2
***P. putida* PaW340 (pWW0::KmR)**	pseud3

The above strains were featured in the study by Rhodes *et al.*,(2014). pseud1 serves as a control whilst pseud2 and pseud3 contain the plasmid pWW0, which carries UV resistance. However, there is thought to be a deletion in pseud2; therefore it is assumed to not carry UV resistance. With respect to the experiments, this means that if the carriage of UV-resistance genes is advantageous during exposure to blue light, then this would be reflected in the inactivation rates of the *P. putida* strains, with pseud3 displaying lower levels of inactivation than pseud1 and pseud2.

2.2 Sample collection and identification

Isolates C1 to E28 were collected and stored on beads in horse serum at -70 °C between October 2010 and June 2012 (Aston 2012; Lancaster University MSc Thesis). These were taken from clinical samples from infected patients at Blackpool Victoria Hospital. All isolates were identified using api®20 E or 20NE (BioMérieux) and further distinguished in some cases by VNTR profiling.

2.3 Maintenance of bacterial cultures

All bacterial strains were recovered and maintained in Tryptone Soya Broth (TSB) (Oxoid Ldt, Basingstoke, Hampshire, England) and Tryptone Soya Agar (TSA) (Oxoid Ldt, Basingstoke, Hampshire, England). For the initial recovery from frozen stocks which comprised beads in glycerol/nutrient broth, the tubes containing the beads were removed from the -70 °C freezer and allowed to thaw before vortexing for

10 seconds. Sterile forceps were used to remove a single bead, and a disposable plastic loop used to roll the ball over the surface of a TSA plate before submerging the bead into the agar using the loop. The plates were left at room temperature and examined after 24 and 48 hours. Following growth, a typical colony was streaked onto a fresh plate to obtain pure colonies. This process was repeated if necessary if purity was not assured. Following this, the plates were stored at 4 °C and sub-cultured on a regular basis onto fresh TSA plates.

2.4 Experiment Preparation

For overnight-grown cultures, a single colony was suspended in 200 µl of filter-sterilized phosphate-buffered saline (PBS) and agitated to form a homogenous suspension. 100 µl of this suspension was inoculated into 4 ml sterile TSB and incubated at 30 °C, shaking at 200 rpm, overnight. Following this, a small sample (~10 µl) was extracted and the OD_{600} value obtained using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Readings were taken in triplicate and the average value used. The value for use in experiments was 0.3 ± 0.1; therefore, the broths were either diluted or given additional incubation time accordingly. 100 µl aliquots of the inoculum were taken for each sample and centrifuged at 17, 000 x *g* for 6 minutes; the time was increased if necessary to obtain a firm pellet. This was washed twice in sterile PBS before re-suspension in 100 µl of sterile PBS. Eight serial dilutions were carried out and either the droplet method (section 2.6.3) or 100 µl spread plates (section 2.6.4 – 2.6.6) of dilution 10^{-6}were performed in at least duplicate. In addition to this, duplicate plates100 µl spread plates for the 10^{-7} and 10^{-8} dilutions were performed to allow the bacterial concentrations to be calculated at a later stage.

2.5 LED Light Source

The light source was designed through collaboration between myself (representative of Lancaster University) and Marl International Limited. Figure 2.1 shows the design and layout of the LED array/module.

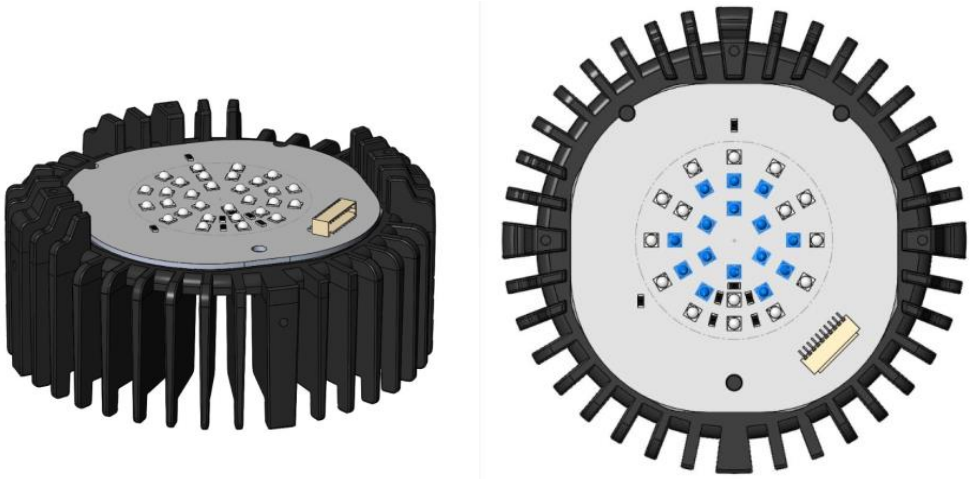


Figure 2.1. Design and layout of the LED module designed for use in the blue light experiments. Whilst the first diagram shows the lateral view of the LED housing, the picture on the right provides information on the number and position of the LEDs. Those shown in blue are the ones that were present, whilst the non-coloured LEDs indicate the available positions for double the number of LEDs present during experimentation.

2.5.1 LED Array Specifications

Table 2.4 summarises the output values for parameters, including peak wavelength, total voltage and total power for continuous mode at 25 °C. For pulsing mode, the input voltage (and therefore total power) was three times greater.

Table 2.4. Electrical specifications of the LED module in continuous mode.

Item	Maximum Rating	Unit
λ_P	405	nanometers
I_{in}	1.4	Amps
P_{total}	20	Watts
n_{leds}	15	Numerical Unit
$\Phi_{v\ led}$	950	milliWatts
$\Phi_{v\ relative}$	1900	milliWatts
$\Phi_{v\ total}$	24.5	Watts

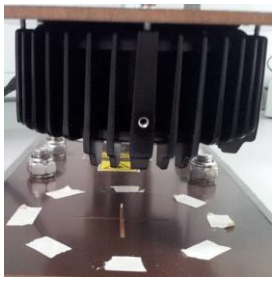
Definitions: λ_P peak wavelength; I_{in} input current; P_{total} total power; nleds number of LEDS; $\Phi_{v\ led}$ luminous flux per LED; $\Phi_{v\ relative}$ and $\Phi_{v\ total}$ total luminous flux of the LED array. The above values are defined at 25 °C.

2.5.2 LED Set-Up

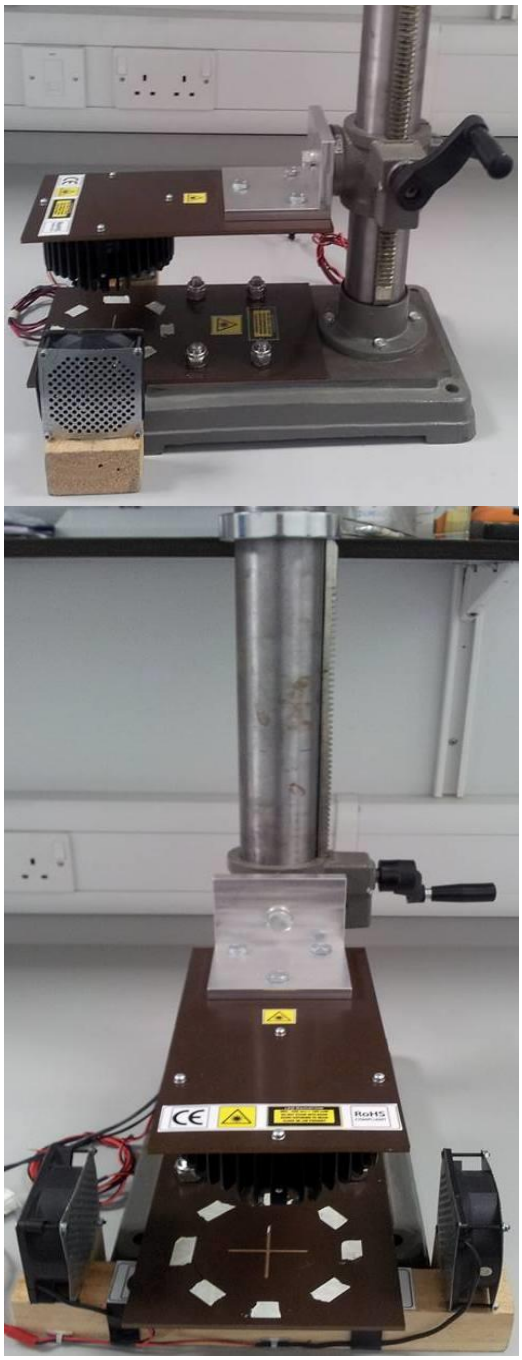
The team at Marl International mounted the LED module (shown in Figure 2.1) onto a mobile platform of wood. This was positioned above the base board, where the marked petri dish position is shown. Whilst the base board was immobile, the upper board carrying the light source could be raised or lowered using the handle to achieve the correct sample distances in the experiments. All experiments were carried out in a dark room, in the absence of visible light. Figure 2.2 is a photograph of the set-up, with labels for the described features.

Continuous modules. The leads from the pulsed modules in this image were connected to the LED board via leads and x connectors.

External fan. To minimize the heating effects of the LED array at the agar surface.



Close-up of LED board. This was surrounded by plastic housing and included a heat-sink.



Pulsed modules. The leads from the pulsed modules in this image were connected to the LED board via leads and x connectors.

Handle. A handle attached to the LED board allowed easy modification of the sample distance from the LED board.

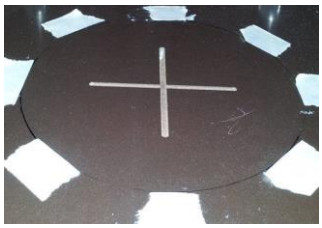


Plate marker. The position of the plate was kept constant by ensuring that the cross was in the centre of a plate and marking the plate outline.

Figure 2.2. Photographs of the components of the LED light module, designed through joint research and work efforts by myself and Marl International respectively.

2.6 Plate layouts

For both the exposure-time and sample-distance experiments described below, the template shown in Figure 2.3 was used. Each dot on the template represents the position of a 2 µl droplet of bacterial suspension on the agar surface. Each plate contained eight serial dilutions of the original bacterial suspension from 10^{-1} to 10^{-8}. Three 2 µl droplets of each dilution were plated as shown in the template in Figure 2.3. These were labelled (droplet) position 1 – 3, starting from the innermost droplet position. The distance between each droplet position was 1 cm. For the 40 °C heat, pulsed vs continuous BL and *Pseudomonas putida* experiments, triplicate 100 µl spread plates of the 10^{-6} dilution were used. UV experiments utilized a modified droplet plate layout, illustrated in Figure 2.5; the droplet size remained 2 µl. All plates were allowed to dry within a Class II Microbiology Safety Cabinet for 30 minutes prior to exposure. Temperature measurements were taken every 10 minutes using an infrared gun. All experiments were repeated in at least duplicate.

2.6.1 Exposure-time experiments

Four different exposure times were used, with a fixed sample distance of 5 cm. The exposure times were 5, 10, 15 and 20 minutes.

2.6.2 Sample-distance experiments

Three sample distances were used, each for a fixed exposure duration of 10 minutes. These were 2, 3, and 4 cm.

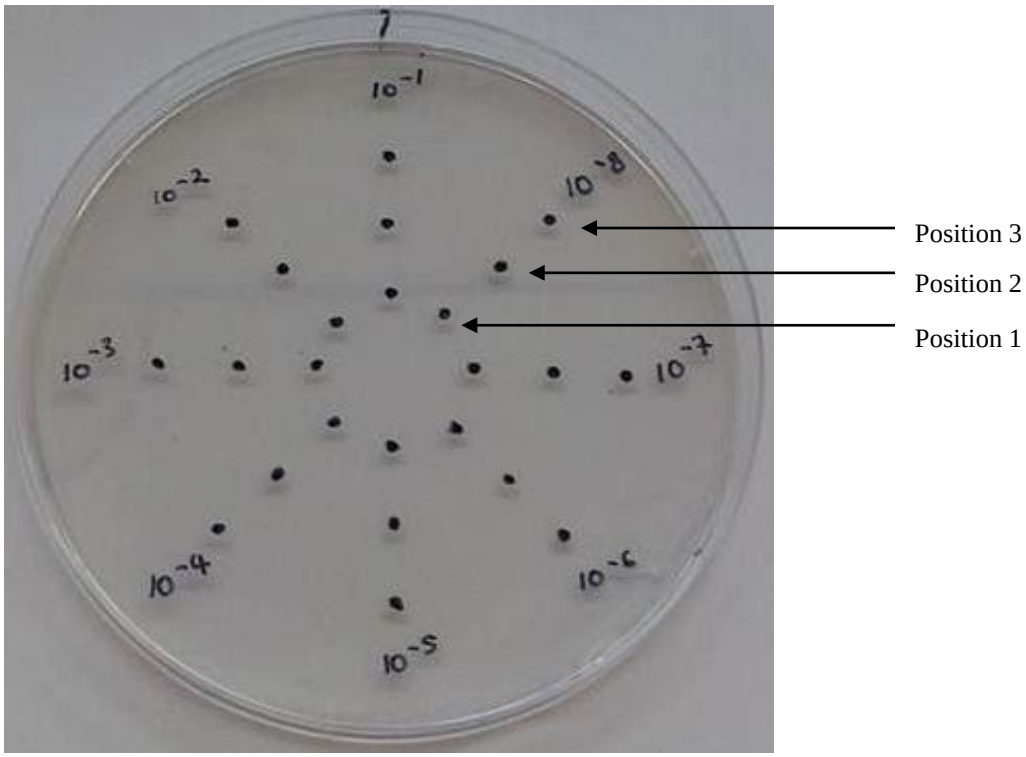


Figure 2.3. Template used for the exposure-time and sample-distance experiments. Each dot represents the position of a 2 µl droplet of bacterial suspension. The dilution of each branch is marked on the perimeter.

2.6.3 Continuous vs Pulsed BL Experiments

100 µl of bacterial suspension at dilution 10^{-6} was spread onto three TSA plates using a disposable plastic spreader, as described in section 2.6. There were three different treatments within these experiments. One plate was exposed to continuous blue light (BL), the second to pulsed BL and the third acted as a control (unexposed) plate. The sample distance was 2 cm and exposure time 10 minutes for the exposed plates. The pulse duration used was 10 milliseconds (ms) with a rest interval of 100 ms resulting in 5, 455 cycles in the 10 minute exposure time. Experiments were repeated in triplicate.

2.6.4 Temperature Experiments

Eppendorf tubes containing 100 µl aliquots of bacterial suspension at dilution 10^{-6} were placed in a heat block at 40 °C for 10 minutes before transferring to a spread plate for enumeration. This was repeated in triplicate.

2.6.5 UV experiments

Square 10 x 10 cm plates (Sterilin, UK) were used for these experiments. Six serial dilutions were used, and the plate divided into four sections of progressing exposure time: 0, 5, 10 and 15 seconds. For experimentation, the plates were inverted onto a UV transilluminator (302 nm) (Figure 2.4). Whilst a more efficient way to provide UV exposure to the bacteria in a controlled manner would have been to use a crosslinker, unfortunately this piece of equipment was not available. However, given that the transilluminator model used states that the UV illumination is uniform, and that the plate took up a fraction of the available surface area, it is unlikely that the distribution of light to the bacterial samples was significantly different across the plate. Three droplets of each dilution acted as triplicates, due to the even spread of UV exposure from the UV transilluminator. The plate layout is in Figure 2.5.

Figure 2.4. UV-transilluminator used in UV experiments.

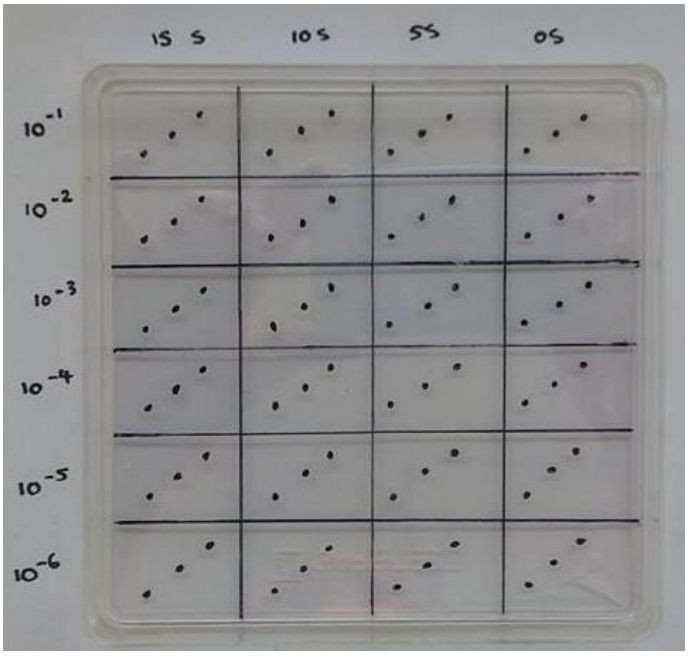


Figure 2.5. Template used within the UV experiments. Each dot represents the position of a 2 µl droplet of bacterial suspension. The dilution of each row is marked on the left; each column represents exposure of that section for the number of seconds specified at the top.

2.6.6 Influence of UV-resistance genes on BL-inactivation of *Pseudomonas putida* strains

The *Pseudomonas putida* strains listed in Table 2.3 were included in the exposure-time and sample-distance experiments. Following the results gained from these, a separate set of experiments on these strains was conducted. 100 µl aliquots of all three strains at dilution 10^{-6} were either:

i) spread onto a TSA plate and exposed to 10 minutes of continuous or pulsed BL at a distance of 5 cm for a duration of 10 minutes, as described for the strains involved in the continuous vs. pulsed BL experiments detailed in section 2.3;

ii) OR transferred into an eppendorf tube and placed in a heat block at 40 °C for 10 minutes before transferring to a spread plate for enumeration.

In all instances, experiments were repeated in triplicate.

2.7 Measurement of Data

2.7.1 Qualitative measurement of BL-inactivation of bacteria – arbitrary rating scale

The impact of BL on the inactivation of a large number of strains was measured qualitatively using the droplet method, combined with a self-defined arbitrary rating scale. This was designed to allow collection of qualitative data, since it was impractical and inaccurate to count the colonies within the 2 µl droplets of bacterial suspension on the agar plates. This ranged from 0-4. Every unit represented a ≤25% droplet reduction. Therefore, 0 = no change, 1 = 0 – 25 %, 2 = 26 - 50%, 3 = 51 - 75% and 4 = > 75 % reductions accordingly. This scale was applied to all experiments using the droplet method. A section of the table containing results from the exposure-time experiments for *Citrobacter freundii* is displayed below in Table 2.5.

Table 2.5. Extract of the table of results for time experiments for the bacterial strain *Citrobacter freundii*. * refers to the location of the 2 µl droplet of bacterial suspension on the agar plate, as shown in Figure 2.3 in the Materials and Methods section.

	Position 1*				Position 2*				Position 3*				
S	5**	10**	15**	20**	5**	10**	15**	20**	5**	10**	15**	20**	D
C4	0	0	0	0	0	0	0	0	0	0	0	0	1
	0	0	0	1	0	0	0	1	0	0	0	0	2
	0	0	1	2	0	0	1	2	0	0	0	1	3
	1	1	2	3	1	1	2	3	0	0	0	2	4
	2	3	3	4	1	2	3	3	1	1	1	3	5
	2	3	3	4	2	2	3	4	1	1	2	4	6
	0	2	3	4	2	2	3	3	1	1	2	3	7
	2	4	4	4	0	-	-	-	-	2	3	4	8

** refers to exposure time in minutes. S = bacterial species/strain. D = dilution used; where the numerical value refers to the number of the ten-fold serial dilution. For example, '1' is equivalent to 10^{-1}. Where a dash with a grey background is present, the value is non-applicable due to the absence of bacterial colonies in that position on the control plate. The colour of the background mirrors the rating scale, from red to blue in ascending order of inactivation.

2.7.2 Quantitative measurement of BL-inactivation of bacteria – spread plate data

Quantitative data was obtained via colony counts from the spread plate experiments outlined in sections 2.6.3 and 2.6.4. The number of colonies that grew on the plates exposed to pulsed BL, continuous BL or heat was compared to the counts on control plates that were not exposed to BL or heat, following incubation of all plates at 30 °C for 18 – 24 hours. For these experiments, only five bacterial strains were used. These were *Enterobacter cloacae*, *Raoultella ornithinolytica*, *Serratia marcescens*, *Acinetobacter* spp. and *Escherichia coli*. Log_{10} reductions were calculated by calculating the CFU/ml of bacteria on the control and experimental plates, which enabled a percentage change to be calculated. This was then converted into a Log_{10} reduction in excel.

2.8 Statistical Methods

A variety of statistical methods were applied to the data. The type of test applied was determined by whether the data was qualitative or quantitative, as detailed in sections 2.7.1 and 2.7.2 above.

2.8.1 Quantitative Data: Chi-squared test of independence and Pearson's Product-Moment Correlation Coefficient

For qualitative data, two statistical analyses were made. The first method tests if there is a significant association between two variables: the Chi-Square test. This produces a *p* value. The significance level chosen for this value was 0.05. The hypotheses were as follows:

i) The droplet position (refer to section 2.6) has no significant effect on the degree of bacterial inactivation

ii) The bacterial concentration has no significant effect on the degree of bacterial inactivation

iii) The sample-distance has no significant effect on the degree of bacterial inactivation

iv) The exposure-time has no significant effect on the degree of bacterial inactivation

v) The mutant strains (OxyR mutants in *S. enterica* and carriage of plasmid pWW0 in *P. putida*) do not show altered inactivation

A *p* value of less than the significance interval (0.05) rejects the null hypothesis, whilst a *p* value of more than 0.05 accepts the null hypothesis.

The second test determined the strength of linear association between two variables. This is known as the Pearson's Product-Moment Correlation Coefficient, which generates an *r* value between two variables. This value ranges from -1 to +1, representative of a perfect negative correlation through to a perfect positive correlation accordingly. A value of or close to zero is therefore representative of no correlation. The equation is shown in Figure 2.6.

$$r = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{[\, n\Sigma x^2 - (\Sigma x)^2 \,][\, n\Sigma y^2 - (\Sigma y)^2 \,]}}$$

Figure 2.6. Equation for the calculation of the Pearson Product Moment Correlation Coefficient. Where n = the number of data pairs, Σ = sum of, x = time and y = degree of bacterial reduction (in accordance with the arbitrary scale used, detailed in section 2.7.1).

2.8.2 Quantitative Data: Paired T-Test

A single statistical test was used in the analysis of the quantitative data. This was the Paired T-Test (two-tailed). This was employed to determine if there is a relationship between two variables, and if the results are statistically significant. The confidence value chosen was 0.05. The hypotheses were as follows:

i) Continuous BL exposure has no effect on bacterial inactivation.

ii) Pulsed BL exposure has no effect on bacterial inactivation.

iii) 40°C heat has no effect on bacterial inactivation.

The result of this test is a *t* value; if this is below the significance interval (0.05), the null hypothesis is rejected. If it is above the significance interval, the null hypothesis is accepted.

Chapter 3: Results

3.1 Irradiance values of the LED module

Within the BL experiments, TSA plates containing the appropriate layout (section 2.6) were illuminated with BL from the LED array for the durations and distances described in sections 2.6.1 – 2.6.3. To calculate the doses of light used in the experiments, it was first necessary to measure the irradiance of the LED module. This was done for a range of heights. Calculation of doses was performed using the following equation:

Dose (J/cm^2) = irradiance (mW/cm^2) x time (seconds)

The irradiance was measured using a spectroradiometer (SR910-A, Macam, Livingston). The results shown in Figure 3.1 are the average values of triplicate readings taken during continuous exposure mode.

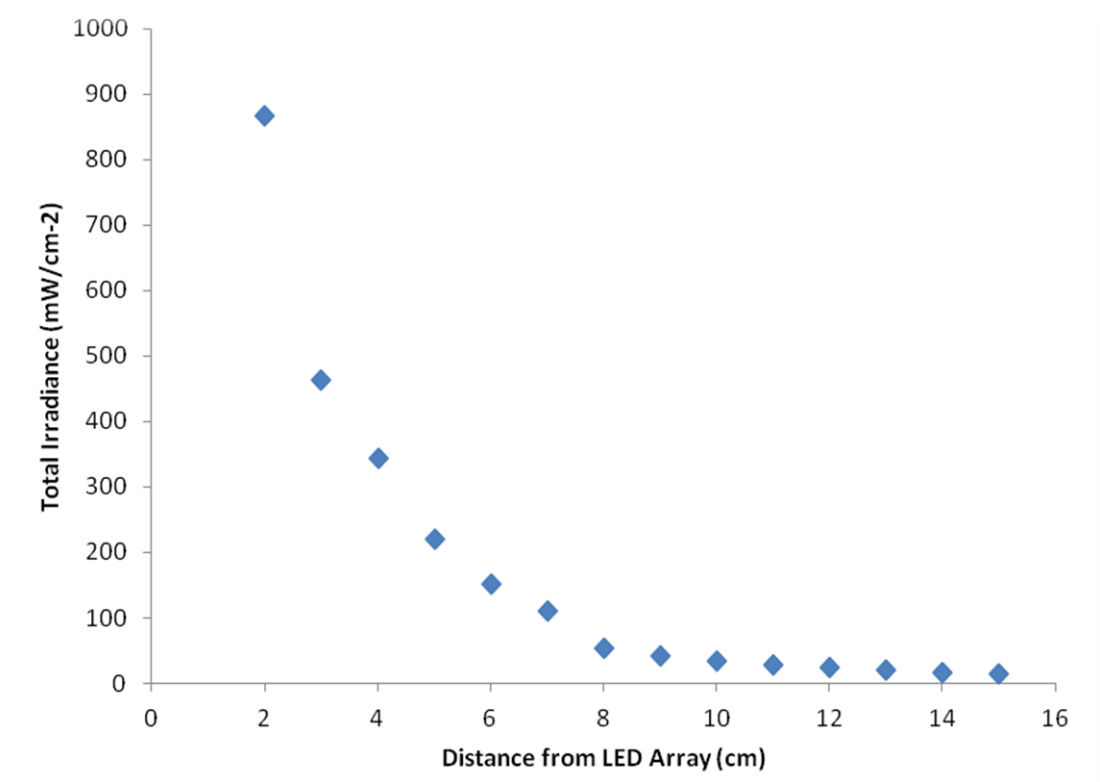


Figure 3.1. Total irradiance values (mW/cm^2) of the LED module for distances ranging from 2 – 15 cm.

From Figure 3.1, the total doses (J/cm²) were calculated for the continuous exposure BL experiments using the equation described in section 3.1. These are displayed in Tables 3.1 and 3.2 for the exposure-time and sample-distance experiments respectively.

Table 3.1. Doses (J/cm²) for each of the exposure durations in the exposure-time experiments. The doses below correspond to a constant distance of 5 cm.

Exposure Time (mins)	Total Dose (J/cm^2)
5	65.8
10	131.5
15	197.3
20	263

Table 3.2. Doses (J/cm²) for each of the distances between the LED array and agar plate used in the sample-distance experiments. The doses below correspond to a constant exposure duration of 10 minutes.

Sample-distance (cm)	Dose (J/cm^2)
2	520.3
3	277.4
4	205.8

3.2 Exposure-time experiments

3.2.1 Objectives

The objectives of the 'exposure-time' experiments were to evaluate the impact of the duration of exposure to BL from the LED array on the degree of inactivation of bacteria. This was mainly assessed using the arbitrary rating scale (section 2.7.1) to determine the relative reductions in the quantity of bacterial colonies within 2 µl droplets of bacterial suspension (section 2.6) on a TSA plate compared to a non-exposed control. Analysis of the results was performed to answer the hypotheses stated in section 2.8.1. These will be discussed in the following sections.

3.2.2 Impact of length of BL exposure on bacterial reduction

The average inactivation values for each bacterial strain (across all dilutions and droplet positions), using the arbitrary rating scale (section 2.7.1) are displayed in Figure 3.2. The average values were used due to the high volume of data produced. It is clear from Figure 3.2 that there is a high degree of variation in the degree of inactivation between bacterial strains. This can be divided on both an inter- and intra-species basis. Two conclusions may be made using the data in Figure 3.2. These are:

i) For the majority of bacterial strains, there is a relationship between length of BL exposure and degree of bacterial inactivation

ii) This represents a positive correlation accordingly.

To test the statistical strength of the above two observations, the Chi-squared test of independence and the Pearson's product moment correlation co-efficient were used respectively.

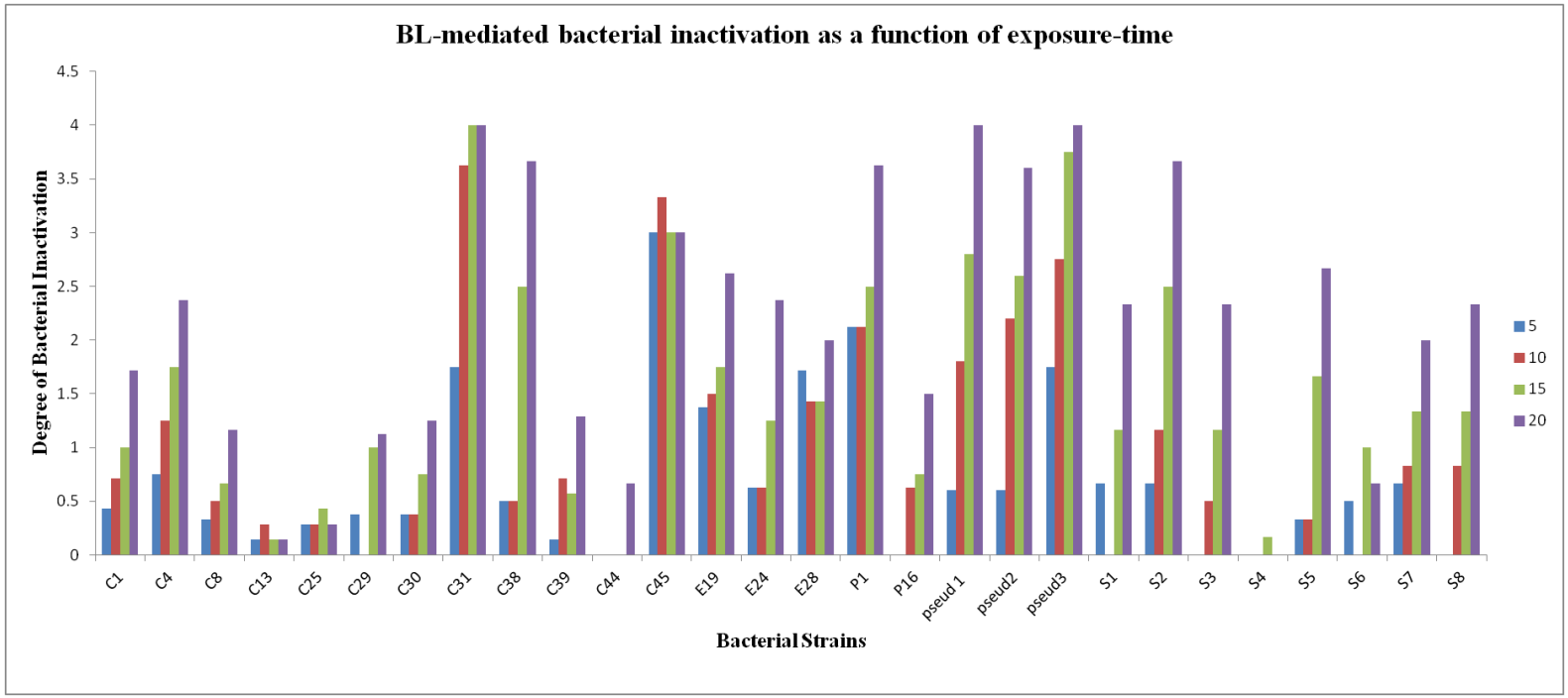


Figure 3.2. Degree of inactivation of all bacterial strains featured in the exposure-time experiments. The legend refers to the exposure time (minutes), at the set height of 5 cm, whilst the y-axis uses the rating scale described in section 3.2. The values presented are the average values of each strain, for both dilution and position on plate. The arbitrary rating scale (2.7.1) was used.

3.2.2.1 Chi-squared test of independence

This statistical test examines if there is a significant association between two variables. In this case, the variables are duration of BL exposure and extent of bacterial inactivation. Table 3.3 is the contingency table generated for this test. The significance level selected for all chi-squared analyses in this chapter is 0.05. Therefore, *P* values less that 0.05 indicate statistically significant results.

Table 3.3. Contingency table for the different exposure times used in the exposure-time experiments, in relation to the degree of bacterial inactivation.

	5 MIN	**10 MIN**	**15 MIN**	**20 MIN**	**Row Totals**
0	116 (81.50) [14.60]	97 (81.50) [2.95]	66 (81.50) [2.95]	47 (81.50) [14.60]	**326**
1	35 (35.50) [0.01]	41(35.50) [0.85]	40 (35.50) [0.57]	26 (35.50) [2.54]	**142**
2	14 (22.75) [3.37]	17 (22.75) [1.45]	31 (22.75) [2.99]	29 (22.75) [1.72]	**91**
3	12 (16.50) [1.23]	11 (16.50) [1.83]	18 (16.50) [0.14]	25 (16.50) [4.38]	**66**
4	8 (28.75) [14.98]	19 (28.75) [3.31]	30 (28.75) [0.05]	58 (28.75) [29.76]	**115**
Column Totals	**185**	**185**	**185**	**185**	**740 (Grand Total)**

The above table contains the values for the observed cell total (e.g. total number of '0' values from the arbitrary scale for all dilutions of all strains for the exposure time of 5

minutes is 116) alongside the 'expected' cell totals (calculated by the statistics software, displayed in round brackets) and the chi-square statistic for each individual cell in square brackets. The *P* value calculated from the above table was <0.00001, and therefore statistically significant. Subsequently, the null hypothesis that exposure time (of BL) has no influence on the degree of bacterial inactivation in the exposure-time experiments can be rejected. Following this result, the chi-square values for pairs of exposure times were calculated. The results are displayed in Table 3.4.

Table 3.4. Chi-square statistics and *p* values for the chi-squared analysis between pairs of BL exposure times.

Exposure Time Pair (mins)	**Chi-square statistic**	***p* value**
5/10	6.98	0.136747
5/15	34.4	<0.00001*
5/20	78.2	<0.00001*
10/15	14.2	0.00683*
10/20	49.0	<0.00001*
15/20	16.3	0.002666*

*statistically significant at $P < 0.05$

With the exception of the difference between 5 and 10 minutes, the differences between the other exposure times are significantly different.

3.2.2.2 Pearson's product moment correlation coefficient

The second test was performed to determine the strength of linear association between two variables, in this case the exposure time and degree of bacterial inactivation. This is known as the Pearson's Product-Moment Correlation Coefficient, which generates an *r* value between two variables. This value ranges from -1 to +1, representative of a perfect negative correlation through to a perfect positive correlation accordingly. A value of or close to zero is therefore representative of no correlation. The results are displayed in Table 3.5.

Table 3.5. Pearson's Product Moment Correlation Co-efficient *r* values for bacterial strains in the Exposure-Time experiments.

Bacterial Genus	**Bacterial Strain**	***r* value**	***p* value**
Pseudomonas	pseud 1	0.99	< 0.00001*
Salmonella	S3	0.98	< 0.00001*
Pseudomonas	P16	0.97	< 0.00001*
Salmonella	S8	0.97	< 0.00001*
Salmonella	S2	0.96	< 0.00001*
Pseudomonas	pseud2	0.96	< 0.00001*
Pseudomonas	pseud3	0.92	0.000024*
Salmonella	S5	0.91	0.000032*
Salmonella	S7	0.90	0.000071*
Pseudomonas	E24	0.89	0.000116*
Serratia	C39	0.88	0.000162*
Citrobacter	C4	0.87	0.00023*
Acinetobacter	C38	0.86	0.000365*
Escherichia	C1	0.85	0.000462*
Salmonella	S1	0.85	0.000503*
Pseudomonas	P1	0.85	0.000509*
Pseudomonas	E19	0.84	0.000707*
Acinetobacter	C31	0.83	0.000891*
Raoultella	C44	0.79	0.002379*
Serratia	C30	0.79	0.002484*
Enterobacter	C29	0.75	0.005001*
Citrobacter	C8	0.56	0.057956
Salmonella	S6	0.51	0.088298
Pseudomonas	E28	0.19	0.548274
Salmonella	S4	0.13	0.676173
Enterobacter	C25	0.09	0.778033
Enterobacter	C13	0.06	0.857418
Serratia	C45	-0.18	0.577777

*statistically significant at $P < 0.05$.

Table 3.5 reveals that a large number of strains used demonstrate an almost perfect positive correlation, with an *r* value of almost +1 (the highest possible value). However, certain strains do not follow this trend. For example, C13 (*Enterobacter cloacae)* has an R value of 0.06. Since this value is closest to 0, this suggests that there is little correlation. Reviewing of the original data reveals that there is minimal reduction for all exposure times for this strain; it is highly resistant to the treatment, and therefore does not follow the overall trend. The majority of *r* values are statistically significant.

3.2.2.3 Inter-species variation in BL inactivation as a function of exposure time

An example of inter-species variation is the contrast between strains belonging to the species *Enterobacter* and *Acinetobacter* respectively. Whilst the degree of bacterial reduction within strains in the former species is low, a strain of the latter species possesses a strong *r* value (C31). This is also demonstrated in Table 3.6, which lists the cumulative reduction values for all four exposure times.

3.2.2.4 Intra-species variation in BL inactivation as a function of exposure time

Table 3.6 demonstrates that there is also intra-species variation in the inactivation data. The most prominent example of this is the data obtained from the *Serratia* strains. Whilst there is negligible difference between the two *Serratia liquifaciens* strains (C30 and C39), there is a significant difference in the inactivation rates between these and the *Serratia marcescens* strain (C45). In addition to this, there is also a broad range of inactivation efficiency within the *Pseudomonas aeruginosa* strains, with the smallest value of 2.88 and the largest value of 12.25. The size of the difference between the *Salmonella* mutants is comparable with the *Pseudomonas*

mutants, with the differences between the highest and lowest values being 9.38 and 7.83 respectively.

Table 3.6. Cumulative values of the average* degree of inactivation for all exposure times.

Strain	Genus	Total Inactivation Score*
C31	*Acinetobacter*	13.38
C45	*Serratia*	12.33
pseud3	*Pseudomonas*	12.25
P1	*Pseudomonas*	10.38
pseud1	*Pseudomonas*	9.20
pseud2	*Pseudomonas*	9.00
S2	*Salmonella*	8.00
E19	*Pseudomonas*	7.25
C38	*Acintobacter*	7.17
E28	*Pseudomonas*	6.57
C4	*Citrobacter*	6.13
S5	*Salmonella*	5.00
E24	*Pseudomonas*	4.88
S7	*Salmonella*	4.83
S8	*Salmonella*	4.50
S1	*Salmonella*	4.17
S3	*Salmonella*	4.00
C1	*Escherichia*	3.86
P16	*Pseudomonas*	2.88
C30	*Serratia*	2.75
C39	*Serratia*	2.71
C8	*Citrobacter*	2.67
C29	*Enterobacter*	2.50
S6	*Salmonella*	2.17
C25	*Enterobacter*	1.29
C13	*Enterobacter*	0.71
C44	*Raoultella*	0.67
S4	*Salmonella*	0.17

*average value across all dilutions and droplet positions, as described in section 2.6.

The above table lists the cumulative values for the inactivation rates for all of the exposure times (5, 10, 15 and 20 minutes) combined for each species, in descending order.

3.2.2.5 Impact of different OxyR mutations on inactivation efficacy of *Salmonella enteric* serovar Typhimurium

To assess the involvement of the correct functioning of the transcriptional regulator OxyR in *Salmonella enterica* serovar Typhimurium, a variety of mutant strains provided by Dr John Roth (University of California) were included in the exposure-time experiments (refer to table 2.2 for the list of mutants). By reviewing the values in Table 3.6, it can be seen that the *Salmonella* mutant strains display a range of sensitivities, but not within the predicted trends. From Table 2.2, the strains where the Tn10 insertions are located near to the mutant *oxyR* gene are S6 and S7. These mutant *oxyR* genes are oxyR1 and oxyR2 respectively. These are the result of dominant mutations in *oxyR* that lead to constitutive expression of *oxyR*. Subsequently, strains with these mutations are highly resistant to oxidative stress. Whilst this appears to be the case for S6, S7 is the third most sensitive mutant strain, and has a total inactivation value that is within the mid-range of the spectrum for all of the bacterial strains. Conversely, strain S4 is the most resistant of both the *Salmonella* mutant strains and all of the bacterial strains featured in the experiments. This is a deletion mutant, where the deletion ranges from *oxy1* to *argH*. Therefore, it was predicted to display high sensitivity to oxidative stress. However, strain S2 shows sensitivity to oxidative stress, as the most sensitive *Salmonella* strain. This mutant features disruption to the *oxyR* gene due to the incorporation of Tn10.

3.2.2.6 Influence of incorporation of a UV-resistance plasmid

To investigate the influence of UV-resistance on BL inactivation, *Pseudomonas putida* mutant strains provided by Dr Glenn Rhodes (CEH, Lancaster) were also included in the experiments. From Table 3.6 it can be seen that all three *P. putida* strains were the most sensitive *Pseudomonas* strains. Furthermore, the strain with the pWW0 plasmid (pseud3) is more sensitive than the wild-type and deletion strains (pseud1 and pseud2 respectively). Therefore, it can be concluded that the carriage of a UV resistance gene is not advantageous during BL exposure.

3.2.2.7 Impact of bacterial concentration on BL-inactivation as a function of exposure time

To test the impact of bacterial concentration of inactivation efficiency of BL, eight dilutions of each strain were included in the exposure-time experiments. To aid in the analysis, the dilutions performed were 1 in 10 serial dilutions, ranging from 10^{-1} to 10^{-8}. All dilutions of each strain were present on the same plate. The arbitrary rating scale (section 2.7.1) was used to define the inactivation efficacy and a Chi-squared test of Independence was used to either accept or reject the null hypothesis that bacterial concentration has no impact on bacteria inactivation by BL (section 2.8.1). This was performed independently for each of the exposure times; because the droplet position was later shown to have no statistically significant effect on the degree of bacterial inactivation (refer to section 3.2.2.8), the values used for each exposure time were the averaged values across all three droplet positions (section 2.6 and Figure 2.3). The Chi-squared and *p* values for each exposure time are shown in Table 3.7.

Table 3.7. Chi-square statistics and *p* values for the chi-squared analysis for the impact of bacterial concentration for individual BL exposure times.

Exposure Time (minutes)	**Chi squared value**	***p* value**
5	61.6	0.000253*
10	69.3	0.000023*
15	91.6	<0.00001*
20	67.5	0.000041*

*Statistically significant at P < 0.05

The *p* value for the analyses conducted for each exposure time are below the significance level of 0.05. Therefore, the impact of bacterial concentration on inactivation efficacy for all exposure times is statistically significant, and the null hypothesis can be rejected.

3.2.2.8 Impact of droplet position on BL inactivation as a function of exposure time

To test the impact of droplet position on inactivation efficiency of BL, three droplet positions of each dilution were featured on the plate (refer to section 2.6 and Figure 2.3). A Chi-squared test was performed on the inactivation of strains in each position (1-3) for each exposure time. The results are displayed in Table 3.8.

Table 3.8. Results from the chi-squared analysis of the impact of droplet position on bacterial inactivation by BL, for the range of exposure-times used.

Exposure Time (minutes)	**Chi-squared value**	***p* value**
5	12.0	0.151651
10	6.37	0.605663
15	8.60	0.377244
20	4.39	0.820327

The *p* value for the analyses conducted for each exposure time are all above the significance level of 0.05. Therefore, the impact of droplet position on inactivation efficacy for all exposure times is statistically insignificant, and the null hypothesis that droplet position has no impact of bacterial inactivation can be accepted.

3.3 Sample-distance experiments

3.3.1 Objectives

The objectives of the 'sample-distance' experiments were to evaluate the impact of the distance between the surface of the agar plate and the LED array described in section 2.5 on the level of bacterial inactivation. This was mainly assessed using the arbitrary rating scale (section 2.7.1) to determine the relative reductions in the quantity of bacterial colonies within 2 μl droplets of bacterial suspension (section 2.6) on a TSA

plate compared to a non-exposed control. In addition to this, analysis of the results was performed to answer the hypotheses stated in section 2.8.1. These will be discussed in the following sections.

3.3.2 Impact of sample-distance on bacterial reduction

The average inactivation values for each bacterial strain (across all dilutions and droplet positions), using the arbitrary rating scale (section 2.7.1) are displayed in Figure 3.4. The average values were used due to the high volume of data produced. The results displayed in Figure 3.4 show comparable trends to those in Figure 3.2. These are:

i) There is a high degree of variation in the degree of inactivation between bacterial strains. This can be divided on both an inter- and intra-species basis.
ii) There is a relationship between sample-distance and degree of bacterial inactivation.
iii) There is a correlation between the two variables accordingly.

To test the statistical strength of the above two observations, the Chi-squared test of independence and Pearson's product moment correlation coefficient were used accordingly.

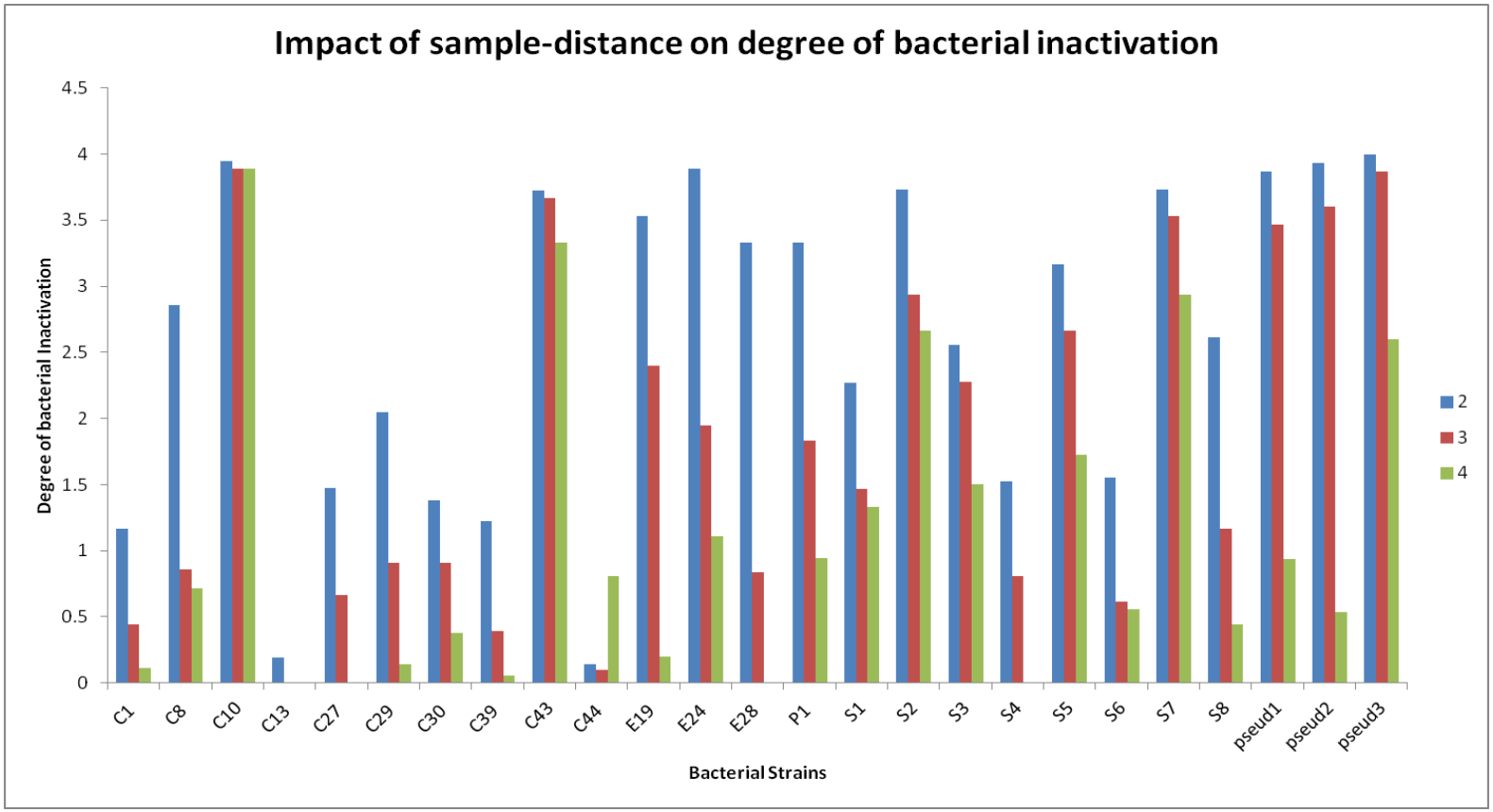


Figure 3.3. Degree of inactivation of the bacterial strains included in the sample-distance experiments (section 2.9.2). The legend refers to the sample-distance (cm). Bacteria were exposed to BL at the described heights for a period of 10 minutes. The y-axis details the results using the arbitrary scale (section 3.2). The values presented are the average values of each strain across all dilutions and droplet positions.

3.3.2.1 Chi-squared test of independence

To test if the relationship between the distance between the sample and LED array and the degree of bacterial inactivation was significantly different, a chi-squared test was applied to the data for each droplet position.

Table 3.9. Results from the Chi-squared analysis of the impact of sample-distance on degree of bacterial inactivation for all three droplet positions.

Droplet Position	Chi-square value	*p* value
1	77.0	<0.00001*
2	68.7	<0.00001*
3	39.0	<0.00001*

*statistically significant at P < 0.05

The *p* values for the analyses conducted for each droplet position regarding the relationship between sample-distance and degree of inactivation efficiency are below the significance level of 0.05. Therefore, they data is statistically significant and the null hypothesis that sample-distance has no impact on inactivation efficacy can be rejected, for all three droplet positions.

3.3.2.2 Pearson's product moment correlation coefficient

The second test was performed to determine the strength of linear association between two variables, in this case the exposure time and degree of bacterial inactivation. This is known as the Pearson's Product-Moment Correlation Coefficient, which generates an *r* value between two variables. This value ranges from -1 to +1, representative of a perfect negative correlation through to a perfect positive correlation accordingly. A value of or close to zero is therefore representative of no correlation. The results are displayed in Table 3.10.

Table 3.10. Pearson's Product Moment Correlation Co-efficient *r* values for bacterial strains in the sample-distance experiments.

Bacterial Genus	**Bacterial Strain**	***r* value**	***p* value**
Salmonella	S8	-0.94	0.00013*
Enterobacter	C29	-0.90	0.000821*
Salmonella	S3	-0.88	0.00185*
Pseudomonas	pseud2	-0.87	0.002118*
Escherichia	C1	-0.85	0.004134*
Pseudomonas	P1	-0.84	0.00441*
Pseudomonas	pseud1	-0.84	0.00489*
Pseudomonas	E19	-0.84	0.00499*
Serratia	C39	-0.83	0.005407*
Pseudomonas	pseud3	0.83	0.005515*
Pseudomonas	E24	-0.83	0.00585*
Pseudomonas	E28	-0.82	0.00643*
Salmonella	S1	0.82	0.00643*
Salmonella	S2	-0.80	0.009008*
Citrobacter	C8	-0.76	0.017472*
Enterobacter	C27	-0.75	0.020201*
Salmonella	S5	-0.67	0.049711*
Enterobacter	C13	-0.65	0.056031
Salmonella	S4	-0.61	0.080468
Salmonella	S7	-0.60	0.085634
Serratia	C30	-0.58	0.09943
Salmonella	S6	-0.55	0.124977
Acinetobacter	C43	-0.33	0.390381
Acinetobacter	C10	-0.16	0.675204
Raoultella	C44	0.49	0.182308

*Statistically significant at $P < 0.05$

Table 3.10 contains the *r* values for the bacterial strains in the sample-distance experiments. These were calculated in the same manner as those *r* values for the exposure-time experiments in section 3.2.2.2. From Table 3.10, the majority of strains have a strong negative R value. This illustrates that the photoinactivation efficiency of the majority of the strains decreased with increasing distance. However, there are anomalies present within the data. C44 shows a positive R value of 0.49. By referring to Figure 3.6, it can be seen that this is in line with the fact that the degree of inactivation for 4 cm is higher than that for 2 and 3 cm. Since there is not a clear trend on the graph, and the degrees of inactivation for all heights are ≤ 25 %, it can be concluded that this result is due in part to the resistance of this strain to BL. Pipetting

error could account for the differences in the size of the 2 µl droplets, making it appear that there are differences affected by the light. The relative resistance of other bacterial strains to BL, such as S6, is also reflected in the *r* value. Where there is little degree of inactivation, the trend will be to a lesser extent than for the more sensitive strains, with the exception of C10 and C43, which are so sensitive a trend is not apparent due to consistent high-level inactivation.

3.3.2.3. Inter- and intra-species related differences in inactivation efficacy

Table 3.11. Cumulative values of the average* degree of inactivation for all strains involved in the sample-distance experiments.

Strain	Genus	Total Inactivation Score*
C10	Acinetobacter	70.33
C43	Acinetobacter	64.33
pseud3	Pseudomonas	52.33
S7	Salmonella	51
S2	Salmonella	46.67
S5	Salmonella	45.33
E24	Pseudomonas	41.67
pseud1	Pseudomonas	41.33
pseud2	Pseudomonas	40.33
S3	Salmonella	38
P1	Pseudomonas	36.67
E19	Pseudomonas	30.67
C8	Citrobacter	30
S1	Salmonella	25.33
S8	Salmonella	25.33
E28	Pseudomonas	25
C29	Enterobacter	21.67
C30	Serratia	18.67
S4	Salmonella	16.33
S6	Salmonella	16.33
C27	Enterobacter	15
C1	Escherichia	10.33
C39	Serratia	10
C44	Raoultella	7.33
C13	Enterobacter	1.33

*average value across all dilutions and droplet positions, as described in section 2.6.3.

From table 3.11, some of the trends that existed for the exposure-time experiments are present within the sample-distance results. For example, the relative sensitivities of *Acinetobacter* and *Enterobacter* remain highly sensitive and resistant respectively. The strains belonging to the *Salmonella* and *Pseudomonas* species display a sensitivity spectrum, as was observed in the exposure-time experiments. However, there are some differences in the patterns in bacterial inactivation in this set of experiments. Whereas mutant *Salmonella* strain S4 was the most resistant organism for the exposure-time experiments, this is no longer the case. In addition to this, the total inactivation score for S4 is equalled by S6. Therefore, whilst the former strain has become less resistant compared to the dataset as a whole, the opposite has occurred to the latter strain. Whilst there was considerable difference between the pure strain *Acinetobacter baumannii*(C31) and the un-defined *Acinetobacter* spp. strain (C38) in the exposure-time experiments, both *Acinetobacter* strains are of similar inactivation efficiency in this set of experiments. The *Enterobacter* strains appear to be more widely distributed amongst the inactivation spectrum in these experiments compared to the former set of experiments.

3.3.2.4 Impact of different OxyR mutations on inactivation efficacy of *Salmonella enteric* serovar Typhimurium

As with the exposure-time experiments, the *Salmonella* mutant strains were also included in the sample-distance experiments. The objectives remained the same. The order of sensitivity of the mutant strains was different during this set of experiments. Whilst S4 was still the most resistant *Salmonella* mutant strain, it was not the most resistant strain overall, as discussed in the previous section. Furthermore, the total inactivation score for S6 was tied with that for S4. Strain S7 displayed increased sensitivity, taking the position of the most sensitive *Salmonella* mutant strain. The only strain to remain in the same position on the sensitivity spectrum of the *Salmonella* strains was strain S1. These inconsistencies introduce difficulty in assessing the impact of OxyR mutations on inactivation via BL.

3.3.2.5 Influence of incorporation of a UV-resistance plasmid

In contrast to the inconsistency of the results for the *Salmonella* mutant strains, the *P. putida* strains illustrated the same order of sensitivity to BL in the sample-distance experiments as that for the exposure-time experiments. This reinforces the conclusion made in section 3.2.2.6 that the carriage of a UV resistance gene is not advantageous during BL exposure.

3.3.2.6 Impact of bacterial concentration on BL inactivation as a function of sample-distance

The impact of bacterial concentration on inactivation rates was assessed by analysing the degree of bacterial inactivation over the range of dilutions described in sections 2.6 and 3.2.2.7. The arbitrary rating scale was used to define the inactivation efficacy and a Chi-squared test of Independence was used to either accept or reject the null hypothesis that bacterial concentration has no impact on bacteria inactivation by BL (section 2.8.1). This was performed independently for each of the sample-distances featured (section 2.6.2), within each of the droplet positions (section 2.6). The results are displayed in Table 3.12.

Table 3.12. *p* values from the chi-squared analyses of impact of bacterial concentration, for i) the different sample-distances (2-3 cm) and ii) droplet positions (section 2.6.3).

	Position 1	**Position 2**	**Position 3**
2 CM	0.038084*	0.083	0.091
3 CM	0.038*	0.093	0.24
4 CM	0.06	0.214	0.17

*statistically significant at $P < 0.05$

From Table 3.12, the majority of the *p* values are not statistically significant. This indicates that in these cases, bacterial concentration did not have a significant effect on bacterial inactivation, and the null hypothesis can be accepted. The null hypothesis can be rejected for the inactivation data for the sample-distances of 2 cm and 3 cm in droplet position 1, where the *p* values are statistically significant.

3.3.2.7 Impact of droplet position on BL inactivation as a function of sample-distance

To test the impact of droplet position on inactivation efficiency of BL, three droplet positions of each dilution were featured on the plate (refer to section 2.6 and Figure 2.3). A Chi-squared test was performed on the inactivation of strains in each position (1-3) for each exposure time. The results are displayed in Table 3.13.

Table 3.13. *P* values of the impact of droplet position on inactivation efficacy on bacterial inactivation for each sample-distance.

	2 cm	**3 cm**	**4 cm**
POS1	0.000067*	<0.00001*	0.689615
POS2	0.000067*	<0.00001*	0.689615
POS3	0.000067*	<0.00001*	0.689615

*statistically significant at $P < 0.05$

From Table 3.13, the *p* values for the sample-distances 2 cm and 3 cm, across all droplet positions, are below the significance level. They are therefore statistically significant, and the null hypothesis that bacterial concentration has no impact on bacterial inactivation can be rejected for these parameters. However, none of the *p* values for the sample distance of 4 cm are statistically significant. Consequently, the null hypothesis can be accepted for the sample-distance 4 cm within all droplet positions.

Following on from this, the relationship between pairs of distances was assessed using the Chi-squared analysis. The results are shown in Table 3.14.

Table 3.14. *p* values obtained from the Chi-squared analysis of the difference in bacterial inactivation between pairs of the distance samples used. Separate analyses were performed for each droplet position (1-3).

	2/3 cm	**2/4 cm**	**3/4 cm**
POS1	0.08	<0.00001*	0.000011*
POS2	0.000139*	<0.00001*	0.007*
POS3	0.004*	<0.00001*	0.084

*statistically significant at $P < 0.05$

From Table 3.14, it can be seen that the majority of the *p* values between the pairs of sample-distances are statistically significant. The exceptions to this are for the 2/3 cm pair for droplet position 1 and the 3/4 cm pair for position 3. These results reinforce the conclusion drawn from Table 3.13.

3.4 Continuous versus Pulsed Experiments

3.4.1 Overview

In this set of experiments, the efficacy of two modes of BL was assessed. These were:

i) Continuous BL exposure mode
ii) Pulsed BL exposure mode

In the pulsed mode, the input voltage value was three times greater than that for continuous mode. The purpose of this was to determine if the degree of inactivation of bacteria could be achieved within a shorter period of time following exposure to short, powerful pulses of BL as opposed to longer durations of continuous BL exposure. This was assessed using spread plates as described in section 2.6.3. The organisms used were a representative sample of the sensitivity spectrum within the

population of strains previously used in the BL experiments. Therefore, they ranged from very sensitive (*Acinetobacter* spp.) to very resistant (*Raoultella ornithinolytica*), with strains of intermediate sensitivity included. The results from the exposure-time and sample-distance experiments were assessed to allow the optimum sample distance and exposure time parameters to be selected. These were a distance of 2 cm for a duration of 10 minutes. The dilution used was 10^{-6}, since at this dilution the colonies were within an accurate countable range (30-300). The $\log_{10}$ reductions of the bacterial strains following exposure to continuous and pulsed BL are displayed in Table 3.15.

3.4.2 Impact of continuous and pulsed BL exposure on bacterial inactivation

Table 3.15. Inactivation rates of *Escherichia coli*, *Acinetobacter spp.*, *Enterobacter cloacae*, *Raoultella ornithinolytica* and *Serratia marcescens* following exposure to 10 minutes of continuous or pulsed BL.

Bacterial Strain	Control	Continuous	$\log_{10}$ Reduction	Pulsed	$\log_{10}$ Reduction
Escherichia coli	1.23×10^9	1.6×10^8	0.89*	1.11×10^9	0.04
Acinetobacter spp.	3.9×10^8	1×10^7	1.59*	2.6×10^8	0.18
Enterobacter cloacae	2.15×10^9	1.69×10^8	1.10*	2.07×10^9	0.02
Raoultella ornithinolytica	6.2×10^8	5.4×10^8	0.06*	6.1×10^8	0.01
Serratia marcescens	1.1×10^8	5×10^7	0.34*	7×10^7	0.20

*statistically significant at $P < 0.05$

From Table 3.15, it is apparent that the continuous mode of exposure to BL is more effective than the pulsed. This is reinforced by the fact that only the reductions from continuous light exposure are statistically significant, as calculated using a Paired T–Test (2 tailed). An anomalous result for the continuous exposure was obtained for strain *Enterobacter cloacae*. This was predicted to be the second most resistant, after *Raoultella ornithinolytica*. However, it is the second most sensitive strain for continuous BL. A fact that makes this further surprising is that the starting numbers

for *Enterobacter cloacae* are the highest. Theoretically, this should heighten the resistance of the bacteria due to shielding effects. However, this is not the only anomaly. The *Acinetobacter spp.* was predicted to have the greatest levels of reduction for both types of exposure. Whilst this was the case for continuous exposure, it is the third most susceptible for the pulsed exposure.

3.5 Temperature Experiments

3.5.1 Objective

During the continuous exposure experiments carried out in section 3.4, it was observed that the agar surface reached temperatures up to 40 °C. Subsequently, a set of experiments to determine if exposure of bacterial suspensions to 40 °C heat had a significant effect on bacterial viability were conducted. This was achieved by using the same dilution used for the spread plates for the continuous and pulsed BL exposure experiments in section 3.4. The results from the temperature experiments are recorded in Table 3.16.

3.5.2 Impact of 40 °C heat on bacterial inactivation

Table 3.16. Comparison of inactivation of *Escherichia coli, Acinetobacter spp., Enterobacter cloacae, Raoultella ornithinolytica* and *Serratia marcescens* from 10 minutes of continuous BL and 40 °C heat. Where there is an asterisk, the reduction is statistically significant at $P < 0.05$

Bacterial Strain	Control	Light	Log_{10} Reduction	Heat	Log_{10} Reduction
Escherichia coli	9.1×10^8	1.4×10^8	0.81*	9.3×10^8	-0.001
Acinetobacter spp.	1.38×10^9	1	9.14*	1.15×10^9	0.08
Enterobacter cloacae	2.12×10^9	9.8×10^8	0.34*	2.13×10^9	-0.002
Raoultella ornithinolytica	1.22×10^9	5×10^8	0.39*	1.1×10^9	0.04
Serratia marcescens	3.5×10^8	1×10^8	0.54*	5.2×10^8	-0.17

In contrast to Table 3.15, *Enterobacter cloacae* is the most resistant strain to both the light exposure plates and heat controls, whilst *Acinetobacter spp.* is the most sensitive in both cases. For all of the strains, the change in bacterial count following exposure to 10 minutes of 40 °C heat was not significantly different from the controls. In contrast, all of the counts for the bacteria exposed to continuous blue light for 10 minutes were significantly different from the controls. It can therefore be concluded that despite the increase in temperature during light exposure experiments, the inhibitory growth effects observed from the plate counts are independently caused by BL, with no interference from the temperature change observed for the bacterial strains involved. This is reinforced by the results gained from the *Pseudomonas* experiments in the section below.

3.6 Influence of UV-resistance on BL-mediated bacterial inactivation

3.6.1 Overview

To determine if the fitness trait of UV-resistance was advantageous to BL inactivation, three *Pseudomonas aeruginosa* mutants were included in the study. Two of these carried a plasmid (pWW0) that encoded a UV-resistance gene, whilst the other served as a control. These were included in the exposure-time and sample-distance experiments for an initial indication of the influence of a UV-resistance gene on efficacy of BL inactivation. Following the conclusion that there was no observable influence within both of the BL experiments, spread plate experiments were conducted to determine if the inactivation of the *P. putida* strains was statistically significant.

3.6.2 BL-inactivation of *Pseudomonas aeruginosa* mutants by exposure to both modes of BL exposure and 40°C heat

Table 3.17**.** Comparison of the inactivation of *Pseudomona putida* mutants by continuous BL, pulsed BL and 40 °C heat following an exposure time of 10 minutes. Where there is an asterisk, the reduction is statistically significant at $P < 0.05$.

Bacterial Strain	Control	Continuous Light	Log_{10} Reduction	Pulsed Light	Log_{10} Reduction	Heat (40°C)	Log_{10} Reduction
pseud1	1×10^8	1×10^7	1.10*	6×10^8	-0.68*	6.4×10^8	-0.70
pseud2	8×10^8	1×10^7	1.89*	1×10^8	0.81*	5.1×10^8	0.18
pseud3	5×10^8	2×10^7	1.42*	2×10^8	0.42*	6.6×10^8	+0.10*

For all three *Pseudomonas* strains, the reductions in colony numbers from both types of BL were significantly different ($P < 0.05$). For the temperature experiments, pseud3 was significantly different; however, this is because the cell counts were actually higher than the control plates. These results suggest that 40 °C heat has no adverse effects on the *Pseudomonas* isolates used. The numbers of colonies for the controls varies slightly. This could be attributed to slightly different growth rates among the strains. Alternatively, the inoculation of the strains into broth for liquid culture, using a loop, could have resulted in introduction of fewer or more colonies per inoculum. Finally, there could have been differences in the numbers of bacteria in the colonies of the different strains. The method of using optical density is not precise enough for exact numbers to be calculated. Since the order of magnitude of all three strains (10^8) is equivalent, this was considered acceptable for the purposes of this Masters project.

3.7 UV experiments

3.7.1 Overview

A set of experiments using UV light to inactivate the bacterial strains that featured in the previously described spread-plate experiments were conducted to assess if the inactivation patterns resulting from exposure to UV were similar to those illustrated following BL exposure. As with the exposure-time and sample-distance experiments (sections 3.2 and 3.3 respectively), the droplet method was applied (section 2.6). However, there were changes in the plate layout (section 2.6.5).

3.7.2 UV light inactivation of bacteria as a function of exposure-time

The results of these experiments are shown in Figure 3.5. From this, it can be seen that as with BL-inactivation, there is a relationship between the degree of bacterial inactivation and the duration of exposure-time. The statistical tests used to determine the significance of this relationship, along with strength of linear association between the two variables being analyzed, were applied to this data set.

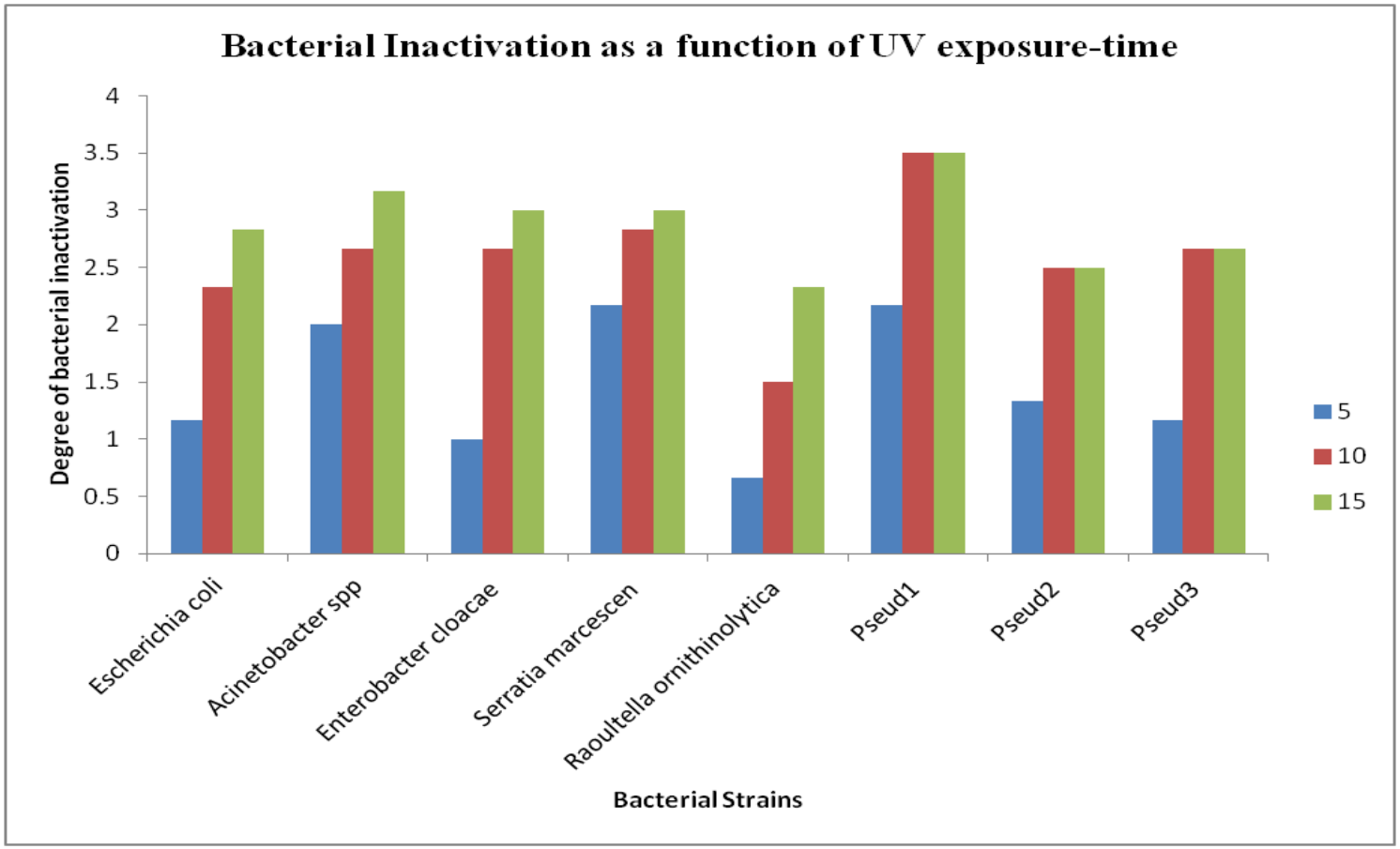


Figure 3.4. Bacterial inactivation (in accordance with the inactivation scale described in section 2.7.1) following exposure to 5, 10 and 15 seconds of exposure to UV light (302 nm) from a transilluminator.

3.7.2.1. Chi-squared test of independence

The Chi-squared test of independence was conducted on the variables exposure-time and degree of bacterial inactivation following exposure to a UV transilluminator (302 nm). The results are displayed in Table 3.18.

Table 3.18. Contingency table for Chi-squared analysis of impact of sample-distance on UV-inactivation of bacteria.

	5 SECS	**10 SECS**	**15 SECS**	**Row Totals**
0	17 (10.33) [4.30]	8 (10.33) [0.53]	6 (10.33) [1.82]	**31**
1	11 (8.00) [1.12]	8 (8.00) [0.00]	5 (8.00) [1.12]	**24**
2	9 (6.00) [1.50]	3 (6.00) [1.50]	6 (6.00) [0.00]	**18**
3	3 (4.00) [0.25]	6 (4.00) [1.00]	3 (4.00) [0.25]	**12**
4	8 (19.67) [6.92]	23 (19.67) [0.56]	28 (19.67) [3.53]	**59**
Column Totals	**48**	**48**	**48**	**144 (Grand Total)**

The chi-square value was 24.41 and the p value was 0.001954. The differences in UV-inactivation for the sample-distances are therefore statistically significant, and the null hypothesis can be rejected. This led to the analysis for pairs of exposure times. The results are shown in Table 3.19.

Table 3.19. *p* values obtained from the Chi-squared analysis of the pairs of exposure times of bacterial strains to UV (302 nm).

Pairs of exposure times (seconds)	***p* value**
5 and 10	0.004701*
5 and 15	0.000718*
10 and 15	0.482455

*statistically significant at $P < 0.05$

From Table 3.19, it can be seen that the only pair of exposure times to not yield a significant *p* value was 10 and 15 seconds of UV exposure. By consulting Figure 3.5, it can be seen that the inactivation scores for 10 seconds of exposure are either very close or equal to those for 15 seconds of exposure for the *Pseudomonas* strains; however, this is not the case for the Enterobacteriaceae.

3.7.2.2 Pearson's product moment correlation coefficient

The results of the Pearson's product moment correlation coefficient performed for the range of exposure-times for the UV experiments are displayed in Table 3.20.

Table 3.20. *r* values from the Pearson's product moment correlation coefficient for UV exposure times and bacterial inactivation.

Strain	***r* value**	***p* value**
Escherichia coli	0.962504	0.000032*
Acinetobacter spp	0.996616	< 0.00001*
Enterobacter cloacae	0.933257	0.000238*
Serratia marcescen	0.944911	0.000122*
Raoultella ornithinolytica	0.99705	< 0.00001*
pseud1	0.866025	0.002538*
pseud2	0.866025	0.002538*
pseud3	0.866025	0.002538*

*statistically significant at $P < 0.05$

All of the above strains illustrated high *r* values, indicating that there is a strong (positive) linear relationship between exposure-time of bacteria to UV light and degree of inactivation.

3.7.2.3 Inter-species variation of UV light-induced inactivation of bacteria

The difference in total inactivation rates between the bacterial strains can be observed from the values in Table 3.21.

Table 3.21. Cumulative values of the average* degree of inactivation for all strains involved in the sample-distance experiments using UV light.

Bacterial Strain	Total Inactivation Score*
pseud1	9.17
Serratia marcescen	8
Acinetobacter spp	7.83
Enterobacter cloacae	6.67
pseud3	6.5
Escherichia coli	6.33
pseud2	6.33
Raoultella ornithinolytica	4.5

*average value across all dilutions, as described in section 2.6.5.

3.7.2.4 Impact of bacterial concentration on bacterial inactivation by UV light

The impact of bacterial concentration on inactivation rates was assessed by analysing the degree of bacterial inactivation over the range of dilutions described in sections 2.6 and 3.2.2.7. The arbitrary rating scale was used to define the inactivation efficacy and a Chi-squared test of Independence was used to either accept or reject the null hypothesis that bacterial concentration has no impact on bacteria inactivation by UV (section 2.8.1). This was performed independently for each of the sample-distances featured (section 2.6.6).

Table 3.22. Results from the Chi-squared analysis of the impact of bacterial concentration on bacterial inactivation by UV light as a function of exposure-time.

Exposure time (seconds)	Chi-squared value	*p* value
5	67.7	<0.00001*
10	76.3	<0.00001*
15	75.2	<0.00001*

*statistically significant at $P < 0.05$

The *p* values for all of the exposure-times were statistically significant. The null hypothesis that bacterial concentration has no impact on bacterial inactivation (via exposure to UV) can therefore be rejected.

3.8. Summary of results

The following facts have been established following analysis of results obtained from the sets of experiments described in the above sections. In summary:

- The relationship between length of exposure of bacteria to both BL and UV light is a statistically significant.
- For the majority of strains, high positive *r* values obtained from the Pearson's product moment correlation coefficient indicate a strong linear relationship between length of light exposure and degree of bacterial inactivation.
- There is a high amount of both inter- and intra-species variation in level of BL-inactivation for both the exposure-time and sample-distance experiments.
- The impact of bacterial concentration on bacterial inactivation by BL is statistically significant for each exposure time in the exposure-time experiments.
- However, the influence of droplet position is not statistically significant within the exposure-time experiments.
- The impact of the distance between the sample and LED array is statistically significant for all sample-distances, across all droplet positions.

- The majority of strains possess high negative *r* values obtained from the Pearson's product moment correlation coefficient indicate a strong linear relationship between sample-distance and degree of bacterial inactivation.
- The impact of bacterial concentration on BL-inactivation was only statistically significant for the sample-distances of 2 cm and 3 cm within position 1. The rest of the combinations of droplet position and sample-distance were not.
- Furthermore, the impact of droplet position on BL-inactivation of bacteria was only statistically significant at the sample-distances 2 cm and 3 cm.
- Continuous BL exposure was more effective for the bacterial inactivation of the five representative strains than pulsed BL; this was reflected in the results of the t-test, in which only the reductions obtained from continuous BL exposure were statistically significant.
- Exposure of these strains to 40 °C heat had no statistically significant impact on bacterial viability
- Exposure of the *Pseudomonas putida* mutant strains carrying UV-resistance genes resulted in statistically significant reductions for both continuous- and pulsed-BL exposure; therefore the UV-resistance do not confer an advantageous fitness trait in these circumstances
- The differences in UV-inactivation for the sample-distances were statistically significant.
- In comparison to the results gained from the BL-inactivation experiments, the total inactivation scores for UV-inactivation of the bacterial strains were on a smaller range.
- The differences in UV-inactivation for the exposure-times were statistically significant.

Discussion

4.1 Overview

The purpose of this project was to design and conduct sets of experiments to determine the following:

- The range of exposure times to Blue light (BL) that generates adequate bacterial reduction across the majority of a large number of bacterial strains within time scales that are practical for industrial applications;
- The range of distances between the light source and bacteria that is practical for industrial applications:
    - Whilst also keeping the exposure time practical;
    - For the majority of bacterial strains featured in the experiments.
- Impact of bacterial concentration on inactivation by BL.
- Within the sample distances used, is the light intensity evenly distributed over the area (agar plate) involved in the experiments?
- Do UV-resistance genes confer advantageous traits to bacteria that possess them?
- Are the bacterial sensitivities/resistances to BL reflected in the inactivation kinetics for UV inactivation?

The results obtained from the experiments will be discussed in the following sections, in addition to how these contribute to current understanding of BL-inactivation of bacteria, interpretation of the results in the context of practical applications and future work.

4.2 Choice of bacterial strains

From 2008, the five most common Gram negative human pathogens were named as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter spp and Acinetobacter baumannii* (Steven & Luis 2013). Four out of five of these species have been selected for use in the experiments in this project, in reflection of

the need to find alternatives to antibiotics to target these pathogens. In addition to this, all of the strains belonging to the *Enterobacteriaceae* family were isolated from patient samples and various target sample sites within the Blackpool Victoria Hospital. They are, therefore, recent isolates that are representative of a diverse set of environmental and clinical backgrounds. Following on from this, the accuracy of using reference strains (from national or private culture collections) to investigate bacteria has been previously questioned (Fux *et al.*, 2005). This is due to the evolving of the genomes of these strains due to decades of sub-culturing within laboratory conditions, where many genes are made redundant resulting in expression of a different genotype (Fux *et al.*, 2005) thus they no longer represent bacteria in the environment. It was highlighted by Fux *et al.*, (2005) that this could lead to the exclusion of pathophysiological mechanisms present within the wild-type strains, rendering research on the pathology of laboratory strains incomplete. Therefore, the use of wild-type strains as opposed to reference strains could have potentially widened the scope of the experiments.

4.3 Choice of wavelength and light source

Reviewing the scientific literature revealed a clear pattern with regards to the wavelength of choice in the blue region of the electromagnetic spectrum; the majority of studies investigating the impact of blue light on bacteria chose the wavelength 405 nm (refer to section 1.2.1 and table 1.2). A theory behind the effectiveness of this wavelength is that most porphyrins are said to contain a sorbet band of 380 – 500 nm (Fyrestam et al. 2015) with peak wavelengths of 400 – 410 nm (Touma et al. 2004). This explains why although 405 nm appears to be the most effective wavelength, there are reports of 470 nm exerting similarly detrimental effects on some bacteria. Due to the quantity of studies using 405 nm, it was logical to use this wavelength in the present study. This light was delivered via a light-emitting diode (LED) array, which was the product of joint work between myself and Marl International Limited, with whom I collaborated with for this project. It is worth noting that the year this project commenced (2014), the Nobel Prize in Physics was awarded to Isamu Akasaki, Hiroshi Amano and Shuji Nakamura, for Blue LED innovation (MLA 2014). They achieved this by overcoming the challenge of creating a blue LED, which had previously presented difficulties for three decades prior to their break-through. The

blue LEDs used to construct the LED array were provided from the company at which Shuji Nakamura is employed.

4.4 Experimental results

4.4.1 Dose-dependent bacterial inactivation

4.4.1.1 Exposure time of bacteria to BL

Referring back to the summary presented in section 3.8, the first observation is that increased exposure time results in an increased degree of inactivation of the majority of bacterial strains. The relationship between the length of exposure of bacteria to BL and degree of inactivation was shown to be statistically significant from the Chi-squared analysis of all of the exposure times collectively. Further analysis between pairs of exposure times revealed that the only pair of exposure times that did not product significantly different results in the degree of bacterial inactivation were the 5 and 10 minute exposure times. An explanation for this could be that the initial doses did not result in adequate production of ROS to overcome the oxidative stress threshold in the majority of the bacterial strains. This correlates with the inactivation kinetics of the organism *Staphylococcus aureus* in the experiments by Farrell *et al.*, (2010), which displayed a shoulder effect within a sigmoidal inactivation plot following exposure to pulsed light (PL) within the visible range of the EMS.

4.4.1.2 Sample distance between bacterial sample and LED

Following on from the above section, it was observed that decreased sample distance led to increased bacterial inactivation rates. As with the exposure time experiments, the relationship of the distance between the bacterial samples and LED array was shown to be statistically significant from the Chi-squared analysis of all of the sample-distances collectively. Inspection of the difference in *p* values illustrates that there is an inactivation pattern common to positions 2 and 3, whereby the difference between 2 cm and 3 cm has a higher degree of statistical significance than the difference between 3 cm and 4 cm (refer to Table 3.14). This pattern is not true for droplet

position 1. This could be due to the fact that the chi-squared value was the greatest for position 1 (Table 3.9), indicating greater statistical significance that is not obvious from the *p* values (all *p* values were <0.00001). Since the dose of BL exposure (J/cm^2) increased with a decrease in sample distance, and the *p* value for bacterial reduction in accordance with all of the sample-distances collectively was statistically significant, the inactivation behaviour within this set of experiments can also be said to be dose-dependent.

4.4.1.3 UV experiments

To determine if there were any parallels in the inactivation kinetics between BL- and UV-inactivation, bacteria were exposed to three durations of exposure: 5, 10 and 15 seconds respectively. Chi-squared analysis of the relationship between exposure time and degree of bacterial inactivation showed that this was statistically significant (Table 3.9). Further analysis into the difference between pairs of exposure times, as for BL-inactivation, revealed that not all pairs of exposure times yielded significant *p* values. In the case of the UV experiments, the difference in bacterial inactivation rates between 10 and 15 seconds of UV exposure was not statistically significant (Table 3.10). UV inactivation of bacteria has been reported to involve a 'tailing phenomenon' at the latter part of the inactivation curve (Ȧ *et al.*, 2014). The cause of this is a much debated topic. However, this tailing effect may be responsible for the statistically insignificant *p* value for the differences in inactivation rates between 10 and 15 seconds of UV exposure. Regardless of the *p* value for the difference between 10 and 15 seconds of UV exposure, the overall *p* value for the impact of exposure time on degree of bacterial inactivation was significant and the bacterial inactivation in the UV experiments was shown to be dose-dependent.

4.4.1.4 Summary

In conclusion, the inactivation of a variety of important human pathogens were inactivated by both BL and UV in a dose-dependent manner. This dose-dependent

inactivation behaviour has been demonstrated in a number of other studies (Bumah *et al.*, 2015; Lipovsky *et al.*, 2009).

4.4.2 Inter- and intra-species related differences in BL-inactivation of bacterial strains

4.4.2.1 BL-inactivation experiments

Another observation is that there are differences in the inactivation efficiency of BL on both an inter- and intra-species level. This was shown for both the exposure-time and sample-distance experiments using BL. This difference in BL sensitivity among bacterial species has been reported from numerous studies involving photoinactivation of bacteria (Soukos *et al.*, 2005; Murdoch *et al.*, 2012; Maclean *et al.*, 2009; Maclean *et al.*, 2014; Dai *et al.*, 2012). Subsequently, investigation of the cause of this has been conducted by some studies (Nitzan *et al.*,2004; Shu *et al.*,2013).

4.4.2.1.1 Porphyrins within bacteria

Porphyrins are abundant compounds that have been suggested to act as endogenous photosensitizers when exposed to BL (Almeida *et al.*,2011). As mentioned in section 1.2.2, the relative quantity and type(s) of porphyrin within bacteria has previously been regarded as an important factor in BL-inactivation efficacy (Soukos *et al.*,2005; Ashkenazi *et al.*,2003; Borelli *et al.*,2006; Nitzan *et al.*,2004). This is because the production of ROS through excitation of endogenous porphyrins is the proposed mechanism for BL-inaction in bacteria. This led to the investigation into the differences in type and relative quantities of porphyrins within a selection of Gram-positive and Gram-negative bacterial strains was determined by Nitzan *et al.*, (2004). It was shown that there was a slight difference in the emission peaks of porphyrins between Gram positive and Gram negative cells. These were 622 nm and 630 nm respectively. In addition to this, whilst the Gram positive species *Staphylococcus* possessed a predominant porphyrin type, this did not exist for Gram negative bacteria (Nitzan *et al.*,2004). These factors could contribute to the relative resistance of Gram negative bacteria compared to Gram positive bacteria. The study concluded that the

differences in inactivation rates of 9 bacterial species, following exposure to 407-420 nm light, was due to the porphyrin amounts and distribution (Nitzan *et al.*, 2004). However, there are contradictory reports on the types and amounts of porphyrins present within some of the studied bacterial species, such as *Propionibacterium acnes* (Masamitsu *et al.*,2012). Therefore, more research on this topic is required before full conclusions can be made.

4.4.2.1.1 Bacterial fitness traits

The differences in the sensitivity of different bacterial species could also be due to species- and strain-specific physiological or genotypic traits that are advantageous to survival of BL-inactivation. For example, the ability of *Enterobacter* species to detoxify antibiotics through the expression of specialist enzymes, along with acquisition of genetic mobile elements (Davin-Regli & Pagès 2015), may also enhance their survival during and following exposure to BL. In addition to this, *Enterobacter* species display adaptability to a broad range of conditions and hosts, thus being presented with various environmental stresses and unsavoury conditions (Davin-Regli & Pagès 2015). This, along with a lack of endogenous porphyrins, could explain the high resistance to BL illustrated by the *Enterobacter* species in the experiments in this project.

4.4.2.1.2 Differences in previous bacterial habitat

Following on from the above section, the environment that the bacteria were sampled from could influence BL-sensitivity. The bacterial strains collected by Aston (2012, Lancaster MSc Thesis) were isolated from both biotic and abiotic environments (Table 2.1). Whilst all of the strains belonging to the *Enterobacteriaceae* family are exclusively from patient samples, the *Pseudomonas* strains are a mixture of patient- and environment-derived isolates. It is not clear if this has an impact from the experiments in this project. However, this is an important topic that should be investigated in future experiments.

4.4.2.2 UV experiments

It was also observed that there were differences in the susceptibility of bacterial strains exposed to UV light. However, by referring to Table 3.20, it is clear that the degree of variation is not as high as for the BL experiments (Tables 3.6 and 3.11). This could be due to the inactivation mechanisms behind the two types of photoinactivation effected by UV and BL. The mechanisms of damage that occur due to BL-inactivation are detailed at length in section 1.4. Whereas there are multiple damage sites and damaging components (ROS) involved in BL-inactivation, damage caused by UV is mostly DNA damage (Ozer & Demirci 2006).

4.4.3 Bacterial concentration can affect the effectiveness of bacterial photoinactivation

These experiments showed that bacterial concentration can significantly affect the efficacy of photoinactivation of bacteria by both BL and UV. The exceptions to this were within the sample-distance experiments; all distances within positions 2 and 3 yielded insignificant results, along with the sample-distance 4 cm within position 1. By reviewing the raw data (see appendices), it can be observed that the inactivation ratings tend to be weighted at the extreme ends of the arbitrary rating scale for the majority of strains. There is also a much lesser degree of intermediate ratings, for most of the dilutions, compared with the results gained from the exposure-time experiments. Two examples of this are strain C13 (*Enterobacter cloacae*) and strain C10 (*Acinetobacter baumannii*). These are shown in Table 4.1.

Table 4.1. Inactivation scores for the strains C13 and C10 within the sample-distance experiments.

	Position 1			Position 2			Position 3			
Strain	2 cm	3 cm	4 cm	2 cm	3 cm	4 cm	2 cm	3 cm	4 cm	Dilution
C13	0	0	0	0	0	0	0	0	0	**1**
	0	0	0	0	0	0	0	0	0	**2**
	0	0	0	0	0	0	0	0	0	**3**
	0	0	0	0	0	0	0	0	0	**4**
	0	0	0	0	0	0	0	0	0	**5**
	2	0	0	2	0	0	0	0	0	**6**
C10	4	4	4	4	4	4	3	3	3	**1**
	4	4	4	4	4	4	4	3	3	**2**
	4	4	4	4	4	4	4	4	4	**3**
	4	4	4	4	4	4	4	4	4	**4**
	4	4	4	4	4	4	4	4	4	**5**
	4	4	4	4	4	4	4	4	4	**6**

Table 4.1 illustrates the reason for the unexpected result that bacterial concentration largely was not significant in affecting the inactivation rates. Because a much lesser portion of the strains demonstrated a range of inactivation scores, the *p* values obtained from the chi-squared analysis were not statistically significant.

4.4.3.1 The 'shielding effect'

Where the bacterial concentration exerted a statistically significant impact on BL-inactivation, this could be attributed to a phenomenon known as the 'shielding effect'. This was described by Farrell *et al.*, (2010), who investigated factors affecting the efficiency of bacterial inactivation using pulsed light. Among these was the effect of population size on inactivation. Similarly to this study, a range of dilutions of bacterial suspension were exposed to a set exposure of light treatment. In parallel to the results seen in this thesis, with an increase in population density is a decrease in inactivation effectiveness. This trend is readily observed upon inspection of the plates used in the current experiments; since all dilutions are present on the same dish, this makes for a quick, easy and immediate comparison. The shielding effect describes the

event in which the greater the number of bacterial cells present, the lower the proportion of these that are exposed either fully or at all to the inactivating light.

4.4.3.1.1 Impact of dose on the shielding effect

An interesting observation regarding the shielding effect within the experiments in this project is that whilst it is a clear (and statistically significant) pattern at the lower doses, it is less prominent at greater doses. An example for this is illustrated by the strain of *Pseudomonas aeruginosa* referred to as 'E28'. This is best illustrated using Figure 4.1. At the lowest dose (4 cm) in the sample-distance experiments, there is no reduction in colonies for any of the dilutions. The shielding trend is more noticeable for the next highest dose; however, for the greatest dose, all dilutions were drastically reduced at the same level. This could be a reflection of the events occurring at the molecular level. If the widely accepted theory behind BL-mediated inactivation is correct, it is plausible that the disruption to the membranes caused by ROS could lead to breakdown of the bacterial cells sufficiently to allow light penetration through these cells if the dose is great enough. This would explain why, at the higher doses, dilutions previously offering shielding at lower doses are devoid of this protection.

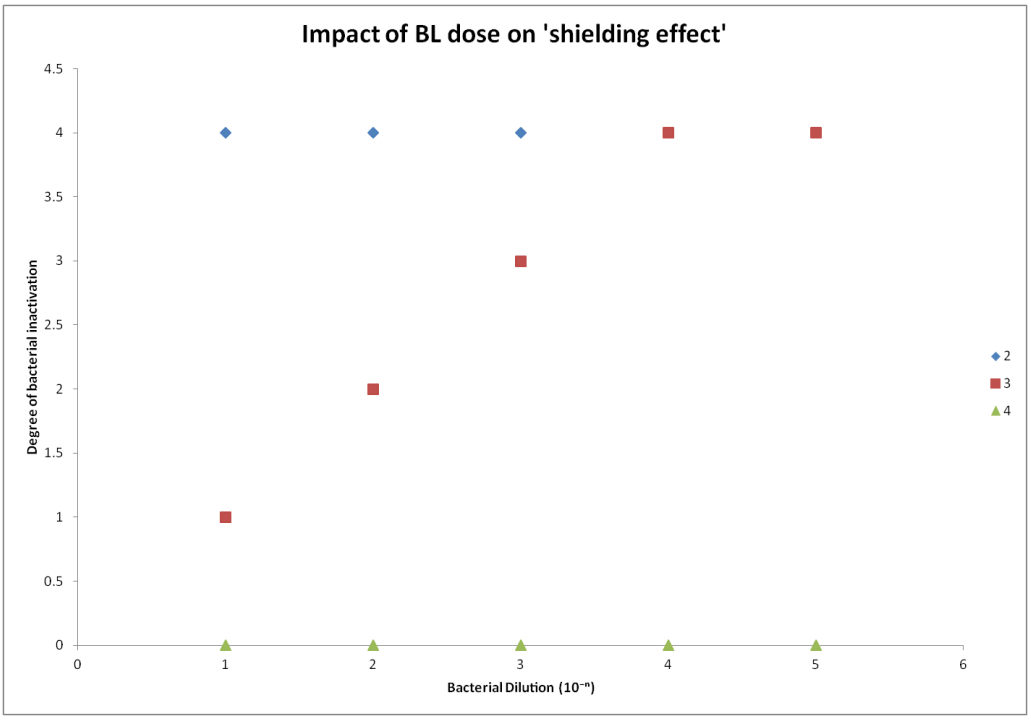


Figure 4.1. Impact of BL dose on the 'shielding effect' of bacterial concentration. Degree of inactivation corresponds to the arbitrary scale discussed in section 2.7.1. The dilution of the bacterial suspension used is a ten-fold serial dilution, where n corresponds to the numbers on the x-axis.

4.4.4 Difference in efficacy of continuous versus pulsed BL

In addition to the exposure-time and sample-distance experiments, determination of the efficacy of continuous and pulsed BL was determined for a set of strains that ranged from very sensitive to very resistant with regards to BL inactivation, in addition to the three *P. putida* strains featured in the project. As described in section 2.6.3, this was assessed by exposing spread plates of each of the bacterial strains involved to 10 minutes of either continuous or pulsed BL, with the distance between the LED array and samples set at 2 cm. By consulting table 3.15, it can been observed that whilst the continuous exposure resulted in statistically significant reduction in the number of CFU/ml of every strain belonging to the Enterobacteriaceae family, the pulsed-BL exposure did not result in statistically significant colony reductions, despite the power output being 3-fold higher (Table 2.4). This can be explained through the dose delivery of these two different modes of BL exposure. Whilst it was possible to accurately measure the irradiance of the LED array during operation in continuous mode, this was not possible in pulsed mode due to the limitations on the pulse length

of the LED array. The pulse length used within the experiments was 10 ms; the equipment used to measure the irradiance would not have been able to accurately measure the irradiance within such a minute time interval. Subsequently, the best possible compromise was to make a comparison of the two modes of light exposure based on a set exposure time and sample-distance. However, this is not truly representative of the equivalence in efficacy, since due to the required rest intervals of 100 ms (that took place following every pulse) the actual dosage delivered during the pulsed mode of exposure would have been lower than expected. Therefore, if equipment had been available to measure the irradiance of the LED array during pulsed mode, a more accurate comparison would be based on a set dose instead. Despite this, all three *P. putida* strains were inactivated to statistically significant levels by both continuous and pulsed BL. By referring to the cumulative values of the average degree of inactivation for all exposure times and sample distances (Tables 3.6 and 3.11 respectively), it can be seen that these strains are very sensitive to BL-inactivation. Therefore, a lower dose of BL could result in sufficient inactivation within these strains compared to the Enterobacteriaceae strains.

4.4.5 Differences between BL- and UV-inactivation of bacteria

4.4.5.1 UV-resistance plasmid *Pseudomonas* strains

To investigate the extent of similarity behind the inactivation mechanisms of UV and BL, two different sets of experiments were conducted. The first involved the inclusion of *Pseudomonas* strains carrying plasmid pWW0, provided by Dr Glenn Rhodes (CEH, Lancaster). The plasmid pWW0 belongs to the IncP-9 plasmid family, which consists of a group of self-transmissible plasmids primarily located within pseudomonads containing resistance genes, such as antibiotic resistance (Rhodes *et al.*,2014). The core backbone of pWW0 possesses *rulAB* homologue genes. These genes have been previously been shown to confer fitness traits to *Pseudomonas putida*. Rhodes *et al.*,(2014) reported the integration of a group of integron-like elements (ILEs) into pWW0. Furthermore, the role of insertion of ILEs into the rulAB operon within other genera was discussed, in terms of relevance to bacterial adaptation and survival. Therefore, three strains from the study by Rhodes *et al.*,(2014) were included in the experiments within this project, to determine if the plasmid pWW0 conferred fitness traits via UV resistance genes during BL inactivation. Strain *P. putida* PaW340 did not contain pWW0, so served as a control. In addition to this, pseud2 was considered to possess a deletion within pWW0; therefore it was predicted to not carry resistance to UV. For both the exposure-time and sample-distance experiments, the strain carrying the UV resistance was the most sensitive; therefore, UV resistance was shown to not influence the survival of *P. putida* within the BL experiments and was not advantageous.

4.4.5.2 Exposure of representative bacterial strains to UV light (302 nm)

The second approach to determine the similarity in BL and UV inactivation was a set of experiments involving the bacterial strains featured in the qualitative experiments (section 2.7.2), in addition to the *Pseudomonas putida* strains provided by Dr Glenn Rhodes (CEH, Lancaster). The purpose of this was to determine if the levels of photoinactivation rates for UV light were comparable to those for BL. Although the results cannot be directly compared due to experimental differences, the relative sensitivities of the strains in relation to each other can be compared. This revealed that although the majority of strains displayed similar inactivation patterns within both types of light inactivation, *Enterobacter cloacae* displayed greater sensitivity towards UV compared to BL. This indicates that DNA damage is an important factor in the inactivation of this strain. Furthermore, the levels of endogenous porphyrins within *Enterobacter* species are unknown. Therefore, the relative resistance to BL compared to UV could be due to minimal, or lack of, endogenous porphyrins. Therefore, damage to the biological components would not occur due to BL exposure in this case.

4.4.6 Influence of OxyR mutations in the BL-inactivation of *Salmonellla enterica* serovar Tyyphimurium mutants

To test the role of OxyR in protection from BL-inactivation in *Salmonellla enterica* serovar Typhimurium, the mutant strains provided by Dr John Roth (University of California) were included in the exposure-time and sample-distance BL experiments. Different types of mutation were featured in the strains used. It was therefore predicted that the deletion strains would show enhanced sensitivity compared to the strains where a Tn10 transposable element was located near to the *oxyR* gene (refer to Table 1.2 for the genotypes). Furthermore, the *oxyR* gene within the latter strains was mutated, resulting in constitutive expression of OxyR. Despite this, within the exposure-time experiments, one of these strains (S7) was the third most sensitive mutant strain, and has a total inactivation value that is within the mid-range of the spectrum for all of the bacterial strains. Conversely, a deletion mutant strain (S4) was the most resistant of both the *Salmonella* mutant strains and all of the bacterial strains featured in the experiments. The apparent lack of predictability of the sensitivity of

the OxyR mutant strains also occurred within the sample-distance results. Whilst S4 was still the most resistant *Salmonella* mutant strain, it was not the most resistant strain overall. Furthermore, the order of BL sensitivity of the mutant strains in relation to one another was altered; the only strain to remain in the same position on the sensitivity spectrum of the *Salmonella* strains was strain S1. These inconsistencies introduced difficulty in assessing the impact of OxyR mutations on inactivation via BL. Another issue was that a control (i.e. a non-mutant strain of *Salmonellla enterica* serovar Typhimurium) was not available. Comparison of the inactivation of the mutants in relation to a strain that has a correctly-functioning OxyR protein would have been extremely useful. It can be concluded from these sets of results that further experiments on these mutant strains, with the inclusion of a positive control, is required before any stable conclusions may be taken from the results. Furthermore, confirmation that the mutant strains listed still hold the correct mutations is necessary.

4.5 Further factors that could have affected the results

4.5.1 Impact of population age on bacterial inactivation

When reviewing stress treatments on bacteria, an important factor is the phase within the growth cycle of the organisms. However, growth rate is equally as important. A change in physiology or phenotype may occur as a result of different growth conditions (Berney *et al.,* 2006). Therefore, it is of utmost importance to evaluate the impact of growth phase, growth rate and growth conditions on the efficacy of a treatment on any one bacterial species. There are a number of factors that corroborate with the theory that bacteria in the stationary growth phase elicit higher levels of resistance compared with those in exponential. For example, the slower or more non-specific the growth rate, the higher the levels of dps and induction of the global stress response, mediated via RpoS, in *E. coli* (Berney *et al.,* 2006). Due to the nature of the experiments within this project, in addition to time limitations, it was not possible to determine the precise growth cycles of each bacterial strain used. Instead, through extensive literature searches on the bacterial strains and experiments with a similar set-up, the incubation time and desired OD_{600} value were determined.

4.5.2 Impact of culture medium

The medium is another factor that can impact oxidative stress resistance of cells (De Spiegeleer *et al.,* 2004). It can impact heat shock protein (HSP) expression in *E. coli* (Berney *et al.,* 2006), which in turn may lead to increased resistance to oxidative stress. Furthermore, in any instance where bacteria have been exposed to a stress, the choice of media can affect the outcome of which damaged cells are able to recover and grow. Oxidative stress specifically has been identified as an important factor in the recovery and growth of injured bacterial cells (De Spiegeleer *et al.,* 2004). The hypothesis that following stress, cells have increased sensitivity to ROS due to an imbalance in cell metabolism, resulting in greater endogenously-produced ROS levels, was raised previously by Bloomfield *et al.,* (1998).

Following on from this, it was demonstrated in the study by Stephens *et al.,* (2000) that the recovery performance of *Salmonella typhimurium* varied significantly as a consequence of the peptone component used. This was illustrated by the difference in the numbers of recovered cells, whereby the maximum difference exceeded three orders of magnitude. In all of the experiments in this project, Tryptone Soya Broth (TSB) was used. The study by De Spiegeleer *et al.,* (2004) reported that the source of tryptone within the broth (LB) used to culture *E. coli* MG1655 affected the sensitivity of organisms to oxidative stress. Whilst the growth of *E. coli* MG1655 was almost identical in both broths used in the study, LBx and LBy, the physiological properties of the organisms grown were significantly different between the broths. Whilst those grown in LBx were more susceptible to growth inhibition to lactoperoxidase and oxidative stress, those grown in LBy were significantly less so. It is therefore possible that if different media had been used in the experiments in this project, varying results may have been obtained.

4.6 Future applications

4.6.1 Why is there a requirement to develop new antimicrobial technology?

Since their introduction, the use of antibiotics has been accompanied with the development of resistance among bacteria (Davies & Davies 2010). This issue has become more problematic as the discovery of new antibiotics has gradually decreased, whilst rates of antibiotic resistance among important human pathogens world-wide has increased. There is therefore a requirement to fulfil the following objectives:

- Conduct an extensive, regulated approach to determine:
    - The percentage of a large range of nosocomial human pathogens that is resistant to the different classes of antibiotic.
    - The distribution of antibiotic resistance levels world-wide.
    - Co-ordinated surveillance of antimicrobial resistance world-wide, for both the types of antimicrobials involved and the resistant microbes themselves.
- Search for new antibiotics.
- Development of new strategies for the control of infections and microorganisms within environments such as hospitals and the food industry.

The above points were outlined and discussed in the 'Global Report of Surveillance of Antimicrobial Resistance' compiled by the World Health Organisation in 2014 (WHO 2014). It was acknowledged in the report that the extent of antimicrobial resistance (AMR) is not fully known and that for this problem to be properly addressed and resolved, more information is required. Regarding the last bullet point, a similar research project was conducted at the University of Strathclyde; this project was discussed by Maclean *et al.*, (2010). This study is discussed in detail in section 1.7.2.5.

4.6.2 Relevance of BL-inactivation of bacteria

As outlined in the above section, there is a requirement to investigate and develop new technologies for the control of microorganisms. An important aspect of this is the

effective disinfection of target environments, such as hospitals and the food industry. Desirable qualities of such technologies for a light-based environmental decontamination system include:

- Low cost per unit.
- Efficiency of bacterial inactivation, regarding:
    - Treatment time.
    - Practical effective distance range from the device.
    - Effectiveness for a wide range of important human pathogens.
    - Operational cost.
- Easy transportation.
- Minimum labour-intensity/maximum automation.
- Safe for use in the presence of humans.

Following on from this, the paper by Abreu *et al.,* (2013) addressed the requirement of new disinfecting technologies for application in hospital environments. Whilst the use for traditional light wavelengths, such as UV, was mentioned, the issues with this were also addressed. These include the requirement of the room to be vacated during treatment, high acquisition cost and increased room turnover were highlighted (Abreu *et al.,* 2013).

BL could offer a solution for the need for new antimicrobial approaches. Whilst the older studies on aPDT and photoinactivation using visible light featured traditional light sources, such as halogen lamps, a range of recent studies have used light-emitting diodes (LEDs) as the light source. The evolution of LEDs to produce white light in 1993 expanded their horizons (Barolet 2008). These are superior in terms of energy-efficiency (Thakuri *et al.,*2011), using up to 90 % less energy that halogen equivalents (TheGreenAge, 2015). They emit less heat due to the lower energy consumption, and have an approximate lifetime of 25, 000 hours TheGreenAge, 2015). Furthermore, they are more concise than traditional lamps and are more robust. This host of desirable qualities has resulted in increasing and widespread use of LEDs in modern scientific studies.

4.6.3 Current and future possible applications

There are currently some applications. In addition to this, a device designed specifically for the environmental disinfection of hospital rooms was investigated and discussed by Maclean *et al.*, (2010). This produced promising results with regard to the effectiveness of keeping levels of important nosocomial pathogens within the hospital environment low, even in the presence of patients carrying high levels of infectious agents.

4.7 Future work

The inactivation of bacteria by BL is a promising strategy as a control method and potentially within medical applications involving treatment of infected patients. However, there are extensive gaps in the current literature that need to be addressed to enable further development of this antimicrobial approach.

4.7.1 Resistance development

One of the most prominent, and over-looked, aspects of BL-inactivation of bacteria is the development of resistance to BL. Many studies have stated that the risk of resistance development is low, due to the multi-target, non-specific nature of the ROS produced as a result of BL-exposure (Tavares *et al.*,2010). In addition to this, in the case of aPDT, the photosensitizers have been said to mainly affect the cell walls and cell membranes (Tavares *et al.*,2010; Thakuri *et al.*,2011). Therefore, it is presumed that in the event that bacteria acquire efflux systems as a defence against photosensitizers, adequate damage will still result from their action from outside of the cell. However, this is a short-sighted view, especially considering that although the attack of ROS is non-specific, the nature of the attack is singular: oxidative stress.

Therefore, heightened cellular defences, and therefore resistance to, oxidative stress would be equivalent to resistance development towards BL-inactivation.

4.7.1.1 Studies that have investigated resistance development of bacteria to photoinactivation

Some studies investigating the development of resistance against both aPDT and BL-inactivation have been performed and reported (Thakuri *et al.*,2011; Lauro *et al.*,2002; Tavares *et al.*,2010). One of these was the study by Lauro *et al.*,(2002), in which resistance development of the organisms *Prevotella intermedia, Fusobacterium nucleatum, Peptostreptococcus micros* and *Actinobacillus actinomycetemcomitans* to aPDT was assessed. This was achieved through repeated exposure experiments, using the same conditions, on surviving organisms from previously exposed plates in successive light exposure experiments. The levels of inactivation between each individual exposure experiment (1-10) were compared. The results were not significantly different for all of the strains, and therefore did not suggest resistance development. However, since this conclusion is based on the ability of bacteria to grow on solid media following light exposure, it is not sensitive to the possibility of bacteria residing in the viable but non-culturable (VBNC) state. Subsequently, if this occurred following the light exposure experiments, then the proportions of bacteria in this state could have been different for the repeated experiments. A study that applied a technique that measured metabolic activity following aPDT treatment, as opposed to culture viability, was conducted by Tavares *et al.*,(2010). As with the study by Lauro *et al.*,(2002), multiple repeated exposures featured in the evaluation of bacterial resistance development. The efficacy of inactivation was not shown to be affected by the repeated exposures.

4.7.1.2 Adaptation to oxidative stress

The ability of two strains of *S. aureus*, termed 101 and 500 respectively, to adapt to oxidative stress was investigated by (Lipovsky *et al.*, 2009). Following determination of the strain-specific minimum inhibitory concentration (MIC) of hydrogen peroxide, the strains were exposed to sub-inhibitory concentrations of hydrogen peroxide. This

resulted in a 4-fold increase in the MIC of strain 500; strain 101 was unable to adapt. Strain 500 possessed a number of advantageous fitness traits that may have enabled oxidative stress adaptability. These include a 10-fold lower porphyrin concentration, significantly greater level of carotenoid pigment and lesser production of hydroxyl radicals following illumination. It is therefore plausible that the traits were exploited by strain 500 to allow adaptation to oxidative stress. Furthermore, it was found that bacteria displayed heightened resistance to BL therapy following the first exposure in the study by (Dai *et al.,* 2013).

4.7.1.3 Implication of overlap between stresses in bacteria

The fact that carbon starvation is known to increase resistance to certain stresses within *E. coli* (Battesti *et al.,* 2011; Youn *et al.,* 2001) means that practical applications of using BL for disinfection may be compromised by the fact that the most common state of bacteria in environments such as hospitals is the stationary phase, due to lack of nutrients. In addition to this, the regulon for oxidative stress in *Eschericia coli and Salmonella* spp. also regulates heat shock proteins (Youn *et al.,* 2001). It was concluded in the study by Dai *et al.,, (*2011) that a possible route for resistance development to aPDT is through heat shock proteins. Prior to drawing this conclusion, they conducted a series of experiments to determine the extent of involvement of HSPs during aPDT treatment. This was focussed on two particular HSPs, GroEL and DnaK. These are part of two major HSP families: GroEL/GroES and DnaK/DnaJ/GrpE. These have been conserved across a range of bacterial species and have remained through bacterial evolution (Chamberlain *et al.,,* 2007). Since these proteins are responsible for protein repair and lipid membrane stabilisation, in addition to providing protection from oxidative stress, it is essential that these be included in studies assessing resistance development in bacteria in response to external stresses. This is reinforced by the fact that expression of HSPs has been previously demonstrated to be up-regulated in response to external stresses and they have been implicated in the development of antibiotic resistance.

4.7.2 Success of inactivation of bacterial biofilms

It has been estimated that up to 80% of infections are biofilm-associated (Lebeaux *et al.*, 2013). Consequently, there has been development of numerous *in vitro* biofilm models to enable a better understanding of the functioning and behavioural aspects of bacteria in these systems. Whilst research in the safety of exposure of mammalian cells to BL is currently incomplete (refer to section 1.7.1.1), the effectiveness of BL-inactivation of biofilms within living systems and abiotic environments is a topic of high-importance to ensure that inactivation systems are effective towards bacteria in this state.

4.8 Limitations and lessons learnt from this study

The experiments in this study have provided valuable information regarding bacterial inactivation of a range of important human pathogens using BL. Results were gained for each of the objectives listed in section 4.1. Due to time restraints, there are a variety of further parameters that should be included in future investigations of BL-inactivation of bacteria. These are detailed in section 4.7.

Following on from this, the experiments featured in this project should be expanded to provide a more in-depth investigation into the topic. An issue that was presented during experimentation was that the limited time allocated for experiments resulted in the use of methods that would allow all of the desired strains to be included in the experiments within the time scale provided. Combined with that fact that only one custom-made light source was available, this resulted in the selection of a method that would allow rapid screening of the range of bacterial strains to BL. Furthermore, a variety of parameters were investigated, including the impact of exposure-time, sample-distance and bacterial concentration on BL-inactivation of bacteria. The method devised for this was the droplet method (section 2.6). This method allowed eight dilutions of a bacterial suspension to be measured simultaneously, speeding up the screening process.

However, a disadvantage to this method is that accurate and reliable colony counting is not possible; the data is therefore arbitrary as opposed to qualitative. To overcome this, a smaller selection of strains was selected following the general screening. This contained strains ranging from very sensitive to very resistant. Spread plates were performed for these strains, under both pulsed and continuous conditions. The data generated from these experiments provided data that was more statistically reliable.

4.9 Conclusions

A number of conclusions regarding the inactivation of a variety of clinically relevant bacterial pathogens using BL can be drawn from the results of this study. Some of these have parallels with results gained from other studies investigating the photoinactivation of bacteria. This study has directed research into photoinactivation of bacteria using visible light wavelengths, and has built upon some fundamental discoveries made by scientists in the mid- to late-19th century (Reed 1974; Mitton & Ackroyd 2005; Rajesh et al. 2011). Namely, the relationships between bacterial inactivation and properties of the light used, including wavelength, intensity and duration of exposure by Downes and Blunt in 1877 (Reed 1974). This chapter has discussed the implications of the results gained from the experiments in this study, why the development of new antimicrobial technologies, such as BL-inactivation of bacteria, are required, and areas for future research. The inactivation of bacteria by BL is highly complex. From the literature, there are a large number of factors that can influence the success of BL-inactivation. Therefore, a large, coordinated research effort using standardised techniques is necessary to attain comparable inactivation results of a diverse selection of bacterial species, under different conditions, in different physiological states. Determination of true bacterial inactivation is essential, since transition into the VBNC state (section 1.6) could result in inaccurate inactivation danger and present a risk to the public. Although there is a range of potential industrial and medical applications of BL-inactivation, the aforementioned parameters must be thoroughly investigated prior to the design of these.

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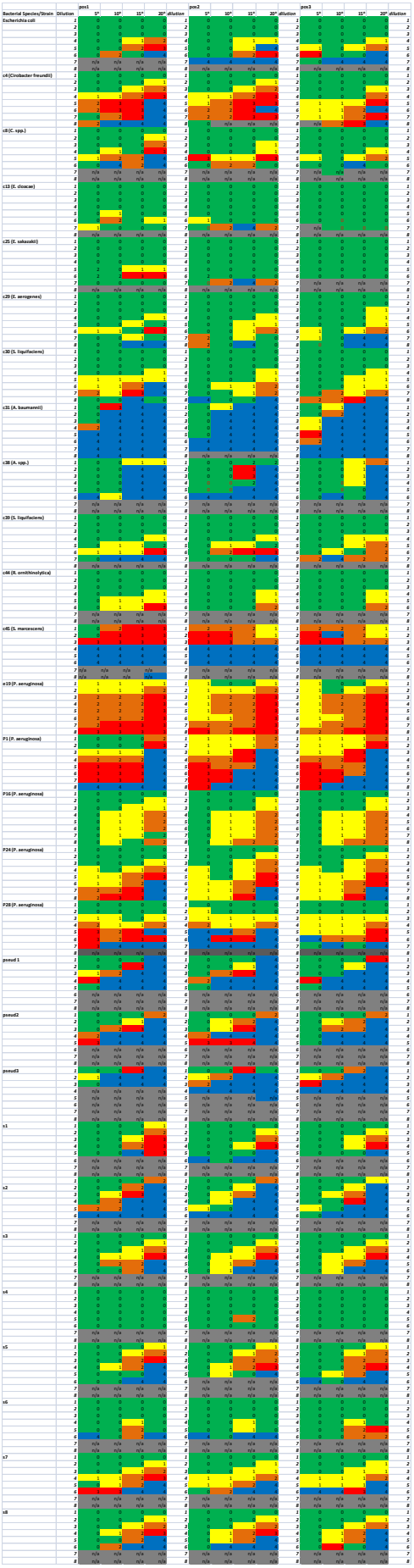
Appendices

The tables constructed in excel for the sample-distance and exposure-time are displayed on the next two pages.

	Position 1*			Position 2*			Position 3*			
Bacterial Strain	2**	3**	4**	2**	3**	4**	2**	3**	4**	Dilution
Escherichia coli	0	0	0	0	0	0	0	0	0	1
	0	0	0	0	0	0	0	0	0	2
	1	0	0	0	0	0	0	0	0	3
	2	1	0	1	0	0	1	0	0	4
	3	1	0	2	1	0	1	0	0	5
	3	3	2	3	2	0	4	0	0	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
Citrobacter spp.	2	0	0	1	0	0	0	0	0	1
	3	0	0	2	0	0	0	0	0	2
	4	0	0	3	0	0	1	0	0	3
	4	1	0	4	0	0	2	0	0	4
	4	1	1	4	0	0	3	0	0	5
	4	3	2	4	0	0	3	1	0	6
	4	4	4	4	4	4	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
Acinetobacter spp.	4	4	4	4	4	4	3	3	3	1
	4	4	4	4	4	4	4	3	3	2
	4	4	4	4	4	4	4	4	4	3
	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	5
	4	4	4	4	4	4	4	4	4	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
Enterobacter cloacae	0	0	0	0	0	0	0	0	0	1
	0	0	0	0	0	0	0	0	0	2
	0	0	0	0	0	0	0	0	0	3
	0	0	0	0	0	0	0	0	0	4
	0	0	0	0	0	0	0	0	0	5
	2	0	0	2	0	0	0	0	0	6
	n/a	n/a	n/a	n/a	n/a	n/a	0	0	0	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
Enterobacter spp.	0	0	0	0	0	0	0	0	0	1
	0	0	0	0	0	0	0	0	0	2
	1	1	0	1	0	0	0	0	0	3
	2	1	0	1	0	0	0	0	0	4
	4	2	0	4	1	0	0	0	0	5
	n/a	n/a	n/a	4	0	0	0	0	0	6
	n/a	n/a	n/a	n/a	n/a	n/a	4	3	0	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
Enterobacter aerogenes	0	0	0	0	0	0	0	0	0	1
	1	0	0	0	0	0	0	0	0	2
	2	0	0	1	0	0	0	0	0	3
	3	1	0	2	1	0	0	0	0	4
	4	2	0	4	2	1	2	1	0	5
	4	4	0	4	4	2	4	1	0	6
	n/a	n/a	n/a	n/a	n/a	n/a	4	1	0	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
Serratia liquifaciens	0	0	0	0	0	0	0	0	0	1
	0	0	0	0	0	0	0	0	0	2
	1	0	0	1	0	0	0	0	0	3
	2	1	0	1	1	0	1	0	0	4
	3	1	0	2	1	0	1	0	0	5
	4	2	1	2	1	0	2	0	0	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
Acinetobacter baumannnii	4	4	3	4	4	3	1	1	1	1
	4	4	4	4	4	3	2	2	1	2
	4	4	4	4	4	4	4	3	2	3
	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	5
	4	4	4	4	4	4	4	4	4	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
Raoultella ornithinolytica	0	0	0	0	0	0	0	0	0	1
	0	0	0	0	0	0	0	0	0	2
	0	0	0	0	0	0	0	0	0	3
	0	0	0	0	0	0	0	0	0	4
	0	0	0	0	0	0	0	0	0	5
	3	0	2	0	0	2	1	2	1	6
	n/a	n/a	n/a	n/a	n/a	n/a	0	0	0	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
Pseudomonas aeruginosa	4	2	0	4	1	0	0	0	0	1
	4	3	0	4	2	0	2	0	0	2
	4	4	0	4	2	0	3	0	0	3
	4	4	0	4	4	0	4	2	0	4
	4	4	2	4	4	0	n/a	n/a	n/a	5
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
S1	1	0	0	1	0	0	1	0	0	1
	2	1	0	1	0	0	1	0	0	2
	2	2	1	2	2	1	2	1	1	3
	3	3	2	4	3	3	2	1	1	4
	4	2	2	4	3	4	4	4	4	5
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
S2	4	3	1	4	1	1	3	1	0	1
	4	4	2	4	2	2	2	1	1	2
	4	4	4	4	4	3	4	2	2	3
	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	5
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
S3	1	0	0	1	0	0	0	0	0	1
	1	1	0	1	1	0	1	1	0	2
	2	2	1	2	2	1	2	2	1	3
	4	4	1	4	4	1	3	3	1	4
	4	1	1	4	4	4	4	4	4	5
	4	4	4	4	4	4	4	4	4	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
S4	1	0	1	1	0	0	0	0	0	1
	1	0	0	1	0	0	0	0	0	2
	1	1	0	1	0	0	0	0	0	3
	2	2	0	2	0	0	0	0	0	4
	3	3	0	2	0	0	0	0	0	5
	4	4	0	3	1	0	0	0	0	6
	n/a	n/a	n/a	4	2	0	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
S5	4	2	0	2	2	0	1	1	0	1
	4	3	1	3	2	1	1	1	0	2
	4	4	3	4	2	1	1	1	1	3
	4	4	2	4	3	2	2	1	1	4
	4	4	4	4	4	2	4	2	2	5
	4	4	4	4	4	4	4	4	4	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
S6	0	0	0	0	0	0	0	0	0	1
	1	0	0	0	0	0	0	0	0	2
	2	1	0	1	0	0	0	0	0	3
	3	1	0	2	1	0	0	0	0	4
	4	2	1	3	1	2	2	1	2	5
	4	4	0	4	0	2	3	1	2	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
S7	4	4	2	4	3	1	2	2	1	1
	4	4	3	4	4	2	3	2	2	2
	4	4	4	4	4	3	3	3	3	3
	4	4	4	4	4	4	4	3	3	4
	4	4	4	4	4	4	4	4	4	5
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
S8	2	0	0	1	0	0	1	0	0	1
	2	0	0	1	0	0	1	0	0	2
	3	1	0	2	1	0	1	1	0	3
	4	2	1	3	2	2	2	1	2	4
	4	4	1	4	3	1	4	3	1	5
	4	4	4	4	4	4	4	4	4	6
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8
pseud1	4	4	0	4	4	0	3	1	0	1
	4	4	4	4	4	0	3	1	0	2
	4	4	1	4	4	0	4	2	0	3
	4	4	3	4	4	0	4	4	2	4
	4	4	4	4	4	0	4	4	4	5
pseud2	4	4	0	4	4	0	3	1	0	1
	4	4	0	4	4	0	4	2	0	2
	4	4	2	4	4	0	4	2	0	3
	4	4	3	4	4	0	4	4	0	4
	4	4	1	4	4	1	4	4	1	5
pseud3	4	4	1	4	4	1	4	3	1	1
	4	4	1	4	4	1	4	3	1	2
	4	4	4	4	4	2	4	4	1	3
	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	5


pos1
pos2
pos3
Bacterial Species/Strain
Dilution
5^
10^
15^
20^
dilution
Escherichia coli
c4 (Cirobacter freundii)
c8 (C. spp.)
c13 (E. cloacae)
c25 (E. sakazakii)
c29 (E. aerogenes)
c30 (S. liquifaciens)
c31 (A. baumannii)
c38 (A. spp.)
c39 (S. liquifaciens)
c44 (E. omithinolytica)
c45 (S. marcescens)
e10 (P. aeruginosa)
P1 (P. aeruginosa)
P16 (P. aeruginosa)
P24 (P. aeruginosa)
P28 (P. aeruginosa)
pseud 1
pseud2
pseud3
s1
s2
s3
s4
s5
s6
s7
s8